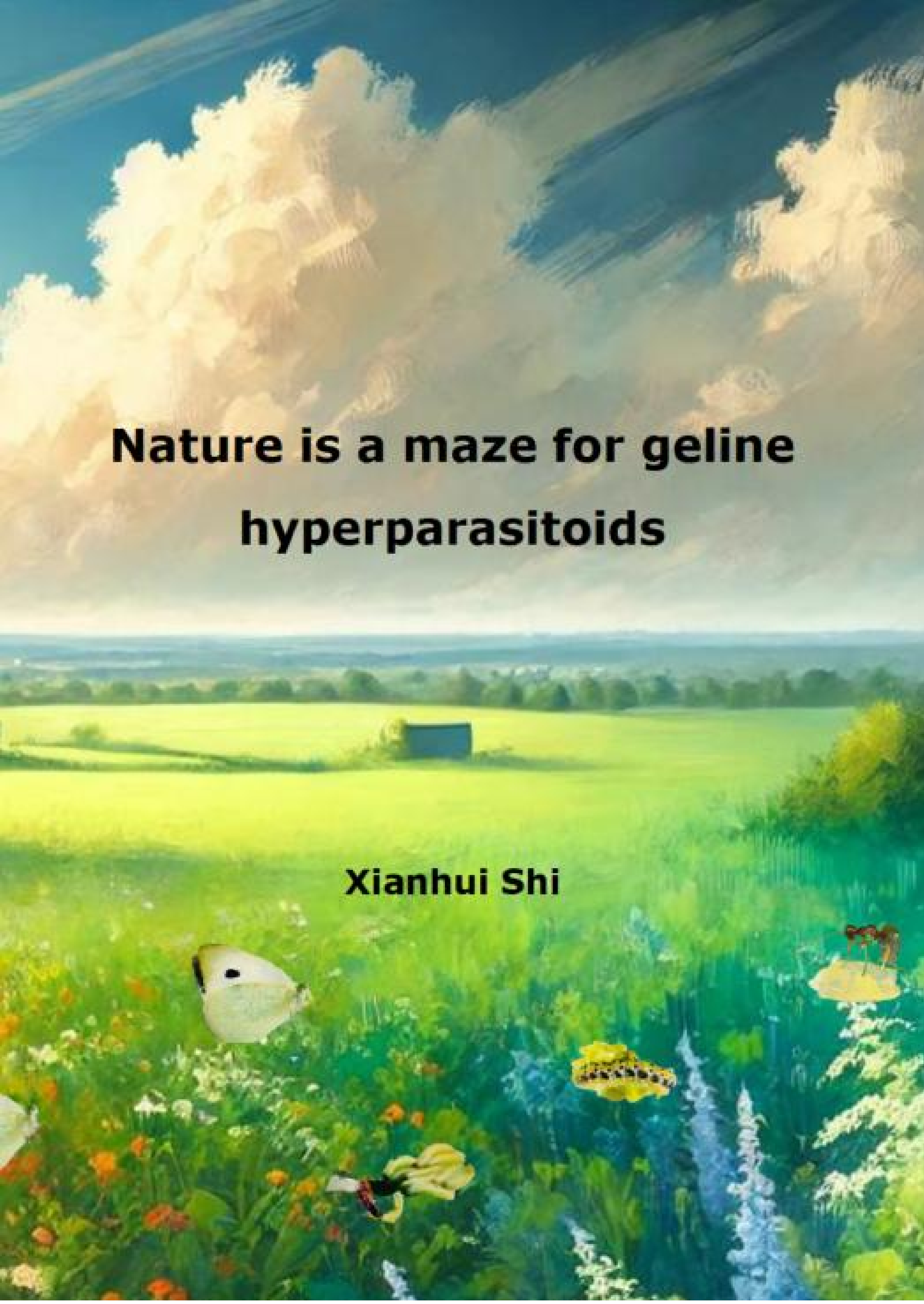

# Nature is a maze for geline hyperparasitoids

Xianhui Shi

**Propositions**

1. The effect of small habitat patches on biodiversity conservation has been overlooked.

   (this thesis)

2. Wingless hyperparasitoids are adapted to finding scarce hosts in complex landscapes.

   (this thesis)

3. Inclusive participation of scientific congresses is limited by narrow registration deadlines.

4. Integrating laboratory and fieldwork enhances the generalizability of ecological research.

5. Live music alleviates stress to listeners more than on radio.

6. In countries where English is widely spoken, requiring local language proficiency for employment is unnecessary.

Propositions belonging to the thesis, entitled

“Nature is a maze for geline hyperparasitoids”

Xianhui Shi

Wageningen, 15 November 2024

# Nature is a maze for geline hyperparasitoids

Xianhui Shi

## Thesis committee

### Promotors

Prof. Dr W. H. van der Putten
Special Professor, Functional Biodiversity
Wageningen University & Research
Netherlands Institute of Ecology, Wageningen

Prof. Dr J. A. Harvey
VU Universiteit Amsterdam
Netherlands Institute of Ecology, Wageningen

### Co-promotor

Dr J. G. de Boer
Netherlands Institute of Ecology, Wageningen
Aeres Hogeschool, Wageningen

### Other members

Prof. Dr B. Wertheim, Wageningen University & Research
Prof. Dr E. H. Poelman, Wageningen University & Research
Dr F. Bianchi, Wageningen University & Research
Dr B. Visser, University of Liège, Belgium

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# Nature is a maze for geline hyperparasitoids

## Xianhui Shi

### Thesis

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# Chapter 1

## General introduction

## The evolution of life-history strategies and trade-offs among traits

Life history theory seeks to explain how evolution shapes organisms to maximize reproductive success, taking into account environmental pressures and inherent constraints within the organism (Stearns, 2000). To approach life history evolution as an optimality problem, one starts by defining fitness, establishing how traits relate to fitness, and identifying trade-offs between traits. The goal is to determine the combination of traits that maximizes fitness (Stearns, 2000). In a perfect world, an organism would live very long, produce offspring continually over its lifespan at regular intervals, would be a strong competitor and is also able to resist attack from a range of natural enemies including parasites, pathogens and predators (Begon et al., 1996). However, no such ‘perfect’ organism exists, because all animals have limited metabolic resources for allocation to an array of biological functions, leading to inevitable trade-offs between fitness functions (Zera and Harshman, 2001). Trade-offs are a fundamental parameter in optimality models of life history evolution, offering a crucial explanation for the diverse range of life history traits observed in natural populations (Stearns, 1992). Prominent and well-studied life-history trade-offs in animals include those between reproduction and survival and between longevity and fecundity (Bartke et al., 2013). Moreover, selection for individual differences in animal personality traits are favored when these traits consistently influence productivity, including growth and reproduction (Biro and Stamps, 2008).

### *Body size as a proxy of fitness*

In many animals, important fitness-related traits like reproduction and longevity are strongly related to body size (Barneche et al., 2018; Bekoff et al., 1981; Blanckenhorn, 2000; Bonnet et al., 2000; Cronin, 2007; Lindstedt and Calder, 1981). Intra-specific differences in body size are common among animals and can vary depending on a range of factors within some species. For instance, body size in honeybees varies considerably among different castes (queen, worker, drone) and is influenced by factors such as temperature, brood cell size and diet acquired by the larvae (Chole et al., 2019). Body size has also been shown to influence foraging behavior, reproduction and survival in many other organisms (Blanckenhorn, 2000; Honěk, 1993; Kerr et al., 2019).

Mammals have evolved more specialized lifestyles and there are divergent mass-specific production rates: increased in groups that specialize on sufficient foods: grazing and browsing herbivores and flesh-eating marine mammals; and declined in groups that have lifestyles with decreased death rates (Sibly and Brown, 2007). A positive relationship between female body size and fecundity has already been shown in many vertebrates such as in mammals (Cassini, 2017; Iskjaer et al., 1989), birds (Böhning-Gaese et al., 2000), reptiles and amphibians (Green, 2015; Shine, 2005) and invertebrates such as insects (Akman and Whitman, 2008; Calvo and Molina, 2005; Kajita and Evans, 2010; Marshall et al., 2013; Pincheira-Donoso and Hunt, 2017; Richardson and Baker, 1997). A meta-analysis of 33 studies showed that there is a general fitness benefit to large size in odonates, and significant positive effect of body size on survivorship of males (Sokolovska et al., 2000).

### *Paternal provisioning of offspring*

According to the "nature versus nurture" paradigm, the phenotype of offspring in most organisms is influenced by a combination of genetically inherited traits from both parents and the resources provided by the parents during their development (Bartelt et al., 2014; Pinker, 2004; Wachs, 1992). Parental (usually maternal) provisioning is showed in many animals, such as insects, birds, sea urchin and fish (Berg et al., 2001; Byrne et al., 2008; Mas and Kölliker, 2008; Nakahira et al., 2013; Ridley, 2007). Maternal effects often provide a mechanism for adaptive transgenerational phenotypic plasticity. Maternal effects refer to the influence of the mother's phenotype on the offspring's phenotype, independent of the mother's genetic contribution. Maternal effects frequently serve as a mechanism for adaptive transgenerational phenotypic plasticity (Hagmayer et al., 2018). Maternal nutrient provisioning to offspring, for instance, can significantly impact offspring size at birth, which in turn affects their survival (Mousseau and Fox, 1998).

## Nature is characterized by structural and chemical complexity

Landscape heterogeneity affects the community composition and the functioning of ecosystems (Wiens, 2002). Habitat loss and degradation of natural ecosystems are major causes of the global biodiversity crisis (Bongaarts, 2019; Newbold et al., 2016). Permanent grasslands are an important land use in Europe that contain high species richness (Feurdean et al., 2018; Wallis-DeVries et al., 2002). The size, shape and composition of different landscape units and the spatial (and temporal) connection between them have been shown to profoundly influence factors such as animal movement and dispersal, population persistence and species interactions (Fahrig, 2007; Fraterrigo et al., 2009; Polis et al., 2004). The negative effects of habitat loss may be reduced when agricultural fields are bordered by strips of natural vegetation consisting of a diverse mixture of wild, flowering plants or by hedgerows of woody shrubs.

Habitat structure and complexity at different scales affects individual organisms, communities, and species interactions. In various habitats, the species richness of herbivorous insects follows a declining trend in the sequence: woody shrubs > perennial herbs > weeds and other annuals > monocots (excluding grasses, as many host records for grasses are unreliable) (Lawton and Schroder, 1977; Lawton and Schröder, 1978). Life-history characteristics of fragmentation-sensitive species reveal that those most negatively affected are typically rare, resource or habitat specialists, and have limited dispersal capabilities. Additionally, species with high population variability and those occupying higher trophic positions are often more negatively impacted by the simplification of their habitats than species in lower trophic levels (Tscharntke et al., 2002) and are greater risk of (local) extinction (Holt et al., 1999; Lawton and May, 1995; Pimm, 1991). Habitats with greater structural heterogeneity offer refuges to organisms whereby they can more easily escape predation or parasitism, and thus find ‘enemy-free space’ (Jeffries and Lawton, 1984). This is vitally important in generating long-term stable cycles between resources and consumers, such as between predators and prey, because it enables some prey to survive and this provide a resource pool for future generations of predators (Sih, 1987). Therefore, the presence of more complex and diverse habitat structures can positively influence the survival rate of multiple species across different trophic levels.

Food resources and reproduction are vitally important for all living organisms. Food offers energy, protein, fat, and other nutritional compounds for the organism to develop (Roff, 2002). Moreover, food enables reproduction, which maintains populations and allows a species to persist (Harvey, 2005; Jervis et al., 2008; Van Noordwijk and de Jong, 1986). Animals need to acquire sufficient resources to sustain growth and development and reach the adult stage. During the adult stage resources are needed for metabolism or reproduction (which often incurs trade-offs between these parameters) (Iwao and Ohsaki, 1996; Roff, 2002; Stearns, 1992). Limited resources can result in competitive interactions among individuals, generating two main outcomes. Competitors can engage in scramble competition (through resource partitioning) when all competitors equally distribute the finite resources, resulting in decreased fitness for all competitors (Royle et al., 2002). Meanwhile, in contest competition successful competitors monopolize all of the resources obtained for survival and reproduction and will thus not share resources with competitors (Wise, 2006). A meta-study showed that predator abundance was higher in complex habitats (Langellotto and Denno, 2004) and showed that highly complex habitats can provide predators refuge from intraguild predation, increase successful prey capture and provide alternative food resources, such as alternative prey, pollen and nectar.

### *The importance of hedgerows in the study of multitrophic interactions*

As discussed above, the structural heterogeneity of habitats is important in that it provides refuges for animals against a range of biotic (i.e. predators) and abiotic (i.e. climate extremes) threats. In a meta-analysis, it indicated that predator abundance was higher in complex habitats. More complex habitats can provide animals with refuge from intraguild predation, influence the rate of predation and offer alternative food sources, such as alternative prey, pollen and nectar (Langellotto and Denno, 2004). Hedgerows bordering meadows and agricultural fields have received considerable attention in enhancing our understanding of important ecological concepts such as niche and refuge theory, food webs and trophic interactions, as conservation corridors and in the maintenance of ecosystem services (Alignier et al., 2020; Montgomery et al., 2020). In intensively managed European agricultural landscapes, hedgerows are dominated by two, small woody trees, the blackthorn (*Prunus spinosa*) and hawthorn (*Craetagus* spp.) (French and Cummins, 2001). Hedgerows offer animals a wide variety of microhabitats, and also serve as nesting and hibernation sites, facilitate dispersal across landscapes, and provide food resources for a diversity of species (Besnard and Secondi, 2014; Griffiths et al., 2007; Staley et al., 2012). Hedgerows also help to buffer against climatic extremes, such as extreme heat, cold, and floods (Wolton et al., 2014). Moreover, hedgerows have been shown to benefit predatory beetles (Staphylinidae) by reducing habitat disturbance and edge effects (Maudsley et al., 2002).

### *Niche overlap and species coexistence*

A significant challenge in ecology is understanding how the vast array of species on Earth is structured into biological communities. Underpinning this is the concept of the niche. It was originally proposed by the pioneering ecologist G. Evelyn Hutchinson (Takola and Schielzeth, 2022), and in its most simple form is defined as ‘the match of a species to specific environmental conditions’ (Polechová and Storch, 2008). The niche concept has been further broken down into two subcategories: the ‘fundamental’ niche, which describes the larger habitat area in which an organism can survive and reproduce, and the ‘realized’ niche, which is the actual habitat (usually, though not always smaller than the fundamental niche) in which an organism is found (Soberón and Arroyo-Peña, 2017). The latter incorporates factors such as competition among and between species, both of which function to reduce niche breadth and drive the specialization of traits. One key organizing mechanism facilitating coexistence among species sharing resource space is through niche partitioning, which enables multiple species to persist within the same trophic level within a complex web of species interactions (Siepielski and McPeek, 2010). This reduces niche overlap and thus promotes species coexistence. Niche overlap is affected by many factors such as body size. Predator size often influences dietary overlap, with ontogenetic shifts often having a greater impact than taxonomic differences. For example, small predators, irrespective of species, often have the narrower diets than larger species because they are restricted to a more limited range of prey sizes. Niche overlap was shown to decrease as differences in body size increased both among and within species (Woodward and Hildrew, 2002). Species coexistence can occur when niche partitioning of resources occurs at different spatial and temporal scales (Albrecht and Gotelli, 2001). Animals compete for the same resources when these resources are scarce. Under these situations, coexistence or competitive displacement are driven by niche overlap and competitive ability differences, and these ultimately determine whether species within the same functional guilds can maintain sympatry or whether one excludes the others (Mayfield and Levine, 2010).

## Parasitoids and hyperparasitoids as model systems in ecological and evolutionary research

Parasitoids are insects whose larvae feed and grow within or on the bodies of other arthropods, typically other insects, and rely on these hosts for their development and survival. In contrast, adult parasitoids are free living and depend on exogenous sources of carbohydrates, such as floral nectar, to extend their longevity and maximize reproductive output (Bianchi and Wäckers, 2008; Heimpel, 2019; Wäckers, 2001). Parasitoid wasps exhibit an array of life-history and development strategies. For instance, parasitoid larvae can develop externally (ectoparasitism) or internally (endoparasitism) on or inside their hosts (Godfray, 1994). Idiobiont parasitoids specialize on parasitizing non-growing host stages, such as eggs or (pre)pupae, or impede the host's further development through envenomation at the time of parasitism (Askew, 1986). Idiobionts paralyze the host during the oviposition sequence, thus preventing its further development (Godfray, 1994; Harvey, 2005; Pennacchio and Strand, 2006). These hosts are ostensibly 'static' resources that 'remain in place'. By contrast, koinobiont parasitoids attack hosts that continue feeding, growing, and molting throughout the course of parasitism (Godfray, 1994; Harvey, 2005; Pennacchio and Strand, 2006). Hosts parasitized by koinobionts represent potentially 'dynamic' resources that 'do not remain in place'.

Most hosts attacked by parasitoids are not much larger than the attacking parasitoid female (Harvey, 2005). Parasitoid wasps are thus model systems to study resource allocation as they depend on individual hosts for their development and reproduction (Godfray, 1994). Because host resources are limited for parasitoids, this drives the selection to optimize host-finding behavior and outcompete other parasitoids that also attack the same host species and stage. To maximize reproductive success, parasitoids need to be efficient foragers and effective competitors as hosts may be attacked by other parasitoids, inter- and intraspecifically (Harvey et al., 2013a; Hochberg, 1991; Iwao and Ohsaki, 1996; Mohamad et al., 2015; Ode et al., 2022; Tian et al., 2008). For extrinsic competition, the consequence can be influenced by species differences in host-searching efficiency, reproductive ability (i.e. egg number), and phenological asynchronisation among different parasitoid species that share the host (Lei and Hanski, 1998; Tillman and Powell, 1992; Tumlinson et al., 1993; Ode et al., 2022). For comparison, the outcome of intrinsic competition is often affected by parasitoid growth rate, solitary or gregarious life history, developmental stage of the host, the order of oviposition events, and host quality (Harvey et al., 2013a; Jervis et al., 2001; Poelman et al., 2014). In addition, a range of factors that affect the development and reproduction of parasitoids across different species and with variable life-history strategies have been well studied over the past several decades (Godfray, 1994; Jervis et al., 2023, 2008). Despite that, there are still many gaps of our understanding of the biology and ecology of parasitoids in many species. For obvious reasons, most of the best-studied parasitoids are also important biological control agents of important pests in agro-ecosystems. This leaves a huge number of species of limited direct economic importance of which little or nothing is known about various aspects of their life-histories and ecologies. This information can help us to fill in many gaps in our understanding or processes in parasitoid evolution, phylogeny and the roles that these fascinating insects play in maintaining the functioning of ecosystems in nature.

For predator or parasitoid species, it is crucial to not overexploit their host or prey to ensure their progeny have sufficient remaining resources (Beddington and Hammond, 1977). Whereas most parasitoids are fully winged and able to disperse over reasonably large distances when searching for hosts, a small number of parasitoids in several subfamilies are wingless and thus their foraging ability is often greatly restricted to small habitat patches. Foraging behavior of parasitoids is constrained by limited metabolic resources that need to be allocated to vital and potentially competing fitness functions such as foraging, reproduction, and survival (Tian et al., 2008). They are also under intense selection and depend on many biotic and abiotic factors including local microclimates, the risk of predation, host abundance and other parameters, and for most parasitoids the optimal phenotype is determined by tradeoffs in life-history traits (Lei and Hanski, 1998; Mohamad et al., 2015; Tumlinson et al., 1993).

Prior to oviposition, many ectoparasitoids physically damage hosts with their ovipositors and drink hemolymph at seeps from the resulting wound (Jervis and

Kidd, 1986). ‘Host-feeding’ is obligate behavior for adult females to acquire proteins that are essential for the completion of oögenesis  (Jervis and Kidd, 1986; Visser et al., 2016). Host-feeding generally leads to the production of large, ‘yolky’ eggs - called ‘anhydropic’- that take a considerable time to lay because they must be significantly compressed down the narrow lumen of the ovipositor. Most endoparasitoids, by contrast, produce mostly yolkless ‘hydropic’ eggs, where the necessary proteins for oogenesis are contained in host hemolymph and are absorbed through the porous membrane of the eggs into the embryo (Grbić and Strand, 1998). In parasitoids, differences in the number of mature eggs at eclosion relative to potential fecundity, and the ability to mature ripe eggs during adult life are defined by an ‘ovigeny index’ whereby most species are partially or wholly ‘synovigenic’ (those species emerging with a full complement of eggs are defined as ‘pro-ovigenic’) (Jervis et al., 2001). Parasitoids that produce anhydropic eggs can generally only produce low numbers of progeny daily (Jervis and Kidd, 1986).

In parasitoids, adult body size at eclosion is affected by many factors during development, including host stage, age or size at parasitism, the nutritional status of the host, the presence or competing organisms such as pathogens or other parasitoids, and weather-related factors such as temperature or moisture (Da Rocha et al., 2007; Gagnon et al., 2024; Harvey, 2005; Harvey and Malcicka, 2016; Klapwijk et al., 2010). In turn, differences in adult parasitoid size can affect demographic parameters such as mate- and host-finding efficiency, dispersal capacity, competition, longevity, fecundity, offspring sex ratio, and susceptibility to other natural enemies such as predators and hyperparasitoids (Godfray, 1994). The ‘size-advantage hypothesis’ (Charnov, 2020) predicts that larger hosts are of higher quality for parasitoids because parasitoids emerging from larger hosts are themselves larger (Salt, 1941), and enjoy higher fitness-related benefits (Godfray, 1994). As stated above, these hosts are often not much larger than the adult parasitoids so parasitoids have evolved amazing efficiency at resource exploitation, utilization and allocation to different fitness functions (Jervis et al., 2008).

### *Hyperparasitoids in the genus* Gelis *as my study system*

Hyperparasitoids in the fourth and higher trophic levels parasitize the larvae or pupae of their primary parasitoid hosts (Poelman et al., 2022; Sullivan, 1987; Sullivan and Völkl, 1999). They are important components of food chains and often play a crucial top-down role in the shaping of multitrophic communities (Poelman et al., 2022). The lifestyles of hyperparasitoids can be categorized into two types: true- and pseudo-hyperparasitoids. True-hyperparasitoids attack larvae of their parasitoid host during their development, while pseudo-hyperparasitoids attack cocoons of parasitoids that are formed when parasitoid larvae have emerged from their host (Poelman et al., 2022; Sullivan and Völkl, 1999).

*Gelis agilis* is a solitary ectoparasitoid (Elzinga et al., 2007) that hyperparasitizes the cocoon of gregarious primary endoparasitoid *Cotesia glomerata* and related species (Harvey et al., 2006; van Nouhuys et al., 2012; van Nouhuys and Hanski, 2005). *Cotesia glomerata* is in turn a specialist koinobiont parasitoid that attacks caterpillars of several closely related butterfly species in the family Pieridae (Feltwell, 1982). Female *C. glomerata* lay broods of up to 50 eggs in first or second-instar larvae of *Pieris brassicae* (Gu et al., 2003; Harvey, 2000). Parasitized caterpillars feed and grow until the final instar when the mature parasitoid larvae perforate holes in the cuticle, emerge and spin cocoons. *Gelis agilis* is an asexually-reproducing species whose females are wingless and mimic ants behaviorally, chemically and morphologically (Harvey et al., 2018). Adult *Gelis* wasps must first host-feed on (pre)pupae of *C. glomerata* in order to obtain proteins crucial for egg production (Harvey et al., 2012). Up to 60 species of *Gelis* occur in Europe (Schwarz and Shaw, 2000), and many are hyperparasitoids of fully cocooned pre-pupae or pupae of other parasitoids (Harvey et al., 2019), whereas others are parasitoids of egg sacs of predators like spiders (Fei et al., 2023).

Although many *Gelis* species are ubiquitous and abundant in many habitats, the biology and ecology of most species has been thus far little-studied.

In nature, food such as nectar and hosts are often physically separated for parasitoids, making parasitoids excellent models to test trade-offs between life-history traits such as reproduction and longevity. When hosts are scarce, or else there is intensive intraspecific competition for hosts, selection for finding and exploiting hosts presumably gets stronger. Herbivore-induced-plant-volatiles (HIPVs) may be able to provide hyperparasitoids with cues about the presence of suitable hosts. This was demonstrated in a related cryptine ichneumonid, *Lysibia nana*, which is also a hyperparasitoid that utilizes HIPVs locate caterpillars parasitized by *C. glomerata* (Poelman et al., 2012). However, *Lysibia nana* does not host-feed and has wings, making it able to cover a much larger habitat area than wingless gelines.

In the field, searching for limited suitable hosts in complex environments is a major challenge for *G. agilis* and related species because they are constrained by traits such as winglessness and competition for hosts. Gelines studied in this thesis are nominal specialists and require parasitoid cocoons on which to reproduce (and host-feed). *Gelis* species may differ in their competitive (intrinsic and extrinsic) abilities, with the better competitor producing more progeny with higher per capita fitness (Honěk, 1993). The (pre-)pupae of parasitoids such as *C. glomerata* serve as excellent hosts and have virtually no physical or internal defenses against parasitism once they are located. However, they have evolved several antipredator/parasitoid strategies against natural enemies. The most common strategy is through avoiding encounters with enemies by manipulating the behavior of parasitized *P. brassicae* caterpillar which wanders from the natal foodplant at maturity and eventually pupates on structures such as wall, tree trunks and other locations. Parasitized caterpillars sometimes attend cocoons of parasitoids that have emerged from them, and act as surrogate ‘bodyguards’, aggressively attacking any predators or hyperparasitoids that attempt to gain access to the cocoons (Grosman et al., 2008; Harvey et al., 2011a, 2008; Maure et al., 2011).

## Ecological questions addressed in this thesis

In 2013, esteemed scientists collaborated in an effort that produced 100 fundamental ecological questions (Sutherland et al., 2013). Questions posited related to life history, population traits, trade-offs and landscape configuration interact and provided an inspiring foundation on which future research could be constructed. Understanding the basic biology, ecology and phylogeny of an organism is vitally important when placing it into a broader community-related framework. This fundamental point is often lost in the fields of evolution and ecology and hinders the ability of researchers to place their findings in a broader framework. Gelines are an abundant component of most terrestrial ecosystems in western Europe but have been virtually unstudied, and yet, as earlier lab-based studies by Harvey and colleagues (Harvey, 2008; Harvey et al., 2015a, 2019; Visser et al., 2016, 2014) have shown they make a good model for studying trade-offs between food resource and host availability.

To successfully reproduce, parasitoids need to forage for food for maintenance and hosts for reproduction. For wingless geline species like *G. agilis*, finding these resources is a major challenge. To simulate how these hyperparasitoids deal with spatially separated hosts and food in nature, I constructed arenas with mazes of varying complexity that were specifically designed to mimic the complex nature of natural habitats (dense swards of vegetation in meadows). Another profoundly important factor that affects species composition and life-history evolution is competition among different sympatric geline species that exhibit overlapping spatial and temporal niches. In this thesis, I examined various components of the life-history, development, biology and ecology of *G. agilis* and other geline parasitoids that are native to the Netherlands.

The aims of my research are to address hitherto unstudied aspects in *G. agilis* and congeneric hyperparasitoids. Initially, I intended to determine if maternal size affects offspring size in hosts of different size (and hence quality). Very few studies, however, have determined if maternal or paternal effects – such as body size or physiological status – can influence the development of their progeny. This aspect has not yet been examined in an idiobiont parasitoid. Second, given that the foraging behavior of *G. agilis* is likely to be affected by small-scale habitat heterogeneity and thus complexity, I then examined this process in microcosms in arenas with specially constructed mazes that create heterogeneity at very small scales. In predator-prey and host-parasitoid interactions, it is important for the persistence of both parties that the higher trophic level natural enemy does not overexploit its prey or host, because this may destabilize the dynamics of tightly interacting species and lead to unpredictable outcomes, such as local extinction (Begon et al., 1995; Wang and Wang, 2012). Instead, habitat complexity enables some prey or hosts to escape from predation or parasitism via the presence of structural, chemical, spatial or temporal refuges. I wanted to determine if this could be achieved in microcosm experiments. Finally, I studied communities of geline parasitoids in two field studies where habitat heterogeneity varied across a landscape. This was also aimed at determining if niches are partitioned at larger scales, thus reducing the impact of competition. This research contributes to our understanding of the bionomics of *G. agilis* and related species.

## Thesis outline

In **Chapter 2**, I investigated maternal and host factors on offspring by using body size as a proxy for fitness. To explore these effects, I manipulated maternal and host sizes by providing each mother with host cocoons of varying sizes daily. The resulting offspring sizes were then recorded and analyzed.

In **Chapter 3**, I examined the foraging behavior and potential trade-offs among fitness-related traits in *G. agilis* in small-scale microcosms (mazes) of variable complexity with manipulated host and food resources. Given that *G. agilis* has low fecundity and is wingless, their dispersal and host-location abilities are limited, even over small distances. To address this, I designed arenas with different levels of complexity, ranging from single arenas to tube-connected arenas. Host cocoons and food resources were placed in separate compartments, with some compartments directly accessible from a central area, while others required passage through intermediate compartments via the tube. The fate of cocoons offered in arenas were recorded as well as longevity and fecundity of *G. agilis*.

In **Chapter 4**, I conducted fieldwork in a local grassland-hedgerow habitat to compare communities of *Gelis* species and determine possible habitat preferences as well as susceptibility of hosts to parasitism and predation. Over two consecutive years, at 6-week intervals, cocoon clusters of *C. glomerata* were placed in these fields for one week and then retrieved back to lab, emerging species were recorded.

In **Chapter 5**, I extended the study described in **Chapter 4** to a large-scale field encompassing three distinct types of habitats: forest, production grassland, and conservation grassland, all bordered by a hedgerow. The research was carried out in the Maasheggen, a UNESCO biosphere reserve located in North Brabant, the Netherlands. This chapter focused on how management practices, habitat heterogeneity, and distance from the hedgerow might influence hyperparasitoid species composition, niche overlap, or competition, as well as the susceptibility of *C. glomerata* cocoons to predation. The same method as described in **Chapter 4** was used**.**

In **Chapter 6**, I summarized the main findings of my thesis and discussed future research directions related to hyperparasitoids.

# Chapter 2

## Maternal size does not influence provisioning ability for offspring development in a hyperparasitoid wasp

Xianhui Shi, Rieta Gols, Jetske G. de Boer, Jeffrey A. Harvey

Under review

## Abstract

The ‘nature versus nurture’ paradigm underpins our understanding of phenotypic variation in the expression of traits in animals. Whereas traits like growth and adult size are often heritable and are passed on from one generation to the next, these can be significantly affected by the quality and quantity of resources provided by one or both parents to their offspring. In many vertebrates, such as birds and mammals, parents raise their young until adult, providing food, shelter and protection. On the other hand, in insects, there is often little or no parental care, and the young are left to fend for themselves. Despite that, some insects can enhance the growth of their offspring. In parasitoid wasps, for example, mothers inject biochemical factors, including venoms, teratocytes, and virus-like particles into the host that increase host quality by regulating the nutritional milieu. However, it is not known whether maternal size is positively correlated with host regulation. Here, we evaluate maternal and host size-related effects on the development of an asexually reproducing (= female only) secondary idiobiont ectoparasitoid, *Gelis agilis* on pre-pupae in cocoons of its host, the primary parasitoid, *Cotesia glomerata*. Females *G. agilis* from two adult size classes, ‘small’ (mean 0.7 mg) or ‘large’ (mean 1.2 mg), were allowed to parasitize cocoons of differing size along a continuum from ~1.2 mg to ~4.0 mg and the body size and development time of their offspring were measured. In both body size classes of *G. agilis* mothers, there was a strong correlation between host size and offspring size. However, there was no effect of adult *G. agilis* size on this parameter: for a given host size, the size of *G. agilis* offspring did not differ between small and large mothers. Our results reveal that host quality is mostly pre-determined, irrespective of maternal size.

**Key words:** *Cotesia glomerata*, *Gelis agilis*, host quality, host size, venom

## Introduction

The ‘nature versus nurture’ paradigm underpins our understanding of phenotypic variation in the expression of traits in animals. Offspring phenotype in most organisms is mediated by genetically-based traits inherited from both parents, as well as through parental provisioning. Whereas in many organisms, morphological and developmental traits are heritable and are passed on from one generation to the next, these can be significantly affected by environmental factors as well as the quality and quantity of resources provided by one or both parents to their offspring. In vertebrates, such as mammals and birds, parents may provide food and shelter for their offspring until they attain maturity (Balshine, 2012; Clutton-Brock, 1991; Cockburn, 2006; Gross, 2005). Parental (usually maternal) provisioning is also sometimes observed in invertebrates, such as insects, and other arthropods. In some spiders, for example, mothers increase offspring survival and fitness through extended guarding and progressive provisioning of prey for up to several weeks during the gregarious phase (Gundermann et al., 1988; Ruch et al., 2014; Yip and Rayor, 2014). Another, more extreme mode of provisioning in some insects and spiders is matriphagy, whereby the mother sacrifices herself as food to her newly-emerged offspring (Kim et al., 2000; Suzuki et al., 2005; Toyama, 2001).

In many arthropods, newly emerged young are left to fend for themselves and thus there is no visually apparent parental care (Gilbert and Manica, 2010). However, even when they do not provide food or directly care for their offspring after eclosion, mothers can still invest in physiological provisioning by providing metabolic resources towards i.e. larger eggs that store more proteins or lipids and thus provide more nutrients for the developing embryo(s) (Beukeboom, 2018). Furthermore, the timing and location of maternal oviposition may profoundly influence offspring fitness. Optimization models predict that mothers should choose the most suitable environment or resources for offspring development, a process known as the ‘preference-performance’ or ‘mother knows best’ hypotheses

(Gripenberg et al., 2010). In herbivorous insects, for instance, the natal plant on which the mother lays her egg(s) may differ in terms of the amount and quality of resources available for her progeny (Knolhoff and Heckel, 2014). Consequently, our understanding or parental care and provisioning for offspring needs to incorporate a wide array of behavioural, physiological and ecological criteria.

One aspect of maternal physiological provisioning in insects that has thus far received less attention is how adult female size may affect progeny growth and development. If larger eggs are assumed to enhance offspring fitness by containing higher levels of nutrients, it follows that egg size may be positively correlated with adult female size. This has indeed been shown for some insects such as Diptera (Berrigan, 1991) but not in some beetles or butterflies (Church et al., 2019). Discrepancies in the body size-egg size correlation may occur because, instead of investing in larger eggs, larger females may have evolved to invest in producing more eggs, thus balancing selection between *per capita* offspring fitness and offspring number (Church et al., 2019; Maeta et al., 1998).

Parasitoid wasps are model systems to study how maternal provisioning may affect offspring development and fitness. Parasitoids are insects whose larvae develop outside (ectoparasitoids), or inside (endoparasitoids), the bodies of other arthropods, whereas the adults are free-living (Godfray, 1994). Unlike almost all other insects, parasitoids depend on finite resources contained in individual hosts that are often not much larger than the adult parasitoid for their development and reproduction (Godfray, 1994). Life history traits in parasitoids are thus strongly influenced by host size and hence quality (Godfray, 1994), and they have evolved mechanisms to maximize the utilization efficiency and allocation of limited resources to various fitness-related functions (e.g. growth and reproduction) (Harvey, 2005; Jervis et al., 2008).

Venoms injected by ectoparasitoids have an array of complex effects on host physiology and metabolism. Their primary function was long thought to be the induction of long-term host paralysis and developmental arrest, thus preventing displacement of the parasitoid larvae through host activity or molting (Asgari and Rivers, 2011). However, research with the pupal ectoparasitoid, *Nasonia vitripennis*, reveals that venom plays an important provisioning role by increasing host lipid levels and helping to release nutrients such as amino acids stored in host tissues that are otherwise inaccessible to the parasitoid larvae (Alvarado et al., 2020; Danneels et al., 2010; Moreau and Asgari, 2015; Mrinalini et al., 2015; Rivers and Denlinger, 1995). Nakamatsu et al. (2007) found that venom, in combination with a parasitoid-derived polydnavirus (PDV), that were injected into their caterpillar host by the gregarious endoparasitoid *Cotesia kariyai*, significantly increased levels of trehalose and lipids in the host hemolymph. Thus, parasitoid venoms can profoundly affect host nutritional quality.

Thus far, numerous studies have examined how host-related factors, such as size or stage at parasitism, affect adult parasitoid size (Godfray, 1994; Harvey, 2005; Jervis et al., 2023). In the vast majority of these studies, however, the body size of the parasitoid mother was completely ignored, and therefore it was not possible to determine if larger mothers are able to manipulate host quality more effectively than smaller mothers. Larger female parasitoids may produce larger eggs or inject higher concentrations of venom than smaller females, and this may enhance their ability to supplement or manipulate the quality and quantity of host resources. In this study, using ‘large’ and ‘small’ females, we compared the development of the solitary ecto-hyperparasitoid *Gelis agilis* Fabricius (Hymenoptera: Ichneumonidae, Cryptinae) in differently sized cocoons of its host, the primary parasitoid, *Cotesia glomerata* L. (Hymenoptera: Braconidae, Microgastrinae). As far as we know, this is the first study to examine the role of maternal size in an idiobiont parasitoid on offspring performance. *Gelis agilis* has distinctively large venom glands and injects venom into the host before oviposition. Previous studies with this association had shown that adult body mass in *G. agilis* was strongly correlated with host cocoon mass at oviposition (Harvey, 2008; Harvey et al., 2015a). However, in these studies the age and body size of the mothers was not determined. Here, both parameters were controlled. Large females weighed, on average, ~80% more than small females.

The main aim of this study is to determine if maternal size affects offspring size when developing across a range of host (cocoon) sizes at oviposition. Thus, can larger mothers enhance host quality through provisioning more effectively than smaller mothers? We hypothesize that larger mothers (1) lay larger eggs than small mothers, and (2) more efficiently regulate host quality by injecting higher amounts of venom and thereby benefiting offspring development. The intimate ways in which hymenopteran parasitoids manipulate their hosts through provisioning is discussed.

## Materials and Methods

### Insects

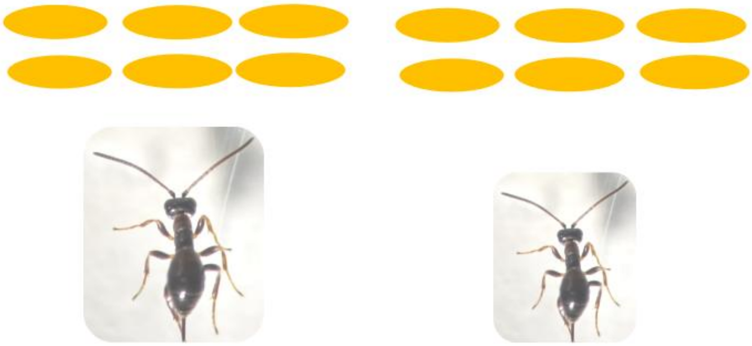
*Gelis agilis* is an asexually-reproducing ectoparasitoid (Harvey, 2008) that parasitizes the pre-pupae and pupae in cocoons of other primary parasitoids including *Cotesia glomerata*. Like most other species in the genus, *Gelis agilis* is wingless and mimics ants behaviorally, chemically and morphologically (Harvey et al., 2018; Malcicka et al., 2015). Adult female wasps destructively host-feed (Jervis and Kidd, 1986) to obtain proteins crucial for egg production before they can parasitize hosts (Harvey et al., 2012). *Gelis agilis* is an idiobiont parasitoid that paralyzes the host during the oviposition sequence, thus preventing its further development (Godfray, 1994; Harvey, 2005; Pennacchio and Strand, 2006). These hosts are ostensibly ‘static’ resources that ‘remains in place’. By contrast, koinobiont parasitoids attack hosts that continue feeding, growing, and molting throughout the course of parasitism (Godfray, 1994; Harvey, 2005; Pennacchio and Strand, 2006). Hosts parasitized by koinobionts represent potentially ‘dynamic’ resources that ‘do not remain in place’.

*Gelis agilis* was originally collected in fields in the vicinity of Wageningen from cocoons of *C. glomerata* and were maintained in a climate cabinet at 10 ± 2°C under a 16: 8 h L: D regime with a relative humidity of 50% at the Netherlands Institute of Ecology (NIOO-KNAW). For rearing and experimental work, all parasitoids were maintained at 22 ± 2°C under a 16: 8 h L: D regime with a relative humidity of 50%. Cocoons of the primary endoparasitoid *C. glomerata* provide suitable resources for *G. agilis* for host-feeding and progeny development. *C. glomerata* is a gregarious koinobiont endoparasitoid that attacks young larvae of the large cabbage white butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae) and other pierids. A single *C. glomerata* female lays up to 50 eggs per host (Harvey, 2000). Parasitized caterpillars feed and grow until the final instar when the mature parasitoid larvae perforate holes in the cuticle, emerge and spin cocoons. *Cotesia glomerata* were reared on first- or second-instar larvae of *P. brassicae*, which were obtained from the general insect rearing of the Laboratory of Entomology at Wageningen University, the Netherlands. The larvae of *C. glomerata* take approximately two weeks to develop inside their host depending on the temperature before they emerge from the host and spin cocoons.

Parasitized *P. brassicae* hosts were maintained in rearing cages (100 × 60 × 60 cm) and provided daily with fresh Brussels sprout plants (*Brassica oleracea*) as food until the parasitoid larvae egressed from their hosts and spun cocoons. Cocoon clusters < 24 h-old were collected and presented to adult *G. agilis* in cages (35 × 35 × 35 cm) for 3 days in which they were provided with honey and water. After that, the cocoons were collected and transferred to Petri dishes (ø = 12 cm) until eclosion of either *C. glomerata* or the hyperparasitoids. Newly emerged hyperparasitoids were collected and placed in cages as described above. Some wasps were used for the experiments or rearing. *Gelis agilis* females can produce eggs within 2-3 days following host-feeding.

## Experimental design

Previous research has shown that *G. agilis* females vary in size between 0.5 mg and 1.8 mg when developing on differently sized cocoons of *C. glomerata* (Harvey, 2008). Based on this, newly emerged females weighing 1.2 mg or more (representing 'large' females) and 0.7 mg or less (representing 'small' females) were used for the experiment. The aim was to ensure that the body size ratio between large and small females approached 2:1. Fifty small and fifty large *G. agilis* females were selected, weighed on a microbalance (Mettler-Toledo, accuracy 1 µg), and then placed individually into small Petri dishes (ø = 5.5 cm). Female *G. agilis* were initially narcoticized by exposing them to $CO_2$ for ~10 min. Honey droplets and water-saturated cotton wool were then supplied in the Petri dishes as an energy and water source, respectively. Size variation of host cocoons was generated by collecting multiple broods of *C. glomerata* cocoons and separating them carefully using forceps and a caecum. Six *C. glomerata* cocoons were placed in each Petri dish to allow *G. agilis* to host-feed. When the females were 'primed' with mature eggs (this takes approximately 48 h), these cocoons were removed and each small or large *G. agilis* was offered individual cocoons of *C. glomerata* < 24 h old over 6 consecutive days (*G. agilis* females were therefore all between 3 and 8 days old) (Fig. 2.1). Freshly separated cocoons for the parasitism assay were weighed on the microbalance. Oviposition assays were conducted between 0900 and 1700 and parasitism was visually determined. Parasitized cocoons were then placed individually in small vials labelled with mother's ID and cocoon mass. After 10 days, host cocoons were checked several times daily for the presence of newly emerged wasps. Adult fresh biomass (described above) and development time in days were recorded. The fate of cocoons that did not produce *G. agilis* or *C. glomerata* were recorded as 'dead cocoons. As control, the fate of forty unparasitized *C. glomerata* cocoons that differed in size (1.0-4.0 mg) was determined. To measure the egg length, fifteen small and large newly emerged *G. agilis* were selected and each was offered with three cocoons for host-feeding for 2 days. Then these *G. agilis* were dissected, the length and width of the eggs were measured using Leica M205 C microscopes to one μm.



Large *Gelis agilis* mother

Small *Gelis agilis* mother

**Figure 2.1.** The experimental design showing different sizes of *Cotesia glomerata* host cocoons offered to large and small *Gelis agilis* mothers.

### Statistical analysis

The distributions of the variables were checked before further analysis by calculating quantiles of each variable and then plotting them against theoretical quantiles (q-q plot) to confirm that the empirical quantiles sat within the borders of the suitable theoretical distribution. Wilcoxon Rank Sum Tests were used to analyze the not normally distributed data such as egg length, oviposition time, offspring size and offspring development time. Linear regression models were utilized to analyze the effect of maternal size on offspring size while controlling for cocoon size, and vice versa. Kaplan-Meier survival analysis was conducted to compare parasitism duration between small and large mothers. Pearson's Chi-squared test was done to test the cocoon fate relationship among different groups. All analyses were performed using R statistical software, version 4.2.2 (R Core Team, 2023).

## Results

*Gelis agilis* produces large, yolky (= anhydropic) eggs that are produced in only very small numbers that are stored beneath the ovaries. Most females carry only one or two eggs at a given time, although in some wasps there can be as many as four (Fig. 2.2 A, 2.2 B). There was no significant difference in mean egg length of eggs produced by small (695.4±31.4 μm) or large mothers (742.4±23.4 μm) ($W=117$, $P>0.05$) (Fig. 2.2 C), or between the oviposition time of large (30.0±2.6 min) and small (35.8±6.9 min) mothers ($W=1746$, $P>0.05$). Across all cocoon sizes at parasitism, there was no significant difference between the mean body sizes (±SE) of offspring from large (1.08±0.30 mg) or small (1.06±0.31 mg) mothers ($W=1634$, $P>0.05$). Fig. 2.3 shows that the regression lines of offspring sizes from small and large *G. agilis* mothers across a range of *C. glomerata* cocoon sizes almost exactly overlie one another. Offspring size increased linearly with host cocoon size at parasitism. Offspring adult body size showed strong linear relationships with host cocoon size in both large and small mothers ($R^2=0.56$, $P<0.001$ and $R^2=0.72$, $P<0.001$) respectively. There was significant difference in oviposition to adult development time in offspring from the small (17.1±0.2 d) and large (16.5±0.2 d) mothers ($W=1262$, $P<0.05$). There were significant differences in cocoon fate (*G. agilis* emergence, *C. glomerata* emergence, dead cocoon from which neither species emerged) from the three groups: cocoons presented to small or large mothers and unparasitized cocoons ($\chi^2=239.8$, df=4, $P<0.001$). The fate of the parasitized cocoons and control cocoons is shown in Fig. 2.4. However, approximately 85% of unparasitized (= control) cocoons produced adult *C. glomerata*, which strongly contrasts with cocoons presented to *G. agilis*.

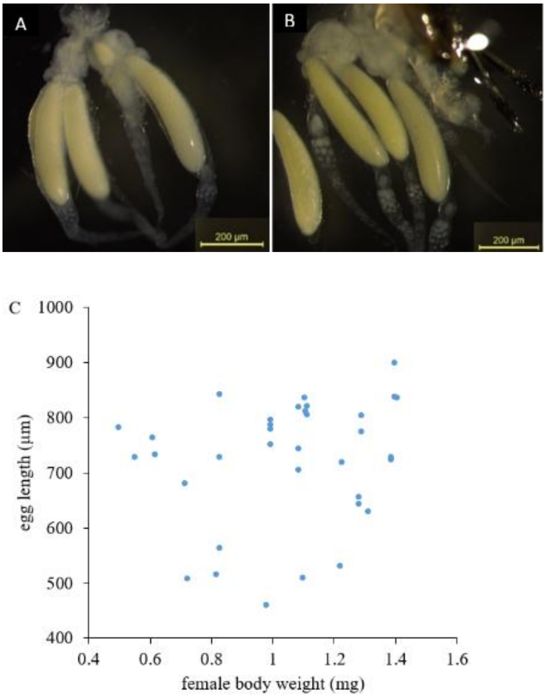


**Figure 2.2.** Dissection of *Gelis agilis* females showing 3 (A) and 4 (B) mature eggs from each *G. agilis* mother. (C) Scatterplot showed the egg length related to *G. agilis* mother weight ($P > 0.05$).

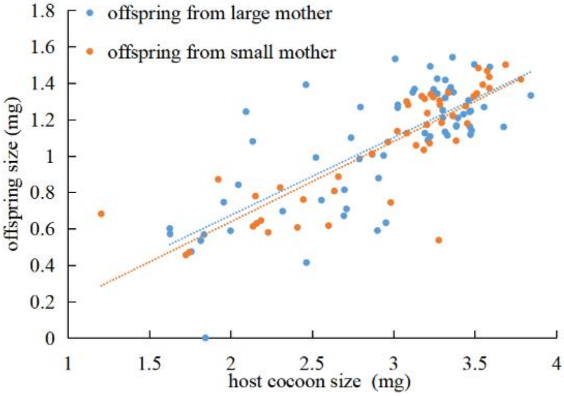


**Figure 2.3.** Relationship between *Gelis agilis* offspring size and cocoon size from large and small *G. agilis* mothers. The linear regression lines of offspring from large and small *G. agilis* mothers were $y = 0.42x - 0.18$, $R^2 = 0.56$ and $y = 0.44x - 0.24$, $R^2 = 0.72$.

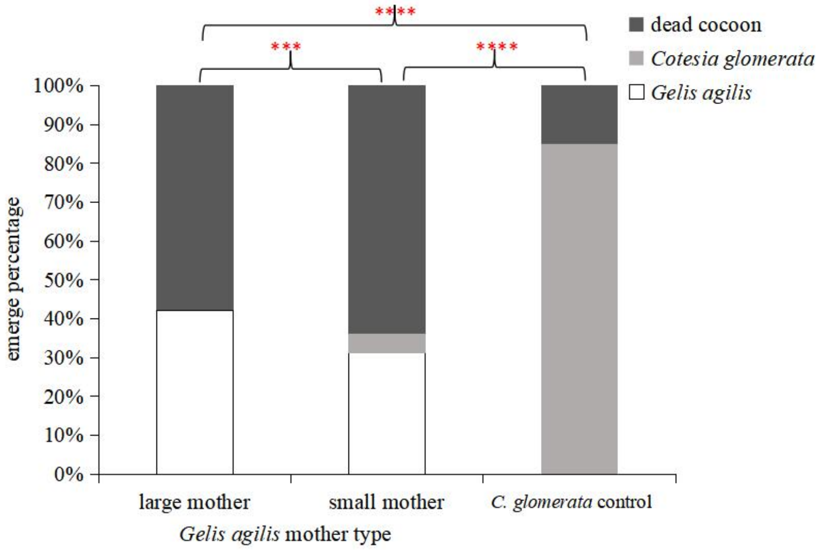


**Figure 2.4**. Fate of *Cotesia glomerata* cocoons visually stung by large and small *Gelis agilis* mothers compared with controls (cocoons not presented to *G. agilis* females). Red asterisks indicated significance.

## Discussion

The results of this study revealed that large and small mothers of *G. agilis* successfully parasitized a wide range of host (*C. glomerata*) cocoon sizes. The fate of host cocoons presented to large or small mothers differed significantly among the three groups. There was, however, a tendency for larger mothers to produce larger eggs, especially among the largest females, although overall this trend was marginally insignificant. Offspring development time differed with maternal size. Most importantly, the body size of the *G. agilis* mother had little or no effect on the body size of her offspring. Offspring size increased linearly across a range of host cocoon sizes at parasitism, and there was almost complete overlap in the offspring size of large and small mothers on host cocoons of similar sizes. This reveals that host size, a proxy of quality, is by far the prime determinant of offspring size in *G. agilis*, and that maternal physiological provisioning ability through egg size or venom injected into the host does not apparently increase with female size.

Oviposition-to-adult development time was, however, significantly longer in the offspring of small than large mothers.

*Gelis agilis*, like other ectoparasitoids, possesses a comparatively large venom gland, and injects venom into its host before oviposition. This leads to rapid host paralysis, and this is important for ectoparasitoids, because hosts that continue to grow or which remain active during parasitism can displace the parasitoid larvae feeding on them. Unlike koinobont endoparasitoids, which usually lay tiny, 'hydropic' eggs into their hosts, idiobiont ectoparasitoids like *G. agilis* generally lay large, 'anhydropic' eggs that are attached to the surface of the host body (Jervis and Kidd, 1986; Ralec, 1995). Hydropic eggs in parasitoids are unique in that they possess an 'extra-embryonic membrane' that enables the embryo to uptake proteins directly from host hemolymph (Grbić and Strand, 1998). During embryogenesis, hydropic eggs may therefore increase dramatically in size, and this strategy allows koinobionts to carry large complements of eggs that can be laid rapidly into their host (sometimes in less than a second!) and enables koinobiont endoparasitoid to exploit abundant or easily-accessible hosts (Price, 1972). Anhydropic eggs, on the other hand, must contain all of the necessary resources for embryological development, and this means that ectoparasitoids generally produce far smaller numbers of them. Moreover, they often take considerable time to lay, as they must be compressed considerably while passing through the lumen of the female ovipositor. *Gelis agilis*, for example, takes up to an hour, and sometimes even longer, to lay a single egg onto the host. Extended handling time might be costly if it reduces parasitism efficiency (Wu et al., 2011), so this is an area that merits further investigation.

It has been known for many years that ectoparasitoid idiobionts like *G. agilis* have evolved various ways of physiologically regulating host quality in ways that benefit offspring fitness. As molecular tools have improved rapidly over the past few decades, our understanding of the physiological function(s) of venom has improved dramatically. It is now possible to determine exactly how venom produced by some ectoparasitoid species affect their hosts. For instance, genomic sequencing of the jewel wasp, *N. vitripennis*, a pupal parasitoid of filth flies (Muscidae) has enabled the precise function of its venom to be elucidated (Danneels et al., 2010; Moreau and Asgari, 2015; Sim and Wheeler, 2016). For instance, *N. vitripennis* venom stimulates differential gene expression in the hemocytes in pupae of its host, *Musca domestica* (Qian et al., 2013). The altered genes are mostly related to a range of biological functions including nutrition, host immunity, stress responses and regulation of transcription/translation.

Body size and development time are important proxies of fitness in parasitoids and most other insects (Beukeboom, 2018; Chown and Gaston, 2010; Godfray, 1994; Harvey, 2005; Jervis et al., 2023, 2008). These parameters have been shown to affect demographic processes such as reproduction and longevity, as well as resource-finding and dispersal ability, extrinsic competition, mating success and defense (Charnov et al., 1981; Chown and Gaston, 2010; Gross, 1993; Ode et al., 2022). In parasitoids, numerous studies have focused on the importance of host-related factors such as age or stage of development at parasitism, size and nutritional status, on parasitoid development (Godfray, 1994; Harvey, 2005; Jervis et al., 2023). Parasitoids are under strong selection for optimal allocation and utilization of ostensibly finite host resources to different and potentially competing fitness functions such as reproduction and survival (Jervis et al., 2008). Because of this, host-parasitoid interactions are physiologically intimate and represent a 'nutritionally integrated system' (Brodeur and Boivin, 2004; Harvey and Malcicka, 2016; Pennacchio and Strand, 2006). We hypothesized that a potential extra benefit of size would be that larger *G. agilis* mothers may be better able to provision their offspring early in development than smaller mothers, perhaps by injecting larger quantities of venom. As with *N. vitripennis* and other parasitoids, *G. agilis* venom is likely to have multiple functions that include increasing the access of stored nutrients, such as lipids and amino acids, to their larvae. However, as our study has shown, small mothers and their progeny are clearly able to regulate and exploit a given amount of host resources as effectively as large mothers and their progeny.

In summary, our study shows that host size is by far the most important factor affecting offspring size in the solitary idiobiont hyperparasitoid, *Gelis agilis*, and that this parameter is not correlated with any maternal size-related provisioning or regulatory abilities. Despite that, more studies are needed to examine the role of parental size in parasitoids on offspring development from both a provisioning and genetic perspective. Both areas have thus far been little studied, and trends can only be elucidated when much more data is available. Several studies have reported that some traits in parasitoids are heritable (Kraaijeveld et al., 1998; Martinez et al., 2012; Olson and Andow, 2002; Wajnberg et al., 2012). A recent study by Rasekh et al. (2022) found some evidence for heritability for offspring size in a solitary koinobiont endoparasitoid based on maternal and paternal size. Future studies with parasitoids should aim to examine both the influence of heritability and provisioning (nature versus nurture) in the evolutioin of developmental strategies.

## Authorship statement

XS, RG and JAH conceived the ideas and designed the experiment. XS did the experiment and collected the data. JGdB and RG advised on data analysis. XS wrote the initial manuscript with inputs from all co-authors.

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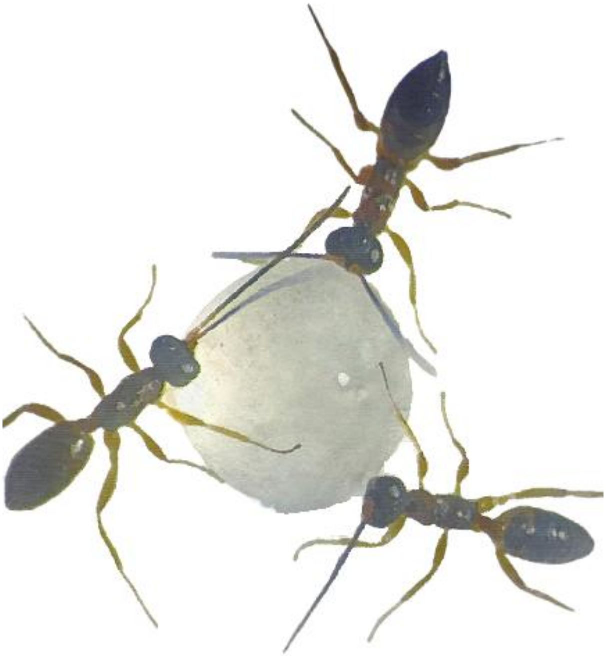

# Chapter 3

## Heterogeneous microcosm mazes affect reproduction and survival in a wingless hyperparasitoid wasp

Xianhui Shi, Rieta Gols, Jetske G. de Boer, Jitske Spee, Irla Hanum, Sergio A. Pelaez-Plazas, Jocelyn da Cruz Freitas, Jeffrey A. Harvey

Under review

## Abstract

In nature, resources necessary for reproduction and survival are often spatially distributed or scarce. Animals may need to utilize stored metabolic resources to locate critical resources. Parasitoid wasps exploit hosts for reproduction, but also rely on carbohydrates, obtained from floral nectar, for maintenance. In the field these resources are not necessarily located in close proximity to one another but may occur in spatially discrete patches, leading to potential trade-offs in fitness related functions such as fecundity and survival. Here, using plastic mazes of varying complexity in microcosms, we compared reproduction and longevity in an ectoparasitic hyperparasitoid wasp, *Gelis agilis*, utilizing cocoons of the primary parasitoid *Cotesia glomerata* as host. *Gelis agilis* is wingless and forages for hosts on or close to the ground in nature. Cocoons of *C. glomerata* were placed together or separated from food (honey and water) in two experiments. The fate of host cocoons (survival to emergence of the primary parasitoid, emergence of the hyperparasitoid, or precocious death) was also recorded. Irrespective of maze complexity, there was not a significant effect on hyperparasitoid fecundity, longevity or *C. glomerata* survival in single Petri dishes when cocoons and food were placed together. However, when food and hosts were spatially and temporally separated in 2 separate Petri dishes connected by a plastic tube, then trade-offs in *G. agilis* traits were observed, and survival of *C. glomerata* was affected. Our findings are discussed within the contexts of habitat heterogeneity and resource-consumer interactions, and refuge theory.

**Key words**: trade-off, adaptation, microcosm, hyperparasitoid, refuge, reproduction

## Introduction

In nature, the vast majority of animals, and especially insects, have fairly specialized diets, meaning that they preferentially feed on only one or a few types of resources that exhibit strong phylogenetic affinity (Loxdale et al., 2011; Loxdale and Harvey, 2016). The resources that are essential for reproduction and survival, such as specific plant taxa, are often embedded among unsuitable resources, meaning that they may be spatially distributed or locally scarce (Klironomos, 2002). Plant communities may consist of many species, each exhibiting its own physical and chemical profile, and this creates profound challenges for small insects to navigate in order to successfully find and locate a specific suitable plant (Aartsma et al., 2019; Barbosa et al., 2009; Gibb & Parr, 2010; Kostenko et al., 2015; Bezemer et al., 2010). Structural heterogeneity in plant communities has been found to affect the foraging efficiency and mate finding ability of different insect taxa (Bezemer et al., 2010; Finch & Collier, 2012; Halali et al., 2020; Vilela et al., 2020), although this often varies according to insect-related traits such as the presence or absence of wings and body size (Alyokhin and Sewell, 2003). For instance, insects foraging on the ground may have to navigate through densely packed vegetation such as grass swards in order to find vital resources, whereas flying insects are less constrained by plant-related heterogeneity. Nevertheless, when food resources are difficult to find, animals may need to utilize internally stored metabolic resources or to trade-off fitness related functions such as reproduction and survival (Roff, 1993; Roff and Fairbairn, 2007; Stearns, 1989).

Parasitoid wasps are model organisms for examining the role of ecological complexity on trade-offs in fitness functions in animals. Parasitoids are insects whose larvae develop on or inside the bodies of other arthropods (Godfray, 1994; Harvey, 2005) whereas the adults are free-living and obtain nutrition in the form of carbohydrates primarily from exogenous plant sources such as nectar (Heimpel, 2019; Jervis et al., 2008, 1993; Wackers and Steppuhn, 2003). Importantly, host resources utilized for reproduction and floral resources utilized for adult maintenance may be spatially separated and occur in discrete patches in nature. This means that female parasitoids need to find mates, hosts and flowers in order to reproduce and achieve optimal fitness. Insect predators are usually much less constrained in finding resources, because they are typically generalists than can maintain themselves on a wide range of prey. Under extreme conditions, such as starvation, female parasitoids have been shown to ignore host-containing patches and to prioritize foraging for nectar or other carbohydrate sources until their hunger is satiated (Desouhant et al., 2005; Lewis et al., 1998; Siekmann et al., 2004).

Another major factor constraining parasitoid host-finding ability is that, owing to longstanding co-evolutionary selection pressures imposed by a suite of behavioral, ecological and physiological host traits, most parasitoids are fairly specialized and thus attack only one or a few closely related species of hosts in nature (Heimpel et al., 2021). This is especially true for many endoparasitoids that lay their eggs inside of the bodies of their hosts. Numerous studies have shown that endoparasitoids from different lineages have evolved a range of strategies to avoid or abrogate internal host immune defenses, but this has inevitably led to a reduction in the host range of many parasitoid species because host immune defenses are phylogenetically conserved (Pennacchio and Strand, 2006; Strand and Pech, 1995). Despite not having to deal with the internal metabolic defenses of their hosts, the host ranges of many ectoparasitoids, which attack non-growing or paralyzed hosts, is also often small. This is due to factors other than host immunity, such as the susceptibility of the host to parasitoid venom (Asgari and Rivers, 2011) or competition with other parasitoids, leading to refined host niche and stage specialization (Price, 1972).

Parasitoids have evolved highly refined mechanisms for locating hosts in the field, and these are often based around responses to specific chemical, tactile and visual stimuli (Fischer et al., 2001; Heimpel and Casas, 2008). Plants damaged by insect herbivores release volatiles that serve as long-range attractants for parasitoids (Turlings and Erb, 2018; Vet and Dicke, 1992). Other parasitoids may detect hosts using chemical cues released by their hosts, such as kairomones from frass and sex pheromones (Fatouros et al., 2008). Lab-based studies with parasitoids have shown that structural complexity can influence foraging efficiency for hosts. For instance, host-finding ability in the solitary endoparasitoid, *Diadegma semiclausum*, was hindered when non-host foodplants were placed into cages along with foodplants containing caterpillars of their host, *Plutella xylostella* (Bukovinszky et al., 2007; Gols et al., 2005). These studies show that even minor structural or chemical impediments can inhibit parasitoid foraging ability, potentially leading to cohorts of hosts that escape parasitism.

At the population level, ecological complexity is important because it determines how susceptible a host population is to its various parasitoid guilds. For example, habitat heterogeneity creates physical, structural and chemical refuges for hosts than reduce the chance of them being overexploited by their parasitoids (Andow & Prokrym, 1990; Begon et al., 1995; Fernandes et al., 2022; Hochberg & Holt, 1995; Holt & Hassell, 1993). On the other hand, if the structural heterogeneity of a habitat become too complex, this might lead to local extinction of parasitoids that are unable to find their hosts (Cronin and Reeve, 2005; Vos et al., 2001). Refuge theory has long underpinned our understanding of multitrophic interactions and community structure (Berryman et al., 2006). However, there are still important knowledge gaps in our understanding of how refuges function across different spatial scales. Lab-based experiments can help by enabling the size, structure and complexity of habitats at very small scales to be controlled and manipulated. This can be done using specially constructed plastic mazes that are placed into containers, such as boxes or Petri dishes, where the foraging and mating behavior of small organisms, like insects, can be closely monitored (Gilad et al., 2023; Mirwan and Kevan, 2015; Stringer et al., 2020).

The ichneumonid subfamily Cryptinae contains species that are fairly specialized ectophagous hyperparasitoids of (pre)pupae of primary parasitoids in cocoons (Schwarz and Shaw, 1998). The genus *Gelis* is one of the most important genera in the Cryptinae, with ~270 species described worldwide and up to 60 in western Europe (Schwarz and Shaw, 2000). Most species are wingless, and are commonly found in habitats such as forests, hedgerows and meadows (Harvey et al., 2014; Heinen and Harvey, 2019). *Gelis* species exhibit traits highly indicative of k-selection (Parry, 1981; Pianka, 1970): comparatively long lifespans which are traded-off against low daily and lifetime fecundity. Parasitoids in this genus also habitually 'host-feed' (Briggs et al., 1995; Jervis and Kidd, 1986) by using the ovipositor to inflict wounds in some hosts that seep hemolymph which is sipped by females in order to obtain exogenous proteins that are primarily (although not exclusively in some species) allocated to the production of large, anhydropic eggs (Flanders, 1942; Ralec, 1995). Like other parasitoids, *Gelis* species also rely on carbohydrates obtained from floral nectar or honeydew excreted by aphids to optimize longevity and hence fitness (Harvey et al., 2012; Van Neerbos et al., 2020).

Here, in a microcosm lab experiment, we examine the effects of structural complexity on reproduction and survival in the asexually reproducing hyperparasitoid wasp, *Gelis agilis* Fabricius (Hymenoptera: Ichneumonidae, Cryptinae). *Gelis agilis* is a specialist hyperparasitoid of primary parasitoid cocoons, including species such as *Cotesia glomerata* L. (Hymenoptera: Braconidae), in the braconid subfamily Microgastrinae. Reproduction and longevity in *G. agilis* and the fate of host cocoons were compared by rearing adult females in individual or connected Petri dishes containing plastic mazes of differing complexity and where host (*C. glomerata*) cocoon and food (honey + water) access were spatially and temporally manipulated. The main aims of the experiment were to determine if trade-offs in life-history and reproductive traits in *G. agilis* can be produced in microcosms, and if increased complexity reduces parasitism success, thus creating a refuge for *C. glomerata*. We hypothesize that mazes can be used in elucidating trade-offs in life-history characters in *G. agilis* and other insects, and that they can aid as simple tools in explaining the principles of ecological theories such as the concept of refuges.

## Materials and Methods

### *Insects*

All *G. agilis* wasps were maintained in a climate compartment at 22±2°C, under a 16:8 h light-dark regime with 50% relative humidity. The *G. agilis* culture originated from cocoons of *C. glomerata* that were placed into two field locations in Gelderland Province, The Netherlands. *Gelis agilis* was thereafter reared on this host. *Cotesia glomerata* cocoons were obtained from the general insect rearing facility at the Department of Entomology, Wageningen University, The Netherlands. Caterpillars (of *P. brassicae*) parasitized by *C. glomerata* were maintained in rearing cages (dimensions: 100 × 60 × 60 cm), in a greenhouse at 22 ± 2°C, under a 16:8 h light-dark regime with 50% relative humidity, where they were provided fresh Brussels sprouts plants (*Brassica oleracea*) daily for nourishment until the parasitoid larvae emerged and spun cocoons. The cocoons of *C. glomerata* were used in the experiments.

### *Experimental Design (general protocol)*

Reproduction and longevity of *G. agilis* were compared in arenas that differed in complexity. Each arena was constructed from a large Petri dish (ø = 13.5 cm) that was compartmentalized using a custom-made 3D-printed inlay made from polylactic acid (PLA) filament. In this way, foraging arenas were created with 2, 4, 8 or 16 compartments and a central access compartment (Fig. 3.1). Small holes in the central circular compartment allowed *G. agilis* females access to each compartment. Petri dishes without an inlay – thus consisting of a single compartment – were used as a control. Honey drops and a ball of cotton wool with absorbed water were also provided in the arena as food and a source of liquid. Provisioning of cocoons, and honey plus water was varied in the different experiments (see below). Four pre-weighed *C. glomerata* cocoons (> 1.00 mg) were evenly distributed over the arena compartments. Cocoons were less than three days old since their formation to ensure they were of optimal suitability for *G. agilis* development.

Newly emerged (< 24h) *G. agilis* were kept individually in small Petri dishes (ø = 5.5 cm) with 3 *C. glomerata* cocoons and allowed to host-feed for 2 days (*G. agilis* is anautogenous and can only lay eggs after host-feeding). Individual females thereafter were released into the central part of the experimental arenas with 4 *C. glomerata* cocoons each round and allowed to forage for host cocoons, honey and water. Cocoons were replaced after 48 h as *G. agilis* can typically parasitize no more than 2 or 3 cocoons of *C. glomerata* over 24 h (Harvey, 2008). After 2 days, the 4 cocoons were collected and placed in labeled vials. Experimental arenas and all collected cocoons were maintained in a climate compartment at 22 ± 2°C, under a 16:8 h light-dark regime with 50% relative humidity. The vials were checked daily to record the emergence of *C. glomerata* or *G. agilis.* If neither species emerged from the cocoons after a month, they were noted as ‘dead’. To check for the viability of *C. glomerata* cocoons, emergence of the primary parasitoid was recorded in an additional cohort of 40 cocoons that were placed in vials and were maintained under the same conditions as the cocoons in the arenas.

To explore whether compartmental complexity would influence longevity and or reproduction of *G. agilis* females, and how this could be affected by food or host cocoon availability, two experiments were designed.

*Experiment 1. Alternating provisioning of cocoons or honey plus water*

We first compared lifetime reproduction of *G. agilis* in arenas with 1, 2, 4, 8 or 16 compartments. Four fresh cocoons were placed in each arena at locations 1-4 as indicated in Fig. 3.1. A ball of cotton with absorbed water, and one separate drop of honey, were placed in the same compartment as cocoon nr. 1. In a pilot study, we found that provisioning of cocoons and food did not affect the foraging efficiency of *G. agilis* (data not shown). Therefore, provisioning of cocoons and honey plus water were alternated every 48h. Thus, hyperparasitoids were never provided with cocoons and food at the same time. This routine was repeated in each Petri dish until the female died. Each arena was replicated with 10 *G. agilis* females.

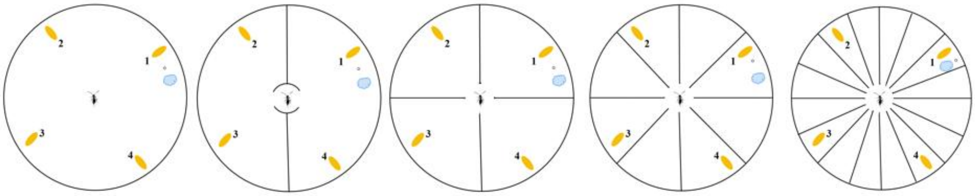

**Figure 3.1**: Experimental arenas with increasing numbers of compartments (1 to 16). The yellow ovals with the numbers represent the four *C. glomerata* cocoons, the blue circle in each arena represents cotton wool with water and the grey dot represents a droplet of honey. Provisioning of cocoons and honey plus water were alternated every 48 h. Females of *G. agilis* were released in the center of the arenas (one female per arena, n = 10 for each arena type). Hosts, honey and water were replaced until the hyperparasitoid died. Collected cocoons were monitored for parasitoid emergence.

*Experiment 2. Two dishes connected by a tube*

In this experiment (Experiment 2.1), we increased complexity even more using 2 Petri-dish arenas that were connected with a 20-cm tube. Arenas on either side of the tube had the same number of compartments. Cocoons were provided in one dish and honey plus water in the other (Fig. 3.2). This set-up required *G. agilis* to travel through the tube. Arenas were constructed with barriers creating compartments as described above, and we used three degrees of habitat complexity in this experiment: 1, 4 or 9 compartments per dish. The 9-compartment dishes were constructed using the 16-compartment inlays, but here 7 of the compartments were closed off completely (Fig. 3.2, grey compartments), 4 compartments could be entered through the center release compartment and 4 could only be accessed through holes in the wall of other compartments (Fig. 3.2, blue lines). Cocoons were placed in locations in such a way that 2 of the 4 cocoons could be accessed directly through the central compartment, whereas the other two could only be reached by going through 2 other empty compartments first (Fig. 3.2). Food was placed in a compartment that also only could be accessed by going through 2 empty compartments. Female *G. agilis* were released through holes in the middle of the 20 cm tube connecting the dishes. When cocoons and food were replaced every 2 days, the *G. agilis* female was collected and temporary placed into a small Petri dish and returned back into the arena through the hole in the tube. Replacement of cocoons continued until the female died. There were 30 replicates per arena type.

In addition to this experiment, three other experiments were conducted in which only water was provided in one of the two dishes (Experiment 2.2), only cocoons were provided in one of the two dishes (Experiment 2.3), or nothing was provided (Experiment 2.3). All these experiments continued until the female died. Each experiment was with n=30 per arena type.

### *Statistical analysis*

The fate of cocoons was classified as hyperparasitized when *G. agilis* emerged, *C. glomerata* when the primary parasitoid emerged and dead when nothing emerged within a month. Based on these data, daily fecundity and daily cocoon mortality were calculated per dish. Daily fecundity was calculated as the total number of hyperparasitoids produced by a female divided by the number of days she lived in the experiment. Similarly, daily cocoon mortality was calculated as the total number of dead cocoons divided by female longevity. Both variables, as well as female longevity, were analyzed, using a general linear model with arena complexity as explanatory variable. Levene's tests were conducted to check assumptions of equal variance among groups and data were log transformed when the test was significant. Post hoc comparisons were performed using Tukey's multiple comparison tests when the effect of arena complexity was significant.

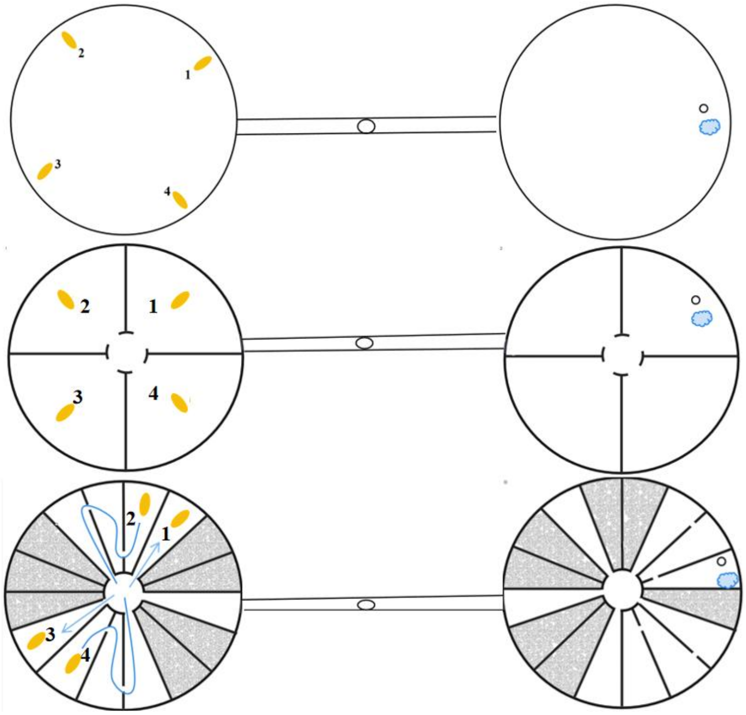

**Figure 3.2**: Arena set ups of the tube experiment 2.1 with two dishes with 1, 4, or 9 compartments connected by a 20 cm plastic tube. Four cocoons of *C. glomerata* were placed in compartments numbered 1 to 4 in one of the two dishes and water (absorbed into cotton) plus honey in one compartment were placed in the other dish. In the 9-compartment dishes, 7 of the compartments were closed off completely (grey compartments), 4 compartments could be entered through the center release compartment and 4 could only be accessed through holes in the wall of other compartments (blue lines). Cocoons were placed in locations in such a way that 2 of the 4 cocoons could be accessed directly through the central compartment

(compartments 1 and 3), whereas the other two could only be reached by going through 2 empty compartments first (compartments 2 and 4). Honey (blue circle) and water (black dot) were placed in a compartment that also only could be accessed by going through 2 empty compartments. *Gelis agilis* females were released through a single central hole in the tube. Cocoons, water and honey were refreshed every 2 days until death of the female in each arena.

In the tube experiments, longevity was greatly reduced in the experiments in which females were released in arenas with only cocoons or nothing at all. To test whether these experimental designs had a significant effect on longevity data were subjected to a single analysis with treatment (cocoons only or nothing) and arena complexity as explanatory factors. All statistics were conducted using R (version 4.2.3) (R Core Team, 2023).

## Results

### *Experiment 1. Alternating provisioning of cocoons or honey plus water*

Although daily fecundity appeared to be higher in the 2-compartment arena (Fig. 3.3A) and longevity (Fig. 3.4A) in the 4-compartment arena, the overall effects of arena complexity on daily fecundity and longevity were not statistically significant across all treatments ($F_{4,45} = 2.45$, $P = 0.059$, $F_{4,45} = 2.18$, $P = 0.086$, respectively). The number of 'dead' cocoons per day did not differ among arena types ($F_{4,45} = 1.32$, $P = 0.27$) (Fig. 3.5A). On average, 0.42 ± 0.02 cocoons were found to be dead per day per female. Of the 2224 cocoons offered in this experiment, ignoring arena type, 46% did not produce parasitoids. The mortality of cocoons in the control without hyperparasitoid exposure was only 15 % (this is equivalent to 0.30 cocoon per day). Thus, some of the cocoons were clearly killed by host-feeding or through the unsuccessful development of the hyperparasitoid.

### *Experiment 2. Two dishes connected by a tube*

When female *G. agilis* foraged for hosts and food offered in dishes separated by a tube, daily offspring production decreased with increasing complexity of the arenas (Fig. 3.3B). Significantly fewer offspring were produced in the 9-compartment set-up than in the other two. Cocoon mortality, on the other hand, decreased with increasing arena complexity (Fig. 3.5B), especially in the 9-compartent arena. Longevity of the females was unaffected by arena type (Fig. 3.4B).

Importantly, longevity was overall much shorter in the two-dish experiments than in the single dish experiments (48 days [grand mean] in experiment 1-Fig. 3.4A and 26 days in experiment 2.1-Fig. 3.4B). Moreover, when females were only provided with honey plus water in the two-dish set-up, arena complexity further reduced longevity ($F_{2, 27} = 8.8$, $P = 0.001$) but only in the 9-compartment arena (Fig. 3.4C). There was no significance in longevity between the same complexity arena set up when offered both cocoons and honey/water vs. only honey/water ($P>0.05$). Longevity of females in the 9-compartment set-up was almost halved compared to the single and four-compartment arenas.

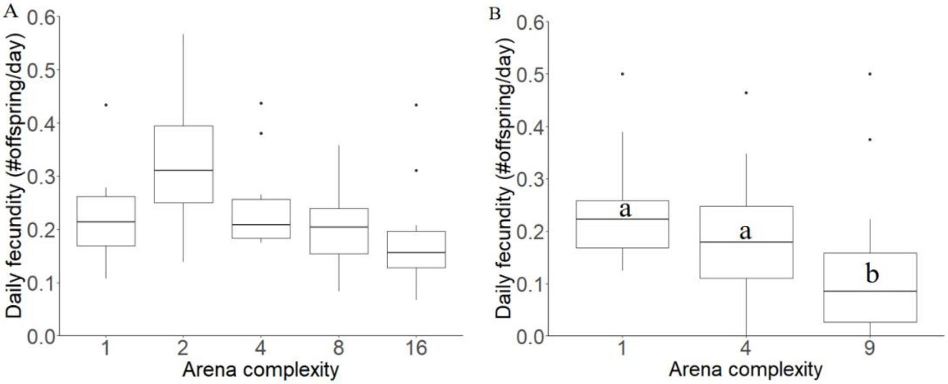

**Figure 3.3.** Daily fecundity of experiment 1 (A) (Alternating provisioning of cocoons or food) and 2.1 (B) (Tube arenas: one side cocoons vs. one side food). Different letters indicated significance.

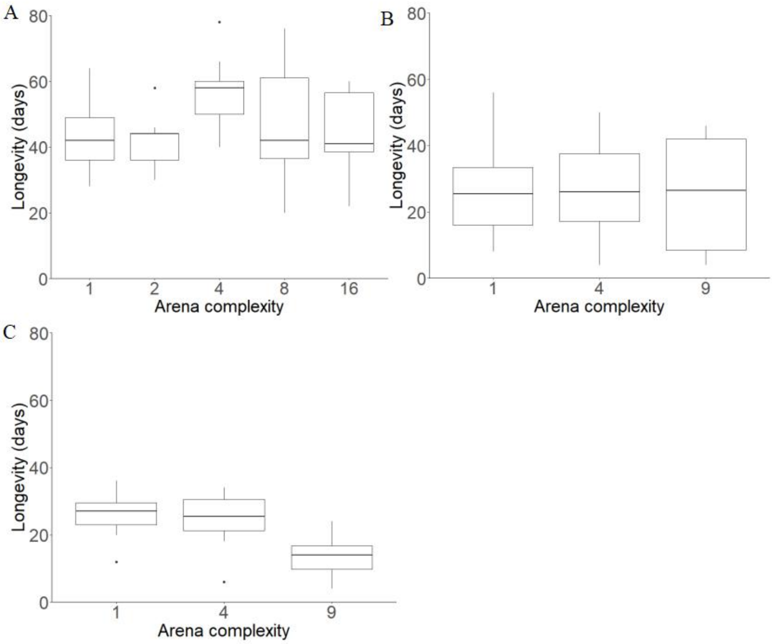

**Figure 3.4.** Female *Gelis agilis* longevity of experiment 1 (A) (Alternating provisioning of cocoons or food), 2.1 (B) (Tube arenas: one side cocoons vs. one side food), and 2.2 (C) (Tube arenas: one side nothing vs. one side food).

In arenas with only cocoons or nothing at all, longevity (range: 1 – 3 days) was greatly reduced compared to the two other tube experiments (range: 4 – 56 days). Still, providing females with cocoons significantly extended their life expectancy by 0.4 days ($F_{1, 56}$ = 9.95, P = 0.002) and also complexity of the arena decreased life expectancy ($F_{2, 56}$ = 5.04, P = 0.0096). Females in the 9-compartment lived 0.4 days less than in the single and 4-compartment arena set-ups (Tukey tests: 4-16, P = 0.03; 1-16, P = 0.015; 1-4, P = 0.94). The interaction between these two explanatory variables was not significant ($F_{2, 56}$ = 0.20, P = 0.81) and, therefore, this term was removed from the analyses.

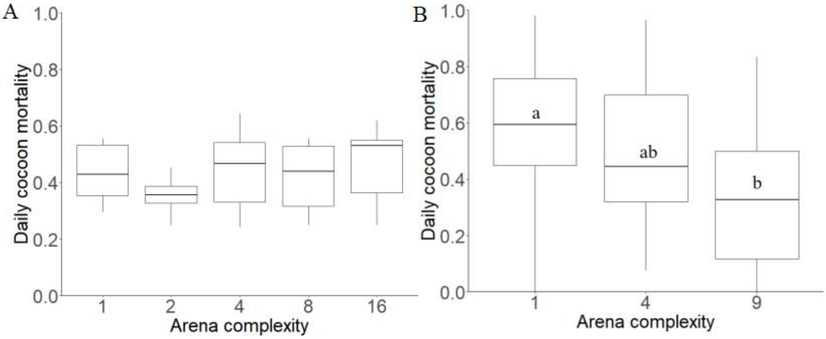

**Figure 3.5.** Daily cocoon mortality of experiment 1 (A) (Alternating provisioning of cocoons or food) and 2.1 (B) (Tube arenas: one side cocoons vs. one side food). Different letters indicated significance.

## Discussion

The results of this study show that *G. agilis* is well adapted at locating and exploiting both hosts (cocoons of *C. glomerata*) and food (honey and water) that were placed in microcosm mazes of variable complexity. Irrespective of how many compartments the mazes contained, when arenas were restricted to single Petri dish and both hosts and food were available at the same time, there was not a significant effect of maze structure on longevity or fecundity in *G. agilis*, or, conversely, on the survival of its host, *C. glomerata*. However, when the complexity of the microcosm mazes was increased by connecting two Petri dishes through a plastic tube and manipulating temporal and spatial food and host availability, reproduction, longevity and host-finding ability of *G. agilis* were negatively affected. Thus, by using mazes, we were able to create sufficient complexity at even very small scales to generate trade-offs in fitness functions in *G. agilis* and to at least create partial refuge conditions for *C. glomerata*.

The high efficiency of *G. agilis* to overcome structural complexity was initially surprising to us, as we had anticipated that even a small, incremental increase in complexity of the arenas would hinder their foraging success. However, we clearly underestimated the host-finding ability of this hyperparasitoid, and the fact that hosts and food were in the same compartment virtually eliminated any physiological costs of foraging. In nature, *Gelis agilis* and other wingless gelines generally occur in habitats where the structural heterogeneity of the vegetation at ground level can be very high, especially in meadows characterized by the presence of dense swards of grasses and forbs. Under these conditions, suitable hosts may be very scarce and sources of nutrition, such as nectar, require the wasps to leave the ground to feed or oviposit, which becomes more complicated when hosts and food are located on different individual plants. Despite that, our results and those obtained from field experiments (Harvey et al., 2014; Heinen and Harvey, 2019) clearly demonstrate that multiple species of geline hyperparasitoids have evolved highly efficient strategies of finding hosts and thriving in meadow and forest edge habitats.

To locate and successfully exploit hosts in the field, primary parasitoids have evolved enhanced visual, tactile and olfactory acuity (Randlkofer et al., 2010; Wäschke et al., 2013). Much attention has focused on the role of herbivore-induced-plant volatiles as long-range attractants (McCormick et al., 2012; Turlings and Erb, 2018; Vet and Dicke, 1992), but at close range parasitoids forage visually or are highly sensitive to host movements or vibrations (Fischer et al., 2001). For hyperparasitoids, which attack hosts in the third trophic level that are presumably less common, and thus more difficult to find than herbivores in the second, host-finding strategies have become increasingly refined (Aartsma et al., 2019; Cusumano et al., 2020, 2019; Poelman et al., 2022; Poelman et al., 2012). The effectiveness of these mechanisms is enhanced through parasitoid learning, where odors of hosts and other host-related cues enable females to more easily to detect hosts or food and to memorize crucial aspects of the local habitat (Giunti et al., 2015; Haverkamp & Smid, 2020; Segura et al., 2007; Vet & Groenewold, 1990).

For wingless hyperparasitoids with a limited host range, and that occur primarily on or close to the ground, learning and memory may also play a vital role in optimal foraging. In our experiment, they may have played an important role in the ability of *G. agilis* to effectively exploit hosts in single Petri dishes with mazes of varying complexity. Only when complexity was increased by adding a second Petri dish and by spatially and temporally manipulating food and hosts was reproduction and survival partially affected, leading to potential trade-offs in these fitness functions. Bear in mind that *G. agilis* do not only require hosts as resources for offspring development, but also that many hosts are killed through host-feeding which is a necessary pre-requisite for acquiring sufficient proteins for the production of yolky 'anhydropic' eggs (Jervis and Kidd, 1986). In some parasitoids, host-feeding can account for over half of host mortality in the field (Kapranas and Luck, 2008), which may explain why cocoon mortality shown here in all experimental treatments was generally higher than parasitism and survival of *C. glomerata*.

The survival of *C. glomerata* to eclosion (or, conversely, their ability to evade parasitism by *G. agilis*) was only significantly increased in the most complex maze setup involving 2 connected Petri dishes with 9-chamber mazes with only indirect access to hosts. Habitat heterogeneity plays a crucial role not only in influencing the stability of both the dynamics of predator-prey and host-parasitoid interactions, but in determining the risk of over- or under-exploitation of resources by consumers. Underpinning the effects of habitat complexity on the success of natural enemies to find and exploit their prey, or for prey to escape from their natural enemies, is refuge theory (Berryman et al., 2006). A refuge is defined as a space within a habitat where an organism gains protection from its natural enemies and where it is either inaccessible or very difficult to find, thus providing a form of enemy-free space (Jeffries and Lawton, 1984; Sih, 1987). Refuges can be facilitated by physical, temporal or chemical characteristics of a habitat (Gols et al., 2005). Hypothetically, when a habitat becomes so structurally or chemically complex that prey or hosts are able to fully escape from their natural enemies, this can lead to localized extinction higher up the food chain and the over-exploitation of resources lower down the food chain (Ruxton, 1995; Sih, 1987). Alternatively, if a habitat is so simple that prey or hosts cannot find any types of refuges, and are thus decimated by their natural enemies, then this can lead to ‘boom-bust’ dynamics that may also precede localized extinction (Ruxton, 1995; Sih, 1987).

The importance of refuges in the dynamics of resource-consumer interactions by generating stable-limit cycles has long interested both theoretical and empirical ecologists (Albrecht et al., 1973; Erbach et al., 2013; Hassell, 1978; Hassell and May, 1974; Lotka, 1920; Nicholson and Bailey, 1935; Sih, 1987; Volterra, 1926). The ideal situation to dampen trophic oscillations in resource-consumer interactions and to enhance long-term persistence is that some prey and hosts survive and produce offspring for future generations, whereas their natural enemies are also able to find sufficient prey or hosts to do the same (Begon et al., 1995). As we have shown here, this can be demonstrated at even very small scales (i.e. microcosms), using specially constructed mazes that are complex enough to limit the ability of one organism to overexploit another. We can speculate that it may be possible to create mazes that are so structurally complex that the other end of the continuum (i.e. complete enemy-free space) is achieved. Mazes are therefore useful tools in exploring longstanding concepts in ecology, particularly with smaller organisms like insects.

## Authorship statement

XS, JS, RG and JAH conceived the ideas and designed the experiment. XS, JS, IH, SAPP and JdCF did the experiment and collected the data. JGdB and RG advised on data analysis. XS wrote the initial manuscript with inputs from all co-authors.

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# Chapter 4

## Seasonal differences in rates of predation and hyperparasitism of parasitoid cocoons in meadows and adjacent hedgerows

Xianhui Shi, Rieta Gols, Jetske G. de Boer, Qiang Yang, Jeffrey A. Harvey

Submitted

## Abstract

Small habitat patches are often neglected for biodiversity conservation although they may contain high species diversity and intricate trophic interaction networks. Hedgerows bordering fields are important in that they function as dispersal corridors and provide important microclimatic and niche-related refuges. Here, we study hyperparasitoids and arthropod predators that are integral components in multitrophic plant-insect communities. We compared rates of hyperparasitism and predation of *Cotesia glomerata* cocoons that were placed in clusters under wire and plastic netting on the ground surface under hedgerows and in adjacent meadows over two consecutive years. Cocoons were collected after one week and reared out to see what emerged from them. Missing cocoons were assumed to have been taken by invertebrate predators. Hyperparasitism levels varied with habitat and season and was higher under hedgerows than in meadows. The species complex of hyperparasitoids also changed over time, although most were closely related members of the same subfamily and genus (Cryptinae, *Gelis* spp.). Predation also varied over time and, in contrast with hyperparasitism, was typically higher in the meadow than under the hedgerow. Our results show that multiple *Gelis* species co-exist at small spatial scales by partitioning resources (host cocoons) temporally. Moreover, differences in rates of hyperparasitism and predation in closely located habitats reveals a partial division in habitat occupancy by these feeding guilds. Our results highlight the importance of environmental factors such as habitat structure in the study of multitrophic interactions involving hyperparasitoids and predators sharing common prey or hosts.

**Key words**: hyperparasitoid, hedge, grassland, habitat structure, microclimate, niche, refuge

## Introduction

Agricultural intensification across much of the biosphere has had one of the most significantly negative effects on biodiversity by eliminating diverse natural habitats consisting of a wide array of plant species and replacing them with crop monocultures that receive frequent applications of fertilizer and pesticides (Emmerson et al., 2016; Firbank et al., 2008; Tscharntke et al., 2012a, 2005). A study in German nature reserves bordering agricultural fields showed that arthropod biomass decreased by up to 76% over only 26 years (Hallmann et al., 2017). Another study found massive declines in grassland and forest insects in Germany over less than 20 years, and the decline was consistent across trophic levels but mainly affected rare species (Seibold et al., 2019). The negative effects of habitat loss may be reduced when agricultural fields are bordered by strips of vegetation consisting of a diverse mixture of wild, flowering plants or by hedgerows of woody shrubs (Litza and Diekmann, 2020). Hedgerows offer animals a wide variety of microhabitats, and also serve as nesting and hibernation sites, facilitate dispersal across landscapes, and provide food resources for a diversity of species, especially insects and other arthropods (Besnard and Secondi, 2014; Davies and Pullin, 2007; Lecq et al., 2017; Staley et al., 2012). They also help to buffer against climatic extremes, such as extreme heat, cold, and floods (Wolton et al., 2014). Hedgerows harbor a variety of invertebrate taxa, including Araneae (spiders), Coleoptera (beetles), Diptera (true flies), Hemiptera (true bugs), Lepidoptera (butterflies and moths) and Hymenoptera (bees, wasps, ants) (Besnard and Secondi, 2014; Davies and Pullin, 2007; Griffiths et al., 2007; Lecq et al., 2017). Moreover, hedgerows have been shown to increase presence and activity of arthropod predators by reducing habitat disturbance and edge effects (Ferrante et al., 2024; Garratt et al., 2017; Maudsley et al., 2002).

As another type of habitat, grass-dominated meadows are often utilized for multiple purposes such as for grazing livestock *in situ* or as feed after harvesting, cultural and recreational needs, conservation of specialized grassland-inhabiting species and as carbon sinks to mitigate greenhouse gas emissions (Boval and Dixon, 2012). In meadows containing a diverse mixture of grasses and flowering forbs, species diversity and structural heterogeneity can benefit insects by providing abundant sources of carbohydrates via nectar as well as food for specialist herbivores, which invariably attract predators and parasitoids higher up the food chain (Luong et al., 2019; Montgomery et al., 2020; Noordijk et al., 2009; Öckinger and Smith, 2007). They can also create structural heterogeneity at small scales that provide protective refuges against competitors or enemies. For example, perennial meadows were shown to function as refuge habitats that promoted native ladybird beetles by resisting the invasion of exotic species, such as the Asian lady beetle *Harmonia axyridis* (Gardiner et al., 2009).

Both hedges and natural grasslands are being threatened by the expansion and intensification of agriculture as well as by urbanization (Montgomery et al., 2020). The loss of hedgerow habitats has undoubtedly contributed to the ongoing decline in biodiversity in Europe and North America (Sklenicka et al., 2009). In France, over 1,000,000 km of hedges have been destroyed in less than 50 years (Pointereau et al., 2001). In the Netherlands, 225, 000 km of hedgerows have been removed since 1900 (Dirkmaat, 2006). In the United Kingdom, hedgerows were managed traditionally every 20-40 years, but over the past 70 years agriculture has intensified to maximize productivity, and, as a result, hedgerow management has intensified and has been mechanized (Alduncin, 2018). This has reduced plant diversity of hedgerows and their refuge potential.

In nature, multiple species such as predators or parasitoids can attack one species of prey or host (Bográn et al., 2002). Parasitoids, unlike predators, typically target specific stages of a few host species (Godfray, 1994), whereas predators are more indiscriminate and will attack prey species based on availability and ease of detection, visually or by olfaction. Species coexistence can occur when niche partitioning of resources occurs at different spatial and temporal scales (Albrecht and Gotelli, 2001). However, when hosts are limited, multiple parasitoids from the same or different species may attack the same individual hosts (Harvey et al., 2013a). Likewise, predators can compete for the same host or prey, particularly if these resources are scarce.  In these scenario's, coexistence or competitive displacement are driven by niche overlap and competitive ability differences, and these ultimately determine whether species within the same functional guilds can maintain sympatry or whether one excludes the others (Mayfield and Levine, 2010).

Hyperparasitoids in the fourth and higher trophic levels parasitize the larvae or pupae of their primary parasitoid hosts (Poelman et al., 2022; Sullivan and Völkl, 1999; Sullivan, 1987). They are important components of food chains that often play a crucial top-down role in shaping multitrophic communities (Poelman et al., 2022). This study compares levels of cocoon hyperparasitism and predation of the primary parasitoid, *Cotesia glomerata* L. (Hymenoptera: Braconidae) in hedgerows and adjacent managed grasslands over the course of two years in the Netherlands. Cocoon clusters were placed on the ground and covered with protective wire and nylon mesh netting under hedgerows and in adjacent grass-dominated meadows along linear transects in the spring to autumn seasons of 2022 and 2023. They were retrieved after one week and reared out to determine what emerged from them. Rates of predation (based on the proportion of cocoons recovered) and hyperparasitism were determined. The main aims of the study were to (1) identify the hyperparasitoids composition in two field types, (2) determine if predation and hyperparasitism rates differ between grasslands and hedgerows. We argue that more research on focal groups of organisms or species interactions is needed to fully appreciate the importance of hedgerows and adjacent habitats for biodiversity conservation.

## Materials and Methods

### *Study sites*

The Netherlands Institute of Ecology, situated in Wageningen, is nestled amongst hedgerows and boasts a variety of meadows within its premises (Fig. 4.1). The designated locations were spaced 20 meters apart. The hedgerows were planted in 2011 including tree and shrub species such as *Cornus sanguinea* (Cornaceae), *Corylus avellana* (Betulaceae), *Crataegus monogyna*, *Euonymus europaeus* (Celastraceae), *Fraxinus excelsior* (Oleaceae), *Prunus padus, P. spinosa, Quercus robur* (Fagaceae), *Sorbus aucuparia* (Rosaceae), and *Viburnum opulus* (Adoxaceae). The meadows consisted of grasses or grassy species (including: *Anthoxanthum odoratum*, *Bromus hordeaceus, Elymus repens, Holcus lanatus, Juncus acutiflorus, Phragmites australis*, *Poa trivialis*), and herbaceous species (including *Centaurea jacea* (Asteraceae), *Crepis biennis*, *Equisetum arvense* (Equisetaceae), *Iris pseudacorus* (Iridaceae), *Mentha rotundifolia* (Lamiaceae), *Plantago lanceolata* (Plantaginaceae), *Rhinanthus minor* (Orobanchaceae), *Saxifraga granulate* (Saxifragaceae), *Typha latifolia* (Typhaceae)). The meadows were mowed seasonally. A total of 26 locations were selected for the study, with locations 1-13 positioned along the hedgerows, and the other 13 locations in the meadow areas (Fig. 4.1).

### *Predation and hyperparasitism of* Cotesia glomerata *cocoons*

*Gelis* hyperparasitoid wasps emerge from diapause in late spring and remain active from June to October in the Netherlands, with peak activity in July and August (Harvey et al., 2014). In the Netherlands, cocoons of the primary parasitoid *C. glomerata* can serve as host for *Gelis* species and different *Gelis* species co-occur in the same small (micro)habitats (Harvey et al., 2014; Heinen and Harvey, 2019). A single *C. glomerata* female can lay up to 50 eggs within early larval stages of *brassicae* hosts. When their larval development is completed, *C. glomerata* form cocoon clusters and these were used in our study to survey hyperparasitism and predation in the field study. *Pieris brassicae* caterpillars parasitized by *C. glomerata* were obtained from the general insect rearing facility at the Laboratory of Entomology, Wageningen University, Netherlands. Parasitized caterpillars were maintained in rearing cages (dimensions: 100 × 60 × 60 cm) in a greenhouse at 22 ± 2°C, 50 % relative humidity, and a 16:8 h light-dark regime. They were provided with Brussels sprout plants (*Brassica oleracea*) as food until the parasitoid larvae emerged and pupated.

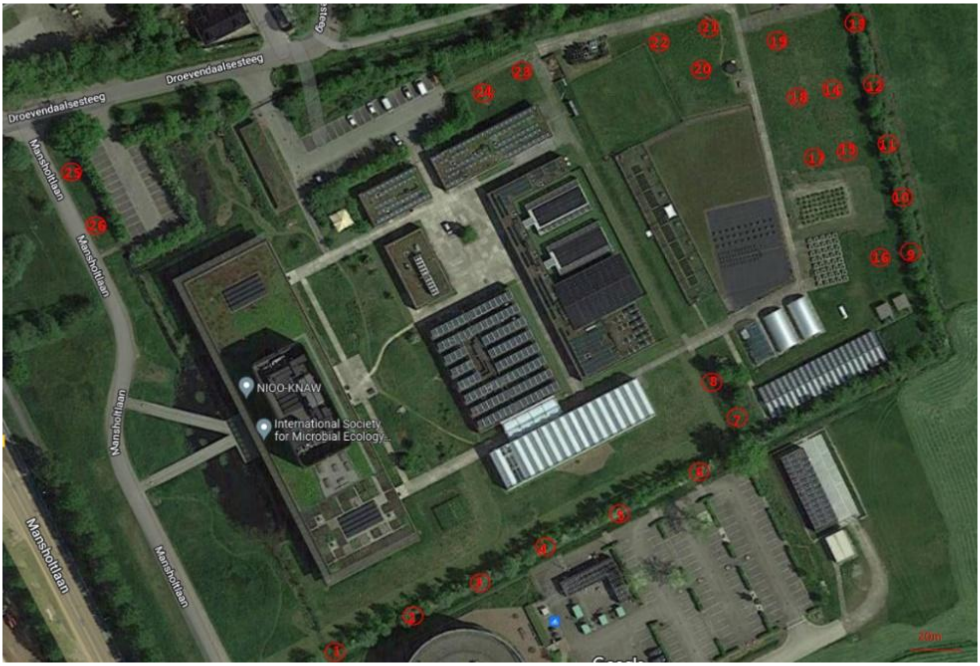

**Figure 4.1.** Map of sampling locations (numbered red circles) near the NIOO-KNAW institute. Thirteen locations (1 - 13) were along hedgerows and the other 13 (14 – 26) were in the meadows.

For placement in the field, clusters of 30 (in 2022) or 20 (in 2023) *C. glomerata* cocoons were attached to a wooden stick covered with baking paper, which helped to keep the cocoons near the stick on the ground. Care was taken to not injure the cocoons. These clusters were protected under a dome of chicken wire and mesh (10 cm x 10 cm) to prevent disturbance by animals such as larger ground beetles, mammals and birds. After one week, cocoons from each sampling location (26 in total) were recollected, placed into labelled vials (one cluster per vial) and returned to the lab. Vials were maintained in a climate chamber (22 ± 2°C, 16:8 h light-dark regime, 50% relative humidity) and hyperparasitoid emergence was recorded daily. Missing cocoon clusters were also counted and assumed to be lost due to predation. At the time of retrieval, all primary parasitoids that had not been attacked by hyperparasitoids had already egressed. Emerging hyperparasitoids were identified to species with sex. Cocoons yielding no wasps after a month were recorded as 'dead cocoons'. Cocoons were also counted to determine partial predation of a cluster. When mowing happened and caused loss of the cover dome in locations (20 and 21), the cocoons retrieved from these locations were not included in the analyses. New cocoons were placed in the field every 2 weeks. This study was conducted from July 5 until September 17 in 2022 (6 rounds), and from April 11 until September 20 in 2023 (8 rounds).

### *Statistical analysis*

In total, there were 26 sampling locations per sampling round distributed over two field types (hedgerow and meadow) over two consecutive years. Hyperparasitism and predation were monitored in all rounds in both years. The response variables obtained for these 26 sampling locations were number of cocoons retrieved out of the 30 (2022) or 20 (2023) cocoons that were placed in the field one week earlier and the number of hyperparasitoids (total and per species) that egressed from the retrieved cocoons. Since no hyperparasitoids emerged from the last two rounds in 2023, the data from these two rounds were combined with one round beforehand, then there were also 6 rounds in 2023. Cocoon retrieval and hyperparasitism proportion among retrieved cocoon clusters was highly variable (Fig. 4.2). Moreover, relatively few hyperparasitoids emerged from the retrieved cocoons; on average less than 6% of the retrieved cocoons produced hyperparasitoids. To reduce the amount of variation and zero entries, we pooled data, both cocoon retrieval and parasitism by hyperparasitoids, over the 6 rounds in each year and focused on differences in distribution of these two variables in relation to field type. To analyze these proportional data, we used a generalized linear model with a binomial distribution of the error terms and a logit link function with field type, year, and their interaction as explanatory variables. Both models displayed overdispersion and to correct for this, quasibinomial models were used, and in both models, interaction terms were not significant, models were rerun without the interaction terms. All statistics were conducted using R (version 4.2.3) (R Core Team, 2023).

## Results

In 2022, 4680 cocoons were placed in the field of which 1380 (29%) were retrieved and of these 183 (13.4%) produced hyperparasitoids. These numbers were 3120, 1045 (33.4%) and 116 (11.1%), respectively, for 2023. Both cocoon retrieval and levels of hyperparasitism were highly variable across sampling locations and sampling rounds (Figs. 4.3 and 4.4). Nevertheless, cocoon retrieval, as a proxy for predation, differed significantly between the two field types ($\chi^2 = 14.7$, df = 1, $P < 0.001$), with fewer cocoons retrieved from the meadows than hedgerows (Fig. 4.2A). Cocoon retrieval, however, was not significantly different between the two years ($\chi^2 = 0.56$, df = 1, $P < 0.45$, Fig. 4.3). Although mean levels of hyperparasitism tended to be higher under hedgerows than in the meadows (2022: 13% vs. 7%; 2023: 12% vs. 7%), these differences were not statistically significant ($\chi^2 = 0.25$, df = 1, $P = 0.62$, Fig. 4.2B). This was mainly caused by the fact that relatively high levels of hyperparasitism with more than half of the retrieved cocoons producing hyperparasitoids occurred incidentally (n =11, darker fields in Fig. 4.4) and these incidents were not restricted to locations along the hedgerows (n = 4). Seven hyperparasitoid species emerged from the retrieved cocoons placed in the field through 2022 and 2023 (Table 4.1).

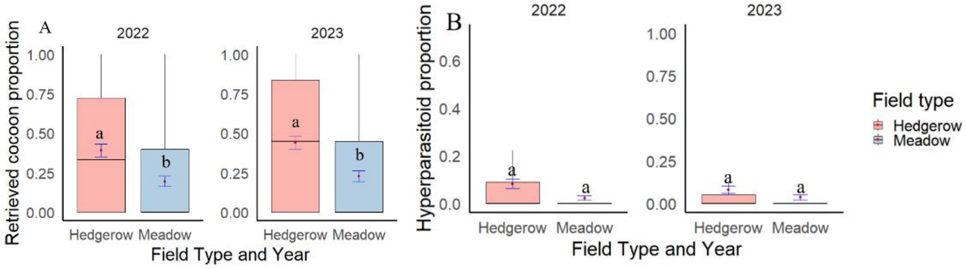

**Figure 4.2.** Retrieval (A) and hyperparasitoid emergence (B) from *C. glomerata* cocoons placed in meadows or near hedgerows in 2022 and 2023. Clusters containing 30 (2022) or 20 (2023) cocoons and were placed in the field for one week in six rounds in each year. Box plots depict proportions of cocoons that were retrieved (A) and the proportion of retrieved cocoons that produced hyperparasitoids. (B). The experiment started in week 27 and ended in week 36 in 2022, and in week 15 until week 37 in 2023. Different letters indicate significances between proportions within a year.

The majority of the hyperparasitoids (88%) were either *Gelis proximus* (n=130), *Gelis hortensis* (n=85) or *Gelis acarorum* (48) (Ichneumonidae: Cryptinae) (Fig. 4.5A-C). Two other geline species eclosed from retrieved cocoons, albeit in low numbers: the asexual species *G. agilis* (n=10) and *Gelis spurius* (n=3). Finally, two other cryptine hyperparasitoid species emerged: *Theroscopus pedestris* (Hymenoptera: Ichneumonidae) (n=12) and *Bathythrix aerea* (Hymenoptera: Ichneumonidae) (n=11). In 2022, *Gelis proximus* and *G. hortensis* were predominant, but fewer of these two species emerged in 2023 when *G. acarorum* became more prevalent. There was no *Gelis spurius* in 2022 while it occurred in 2023. More *B. aerea* and *T. pedestris* emerged in 2023. Parasitoid species composition varied between 2022 and 2023, and several species sometimes emerged from the same cocoon cluster (Fig. 4.5). Potential generalist predators of *C. glomerata* cocoons found in theses habitats could be *Pardosa agrestis* (wolf spider), *Lasius niger* (black ant) and *Limax cinereoniger* (slug) (Fig. 4.6).

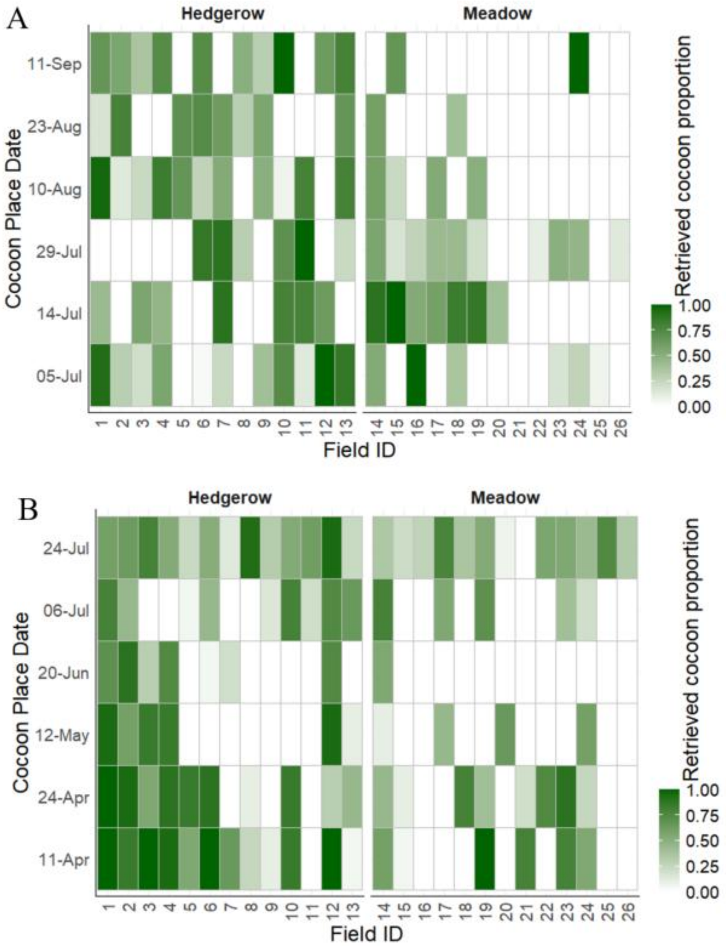

**Figure 4.3.** Heat map displaying retrieved cocoon proportion in 26 locations (from hedge and meadow) through year 2022 (A) and 2023 (B).

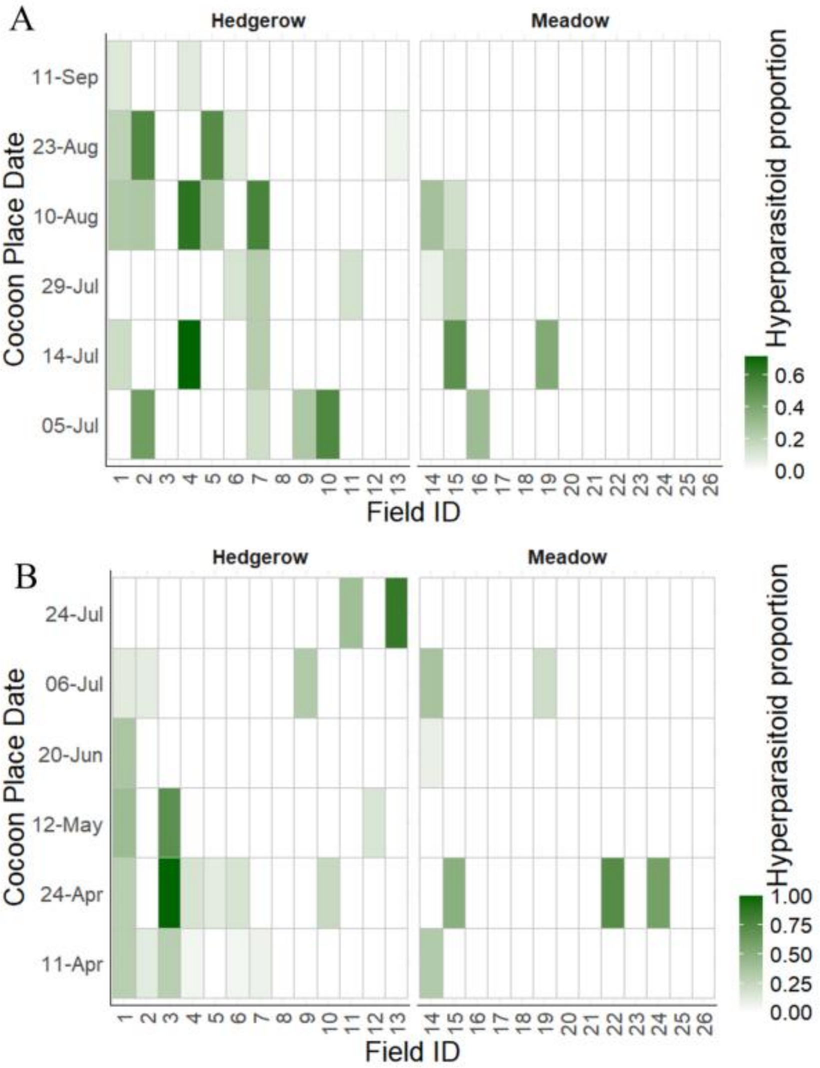

**Figure 4.4.** Heat map displaying proportion of emerged hyperparasitoids from 26 locations (from hedge and meadow) through year 2022 (A) and 2023 (B).

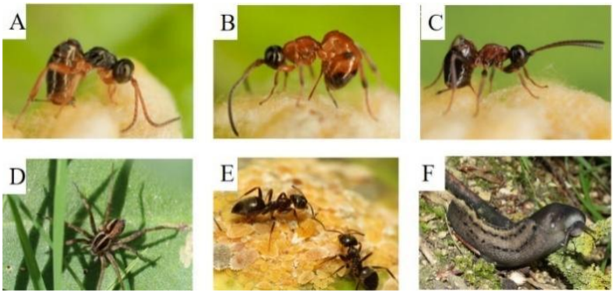

**Figure 4.5.** Hyperparasitoid species that emerged from retrieved *Cotesia glomerata* cocoons (A - C) and potential predators (D - F). (A) *Gelis proximus,* (B) *Gelis hortensis*, (C) *Gelis acarorum,* (D) *Pardosa agrestis*, (E) *Lasius niger*, and (F) *Limax cinereoniger*. Photo credits: Tibor Bukovinszky (A-C), AfroBrazilian (D), Katja Schulz (E), Holger Krisp (F).

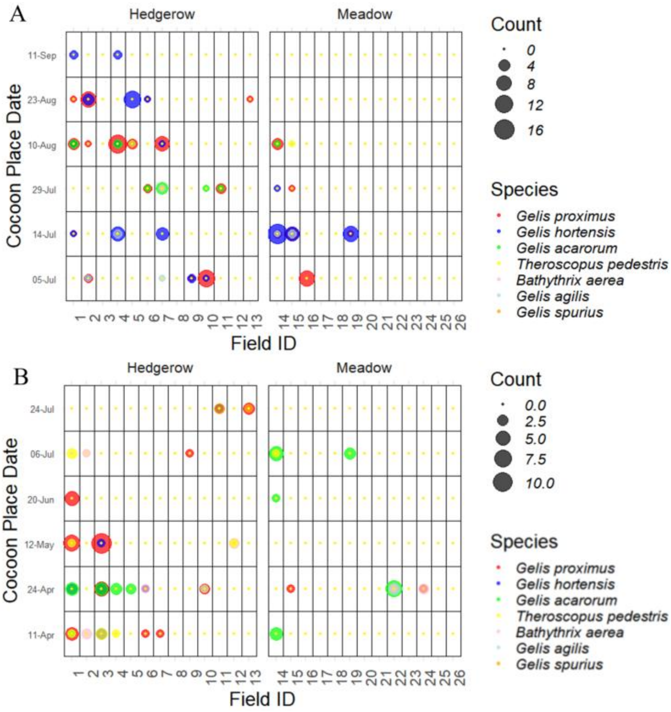

**Figure 4.6.** Heat map displaying species of emerged hyperparasitoids from 26 locations (from hedge and meadow) through year 2022 (A) and 2023 (B).

**Table 4.1**. Hyperparasitoids emerged from *Cotesia glomerata* cocoons placed in meadows and along hedgerows in 2022 and 2023.

| Hyperparasitoid species | 2022 | | 2023 | |
|---|---|---|---|---|
| | hedgerow | meadow | hedgerow | meadow |
| *Gelis proximus* | 62 (50♀: 12♂) | 23 (18♀: 5♂) | 43 (22♀: 21♂) | 2♀ |
| *Gelis hortensis* | 42 (39♀: 3♂) | 33 (28♀: 5♂) | 10 (8♀: 2♂) | 0 |
| *Gelis acarorum* | 12♀ | 1♀ | 15 (8♀: 7♂) | 20 (13♀: 7♂) |
| *Gelis agilis* | 4♀ | 3♀ | 0 | 3♀ |
| *Gelis spurius* | 0 | 0 | 3♀ | 0 |
| *Bathythrix aerea* | 1♀ | 0 | 7♂ | 3♂ |
| *Theroscopus pedestris* | 1♂ | 1♂ | 9 (1♀: 8♂) | 1♂ |

## Discussion

The results of this study show the abundance and species composition of hyperparasitoids emerging from cocoons of their host, *C. glomerata*, varied seasonally. The majority of hyperparasitoids emerged in late July and August of 2022, and before early May of 2023. Although there was temporal overlap in emergence among the different gelines, the species composition of hyperparasitoids exhibited a tendency to fluctuate monthly. For example, in 2022, *G. hortensis* predominated in late July and August, whereas *G. proximus* was more prevalent during other months. In 2023, *G. acarorum*, which was less commonly found in 2022, was the predominant species emerging from cocoons until June, followed by *G. proximus*. These results suggest that niche occupancy and segregation may be somewhat ‘finely-tuned’, enabling multiple species to co-exist within the same localized habitat. On the other hand, different *Gelis* species occasionally emerged from cocoons within the same cluster, revealing that there is still significant niche overlap amongst them. The three most prevalent *Gelis* species are all closely related (Harvey et al., 2018), of similar size, and exhibit broadly overlapping traits (Harvey et al., 2019; Visser et al., 2016, 2014). These convergent traits and overlapping niches among the gelines raise important and fundamental questions about what factors enable them to co-exist in small scale, localized habitats.

Our results also show that very few hyperparasitoids emerged from cocoons placed into the meadow habitats, although the difference between this and cocoons placed under hedgerows was not significant. Previous research has also reported higher invertebrate diversity within hedgerows than elsewhere in adjacent agroecosystems (Merckx et al., 2012; Morandin et al., 2014; Paoletti et al., 1997). Agricultural fields are often dominated by a single crop monoculture, whereas regularly mown lawns or meadows may also be dominated by only a few species (primarily grasses) of plants that do not grow large enough to sustain long food chains. On the other hand, larger forbs often grow under and adjacent to hedgerow shrubs (Boutin et al., 2022; Forman and Baudry, 1984), and these in turn may harbor more insect species. For example, wild mustard plants, such as *Brassica nigra* and *Sinapsis arvensis*, that act as food plants for the large cabbage white butterfly *P. brassicae*, often grow along field and hedgerow margins (Gladbach et al., 2011; Navntoft et al., 2011; Wilson, 2019). Late-instar caterpillars of *P. brassicae* wander at maturity and seek pupation sites nearby, possibly on the natal foodplant or on adjacent vegetation including the hedgerow. Here mature larvae of *C. glomerata* emerge from parasitized caterpillars and spin cocoons that are physically attached to the substrate. Hyperparasitoids that exploit these cocoons will clearly optimize their foraging by remaining in habitats where their hosts are more likely to be found.

Furthermore, they also need exogenous sources of carbohydrates on which to feed. This can come in the form of plant-derived nectar (Araj et al., 2011; Harvey et al., 2012; Poelman et al., 2022) or honeydew excreted by aphids and mealybugs (Buitenhuis et al., 2004; Van Neerbos et al., 2020) that may also be more abundant in hedgerows and adjacent vegetation.

A study conducted in the same general habitat area a decade earlier using *B. nigra* as focal host plant of *P. brassicae* and *C. glomerata* (Harvey et al., 2014) found that *G. acarorum* was the most abundant wingless hyperparasitoid species, while *G. proximus* and *G. hortensis* were completely absent. This demonstrates that hyperparasitoid species composition in the same (micro) habitats can vary over time. Another study (Shi et al., in preparation) explored the diversity and abundance of ground-dwelling hyperparasitoids of the Maasheggen, in Brabant, Netherlands using the same primary parasitoid host (*C. glomerata*). This area is designated as a UNESCO Biosphere reserve (https://en.maasheggenunesco.com) and is characterized by several grassland and forest habitats that are bordered by hedgerows consisting of hawthorn (*Crataegus* spp.) and blackthorn (*Prunus spinosa*). The more open forested habitat and hedgerows contained a higher abundance of hyperparasitoids than the grassland habitats, with *G. hortensis* and *G. proximus* also being the dominant species.

Significantly less cocoons were recovered after being in the field for one week in the meadows than adjacent to the hedgerows. As the cocoons were placed under a combination of wire and nylon mesh, this reveals that the cocoons were inevitably removed by invertebrate predators that were small enough to be able to gain access to them. Generalist predators or omnivores, including ants, ground beetles, spiders and even slugs are often abundant in meadows, and may have accounted for the higher loss of *C. glomerata* cocoons placed there than in the more open sites adjacent to the hedgerows. Dense swards of grasses provide an impediment to dispersal, hindering the movement of larger, visually foraging predators in meadows. However, smaller insect predators (i.e. ants), or slow-moving omnivores (i.e. slugs) might be more abundant in meadows with tall and dense vegetation, where microclimatic conditions (moisture, temperature) are more stable and hence optimal (Eggenschwiler et al., 2013; Holec et al., 2006).

Large habitat patches have been traditionally prioritized over small patches for biodiversity conservation, probably because focus has been on re-establishing apex predators, such as wolves, mountain lions and grizzly bears at the terminal end of the food chain (Huck et al., 2010; Mbuh and Vruno, 2018; Noss et al., 1996; Shepherd and Whittington, 2006). These species require large expanses of habitat to sustain viable populations and for dispersal between habitats, although dietary breadth and landscape matrix play an important role in their conservation and recovery (Wolfe et al., 2022). One of the shortcomings of this approach has been to neglect the importance of smaller habitats that are embedded within the larger ones. Small-scale habitats, such as meadows or hedgerows that were the focus of this study, are clearly important for maintaining healthy populations of smaller vertebrates as well as a large number of invertebrate species, many of which (like hyperparasitoids) have thus far been little studied. Many of these species complete their life-cycles in habitats at very small scales, such as under rocks or logs, on individual plants, or in structurally heterogeneous plant patches. For example, smaller habitat patches sometimes harbor a more diverse array of species than an equivalent area consisting of a few large habitat patches (Riva and Fahrig, 2022). More importantly, habitats at smaller scales can help to counteract the negative impacts of habitat fragmentation and climate change on biodiversity by providing microclimatic refuges or by enhancing the functional connectivity to other habitats across landscapes (Vanneste et al., 2020a). It is hoped that more studies will examine little studied but hitherto important taxonomic or functional groups of arthropods in hedgerows and adjacent habitats and place their findings in the context of processes such as microclimatic variation in a warming world.

## Author contributions

XS, RG and JAH conceived the ideas and designed the experiment; XS collected the data; JGdB,QY and RG advised on data analysis. XS wrote the initial manuscript with input from all co-authors.

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# Chapter 5

## Temporal variation in predation and hyperparasitism rates of parasitoid cocoons in three habitats bordered by hedgerows

Xianhui Shi, Rieta Gols, Jetske G. de Boer, Elisa Mittelstorb, Chloe M. Connolly, Anouk den Ouden, Qiang Yang, Jeffrey A. Harvey

Submitted

## Abstract

Habitat heterogeneity affects species composition, community structure and ecosystem functioning. Heterogeneity has been reduced across many agricultural landscapes. Hedgerows, that border agricultural fields, are profoundly important for insects because they increase heterogeneity by providing refuges, food, and as corridors for dispersal between habitats. Here, using cocoons of a primary parasitoid (*Cotesia glomerata*) as a food resource, we compared levels of predation and hyperparasitism in three distinct habitats embedded in an agricultural landscape in the Netherlands, the 'Maasheggen', a designated UNESCO biosphere reserve: production grassland (PG), which is managed for livestock feed; conservation grassland (CG), which is managed to create plant species-rich meadows, and a deciduous forest. Between June and October 2023, parasitoid cocoons were placed under protective netting in linear transects at 10-meter intervals, from under a hedgerow into the field or forest, and collected a week later. Those that were not found after one week were assumed to have been removed by invertebrate predators. Retrieved cocoons were monitored for the emergence of the primary parasitoid, hyperparasitoids, or no emergence (= death). Hyperparasitism was mostly by two wingless *Gelis* species and was highest early in summer, primarily in cocoons placed under hedgerows or inside the forest. Alternatively, cocoon predation rate was independent of location and increased significantly later in summer. Our results show that hyperparasitoids preferred more open habitats under hedgerows or on the forest floor, whereas generalist predators were more evenly distributed. This study underscores the importance of hedgerows as critical refuges for some insect taxa. More broadly, hedgerows play an important role in landscape-level conservation of biodiversity.

**Key words**: hyperparasitoid, habitat structure, heterogeneity, microclimate, niche overlap, refuge

## Introduction

Landscape heterogeneity affects the community composition and the functioning of ecosystems (Wiens, 2002). It is broadly defined as the size, shape and composition of different landscape units and the spatial (and temporal) connection between them (Fahrig et al., 2011). These parameters have been shown to profoundly influence factors such as animal movement and dispersal (Fahrig, 2007), population persistence (Fraterrigo et al., 2009) and species interactions (Polis et al., 2004). In turn, heterogeneity has been divided into two types: composition heterogeneity (the number and proportions of different cover types) and configuration heterogeneity (the spatial arrangement of the cover types) (Fahrig and Nuttle, 2005). These are both essentially versions of the initial two elements of 'Duelli' s mosaic concept', whereby regional biodiversity focuses on structural parameters that include habitat diversity (the number of biotope types per unit area) and habitat heterogeneity (the number of habitat patches and ecotone length per unit area), respectively, while functionally focusing on metacommunity dynamics (Duelli, 1997).

There is abundant evidence showing that habitat structure enhances species diversity in communities. Three vegetational components of habitat structure play a vital role in enhancing species richness at local scales: the diversity of plant species, their spatial distribution and the architectural complexity of the plants within the habitat. For example, in the Argentine Chaco, a mosaic of grassland and open forest habitats, arthropod diversity was smaller on sites with reduced structural complexity, and the guild-size ratio of non-predatory arthropods affected the diversity of other trophic levels (Gardner et al., 1995). However, thus far, landscape heterogeneity has been little studied in agricultural landscapes as a consequence of the predominance of the 'habitat–matrix paradigm in landscape ecology' in which landscapes include habitats and hostile matrices (Fischer and Lindenmayer, 2006).

Currently, approximately 40% of the land globally is allocated for agriculture and this has resulted in the loss of terrestrial ecosystems such as forests and grasslands (Foley et al., 2005). Furthermore, the expansion of intensive agriculture has led to the fragmentation of remaining ecosystems, further leading to their degradation (Diekötter and Crist, 2013). The loss and fragmentation of natural ecosystems, that has been exacerbated by other human-mediated stresses such as invasive species, various types of pollution, overharvesting and climate change, has led to a marked decline in species and genetic richness of plants and animals across the biosphere (Ceballos and Ehrlich, 2006; Dirzo et al., 2014; Newbold et al., 2015). Insects are especially susceptible to anthropogenic changes occurring in nature that are transpiring across variable spatial and temporal scales (Wagner et al., 2021).

Hedgerows border agricultural landscapes in parts of the world and numerous studies have shown that they provide important habitats for many species of arthropods (Cobbold and Supp, 2012; Griffiths et al., 2007; Lecq et al., 2017; Maudsley et al., 2002; Staley et al., 2012). For example, they buffer organisms against climatic extremes, such as temperature fluctuations, wind and heavy rainfall (Herbst et al., 2006; Leroux et al., 2022; Vanneste et al., 2020b), and also provide structural refuges against predation and parasitism, thereby stabilizing multi-trophic interactions and species food webs (Dunn et al., 2016; Facey et al., 2014). Hedgerows are known to function as dispersal corridors, and facilitate species movement across landscapes and between different habitats (Davies and Pullin, 2007). Moreover, hedgerows comprised of young trees or bushes also provide food resources, such as nectar and plant tissues, that may enhance the fitness of insects that depend on these resources. In this way they may boost landscape-level biodiversity (Diekötter and Crist, 2013). The presence of hedgerows bordering agricultural fields can also enhance biological control programs by providing food and shelter for natural enemies of crop pests (Garratt et al., 2017; Morandin et al., 2014).

Among insects, parasitoid wasps (Hymenoptera) are excellent organisms to examine the effect of habitat heterogeneity on species richness and abundance. Parastioids are insects that develop inside, or on the bodies of other arthropods, whereas the adults are free-living (Godfray, 1994). Most parasitoids are specialists that attack only one or a few closely related species of hosts (Gagic et al., 2016; Korenko et al., 2018), and therefore they are dependent on finding hosts that only occur in specific (micro)habitats or which are intimately associated with certain species of plants. In searching for hosts, parasitoids utilize olfactory chemical cues that are important for foraging in structurally and chemically complex habitats (Poelman et al., 2012; Quilici and Rousse, 2012; Tentelier and Fauvergue, 2007). Parasitoids can occupy two or more trophic levels: primary parasitoids (in the third trophic level) attack various stages of herbivore hosts, whereas hyperparasitoids (in the fourth or higher trophic levels) attack larval or pupal stages of parasitoid hosts (Harvey et al., 2009; Poelman et al., 2022).

Parasitoids in the genus *Gelis* (Hymenoptera: Ichneumonidae, Cryptinae) are abundant in the Palearctic and Nearctic realms (Harvey, 2008; Harvey et al., 2017; Schwarz and Shaw, 2000; van Nouhuys and Hanski, 2000). Many species are wingless, and exhibit behavioural, morphological and chemical traits that mimic ants, presumably as a means of defence against predators such as wolf spiders (Harvey et al., 2018; Malcicka et al., 2015). Being wingless means that these parasitoids probably take a considerable time navigating through vegetation in order to successfully find mates and hosts. In nature, time is a costly currency that can exert significant fitness costs on an organism in terms inability to optimize reproduction (Rosenheim et al., 2008). Despite that, the host range of some *Gelis* species appears to be limited. For instance, some species of *Gelis* are specialist natural enemies of spider egg sacs (Fei et al., 2023) whereas others are hyperparasitoids of parasitoid cocoons (Harvey and Witjes, 2005). These parasitoids appear to have evolved life-history strategies, such as extremely long-lifespans and low fecundity, that enable them to thrive in different habitats (Harvey, 2008; Visser et al., 2016).

Previous studies reported that several *Gelis* species in the Netherlands are hyperparasitoids of cocoons of the primary parasitoid *Cotesia glomerata* and that they co-occur in the same small (micro)habitats (Harvey et al., 2014; Heinen and Harvey, 2019). This suggests that there is some niche overlap among them and that they may compete for hosts under certain conditions (Harvey et al., 2019). In this study we compared the diversity and abundance of *Gelis* species over the course of a growing season in relation to the presence of hedgerows and in habitats under different management schemes, as well as the predation of their host. Experiments were carried out in the Maasheggen, a UNESCO designated biosphere reserve located in the Netherlands. This area is comprised of an historically cultivated landscape bordered by hedgerows that were planted around 1100 AD close to the River Maas in the southeast of the Netherlands on the border between the provinces of Noord-Brabant and Limburg. The area is characterized by hedges bordering small fields that are either managed for crops (beet, maize), grass production (production grassland, PG) for cattle feed, or conservation of diverse floral meadow plants (conservation grassland, CG). The landscape also contains several small deciduous forests and wetlands that border the fields, thus, creating a heterogeneous landscape. Despite their known ecological importance, information on the effects of hedgerows and landscape management programs on many arthropod groups is scarce (Albrecht et al., 2020).

To elucidate how landscape heterogeneity and structural complexity influence *Gelis* species composition in different habitats, as well as to measure predation rates of their host, we placed clusters of cocoons of *C. glomerata* on the ground under finely meshed nets along transects from hedgerows into PG, CG and forests in the Maasheggen. Cocoons were placed in the field over the course of several months at three weeks interval for 7 days and were then collected and reared out to determine their fate (dead cocoon, primary parasitoid or hyperparasitoid). Cocoons that were not retrieved were presumed to have been removed by a predator, and this was also recorded. The major aim of the study was to compare rates of hyperparasitism or predation of parasitoid cocoons placed into habitats under different management schemes in the Maasheggen. We predict that habitat complexity (hedgerow, or habitat type) will affect parasitism and predation rates due to a combination of differences in microhabitats and refuges present and that the dynamics of these may change over the course of a growing season.

## Materials and Methods

### *Study sites and experimental fields*

The Maasheggen area (~2,000 hectares) consists of various small fields (< 4 ha) that are used for various agricultural or conservation purposes and are characteristically separated by hedges that have been established in the 12th century (https://www.maasheggenunesco.com/). This study focused on three types of fields subjected to different management practices: intensively managed production grasslands, extensively managed conservation grasslands prioritizing floral diversity, and forest (Fig. 5.1A). Production grasslands consisted of fertilized fields dominated by plants that were regularly mowed (approximately every 6 weeks), such as *Lolium perenne*, *Trifolium pratense* (Red Clover) and several daisy species and others, depending on season. Conservation grasslands contained a mosaic of monocot and dicot species including *Taraxacum officinale* (Common Dandelion), *Rumex obtusifolius* (Broad-leaved Dock), *Holcus lanatus* (Yorkshire-fog), *Ranunculus acris* (Meadow Buttercup), *Alopecurus pratensis* (Meadow Foxtail). Forests consisted mainly of *Salix* alba (White Willow), *Rubus caesius* (Dewberry), *Quercus robur* (Pedunculate Oak) with some understory on the forest floor. Hedgerows consist primarily of *Crataegus* species (hawthorn) and *Prunus spinosa* (blackthorn), intermitted with *Fraxinus excelsior* (Ash), *Euonymus europaeus* (European spindle), *Sambucus nigra* (elderberry) with *Urtica dioica* (Common

Nettle) and other dicots at the bottom. Five locations were selected in each field type (=replicates) and paired sampling locations were established in each field at three distances from one of the hedges: 0, 10 and 20 m, respectively (Fig. 5.1E). The six sampling locations in each field were marked with flags. In total there were 3 (field types) x 5 (replicates) x 3 (distances) x 2 (paired) sampling locations.

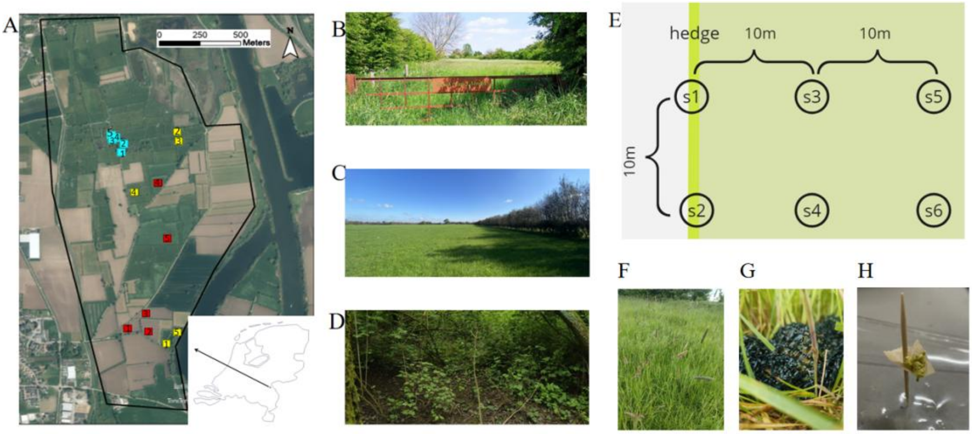

**Figure 5.1.** Experimental design. (A) Map of habitat sites in the Maasheggen. Three types of sites were set as representatives for different habitats, production grasslands (red plots), conservation grasslands (yellow plots), and forests (blue plots) (Credit: Anouk den Ouden). (B) Conservation grasslands, (C) Production grasslands, and (D) Forests, (E) Scheme of sampling locations in each field at 0, 10 or 20 m from the hedge (bright green strip). In each field, there were two paired rows at 10 m distance. Data at the same distance from the hedge were combined, resulting in three data points per field, with five replicates per field type. (F) Vegetation density in the PG field. (G) Ground view of the trap. (H) Cocoon cluster set.

### *Predation and hyperparasitism of C. glomerata cocoons*

To serve as prey and hosts in the experimental fields, fresh cocoon clusters of *C. glomerata* were placed and retrieved seven times from June 2 to October 10 in 2023 at three-week intervals. In nature, cocoon clusters of the gregarious primary parasitoid *C. glomerata* serve as hosts for a range of hyperparasitoids including

*Gelis* species (Heinen and Harvey, 2019). *Gelis spp* wasps emerge from diapause in late spring and are active from June to October in the Netherlands, with peak activity in July and August (Harvey et al., 2014). Pupae and cocoons of insects are also prey for predacious arthropods such as spiders and ground beetles (Harvey et al., 2018, 2013b). Cocoon cluster of *C. glomerata* to be placed in the field were reared in the laboratory. Briefly, parasitized first or second instar larvae of *P. brassicae*, which is the preferred host of *C. glomerata*, were provided by Wageningen University, the Netherlands. The parasitized hosts were reared on Brussels sprout plants (*Brassica oleracea*) until the full-grown parasitoids egressed from their hosts for pupation after about 2-3 weeks (Harvey, 2000). Parasitized caterpillars were maintained in rearing cages (100 × 60 × 60 cm), in a greenhouse at 22 ± 2°C, under a 16:8 h light-dark regime with 50% relative humidity. Cocoon clusters were collected within 24 h after formation and were used immediately or were stored for a of maximum of three days at 5°C in a refrigerator to slow-down their development. Clusters of twenty *C. glomerata* cocoons were prepared by counting and removing excess cocoons. Clusters were placed on a small piece of baking paper (1 by 1 cm). A cocktail stick was used to attach the cocoons to the baking paper and hold the cocoon in place on the ground in the field (Fig. 5.1H). Care was taken to not injure the cocoons. Each cluster was placed under a dome created by chicken wire and plastic mesh (10 x 10 cm) to prevent predation by larger animals such as larger ground beetles, mammals and birds. After one week, cocoons were retrieved from all 90 sampling locations. Missing cocoon clusters were also counted and assumed to be lost due to predation. Retrieved cocoons were placed in vials (one cluster per vial) and returned to the lab where they were maintained in a climate chamber at 22±2°C, under a 16:8 h light-dark regime with 50% relative humidity. Emergence of hyperparasitoids was monitored daily for one month. At the time of retrieval all primary parasitoids that had not been attacked by hyperparasitoids had already egressed. Emerging hyperparasitoids were counted and identified. Cocoons were also counted to determine partial predation of a cluster.

*Statistical analyses*

In total there were 45 sampling units per sampling round distributed over three field types (CG, PG and forest) each with 5 replicates. Within each replicate there were three paired distance-from-hedge locations (0, 10 and 20 m). The data for each paired distance-sampling unit were combined into a single data point. The variables obtained for these 45 sampling units were the number of cocoons retrieved out of the 40 cocoons that were placed in the field one week earlier and the number of hyperparasitoids (total and per species) that egressed from the retrieved cocoons. Since relatively few hyperparasitoids emerged from the retrieved cocoons (164 out of 3185 retrieved cocoons produced hyperparasitoids), we focused in the analysis on the distribution of the total number of hyperparasitoids in relation to field type and distance from the hedge. During the last sampling round in October, most of the fields were mown, destroying the majority of the cocoon clusters. This round was, therefore, omitted from the data set. Thus, the data contained 6 sampling rounds instead of 7. The structure of the data with relatively few replicates (n=5) limited the possibility to analyze temporal effects and those related to the field (type and distance from the hedge) in a single analysis (i.e., mixed model approach with random and fixed factors). Therefore, we analyzed temporal effects and field-by-distance effects in separate analyses. To statistically analyze the effect of sampling time on cocoon retrieval and parasitism by hyperparasiotids, we compared the proportion of cocoons that were retrieved and the proportion of hyperparasitoids emerging from these cocoons using a generalized linear model with a binomial distribution of the error terms and a logit link function. In rounds 5 and 6, only 1 and 0 hyperparasitoids, respectively, emerged from retrieved cocoons. To reduce the number of zeros in the data set, results on hyperparasitoids for the last two rounds were combined. Field type (fixed factor) and round number (covariate) served as explanatory variables in both models, i.e., model for cocoon retrieval and the model for hyperparasitoids. In this analysis we ignored the effect of distance from the hedge and only included field type.

In a second analysis, we explored the effect of field type and distance from the hedge. Results for each field-type-by-distance-from-hedge combination were pooled over all sampling rounds. We subjected cocoon recovery and hyperparasitoid emergence to similar generalized linear models as described for temporal effects, but here with field type (fixed factor) and distance from the hedge (covariate) as explanatory variables. Data in all models displayed overdispersion and to correct for this quasibinomial models were used. In all models, interaction terms were not significant, and models were rerun without the interaction terms. Post hoc Tukey HSD tests were performed when the effect of field-type was significant. All statistics were conducted using R (version 4.2.3) (R Core Team, 2023).

## Results

### *Cocoon retrieval as a proxy for predation*

The number of cocoons retrieved during the sampling period from the beginning of June (wk 22) to mid-September (wk 38) decreased with time ($\chi^2_1 = 34.8$, $P < 0.001$, Fig. 5.3). While in wk 22 on average 50% of the cocoons were retrieved, this percentage dropped to less than 20% during the last three rounds. Temporal patterns of cocoon retrieval did not significantly differ across field types ($\chi^2_2 = 3.98$, $P = 0.136$). When cocoon retrieval was summed over the experimental period, it did not differ across the field types ($\chi^2_2 = 4.70$, $P = 0.095$), nor was it affected by distance from the hedge ($\chi^2_1 = 0.47$, $P = 0.49$).

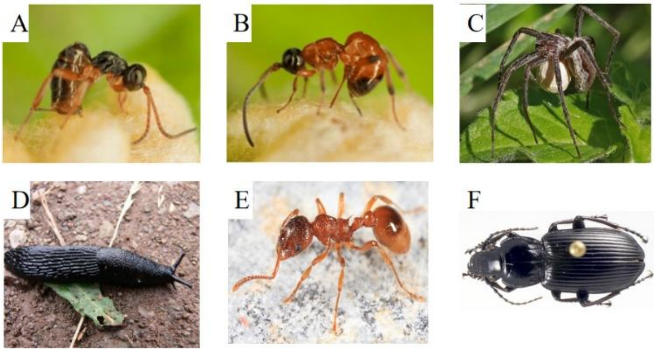

**Figure 5.2.** Hyperparasitoid species that emerged from retrieved *Cotesia glomerata* cocoons (A - B) and potential predators (C - F). (A) *Gelis proximus*. (B) *Gelis hortensis*. (C) *Pisaura mirabilis*. (D) *Arion ater*. (E) *Myrmica rubra*. (F) *Pterostichus melanarius*. Photo credits: Tibor Bukovinszky (A-B), Charles J. Sharp (C), Prashanthns (D), Tom Murray (E), Kipling Will (F).

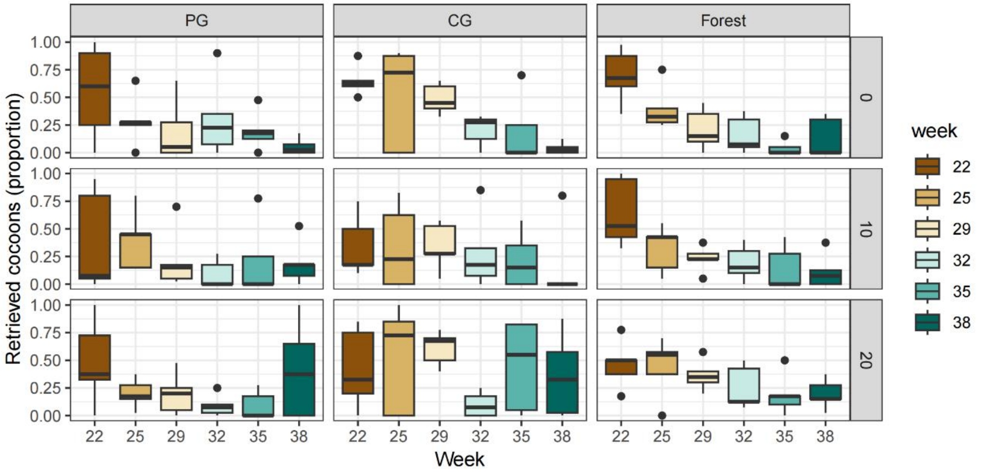

**Figure 5.3.** Cocoon retrieval from production grassland (PG), conservation grassland (CG) and forest in the summer and autumn of 2023 in the Maasheggen. Cocoon clusters of *C. glomerata* were placed for one week in the three habitat types (panels from left to right) at 0, 10 or 20 m from the hedge (panels from top to bottom) at three-week intervals. Boxplots depict the proportion of cocoons retrieved of the original two cocoon clusters containing 20 cocoons each (see Figure 5.1).

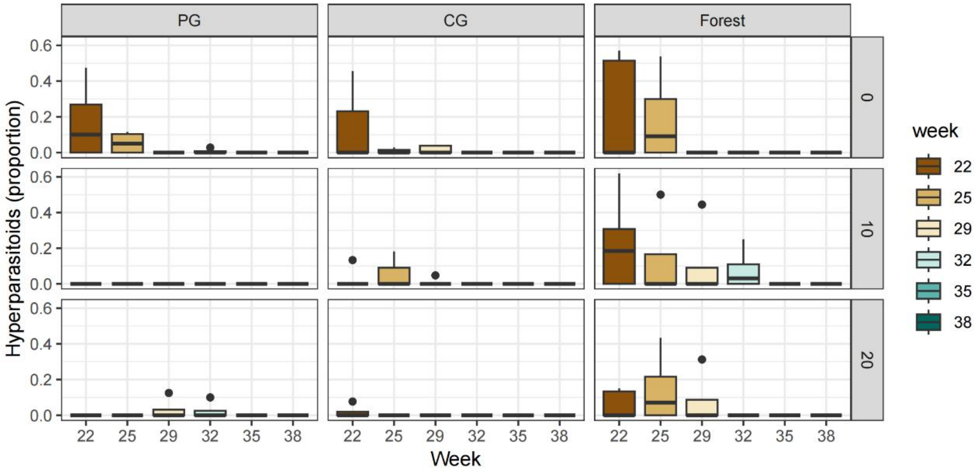

**Figure 5.4.** Parasitism by hyperparasitoids in production grassland (PG), conservation grassland (CG) and forest in the summer and autumn of 2023 in the Maasheggen. Cocoon clusters of *C. glomerata* were placed for one week in the three habitat types (panels from left to right) at 0, 10 or 20 m from the hedge (panels from top to bottom) at three-week intervals. Retrieved cocoons were monitored for hyperparasitoid emergence. Boxplots depict the proportion of retrieved cocoons that were hyperparasitized in each week. The design of the experiment is explained in Figure 5.1.

### *Hyperparasitism*

In total 164 hyperparasitoids emerged from the cocoons that were recollected after one week exposure in the field. The majority of the hyperparasitoids (85%) were either *Gelis proximus* (n=77) or *Gelis hortensis* (n=65) (Ichneumonidae: Cryptinae). Three other geline species eclosed from retrieved cocoons, albeit in low numbers: *Gelis acarorum* (n=6), *Gelis spurius* (n = 1), and the asexual *G. agilis* (n = 8). One non-geline phygadeuontine hyperparasitoid species emerged: *Theroscopus pedestris* (n=6) and one non-ichneumonid species *Anacharitinae sp.* (n=1), belonging to the Figitidae. Over the experimental period, hyperparasitism differed among the field types ($\chi^2_2 = 15.0$, $P < 0.001$, Fig. 5.4). It was higher in the forest than in both grassland types (Tukey test, $P<0.05$). Hyperparasistim in CG and PG was similar (Tukey test, P<0.99). Moreover, hyperparasitim decreased with distance from the hedge (Fig. 5.4). Parasitism by hyperparasitoids decreased from almost 10% in wk 22 to less than 1% in the last three rounds combined ($\chi^2_1 = 64.3$, $P < 0.001$, Fig. 5.4).

## Discussion

Irrespective of parasitism status, *Pieris brassicae* caterpillars often wander away from the natal foodplant when they are mature in order to pupate (the so-called ‘wandering phase’) (Smallegange et al., 2007). Pupation may occur on the ground or on an elevated site, such as a tree trunk, that may be located a considerable distance from the foodplant, and has been suggested as a means of escaping co-evolved enemies that use cues from the host plant as a means to escape predation or parasitism (Lindstedt et al., 2019). Despite that, we found that a total of 164 hyperparasitoids, representing 7 species, emerged successfully from field-placed cocoon clusters of *C. glomerata* over the duration of the experiment. The two most prominent *Gelis* species parasitizing cocoons of *C. glomerata* were the wingless species *G. proximus* and *G. hortensis* (Fig. 5.2). Both species occurred in all three habitat types, although they were more common emerging from cocoons placed inside the forest and adjacent to the hedgerows, possibly because the ground habitats were more open there. Furthermore, the ground habitats of the forest and hedgerow sites were similar in terms of openness, and shade availability. By contrast, very few emerged from cocoons placed directly into the PG and CG fields.

A remarkable aspect of the biology of wingless *Gelis* species is that they are able to navigate structurally and chemically complex habitats on the ground while foraging for hosts (Harvey et al., 2014). Winged (hyper)parasitoids that are efficient fliers are not confronted with the same kind of physical impediments and can disperse over an area in minutes that would take wingless parasitoids many hours. In the PG and CG meadows, vegetation on the soil interface is tightly matted in thick swards throughout the growing season, which creates a veritable maze for ground foraging insects to traverse (Dennis et al., 1998). Furthermore, host (cocoon) abundance is likely to be higher in wooded habitats, where trees and shrubs contain more abundant resources for herbivorous insects and their primary parasitoids than meadows dominated by grasses and a few species of relatively small forbs. Previous studies have shown that parasitoids can detect host cocoons using kairomones via highly refined antennal sensilla (Bénédet et al., 2002; Jumean et al., 2005). Although factors attracting gelines to their hosts have not been investigated, it is clear that chemical cues associated with the cocoons of their hosts probably play an important role.

Despite being placed under wire mesh and plastic netting, many of the cocoon clusters placed into each of the three habitat types were removed over the course of the following week throughout the experiment. A substantial proportion (up to 69%) of cocoons disappeared, presumably due to small-sized predators. It was assumed that the nets placed over the cocoons would reduce the risk of removal by birds or, more likely, ground-dwelling mammalian predators like shrews or even mice (which are omnivores). Since the cocoons are immobile, their removal was caused by a potentially wide range of small-sized ground-dwelling predators and omnivores, some of which also scavenge. Indeed, this is a common phenomenon, supported by other field studies on immobile insects. Generalist predators destroyed ~30% of cocoons of the moths *Acharia stimulea* and *Euclea delphinii* in the field (Murphy & Lill, 2010). A recent study also found that immobile aphids, which were glued to predation cards and placed on the soil surface were attacked by a number of insect and other invertebrate taxa with generally long handling times, including harvestmen, spiders, gastropods and ground beetles (Gardarin et al., 2023). Ants are also major predators or scavengers of immobile or dead invertebrates (Fellers and Fellers, 1982; Retana et al., 1991; Tan and Corlett, 2012). Most species of ants use tactile or olfactory cues to hunt their prey (Binz et al., 2016; Traniello, 1989). The fact that parasitoid cocoons were often removed completely in our study (with no sign of residues) implicates ants as the major predators of *C. glomerata* cocoons because they would not consume them *in situ* but take them back to their colony as food for developing larvae.

The proportion of *C. glomerata* cocoons producing hyperparasitoids was highest in June and decreased thereafter through the summer into autumn months, whereas the proportion of cocoons retrieved also decreased dramatically over time across all habitats, irrespective of their proximity to the hedgerows. This suggests that the abundance of cocoon hyperparasitoids may peak earlier in the season, whereas the abundance and diversity of invertebrate predators presumably increases over time until autumn, and that predators are more evenly distributed across the landscape. For instance, ant colonies grow rapidly over the course of summer, and contract in winter (Heller and Gordon, 2006; Markin, 1970). The abundance of ground dwelling macro-invertebrate arthropods in an Appalachian ecosystem was found to be higher in summer and autumn than in spring (Greenberg and Forrest, 2003). Recent climate warming may also enhance the number of generations of arthropods, including predators (Macgregor et al., 2019), thus enabling them to build up their populations. Habitat management plays an important role in the structure and abundance of the insect communities across different seasons (Landis et al., 2000). Our results suggest that generalist predation pressure can increase rapidly over the course of the summer in different habitats bordered by hedgerows.

The importance of hedgerows as micro- and macroscopic refuges and dispersal corridors for a wide range of species is well-documented (Boughey et al., 2011; Graham et al., 2018; Kratschmer et al., 2024). Hedgerows contain several characteristics that are important to a wide range of vertebrate and invertebrate taxa. First, they are often far more structurally complex, both vertically and horizontally, than adjacent fields, which are often sown with a single crop species or else are managed as feed for livestock and/or heavily grazed (Hinsley and Bellamy, 2000; Kratschmer et al., 2024). Hedgerows often allow wild flowering plants to grow under the canopy of the dominant hedgerow plant species (hawthorn, *Crataegus* spp., and blackthorn, *Prunus spinosa*) where they provide food and shelter for herbivores (Staley et al., 2012). These, in turn, attract natural enemies, such as predators and parasitoids, that may provide important ecological services in adjacent crop fields (Montgomery et al., 2020) or act as carbon storage sites (Thiel et al., 2015). Small mammals and birds will also utilize hedgerows as breeding sites or to find cryptic preys (Hinsley and Bellamy, 2000; Michel et al., 2006). Hedgerows mitigate climatic extremes, such as heat and drought, that can decimate populations of insects and other arthropods (Vanneste et al., 2020b).

A major aim in European countries has been to implement policies protecting 'linear' landscape elements such as riparian banks, field margins and hedgerows (Aschi et al., 2023). In urban and agricultural landscapes in the Netherlands, various initiatives, such as the Delta Plan biodiversity restoration, are now being implemented in an attempt to 'bend the curve' (Leclère et al., 2020) and restore species diversity and abundance (Baptist et al., 2019). In our study, using hyperparasitoids and their primary parasitoid host as a model system, we have shown that hedgerows in the Maasheggen and adjacent forests are important habitats for a group of fascinating but hitherto little studied specialist (hyper)parasitic wasps in the Cryptinae. We stress, however, that in order to elucidate demographic trends in these habitats that collections need to be carried out over many years and with multiple groups of ground-dwelling arthropods. In this vein, research at the Maasheggen is examining the effects of hedgerows on other arthropod groups, as well as soil micro-and macrobiomes in hedgerow soil and soil in PG and CG. It is envisaged that these data will shed more light on the important role that the Maasheggen, a UNESCO world heritage site, and other hedgerows, play in conserving a wide range of animal and plant taxa. Given that biodiversity is seriously threatened by intensive agricultural practices across Europe (Kleijn et al., 2009), more studies are needed to elucidate the importance of hedgerows across landscapes in different countries.

## Authorship statement

XS, RG and JAH conceived the ideas and designed the experiment. XS, EM, CC and AtO did the field and laboratory work. JGdB and RG advised on data analysis. XS wrote the initial manuscript with input from all co-authors.

## Acknowledgements

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Gwerke van J. Verdijk

# Chapter 6

## General Discussion

In 2013, a group of scientists, led by British ecologist William Sutherland identified 100 ecological questions that were important for fundamental ecology (Sutherland et al., 2013). Many of the 100 questions focused on the understanding multiple aspects of organisms and ecosystems. However, one issue that hinders research is when poor knowledge of phylogeny leads researchers to study traits that are not applicable to their own study organisms (Harvey et al., 2015). Several of the questions raised by Sutherland et al. (2013) were addressed in this thesis using geline hyperparasitoids as a model system. Specifically, with *Gelis agilis* as my main study organism along with cocoons of its host, the endoparasitoid *Cotesia glomerata*, I started my research with two lab-based experiments that examined maternal effects on offspring development, foraging behavior and trade-offs in fitness-related traits. Then, I conducted two field studies in habitats that varied in structural heterogeneity to compare geline communities between these habitats over time and to partially test refuge theory with respect to the susceptibility of *C. glomerata* to parasitism and predation.

Question 20 of Sutherland et al. (2013) asked to what degree do trans-generational effects on life histories, such as maternal effects, impact on population dynamics? In **Chapter 2**, I explored the possible effect of different sizes of female parasitoids and host cocoons on offspring size and development time. Thus far, maternal (and paternal) size has been almost completely excluded in studies of host-parasitoid developmental interactions. Instead, the focus has been exclusively on effects of host-related attributes – size, age, stage of development etc. – on parasitoid offspring development. Here, I found that there was no significant effect of maternal size on offspring size. Thus, in hosts of similar size, big mothers and small mothers produce similarly sized offspring. Large mothers do not possess an inherent provisioning advantage compared with smaller mothers.

Question 10 of Sutherland et al. (2013) asked what are the physiological bases of life-history trade-offs? In question 16 they asked how do organisms make movement decisions in relation to dispersal, migration, foraging or mate search? In **Chapter 3,** using specially designed mazes in microcosms that created foraging arenas of variable complexity, I examined fitness-related effects on *G. agilis*. In these arenas host cocoons (for reproduction) and honey/water (for food) were placed in different compartments and at different time intervals. In more complex set-ups, I expected trade-offs in fitness functions, such as between longevity and fecundity. These indeed were found in the most complex experimental habitat, but the results also demonstrated that hyperparasitoids are well-adapted at finding hosts and food under what I perceived to be more complex scenarios. Question 13 of Sutherland et al. (2013) asked how do species and population traits and landscape configuration interact to determine realized dispersal distances? In order to address that question, in **Chapters 4 and 5**, I conducted two field studies where different habitats were included to check the effect on hyperparasitoid traits and diversity. Two different nature systems consisted of NIOO-KNAW institute with meadows and hedgerows, Maasheggen with three different habitat types.

One of the questions that is surprisingly missing from the Sutherland et al. (2013) study – and it is quite an alarming omission – is how the physical environment affects species and species interactions. The paper reveals a laser-like focus on organisms but largely neglects the environment in which these organisms live and the important role this plays in driving the evolution of traits in organisms and its importance under anthropogenic change. A more recent paper proposing 100 questions in conservation biology (Cooke et al., 2021), asked a relevant question: How can the complexity of natural environments and interacting stressors be better incorporated into conservation physiology research? There is abundant research showing that simplified habitats – such as agro-ecosystems, or habitats that have been significantly damaged – generate unstable trophic interactions characterized by boom-and-bust cycles. In agro-ecosystems, food resources (the crop) are often grown in monocultures, greatly reducing the ability of herbivores to find their food plants. In turn, the limited chemical and physical heterogeneity of cropping systems makes it much easier for natural enemies such as predators and parasitoids to find their prey and hosts respectively. Long underpinning ecological theory and our understanding of processes that generate long-term stability in species interactions is the concept of refuges, which are parts of habitats where organisms can escape from their natural enemies (Selwood & Zimmer, 2020; Sih, 1987). In this thesis **(Chapters 2-4)** I also examined the susceptibility of host cocoons to parasitism and predation, revealing which small-scale habitats are hot-spots for natural enemies, and which might serve as trial refuges.

In this chapter, I will focus on how hyperparasitoids are able to adapt to complex field habitats under strong selection pressure on how they utilize and allocate spatially partitioned resources. This will be explored from discussing the above questions and linking the questions to different experimental chapters. In the conclusion, I will summarize how the results from this thesis could be contributed to further research focusing on high trophic level organisms and will offer the suggestions for both entomologists and farmers to attain better knowledge of combining landscape types with biological pest control.

### To what degree do trans-generational affect life histories?

My study indicated that in the solitary idiobiont hyperparasitoid, *Gelis agilis*, mothers had minimal impact on the body size of their offspring (**Chapter 2**). When developing in large host cocoons, *Gelis agilis* offspring body size was occasionally more than twice as heavy as the body size of small mothers (and *vice-versa*), indicating that maternal physiological provisioning ability played at most a negligible role in reproduction. Large mothers produce small offspring on small hosts and small mothers produce large offspring on large hosts. Irrespective of maternal size, offspring body size was very strongly correlated with host size. This showed that host size is the primary determinant of offspring size which overrides maternal size-related provisioning ability even though there was a trend suggesting larger mothers produce somewhat larger eggs. Both large and small *G. agilis* could parasitize a wide range of host cocoon sizes (**Chapter 2**). In contrast with our results, a recent study by Rasekh et al. (2022) found positive parental influences of body size, development time and egg load in the solitary aphid endoparasitoid, *Lysiphlebus fabarum*. This study was weak, however, in that the number of stings (eggs laid) was not controlled and that development of koinobionts is largely dependent on future host quality, based on the final size of the host when it is killed by the parasitoid larva(s) (Harvey, 2005). Additionally, I also found that maternal size did not affect the parasitism duration of hosts (**Chapter 2**). To manipulate embryonic development, female insects can influence carbohydrates and polyols contents of the egg through hormonal regulation (Denlinger, 2002; Yamashita et al., 2001). They can also invest in physiological provisioning by allocating metabolic resources to produce larger eggs, which store more proteins and lipids, thereby offering greater nutritional support for the developing embryos (Beukeboom, 2018). Possible nutritional provisioning through venom proteins has been described in the jewel wasp, *Nasonia vitripennis* (Danneels et al., 2010).

Maternal effects have the potential to influence a wide range of ecological and evolutionary processes. Mothers can influence offspring traits in several ways (**Chapters 2, 3**), including per-capita provisioning (Dugas et al., 2016), host or oviposition-site selection (Mitchell et al., 2013), and hormone transfer (Sheriff et al., 2010). Maternal effects can lead to variations in offspring due to differences in energetic provisioning, proteins, nutrients, hormones, and toxins (Wolf & Wade, 2009). To understand the significance of maternal effects, studies have thus far mainly investigated either the total variance attributed to maternal effects or the specific non-genetic mechanisms through which mothers influence the traits of their offspring (McAdam et al., 2014). However, here maternal size did not influence offspring size of development time (days from oviposition-to-adult). If mothers do provision their offspring with i.e. venom, it is independent of maternal size, at least in *G. agilis*. (**Chapter 2**). Parasitoids utilize cues such as volatile, visual, and physical signals to assess host quality and make oviposition decisions. Factors such as host size, shape, color, and movement all play influential roles in this process (Henry et al., 2005; Mackauer et al., 1996).

Maternal oviposition decisions can also be affected by factors such as age and prior experience, which affect a female's preference for selecting the environment where her offspring will develop (**Chapter 2, 3, 4, 5**). This concept is defined by the preference-performance or "mother knows best" hypothesis (Gripenberg et al., 2010). Increased reproductive investment into hydropic eggs may negatively impact egg quality due to reduced energetic provisioning for embryos (bearing in mind that anhydropic eggs contain all of the pre-packaged proteins necessary for successful embryogenesis to hatching). The lifetime reproductive success of a female parasitoid is constrained by either host availability (or time) and egg production (Heimpel & Rosenheim, 1998; Keinan & Keasar, 2019). There are two primary theories regarding how organisms optimize their age-related reproductive investment: terminal investment and reproductive restraint. According to the terminal investment theory, organisms are predicted to increase their reproductive investment as they near the end of their lifespan (Clutton-Brock, 1984; Isaac & Johnson, 2005). Anhydropic eggs in most parasitoids can only be produced slowly and in limited quantities. Additionally, injecting these eggs onto or into the host requires considerable handling time for each egg (Harvey, 2008). In parasitoids that produce them, there is no evidence that an increase of investment into reproduction occurs towards the end of a female's life (see Harvey et al., 2019).

Body size is an important fitness proxy for parasitoids as it is generally positively correlated with longevity, host finding and fecundity (**Chapters 2, 3**). My study showed that the offspring development time was fairly uniform across a range of host sizes (**Chapter 2**). Body size affects demographic processes such as reproduction and longevity as well as host-finding and dispersal ability, extrinsic competition for hosts and mates and physical defense against other natural enemies such as predators (Charnov et al., 1981; Harvey et al., 1994). Moreover, it has been demonstrated in various studies that the size of parasitoid offspring is primarily determined by the size of the host (Qi et al., 2024; Santolamazza-Carbone et al., 2007; Ueno, 2015). However, body size and life span are showed to be negatively correlated with the ovigeny index (the ratio of the number of mature eggs at adult eclosion against the potential maximum number of mature eggs produced during a lifespan) in cross-species analyses (Abram et al., 2016; Ellers & Jervis, 2003; Jervis et al., 2003).

When hosts are scarce or difficult to find, selection might favour a reduction in egg production (so-called ‘r’ versus ‘k’ selection, Pianka, 1970). Research with parasitoids attacking different stages of the jack pine sawfly, *Neodiprion swanei*, supports this hypothesis (Price, 1972). Resources carried over to the adult stage from the larvae in parasitoid wasps, which fuel both ovigenesis and somatic functions, are determined by a combination of environmental factors. These factors include host size and quality, the duration of larval development, and the intensity of larval competition within the host. These environmental influences affect metabolic rates, lipid synthesis, and other physiological processes in developing parasitoids (Ismail et al., 2012; Moiroux et al., 2018).

## How do organisms make movement decisions?

Unlike most other insects, parasitoids rely on the limited resources contained within individual hosts for their development and reproduction. These hosts are often not much larger than the adult parasitoid itself (Godfray, 1994). Many species exploit resources distributed in distinct patches in the environment. Hyperparasitoids in the fourth trophic level like *G. agilis* and related species that are wingless but fairly host-specialized are under extremely intensive selection pressure to find and locate the cocoons of primary parasitoid hosts that are probably quite scarce. These factors may have driven the evolution of sophisticated strategies for locating their hosts in complex environments (Harvey et al., 2011). Organisms make behavioral decisions based on a variety of environmental cues and internal factors. Egg and pupal parasitoids in particular need to refine their host-searching ability for hosts within limited temporal developmental windows. Odor cues associated with the plant-host complex are crucial in guiding females toward their hosts. However, the response of parasitoids is not always consistent within the same genotype and can be also influenced by environmental factors. For parasitoids, foraging decisions are influenced by community diversity and complexity (**Chapters 3, 4, 5**). When ectoparasitoid idiobionts attack their hosts, venom will first be injected to paralyze the hosts to prevent the hosts from moving and developing which is essential to maintain optimal host quality (**Chapters 2, 3**).

In natural ecosystems, parasitoids forage in habitats that can be highly complex comprising diverse plant and herbivore communities (Dicke et al., 2009). Nonhost herbivores can affect the efficiency of host location by parasitoids, similar to various other biotic and abiotic factors (**Chapters 3, 4, 5**), both during their response to herbivore-induced plant volatiles (HIPVs) and their behavior on the food patch (Dicke et al., 2009; Gouinguené & Turlings, 2002). The presence of other herbivores in these habitats can strongly impact parasitoid foraging behavior at multiple levels. These levels range from finding the plant where the host is feeding to locating the host on the plant and deciding whether or not to parasitize the host, with each decision phase being crucial to parasitoid fitness (McArthur & Planka, 1966; Van Alphen et al., 2003). In my first field study, hyperparasitism rates were marginally, though not significantly higher in cocoons placed under hedgerows than in meadows (**Chapter 4**). I had initially assumed that denser vegetation would make it more difficult for hyperparasitoids to find host cocoons in meadows than in the more open bare ground under the hedgerows, but this was not the case. This reveals that gelines are more evenly distributed on the ground in some grass-dominated habitats. Then in my second field study, hyperparasitism rates were higher in forest than in production grassland or conservation grassland (**Chapter 5**) where forest consisted of more tree species which allowed the forest floor to be ‘open’ and less of an impediment for parasitoid movement.

In these two field experiments **(Chapters 4, 5),** cocoon retrieval rates were used as a proxy for predation. Given that the cocoons were placed under a combination of wire and nylon netting, they were only accessible to invertebrate predators such as ants, spiders, ground beetles and omnivores like slugs. Several trends were apparent. First, predation rates differed in meadows and hedgerows **(Chapter 4)** and were higher in the former habitat. Second, in the Maasheggen, cocoon retrieval rates decreased over the course of different seasons which indicated that predators were more active later in the year or predator density increased, which is the more likely explanation (**Chapter 5**). There was, however, no significance among the field types or the distance from the hedgerows. This might be due to predators not being as strongly influenced by habitat complexity as hyperparasitoids, or that different predator species are adapted to variable habitat complexity (**Chapters 4, 5**). When cocoon clusters were placed in the field for one week, they were sometimes attacked by several hyperparasitoid species, and more than one species (both winged and wingless) occasionally emerged from the same cocoon cluster, which reveals that there is close niche overlap and competition among these species. However, given that most cocoons were not parasitized, presumably because of the overall scarcity of hyperparasitoids, niche overlap and competition amongst the species is diluted (**Chapters 4, 5**). In some hyperparasitoids, such as the cryptine *Lysibia nana*, a solitary winged ectoparasitoid, females sometimes parasitize most or even all host cocoons in a single oviposition bout, a process that can take up to several hours (Harvey et al., 2006). The hyperparasitoid *Trichomalopsis apanteloctena* can monopolize and parasitize almost an entire brood of *Cotesia kariyai* cocoons over the course of up to 4 days (Takai et al., 2008). Functional responses to host availability are much slower than in *L. nana* and related species like *Acrolyta nens*, because their host handling times are much longer (Chen et al., 2019). This reduces the ability of gelines to monopolize cocoon broods of their hosts and opens up the possibility of resource partitioning amongst them.

## What are the physiological factors accounting for life-history trade-offs?

Life-history trade-offs arise from the finite resources that organisms can allocate to various functions such as growth, reproduction, and survival. The physiological factors underpinning the expression of trade-offs are rooted in the allocation of energy and nutrients to competing physiological processes. Life-history theory suggests that organisms must optimally trade-off limited energetic and time resources between life-history traits to maximize fitness (Roff, 2002; Stearns, 1998). Lifetime reproductive success in parasitoid wasps is constrained by the number of eggs available for oviposition at any given time and the time available for locating hosts (Heimpel & Rosenheim, 1998). A key trade-off in life-history strategies is the energetic cost associated with reproduction (Stearns, 1989). Based on this theory, in order to produce more offspring, *Gelis agilis* need to increase host-feeding bouts on *C. glomerata* cocoons to obtain sufficient nutrients for egg maturation, but this will come at the expense of longevity, as resources for somatic and germinal tissue are divided (**Chapters 2, 3**). *Gelis agilis* kills hosts on which it host-feeds, and selects others for oviposition (described by Jervis & Kidd [1986] as the 'destructive, non-concurrent strategy'). In **Chapter 3**, the number of cocoons presumably killed by host-feeding per day did not differ significantly among arena types in single Petri dishes. However, in the two-Petri dish arenas, host-feeding induced cocoon mortality was lower in the most complex maze set-up, showing that hosts are more likely to escape predation and parasitism once a critical complexity threshold is reached. This also has implications for the expression of life-history trade-offs between reproduction and survival.

Hyperparasitoids face extra constraints as a consequence of limited host resources and sparsely distributed than the herbivorous hosts of primary parasitoids further up the food chain (Brodeur, 2000; Harvey, 2008). When hosts are scarce and aggregated, selection can lead to divergent host exploitation strategies. If the primary constraint to a population's fitness is host availability, the evolution of increased longevity and/or foraging efficiency is to be favored by selection (Price, 1972; Rosenheim et al., 2010). In **Chapter 3**, offspring production was unaffected by maze complexity, which may have been because hosts and honey were in the same compartment. However, when these resources were spatially separated in two Petri dishes connected by a tube, reproduction and longevity were negatively affected, but only in the most complex (9-compartment) maze.

Egg limitation is a major constraint on the functional response of parasitoids like *G. agilis*. It has an OI of 0, must host-feed to mature eggs (meaning it is anautogenous) and can only mature a few eggs per day (**Chapters 2, 3**). Pro-ovigeny, or a high OI, maximizes the number of eggs available early in life but reduces the female's reproductive plasticity, limiting her ability to match egg supply to varying host availability during her lifetime. Conversely, a low OI (i.e. synovigeny), such as in *G. agilis* (**Chapters 2, 3**), means only a small fraction of the lifetime potential egg complement is available for laying at any one time, but it provides greater reproductive plasticity over the female's lifespan (Ellers et al., 2000; Jervis et al., 2001; Jervis & Ferns, 2004). Synovigenic parasitoids can adjust their egg loads in response to host availability, potentially reducing the risk of egg or host limitation (Ellers et al., 2000; Jervis et al., 2001). In **Chapter 3**, host-feeding induced cocoon mortality decreased with increasing arena complexity. When only cocoons were presented in one side of arena, longevity was greatly reduced compared to the other tube experiments. This strategy is clearly adaptive in stochastic environments and is the most common egg maturation strategy in parasitoid wasps (Jervis et al., 2001; Wajnberg et al., 2012). In the absence of hosts, synovigenic parasitoids that produce anhydropic eggs have been shown to ‘resorb’ proteins from the egg, a process known as ‘phasic castration’ (Rivero-Lynch & Godfray, 1997; Rosenheim et al., 2000; Sandlan, 1979). Resorption is a process that, when hosts are scarce, parasitoids reallocate egg proteins towards metabolic maintenance. Undoubtedly, *Gelis agilis* can resorb eggs as well when hosts are unavailable. Synovigenic parasitoids also benefit by not requiring heavy investment in egg production during pre-adult stages. Nonetheless, providing *G. agilis* females with fresh cocoons every two days enabled them to produce many more offspring than if they had been presented less frequently (**Chapter 3**). Other gelines in the field also presumably resorb egg proteins in the absence of hosts, thus extending their longevities as well.

## How do species traits and landscape configurations interact?

A core principle in landscape ecology is that spatial heterogeneity influences ecological systems (Wiens, 2002). In human-dominated regions, particularly in agricultural landscapes, it is crucial to understand how spatial heterogeneity affects biodiversity for effective conservation (Tscharntke et al., 2005). Species traits and landscape configuration interact in various ways to influence the foraging behavior and overall fitness of organisms. Structurally complex landscapes can harbor more species by providing additional resources and higher diversity, providing temporal refuges (Rusch et al., 2011; Tscharntke et al., 2012). Refuges have a stabilizing effect on predator-prey systems (Sih, 1987) and help to maintain community resilience and function. In **Chapters 2-4**, the variable survival of *C. glomerata* under different maze complexity or in different habitats show that structural complexity plays an important role in affecting the susceptibility of the parasitoid cocoons – which were ostensibly defenceless – to both hyperparasitoids and predators. The persistence of trophic interactions depends crucially on the survival of enough hosts/prey to sustain higher trophic levels, which refuges provide. At the same time, some of the host/prey population need to be vulnerable to their natural enemies for them to be able to persist as well. Insects forage in habitats of variable complexity where resources are interspersed with non-resources. The interplay between species traits and landscape configuration significantly influences foraging behaviour and overall fitness. Prior experience and learning are vitally important in shaping foraging strategies, because this allows animals to collect, store, and utilize information effectively (Eliassen et al., 2009).

Parasitoids are especially vulnerable to agricultural intensification due to their small size, limited dispersal capabilities, high host specificity, and reliance on diverse habitats for both arthropod hosts and floral resources (Landis et al., 2000). Parasitoids forage for hosts to lay their eggs, and thus foraging behavior is directly linked to fitness, making them ideal model organisms for studies of optimal foraging theory (Thiel & Hoffmeister, 2009; van Baalen & Hemerik, 2008). For parasitoids, the quality of their environment is determined by both the suitability and abundance of hosts, which fluctuates across different seasons (Vercken et al., 2015). Resources like nectar, that are also vitally important for parasitoid maintenance and enhance longevity, can also vary over time. Once host and food resources become highly limiting, this can negatively affect their performance. The lack of suitable sources of carbohydrates in simple habitats, such as cropping systems or semi-urban landscapes, can have significant impacts on insects like parasitoids. As I demonstrated here, in tiny microcosms (Petri dishes), when females were provided only with honey plus water in the two-dish setup, longevity was reduced significantly (**Chapter 3**). Longevity of females in the 9-compartment setup was almost halved compared to the single and 4-compartment arenas. I would imagine that natural systems are often much more complex with resources much more profoundly partitioned than in my most complex maze. This gives some indication what amazing organisms gelines are, to be able to exist and thrive as they do across Dutch landscapes.

Phenotypic plasticity enables a single genotype to generate a variety of phenotypes in response to different environmental conditions (Auld et al., 2010). For instance, alternative life histories can emerge due to maternal effects, also known as transgenerational plasticity. In this process, the proportion of individuals exhibiting a particular phenotype is influenced by the environmental conditions experienced by the previous generation (Mousseau & Dingle, 1991). Learning through prior experience is crucial in shaping animal foraging behavior, as it allows animals to gather, store, and use information (Eliassen et al., 2009). Parasitoid wasps are renowned for utilizing associative learning to enhance their foraging efficiency. By linking environmental cues, such as herbivore-induced plant volatiles, to the presence of hosts, female wasps can effectively locate subsequent plants that harbour potential hosts (Kruidhof et al., 2015; Papaj & Vet, 1990). In **Chapter 3**, I found that *G. agilis* was quite adept at finding hosts in even the most complex mazes. This could be because *G. agilis* learns from their previous foraging experience, and then utilized this memory to locate hosts later placed in the same compartments in the arenas.

Parasitoids have developed behavioural adaptations to manage both intra- and interspecific competition for hosts. These adaptations serve as indicators of resource availability and help assess the risk of superparasitism. By observing these behaviours, researchers can infer the density and quality of available hosts as well as the competitive pressures within the parasitoid community (Brodeur & Boivin, 2006; Cusumano et al., 2016). My study provided new insights into how animals use previously obtained information in complex foraging situations, confirming that the costs and benefits of learning depend on the environment in which animals forage (**Chapters 3, 4, 5**). Despite the challenges of studying small animals in natural habitats, it is evident that dynamic conditions in nature have led to the evolution of behavioural traits allowing animals to use information on local circumstances and adjust their behaviour accordingly, such as through learning. However, existing studies have thus far offered limited detail on how many hyperparasitoids interact with their environment in the field which led to my **Chapters 4 and 5**.

Two studies on *C. glomerata* and its hyperparasitoids indicated that host quality is a linear function of host age (Harvey et al., 2006; Chen et al., 2019). About 60 hours after cocoon formation, the parasitoid pupae undergo significant developmental changes, such as tissue differentiation in wings, antennae, and legs, and cuticle sclerotization. These changes make host tissues less palatable for hyperparasitoid offspring. In specialist host-parasitoid-hyperparasitoid systems, resources become less abundant to foraging insects at higher trophic levels up the food chain. It’s hypothesized that hyperparasitoids are involved in more complex foraging decisions involving multiple steps to locate food plants and hosts (Poelman et al., 2012). This intricate process underscores the importance of examining landscape complexity to better understand more complex and higher trophic level communities (Pak et al., 2015).

Agricultural lands are often viewed as ‘biodiversity deserts’. However, high-quality agricultural matrices play a crucial role in the migration and persistence of biodiversity (Perfecto et al., 2019; Vandermeer et al., 2010). In my study, field locations were chosen close to hedgerows and included production grassland that functions as a food source for overwintering livestock. When cocoon clusters were placed into these fields for one week, many were predated. The proportion of retrieved cocoons varied significantly between meadows and hedgerows, with a higher predation rate observed in meadows. These cocoons could be preyed by predators such as ants, spiders and slugs (**Chapters 4, 5**). The fields all consisted of hedgerows, and it has been shown that increased field margins greatly enhance slug activity (Eggenschwiler et al., 2013). Most plants, which are insect-pollinated, provide essential floral resources like nectar and pollen, serving as critical nutritional sources for adult parasitoids. Additionally, both forest and grassland habitats often host a diverse array of arthropod species, providing abundant and varied hosts.

## Summary and future perspectives

Biodiversity hotspots have lost more than 70% of their natural habitat (Myers et al., 2000), prompting the need for biodiversity conservation within the context of "eco-agriculture." This includes creating integrated conservation-agriculture landscapes (Scherr & McNeely, 2008). The biodiversity of an agroecosystem is maintained at different levels across various landscape components. Some generalist species, like ladybeetles, can occur throughout the entire landscape (Magagula & Samways,

2001) while other species cannot. Conservation management should consider environmental complexity at multiple scales. Enhancing naturally occurring populations of biocontrol agents, such as parasitoid wasps, could reduce reliance on synthetic insecticides. As the social, environmental, and economic costs of synthetic pesticides rise, biological control becomes increasingly important. Proactive management of farms and landscapes to benefit natural enemies is a cost-effective pest control strategy with numerous environmental and social benefits (Kremen & Miles, 2012).

Parasitoids have long been recognized as effective biocontrol agents against insect pests and have been widely employed for this purpose (Van Lenteren et al., 2018). When biological control efforts fail, the reason could be that the role of natural enemies in regulating host populations within complex food webs has been overlooked (Goldson et al., 2014). Intraguild interactions among parasitoid species—species that share the same host—illustrate the complexity of food web dynamics and the challenges in developing successful biological control programs (Traugott et al., 2008). Hyperparasitoids are specific natural enemies of parasitoids, and in agriculture, they are seen as the 'bad guys' because they parasitize the parasitoids that are used as biocontrol agents (Cusumano et al., 2020; Poelman et al., 2022). Hyperparasitoids play a significant top-down role in shaping multitrophic communities (Poelman et al., 2022). The influence of higher trophic levels, such as hyperparasitoids, on pest population regulation has often been overlooked compared to other species interactions, such as competition. Hyperparasitoids act as top carnivores and can significantly disrupt biological pest control by reducing host parasitoid populations, leading to pest outbreaks (Cusumano et al., 2020). They impact their host parasitoids in several ways. Firstly, they parasitize the host offspring. Sixteen species of hyperparasitoids were found to attack *Cotesia melanoscela*, causing almost 50% mortality of the primary parasitoid (Cusumano et al., 2020). Hyperparasitism of *Cotesia rubecula*, a parasitoid of *Pieris rapae*, was up to 40% in collard fields in west Virigina

(Mcdonald & Kok, 1991). Host-feeding parasitoids often kill many more hosts than they parasitize, suggesting that idiobiont hyperparasitoids may kill far more primary parasitoids than has thus far been reported.

This study system is fascinating because traits found in gelines—such as winglessness, low fecundity, and low dispersal capacity—make them ideal models for studying fundamental ecological questions. However, among the millions of species coexisting on Earth, little is known about their biology and the ecological roles they pay in the functioning of ecosystems, all while a number of human activities continue to simplify and destroy natural systems with alarming speed. Beyond economically important species, research should also focus on those species that do not directly interact with humans but are crucial components of healthy, functioning ecosystems, such as species in higher trophic levels, hyperparasitoids. Future research needs to expand our knowledge of the many intriguing aspects of nature, helping humanity appreciate the critical niches filled by various species that might otherwise remain unstudied. The field of ecology therefore needs to embrace diversity in its most holistic forms, and to look beyond economic valuation and societal relevance to better understand and appreciate the importance of all living things, from microbes to whales, including even the hyperparasitoids.

## Conclusion

In this discussion, I investigated development, reproduction and biology of geline hyperparasitoids related to factors such as maternal provisioning, habitat heterogeneity and spatially segregated resources. Firstly, I studied maternity and host size effect on offspring development and found that there is strong relationship between host size and offspring size, independent of maternal size. Then to explore hyperparasitoid foraging, maze arenas with different complexities were designed to mimic natural complexity at small scales. Hyperparasitoids efficiently located food resources in even more complex habitats, but there were trade-offs in life-history traits found. To better understand how hyperparasitoids survive in more complicated habitats, field experiments were conducted. One study focused on hedgerows and meadows over two consecutive years at a localized small scale. Then more expansive habitats were chosen with three field types: production grassland (PG, intensively managed), conservation grassland (CG, extensively managed) and a mature deciduous forest. All were bordered by hedgerows and extended into grasslands or open fields. Both field studies showed that there was significant niche overlap among the gelines and that different species co-existed and exploited the same cocoon cluster. Dominant species changed over time and there was more predation of host cocoons in meadows. More hyperparasitoids emerged from cocoons placed under hedgerows, probably because these were more open (as in forests).

These studies show that habitat structure and complexity influences hyperparasitoid diversity and abundance. This study linked aspects of life history and trait expression in species in higher trophic levels, specifically *Gelis* hyperparasitoids, to local habitat and landscape-level complexity. It also enhanced our understanding of interactions between primary parasitoids and hyperparasitoids, and revealed how resilient these organisms are in nature, perhaps explaining why the genus is quite speciose in western Europe. In summary, my study demonstrates that geline hyperparasitoids are abundant and ubiquitous in Dutch landscapes. Moreover, the research highlights the significance of hedgerows as habitats for little-studied organisms such as wingless hyperparasitoids. This study enhances our understanding of the role habitat structure and type play in maintaining populations of these organisms.

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## Summary

Life history theory underpins our understanding of trait expression in organisms, with particular emphasis paid on the evolution and expression of reproductive strategies. Unlike most insect predators, foraging and reproduction are directly linked in parasitoid wasps, and different resources are used for metabolic maintenance and reproduction. For instance, parasitoids reproduce by laying their eggs on, or in the bodies of other arthropods, whereas the adults are free-living and obtain nutrients from sources like floral nectar. Some parasitoids are wingless and have limited dispersal ability compared with fully-winged species. These species are especially challenged in habitats where hosts are scarce or where they are spatially separated from food. Many factors influence parasitoid life-history traits such as development, reproduction and survival, and include host quality and offspring paternity, although the latter parameter has thus far been little-studied. Furthermore, host availability profoundly affects the evolution of traits in parasitoids, and this is in turn affected by factors such as host stage attacked and environmental heterogeneity. Habitat complexity at different scales, for instance, is an important component which greatly influences the dispersal ability in insects, and it can also affect the ability of parasitoids to find and exploit their hosts. At the same time, habitat heterogeneity is an important factor stabilizing the dynamics of predator-prey and host-parasitoid interactions, by providing refuges that prevent the overexploitation of one trophic level by another. Hyperparasitoids, in the fourth and higher trophic levels, make excellent model organisms for examining various life-history theories in organisms.

In my thesis, I tested research questions related to the expression of life-history traits in hyperparasitoids through both laboratory experiments (**Chapters 2, 3**) and field studies (**Chapters 4, 5**). In lab experiments I used the solitary asexual species, *Gelis agilis*, a specialist secondary hyperparasitoid of primary parasitoid cocoons (*Cotesia glomerata*). Maternal and host effects on development were tested using different sizes of both mothers (large, small) and host cocoons (along a continuum).

Offspring size was set as the proxy for fitness. Habitat effects on foraging and survival were investigated in the laboratory by designing arenas with increasing complexity, in single Petri dishes with mazes and tube-connected ‘double arenas’, also with mazes. I also placed cocoon clusters of *C. glomerata* into different field habitats and monitored both the identity and number of emerging hyperparasitoids but also cocoon mortality and survival.

In **Chapter 2**, I examined the effects of both maternal and host size on offspring in the laboratory by manipulating and offering different sizes of cocoons to mothers in two size classes daily. I found that host size, and not maternal size, affected offspring size and development time. This reveals that larger mothers are unable to enhance offspring size by possessing better provisioning ability, through the quality or quantity of factors like venom. These results underpin the importance of host size on parasitoid development.

In **Chapter 3**, I designed foraging arenas for *G. agilis* in which small-scale habitat complexity was manipulated through the insertion of specially-constructed plastic mazes. Hosts and/or food plus water were placed into different ‘compartments’, and female wasps were allowed to search for them over two-day periods. I aimed to see if it was possible to generate trade-offs between fitness related factors such as reproduction and survival in mazes of varying complexity. The results showed that in single arenas the foraging and reproduction in *G. agilis* was unaffected by maze complexity. Even in 16-compartment arena where food and host resources were separated into different compartments, hyperparasitoids could efficiently utilize both resources for survival and reproduction. However, when arenas were more complicated with a tube connecting two Petri dishes, and the 16-compartment were designed with indirect access to two of the cocoons, daily fecundity decreased, and early mortality was higher. Significantly fewer offspring were produced in more complex arenas compared to simpler ones. Thus, even in small-scale microcosms, it is possible to create sufficient complexity to compromise maximum potential performance in *G. agilis*.

**Chapters 4 and 5** were field studies that aimed to compare *Gelis* species assemblages in natural habitats and to more explicitly test how habitat heterogeneity influences hyperparasitoids over the course of different seasons. Two types of fields were selected: a local, small-scale field that consisted of elevated hedgerows and grass-dominated meadows, and a larger, and a much older site (Maasheggen) with a network of interconnected habitats via hedgerows of buckthorn and hawthorn that is a UNESCO biosphere reserve. There, three habitats present are production grassland (managed for livestock feed), conservation grassland (managed to create floral diversity) and a mature deciduous forest adjacent to a wetland. In the first field study, I found that more *C. glomerata* cocoons were removed by invertebrate predators in the meadow, whereas marginally more hyperparasitoids emerged from cocoons placed under the hedgerows. Most hyperparasitoid species were wingless gelines (Cryptinae), and sometimes two species emerged from the same cocoon cluster, indicating some niche overlap amongst them. In the Maasheggen, a higher proportion of host cocoons were removed by predators in the production grassland whereas more hyperparasitoids emerged from cocoons placed inside the forest and under the hedgerows. This suggests two things: first, that hosts are more likely to be found in nominally 'woody' habitats, or second, that open habitats reduce impediments on foraging for wingless gelines.

In summary, my thesis demonstrated that host (and not maternal) size was the main determinant affecting offspring size, highlighting the critical role of host resources in hyperparasitoid development. My research also revealed that habitat complexity influences the foraging efficiency, dispersal ability and species composition of hyperparasitoids that live mostly on the ground. More complicated habitats impede dispersal for wingless species. Additionally, different hyperparasitoid species clearly share small-scale habitats and co-exist, although there are seasonal gradients in the abundance of different species. This thesis provided me with detailed information on the natural constraints affecting the development, reproduction, and survival of hyperparasitoids, with *G. agilis* being a model species for lab-based studies. Furthermore, the field studies elucidated seasonal and habitat-related differences within the *Gelis* species community. These findings offer valuable insights into higher trophic level species and their interactions with their resources and the environment. It is also hoped that they will stimulate more research on what are often perceived to be innocuous little organisms, Wbut which are abundant and ubiquitous in nature.

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## About the author

Xianhui Shi was born on 6th May 1993 in Qixia city, Shandong Province, China. Growing up in a village where every family cultivated apple trees for decades, she spent considerable time in nature and developed a deep obsession with it. She started her bachelor in 2012 in Shandong Agricultural University studying Forest Protection, where she growed her intrigue in insects. To further explore the fascinating world of insects, she pursued her master in Forest Protection in South China Agricultural University in Guangzhou in 2016, under the supervision of prof. Xiujun Wen. Her research focused on forest pests and chemical communication in moths. She conducted fieldwork in several mountainous regions in southern China, which expanded her understanding of ecology and biocontrol. During her master's project in 2018, she received a scholarship to visit Rutgers University in New Jersey, USA, supervised by prof. Changlu Wang. Her journey there focused on urban pests such as cockroach and bed bug in apartments in both New Jersey and New York. This journey broadened her academic perspective. After completing her master's, she worked as a research assistant for a year, engaging in various fieldwork projects that deepened her love for nature.

Inspired by her research experiences, Xianhui decided to pursue a PhD abroad to delve deeper into the study of insects. She secured funding from the China Scholarship Council to continue her studies at Wageningen University. She began her PhD in October 2020 and started a new project at NIOO-KNAW in December 2021, supervised by prof. Wim van der Putten, prof Jeffrey Harvey, and dr. Jetske de Boer. Her project, entitled "Nature is a Maze for the Hyperparasitoids" explored higher trophic levels in food webs, providing her with a broader view of science.

## List of publications

Shi X., Gols R., de Boer J.G., Harvey J.A. 2024. Host size overrides maternal effects on development of a secondary hyperparasitoid wasp. Under review.

Shi X., Gols R., de Boer J.G., Spee J., Hanum I, Pelaez-Plazas S.A., da Cruz Freitas J, Harvey J.A. 2024. Heterogeneous microcosm mazes affect reproduction and survival in a wingless hyperparasitoid wasp. Under review.

Shi X., Gols R., de Boer J.G., Yang Q, Harvey J.A. 2024. Seasonal differences in rates of predation and hyperparasitism of parasitoid cocoons in meadows and adjacent hedgerows. Submitted.

Shi X., Gols R., de Boer J.G., Mittelstorb E., Connolly C.M. , den Ouden A, Yang Q, Harvey J.A. 2024. Temporal variation in predation and hyperparasitism rates of parasitoid cocoons in three habitats bordered by hedgerows. Submitted.

Shi X., Ma T., Zhang S., Sun Z., Chen X., Wang C., Jia C., Liang Y., Zhu Y., He Y., Wen X. 2018. Calling and mating behavior of *Diaphania angustalis* (Lepidoptera: Crambidae). Journal of Economic Entomology, 111(5): 2250-2254.

Ma T., Shi X., Shen J., Wang C., Zhang S., Lu X., Sun Z., Chen X., Wang C., Xie W., Wen X. 2018. Field Evaluation of Commercial Attractants and Trap Placement for Monitoring Pine Sawyer Beetle, *Monochamus alternatus* (Coleoptera: Cerambycidae) in Guangdong, China. Journal of Economic Entomology, 111(1):239-246.

Ma T., Lin N., Shi X., Zhou L., Zhou Q., Wang C., Zhang S., Liu Z., Sun Z., Chen X., He Y., Wen X. 2018. Female *Diaphania glauculalis* (Guenee) calling behavior diel rhythm and temporal change in pheromone. Journal of Entomological Science, 53(4): 407-421.

## PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE & RC) which comprises of a minimum total of 30 ECTS (=22 weeks) of Training and Education activities have been collected.

### Review/project proposal (6 ECTS)

- The effect of context and trait-dependent factors on development and reproduction of the secondary parasitoid, *Gelis agilis*

### Post-graduate courses (7.2 ECTS)

- Comparative Biology & Systematics, Biosystematics (2021)
- What’s bugging you, PE&RC (2023)
- Bridging Science to Practice: Exploring and developing Decision Support Systems for Ecological Intensification of Agricultural systems, PE&RC (2024)
- Linear Models, PE&RC (2023)
- Introduction to R and R Studio, PE&RC (2023)
- Generalized Linear Models, PE&RC (2023)

### Deficiency, refresh, brush-up courses (0.6 ECTS)

- Searching and organising literature for PhD candidates (2021)
- Intercultural communication training (2021)

### Competence, skills and career-oriented activities (3.9 ECTS)

- Critical thinking and argumentation, WGS (2021)
- Project and Time Management, WGS (2021)
- Presenting with impact, WGS (2021)
- The Essentials of Scientific Writing, WGS (2022)

### Scientific Integrity/Ethics in science activities (0.6 ECTS)

- Scientific Integrity, WGS (2021)

## PE&RC Annual meetings, seminars and PE&RC weekend/retreat (1.8 ECTS)

- PE&RC First years retreat (2021)
- PE&RC Midterm retreat (2022)
- PE&RC Day (2023)

## National scientific meetings, local seminars, and discussion groups (5.7 ECTS)

- Wageningen Evolution and Ecology Seminars (2020-2024)
- Nederlandse Entomologendag-Sectie Entomologendag (2020-2024)
- NLSEB Annual Meeting (2023)
- Netherlands Annual Ecology Meeting (2022)
- Open Science Festival (2021)
- Symposium “The Anthropocene biosphere - transforming for sustainable future” (2022)

## International symposia, workshops and conferences (5 ECTS)

- XII European Congress of Entomology, Crete (2023)
- 7th European Congress of Conservation Biology “Biodiversity positive by 2030”, Bologna (2024)

## BSc/MSc thesis supervision (6 ECTS)

- Research topic 1: The effect of small-scale spatial complexity on host finding and reproduction of the hyperparasitoid *Gelis agilis* (Hymenoptera: Ichneumonidae)
- Research topic 2: The impact of increasingly complex environments on longevity and reproductive success of *Gelis agilis* (Hymenoptera: Ichneumonidae)
- Research topic 3: Trade-off between reproductive investment and longevity in the hyperparasitoid wasp *Gelis agilis*
- Research topic 4: Competition within Dutch hyperparasitoids for *Cotesia glomerata* in a semi-urban environment
- Research topic 5: The trade-off in longevity and reproductive investment for the hyperparasitoid *Gelis agilis* in different habitat complexities
- Research topic 6: Species and abundance of the hyperparasitoid wasp of the genus Gelis in different microhabitats
- Research topic 7: Tertiary hyperparasitism in a Generalist pupal hyperparasitoid, *Gelis agilis* (Hymenoptera: Ichneumonidae)
- Research topic 8: Prevalance of hyperparasitoid in different habitat

## Colophon

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