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# Spatial and temporal diversity in hyperparasitoid communities of *Cotesia glomerata* on garlic mustard, *Alliaria petiolata*

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**Abstract.** 1. Interactions between two trophic levels can be very intimate, often making species dependent on each other, something that increases with specialisation. Some specialised multivoltine herbivores may depend on multiple plant species for their survival over the course of a growing season, especially if their food plants are short-lived and grow at different times. Later generations may exploit different plant species from those exploited by previous generations.

2. Multivoltine parasitoids as well as their natural enemies must also find their hosts on different food plants in different habitats across the season. Secondary hyperparasitoid communities have been studied on cocoons of the primary parasitoid, *Cotesia glomerata* (Hymenoptera: Braconidae), on black mustard (*Brassica nigra*) – a major food plant of its host, the large cabbage white (*Pieris brassicae*) – which grows in mid-summer.

3. Here, hyperparasitoid communities on *C. glomerata* pupal clusters were studied on an early-season host, garlic mustard, *Alliaria petiolata*, over ‘time’ (one season, April–July) in six closely located ‘populations’ (c. 2 km apart), and within two different ‘areas’ at greater separation (c. 100 km apart). At the plant level, spatial effects of pupal ‘location’ (canopy or bottom) on the plant were tested.

4. Although large-scale separation (area) did not influence hyperparasitism, sampling time and small-scale separation (population) affected hyperparasitism levels and composition of hyperparasitoid communities. Location on the plant strongly increased proportions of winged species in the canopy and proportions of wingless species in bottom-located pupae.

5. These results show that hyperparasitism varies considerably at the local level, but that differences in hyperparasitoid communities do not increase with spatial distance.

**Key words.** *Alliaria petiolata*, *Cotesia glomerata*, hyperparasitism, parasitoid, *Pieris brassicae*, resource partitioning.

## Introduction

Natural communities are made up of different species of organisms that often influence (or interact with) each other, often in large networks or food webs consisting of multiple trophic levels. Many species of consumers (e.g. herbivores and their

natural enemies) are highly specialised and therefore exploit only a small range of resources in the trophic levels beneath them (Loxdale *et al.*, 2011; Ali & Agrawal, 2012; Loxdale & Harvey, 2016). In many ecological communities, specific kinds of resources are utilised by multiple species. This may generate competition among them, particularly if these resources are scarce or limiting (Hairston *et al.*, 1960). Ecological theory predicts that when two highly similar species co-occur on the same resource, one will eventually be outcompeted by the stronger species. However, there are numerous examples in nature where multiple species are effectively able to partition

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the same resource and thus to coexist (Inouye, 1978; Peck, 1989; Kasahara & Katoh, 2008). For example, bumblebees alter their foraging strategies in flower meadows to a narrower range of species, when a competing species of the same genus is present in the same area (Inouye, 1978). Peck found that insectivorous bird species in Great Britain coexist in the same habitats by occupying different microhabitats along a spatial (vertical) gradient in the forest canopy. Moreover, kingfishers in the Neotropics coexist by exploiting fish prey of different sizes in different habitats (e.g. from forest pools to lakes), which, in turn, correlates with kingfisher body size (Remsen, 1991; Kasahara & Katoh, 2008).

Parasitoid wasps are hymenopterous insects that lay their eggs in or on the body of an insect host, consuming and killing them in the process (Godfray, 1994; Quicke, 2014). As they are often quite specialised and intimately tied to a narrow range of insect hosts, this makes them an interesting group of organisms in which to study coexistence processes. Insect herbivores may be hosts to many parasitoid species (e.g. Price, 1972). Inevitably, there may be strong competition among parasitoid assemblages for the limited resources of insect hosts under certain conditions. Not surprisingly, parasitoids deploy resource-partitioning strategies to enable coexistence and species survival (Price, 1970, 1974; Mopper *et al.*, 1990). For example, many insect herbivores harbour parasitoid ‘guilds’ consisting of species that are specialised in attacking different host stages, e.g. eggs, larvae or pupae (Godfray, 1994; Quicke, 2014). Furthermore, most species of primary parasitoids are attacked by hyperparasitoids that also occur in two guilds. Primary hyperparasitoids attack the parasitoid larvae inside the body of the herbivore host, whereas secondary hyperparasitoids attack the pre-pupae or pupae of the primary parasitoid once it has emerged from the herbivore host. Both examples represent a kind of ‘temporal partitioning’ of the host life cycle between different parasitoid species (Godfray, 1994), which is often necessary for multiple species to coexist on one host species (Haigh & Smith, 1972).

Another striking example of a community and its variability can be found in the many species of secondary hyperparasitoids attacking pupae of *Cotesia* species (Weseloh, 1978, 1986; Stefanescu *et al.*, 2009; Harvey *et al.*, 2014). A recent study revealed that small-scale spatial as well as temporal aspects play an important role in the use of resources (being the parasitoid pupae) by the various secondary (pupal) hyperparasitoids of the large cabbage white butterfly parasitoid (*Cotesia glomerata* L.). In nature, *C. glomerata* typically lays broods of between 15 and 50 eggs into young larvae of *Pieris brassicae* and these are, in turn, attacked by as many as 10 species of secondary hyperparasitoids (Poelman *et al.*, 2012; Harvey *et al.*, 2014). Harvey *et al.* (2014) found that pupal clusters of approximately 30 *C. glomerata* placed near the ground on black mustard (*Brassica nigra* Koch) plants attracted mainly wingless parasitoids, whereas cocoons placed higher up in the canopy attracted mostly winged species. Moreover, the composition of hyperparasitoid assemblages varied throughout the growth season, with distinct peaks for the more specialised species and a seemingly more constant presence, but low abundance, of more generalistic species (Harvey *et al.*, 2014).

An important factor that underpins multitrophic interactions is phenology. Phenological events are especially important in specialised (or oligophagous) insect herbivores that have multiple generations per growing season, and even more so on species that feed on short-lived annual plants. After a first generation of insects, the initial food plant may be senescing, or may no longer be present. As a result, even oligophagous multivoltine herbivores often have no other choice than to deposit eggs on a different (but closely related) host plant from the one on which they developed. For example, specialist flea beetles feeding on brassicaceous hosts have been shown to switch hosts throughout the season, choosing winter cress (*Barbarea vulgaris* Aiton) as early- and late-season host plants to feed on and overwinter near to, whilst feeding on other available brassicaceous hosts during the summer months (Root & Tahvanainen, 1969). As insect communities vary between individual plants (Mooney & Agrawal, 2008) and vary even more strongly between plant species, and this, in turn, affects insects higher up the food chain (e.g. Lill *et al.*, 2002), it is likely that such host switches may influence hyperparasitism.

The large cabbage white butterfly, *P. brassicae* L. is a multivoltine herbivore that has up to three generations per year in northern Europe and four in the south (Feltwell, 1982). It develops on several brassicaceous host plant species that are usually large and grow in dense, tightly packed assemblages, which is a prerequisite for survival because a brood may require at least several plants to sustain their complete larval development (Chew & Renwick, 1995; Fei *et al.*, 2014, 2016). Because the food plants of *P. brassicae* are mostly ephemeral (fast-growing) annuals and biennials and are found in discrete parts of the growing season (April–October), the herbivore depends on multiple host plant species for its subsequent generations (Fei *et al.*, 2016; Heinen *et al.*, 2016). Black mustard, which was used in previous studies, is an important host plant (Blatt *et al.*, 2008) that grows in assemblages and can be found in the field in mid-to-late summer (e.g. July–August). The cabbage white butterfly’s specialised gregarious parasitoid, *C. glomerata*, is also multivoltine and has two to three generations per year in the field (Clausen, 1940; Van Der Meijden & Klinkhamer, 2000). Early generations of *P. brassicae* are present in the field from late April, long before black mustard is present. The only suitable wild brassicaceous host plants that are present at this time are field mustard, *Brassica napa* and garlic mustard, *Alliaria petiolata* (Bieb.). *Pieris brassicae* caterpillars have been found feeding on *A. petiolata* in the field (e.g. J. A. Harvey, pers. obs.). Additionally, cocoon clusters of *C. glomerata* and a primary hyperparasitoid, *Mesochorus gemellus*, have also been collected from this plant species (Harvey *et al.*, 2016).

Here, we examine the composition of secondary hyperparasitoid communities on *C. glomerata* pupal clusters placed on *A. petiolata* over the course of one growing season during which time the plant is found naturally in the field (April–July). Cocoon clusters of *C. glomerata* were placed on garlic mustard plants in naturally occurring populations in two different parts of the Netherlands, at different heights (ground versus canopy) and in six different forest/forest edge locations, in order to follow hyperparasitoid community composition on an early brassicaceous host plant through time. We hypothesised: (i)

that different garlic mustard populations will harbour different parasitoids and that hyperparasitoid communities would differ more on plants between than within the two areas (Amsterdam and Wageningen, the Netherlands); (ii) that the composition of hyperparasitoid communities would change over the course of a growing season, with species occupying specific temporal niches; and (iii) that there would be an effect of cluster placement on the host plant on the functional types that dominate the respective hyperparasitoid communities; cocoon clusters placed at the base of the host plant would yield more wingless species, and those placed at the top would yield more winged species.

## Materials and methods

### Plants

Garlic mustard (*A. petiolata*) is an herbaceous plant native to Eurasia. In the Netherlands, it grows abundantly in patches along roadsides and often under thick forest canopy. It prefers shady habitats. Garlic mustard is a biennial plant that forms a rosette in the first year, which dies during winter. A new rosette is formed in the second year, from which a flower stalk grows, usually from late April. Garlic mustard flowers and sets seed from April to early July, after which the plant usually starts senescing. Garlic mustard is also highly invasive in large parts of North America, where it lacks natural enemies and outcompetes native plants (Cavers *et al.*, 1979).

### Insects

*Pieris brassicae* L. is a multivoltine butterfly species that is a specialist of brassicaceous host plants. It can have three to four generations per year, depending on the conditions in the field. The butterfly lays eggs in clusters, often producing clusters of 50 eggs or more. During their larval stages, the caterpillars feed gregariously, often stripping host plants entirely. *Pieris brassicae* is dependent on plants that grow in assemblages, in order to provide the biomass needed to fulfil the larval development. The butterflies can often be found from April to October. *Pieris brassicae* is the preferred host of the parasitoid *C. glomerata*.

*Cotesia glomerata* were maintained in a climate chamber at  $22 \pm 2$  °C at the Netherlands Institute of Ecology (NIOO) and were reared from *P. brassicae* feeding on *Brassica oleracea* var. *Cyrus* (Harvey, 2000). *Cotesia glomerata* usually deposits 20–40 eggs in L1–L3 caterpillars. The larvae develop in the living host, feeding on host tissue until the host reaches its final instar. The mature larvae chew their way out through the caterpillar's cuticle and spin cocoons in clusters in close proximity to the dying host (Harvey, 2000). New clusters were collected from the cage daily and stored in the refrigerator. *Pieris brassicae* were kindly provided by the insect-rearing facilities at Wageningen University. Both the herbivore and its parasitoid were originally collected from agricultural fields around Wageningen University and had been in culture for many years.

### Experimental protocol

To test the effects of large- and smaller-scale spatial separation of populations on the parasitisation of *C. glomerata* by hyperparasitoid wasps, three populations in the area of Wageningen (Bennekom, Wageningen Hoog, and Wageningen Zoom) and three populations in the area of Amsterdam (Amsterdamse Bos forest, Amsterdamse Bos parking and Amsterdamse Bos road) were selected in which *A. petiolata* patches were abundant during the spring of 2015. The populations within each area were roughly 2 km apart (see Supporting information, Fig. S1a,b). The two sampling areas were roughly 100 km apart and climatically quite different. Amsterdam lies close to the North Sea and, as such, generally experiences a milder climate, with relatively higher winter temperatures and lower summer temperatures. By contrast, Wageningen is located further away from the coast and, as a result, often has relatively lower winter temperatures and higher summer temperatures. Garlic mustard generally grows best in half-open to shaded conditions. The populations within each area differed in the amount of sun received, with one population being fully shaded and two being in half-open areas that received sunlight throughout parts of the day. In each population, at every time point (every 2 weeks), 10 random plants were selected and labelled. In the first repetition (April), one *C. glomerata* cocoon cluster was attached only to the basal rosette of each garlic mustard plant as, in this stage, the plants had not yet developed flowering stalks. For the population in Wageningen Zoomweg, this was repeated in the second temporal replicate as plants were still only rosettes (see Table S1 for full sampling scheme). In all following temporal replicates (May–June), an additional cluster was placed on the stems of each plant at a height of 40–60 cm, depending on the height of the plants. Parasitoid clusters were left in the field to be hyperparasitised for 1 week. The cocoon clusters were collected and placed in a climate chamber at  $22 \pm 2$  °C upon emergence of wasps. The emerging species were identified using a stereomicroscope. The cocoons in each cluster were counted after the experiments, because tight clustering makes it impossible to count them without separating the pupae and destroying the cluster's integrity. On average, a cocoon cluster contained around 20 individual pupae (specific numbers per location are given in Table 2). The experiment was replicated every 2 weeks until the garlic mustard shoots died, which happened after the late June sampling.

### Data analysis

*Effects of sampling time and interactions with smaller-scale separation (population) and larger-scale separation (area) on parasitism of Cotesia glomerata on garlic mustard.* To investigate the main effect of time and the interactions among time, area and population, we tested their effects on 'proportion of pupal clusters hyperparasitised', 'number of winged hyperparasitoids', 'number of wingless hyperparasitoids', and 'number of species' using a generalised linear model with time, area and population and their interactions as fixed effects. Proportional data were fitted with binomial error distribution (link = logit) and count data with Poisson error

**Table 1.** An overview of the species of hyperparasitoids recorded, with their observed occurrence in the season and their life-history strategy.

| Species:                  | Morphology | Occurrence              | Life-history strategy | Numbers |
|---------------------------|------------|-------------------------|-----------------------|---------|
| <i>Pteromalus semotus</i> | Winged     | Late June               | Large number of eggs  | 101     |
| <i>Lysibia nana</i>       | Winged     | Late May                | Large number of eggs  | 7       |
| <i>Acrolyta nens</i>      | Winged     | Late April–late May     | Large number of eggs  | 207     |
| <i>Bathythrix aerea</i>   | Winged     | Late May–early June     | Unknown               | 27      |
| <i>Gelis aerator</i>      | Winged     | Late May–early June     | Few eggs              | 15      |
| <i>Gelis hortensis</i>    | Wingless   | Throughout study period | Few eggs              | 26      |
| <i>Gelis proximus</i>     | Wingless   | Throughout study period | Few eggs              | 264     |
| <i>Gelis spurius</i>      | Wingless   | Throughout study period | Few eggs              | 37      |

Total numbers found in this study are presented for each species.

distribution (log-link). As data were overdispersed, data were fitted with quasi-likelihood.

*Effects of larger-scale separation (area) and smaller-scale separation (population) of plant populations on parasitism of Cotesia glomerata on garlic mustard.* The effect of location (Wageningen/Amsterdam populations) on plant on parasitism of *C. glomerata* was analysed using generalised linear mixed models, with ‘proportion of pupal clusters hyperparasitised’, ‘number of winged parasitoids’, ‘number of wingless parasitoids’, and ‘number of species’ as response variables. Area was incorporated as a fixed effect, with ‘individual plant’ nested in ‘population’ (to account for the fact that bottom and top clusters were placed on the same plant) and ‘time’ as random effects.

The effect of population (six populations; three near Wageningen, three near Amsterdam) on plant on parasitism of *C. glomerata* was analysed using generalised linear mixed models, with ‘proportion of pupal clusters hyperparasitised’, ‘number of winged parasitoids’, ‘number of wingless parasitoids’, and ‘number of species’ as response variables. Population was incorporated as a fixed effect, with ‘individual plant’ (to account for the fact that bottom and top clusters were placed on the same plant) and ‘time’ as random effects.

*Effects of cluster placement on plant on parasitism of Cotesia glomerata on garlic mustard.* The effect of pupal cluster placement (top/bottom) on plant on parasitism of *C. glomerata* was analysed using generalised linear mixed models, with ‘proportion of pupal clusters hyperparasitised’, ‘number of winged parasitoids’, ‘number of wingless parasitoids’, and ‘number of species’ as response variables. Placement was incorporated as a fixed effect, with ‘individual plant’ nested in ‘population’ (to account for the fact that bottom and top clusters were placed on the same plant) and ‘time’ as random effects.

All generalised linear (mixed) models were performed in R STUDIO version 1.1.419, R version 3.4.3 (R Core Team, 2018), using the ‘lme4’ package (Bates *et al.*, 2015).

*Effects of sampling time, population and cluster placement on the plant on hyperparasitoid community composition.* We used a multivariate analysis to assess how sampling

**Table 2.** Average number of pupae and number of pupae parasitised in the retrieved pupal clusters separated per location.

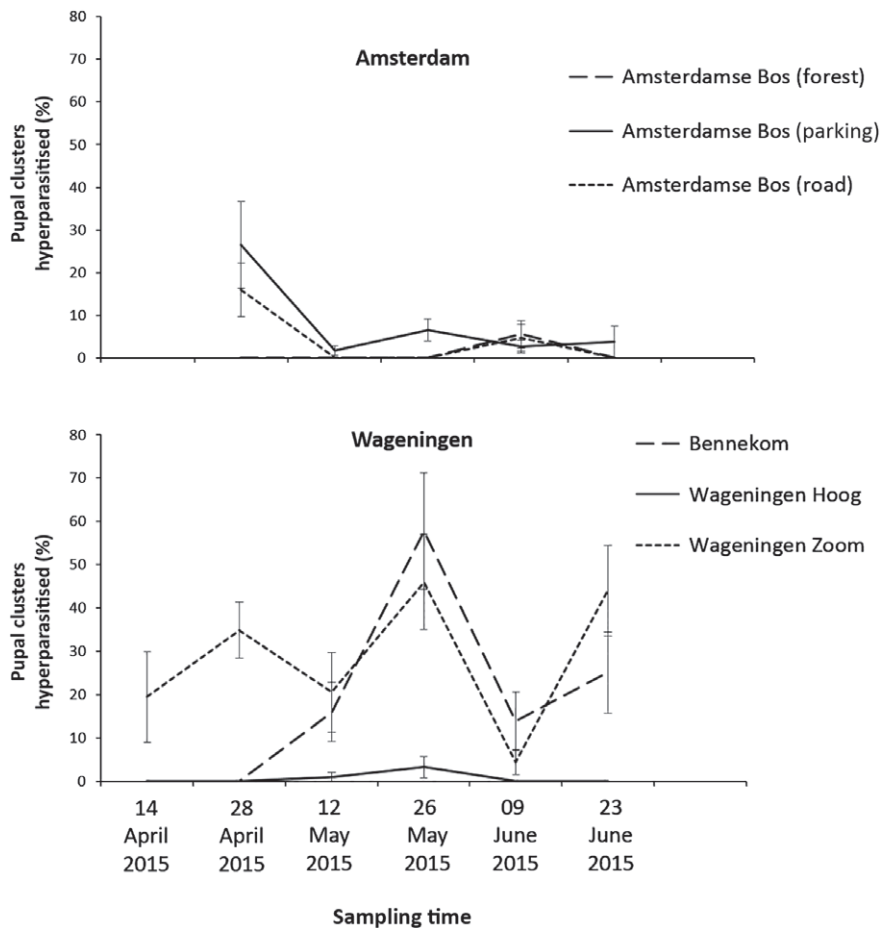
| Population            | Pupal cluster sample size | Mean number of pupae per cluster | Mean number of parasitised pupae per cluster |
|-----------------------|---------------------------|----------------------------------|--|
| Bennekom              | 64                        | 19.34 (1.42)                     | 2.75 (0.59)                                  |
| Wageningen Hoog       | 79                        | 18.73 (0.97)                     | 0.14 (0.08)                                  |
| Wageningen Zoom       | 58                        | 19.79 (1.52)                     | 5.71 (0.90)                                  |
| Amsterdam Bos road    | 51                        | 22.88 (1.80)                     | 1.61 (0.71)                                  |
| Amsterdam Bos parking | 56                        | 23.05 (1.51)                     | 1.41 (0.39)                                  |
| Amsterdam Bos forest  | 53                        | 21.62 (1.39)                     | 0.23 (0.15)                                  |

Standard errors are shown in brackets.

time, population and cluster placement on the plant affected hyperparasitoid community composition. The values for species variables consisted of the number of eight hyperparasitoids present in each of the sampled pupal clusters. The environmental variables that were used to explain variation in communities were time (six sampling time points), population (six populations) and placement (clusters placed at the top or bottom). We first used an (unconstrained) principal component analysis to visualise how the multivariate communities were distributed in ordination space. Then, we performed three individual (constrained) redundancy analyses in which we used the explanatory variables time, population or placement on the plant to explain variation in the hyperparasitoid community structure. Predictor effects of the explanatory variables were tested with ‘all constrained axes tests’, based on 499 unrestricted permutations. All multivariate analyses were performed in CANOCO version 5.03 (Microcomputer Power, Ithaca New York).

## Results

Overall, out of 7470 individual retrieved pupae, 691 of them (roughly 9%) were parasitised. We found eight different species of hyperparasitoid [species and total number: *Lysibia nana* (7), *Acrolyta nens* (207), *Gelis proximus* (264), *Gelis aerator* (15), *Gelis hortensis* (26), *Gelis spurius* (37), *Bathythrix aerea* (27)



**Fig. 1.** The percentage of *Cotesia glomerata* pupal clusters that have been hyperparasitised by secondary hyperparasitoids over time for three locations near the Amsterdam area and three locations near the Wageningen area. Both the locations and the populations within them were sampled on the same day.

and *Pteromalus semotus* (101), respectively]. Of these species, five were winged and three were wingless (see Table 1; Figs S2, S3). The three wingless species of *Gelis*, namely *G. spurius* Forster, *G. proximus* Forster, and *G. hortensis* Christ, had, to our knowledge, not been recorded previously as hyperparasitoids of *C. glomerata*.

*Effects of sampling time and interactions with smaller-scale separation (population) and larger-scale separation (area) on parasitism of Cotesia glomerata on garlic mustard*

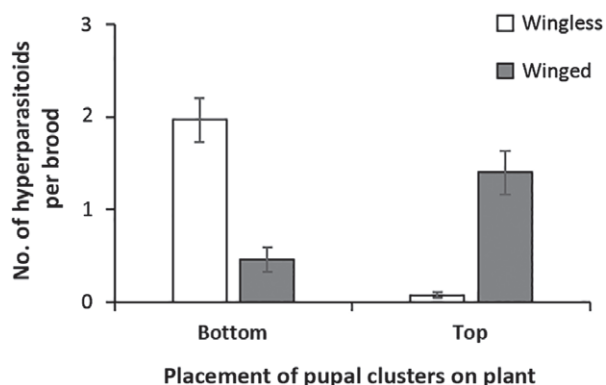
Time did not significantly affect the number of wingless hyperparasitoid species recovered in this study. However, the number of winged species showed a strong effect of time ( $t = 2.2$ ,  $P = 0.032$ ), but this effect significantly differed with area ( $t = -2.1$ ,  $P = 0.034$ ). In the Amsterdam area, winged species were more abundant in the earlier samplings and decreased with time, whereas in the Wageningen area, winged species were not present in the early samplings and their numbers increased with time (see Figs S2, S3).

Time had a significant effect on the proportion of pupae parasitised ( $t = -2.6$ ,  $P = 0.010$ ; see Fig. 1) with an overall peak around the fourth sampling time. However, the effect of time significantly varied according to area, with Amsterdam pupae receiving higher proportions of parasitism in the first weeks of April, and Wageningen peaking in parasitism in the later samplings in May and June ( $t = 2.4$ ,  $P = 0.018$ ; see Fig. 1).

The number of hyperparasitoid species that were recovered also significantly differed with time, with the highest numbers in the fourth and sixth samplings in late May and late June reaching four and five hyperparasitoid species per population, respectively; see Figs S2, S3).

*Effects of larger-scale separation (area) and smaller-scale (population) of plant populations on parasitism of Cotesia glomerata on garlic mustard*

The number of winged or wingless hyperparasitoid species in a pupal cluster or the proportion of pupae parasitised was not significantly affected by population. However, there was a significant effect of population on the number of species



**Fig. 2.** Average numbers of winged and wingless hyperparasitoids in pupal clusters placed in the canopy or at the base of a garlic mustard plant, near ground level.

recovered ( $z = -2.3$ ,  $P = 0.02$ ), with Wageningen Zoom and Bennekom and Amsterdamse Bos parking populations having the highest number of species across the experiment (see Figs S2, S3).

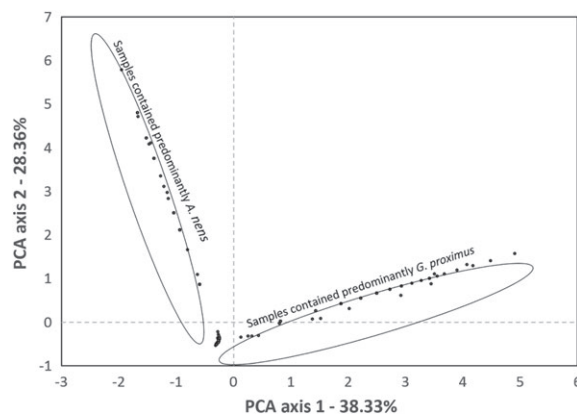
Area did not explain any of the variation in hyperparasitism in any of the measured parameters.

#### *Effects of cluster placement on plant on parasitism of Cotesia glomerata on garlic mustard*

Overall, we found that clusters that were placed near the bottom experienced a higher proportion of parasitism than those that were placed in the canopy (13% and 9%, respectively;  $z = 5.0$ ,  $P < 0.001$ , data not shown). However, we found no significant effects of placement on the plant on the number of species that parasitised a pupal cluster. We found that the number of winged hyperparasitoids per pupal cluster was significantly higher in the top-placed pupal clusters than in those placed at the bottom ( $z = 8.1$ ,  $P < 0.001$ ; see Fig. 2). By contrast, the number of wingless species was significantly higher in bottom-placed pupal clusters than in those placed at the top ( $z = -9.2$ ,  $P < 0.001$ ; see Fig. 2).

#### *Effects of sampling time, population and cluster placement on the plant on hyperparasitoid community composition*

There was strong clustering observed in the multivariate analysis of the hyperparasitoid assemblages. The two biggest clusters are formed by those pupal clusters that are parasitised by *G. proximus* and *A. nens* Hartig, the two hyperparasitoid species that were most abundant in this study (see Fig. 3). The community structure was significantly affected by time (pseudo- $F = 3.9$ ,  $P = 0.002$ ), location (pseudo- $F = 13.0$ ,  $P = 0.002$ ) and placement of the pupal clusters (pseudo- $F = 23.5$ ,  $P = 0.002$ ). Overall, it is evident that species are often temporally restricted, with *A. nens* and *P. semotus* Walker, in particular, correlating strongly with certain sampling times (the early May and late June samplings for *A. nens* and the early July sampling for *P. semotus*; see Fig. 4a).

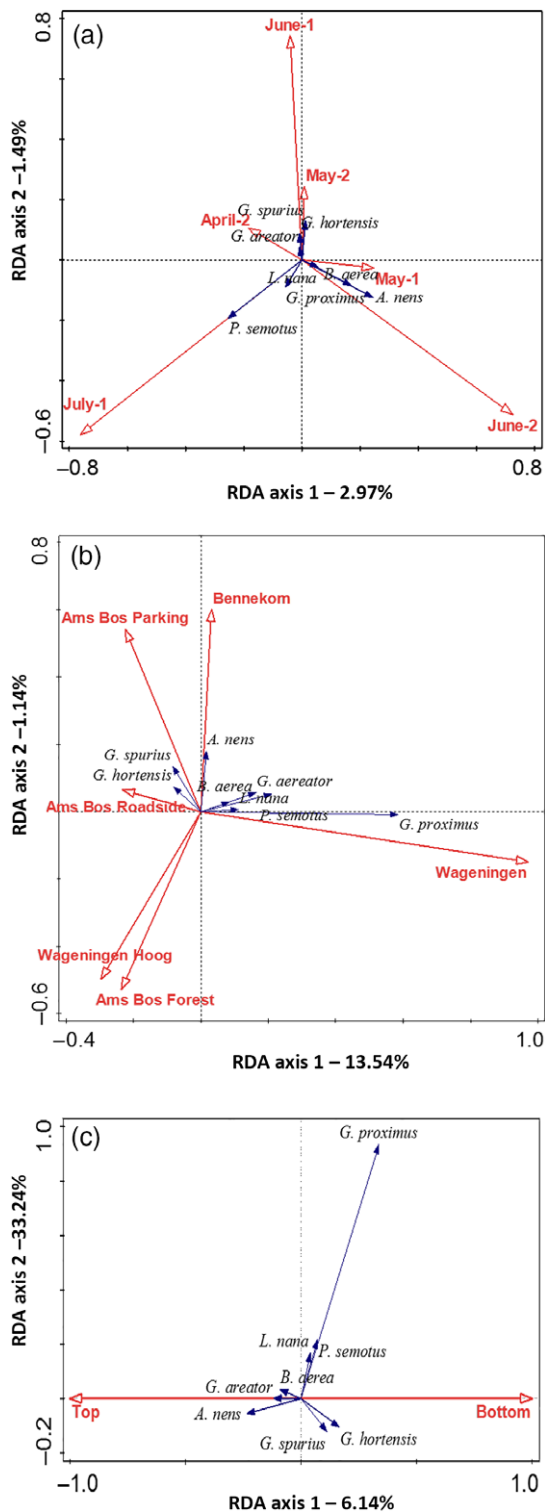


**Fig. 3.** Plot of the first two axes of an unconstrained principal component analysis (PCA). Three apparent clusters are visible. The two elongated clusters represent clusters that were dominated by either *Gelis proximus* or *Acrolyta nens*. The smaller cluster at the base represents samples that were hyperparasitised by other species.

It is also evident that certain species are more closely linked to certain locations. The two wingless species, *G. proximus* and *G. hortensis*, were most strongly associated with two garlic mustard populations near the Amsterdamse Bos parking and Amsterdamse Bos road site, whereas most other species, both winged and wingless, were more strongly associated with the sites Wageningen Zoom and Bennekom. Interestingly, two sites, Wageningen Hoog and Amsterdamse Bos Forest, yielded hardly any hyperparasitoids, which explains why no species are associated with them (see Fig. 4b). Lastly, three winged species, *A. nens*, *G. aereator* and *Bathythrix aerea* Gravenhorst, were more associated with top-placed pupal clusters. However, interestingly, *P. semotus* and *L. nana* Gravenhorst, two winged species, were found in top- and bottom-placed clusters, but they seemed to have a slight preference for the bottom-placed clusters (see Fig. 4c).

## Discussion

A previous study by Harvey *et al.* (2014), performed during the summer months, observed temporal changes in hyperparasitoid assemblages on pupal clusters of *C. glomerata* in its lepidopteran host *P. brassicae* – on a late-seasonal host plant, black mustard (*B. nigra*). Here, we examined hyperparasitoid assemblages from the same parasitoid–host system (*C. glomerata*–*P. brassicae*) on an early-seasonal host plant, garlic mustard, *A. petiolata*. We tested the effect of large-scale spatial separation (sampling areas 100 km apart), smaller-scale separation (populations *c.* 2 km apart) as well as spatial separation on the same plant (placement of pupal clusters near the top or bottom of the plant), and we also tested how these processes varied over seasonal time. We showed that, indeed, hyperparasitoid pressure on *C. glomerata* pupae is already evident early in the growing season and we found that throughout the spring, various hyperparasitoid species are present, including several species that had not been previously recorded for *C. glomerata*, namely three gelines: *G. spurius*, *G. hortensis* and *G. proximus*.



**Fig. 4.** Plots of the first two axes of constrained redundancy analyses testing the effects of time of sampling (a), sampling location (b) or pupal cluster placement (c) on the garlic mustard plant. Red arrows represent the explanatory variables and blue arrows represent the hyperparasitoid species' response. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].

Interestingly, we found that there was no significant effect of large-scale separation of the sampling areas on hyperparasitism, but that there was a strong effect of the smaller-scale separation of host plant populations on percentage parasitism and number of species, indicating that population-level differences still occur, even at relatively short distances. Cocoons placed on garlic mustard populations in Wageningen and Bennekom had the highest parasitisation rates, followed by those on plant populations at the Amsterdamse Bos parking site and the Amsterdamse Bos road site. It is noteworthy that all of these are populations that grow along forest edges and have a certain degree of openness, being located closer to open field areas than the two remaining mustard populations in Wageningen Hoog and Amsterdamse Bos forest. These two sites are located inside deciduous forests and are well away from open fields. Shady forest habitats are considered less suitable for either *P. brassicae* or *C. glomerata* because these species are known to prefer open forest edges or field margins where most of their preferred food plants, such as black, field and charlock mustards and wild radish, most commonly grow (Feltwell, 1982; Harvey *et al.*, 2014). Possible explanations for the observation are that locations inside dense forests do not provide an ideal environment for most (hyper)parasitoids of grassland species, e.g. in terms of dietary resources or even host availability. Moreover, suitable ('grassland species') foraging habitat may become more and more fragmented in forests, and habitat fragmentation is known to strongly influence parasitisation rates (e.g. Roland & Taylor, 1997). Interestingly, *P. brassicae* and *C. glomerata* have been collected during our field samplings in the heavily forested Amsterdamse Bos population (see Harvey *et al.*, 2016), where hyperparasitism turned out to be almost absent. We can speculate that such areas provide enemy-free space for the herbivore and its primary parasitoid, although our study was not designed to test for this specifically. It would be interesting to investigate whether experimental populations along a forest density gradient experience a loss of enemies (Stamp, 2001).

One of the major factors to consider when comparing hyperparasitoid assemblages across season on different food plants is not only habitat preference but also the degree of specialism exhibited by different species of hyperparasitoids in the fourth trophic level. Specialists are evolutionarily required to 'track' their hosts and are therefore under much stronger selection than generalists to locate cocoons of *C. glomerata* occurring in different habitats. For instance, the most frequently recorded winged hyperparasitoids of *C. glomerata*, *L. nana* and *A. nens*, are considered to be specialised on cocoons of *Cotesia* spp., and perhaps even on *C. glomerata* alone (Schwarz & Shaw, 2000; Harvey *et al.*, 2009). By contrast, the *Gelis* species are considered to be extreme generalists that will attack hosts as phylogenetically divergent as spider egg sacs, moth pupae and parasitoid cocoons (Bezant, 1956; Russell, 1987; Cobb & Cobb, 2004). Search area covered per unit of time is clearly limited in wingless species, compared with winged species that can cover larger distances, and stronger specialisation would not be very beneficial. Wingless hyperparasitoids may have evolved to broaden their host range while trading off traits like wings for reduced fecundity and increased longevity (Harvey, 2008; Harvey *et al.*, 2017; Visser *et al.*, 2014, 2016).



Similar to the initial study in *B. nigra*, we found that there is small-scale partitioning of resources (the pupal clusters) between the winged and wingless hyperparasitoid species (Harvey *et al.*, 2014). Similar to the previous study, we noted that the wingless species of the genus *Gelis* are mainly found in the clusters near the ground, whereas the winged species, such as *G. aereator* and *A. nens*, are mostly found in the canopy. This makes sense when you compare the foraging strategies of the two morphologies. Winged species obviously span larger distances, as their wings allow them to do so. Having wings, however, comes with a trade-off, as flying is likely to be more difficult in dense undergrowth, leaving an entire area relatively unexplored. By contrast, wingless species clearly cannot traverse very large distances (as they do not possess wings), but they can easily navigate more dense vegetation and leaf litter, where many *Gelis* species are often found (Harvey *et al.*, 2014, 2015). What is interesting is that the composition of the communities differed quite substantially from the previous study. Although their study was performed in relative proximity to the Wageningen area populations, Harvey *et al.* (2014) found two other species of wingless *Gelis* than we report in the current study, whereas the winged species were exactly the same. Similar to our study, the winged species have distinct peaks in their reproduction, whereas the wingless species seem to reproduce continuously, albeit at considerably lower rates.

An interesting observation is that most of the *Gelis* species are present throughout the season, starting very early in spring, in our study emerging from pupal clusters that were placed in mid-April. Around this time, it is rare to see a cabbage white butterfly adult, let alone their larvae. This raises the question as to where these early hyperparasitoids come from and why they are already active during times when their hosts are not yet present. Previous studies have shown that some species of the *Gelis* genus have life spans of 70 days without hosts at ambient temperatures (Harvey, 2008; Harvey *et al.*, 2015, 2017; Visser *et al.*, 2016). As metabolic rates generally decrease with falling temperatures, it is expected that life span could be even higher under colder temperature regimes, so it could be that these species overwinter during the adult stage and oviposit at the first opportunity that is presented. This is in line with their opportunistic lifestyle, as it is known that many *Gelis* species will accept alternative hosts, even from non-lepidopteran taxa.

Other, mainly winged, parasitoid species show different patterns, often peaking in abundance around one of the sampling dates. For instance, *A. nens* is highly abundant around late May and early June, but then disappears, quickly replaced by *P. semotus*, which is more abundant later in the season (Harvey *et al.*, 2014). It seems that partitioning the available resources over time can also be a successful strategy. This may be even more important in these species, as they produce large numbers of eggs (Harvey *et al.*, 2009) and can often parasitise many pupae in a pupal cluster, leaving very little room for competing species that occur simultaneously. However, examples of even smaller-scale spatial partitioning of resources between different parasitoid species have been seen before in the literature (Price, 1972). A nice example is the exploitation of eucalyptus longhorned borer larvae feeding at different depths in the woody substrate by different parasitoid species that differ in ovipositor

length (Paine *et al.*, 2000). Pupae of *C. glomerata* are generally part of larger clusters that have a clear spatial arrangement. The pupae on the outside of a cluster are more exposed and thus may be more easily available to hyperparasitism than those pupae in the inner layers of the cluster, and it is currently unknown whether different parasitoid species target different pupae in a cluster. It would be interesting to investigate whether such more microscale resource partitioning at the pupal cluster level can enable coexistence of multiple species.

Multivoltine consumers, such as herbivores and natural enemies, depend on multiple hosts throughout one growing season. As presence and quality of hosts may vary with time, especially in the case of herbivores and their host plants, host switching is often necessary. In this study, we follow up on a previous study that investigated pupal hyperparasitoid assemblages on a mid-summer host plant of *P. brassicae* and its parasitoid *C. glomerata*. Here, we investigate such assemblages on an early-season host, garlic mustard, in six plant populations.

In conclusion, we have shown that hyperparasitoid pressure is present from early in the season, with multiple species often occupying one pupal cluster. Levels of hyperparasitism and the composition of hyperparasitoid assemblages are strongly dependent on host plant population. Similar to the previous study, we found a strong partitioning of pupal resources between pupal clusters placed near the ground (wingless, generalist hyperparasitoids) and those placed in the canopy (winged, specialist hyperparasitoids). Lastly, we found that some garlic mustard populations, located in dense forest sites, were free of hyperparasitism, suggesting that this may be enemy-free space for the primary parasitoids. Whether forest or vegetation types may indeed explain the absence of hyperparasitism in some locations should be addressed in future studies.

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## Author contributions

RH and JAH conceived the ideas for the experiment. RH conducted the field experiments in the Wageningen populations and JAH conducted the field experiments in the Amsterdamse Bos populations. RH analysed the data. RH led the writing of the manuscript and JAH contributed substantially to the final version of the manuscript.

## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Schematic overview of the sampling scheme by location and sampling date.

**Fig. S1.** Overview of the garlic mustard populations in Wageningen and Amsterdam.

**Fig. S2.** Average number of hyperparasitoids per pupal cluster in the three Amsterdam sampling locations over time.

**Fig. S3.** Average number of hyperparasitoids per pupal cluster in the three Wageningen sampling locations over time.

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