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***published in***
Basic and Applied Ecology
2020

***DOI (link to publisher)***
10.1016/j.baae.2020.04.007

***document version***
Peer reviewed version

# Shared enemies exert differential mortality on two competing parasitic wasps

Dhaval K. Vyas[a*], Ryan L. Paul[a], Michael W. Gates[b], Tristan Kubik[a], Jeffery A. Harvey[c], Boris C. Kondratieff[a] and Paul J. Ode[a]

[a]Graduate Degree Program in Ecology, and Department of Agricultural Biology, Colorado State University, 1177 Campus Delivery, Fort Collins, CO 80523-1177 USA

[b]Systematic Entomology Laboratory, United States Department of Agriculture-Agricultural Research Service, c/o National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, MRC 168, Washington, D.C. 20013 USA

[c]Netherlands Institute of Ecology, Droevendaalsesteeg 10, 6708 PB Wageningen, The Netherlands

*Corresponding author.

E-mail address: Dhaval.Vyas@du.edu.

Present address: University of Denver, Department of Biological Sciences, 2050 E. Illif Avenue, Denver, CO 80208 USA

**Abstract**

Classical biological control programs introduce primary parasitoids into new geographic regions, often exposing them to existing populations of hyperparasitoids. Hyperparasitoids are frequently implicated in the failure of parasitoid biological control agents to establish and provide control of insect pests. The outcome of competition among two or more parasitoid species may be altered if the parasitoids are differentially attacked by the same hyperparasitoids. A reliable assessment of the hyperparasitoid community is needed to understand how top-down trophic interactions influence the effectiveness of introduced parasitoids. We examined the diversity of hyperparasitoids attacking *Cotesia glomerata* in Colorado (USA), where the congener *C. rubecula* is absent. We compared this diversity with the hyperparasitoid community of *C. glomerata* and *C. rubecula* from Maryland (USA) where both wasps co-occur and use the imported cabbageworm (*Pieris rapae*) as their main host. Field collected *C. glomerata* broods were analyzed to examine the relationship between brood sizes and the adult sex ratio and the likelihood of attack by different hyperparasitoid species. A total of nine hyperparasitoid species were found in Colorado, of which four species also occurred in Maryland. While larger *C. glomerata* broods experienced increased odds of hyperparasitism, *C. glomerata* developing in larger broods had higher per capita survivorship than those developing in smaller broods. The proportion of adult male *C. glomerata* in a brood increased with brood size in both non-hyperparasitized and hyperparasitized broods, suggesting that female *C. glomerata* were not preferentially hyperparasitized. Hyperparasitoids inflicted greater mortality on *C. rubecula* than on *C. glomerata*. This differential hyperparasitism may enable the coexistence of *C. glomerata* with its congener *C. rubecula*, which usually outcompetes and displaces *C. glomerata*.

*Keywords*:

sex ratio

shared enemy

biological control

hyperparasitoid

top-down pressure

## Introduction

A seminal paper by Price et al. (1980) argued that to better understand the dynamics of plant-herbivore interactions, it is imperative to include consideration of natural enemies of herbivores, such as predators and parasitoids, in the third trophic level. Similarly, consideration of the role of predators and hyperparasitoids in the fourth trophic level is necessary to fully understand interactions between herbivores and their natural enemies (Sullivan & Völkl, 1999). Hyperparasitoids are a significant mortality factor for many parasitoids and have been implicated in the failure of several biological control introductions to establish and to provide adequate control (Sullivan, 1987; Stiling, 1993; Sullivan & Völkl, 1999; Chacón, Landis, & Heimpel, 2008; Schooler, De Barro, & Ives, 2011; Frago, Pujade-Villar, Guara, & Selfa, 2012; Nofemala, 2013; Kaser & Ode, 2016). Despite the importance of parasitoids in structuring plant and insect communities and in the implementation of biological control programs, in too few cases do we understand the consequences of hyperparasitoid communities that attack primary parasitoids.

In addition to direct consumptive effects, antagonist species can have important indirect impacts on species at lower trophic levels. There are two ways in which hyperparasitoids can influence the communities inhabited by parasitoids. First, by reducing parasitoid populations, they provide enemy-release for herbivores that can have cascading negative effects on plant fitness through increased herbivory (Rosenheim, 1998; Brodeur & McNeil, 1992; Schooler et al. 2011). Second, hyperparasitoids can result in apparent competition between parasitoid species if they preferentially attack one species over the other (van Nouhuys & Hanski 2000; Morris, Müller, & Godfray, 2001; Acebes & Messing, 2013; Nofemala, 2013; Kaser & Ode, 2016).

Furthermore, the presence of hyperparasitoids can influence foraging behavior and allocation decisions of individual parasitoid adults. Parasitoids may minimize residence times at

patches containing hosts, thereby reducing mortality risks associated with predation and hyperparasitism (Mackauer & Völkl, 1993; Petersen, Matthiesen, Francke, & Wyss, 2000). Because female parasitoids are often larger than males, they may offer more resources as a host for hyperparasitoids and differential hyperparasitism of female parasitoids can result in a more male-biased offspring sex ratio at adult emergence compared to the sex ratio at oviposition (e.g., Chow & Mackauer, 1996; Heinz, 1996; Gómez-Marco et al., 2015).

Parasitoid reproductive strategies may also be under top-down control from hyperparasitoid attacks. The ancestral condition in parasitic wasps is to have one offspring develop per host (Mayhew, 1998), and hyperparasitoids are suspected to influence the evolution of increased clutch size in wasp parasitoids (Godfray, Partridge, & Harvey, 1991). Larger clutches can be costly because they result in smaller parasitoid adults, which have fewer eggs (i.e., fitness costs) (Godfray, 1994; Visser, 1994). However, more offspring increase survivorship when they reduce the per capita mortality (Godfray et al., 1991); thus, the evolution of multiple offspring per host could be a strategy to cope with hyperparasitoid attacks. Given the influence that hyperparasitoids have on parasitoid population dynamics, foraging behavior, and reproductive strategies, characterizing the diversity and abundance of hyperparasitoids is important for understanding the success of parasitoids in regulating their host populations.

Geographic patterns of parasitoid-host relationships are known to affect parasitoid foraging behaviors (Kraaijeveld, Nowee, & Najem, 1995), morphology (Dixon, Craig, & Itami, 2009) and reproductive success (Potting, Vet, & Overholt, 1997). Likewise, geographic variations in parasitoid-hyperparasitoid interactions may also influence parasitoid evolution, yet this topic remains unexplored. As parasitoids establish in novel regions, a common consequence of many biological control programs, they can benefit by escaping hyperparasitoids from their

original habitats (Schulz, Lucardi, & Marsico, 2019). Conversely, an introduced parasitoid can become host to resident hyperparasitoids that are capable of successfully parasitizing the new parasitoid host (Coulautti, Ricciardi, Grigorovich, & MacIsaac, 2004). If the community of hyperparasitoids varies across the distribution of a wide-ranging parasitoid, then we may find population-level differences in life-history traits such as survivorship and reproductive success. Given the importance of hyperparasitoids in influencing the establishment of parasitoids, multiple populations of a parasitoid should be examined in order to fully understand the bottom-up and top-down factors relevant to its survival.

*Cotesia glomerata* (Linnaeus) (Hymenoptera: Braconidae) and *C. rubecula* (Marshall) are parasitoid wasps that originate in Europe where their co-existence in shared habitats is mediated largely by their use of different host species. In Europe, *C. glomerata* primarily attacks *Pieris brassicae* (L.) (Lepidoptera: Pieridae) and occasionally attacks *P. rapae* (L.) (Laing & Levin, 1982; Feltwell, 1982; Ohsaki & Sato, 1990), wheras *C. rubecula* is a specialist on *P. rapae* (Geervliet et al. 2000). In North America, on the other hand, both *Cotesia* species attack *P. rapae* as *P. brassicae* does not occur on the continent. As a specialist, *C. rubecula* is considered a more effective biological control agent of *P. rapae* (Puttler, Parker, Pinnell, & Thekwe, 1970; Parker, Frank, & Pinnel, 1972), but its establishment and success may be reduced because of its vulnerability to the hyperparasitoids that also attack *C. glomerata* (McDonald & Kok, 1991; McDonald & Kok, 1992; Gaines & Kok, 1999). Both *Cotesia* species develop inside the host, but *C. glomerata* is gregarious with multiple larvae emerging from a host and forming a brood of cocoons, while *C. rubecula* is solitary with only one larva making a cocoon after exiting the host. The *C. rubecula* larva is often 5-15% larger than a *C. glomerata* larva, and this size difference continues into the pupal and adult stages (D.K. Vyas & R. L. Paul pers. obs.).

Whether differences in hyperparasitoid assemblages, associated with different *C. glomerata* populations, mediate host use patterns and interactions with competitors such as *C. rubecula* remain open questions.

We explored the impact of hyperparasitoids on *C. glomerata* and its stronger competitor, *C. rubecula*, in a region where they coexist (Maryland, USA [MD]) and in a region where *C. glomerata* exists in the absence of *C. rubecula* (Colorado, USA [CO]). For our first objective, we compared the diversity of hyperparasitoids between CO and MD, and between the two *Cotesia* species. Hyperparasitoids in the region of coexistence may differ from locations where only *C. glomerata* exists. Surveying the diversity of top-down mortality factors would provide information on the possible threats to *C. rubecula* as biological control programs introduce it to new habitats. Our second objective was to determine whether one *Cotesia* species is more vulnerable to hyperparasitism. The single cocoon for *C. rubecula* should be disadvantageous compared to the *C. glomerata* brood, from which some offspring escape hyperparasitoid attacks. In habitats with both *Cotesia* species, shared hyperparasitoids may inflict differential mortality favoring one species over the other. For our final objective, we examined the impact of hyperparasitoids on *C. glomerata* survivorship and sex ratio, two significant life history traits that can be affected by species in higher trophic levels. We expected the likelihood of a given brood experiencing hyperparasitism to increase with brood size; conversely, per capita mortality should decrease if a larger brood increases the chances that any single *C. glomerata* larva escapes hyperparasitism. *Cotesia glomerata* broods often contain both males and females, but pupating and adult females are generally larger than males (Gols, Van Dam, Raaijmakers, Dicke, & Harvey, 2009). If their larger size makes female *C. glomerata* more valuable as hosts for

hyperparasitoids, and if hyperparasitoids preferentially attack female *C. glomerata*, then the brood sex ratio of emerging *C. glomerata* will be more male-biased in hyperparasitized broods.

## Materials and methods

### Study System

In North America, *C. glomerata* and *C. rubecula* have been introduced as biological control agents of *P. rapae,* an important pest of many *Brassica* crops (Wilkinson, 1966; Clausen, 1978; McDonald & Kok, 1992; van Driesche & Nunn, 2002; Wold-Burkness et al. 2005). *Pieris brassicae,* the preferred host of *C. glomerata*, is absent in North America where both *Cotesia* species use *P. rapae* as the primary host. When *C. glomerata* and *C. rubecula* attack the same host individual (multiparasitism), *C. rubecula* invariably wins competition by killing the eggs or larvae of *C. glomerata* (Laing & Corrigan, 1987, Geervliet et al., 2000). In many regions of North America, *C. rubecula* has largely outcompeted *C. glomerata*, resulting in the extirpation of *C. glomerata* (Herlihy et al., 2012); although, some evidence suggests that *C. glomerata* may persist by switching to other pierids such as the mustard white *P. napi oleracea* (Benson, Van Dreische, Pasquale, & Elkinton, 2003) or the checkered white *Pontia protodice* (Van Driesche, Nunn, Kreke, Goldstein, & Benson, 2003; D.K. Vyas & R. L. Paul pers. obs.). There remain parts of North America (e.g., CO) where *C. glomerata* occurs without *C. rubecula* and a few areas where the two species coexist (e.g., MD).

In the Netherlands, *C. glomerata* is parasitized by at least 11 species of hyperparasitoids (Laing & Levin, 1982; Harvey, Snaas, Malcicka, Visser, & Bezemer, 2014; Poelman et al. 2012). North American populations of *C. glomerata* are attacked by a largely different community of hyperparasitoids; of the six hyperparasitoid species emerging from *C. glomerata* cocoons

collected in Virginia (USA), only *Baryscapus* (=*Tetrastichus*) *galactopus* (Ratzeburg) (Hymenoptera: Eulophidae) has been documented to parasitize *C. glomerata* in Europe (Gaines & Kok, 1999). Nevertheless, little is known about the diversity of hyperparasitoids that attack different *C. glomerata* populations across North America, especially with regards to whether *C. rubecula* is also present.

Field sites

Field samples from Colorado (CO) were collected from June 30, 2015 to October 28, 2015 from cultivated *Brassica oleracea* crops grown at six vegetable farms (see Appendix A: Table A.1). Each farm was surveyed every two weeks to ensure the absence of insecticides that harm *P. rapae* and associated parasitoids. Crops were planted before June 2015 at all farms. Planting of cultivars was determined by the owner of each farm, except at Colorado State University's Agriculture Research Development and Education Center (ARDEC) North and ARDEC South where crops were planted by D.K.V, R.L.P & P.J.O. At the commercial farms, plants were grown in rows with a range of 15 to 50 plants per row. Plants were grown in 15 rows at ARDEC North (ca. 60 plants per row) and 10 rows at ARDEC South (ca. 70 plants per row) with 0.30 m spacing between plants and 0.90 m between rows.

Field sampling in MD occurred between August 15-20, 2016, May 24-September 23, 2017, and July 6-9, 2018. Approximately 3-5 crop varieties (see Appendix A: Tables A.1 & A.2) were sampled each visit and a different row of plants was randomly selected to avoid re-sampling the same plants in subsequent weeks. *Cotesia glomerata* and *C. rubecula* pupae were collected from cultivated varieties of *B. oleracea* on seven vegetable farms in 2016 (see Appendix A: Table A.2).

Data collection

At each farm in CO, individual leaves of 20-50 plants per variety were searched for *P. rapae* larvae and *Cotesia* cocoons. Within a row, every third plant was searched for the presence of *C. glomerata* broods (and *C. rubecula* cocoons in the MD samples) that had emerged from their hosts. As each *C. glomerata* or *C. rubecula* larva finishes feeding and emerges from its host, it forms a silken cocoon. Only a single cocoon is made by the solitary *C. rubecula* larva, but in the case of *C. glomerata*, a cluster of individual cocoons from the same host was considered a brood. Sampling concluded for the season when plants were harvested by the owners, when plants became too large to effectively detect *C. rubecula* cocoons or *C. glomerata* broods, or after the onset of freezing temperatures. *Cotesia glomerata* broods and *C. rubecula* cocoons were collected and brought back to the laboratory to rear out any hyperparasitoids. Field collection of *Cotesia* pupae in MD was performed using similar methods as described above for CO, however *P. rapae* were not sampled in the same manner as in CO because we did not have access to facilities for rearing live insect specimens in MD.

*Pieris rapae* caterpillars were reared in 37 ml plastic cups and kept in an environmental chamber at 25 °C and 16L:8D photoperiod until they pupated or parasitoid larvae emerged from the host. Some hyperparasitoids attack their parasitoid hosts while they are inside the herbivore host (Sullivan 1987; Sullivan & Volkl 1999); therefore, *Cotesia* spp. larvae inside *P. rapae* caterpillars were susceptible to hyperparasitism. Dead caterpillars were dissected to determine if they were parasitized by *C. glomerata* or *C. rubecula*.

Each *C. glomerata* brood or *C. rubecula* cocoon was kept individually in 37 ml plastic cups and placed in an environmental chamber at 25 °C and 16L:8D photoperiod. *Cotesia*

*glomerata* broods or *C. rubecula* cocoons were checked daily for the emergence of parasitoid adults and hyperparasitoids. *Cotesia glomerata* broods and *C. rubecula* cocoons were kept in the environmental chamber for at least five months, a period sufficient to allow hyperparasitoids to complete development and emerge as adults. Each *C. glomerata* brood was examined under a stereomicroscope to determine the number of individual cocoons per brood, the sex ratio (proportion male), and the identity and number of emerged and unemerged hyperparasitoid adults. *Cotesia rubecula* cocoons were also assessed under the stereomicroscope to ascertain the sex of unemerged adults and hyperparasitoid identity. Unemerged adult *C. glomerata* and *C. rubecula* were included in the overall sex ratio if their sex could be distinguished.

At times, *C. rubecula* cocoons and *C. glomerata* broods were collected because they appeared intact in the field, but inspections under the microscope showed that they were empty. We distinguished whether the empty cocoons contained a *Cotesia* wasp or a hyperparasitoid based on the nature of the exit hole(s) on the cocoon. When *C. glomerata* or *C. rubecula* exit their cocoon, they always make a straight-lined hole at the cocoon's terminal end (D.K. Vyas, J.A. Harvey & R.L. Paul pers. obs.) (see Appendix A: Fig. A.1A). In contrast, hyperparasitoids usually exit the cocoon through a smaller jagged-edged hole on the sides of the cocoon. Species identification was not attempted for empty hyperparasitized cocoons (see Appendix A: Fig. A.1B).

Unemerged immature hyperparasitoids (both larvae and pupae) could not be reliably identified to species but could be distinguished from unemerged immature *C. glomerata* or *C. rubecula* based on the hyperparasitoids' sizes and number of larvae per cocoon in a brood. Unemerged hyperparasitoid larvae were at least half the size and were generally found to be alive, whereas unemerged *C. glomerata* or *C. rubecula* larvae were generally dead and

desiccated. In the case of *C. glomerata*, each larva spins a single cocoon, so when two or more larvae or pupae are found in an individual cocoon, this indicated that the cocoon was hyperparasitized. Only hyperparasitoid adults were identified to the species.

Ethanol-preserved hyperparasitoids were dehydrated through increasing concentrations of ethanol and transferred to hexamethyldisilazane (HMDS) (Heraty & Hawks, 1998) before point-mounting. All specimens were determined to genus by sight identification or using Gibson, Huber, and Woolley (1997). Specimens were identified to species, when possible, using relevant keys and primary literature listed in Noyes (2018). All species identifications were corroborated through comparison with authoritatively identified specimens in the Smithsonian National Museum of Natural History.

Data Analyses

We assessed multitrophic interactions by relating the total number of *P. rapae* caterpillars per plant to the probability of *C. glomerata* parasitism, as well as the percentage of these hosts that were parasitized by *C. glomerata* to the probability of hyperparasitism. Hyperparasitoid data were analyzed as presence-absence of hyperparasitoids in a brood, the number of hyperparasitoid adults found, and the number of cocoons parasitized out of the total number of *C. glomerata* cocoons in a brood. As described above, hyperparasitoids create distinct exit holes that are easily distinguished from the holes made by *C. glomerata* during eclosion (D.K. Vyas pers. obs.; see Appendix A: Fig. A.2), thus allowing calculation of the proportion of a *C. glomerata* brood that was hyperparasitized. A brood was scored as hyperparasitized if adult or immature hyperparasitoids were observed or if cocoons displayed exit holes indicative of hyperparasitoids. *Cotesia glomerata* brood size was calculated as the number of cocoons per

brood. Proportions of male *C. glomerata* in a brood (i.e., sex ratio) and proportion of adult *C. glomerata* emerged were treated as binomial counts. *Cotesia glomerata* brood size and the proportion of emerged *C. glomerata* adults were compared between non-hyperparasitized and hyperparasitized *C. glomerata* broods using t-tests for brood size and logistic regression for proportion emerged. Site was included as a blocking factor to account for spatial heterogeneity in the CO and MD data. Year was included as an explanatory factor to account for any temporal differences across years for the MD data.

Logistic regression (PROC LOGISTIC; SAS Institute Inc., Cary, NC) was used to examine the relationship between the total number of *P. rapae* caterpillars per plant and the probability that any one of these caterpillars was parasitized, percent of P. rapae parasitized by C. glomerata and probability of hyperparasitism, *C. glomerata* brood size and sex ratio, as well as between brood size and likelihood of hyperparasitism . We found unequal variances when comparing the mean number of *B. galactopus* adults found in MD *C. glomerata* and MD *C. rubecula*, therefore a Welch's t-test was used for this analysis. Unless noted otherwise, all means are reported with standard errors and parameter estimates are reported with 95% confidence intervals. For all statistical analyses, the level of significance was set at p=0.05. Analyses were performed in JMP© PRO, version 14 (SAS Institute Inc., Cary, NC) or SAS™ Studio, version 3.5 (SAS Institute Inc., Cary, NC).

**Results**

Hyperparasitoid communities attacking *C. glomerata* and *C. rubecula*

Between June and October 2015, we collected a total of 605 *C. glomerata* broods from 2307 *B. oleracea* plants across the six CO field sites. The majority of *C. glomerata* broods were

277 collected at ARDEC North (39.83%) and ARDEC South (45.95%) with 85% of all broods
278 collected between August and September (Table 1). Of the 605 broods, 328 were excluded from
279 the analyses because they were found to be empty (e.g., all parasitoids had already eclosed)
280 during field sampling, lacked data on the number of cocoons, or *Cotesia* wasps were absent (e.g.,
281 escaped from container). The remaining 277 broods were analyzed for hyperparasitism and 136
282 (49.09%) of these broods were hyperparasitized. Hyperparasitoid adults emerged from 75%
283 (101/136) of the total number of hyperparasitized broods (Table 1), whereas the remaining 35
284 broods contained unemerged hyperparasitoid adults (n=12) or immature hyperparasitoids (n=23)
285 that were found during dissections. Hyperparasitism of CO *C. glomerata* was detected from July
286 to October, but most of the CO *C. glomerata* broods that were analyzed for hyperparasitism were
287 collected in August (49.28%) and September (42.39%) (Table 1).
288 As the abundance of *P. rapae* increased per plant, so did the odds that any one *P. rapae*
289 caterpillar was parasitized by CO *C. glomerata*; every additional caterpillar resulting in a 6%
290 (95% CI: 4%, 8%) increase in the likelihood that a *P. rapae* was parasitized (log likelihood
291 $\chi^2$=23.6, d.f.=1 p<0.001) (Fig. 1). *Cotesia glomerata* brood sizes ranged between 5 and 75
292 cocoons per brood, and the number of cocoons in a brood was independent of the month when
293 the brood was collected ($r^2$=0.002, p=0.42). Interestingly, the probability that a CO *C. glomerata*
294 brood was hyperparasitized increased with the percentage of *P. rapae* caterpillars per plant that
295 were parasitized by *C. glomerata* (log likelihood $\chi^2$= 35.80, d.f.=1, p<0.001) (Fig. 2). For every
296 additional *P. rapae* parasitized by *C. glomerata*, there was a 4% (3%, 5%) increase in the
297 likelihood of hyperparasitism. The site from which CO *C. glomerata* originated influenced the
298 relationship between probability of parasitism and number of caterpillars per plant (log
299 likelihood $\chi^2$=60.79, d.f.=5, p<0.001), but not the probability of hyperparasitism and the

300 percentage of *C. glomerata* parasitized caterpillars per plant (log likelihood $\chi^2$=2.22, d.f.=4,
301 p=0.69).

302 A total of eight hyperparasitoid species were identified (Table 1) from CO *C. glomerata*
303 broods with 89% (121/136) of the samples dominated by three species: *Baryscapus galactopus*
304 (Ratzeburg) (Hymenoptera: Eulophidae), *Catolaccus aeneoviridis* (Girault) (Hymenoptera:
305 Pteromalidae) and *Trichomalopsis dubia* (Ashmead) (Hymenoptera: Pteromalidae), with *B.*
306 *galactopus* being the most abundant. All three of these hyperparasitoids were found from a
307 similar proportion of CO *C glomerata* broods. In contrast to the eight hyperparasitoid species
308 found attacking *C. glomerata* broods in CO, MD *C. glomerata* broods (n=144) were attacked by
309 three hyperparasitoid species (Table 1). As with CO *C. glomerata*, MD *C. glomerata* were
310 mainly attacked by *B. galactopus* (n=83) (Table 1). The odds of finding *Conura torvina*
311 (Cresson) (Hymenoptera: Chalcididae) were 14.06 (95% CI: 3.17, 62.45) times greater in MD
312 broods than in CO broods (log likelihood $\chi^2$=20.55, d.f.=1, p<0.001) (see Appendix B), whereas
313 the odds of finding *C. aeneoviridis* were 6.38 (95% CI: 2.58, 15.78) times higher in CO *C.*
314 *glomerata* broods than in broods from MD (log likelihood $\chi^2$=22.03, d.f.=1, p<0.001).

315 Hyperparasitoids attacked 176 of the 266 *C. rubecula* cocoons (Table 1), but 10 of these
316 cocoons lacked the hyperparasitoid adults preventing species identification. As with *C.*
317 *glomerata*, the most common hyperparasitoid of *C. rubecula* was *B. galactopus* (n=119),
318 followed by *C. torvina* (n=45), whose odds of occurrence were 24.92 (95% CI: 5.91, 104.91)
319 greater in *C. rubecula* cocoons compared to *C. glomerata* broods in CO (log likelihood $\chi^2$=46.29,
320 d.f.=1, p<0.001), but not compared to broods from MD (log likelihood $\chi^2$=3.37, d.f.=1, p=0.07)
321 (Table 1). *Catolaccus aeneoviridis* was found from only two *C. rubecula* cocoons and neither of
322 the unknown hyperparasitoids seen attacking *C. glomerata* in CO and MD were observed in *C.*

*rubecula* cocoons. When hyperparasitoids emerged from *C. rubecula* cocoons, it was always one individual per cocoon, with the exception of *B. galactopus*, which had an average of 11.48±0.44 individuals emerge from cocoons (see Appendix B). However, the multiple cocoons of *C. glomerata* broods made the brood susceptible to attacks from more than one hyperparasitoid species. Indeed, we found that 24.2% (33/136) of CO *C. glomerata* broods and 15.3% (15/98) of MD *C. glomerata* broods had more than one species of hyperparasitoid (Tables A.3 & A.4).

Hyperparasitism inflicted an obvious fitness costs for *C. glomerata*, which experienced a reduction in the emergence of adults from hyperparasitized broods. The odds of adult CO *C. glomerata* emerging successfully were 1.44 (95% CI 1.10, 1.92) times higher in non-hyperparasitized broods (log likelihood $\chi^2$=6.86, p=0.01, d.f.=1), even though non-parasitized broods were smaller on average (non-hyperparasitized broods n=141: 26.6 ± 0.9; hyperparasitized broods n=121: 31.5 ± 1.3) ($t_{260}$=3.24, p=0.001). MD *C. glomerata* also had higher success emerging from non-hyperparasitized broods with the odds being nearly twice (odds=1.95, 95% CI 1.33, 2.92) as high as in hyperparasitized broods (log likelihood $\chi^2$=12.33, p<.001, d.f.=1), however average brood sizes were similar between non-hyperparasitized (n=46, 24.52 ± 1.94) and hyperparasitized broods (n=98, 25.58 ± 1.33) ($t_{142}$=0.45, p=0.65).

Mortality from hyperparasitoid attacks

When hyperparasitoids attacked *C. rubecula* and *C. glomerata* (from CO and MD), the mortality was more severe for *C. rubecula*, likely because its cocoons develop alone and not in a brood as does *C. glomerata*. Hyperparasitoids rarely attacked the entire *C. glomerata* brood, with only 7% (10/144) of MD *C. glomerata* and 1% (4/277) of CO *C. glomerata* broods

experiencing 100% mortality, whereas 66% (176/266) of *C. rubecula* cocoons died from hyperparasitoid attacks. Although 49.1% (136/277) of CO *C. glomerata* broods and 68% (98/144) of MD *C. glomerata* broods were attacked by hyperparasitoids, the majority of cocoons within these broods escaped hyperparasitism (59% [1444/2459] for CO *C. glomerata*; 72% [2607/3635] for MD *C. glomerata*). The odds of a MD *C. rubecula* cocoon being hyperparasitized were 2.78 (95% CI 2.13, 3.63) times greater than that of a CO *C. glomerata* cocoon (log likelihood $\chi^2$=60.25, d.f.=1, p<0.001) and 4.96 (95% CI: 3.81, 6.46) times greater than that of a MD *C. glomerata* cocoon (log likelihood $\chi^2$=151.35, d.f.=1, p<0.001). Among *C. glomerata*, the odds of a MD brood being hyperparasitized were 2.26 (95% CI: 1.47, 3.44) times greater than that of a CO brood (log likelihood $\chi^2$=14.84, d.f.=1, p<0.001), but the cocoons in a MD brood were half as likely (odds = 0.56; 95% CI 0.50, 0.62) to be attacked as were conspecifics in CO (log likelihood $\chi^2$=110.34, d.f.=1, p<0.001).

Hyperparasitism and brood size of *C. glomerata*

Across all hyperparasitoid species that attacked CO *C. glomerata*, larger broods were more likely to be attacked with the odds of hyperparasitism increasing by 3% (95% CI: 1%, 5%) with each additional cocoon in a brood (Fig. 3A). While larger brood sizes increased the odds of an attack, they reduced per capita hyperparasitism since the proportion of cocoons that were hyperparasitized decreased by 5% (95% CI: 1%, 9%) with each additional cocoon (Fig. 4A). Site in CO was not a significant factor influencing the relationships between brood size and the likelihood of hyperparasitism (log likelihood $\chi^2$=7.77, d.f.=4, p=0.10) or proportion of brood hyperparasitized (log likelihood $\chi^2$=3.92, d.f.=3, p=0.27). The relationships between brood size and hyperparasitism may be regional since MD *C. glomerata* failed to show an increase in the

probability of hyperparasitoid attacks with larger brood size (Fig. 3B), nor was there a relationship between brood size and the proportion of brood hyperparasitized for MD *C. glomerata* (Fig. 4B). The relationship between brood size and probability of hyperparasitism was similar across sites in MD (log likelihood $\chi^2$=10.04, d.f.=5, p=0.07), but it was greater in 2018 than in 2017 (log likelihood $\chi^2$=10.53, d.f.=2, p=0.005). The proportion of cocoons within a brood hyperparasitized was not affected by the site (log likelihood $\chi^2$=3.44, d.f.=5, p=0.63) or year (likelihood of hyperparasitism: log likelihood $\chi^2$=4.48, d.f.=2, p=0.11).

Hyperparasitism and sex ratio of *C. glomerata*

Hyperparasitoids unlikely influenced the *C. glomerata* brood sex ratio as both non-hyperparasitized and hyperparasitized broods had a similar proportion of male *C. glomerata* emerging in CO (proportion of males for hyperparasitized vs non-hyperparasitized broods: 0.43 ± 0.03 vs. 0.37 ± 0.02) (log likelihood $\chi^2$=0.12, p=0.73, d.f.=1) and in MD (0.38 ± 0.05 vs. 0.49 ± 0.06) (log likelihood $\chi^2$=0.74, p=0.39, d.f.=1). For CO *C. glomerata*, increasing brood size, rather than the likelihood of hyperparasitism, was an important predictor of a male-biased sex ratio. With every additional *C. glomerata* cocoon in a brood, male-bias increased by 5% (95% CI: 1%,10%) for non-hyperparasitized broods and by 3% (95% CI: 0.01%, 6%) for hyperparasitized broods (Fig. 5A-B); but, there was no difference in the increase in male-bias between non-hyperparasitized and hyperparasitized broods (log likelihood $\chi^2$= 0.01, d.f.=1, p=0.91). MD *C. glomerata* failed to show a similar increase in sex ratio with brood size for either non-hyperparasitized or hyperparasitized broods (Fig. 5C-D). Site in CO was not a significant factor influencing the relationship between sex ratio and brood size for non-hyperparasitized (log likelihood $\chi^2$= 0.31, d.f.=2, p=0.86) or hyperparasitized (log likelihood $\chi^2$=

392 0.01, d.f.=1, p=0.99) broods. Year of sampling and site in MD both failed to explain the
393 variance in sex ratio and brood size of non-hyperparasitized (year: log likelihood $\chi^2$= 1.45,
394 d.f.=2, p=0.48; site: log likelihood $\chi^2$= 0.82, d.f.=3, p=0.84) and hyperparasitized broods (year:
395 log likelihood $\chi^2$= 0.003, d.f.=2, p=0.95; site: log likelihood $\chi^2$= 0.001, d.f.=3, p=0.95).
396
397 **Discussion**
398 We showed that the hyperparasitoid community in MD was equally likely to attack *C.*
399 *rubecula* cocoons and *C. glomerata* broods, but these hyperparasitoids inflicted higher mortality
400 on *C. rubecula* because its single offspring dies from hyperparasitoid attacks, whereas the
401 gregarious brood of *C. glomerata* ensures some cocoons survive even if siblings become
402 parasitized. Therefore, developing in a group reduces the per capita likelihood of attack from a
403 hyperparasitoid. In support of this per capita dilution effect, brood size plays a significant role in
404 influencing the likelihood of survivorship of individual *C. glomerata*. The per capita risk of
405 hyperparasitism significantly decreases in larger broods. Indeed, gregariousness as a life-history
406 trait may have evolved to decrease the mortality from natural enemies (Ode & Rosenheim, 1998;
407 Mayhew, 1998; Pexton & Mayhew, 2004). Additionally, that *C. rubecula* individuals are larger
408 than individual *C. glomerata* may also contribute to the apparent preference of hyperparasitoids
409 for *C. rubecula*, which is preferred over *C. glomerata* in Europe (Poelman et al., 2012).
410 Furthermore, given that both *Cotesia* species largely share the same hyperparasitoid community,
411 the numerous *C. glomerata* cocoons of its broods are potentially reservoirs for hyperparasitoids
412 that can in turn attack *C. rubecula*, possibly preventing competitive exclusion of *C. glomerata* by
413 *C. rubecula* in a region like MD. Taken together, this asymmetry in the effects of

hyperparasitoids may help *C. glomerata* coexist with *C. rubecula* in locations such as MD and may even prevent the establishment of *C. rubecula* in other regions.

While significantly different from Europe (e.g., Harvey et al., 2014), the hyperparasitoid fauna attacking *C. glomerata* and *C. rubecula* are broadly similar across North America. Like other surveys of hyperparasitoids elsewhere in North America (McDonald & Kok, 1991; Weis, Gray, & Heimpel, 2016), we showed that *B. galactopus* was the most abundant hyperparasitoid of *C. glomerata* and *C. rubecula* in both CO and MD. *Baryscapus galactopus* appears ubiquitous in North America, but regional differences in the other hyperparasitoid species could affect how strongly hyperparasitoids impact *C. glomerata* and *C. rubecula*. While *B. galactopus* is the only hyperparasitoid of these two *Cotesia* wasps that occurs in both Europe and North America, it is less abundant compared to other hyperparasitoids in Europe (Poelman et al. 2012). The intercontinental differences in the natural enemy communities attacking these *C. glomerata* and *C. rubecula* populations is an example of how multitrophic changes occur as a consequence of species moving into new environments (Schönrogge et al., 2011; Carrasco et al., 2018).

The relationship between brood size and higher concentrations of attractant volatiles may explain why the odds of hyperparasitism increased with larger in brood size in CO. Poelman et al. (2012) observed how *C. glomerata* hyperparasitoids were attracted more to herbivore-induced plant volatiles (HIPVs) from plants with *C. glomerata* parasitized *P. rapae* hosts compared to plants with unparasitized *P. rapae* hosts. Our data support this finding by demonstrating an increase in the probability of hyperparasitism with greater percentages of *P. rapae* parasitized by *C. glomerata* in CO (Fig. 2). An increase in the abundance of the herbivore host attracted more parasitoids (Fig. 1), and as more hosts were parasitized, there was an increase in hyperparasitism. Host-parasitoid interactions are mediated by chemical cues derived from plant diets (Poelman et

al. 2012; Poelman, Harvey, van Loon, Vet, & Dicke, 2013; Zhu et al., 2014), and these cues seem to have density-dependent consequences across multiple trophic levels.

If larger *C. glomerata* broods increase the magnitude of volatile emissions, then larger brood sizes may be more attractive and at greater risk to hyperparasitism. However, larger broods are beneficial if they increase the per capita survivorship, as suggested by our data for *C. glomerata*. Gregarious parasitoids can control the number of eggs laid in a host (Godfray, 1994; Pexton & Mayhew, 2005); therefore, females that lay larger broods may reduce costs of high mortality from hyperparasitoids. Gregariousness is a life-history trait that may have evolved to decrease the mortality from host defenses and natural enemies (e.g., predators and hyperparasitoids) (Ode & Rosenheim, 1998; Mayhew, 1998; Pexton & Mayhew, 2004).

Hyperparasitoids may be targeting larger broods, but we found no evidence showing that female *C. glomerata* were preferred by their hyperparasitoids. The relationship we found between male-biased sex ratios and brood size is corroborated by other field studies of *C. glomerata*. Gu, Wang, and Dorn (2003) found that *C. glomerata* broods become more male-biased as brood size increases for *C. glomerata* attacking *P. brassicae* in Switzerland, and Tagawa (2000) reported a similar pattern for *C. glomerata* attacking *P. rapae* in Japan. Female oviposition decisions are likely responsible for higher male-biased sex ratios in larger broods. For *C. glomerata*, like all hymenopterans, unfertilized eggs yield males and fertilized eggs produce females; females can adjust the ratio of fertilized and unfertilized eggs during oviposition. Single ovipositions by *C. glomerata* attacking *P. rapae* usually yield a mean brood size of 26.4±2.2 (Le Masurier, 1991) and a female-biased sex ratio (Tagawa, 2000). However, multiple females can attack the same host, and in these hosts, brood size increases at the cost of smaller-sized individuals as offspring engage in scramble competition for host resources. Since

adult size is correlated with egg load and fecundity in females, smaller sized males should be less costly than small females. Therefore, larger broods (>40 cocoons per brood) likely result from multiple *C. glomerata* attacks and these broods have male-biased sex ratios because females lay more unfertilized eggs into previously parasitized hosts (Gu et al. 2003).

We acknowledge important limitations in our comparisons of *Cotesia* populations between CO and MD. Even though we sampled multiple sites in each region (see Appendix A: Tables A.1 & A.2), the majority of our data came from two sites in CO and two sites in MD. The degree of variability within CO and MD likely resulted from site-dependent factors that influence parasitoid and hyperparasitoid populations. Differences in microclimates and crop management regimes likely affected the abundance and diversity of insects across the different sites. Even though only a couple of sites in each state contributed to most of our results, our data are comparable to previous research that also relied on data from only a few sites (McDonald & Kok, 1991; McDonald & Kok, 1992; Gaines & Kok, 1999; Weis et al., 2016). In addition to spatial variability, temporal variability can affect relationships between insects, especially if there are differences across years in the abundance of a species that heterospecifics require for survival and reproduction. Indeed, annual differences may have contributed to the variance in the relationship between *C. glomerata* brood size and probability of hyperparasitism. Future studies should consider examining the hyperparasitoids of the *C. glomerata and C. rubecula* in regions of North America where their populations are less studied, as well as sampling in more wild-type habitats.

The North American distribution of *C. rubecula* has been partially attributed to asynchronous diapause cycles between *C. rubecula* and *P. rapae* (Nealis, 1985), but differential attack of *C. rubecula* by hyperparasitoids has also been proposed as an explanation (McDonald

& Kok, 1991; Gaines & Kok, 1999). While some biological control programs use species that are able to cope with the new suite of hyperparasitoids, (Agricola & Fischer, 1991; Herren & Neuenschwander, 1991; Neuenschwander & Hammond, 1988), other control efforts are disrupted by the mortality inflicted by this top-down factor (Rosenheim, 1998; Frago et al., 2012; Gómez-Marco et al., 2015). In addition to impacts from direct trophic interactions, hyperparasitoids are known to have indirect effects on parasitoid community ecology. For instance, in South Africa, the presence of hyperparasitoids may have played a role in the apparent competition between *Cotesia vestalis* (Haliday), the dominant parasitoid of diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) and two other primary parasitoids. As hyperparasitism of *C. vestalis* increased, the population of *C. vestalis* declined and populations of the other two primary parasitoids, *Oomyzus sokolowskii* (Kurdjumov) (Hymenoptera: Eulophidae) and *Diadromus collaris* (Gravenhorst) (Hymenoptera: Ichneumonidae), increased (Nofemela, 2013). These studies demonstrate the impact hyperparasitoids can have in influencing the structure of parasitoid communities.

## Conclusion

After moving to North America, *C. glomerata* and *C. rubecula* experienced important changes to their community of natural enemies. The lack of evolutionary history with North American hyperparasitoids may influence the establishment of *C. glomerata* and *C. rubecula*, both of which are from Europe where they have evolved with a different hyperparasitoids community. However, differences in the reproductive strategies of these parasitoids seem to favor *C. glomerata*, which can escape the severe mortality impacts that hyperparasitoids inflict on *C. rubecula*. Given that parasitoid survivorship, population dynamics and community ecology

506 can all be impacted by hyperparasitoids, successful biological control initiatives must consider
507 this guild of wasps before implementing parasitoids as a management strategy.
508
509 **Acknowledgements**
510 We thank numerous organic farmers at each of our field sites in Colorado and Maryland for their
511 cooperation and willingness to grant us access to their farms. We are grateful to Dr. Whitney Cranshaw
512 and his students for planting our desired plant varieties at ARDEC South. Field work was possible thanks
513 to several former students including Stacy Endriss, Aiden Smith, Brian Smith, Evan Smith, Riley Snider,
514 Christopher Tennant, and Adrianna Tompros. This work was funded by the United States Department of
515 Agriculture NIFA AFRI: 2014-67013-2172 to P.J.O. Additional financial support was provided to
516 D.K.V. by the William M. Brown Professional Development Award, Sigma Xi and the Colorado State
517 University Graduate Degree Program in Ecology
518
519 Appendix A. Supplementary data
520 Appendix B. Supplementary data
521 Supplementary data associated with this article can be found, in the online version, at XXXXX.

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Table 1. The hyperparasitoid species that emerged from *Cotesia glomerata* broods (n=277) collected in Colorado from June-October 2015 and from *C. glomerata* broods (n=144) and *C. rubecula* cocoons (n=266) collected in Maryland from August 2016, May-September 2017 and July 2018.

| Family | Subfamily | Hyperparasitoid species | # of Cg broods & Cr cocoons with hyperparasitoid species | | | Number of emerged individuals | | |
|---|---|---|---|---|---|---|---|---|
| | | | CO Cg | MD Cg | MD Cr | CO Cg | MD Cg | MD Cr |
| Chalcididae | Chalcidinae | *Conura torvina* | 2 | 17 | 45 | 23 | 34 | 45 |
| Encyrtidae | n/a | Unidentified | 1 | 0 | 0 | 1 | 0 | 0 |
| Eulophidae | Tetrastichinae | *Baryscapus galactopus* | 42 | 83 | 119 | 874 | 2188 | 1366 |
| Ichneumonidae | Cryptinae | *Gelis* sp. | 1 | 0 | 0 | 1 | 0 | 0 |
| Pteromalidae | Pteromalinae | *Trichomalopsis dubia* | 39 | 0 | 0 | 372 | 0 | 0 |
| Pteromalidae | Pteromalinae | *Catolaccus aeneoviridis* | 40 | 6 | 2 | 264 | 49 | 2 |
| Pteromalidae | Pteromalinae | *Dibrachys cavus* | 1 | 0 | 0 | 36 | 0 | 0 |
| Pteromalidae | Pteromalinae | *Hypopteromalus tabacum* | 3 | 0 | 0 | 21 | 0 | 0 |

CO = Colorado, MD = Maryland, Cg = *C. glomerata*, Cr = *C. rubecula*