PARASITOIDS' ECOLOGY AND EVOLUTION

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PUBLISHED IN: Frontiers in Ecology and Evolution

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-480-4
DOI 10.3389/978-2-88963-480-4

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PARASITOIDS' ECOLOGY AND EVOLUTION

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Citation: Calatayud, P.-A., Clarke, C. W., Sforza, R. F. H., Ndemah, R. N., Nyamukondiwa, C., eds. (2020). Parasitoids' Ecology and Evolution. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-480-4

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frontiers in Ecology and Evolution

EDITORIAL
published: 10 December 2019
doi: 10.3389/fevo.2019.00485

Editorial: Parasitoids' Ecology and Evolution

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Keywords: biological control, host-associated differentiation, coevolution, genetic structure, environmental change

Editorial on the Research Topic

Parasitoids' Ecology and Evolution

OPEN ACCESS

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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *17 September 2019*
Accepted: *28 November 2019*
Published: *10 December 2019*

Citation:
Clarke CW, Calatayud P-A, Sforza RFH, Ndemah RN and Nyamukondiwa C (2019) Editorial: Parasitoids' Ecology and Evolution. Front. Ecol. Evol. 7:485. doi: 10.3389/fevo.2019.00485

Parasitoids are among arthropods that are most widely used in biological control against crop pests, and thus are a significant component of integrated pest management systems. The interaction of parasitoids with the environment (including both hosts insects and plants) have been well-studied for guilds that lay eggs in or on an insect host, followed by larval development, ultimately killing the host (e.g., Godfray, 1994; Kaiser et al., 2017). A better knowledge of parasitoid's biology and ecology is key to successful application of evolutionary principles and determinant to identifying intimate connections with the life history of respective hosts (Roitberg et al., 2001), that often lead to extremely specialized host-parasitoid interactions. Although often overlooked in research and development, ecological and evolutionary considerations are significant to natural control of pests by parasitoids in agricultural systems (e.g., Heimpel and Mills, 2017). The articles in this Research Topic on "parasitoids' ecology and evolution" address fundamental topics in ecology and evolution of parasitoids and their hosts in a context of global changes, i.e., both climate and landscape changes. As in all science disciplines, the purpose of this Research Topic is to showcase current research and unravel new opportunities for future investigations with respect to management of pests by parasitoids.

Why wide-ranging studies on parasitoid's ecology and evolution? Over the years, there has been valid concerns for non-target impacts and the environment in the utilization of biocontrol agents as part of an integrated management strategy. Consequently, the agents are now only released into agricultural systems for regulation of pests following a thorough and extensive host-testing aimed at delimiting the range of candidate biocontrol agents. This approach has undoubtedly made biological control safer and more predictive ecologically. Research focused on host range exclusively, however, lacks a measure of genetic variation in host use and the responses of the respective hosts under different climate scenarios. Subsequently, there remains uncertainties on the co-evolutionary interactions between hosts, parasitoids, plants, and on future establishments.

Parasitoids complete development in other arthropods, mostly insects, leading to their death or sterility and offer an excellent mechanism for natural and sustainable pest control. Though parasitoids may appear generalists, careful ecological studies tell of a hidden complexity with an assemblage of populations having more restricted host ranges. We therefore highlight that

studying parasitoid diversity may benefit a successful implementation for their use in biological control and ignoring their diversity can be damaging. Both Frayssinet et al., and Bredlau et al. sought to understand the intra- and interspecific plasticity with an aim of enhancing our knowledge on how biocontrol agents can be used for the advancement of integrated pest management in fluctuating host densities and seasonal changes. Tougeron et al. further provided perspectives on the implications for biological control based on the composition of host-parasitoid communities in the climate change context.

Factors associated with poor parasitoid performance are attributable to management practices, e.g., chemical applications, and disturbances in agricultural landscapes (Gurr et al., 2017). Chidawanyika et al. exemplified the effect of climate change as a modifying factor of parasitoid physiology and thereby the effectiveness of biocontrol in diverse agroecosystems. Factors such as habitat fragmentation have been referred to as key disruptors of parasitoid assemblages (Cooke and Roland, 2000) and attributable to loss of connectivity in community assembly. In order to maintain the parasitoid genetic heterogeneity for a robust resilient agricultural systems, Branca et al. highlighted the attention and the need to consider the evolutionary capacity at local and area-wide landscapes. Research should also consider identifying parasitoids that are adaptable to changing climates and agricultural landscapes, and those which are able to cope with host evolution despite many additional biotic and abiotic ecological forces, including reproduction manipulators that would be expected to reduce local adaptation to hosts (Branca et al.; Chidawanyika et al.).

The success of a biocontrol program depends on the foraging efficiency of parasitoids which includes their ability to accurately locate, manipulate, and accept their hosts (Vinson, 1976). For classical and augmentative biological control, locating the target pest depends on the interplay between the parasitoid, the target pest and the crop. Foraging can be enhanced by adult pre-release exposure to target pest and host plant volatiles. de Bruijn et al. showed that persistent memories, such as those formed after several experiences spaced in time, can lead to maladaptive foraging behavior if the contained information becomes unreliable. Kruidhof et al. also demonstrated that studies focusing on plant volatiles may be constrained by a weak response to foraging cues originating from a crop environment, and/or an innate tendency for dispersal upon release. These latter factors lead to declining searching efficiency and subsequently, reduction of parasitism rates, can be overcome by optimizing the parasitoids foraging behavior through parasitoid olfactory conditioning (POC). Parasitoids can be trained to become more efficient in the different phases involved in the process of host searching and host acceptance and POC can thus, result in a "foraging efficacy gain" (Kruidhof et al.).

The success of parasitoid biocontrol agents in addition, depends strongly also on host density and timing of seasonal activities (phenology) (Berryman, 1999; Jeffs and Lewis, 2013). For example, any decline of pest density linked to changes in their host plant characteristic and seasonal availability (e.g., enhanced evasive behavior of the plants) can drastically affect the parasitism rates and then the success of the biological control program (Tomasetto et al.). To overcome this risk of parasitism decline, modifications in parasitoid community composition linked to shifts in diapause expression (reduction or arrest of the use of winter diapause) and to host availability throughout the year can occur (Tougeron et al.). Parasitoids tend to show a preference for ovipositing in the host species in which they developed regardless of host identity and the availability of alternative hosts because switching to novel hosts is initially time consuming and costly (Jones et al., 2015). The costs and the time frame over which these are incurred, may influence host selection behavior and host-parasitoid dynamics in multispecies communities. Frayssinet et al. described here the ability of a parasitoid to switch hosts in fluctuating densities of the preferred host, a strategy that would allow the parasitoid to avoid seasonal population collapses.

The ability of parasitoids to counter host immune defenses and forage for hosts is dependent on host maternal factors and is shaped by co-evolution (Kraaijeveld and Godfray, 1999). Parasitoids learn to associate environmental cues and food, with hosts while foraging and use specific signals to discriminate between hosts and non-host species using chemical compounds to locate and accept their hosts. Bichang'a et al. (2018) showed for the first time that an enzyme from oral secretions of the host plays a key role in host acceptance and oviposition by parasitoids. The molecular variations in this enzyme could explain and account for host-range differences between parasitoid species and the evolutionary processes involved in chemically-mediated host specialization (Bichang'a et al.). Bracovirus genes though different across orders of parasitoids are responsible for immune suppressive abilities. Parasitoids could be responsible for maintaining a reproductive isolation across a broad range of host-food plant sources by the mode of maternal factors expressed and presence of multiple hosts (Bredlau et al.). These authors highlighted here an unexplored study area in biocontrol programs that comprises parasitoid population structure among different host-associated populations, their maternal factors and host plant sources at the landscape level.

CONCLUSION AND FUTURE CHALLENGES

Articles published in this Research Topic "parasitoids' ecology and evolution" explore the evolutionary aspects of biological control and opens new areas for future research. For classical biological control that involves importation and introduction of agents from their native range, the choice of biocontrol agents could be based on established relationships with the host in the native range or interactive models that predict the effectiveness of the agents in "new associations" accounting for multiple hosts and scenarios of hosts unavailability. With this Research Topic, we aimed to provide a platform for scientists to share their understanding of mechanisms that drive the ecological and evolutionary interactions between parasitoids and their hosts. The excellent contributions are a demonstration of a still active research community in this and provided an up-to-date

understanding of the intrinsic capacity of parasitoids to adapt in rapidly changing agricultural landscapes.

AUTHOR CONTRIBUTIONS

P-AC came up with the topic idea. CC, RS, RN, and CN contributed to its description and to the overall organization of the special Research Topic. All editors contributed to the overseeing of reviews for this special issue. CC wrote the first version of the cover editorial and all editors contributed to its final version.

ACKNOWLEDGMENTS

We wish to thank all authors of this Research Topic for their contributions.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 16 October 2018
doi: 10.3389/fevo.2018.00160

Costs of Persisting Unreliable Memory: Reduced Foraging Efficiency for Free-Flying Parasitic Wasps in a Wind Tunnel

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Parasitic wasps are known to improve their foraging efficiency after learning of herbivore-induced plant volatiles (HIPVs) upon encountering their hosts on these plants. However, due to spatial and temporal variation of herbivore communities, learned HIPV cues can become unreliable, no longer correctly predicting host presence. Little is known about the potential fitness costs when memories holding such unreliable information persist. Here we studied how persistent memory, containing unreliable information, affects the foraging efficiency for hosts in *Cotesia glomerata*. Wasps were conditioned to associate one of two types of HIPVs with either *P. brassicae* frass, 1 single oviposition in *P. brassicae*, 3 ovipositions in *P. brassicae* spaced in time or they were kept unconditioned. The following day, wasps were allowed to forage in a wind tunnel, in an environment that either conflicted or was congruent with their learned plant experience. The foraging environment consisted of host (*P. brassicae*) and non-host (*Mamestra brassicae*) infested plants. The conflicting environment had non-hosts on the conditioned plant species and hosts on the non-conditioned plant species, whereas the congruent environment had hosts on the conditioned plant species and non-hosts on the unconditioned plant species. Wasps had to navigate through five non-host infested plants to reach the host-infested plant. Since *C. glomerata* wasps do not distinguish between HIPVs induced by host and non-host caterpillars, the conflicting foraging situation caused a prediction error, by guiding wasps to non-host infested plants. Especially wasps given 3 spaced oviposition experiences, tested in a conflicting situation, spent significantly more time on non-host infested plants and showed a high tendency to oviposit in the non-hosts. As a result, they took significantly more time to find their hosts. Conditioned wasps, which were tested in a congruent situation, were more responsive than unconditioned wasps, but there was no difference in foraging efficiency between these two groups in the wasps that showed a response. We conclude that persistent memories, such as formed after 3 experiences spaced in time, can lead to maladaptive foraging behavior if the contained information becomes unreliable.

Keywords: *Cotesia glomerata*, learning, foraging efficiency, unreliable information, non-host, oviposition, prediction error, memory

OPEN ACCESS

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Reviewed by:
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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *31 July 2018*
Accepted: *25 September 2018*
Published: *16 October 2018*

Citation:
de Bruijn JAC, Vet LEM and Smid HM (2018) Costs of Persisting Unreliable Memory: Reduced Foraging Efficiency for Free-Flying Parasitic Wasps in a Wind Tunnel. Front. Ecol. Evol. 6:160. doi: 10.3389/fevo.2018.00160

INTRODUCTION

A high degree of spatial and temporal variation exists in herbivore communities, which makes it challenging for predators to find suitable prey. The way parasitoids use environmental cues to find resources such as hosts is of great importance for their realized lifetime reproductive success, a measure of fitness (Van Baalen and Hemerik, 2007). An inexperienced female parasitic wasp is attracted by a range of environmental cues, which have proven their reliability for host finding over generations through natural selection (Stephens, 1993; Van Alphen and Bernstein, 2008; Hoedjes et al., 2011). Due to the high degree of both spatial and temporal variation within and between generations in the availability, distribution and abundance of both host and host plant species, these cues can be insufficient to guide parasitoids to their hosts (Stephens, 1993; Vet, 2001). Parasitoids can, however, acquire and process information as they forage, thereby learning how to become more efficient foragers. Parasitoids are known to use a wide variety of olfactory, visual, auditory and tactile cues to obtain and store information on local host presence, distribution and abundance (Vet and Dicke, 1992; Turlings et al., 1993; Van Alphen and Bernstein, 2008; Ishii and Shimada, 2009). Acquisition of this information can be achieved through learning, in particular through associative ovipositional learning, where an oviposition in a host becomes associated with various environmental cues, such as herbivore-induced plant volatiles (HIPVs), resulting in associative memory (Bleeker et al., 2006). Even an encounter with host traces, such as silk and feces (frass), without the host themselves, results in learning of HIPV's (Geervliet et al., 1998), albeit that such memories are generally less persistent than after an oviposition experience (Lewis and Martin, 1990; Takasu and Lewis, 2003). With these memories, parasitic wasps can temporarily adapt their foraging strategy to current local host and host-plant availability.

In general, only when multiple learning events occur spaced in time, the learned information is considered reliable enough to adapt foraging behavior accordingly for a prolonged time. It is then stored in robust long-term memory (LTM), which can last for days (Menzel, 1999; Hoedjes et al., 2011). Moreover, LTM formation is costly in terms of energy expenditure (Menzel, 1999; Mery and Kawecki, 2004), because it depends on protein synthesis (Tully et al., 1994), which is another reason why single learning events usually results in the formation of energetically inexpensive, short lasting memory, naturally decaying within minutes to hours (Menzel, 1999; Hoedjes et al., 2011).

The generalist larval parasitoid *Cotesia glomerata*, is well known for its ability to learn in both laboratory and (semi-)field studies (Geervliet et al., 1998; Perfecto and Vet, 2003; Smid et al., 2007; De Rijk et al., 2018; Vosteen et al., manuscript in preparation). Unlike general theory, it consolidates LTM for oviposition events on certain host plants within 4 h after only a single oviposition in its host *Pieris brassicae* (Smid et al., 2007). This direct LTM induction is most likely due to the spatial distribution and gregarious nature of this host, since a single encounter with a gregarious host reliably predicts many oviposition opportunities. Indeed, when this wasp species encounters a solitary host, *P. rapae*, it does not form LTM, but a less persistent memory type, anesthesia-resistant memory (Kruidhof et al., 2012). While LTM of a single oviposition wanes over 5 days, spaced conditioning with 3 ovipositions leads to even more persistent LTM, lasting for more than 5 days (Van Vugt et al., 2015). Thus, experiences with only frass, a single oviposition or three ovipositions spaced in time each induce different memories with increasing levels of persistence.

This memory guides *C. glomerata* to subsequent host patches, but due to the high similarity of HIPV of host and non-host species, these wasps are often unable to discriminate between them (Geervliet et al., 1996; Vos et al., 2001; Bukovinszky et al., 2012), even after oviposition experience (Vosteen et al., manuscript in preparation). The presence of non-host on host plant species has been found to lead to reduced foraging efficiency (Vos et al., 2001; Bukovinszky et al., 2012; De Rijk et al., 2016b; Desurmont et al., 2018; Vosteen et al., manuscript in preparation).

Since environments keep changing, assessment of the reliability of the learned information is a continuous process. Non-hosts might occur on plants previously associated with hosts. Encountering non-hosts on plants that emit HIPVs previously associated with host presence, leads to a predication error; the learned cues do not predict host presence, they have become unreliable. To optimize foraging efficiency, information needs to be processed in an adaptive and integrative way (Hilker and Mcneil, 2008), continuously updating memories and acting according to the most reliable information available.

The different levels of memory persistence described above make these wasps an ideal model to study the risk of maladaptive foraging behavior due to persistent unreliable information. Here we conducted a wind tunnel experiment to study how foraging efficiency is affected in the parasitic wasp *C. glomerata*, when foraging in an environment, which was either conflicting or congruent with previously learnt information varying in persistence. We confronted the wasps with non-hosts on the plant species on which had they previously found their hosts, and hosts on the plant species not encountered before (conflicting) or vice versa (congruent). We expect that with higher levels of memory persistence, wasps will increasingly suffer from reduced foraging efficiency in the conflicting foraging situation, and benefit on the other hand from improved foraging efficiency in the congruent foraging situation.

MATERIALS AND METHODS

Insects

Pieris brassicae (Lepidoptera: Pieridae) and *Mamestra brassicae* (Lepidoptra: Noctuidae) caterpillars were reared on Brussels sprouts plants (*Brassicae oleracea* L. var. gemmifera cultivar Cyrus). Females of the parasitic wasps *C. glomerata* (Hymenoptera: Braconidae) were obtained from a yearly re-established culture, and reared on *P. brassicae* caterpillars, to maintain natural foraging behavior. All insect cultures were maintained at the Laboratory of Entomology, Wageningen University and were reared under the same conditions in a climate-controlled greenhouse with natural light conditions, 21 ± 1°C and 50–70% humidity. First instar *P. brassicae* caterpillars were used for parasitoid rearing. Upon emergence of the parasitoid larvae, cocoons were collected and kept in Petri

dishes which were put in a climate cabinet (21 ± 1°C, L16:D8 photoperiod and 50–70% humidity). Just prior to emergence the cocoons were transferred to cages (40 × 303 × 30 cm, Bugdorm-1 Insect rearing cage, type DP1000, Megaview Science, Taiwan) with honey and water. Two-day-old females were selected from these cages and kept with honey and water until the start of experiments, when females were 3–5 days old.

Plants

For experiments 3–4 weeks old *Brassicae nigra L.* and *Sinapis arvensis L.* plants were used. Plants were watered daily and were supported by a small green wooden stick and a metal ring to ensure upright growth. Induction of both plant species was accomplished by placing 2 batches of 5 *M. brassicae* 48 h prior to experiments, or 2 batches of 5 *P. brassicae* caterpillars 24 h prior to experiments, on the fourth true leaf of a plant with clip cages. Clip cages were kept upright by attaching each of them to a small green wooden stick (30 cm long, 4 mm diameter) to prevent the leaf from breaking due to the weight of the clip cage. Besides the clip cages, some cotton wool was wrapped around the base of the leaf to prevent the spread of caterpillars to other leaves once the clip cages were removed. Early first instar *P. brassicae* and late first instar *M. brassicae* caterpillars were used to infest plants. The difference in age was to obtain similar caterpillar body sizes. *M. brassicae,* however, caused less feeding damage and the induced plants were less attractive to parasitoids after 24 h induction (personal observation), therefore *M. brassicae* was kept on the plant 24 h longer than *P. brassicae* to obtain similar damage and attractiveness of plants. After every 3 h of experiments plants were replaced.

Parasitoid Conditioning

A day before conditioning a *B. nigra* and a *S. arvensis* plant were induced with approximately 200–300 *P. brassicae* caterpillars spread in batches of approximately 50 caterpillars over the plant leaves. A classical (Pavlovian) conditioning procedure was used, which excludes the host-searching phase, adopted from Bleeker et al. (2006), to give wasps an associative learning experience (**Figure 1**). This procedure consists of giving wasps oviposition experience on a plant leaf, where wasps learned to associate plant odors as the conditioned stimulus (CS) with suitable hosts as the unconditioned stimulus (US). This type of conditioning is considered a form of classical (Pavlovian) conditioning, where the host-searching phase is excluded.

A total of 7 different conditioning treatments were conducted; wasps were kept unconditioned or were given conditioning experience on either the induced *B. nigra* or *S. arvensis* plant. Conditioning on these leaves consisted of (A) a single leaf damage experience where a wasp was transferred from a glass vial to a leaf with host feeding damage. The first instar *P. brassicae* host caterpillars had been removed, but their frass was still present. The wasp was allowed to contact the host frass for 20 s, after which it was gently removed with the glass vial. (B) A single oviposition in a first instar *P. brassicae* caterpillar, which was performed as under (A), but now with host caterpillars present. After a single oviposition, the wasp was removed with a glass vial. (C) Spaced conditioning consisting of 3 ovipositions spaced in time. It was performed as 3 sequences of single ovipositions, as described for (B), spaced by intervals of 10 min, during which the wasp remained in the glass vial. Wasps were conditioned individually and only ovipositions lasting longer than 2 s were considered successful (Coleman et al., 1999). **Figure 1** shows an overview of these conditioning procedures. While both 1 and 3 ovipositions are expected to induce LTM, spaced conditioning with 3 ovipositions leads to longer lasting, more robust LTM (Smid et al., 2007; Van Vugt et al., 2015), with stronger memory persistence (**Figure 1**). After conditioning, wasps from all treatment groups were placed in small cages (17 × 17 × 17 cm, Bugdorm type 41515, Megaview Science, Taiwan) supplied with water and honey till testing in the wind tunnel the next day.

Wind Tunnel Set-Up

The experiment was conducted in a wind tunnel as described in Geervliet et al. (1994) with wind speed set to 10 cm/s, a temperature of 24 ± 1°C and a relative humidity fluctuating between 50% and 70%. A glass cylinder (30 cm long, diameter 15 cm) was used as release site and was placed 70 cm upwind from the first plant. Six plants were placed 15 cm apart and 10 cm from the walls of the wind tunnel, five plants infested with the non-host *M. brassicae* and one with host *P. brassicae*, the latter being placed upwind from the five non-host infested plants. Two different foraging situations were created, with either the non-host *M. brassicae* on *B. nigra* and the host *P. brassicae* on *S. arvensis,* or vice versa. On a single experimental day both foraging situations were used, each running for 3 h. Both the order of the foraging situations and the position of the *P. brassicae* plant were alternated daily. The order of the 7 conditioning treatments was randomized, on each experimental day 2 wasps were tested per treatment. The 7 conditioning treatments and the 2 foraging situations lead to a total of 14 treatments, each treatment was replicated 15 times. An overview of the conditioning and test procedure of these various treatments is shown in **Figure 1**.

The wind tunnel was turned on 1–1.5 h prior to experiments to create stable temperature and humidity values. Just prior to the start of the experiment plants were positioned in the wind tunnel, clip cages and their supporting sticks were removed. Caterpillar movement was restricted to the leaf due to the cotton wool wrapped around the base and caterpillars were counted to make sure 10 live caterpillar would be available. Dead caterpillars were replaced by caterpillars of the same size and age.

Upon the start of the experiment a single wasp was transferred to a glass test tube (12 × 75 mm), from its cage into the glass release cylinder in the wind tunnel. Each wasp was given 5 min to initiate flight and leave the cylinder. Those that did not fly out of the glass cylinder were taken out of the experiment. Wasps which directly flew to the ceiling of the wind tunnel were re-released once.

Behavioral Observations

Wasp behavior was recorded on a hand-held computer with The Observer XT 10 software (Noldus Information Technology B.V., Wageningen, The Netherlands) for 15 min or until first

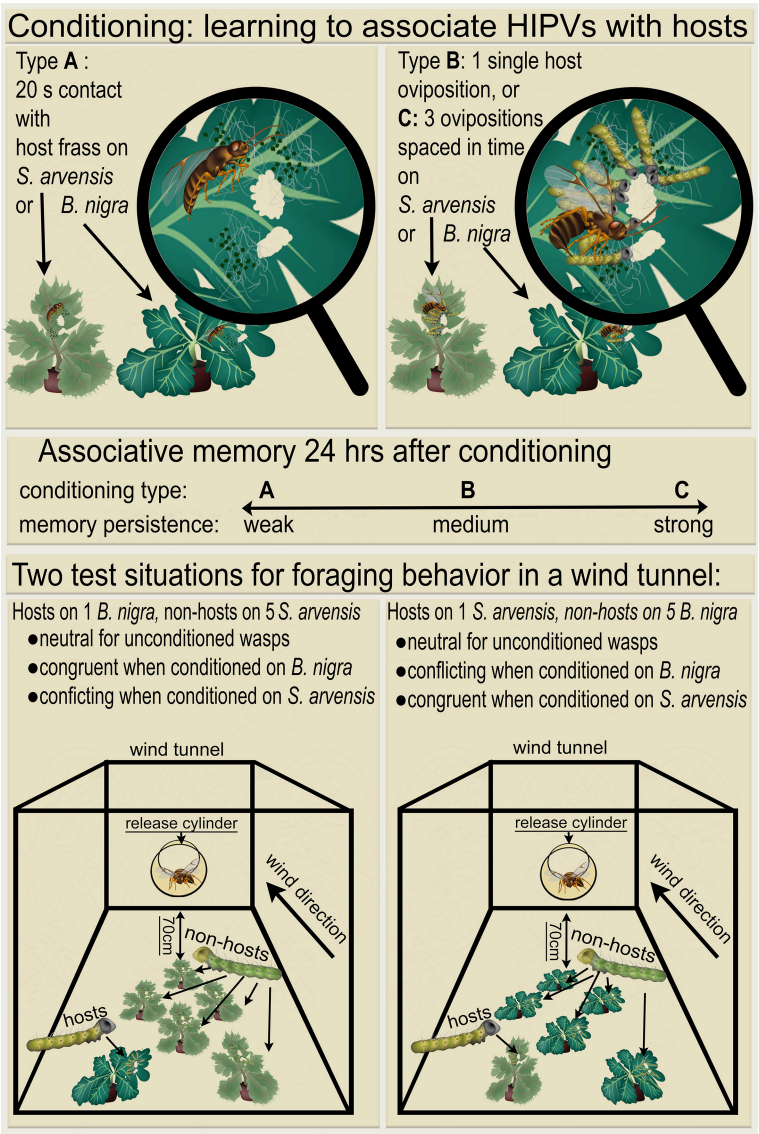

FIGURE 1 | Overview of the conditioning and test procedures. Wasps were given a learning experience **(Top)**, in which they learned to associate herbivore-induced plant volatiles (HIPVs) of *Brassicae nigra* (dark green plants) or *Sinapis arvensis* (light green plants) with either a 20 s host-frass (*Pieris brassicae*) exposure, 1 single host oviposition, or 3 host ovipositions spaced in time. These conditioning treatments resulted in increasing levels of memory persistence (**mid panel**, indicated with weak, medium or strong), for either *B. nigra* or *S. arvensis* as predictor for the presence of *P. brassicae* hosts. The next day these conditioned wasps were tested in foraging situations created in a wind tunnel **(Bottom)**, which were either congruent or conflicting with their memory. A conflicting situation consisted of non-hosts (*Mamestra brassicae*) on five of the plant of the species on which the wasps previously experienced hosts or host-frass, and hosts on only one plant of the alternative plant species, located most upwind from the release point. The congruent situation had the same array of 5 plants with non-hosts and one plant with hosts, but in this case the hosts were present on the same plant species on which the previously were conditioned. Unconditioned wasps were also tested, for which both foraging situations were considered as neutral. Altogether, this results in 14 different treatments.

host oviposition. We used the following behavioral parameters for statistical analysis: foraging time (total time of the behavioral recording), time on non-host patches, number of non-host patch visits and non-host oviposition occurrences. Only behavior on the actual infested leaves was considered. Furthermore, direct flight (the percentage of wasps which only landed on the host plant after flight initiation) and wasp response (the percentage of wasps initiating flight and orientation to the HIPVs) were also used for statistical analysis.

Statistics

All statistical analyses were done in R version 3.4.3 (R Development Core Team 2017). Foraging time was analyzed using survival analysis with a cox regression analysis (coxph from the survival package, (Therneau and Lumley, 2015)), where censored data consisted of wasps not finding their host within 900 s. Data on time on non-host patches and number of non-host patch visits were analyzed using linear mixed models (lme from the nlme package, Pinheiro et al., 2014) with experimental day

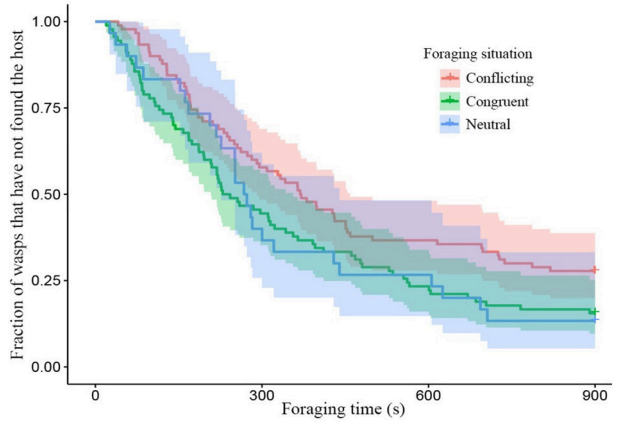

FIGURE 2 | Survival plot of the fraction of wasps which have not found the host with foraging time in different foraging situations. Colored areas around the lines show the 95% confidence interval (neutral $n = 30$, conflicting and congruent $n = 90$). Conflicting and congruent survival curves were significantly different ($z = -2.38$, $p = 0.046$).

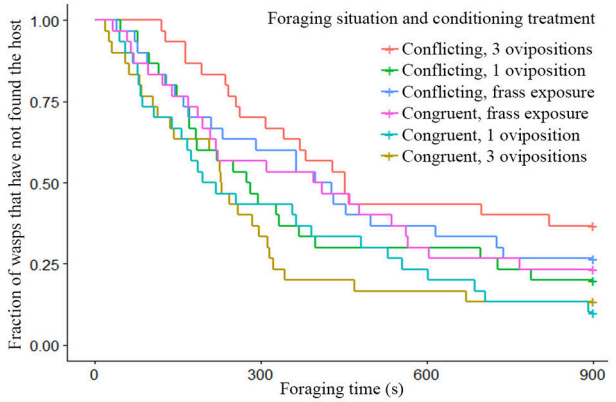

FIGURE 3 | Survival plot of the fraction of wasps which have not found the host within the 900 s foraging time, given different conditioning treatments and foraging situations ($n = 30$). Wasps given 3 spaced ovipositions in combination with conflicting information were significantly different from wasps given 3 spaced oviposition in combination with congruent information ($z = -2.96$, $p = 0.036$).

as a random factor. Data on the number of non-host patch visits was log transformed to account for equal variance, time on non-host patches was square root transformed. Presence/absence data on non-host oviposition, direct flight and response was analyzed with a Bernoulli glmm (glmer from the lme4 package, Bates et al., 2014) with day as a random factor.

The statistical models used foraging situation, test plant species and conditioning treatment as fixed factors. Due to an incomplete factorial design, models including unconditioned wasps/neutral foraging situation were run without conditioning treatment and vice versa. Differences between groups were analyzed with a least-square means *post-hoc* comparison with error correction (lsmeans from the lsmeans package, Lenth, 2016).

RESULTS

The Effect of Foraging Situation and Conditioning Treatment on Foraging Behavior

Wasps given conflicting information had more difficulty finding hosts, than wasps given congruent information as can be seen by the clear divergence of their survival curves in **Figure 2**. While conditioning treatment did not have a strong effect on its own, the combination of foraging situation and the conditioning treatment shows clear effects of spaced conditioning with 3 ovipositions (**Figure 3**). While 3 ovipositions with congruent information made them the fastest group to find the host, 3 ovipositions with conflicting information resulted in wasps being the slowest group to find the host. Since the congruent and conflicting survival curves of frass and a single oviposition show a high degree of overlap, the overall difference between congruent and conflicting foraging situations is mainly explained by the effect of spaced conditioning with 3 ovipositions (**Figure 3**).

Assessment of the underlying behavioral components during the foraging period revealed significant differences in the time wasps spent on non-host patches. Wasps given spaced conditioning with conflicting information stayed more than twice as long on non-host patches, than wasps given spaced conditioning with congruent information (**Figure 4**). The same pattern was observed for non-host oviposition, where wasps given spaced conditioning with conflicting information oviposited 3 times as often in non-hosts, but here the difference between the two spaced conditioning groups had a p-value of 0.063 (**Figure 5**).

Survival analysis of unconditioned wasps, foraging in a neutral situation, switched between the congruent and conflicting conditioned wasps within the first 250 s (**Figure 2**). Thereafter, the unconditioned wasps behaved very similar to congruently conditioned wasps. Overall, wasps foraging in a neutral situation did not behave significantly different from wasps foraging in a conflicting ($z = 1.76$, $p = 0.183$) or congruent situation ($z = 0.031$, $p = 1.000$), due to high behavioral variation show in the 95% confidence interval in **Figure 2**. Unconditioned wasps did make fewer visits to non-host patches, than wasps given a conflicting experience ($f = 3.04$, $p = 0.049$, **Figure 6A**). Furthermore, fewer unconditioned, than conditioned wasps responded to HIPVs in the wind tunnel ($z = -5.19$, $p = 0.000$, **Figure 6B**).

Test Plant Species Effects on Foraging Behavior

The plants species offered during the foraging trail also greatly influenced foraging behavior. In foraging situations when non-hosts were present on *B. nigra* and the hosts on *S. arvensis*, wasps took longer to find the host ($Chi^2 = 4.87$, $p = 0.027$), they spent more time on non-host leaves ($t = -3.38$, $p = 0.001$, **Figure 7A**) and visited non-host leaves more often ($t = -2.61$, $p = 0.010$,

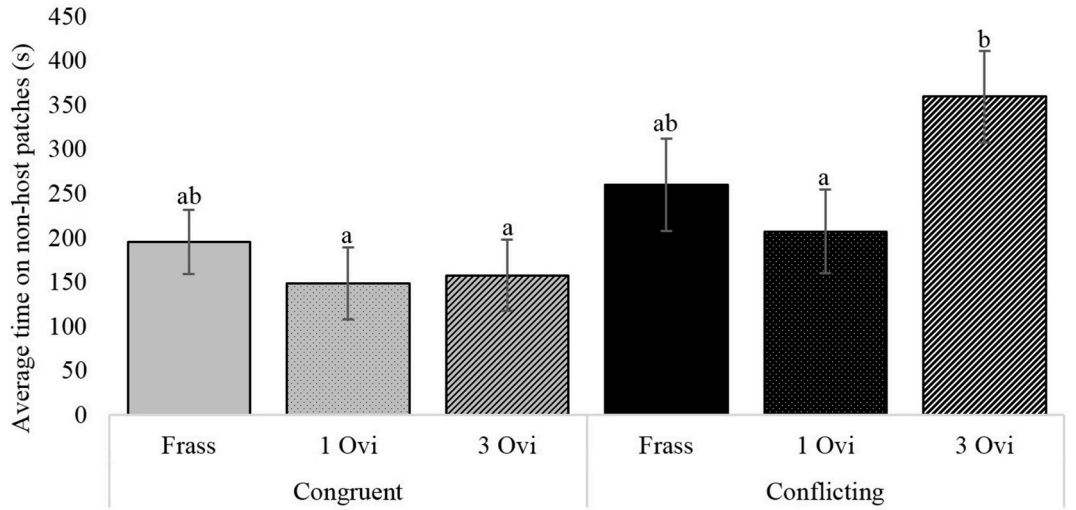

FIGURE 4 | The influence of foraging situation and conditioning treatment on the average time wasps spent on non-host patches. Conditioning treatments consisted of; a 20 s host frass exposure (Frass), 1 oviposition (1 Ovi), or 3 spaced ovipositions (3 Ovi). Bars with different letters are significantly different ($n = 30$, $\alpha = 0.05$), error bars show the s.e.

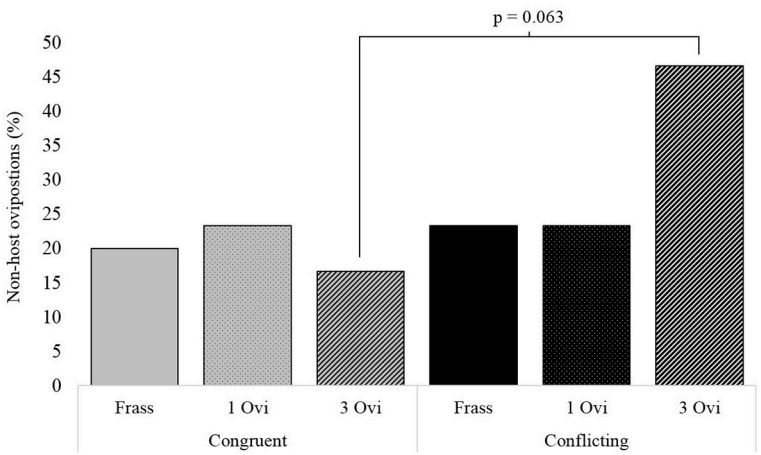

FIGURE 5 | The percentage of wasps oviposting in non-host when given either conflicting or congruent experience with either a 20 s frass experience (Frass), a single oviposition (1 Ovi), or 3 spaced ovipositions (3 Ovi) ($n = 30$).

Figure 7B), than when the host was on *B. nigra* and the non-host on *S. arvensis*. Furthermore, wasps performed more direct flights when the host was found on *B. nigra*, than when it was on *S. arvensis* ($z = 2.79$, $p = 0.005$, **Figure 7C**).

DISCUSSION

Natural environments are ever changing, and as a consequence learned information can become outdated and should be forgotten. Since most parasitoids are time-limited, they should utilize their time as efficiently as possible, since the way they exploit their environment directly determines their realized fitness (Van Baalen and Hemerik, 2007). While most studies focusing on the effect of learning on foraging efficiency provide the wasp with a foraging situation highly similar to what they have been trained in Geervliet et al. (1998), Takasu and Lewis (2003), Bleeker et al. (2006), Smid et al. (2007), Kruidhof et al. (2015), De Rijk et al. (2018), and Desurmont et al. (2018), we tested how both reliable and unreliable information affects foraging efficiency in a foraging situation with attractive odor plumes of both hosts and non-hosts. As expected, we found maladaptive foraging behavior after providing wasps with conflicting information, especially when the information has previously proven to be reliable through spaced conditioning. It seems that a 3 spaced oviposition experience does not only result in longer lasting memory (Smid et al., 2007; Van Vugt et al., 2015), but also results in a stronger focus on the memory content during foraging as the information is considered more reliable.

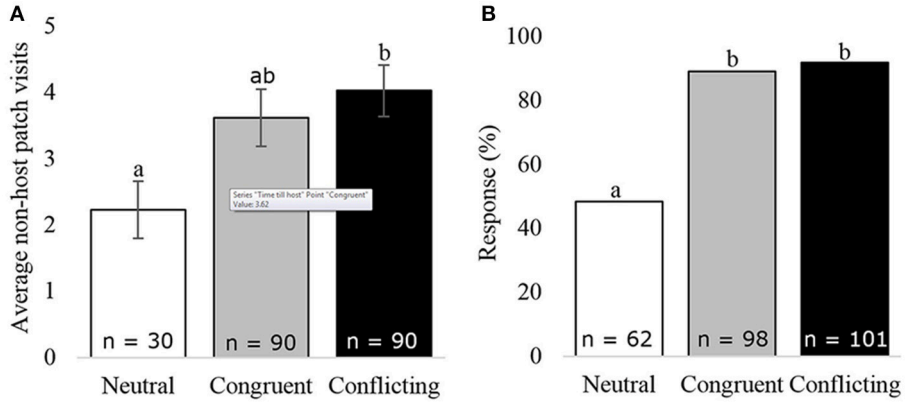

FIGURE 6 | Wasp behavior under different foraging situations: **(A)** average non-host patch visits, **(B)** the percentage of wasps responding to the HIPVs in the wind tunnel. Bars with different letters are significantly different ($\alpha = 0.05$), error bars show the s.e.

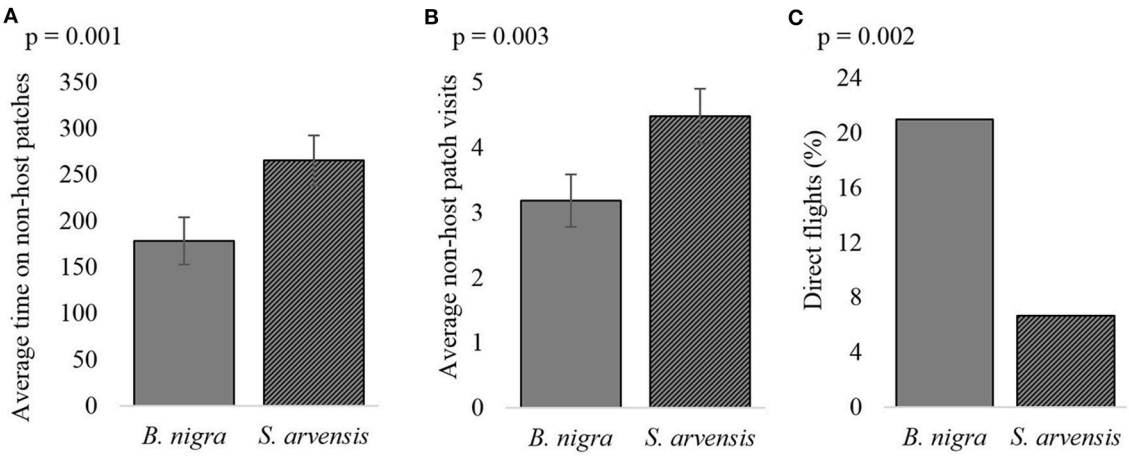

FIGURE 7 | The effect of different host plant species on **(A)** the average amount of time wasps spent on non-host patches, **(B)** the average number of visits to non-host patches and **(C)** the number of direct flights, when the host is found on either *Brassica nigra* or *Sinapis arvensis*. Error bars show the s.e. ($n = 105$).

This was reflected in wasps taking more time to find hosts and spending more time on non-host patches.

Though learning is generally expected to result in an increase in foraging efficiency, finding more hosts and increasing realized fitness, this most likely only applies if the obtained information is correct. Learning is known to be costly in various ways (Mery and Kawecki, 2004) and our study confirms that persistent unreliable memory involves costs primarily associated with time. However, it is still unclear how the wasp will overcome long-term negative effects of this unreliable information. The encounter of a non-host, upon the response to HIPV's previously associated with a host, causes a prediction error and can be considered as a memory extinction event. This event might trigger the formation of additional memory traces, which will diminish the response to the learned cues faster than by natural memory decay (Exton-Mcguinness et al., 2015).

Hoedjes et al. (2011) suggested that high cue variability and low cue reliability within a generation should favor the formation of short-lasting memory forms such as STM and ARM rather than LTM. Since LTM is formed after a single oviposition in *P. brassicae* it seems likely to assume that the HIPVs cue learned in this experiment are considered to be of high cue reliability under natural conditions. However, *P. brassicae* and *M. brassicae* have overlapping host-plant species ranges and share the same habitats. Co-occurrence of these species occurs under natural conditions, on plants in close proximity, but also on the same plant and even the same leaf (Vos et al., 2001). Therefore, it seems likely that non-hosts such as *M. brassicae* are regularly encountered and the cue reliability would be rather low. However, a single encounter with the gregarious *P. brassicae* caterpillars consists of such a high reward value, due to multiple oviposition opportunities, that this might outweigh potential negative effects of cue variability and still facilitates LTM formation after a single oviposition. As mentioned in Koops (2004), if the benefit of correct information is high relative to the cost of the information being unreliable, then the wasps should still respond, even if the reliability of the information is relatively low.

The observed foraging behavior also varied with plant species. When hosts were present on *B. nigra* and non-host on *S. arvensis,* wasps found the host-infested plants faster, performed more direct flights and spent less time on non-host-infested plants compared with the reciprocal situation. *Sinapis arvensis* and *Brassica nigra* are considered sister species (Agerbirk et al., 2008), yet they are apparently different enough to cause substantial differences in foraging behavior, depending on which plant species contained the hosts or non-hosts. Possibly, *B. nigra* HIPVs are easier to detect, or are more attractive, than HIPVs of *S. arvensis,* making it easier for wasps to find the attractive *B. nigra* host-infested plant among the less attractive HIPVs of *S. arvensis*, than vice versa.

The observation that *C. glomerata* is less efficient at finding host in the presence of non-hosts has already been shown in various studies (Bukovinszky et al., 2012; De Rijk et al., 2016a), yet so far there has been no mentioning of non-host acceptance under (semi-) natural foraging conditions. Under laboratory conditions, however, Vosteen et al. (manuscript in preparation) and Bukovinszky et al. (2012) found occasional non-host oviposition by *C. glomerata* in *M. brassicae* with flight assays. Vosteen et al. (manuscript in preparation) found non-host acceptance levels up to 27%, which seems comparable with our findings. Currently we are investigating to which extent *M. brassicae* is truly a non-host, if these findings are a side-effect of the test setup and which circumstances favor non-host acceptance.

In contrast to what we expected, we found that congruently conditioned wasps behaved very similar to unconditioned wasps. While the study of Kruidhof et al. (2015) showed higher foraging efficiency after associative learning of HIPVs with *C. glomerata*, we did not find this in this study. The main reason why we do not find this positive effect of associative learning is most likely since we discarded wasps which did not respond within 5 min. While response levels of the conditioned wasps were around 90%, only 48% of the unconditioned wasps responded within 5 min. Oviposition experienced wasps are known to be more responsive to HIPVs in general. Giving wasps oviposition experience or exposing them to host frass prior to testing is a general way to increase the responsiveness of parasitoid to HIPVs (Geervliet et al., 1998; Takasu and Lewis, 2003; Bleeker et al., 2006; Peñaflor et al., 2017).

Overall we conclude that learning unreliable information causes maladapted foraging behavior, which reduces foraging efficiency under the conflicting test conditions, compared to the congruent test situation. However, parasitoids do not only learn to associate environmental cues with host while foraging, but also with food (Tertuliano et al., 2004; Wäckers et al., 2006). Hungry parasitoid will primarily respond to cues associated with food, while fed parasitoids will primarily respond to cues associated with hosts (Lewis and Takasu, 1990; Luo et al., 2013). Their environment in combination with their physiological state will determine their foraging behavior and the way they use learned cues. The effect of unreliable memory in relation to food learning and foraging behavior has not been researched in parasitoids so far, but has been researched in honeybees with color learning with a food reward. Similar negative effects of persistent unreliable memory were found; 3 learning events led to longer lasting memory than 1 learning event (Menzel, 1968), and bees with 3 learning events returned more often to the previously rewarding color, which now only supplied tap water, than wasps given 1 learning event (Couvillon and Bitterman, 1980).

By learning how parasitoids integrate different kinds of information from their environment to optimize foraging efficiency, we can greatly advance spatial movement models and biological control efforts (Van Alphen and Bernstein, 2008; Ishii and Shimada, 2009; Wajnberg et al., 2016). Furthermore, the higher response of parasitoids to local HIPVs after learning is interesting for biological control practices (Prokopy and Lewis, 1993; Giunti et al., 2015).

AUTHOR CONTRIBUTIONS

JdB, HS, and LV designed the study. JdB conducted the experiments and analyzed the data. JdB, HS, and LV interpreted the data. JdB prepared the figures and drafted the MS. HS prepared **Figure 1**. JdB wrote the final version based on comments of HS and LV.

ACKNOWLEDGMENTS

This study was supported by NWO open competition grant nr. 824.14.023 to LV.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 29 October 2018
doi: 10.3389/fevo.2018.00173

Rapid Responses of Winter Aphid-Parasitoid Communities to Climate Warming

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Consequences of inter-annual environmental fluctuations, including those associated with climate change, can have a knock-on effect from individual to community scale. In particular, changes in species seasonal phenology can modify the structure and composition of communities, with potential consequences on their functioning and the provision of ecosystem services. In mild climate areas, aphids can be present in cereal fields throughout the winter, which allows aphid parasitoids to remain active. Using a 9-year dataset of aphid-parasitoid winter trophic webs in cereal fields of Western France, we report that the community structure and composition that prevailed before 2011 have recently shifted toward a more diversified community, with the presence of two new braconid parasitoid species (*Aphidius ervi* and *Aphidius avenae*), a few hyperparasitoid species and one aphid species (*Metopolophium dirhodum*). Modifications in minimal winter temperatures and frequency of frost events across the years partially explain observed community changes, although no clear climatic trend can be emphasized. Strong bottom-up effects from the relative abundance of aphid species also determine the relative abundance of parasitoid species each winter. Strong compartmentalization in parasitoid preference for host is reported. We suggest the recent modifications in parasitoid community composition to be linked to shifts in diapause expression (reduction or arrest of the use of winter diapause) and to host availability throughout the year. We highlight the implications for natural biological control in cereal fields. Perspectives are proposed to predict the composition of future host-parasitoid communities in the climate change context.

Keywords: overwintering strategies, diapause, species diversity, competition, biological control

OPEN ACCESS

Edited by:
Paul-André Calatayud, Institut de Recherche Pour le Développement (IRD), France

Reviewed by:
Pablo Almaraz, Instituto de Ciencias Marinas de Andalucía (ICMAN), Spain
Régis Babin, Centre de Coopération Internationale en Recherche Agronomique Pour le Développement (CIRAD), France

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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *20 July 2018*
Accepted: *10 October 2018*
Published: *29 October 2018*

Citation:
Tougeron K, Damien M, Le Lann C, Brodeur J and van Baaren J (2018) Rapid Responses of Winter Aphid-Parasitoid Communities to Climate Warming. Front. Ecol. Evol. 6:173. doi: 10.3389/fevo.2018.00173

INTRODUCTION

Climate change impacts the geographic distribution, diversity, and abundance of organisms (Walther et al., 2002; Parmesan, 2006). In particular, climate warming can strongly influence their seasonal phenology, migration pattern, number of generations per year, and overwintering strategy (Roy and Sparks, 2000; Altermatt, 2010; Bale and Hayward, 2010). In temperate areas temperatures are increasing faster in winter than in summer, leading to overall milder, shorter, and later winter periods (IPCC, 2014). Plastic and adaptive responses of organisms to new thermal environments

could modify species interactions such as competition, predation and parasitism and impact the structure and stability of communities (Hughes, 2000).

In the context of the global diversity crisis, studies increasingly focus on how trophic networks respond to global changes (Parmesan, 2006; Chaianunporn and Hovestadt, 2015). Indeed, species interactions within communities support the majority of ecosystem services and must be considered as study systems *per se* (Montoya et al., 2003). In some cases, food web structure, and composition are quite fragile and are likely to rapidly change in the context of climate warming; understanding how and why these food webs vary in space and time is a central objective in community ecology (Facey et al., 2014). New species appear while others disappear from food webs and changes in species interactions between trophic levels occur (Tylianakis et al., 2008; Chaianunporn and Hovestadt, 2015).

Parasites are omnipresent in almost every food web (Dobson et al., 2008) and their interactions with hosts greatly contribute to ecosystem functioning (Lafferty et al., 2008). Their ecology is tightly associated with their hosts' and likely to be influenced by climate change, threatening the provisioning of ecosystem services such as natural biological pest control by insect parasitoids (Hance et al., 2007; Jeffs and Lewis, 2013). The impacts of climate change or inter-annual variations in climatic conditions on host-parasitoid communities remain little explored compared to other types of food webs (e.g., plant-herbivore networks; Singer and Parmesan, 2010) and there have been few attempts at predicting their future structure and composition under different scenarios of climate change (Jeffs and Lewis, 2013).

In regions characterized by mild winter temperatures, the absence of lethal frosts allows aphids and their parasitoids to remain active and reproduce throughout winter. In cereal crops of Western France, aphid-parasitoid communities in winter were historically (over the past three decades) composed of the two parasitoid species *Aphidius rhopalosiphi* De Stefani-Perez and *Aphidius matricariae* Haliday and the two aphid species *Rhopalosiphum padi* (L.) and *Sitobion avenae* (Fabricius). From late spring to fall, additional species were present, including the parasitoids *Aphidius ervi* Haliday and *Aphidius avenae* Haliday and the aphid *Metopolophium dirhodum* (Walker) (Rabasse et al., 1983; Krespi, 1990; Krespi et al., 1997). These seasonal variations in aphid and parasitoid species occurrence seem to be consistent across Western Europe in cereal crops (Lumbierres et al., 2007; Honek et al., 2018) and likely reflect thermal niche separation (Le Lann et al., 2011; Andrade et al., 2016). The parasitoid *A. avenae* shows less cold resistance and more heat resistance than *A. rhopalosiphi* (Le Lann et al., 2011), while the aphid *R. padi* prefers cooler conditions and is more cold resistant than *S. avenae* (Jarošík et al., 2003; van Baaren et al., 2010; Alford et al., 2016).

Andrade et al. (2016) reported that in host-parasitoid communities of Western France parasitoid species usually not encountered during winter are now being active throughout the season and exploiting anholocyclic aphids (i.e., aphids that have parthenogenetic reproduction all-year-long). In the present study, we have adopted a community-wide approach to analyze the effects of long-term (inter-annual) variations in climatic conditions on aphid and parasitoid species occurrence and relative abundance. Using a 9-year dataset, we explored community assembly rules by first describing temporal changes in winter aphid-parasitoid associations, and then linking these changes to modifications in winter climatic conditions and to other abiotic and biotic factors such as shifts in species interactions. We expect warmer winters to be associated with occurrence and higher abundance of *A. avenae* and *A. ervi* for parasitoids and *S. avenae* and *M. dirhodum* for aphids. We expect colder winters to be associated with the presence of *A. rhopalosiphi* and *A. matricariae* for parasitoids and *R. padi* for aphids.

MATERIALS AND METHODS

Data Collection

Data consist in aphid-parasitoid pairs of species gathered from different studies conducted in the Long Term Ecological Research (LTER) ZA Armorique, France (48°29′ N−1°35′W), each winter from 2009/10 to 2017/18, at variable dates from late-November to mid-March of each year (excepted in 2009/10 when sampling was conducted only in January and February). Data from winter 2009/10 to winter 2012/13 were obtained from Andrade et al. (2016) and Eoche-Bosy et al. (2016), data of 2013/14 from Tougeron et al. (2016), data of 2014/15 from Tougeron et al. (2017), data of 2015/16 from Damien et al. (2017) and data of 2016/17 and 2017/18 from unpublished field results. In winter 2010/2011, no parasitoids nor aphids were found in the fields due to frost conditions during 15 consecutive days in November (Andrade et al., 2016) so this winter was excluded from the dataset to minimize unbalanced analyses on community data.

Mean, mean minimum and mean maximum daily temperature data per sampling year in the LTER were obtained from Météo France (2018). Additionally, we calculated the number of frost events (i.e., occurrence of at least 3 consecutive days with negative mean temperatures, which could be lethal for most species) and mean duration of frost events (days). Highly correlated variables >70% were not used for our analyses. We thus only used the mean minimal temperature (correlated with mean temperature and mean maximum temperatures) and the mean duration of frost events (correlated with the number of frost events) for statistical analyses. Mean temperature was used in graphic representations as a generic measure for interannual climatic variations. Raw data can be found in **Supplementary Table S1**.

Sampling and Quantitative Food-Webs

In each of the studies from which data was collected, sampling was performed following the protocol of Andrade et al. (2016), with differences in the location of sampled fields due to crop rotations. In brief, sampling was conducted every 10 days in six to fifteen cereal fields each year; mainly winter wheat, but also barley and triticale. Sampling effort was consistent across the years. All aphids and aphid mummies (i.e., exoskeleton of dead aphid containing a developing parasitoid) were randomly collected during a 1-h period over an approximate area of 1,000 m^2. Aphid density was very low in winter; around one aphid/m^2.

Accordingly, parasitism rate during all winters was high (60–90%), underlying rarity of hosts and high competition levels among parasitoids. As the aphid-parasitoid network is stable over the winter sampling period (Andrade et al., 2016; Damien et al., 2017), data were pooled for the entire winter season. Live aphids were brought back to the laboratory and reared on winter wheat until mummification or death. All mummies were maintained in gelatin capsules at ambient temperature (17–20°C) until parasitoid emergence. Emerging adult primary parasitoids and aphid hosts were then identified to the species based on morphological characters (Hullé et al., 2006). Hyperparasitoids (secondary parasitoids) were identified to the genus level. Parasitoids and hyperparasitoids that emerged more than 25 days after sampling, representing each year <25% of the total number of sampled mummies, were excluded from the food-web analysis to avoid accounting for diapausing individuals when characterizing winter-active communities. Important differences in sample sizes are due to variations in aphid densities and different climatic conditions among years.

To examine trophic interactions between host and parasitoid species, quantitative food webs using the relative abundance (%) of each species were constructed for each winter following the methodology of Memmott et al. (1994). Hyperparasitoids were assigned to the same trophic level than primary parasitoids because it was impossible to assess in which parasitoid host species they developed.

Analyses

Food webs were compared among years using several quantitative and qualitative metrics calculated using the *bipartite* (Dormann et al., 2009) and the *codyn* R packages (Hallett et al., 2016): Connectance—the overall complexity of the food web (realized proportion of potential links); Web Asymmetry—the balance between numbers of parasitoid and aphid species (negative values indicate more species in higher than in lower trophic-level); H2—the level of specialization within a network, from 0 (no specialization) to 1 (perfect specialization); Generality—the weighted mean number of aphid species exploited by each parasitoid species; Vulnerability—the weighted mean number of parasitoid species attacking a given aphid species.

Then, we performed a non-metric multidimensional scaling (NMDS) analysis to group years by climatic similarities based on a distance matrix; accordingly, years were grouped by four based on their distance to the more extreme years on the NMDS. Following these analyses, years were characterized as either mild or cold winters, and these categories were used to group years on the following Principal Component Analysis (PCA) representation. A first PCA was performed to separate each year of sampling based on selected climatic variables; mean minimal temperatures, and mean duration of frost events. Another PCA was performed to separate each year of sampling based on aphid and parasitoid species relative abundances. Finally, a Canonical Correspondence Analysis (CCA) was performed to assess relationships between aphid and parasitoid species and the climatic variables matrixes. ANOVA-like permutation tests for CCA (*vegan*) were used to assess the significance of constraints. Only primary parasitoids from the genus *Aphidius* were considered for analyses on climatic variations.

As we wanted to account for species co-occurrences in our analyses, species-by-species models were not appropriate. Instead, we used a community approach by analyzing separately the effects of the selected climatic variables across years on parasitoid species (matrix containing the relative abundance of the four parasitoid species) and aphid species (matrix containing the relative abundance of the three aphid species). To do so, Bray-Curtis dissimilarity indexes in species relative abundances of each sampling year were calculated (separately for parasitoids and aphids) and fitted to linear models as response variables using the Adonis-Permanova function from the R package *vegan* (Oksanen et al., 2015) calculating permutation test with pseudo-F ratios. For aphids, the minimal temperature and the mean duration of frost events were used as explanatory variables. For parasitoids, we also included the relative abundance of each of the three aphid species as explanatory factors in the linear models. All analyses were performed using R software (R Core Team, 2017).

RESULTS

Changes in species richness and relative abundances were observed from winter 2009/10 to winter 2017/18 in the aphid-parasitoid food webs, with important inter-annual variations (**Figure 1A**). The year 2009/10 was similar to the past three decades, as described in introduction, with *A. rhopalosiphi* and *A. matricariae* being the only two parasitoid species active in winter and exploiting *S. avenae* and *R. padi*. In addition to these two aphid species, the aphid *M. dirhodum* was present every winter starting 2011/12 at 12%, and it represented up to 68% of the aphid species relative abundance in winter 2015/16 (**Figure 1A**). There was high variability in aphid proportions between years, for each species.

A. avenae was observed for the first time in the winter 2011/12 with a relative abundance of 52%. *A. ervi* was observed in the network in 2013/14 with a relative abundance of 5%. Both species have since remained present in the network, although at variable relative abundances. *A. rhopalosiphi* was present every winter while *A. matricariae* occurrence and relative abundance were highly variable over the years. *Ephedrus plagiator* (Nees) and *Diaeretiella rapae* (M'intosh), two generalist species (Hullé et al., 2006), were anecdotally reported in winter 2011/12 and 2012/13. Hyperparasitoids from the genera *Alloxysta*, *Asaphes* and *Phaenoghyphis* were also detected in two out of eight winters.

Mean temperature varied across years and ranged from 4.2°C in 2009/10, to 7.9°C in 2015/16, and was graphically concurrent with variations in aphid and parasitoid relative abundances (**Figure 1B**).

Food-web metrics are summarized in **Table 1**. The food-web connectance was ≥0.6 every year; all links the first year and almost all potential links the following years between each parasitoid and aphid species were observed. In most years, the food web was asymmetric, with more parasitoid species than aphid species. The degree of specialization within the food-web (H2 index) tended to decrease over the years. Accordingly, the

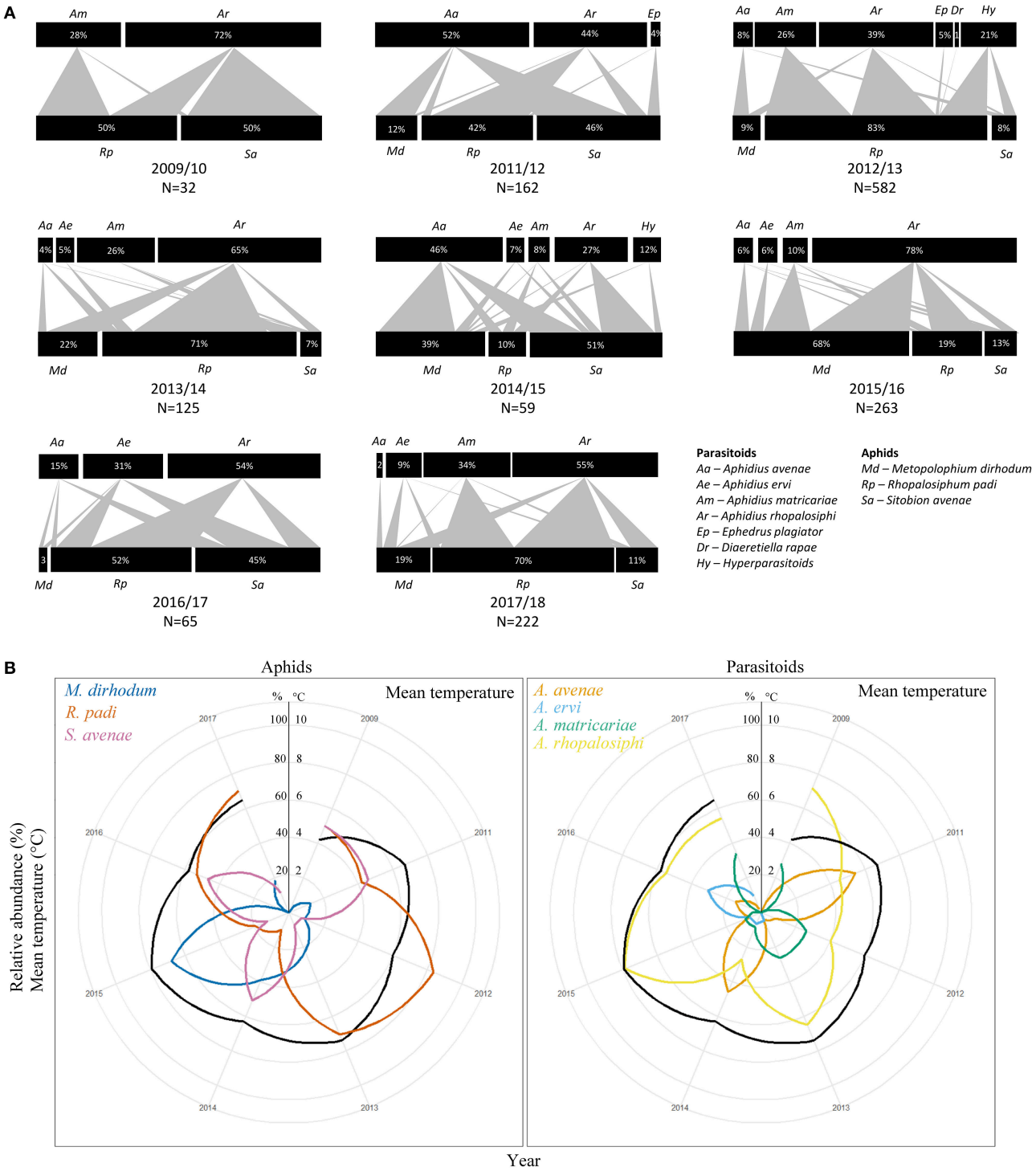

FIGURE 1 | (A) Quantitative food webs of parasitoid and aphid community composition in winters 2009/10 to 2017/18 (there was no insects in 2010/11). Upper and lower bars represent the relative abundance (%) of parasitoid, including hyperparasitoids, and aphid species, respectively. The thickness of the arrow between bars is proportional to the interaction strength between a pair of species. Total number of individuals (N) used to construct each food web is shown for each year.
(B) Rankplots showing the relative abundances (%) of aphids (left panel) and *Aphidius* parasitoids (right panel) each winter from 2009/10 to 2017/18. Mean winter temperature (°C) is shown in black.

TABLE 1 | Number of species in each trophic level (primary parasitoids and aphids) and food-web metrics for each sampled winter.

Year	2009/10	2011/12	2012/13	2013/14	2014/15	2015/16	2016/17	2017/18
Number of primary parasitoid species	2	3	5	4	4	4	3	4
Number of aphid species	2	3	3	3	3	3	3	3
Connectance	1.00	0.89	0.60	0.83	0.92	0.92	0.89	0.83
Web asymmetry	0.00	0.00	−0.25	−0.14	−0.14	−0.14	0.00	−0.14
H2	0.53	0.71	0.41	0.36	0.24	0.22	0.34	0.24
Generality	1.63	1.43	2.18	2.41	2.67	2.26	2.25	2.50
Vulnerability	1.77	2.00	1.96	2.47	2.41	2.73	2.26	2.64

Connectance, the overall complexity of the food web (realized proportion of potential links); Web Asymmetry, the balance between numbers of parasitoid and aphid species (negative values indicate more species in higher than in lower trophic-level); H2, the level of specialization within a network, from 0 (no specialization) to 1 (perfect specialization); Generality, the weighted mean number of aphid species exploited by each parasitoid species; Vulnerability, the weighted mean number of parasitoid species attacking a given aphid species.

generality and vulnerability indexes tend to increase over time; each parasitoid species attacked more aphids and each aphid species was exploited by more parasitoid species over the years, in general (**Table 1**).

NMDS analysis showed that years 2009/10, 2012/13, 2013/14, and 2017/18 can be grouped together as "cold winters" whereas years 2011/12, 2014/15, 2015/16, and 2016/17 can be described as "mild winters." This clustering was supported by 94.7% inertia on the PC1 of the PCA (**Figure 2A**). Graphically, coldest winters (i.e., decreasing minimal temperatures and increasing duration of frost events) were overall associated with the co-occurrence and higher relative abundances of both *A. rhopalosiphi* and *A. matricariae* parasitoids and *R. padi* aphids, while warmest winters were associated with co-occurrence and higher abundance of both *A. avenae* and *A. ervi* parasitoids and *S. avenae* aphids. *M. dirhodum* was highly abundant in the warmest winter (2015/16) (**Figures 1**, **2B**). However, the aphid-parasitoid community PCA was only supported by 43% inertia on the PC1, indicating that species partition on this figure is only partially explained by the sampling year (**Figure 2B**).

We found a marginally non-significant influence of the selected climatic data (mean minimal temperature and duration of frost events) on global aphid and parasitoid relative abundances across years (CCA ANOVA-like permutation test, $F = 1.67$, df $= 2$, $p = 0.06$). In details, aphid abundances were significantly affected by changes in mean minimal temperatures (Permanova, $F = 2.03$, df $= 1$, $R^2 = 0.22$, $p = 0.03$) and in mean duration of frost events across the years ($F = 2.57$, df $= 1$, $R^2 = 0.27$, $p = 0.04$), in the way described in the precedent paragraph. Parasitoid abundances were marginally significantly affected by changes in mean minimal temperatures ($F = 2.67$, df $= 1$, $R^2 = 0.20$, $p = 0.05$) and significantly affected by changes in mean duration of frosts across the years ($F = 14.72$, df $= 1$, $R^2 = 0.15$, $p = 0.01$), as described above. Changes in parasitoid abundances across the years were not affected by the abundances of the aphid *S. avenae* ($F = 1.7$, df $= 1$, $R^2 = 0.16$, $p = 0.16$) but was significantly affected by the abundances of *M. dirhodum* ($F = 35.5$, df $= 1$, $R^2 = 0.37$, p=0.01) and *R. padi* ($F = 17.2$, df $= 1$, $R^2 = 0.18$, $p = 0.009$).

Preferential associations between aphid hosts and primary parasitoid species occurred as shown by the arrows on **Figure 2B** (pooled data across years). The aphid *S. avenae* was mostly associated with the parasitoids *A. avenae* and *A. ervi* while the aphid *R. padi* was mostly associated with the parasitoids *A. rhopalosiphi* and *A. matricariae*. *M. dirhodum* was not preferentially associated with any parasitoid in the food web (**Figure 2B**).

DISCUSSION

Our results illustrate how climatic changes during winter have rapidly, over nine years, translated into modifications in species composition within an aphid-parasitoid community. By contrast with the community typically described over the past decades (Rabasse et al., 1983; Krespi et al., 1997), two parasitoid species *A. avenae* and to a minor extent *A. ervi,* and one aphid species *M. dirhodum* are now active in Western France throughout winter together with other species of the community. The winter trophic network composition in cereal fields is getting similar to what is usually described in spring in this area. The winter food web has become more diversified in aphid and parasitoid species and, while the connectance (realized links) remains stable over time, the degree of specialization tends to decrease, suggesting that parasitoids exploit aphids in function of their relative abundance, as reported in spring (Andrade et al., 2016). This may be due to increasing aphid densities in winter on cereal crops, leading to lower competition pressure among parasitoids.

Changes in occurrence do not arise from recent modifications in distribution range of the species, since *A. avenae*, *A. ervi,* and *M. dirhodum* have been commonly observed for several decades in spring at the same location (Krespi, 1990; Andrade et al., 2016). Our results suggest a recent shift in overwintering strategy in *A. avenae* and *A. ervi* parasitoid populations with some individuals remaining active throughout the winter rather than entering diapause. This hypothesis is supported by results from a laboratory experiment showing that diapause incidence in both parasitoid species was low (<15%), even when parasitoids were reared under fall-like temperature conditions that usually induce high levels of diapause (Tougeron et al., 2017). Variations in species composition in the food web over the years may arise from differences in thermal niches; the most cold-resistant species usually remained active during winter (e.g., *A. rhopalosiphi*, *A. matricariae,* and *R. padi*) whereas less

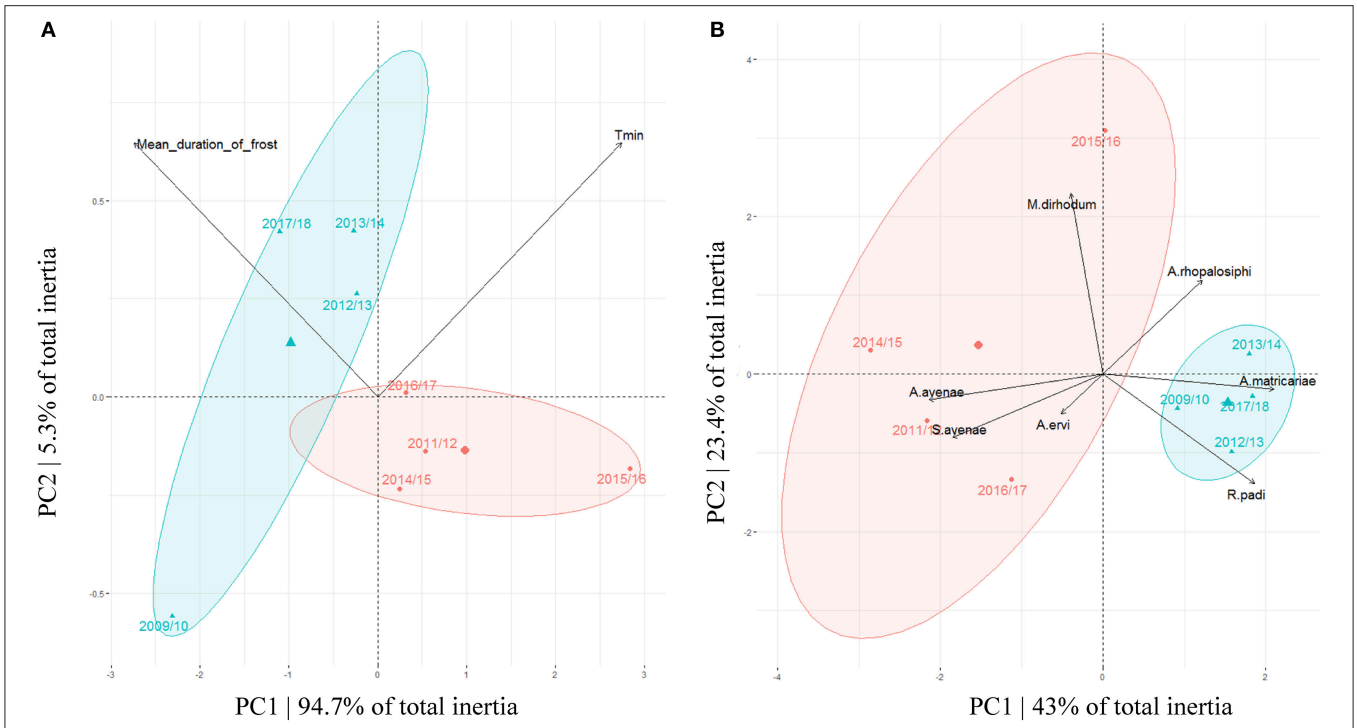

FIGURE 2 | (A) PCA partitioning two climatic variables (mean minimal temperatures "Tmin" and mean duration of frost events) by sampling years. **(B)** PCA partitioning aphid and parasitoid species relative abundances by sampling years. Confidence ellipses are constructed around groups of cold (blue) and mild (red) winters.

cold-resistant species (e.g., *A. avenae*, *A. ervi*, *M. dirhodum*, and hyperparasitoids) were probably in diapause and mostly active from spring to fall (Krespi, 1990; Le Lann et al., 2011; Alford et al., 2016; Andrade et al., 2016; Tougeron et al., 2017). Overwintering temperature may now be warm enough to allow niche overlapping of all these species during winter. No clear trends in temperature change seem to appear over 9 years of study so our data can only lead to partial conclusion on a climate-change effect on aphids and parasitoids. However, we recently showed that from 1976 to 2015, the daily average temperature increased by an average of 1.13 °C in winter. Furthermore, days with cold spell events during winter decreased in frequency since the 1970s (Tougeron et al., 2017). Thus, it is important to state that the interannual variation in climatic conditions observed over 9 years may not be larger than the long-term trend in the study area.

It has been shown that fine-scale intra-seasonal temperature variations (i.e., temperature experienced by the insect during its development) played an important role in shaping local aphid-parasitoid communities in Western France (Andrade et al., 2015, 2016). For example higher developmental temperatures were associated with increasing abundance in *A. avenae, S. avenae* and decreasing abundance in *R. padi* (Andrade et al., 2016). Winter 2016/17 was on average warmer than other winters but important cold spells occurred in December and January, which may have conducted to higher abundances of *R. padi* and *A. rhopalosiphi*, the more cold tolerant species in the food-web (Le Lann et al., 2011; Alford et al., 2016), and quasi-disappearance of *M. dirhodum* from the system, through environmental filtering. Such thermal extremes and microclimatic variations may reduce or eliminate any advantages of global warming for some species (Ma et al., 2015; Sgrò et al., 2016) and may impede evaluation and prediction of climate change effects on community dynamics (Bailey and van de Pol, 2016; Blonder et al., 2017).

We have shown that both mean minimal temperatures and mean duration of frost events over the winter are predictors of winter aphid abundances and of their variation in occurrence among years. Honek et al. (2018) also demonstrated that temperature in winter was an important predictor of maximum abundances of cereal aphids during the weeks following sampling. However, change in mean minimal temperature and duration of frost events only slightly contributed to the trend observed in parasitoid relative abundance changes over the years. Stochastic effects or other environmental variables than temperature such as host-parasitoid interactions may better explain inter-annual variations in species abundances and occurrences during winter. For instance, we showed high level of host-parasitoid compartmentalization within the food web; the variation in relative abundance of some species was highly correlated with abundance of other species, suggesting bottom-up effects on parasitoid abundance. The importance of host species in shaping parasitoid response to climate warming must therefore be accounted (Barton and Ives, 2014). Parasitoids and their hosts may also be influenced by microclimatic refuges in the landscape (Tougeron et al., 2016; Alford et al., 2017), by surrounding plant covers (Gagic et al.,

2012; Damien et al., 2017) or by plant quality (Honek et al., 2018).

Modifications of the parasitoid guild could also be due to shifts in competition for hosts following the addition of new species. Indeed, female parasitoids show seasonal variations in foraging behavior (Roitberg et al., 1992) and can adapt their foraging strategies to competition or host-patch quality (Outreman et al., 2005; Le Lann et al., 2008; Barrette et al., 2010; Moiroux et al., 2015). In winter, it has been demonstrated that female parasitoids adopt generalist strategies due to shortage of optimal hosts, leading to high competition, whereas spring parasitoids usually display specialist strategies by selecting optimal host species (Eoche-Bosy et al., 2016). The recent addition of *A. avenae* and *A. ervi* in the overwintering food web, which are good competitors at exploiting *S. avenae*, may have reduced the abundance of *A. matricariae* and *A. rhopalosiphi* (Le Lann et al., 2012; Andrade et al., 2016; Eoche-Bosy et al., 2016).

Climate warming challenges coexistence and interaction between ecologically related species, as well as community stability and ecosystem functioning (van der Putten et al., 2004; Tylianakis et al., 2008). In cereal fields, overwintering reproduction of aphid parasitoids plays an important role in suppressing early cereal aphid populations in spring (Langer et al., 1997; Plantegenest et al., 2001; Honek et al., 2018). Increasing number of parasitoid species during winter due to climate warming could enhance aphid natural biological control through increasing already high parasitism rate in winter, even if consequences of niche overlapping between parasitoid species via addition of new species in the food-web are difficult to predict. The presence of non-diapausing hyperparasitoids, reported for the first time in winter in Western France in 2012/13 (Tougeron et al., 2017), may reduce the efficiency of biological control in the fields, although hyperparasitoids can sometimes stabilize primary parasitoid populations (Tougeron and Tena, in press). In Spain, characterized by relatively warm winters, hyperparasitism remains high throughout the year which disrupts biological control by primary parasitoids in orchards (Gómez-Marco et al., 2015). With an expected temperature increase from 0.5 to 2°C in the next decades (Karl and Trenberth, 2003), occurrences of new species in food-webs such as hyperparasitoids can be more common, either through shifts in geographic distribution (e.g., biological invasions) or shifts in phenology (e.g., reduction of diapause expression) (Tougeron and Tena, in press).

By drawing from the effects of interannual climatic condition variations over nine years, and based upon studies conducted in this area decades ago, we can observe that changes in host-parasitoid winter communities are not random, which ultimately helps at determining potential actions of long-term climate-change. Based on the data currently available on this host-parasitoid system, we observed the homogenization of winter and spring aphid-parasitoid communities, most likely due to change in phenology after increasing temperatures during winter and decreasing duration of frost events. High variations between years may underline a transition period between two episodes of stable communities, although the food-web could also remain unstable due to variations in climate at fine temporal scale. Data over a longer time period will help at establishing stronger conclusions on climate-change effects on insect communities in this area. Predictive analyses on the community structures should now integrate local changes in overwintering strategies of one or more species to identify the potential effect of climate change on ecosystem services provided by parasitoids.

AUTHOR CONTRIBUTIONS

KT and MD collected and analyzed the data. KT wrote a first version of the manuscript. KT, MD, CL, JB, and JvB made substantial contributions to the manuscript.

ACKNOWLEDGMENTS

We thank all collaborators at Ecobio lab who helped with field work, S. Chollet and J.-S. Pierre for their assistance in analyzing the community data. KT was funded by the French Region Bretagne and the Canada Research Chair in biological control awarded to JB. MD was funded by the FLEUR project and INRA. This study was supported by the LTER France Zone Atelier Armorique.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00173/full#supplementary-material

Supplementary Table S1 | Data on species relative abundance and climatic variations across the years.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

BRIEF RESEARCH REPORT
published: 14 December 2018
doi: 10.3389/fevo.2018.00215

Biological Control Success of a Pasture Pest: Has Its Parasitoid Lost Its Functional Mojo?

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Sustainable and integrated pest management often involves insect parasitoids. However, the effectiveness of parasitoids biocontrol has often failed, frequently for obscure reasons. A parasitoid's success is partly due to its behavioral response to pest density, i.e. its consumer functional response. For many years in New Zealand, a braconid parasitoid, *Microctonus hyperodae* successfully suppressed a severe ryegrass weevil pest, *Listronotus bonariensis*. However, there is now evidence that this has severely declined, but that the extent of decline can depend on the pasture species. Here, we tested whether the current functional responses of *M. hyperodae* to *L. bonarensis* in two of the most common New Zealand pasture grasses *(Lolium multiflorum and L. perenne)* reflect observed differences in field parasitism and whether this functional response has changed over time. Our analysis involved data from 1993 and 2018. We found a type I functional response in *L. multiflorum* in both years, but the slope of the relationship declined over time. There was no evidence for any type of functional response in *L. perenne*. This lack of response in *L. perenne* coincided with consistently found lower parasitism rates on this host plant than in *L. multiflorum*; both in the field and laboratory. Here, we found that apparently declining searching efficiency was correlated with the decline in parasitism. This observation supports the hypothesis that parasitism decline could be the result of evolution of resistance based on enhanced evasive behavior by *L. bonariensis.*

Keywords: attack rates, host density, invertebrate species, predator-prey interaction, natural enemy, weevil

OPEN ACCESS

Edited by:
René F. H. Sforza, European Biological Control Laboratory (USDA-ARS), France

Reviewed by:
Andrea Sciarretta, University of Molise, Italy
Adam J. Vanbergen, INRA UMR1347 Agroécologie, France

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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *12 July 2018*
Accepted: *28 November 2018*
Published: *14 December 2018*

Citation:
Tomasetto F, Casanovas P, Brandt SN and Goldson SL (2018) Biological Control Success of a Pasture Pest: Has Its Parasitoid Lost Its Functional Mojo? Front. Ecol. Evol. 6:215. doi: 10.3389/fevo.2018.00215

INTRODUCTION

Adequate global nutritious and healthy food production in the face of growing human population will require sustainable pest management methods (Bruinsma, 2003; Ramankutty et al., 2018). Insect parasitoids are often a significant component of integrated pest management in agriculture. Also parasitoids are important subjects of behavioral and population studies because they are remarkably common in nature, are frequently easy to raise and to handle and, mostly, key species for the control of many insect pests (Godfray et al., 1994; Fernández-Arhex and Corley, 2003; Begg et al., 2017). However, in general, biological control based on parasitoids has more often failed than succeeded, with difficulties in identifying the reasons (Hawkins and Cornell, 1999; Fernández-Arhex and Corley, 2003).

Among the attributes thought to be related to the success of parasitoid biocontrol agents is the behavior of individual parasitoids in response to host density (Huffaker et al., 1971; Barlow et al., 1993; Berryman, 1999). Such behavior is called 'consumer functional response' and is defined as the relationship between the number of hosts attacked by a predator as a function of prey density (Solomon, 1949; Holling, 1959, 1966). Functional response studies have been used by evolutionary biologists and ecologists to clarify co-evolutionary relationships, and to infer basic mechanisms underlying the interactions of predator-prey or parasitoid-host behavior (Houck and Strauss, 1985). Holling (1959) described three types of functional responses. The type I response describes a linear relationship between the number of hosts parasitised and host density, where the slope is the parasitoid's searching efficiency. The type II response is an asymptotic parasitism curve that constantly decelerates as host numbers increase, due to the time it takes the parasitoid to handle the host. The asymptote reflects the maximum attack rate. The type III response is a sigmoid curve: as host density rises, the response initially accelerates due to the parasitoid becoming increasingly efficient at finding hosts (attack rate increases or handling time decreases) then levels off under the influence of handling time or satiation (Berryman, 1999; Hassell, 2000).

Holling (1959) suggested that the type II response may be typical of invertebrate predators (including parasitoids) because they are limited by a fixed time to search for hosts. However, later work suggested that parasitoids may well-display type III response (Van Lenteren and Bakker, 1975; Hassell et al., 1977) because, at least theoretically, predators that exhibit a type III response points to their ability to regulate their hosts (Bernal et al., 1994). Related to this, the ability of parasitoids to locate hosts at low densities has become one criterion for the selection of candidates for classical biocontrol introductions (Barlow et al., 1993).

For many years the impact of the severe ryegrass pest, Argentine stem weevil (ASW) *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae), on New Zealand's improved grassland ecosystems was successfully suppressed by the South American braconid parasitoid control agent *Microctonus hyperodae* Loan (Hymenoptera: Braconidae) (Barker and Addison, 2006). However, this is no longer the case; that *M. hyperodae* is losing its efficacy has been highlighted by recent systematic field and laboratory studies designed to compare historical and contemporary parasitism rates (Goldson et al., 2014a,b, 2015; Goldson and Tomasetto, 2016; Tomasetto et al., 2017c, 2018). These studies also found significant differences in *L. bonariensis* parasitism rates in two common grasses in New Zealand's improved pastures, tetraploid *Lolium multiflorum* Lam. and diploid *L. perenne* L. The reduced parasitism rate was particularly apparent in *L. perenne* (Tomasetto et al., 2017a,b). The possible mechanisms of this decline over time and the causes of the differences in parasitism rates between grass types, have yet to be fully determined.

In this study, we tested (1) whether the current functional response of *M. hyperodae* to the density of its host pest, *L. bonarensis*, differs between two of the most common pasture grass species where parasitism rates have been shown to differ significantly in New Zealand agroecosystems, and (2) whether the functional response of *M. hyperodae* to *L. bonarensis* density has changed over the last 25 years, potentially being related to the changes in parasitism rates.

MATERIALS AND METHODS

Experimental Design

Weevil adults were collected from a Lincoln farm (−43.6281, 172.4361) using a modified leaf blowing machine (Goldson et al., 2000) between 3rd and 4th January 2018. These were then purged of egg and larval parasitoids by storing them for 41 to 42 days at ambient laboratory temperatures (23 ± 2°C) and 16:8 L:D photoperiod (similar settings to those of Goldson and Tomasetto (2016). The *M. hyperodae* pupae, which emerged from these weevils, were then reared to obtain adult parasitoids for this study while the unparasitised portion of the *L. bonariensis* population was used for the experiment.

Ten 2 × 2 m field cages were set up each over a 2 × 4 m tarpaulin. Inner mesh barriers, attached with strips of Velcro, were set up to divide each cage into four arenas, resulting in 40 total arenas. A spray foam sealant and duct tape were used to seal the cage edges (**Figure S1A**). All experimental work was established on 14th February 2018 using 450 × 340 mm × 640 mm translucent plastic bins. Twenty 550 mm × 305 mm × 45 mm propagation trays of *L. multiflorum* and 20 trays with *L. perenne* were assigned in each bin (**Figure S1B**). We used tetraploid *L. multiflorum* (cv. Hogan) and tetraploid *L. perenne* (cv. Bealy) without endophytes, which can confer upon the grasses resistance to weevils that could affect the interaction with parasitoids. The description of these cultivars amongst New Zealand's pastureland species can be found in Stewart et al. (2014).

We used an experimental regression design for this study (Quinn and Keough, 2002), where we manipulated the density of weevils in each arena for the two different grass species. In each arena, we placed one tray with either *L. multiflorum* or *L. perenne*, and these were stocked with two parasitoids together with one of ten weevil densities ($n_{weevils}$ = 25, 50, 75, 100, 125, 150, 175, 200, 225, 250). These combinations of weevil densities and pasture species were randomly allocated to the arenas. After 10 days, the weevils were removed from the cages by flotation in a concentrated ammonium sulfate solution (Proffitt et al., 1993), and frozen at −20°C prior to being dissected to assess percent parasitism rates (i.e. number of parasitized weevils per total number of weevils dissected).

To determine whether functional responses have changed with time, we used historical data from a similar experiment on *L. multiflorum* (with low levels of fungal alkaloids such as peramine) carried out between October 1991 and February 1993 by Barlow et al. (1993). The latter study was also conducted on flat land, used same soil type, the same time of the year and a virtual monoculture of *L. multiflorum*. However, in their publication their analysis was restricted to the October 1991 data. But, in the interests of seasonal congruency we used Barlow et al. (1993) unpublished February 1993 data to compare with February 2018 parasitism data. Further, we took into account the host density range in Barlow et al. (1993) experiment when designing the experiment described here, to make possible comparisons

between the years (they used 25, 50, 125, and 200 weevils/m^2). In Barlow et al. (1993) however, only a fraction of weevils were found at the end of the experiment. We used the number of recovered weevils from both experiments to compare the results.

Parasitism Rates Comparison and Functional Response Analysis

We used an ANOVA on a generalized linear model to test whether parasitism rates were different between the two species of pasture in the 2018 experiment, and a separate ANOVA on a generalized linear model to test the differences between the *L. multiflorum* experiment of 1993 and 2018. In both models we used "quasi-binomial" error distribution to account for overdispersion of the data.

By dissecting the weevils recovered from the arenas at the end of the experiment we tested for a Type I parasitism response using a generalized linear model with a Gaussian error distribution. The same model was also applied to the historical data (February 1993). To test for Type II and III responses, we fitted Holling (1959) models to the data using the tools in the FRAIR R package (Pritchard et al., 2017) in R version 3.4.1 (R Development Core Team, 2018). These models were optimized using maximum likelihood estimation combined with a robust approach to fitting nonlinear models. A binomial likelihood function was used allowing for optimisation on the basis of arbitrary probability

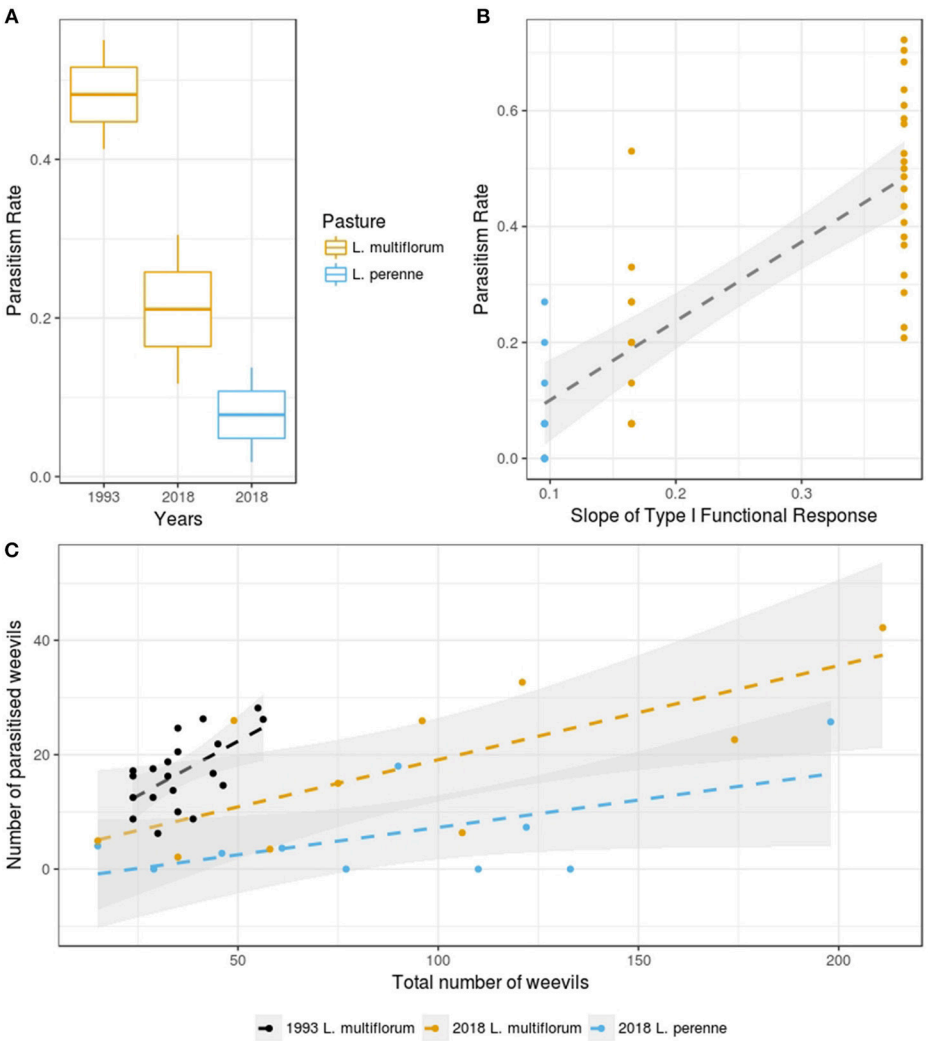

FIGURE 1 | (A) Parasitism rates for the different years and the different pastures of the current experiment. The boxes show the mean and standard error of the data, and the whiskers show the standard deviation of the data (yellow colors indicate *Lolium multiflorum* and blue colors indicate *L. perenne*). **(B)** Relationship between the slope of the Type I functional response and parasitism rates for the combined historical and current data (i.e., 1993 and 2018 data). The 95% confidence intervals are based on bootstrapped model fitting (yellow dots indicate *Lolium multiflorum* and blue dots indicate *L. perenne*). **(C)** Relationship between recovered weevil density and number of weevils parasitized (black dots indicate 1993 data in *L. multiflorum*, yellow dots indicate current data in *L. multiflorum* and blue dots indicate current data in *L. perenne*). The 95% confidence intervals are based on bootstrapped model fitting.

distributions (including Z-statistics and *p*-values; Bolker et al., 2013).

The slope of the Type I functional response represents the parasitoid's searching efficiency (Holling 1959), therefore we tested whether parasitism rates were related to parasitoid searching efficiency by using a generalized linear model (with Gaussian error distribution) of the parasitism rate as a function of the slope of the Type I response.

RESULTS

We found that parasitism rates were significantly higher in *L. multiflorum* than in *L. perenne* under the 2018 experimental conditions ($p = 0.01$, $F = 7.8$, df = 18; **Figure 1A**). Also, overall parasitism rates were significantly higher in the 1993 experiment than in 2018 ($p < 0.005$, $F = 31.5$, df = 28; **Figure 1A**).

We found no evidence in the 2018 data of any functional response in the *L. perenne* treatment. There was also a significant positive relationship between the slopes of the Type I functional responses in the *L. multiflorum* in 1993 and 2018 experiments ($p < 0.005$, $R^2 = 0.62$; **Figure 1B**). We found no evidence of type II and III functional responses in any of the years or pasture types. However, we found a type I responses in *L. multiflorum* in both the historical 1993 data (p-value= 0.005, $t = 3.14$, slope = 0.38) and in 2018 data ($p = 0.015$, $t = 3.07$, slope = 0.16; **Figure 1C**).

DISCUSSION

This study measured ASW parasitism rates by *M. hyperodae* under different host-parasitoid ratios in two common New Zealand ryegrass pasture species under controlled experimental conditions. We found a Type I host-parasitoid functional response using data collected from *L. multiflorum* plants in both 1993 and current experiment. However, the slopes of these functional responses were different, with the historical one being significantly greater than the current one. This suggests a decline in searching efficiency in *L. multiflorum* since 1993, indicating that the efficacy of parasitoid biocontrol had declined in this grass species and corresponds to that which has been generally found elsewhere, especially in *L. perenne* (Goldson and Tomasetto, 2016).

Based on the data they collected in October 1991 (Barlow et al., 1993) did not find a parasitoid functional response in *L. multiflorum*. However, they noted that this was possibly due to the significant weevil mortality in the cages where the weevil populations had declined to such an extent that there was effectively no weevil density treatments (Goldson et al., 1993). ASW collected in October typically show high mortality due to overwintering stress and senescence (Goldson et al., 2011). A similar decline in ASW density did not occur in this study despite the rigors of collection, rearing and release into the cages. This is highly likely to have been because the February collected weevils were only recently emerged.

Under the controlled experimental conditions used in this study, the lack of functional response in the *L. perenne* plots suggests that parasitism rates were not affected by ASW density. Moreover, parasitism rates in *L. perenne* grass were far lower than those found in *L. multiflorum*. This conforms to earlier findings in the field and laboratory experiments which showed that host-plant species did indeed significantly affect parasitism rates (Goldson and Tomasetto, 2016; Tomasetto et al., 2017a,c). As a possible mechanism for this, it has been posited that the tendency for tetraploid *L. multiflorum* to have fewer and larger tillers may offer less opportunity to behavioral-based escape responses by the weevil (Tomasetto et al., 2017b). Such behaviourally-mediated resistance to *M. hyperodae* by ASW was originally suggested by Goldson et al. (2015) based on the observation that the weevils tended to move off the foliage toward the soil when parasitoids were introduced (Gerard, 2000). It is further hypothesized that this sort of response has become enhanced through consistent parasitoid selection pressure and is under investigation elsewhere. In point of fact, the escape-response hypothesis has been supported by this study which showed the significant decline in the slope of the Type I functional response in *L. multiflorum* (from 0.38 to 0.16) since the 1990s, indicating a reduction in searching efficiency (via increased evasion by ASW).

Finally the results presented here are consistent with those in earlier studies where parasitism rates was found to be higher in tetraploid *L multiflorum* than diploid *L. perenne* (Goldson et al., 2015; Goldson and Tomasetto, 2016). Should selection pressure be the underlying mechanism for this decline, we should expect its expression to be most apparent in *L. perenne,* this species being the most common pasture grass in New Zealand.

AUTHOR CONTRIBUTIONS

FT and PC conceived the idea. SB, FT, and PC collected the data. PC analyzed the data with advice from FT. All authors contributed to the writing of the manuscript.

FUNDING

The work was funded by the AgResearch Curiosity Fund granted to FT.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Jason Tylianakis (University of Canterbury) for kindly provided field cages and for his expert technical and editorial advice. Mark McNeill (AgResearch) for providing the historical data. Philippa Gerard and John Kean (AgResearch) for editorial advice. Janet Montgomery (Agriseeds) for kindly provided seeds. Shaun Monk (Grasslanz) for kindly provided the collection sites. John Proffitt assisted energetically with the cage assembling. Malvika Bana contributed to the experiment establishment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00215/full#supplementary-material

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Conflict of Interest Statement: FT, SB, and SG were employed by AgResearch Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 19 December 2018
doi: 10.3389/fevo.2018.00228

Salivary α-Amylase of Stem Borer Hosts Determines Host Recognition and Acceptance for Oviposition by *Cotesia* spp. (Hymenoptera, Braconidae)

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OPEN ACCESS

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Specialty section:
This article was submitted to Coevolution, a section of the journal Frontiers in Ecology and Evolution

Received: 22 June 2018
Accepted: 07 December 2018
Published: 19 December 2018

Citation:
Bichang'a GB, Da Lage J-L, Sambai K, Mule S, Le Ru B, Kaiser L, Juma G, Maina EN and Calatayud P-A (2018) Salivary α-Amylase of Stem Borer Hosts Determines Host Recognition and Acceptance for Oviposition by Cotesia spp. (Hymenoptera, Braconidae). Front. Ecol. Evol. 6:228. doi: 10.3389/fevo.2018.00228

Foraging insect parasitoids use specific chemical cues to discriminate between host and non-host species. Several compounds have been identified in "host location and acceptance." However, nothing is known about the molecular variations in these compounds that could account for host-range differences between parasitoid species. In a previous study, it was shown that during the host-finding process, contact between the braconid *Cotesia flavipes* and its host is crucial, and that α-amylase of oral secretions from the host plays a key role for host acceptance and oviposition by the parasitoid. The present study sought to establish whether the variations in this enzyme could explain specific host recognition in different host-parasitoid associations. Different species and populations of the *C. flavipes* complex specialized on graminaceous lepidopteran stemborers were used. Electrophoresis of α-amylase revealed different isoforms that mediate the parasitoid's oviposition acceptance and preference for a specific host. This discovery opens up new avenues for investigating the evolutionary processes at play in chemically-mediated host specialization in the species-rich *Cotesia* genus.

Keywords: parasitic wasp, *Cotesia flavipes*, *Cotesia sesamiae*, *Cotesia typhae*, protein perception, host specificity, oviposition

INTRODUCTION

Parasitoids comprise the major biological control agents of pest insects (Pimentel et al., 1992; Tilman et al., 2001; Lazarovitz et al., 2007; Godfray et al., 2010). Among them, the Hymenoptera order contains the most diversified species: 50,000 in Hymenoptera, compared with only 15,000 in Diptera, and 3,000 in other orders (Quicke, 1997). To reproduce successfully, the parasitoids need to overcome the behavioral and physiological defenses of their hosts (Kaiser et al., 2017a). The hosts' defense mechanisms, which co-evolved with the parasitoids, may be linked to host range changes and the appearance of host races within different parasitoid species (Kaiser et al., 2017a). These underlying mechanisms provide insight into evolutionary biology, and they might improve the selection of parasitoids in bio-control.

The ability of parasitoids to efficiently utilize cues from their habitat and to efficiently distinguish suitable from unsuitable hosts, determines their field efficiency (Wajnberg et al., 2008;

Wajnberg and Colazza, 2013). When locating hosts, they first use long (i.e., from a distance) and short-range chemicals coming from the host habitat, and secondly those directly present on the host and on its feeding products (Wajnberg et al., 2008; Wajnberg and Colazza, 2013). However, long-range chemicals from the habitat do not generally give them sufficiently reliable information about the host's suitability (Vet, 1999). In contrast, those directly present on the host and on its feeding products are directly used during host-contact evaluation by the parasitoids. These chemicals generally allow them to assess the quality and status of the herbivore's suitability (Lewis and Martin, 1990; Vinson, 1991; Godfray, 1994; Wajnberg et al., 2008; Wajnberg and Colazza, 2013). Moreover, the structure and quantity of these semiochemicals, which vary according to the host's species, the developmental stage of the host, the host's size, condition, and diet, influence host acceptance and selection by the parasitoids (Vinson, 1991; Röse et al., 1997; Wajnberg et al., 2008; Wajnberg and Colazza, 2013).

Among parasitoids, *Cotesia* is one of the most diverse genera in the Braconidae family (Kaiser et al., 2017a). Many *Cotesia* species may appear to have broad host ranges, but careful ecological studies have revealed a hidden complexity with an assemblage of populations with more restricted host ranges (Branca et al., 2011; Kaiser et al., 2017b). Whereas recent studies revealed that variations in virulence genes account for differences in host range and in the degree of specialization toward a host (Gauthier et al., 2018), almost nothing is known about the variations in functions involved in specific host recognition and acceptance.

The *Cotesia flavipes* species-monophyletic group is composed of four sister species: *C. chilonis* (Matsumura), *C. flavipes* Cameron, *C. nonagriae* (Olliff), and *C. sesamiae* (Cameron). They are all gregarious endoparasitoids of crambid, pyralid and noctuid stem borers feeding on Poaceae, Typhaceae, and Cyperaceae species (Kaiser et al., 2017b). These small wasps, after mating, lay egg(s) in a host's body (generally a caterpillar). To inhibit the immune response of the caterpillars, they use a domesticated virus called bracovirus (PolyDNA virus). These viruses are located in the wasp ovaries and are integrated into the genome of the wasp and injected into the caterpillar together with the eggs during the parasitism process (see Kaiser et al., 2017a for review).

Cotesia flavipes Cameron is widespread in Asia and was introduced into Africa to control the invasive Asian crambid *Chilo partellus* Swinhoe (Overholt et al., 1994a,b). It parasitizes the larvae of more than 30 Lepidoptera species, including the crambids *C. partellus* and *Chilo suppressalis* (Walker), as well as the African noctuid *Sesamia calamistis* Hampson, a new association host (https://www.cabi.org/isc/datasheet/5951). The *C. flavipes* population brought into Africa for classical biological control was specific to *C. partellus* in Asia (Muirhead et al., 2012). *Cotesia sesamiae* is widespread in Sub-Saharan Africa and is commonly found on *Busseola fusca* and *S. calamistis* (Kfir, 1995; Kfir et al., 2002), but its parasitism success greatly depends on the host species and parasitoids populations (Mochiah et al., 2002; Gitau et al., 2010). Two factors contribute to the differences and hence to the performance of *C. sesamiae* populations on stem borer pests across Africa, namely, the symbiotic polyDNA viruses, which are responsible for the differences in virulence of *C. sesamiae* population on *B. fusca* (Gitau et al., 2010), and the bacteria *Wolbachia,* by creating cytoplasmic incompatibilities between populations of *C. sesamiae* populations (Mochiah et al., 2002). In contrast to the *C. sesamiae* population from Mombasa/coastal Kenya (*Cs*-Coast), the *C. sesamiae* population from Kitale/inland Kenya (*Cs*-Inland) is able to develop in *B. fusca*, which is predominant in the highlands, whereas both are able to develop in the noctuid *S. calamistis,* the main host of *C. sesamiae* population from Mombasa/coastal Kenya (Ngi-Song et al., 1995). The *Cs*-Inland is mostly present in the highlands and wet regions, where its host *B. fusca* occurs, and is absent in the dry and warmer regions, where *Cs*-Coast and *C. flavipes* predominate (Mailafiya et al., 2010; Mwalusepo et al., 2015). The genetic diversity of these *C. sesamiae* populations, especially regarding their relationships with spatial, biotic, and abiotic ecological factors, is reported by Branca et al. (2018). The authors highlighted the importance of host forces in the evolution of the diversity of parasitoid-host interactions.

Cotesia sesamiae and *C. flavipes* locate their host at a distance by the emission of volatiles from the plants infested by their hosts. However, these volatiles do not convey reliable information on host suitability but are simply indicators of the presence of herbivores in the plant. As a result, *C. sesamiae* and *C. flavipes* might be attracted to plants infested by unsuitable Lepidoptera stemborers (Potting et al., 1993, 1995; Ngi-Song et al., 1996; Obonyo et al., 2008). It is only when approaching and touching the host that *C. sesamiae* and *C. flavipes* are able to identify their hosts properly, relying on specific host-produced signals. The signals particularly arise from oral secretions, which give reliable information on the host identity perceived by the tactile and contact-chemoreception of the parasitoid (Obonyo et al., 2010a,b). These authors observed that host selection and acceptance by the parasitoid females for parasitism is characterized by two behavioral steps: drumming the body of the host with the antennae (antennation), followed by an attempt to oviposit into the host. Recently, Bichang'a et al. (2018) showed that α-amylase present in the oral secretions of *C. partellus* larvae mediates these behavioral responses of *C. flavipes*. The present study investigates whether α-amylase presents variations which allow for recognition and selection of host species or population in *Cotesia* spp. using the two populations of *C. sesamiae* living in Kenya with their respective hosts *B. fusca* and *S. calamistis*, as well as a new species of *Cotesia* described recently as *C. typhae* Fernandez-Triana sp., parasitizing *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera, Noctuidae) (Kaiser et al., 2017a), and the introduced *C. flavipes* and its old association host *C. partellus*.

MATERIALS AND METHODS

Insect Rearing

Females of *C. flavipes,* an inland and coastal population of *C. sesamiae* (hereafter named *Cs*-Inland and Cs-Coast, respectively), as well as that of *C. typhae,* came from laboratory-reared colonies established at *icipe*, Nairobi, Kenya. *Cotesia flavipes* was initially obtained in 2005 from *C. partellus* larvae collected

TABLE 1 | Suitability of lepidopteran stem borer species to different Cotesia species and strains based on field observations and the literature.

	Chilo partellus	*Busseola fusca*	*Sesamia calamistis*	*Sesamia nonagrioides*
Cotesia flavipes	o	w	new	non
Cotesia sesamiae				
Cs-Inland	w	o	o	non
Cs-Coast	new	w	o	non
Cotesia typhae	non	non	w	o

A code was attributed to indicate the level of host suitability, where non, non-host; w, "weak" host association; new, new host association; o, old host association.

from maize fields in Mombasa, coastal Kenya. *Cs*-Inland was initially obtained in 2006 from *B. fusca* larvae infesting maize fields in Kitale, Western Kenya, while the *Cs*-Coast was initially obtained in 2007 from *S. calamistis* larvae infesting maize fields in Mombasa (coastal Kenya). *Cotesia typhae* was initially obtained in 2013 from *S. nonagrioides* larvae infesting *Cyperus dives* at Kobodo near Lake Victoria, Kenya.

Cotesia flavipes, *Cs*-Inland, *Cs*-Coast, and *C. typhae* were continuously reared on larvae of *C. partellus*, *B. fusca*, *S. calamistis,* and *S. nonagrioides*, respectively, as previously described by (Overholt et al., 1994a). Twice a year, all colonies were rejuvenated by field-collected parasitoids.

For each colony, the cocoons were kept until emergence. After emergence, adult parasitoids were fed on a 20% honey/water solution and placed under artificial light for 8 h to mate. In all the behavioral bioassays, 1-day-old naïve (i.e., without oviposition experience), mated females were used. Similar to Overholt et al. (1994a), experimental conditions were at 25 ± 2°C, at 50–80% relative humidity (RH) and with a 12:12 h (L:D) photoperiod.

Different host species that varied in their suitability according to the *Cotesia* species and strains were used (**Table 1**). Old host association (=natural host) was defined according to both the origin of the parasitoid and the host (**Table 1**). For example, *C. partellus* is considered an old host association, since this host is from the same origin of the parasitoid in Asia (Overholt et al., 1994b) and was parasitizing this host before its introduction into Africa, whereas the African *S. calamistis* is a new association.

Chilo partellus and *S. calamistis* were initially collected from maize fields in coastal regions of Kenya, and *B. fusca* from maize fields in Western Kenya (Kitale), while *S. nonagrioides* were initially collected from *Typha domingensis* in Makindu, Kenya. The larvae of *C. partellus* were continuously reared at *icipe* on artificial diets of Ochieng et al. (1985), whereas the larvae of the other species were fed on the artificial diet of Onyango and Ochieng'-Odero (1994). Twice a year, all host's colonies were rejuvenated by field-collected stemborer larvae. **Table 1** gives the host-parasitoid species and strains associations.

Collection of Oral Secretions From Host Larvae

Acceptance of host larvae for oviposition by *Cotesia* parasitoids is enhanced when the host larvae are fed on maize stems for 24 h prior to exposure to parasitism (Mohyuddin et al., 1981; Inayatullah, 1983; Van Leerdam et al., 1985; Potting et al., 1993; Overholt et al., 1994a), most likely because more α-amylase can be found in the oral secretion from larvae after feeding on maize stems than on artificial diets (Bichang'a et al., 2018). Therefore, α-amylase was isolated from third and fourth instar larvae previously fed for 24 h on maize stems. Each larva was squeezed by soft forceps behind the head to collect its oral secretion into a capillary tube and was immediately transferred to an Eppendorf tube which had been placed on ice. This was repeated for at least 100–200 larvae per species to get a sufficient amount of oral secretion (about 500–800 μL per species), estimated by weighing. All samples were preserved at −80°C until further use.

Purification of the α-Amylases

The oral secretions were first centrifuged at 11,000 × g for 5 min in order to remove the undetected debris (grass and undigested food materials). About 600–800 μL of supernatant was transferred to a clean tube and the proteins precipitated using ammonium sulfate salt. To the supernatant, ammonium sulfate salt was gradually added to a final salt saturation of 90% and precipitated overnight at 4°C. The proteins were subsequently pelleted by centrifugation at 12,000 × g for 1 h at 4°C and were then resuspended in HEPES-NaCl buffer (HEPES 20 mM, NaCl 20 mM, $CaCl_2$ 1 mM, pH 7.5) and dialyzed (MWCO 12–14000 Da) overnight at 4°C in the same buffer.

The α-amylase was purified using the glycogen-amylase complex precipitation method described by Loyter and Schramm (1962) with some modifications. Briefly, ice-cold absolute ethanol was added dropwise (2/3 v/v) to the dialyzed samples placed on ice and mixed for 40 min at 4°C. This mixture was centrifuged at 20,000 rpm for 30 min at 4°C to pellet the nucleic acids. To the supernatant, glycogen (Sigma Aldrich) was added to a final concentration of 2.4 mg/ml per sample and mixed for 20 min for *S. calamistis* and *S. nonagrioides*, and 5 min for *B. fusca* and *C. partellus* at 4°C (As observed in previous assays; the different timings allowed for optimum yield of α-amylases). Subsequently, the mixtures were centrifuged for 20 min at 20,000 rpm at 4°C to pellet the amylase-substrate complex, and the pellets were dissolved in the aforementioned HEPES-NaCl buffer. The amylase-substrate complexes were left on the bench for 3 h at room temperature to digest the glycogen in the complexes. The remaining α-amylases were dialyzed (MWCO 12-14000 Da) overnight against the same buffer and kept at −20°C for electrophoresis and bioassays.

Native PAGE and α-Amylase zymogram

For the α-amylases of each host species, electrophoresis was conducted under non-denaturing conditions (native PAGE electrophoresis) as follows: For each host species, ten microliters of purified α-amylase were mixed separately with 10μL buffer (50 mM tris-HCl, pH 6.8, 10% glycerol (v/v), and 1% bromophenol blue) and electrophoresed in the Ornstein-Davis discontinuous buffer system on a 7.5% native polyacrylamide gel at 4°C according to Schrambach and Jovin (1983) and Niepmann and Zheng (2006). After running the gel at a

constant voltage of 150 V and a current of 25 mA for 1 h, and when the dye-containing sample reached the bottom of the glass, the polyacrylamide gel was stained according to Nagaraju and Abraham (1995) with minor modifications. The gel was incubated for 1 h at 37°C in 1% soluble potato starch (Sigma Aldrich) and 1 M $CaCl_2$, washed thoroughly with ddH_20 and subsequently stained with 0.1% of Lugol's iodine solution (I_3K) until white bands against a blue background were visible. The proteins were compared to a molecular mass standard (Sigma Aldrich) containing albumin from bovine serum (Sigma A8654, 132 kDa), albumin from chicken egg white (Sigma A8529, 45 kDa), and α lactalbumin from bovine milk (Sigma L4385, 14.2 kDa). The gel images were acquired using the myECL™ Imager (Thermo) and analyzed using myImageAnalysis™ Software (Thermo).

It was previously observed that the concentration of α-amylase in the extract conditioned the behavioral response of the wasp (Bichang'a, 2018; Bichang'a et al., 2018).

For each host species, the concentration of α-amylase was estimated using a calibration electrophoretic migration obtained from increasing concentrations of between 50 and 1000 μg/mL of α-amylase of *Aspergillus oryzae* from Sigma No A9857 and of *D. melanogaster* produced on the *Pichia pastoris* yeast (**Figure S1**). This calibration electrophoretic migration did not lead us to a precise amount of α-amylase but rather to a range of concentrations. Moreover, it was observed that the optimal range of concentrations of α-amylase to induce host recognition and acceptance for oviposition behaviors by the parasitoids was 300–500 μg/ml (Bichang'a, 2018; Bichang'a et al., 2018). For each of the host species, the concentrations of α-amylase used for the subsequent bioassays was adjusted at 300–500 μg/ml.

Western Blot Analysis of the Purified α-Amylases of Each Host Species

In order to confirm for each stem borer species that the purified proteins were indeed α-amylases, after being used for all bioassays, a western blot was performed using an antibody specific to *Drosophila melanogaster* Meigen α-amylase using the similar protocol of Bichang'a et al. (2018). Ten microliters of each heat denatured protein sample (about 500 ng/μl) were loaded on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and electrophoresis conducted for 1 h at 200 volts in a MOPS buffer. The proteins were then transferred to an iBlot Gel Transfer Nitrocellulose membrane (Invitrogen) using the iBlot Gel Transfer Device (Invitrogen). The membrane was washed in 1X PBS for 20 min, after which it was incubated for 90 min in a milk solution (1X PBS, 0.1% Tween, 5% milk) in order to saturate the membrane with proteins. The membrane was then incubated with the primary anti *Drosophila melanogaster* α-amylase antibody (gift from Dr B. Lemaitre) according to Chng et al. (2014), it was diluted 1,000-fold in a solution of 1X PBS, 0.1% Tween, 1% milk for several hours. After this step, the membrane was washed six times in 1X PBS, 0.1% Tween before incubating with the secondary antibody (anti-guinea pig IgG Peroxidase, Sigma A7289), diluted 1,000-fold in a solution of 1X PBS, 0.1% Tween, 1% milk, for 1 h. The membrane was then washed 3 times in 1X PBS, 0.1% Tween. The peroxidase activity was detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and recorded on an Odyssey FC imager.

Behavioral Bioassays

In this study, the two behavioral steps (antennation + stinging attempt), as shown by Obonyo et al. (2010a,b), were used to confirm host acceptance by *Cotesia* females for oviposition. To test the behavioral activities triggered by different α-amylases, 300–500 μg/ml of α-amylases [the minimal concentration found to mediate a positive response of *C. flavipes* (Bichang'a et al., 2018)] were placed on small pieces of cotton wool and presented to female parasitoids. A small piece of cotton wool was rolled into a spherical shape of around 2 mm in diameter and placed at the center of a Petri dish of 8 cm in diameter without a cover. About 0.5–1 μL of α-amylase was deposited on the cotton wool ball. A single female wasp was introduced near the cotton wool and both were covered with a transparent circular Perpex lid (3 cm wide, 1 cm high) to prevent the parasitoid from flying off, and to allow for observations.

The behavior of the parasitoid in the Petri dish was monitored for a maximum of 120 s. For each female, both antennation and stinging attempts were recorded. The percentage of positive responses (i.e., antennation + stinging) was calculated from 30 females tested per type of α-amylase. The females, the cotton wool balls with tested α-amylase and the arena were replaced after each observation.

According to Obonyo et al. (2010a), all behavioral experiments were carried out in a room at 26 ± 1°C between 10 a.m. and 2 p.m. with a constant source of light to maintain an optimal temperature for the behavioral activities of the female parasitoids.

Statistical Analysis

For each bioassay, Marascuilo's procedure, that is, a pairwise comparison after Pearson's Chi-square test to check the overall significance differences, was used to separate the proportions of wasps that exhibited positive responses (i.e., antennation + stinging attempts) (Marascuilo, 1966).

RESULTS

The α-amylase exhibited species-specific electrophoretic migrations showing different numbers of isoforms using the Lugol test (**Figure 1**). The α-amylase of *C. partellus* exhibited mostly 1 band, whereas α-amylase of *B. fusca* appeared to have two main different isoforms, while that of *S. calamistis* exhibited two thick, highly visible isoforms, and three thinner bands between and three faint bands, which migrated much faster than the others. α-Amylase of *S. nonagrioides* had three thick groups of isoforms, one thin band and a pair of highly visible thin bands migrating faster. We confirmed by Western blot analysis for *S. nonagrioides*, *S. calamistis* and *B. fusca* that these were alpha-amylase proteins (**Figure 2**). In the non-denaturing gels stained using iodine at **Figure 1**, which show white bands where active amylases have migrated, proteins are separated by their electric

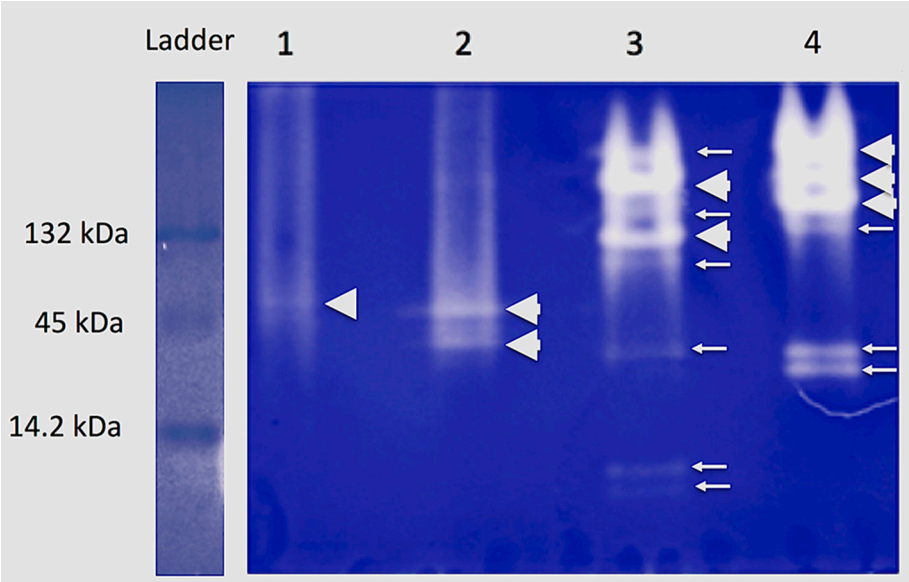

FIGURE 1 | Non-denaturing gel electrophoresis of the amylolytic activity of the purified α-amylases from the oral secretions of larvae of *Chilo partellus* (1), *Busseola fusca* (2), *Sesamia calamistis* (3), and *Sesamia nonagrioides* (4). For each species, the arrows highlight the main isoforms obtained.

charge, which is mostly the result of the difference between (Lys and Arg) and (Asp and Glu) residue numbers. A single gene may exhibit two bands if the two alleles differ in charge. If there are more than two bands, especially if they are separated, e.g., as two pairs of bands, one can infer that there are two active copies. In contrast, in the SDS-PAGE (denaturing) used for Western blot, all the proteins migrate to the same position because they have the same molecular weight. This is the reason why a single labeled band was observed in **Figure 2**. For a mixture of various proteins, migration of **Figure 1** depends on both electric charge and molecular weight (as well as conformation, shape, etc.); but as far as amylases only are concerned, since they all have similar molecular weight, the differences observed in migration distances are due to the differences in electric charges (electromorphs). However, no band was obtained for *C. partellus,* although α-amylase activity was seen in these sample type in **Figure 1**. The amount of protein sample of the *C. partellus* used for western blot was lower compared to amounts of the other species. The limit of protein detection was therefore attained for this sample type by Western blot.

For each parasitoid species and strains used in this study, parasitoid females exhibited different behavior according to the origin of the α-amylase (*C. flavipes*: Chi-square = 13.43; df = 3, $P = 0.0038$; *Cs*-Inland: Chi-square = 27.548; df = 3, $P < 0.0001$; *Cs*-Coast: Chi-square = 8.2458; df = 3 and $P = 0.04119$ and *C. typhae:* Chi-square = 15.239; df = 3 and $P = 0.001623$) (**Figure 3**). For *C. flavipes* females, α-amylases from the larvae of the old association host *C. partellus* and the new association host *S. calamistis* induced the highest positive responses followed by those from *B. fusca,* whereas those from *S. nonagrioides* larvae did not induce any behavior (**Figure 3**). For *Cs*-Inland females, α-amylases from the preferred host *B. fusca* induced the highest positive response, followed by those from the suitable *S. calamistis*, whereas those from the unsuitable hosts *C. partellus* and *S. nonagrioides* did not induce any response (**Figure 3**). For the *Cs*-Coast females, α-amylases from the suitable new association hosts *C. partellus* and the natural host *S. calamistis* induced higher responses than those from the unsuitable *B. fusca* and *S. nonagrioides* (**Figure 3**). For the more specific *Cotesia* species, α-amylase from the suitable host *S. nonagrioides* induced a higher response than those from the unsuitable species (**Figure 3**).

In summary, for each parasitoid species and population there was a strong co-relationship between the behavioral response toward α-amylases of the larvae by the parasitoid female (**Figure 3**) and the level of host suitability (**Table 1**).

DISCUSSIONS

This study revealed that the response of female *Cotesia* to the α-amylase from larval oral secretions depended on both the host and parasitoid species or population, with a strong relationship between the level of response and host preference/suitability. Highest responses were observed with the proteins of the old association host (i.e., most suitable host), whereas protein of unsuitable species triggered little or no response. Variations of host α-amylase between host species would thus allow specific host recognition and acceptance by the parasitoids.

Lepidopteran stemborers in Africa present high ecological and genetic diversity (Le Ru et al., 2006a,b), characterized by a large number of closely related plant-specific species (Le Ru et al., 2006a,b; Moolman et al., 2014; Ong'amo et al., 2014; Goftishu et al., 2018). Correspondingly, Mailafiya et al. (2009) found a

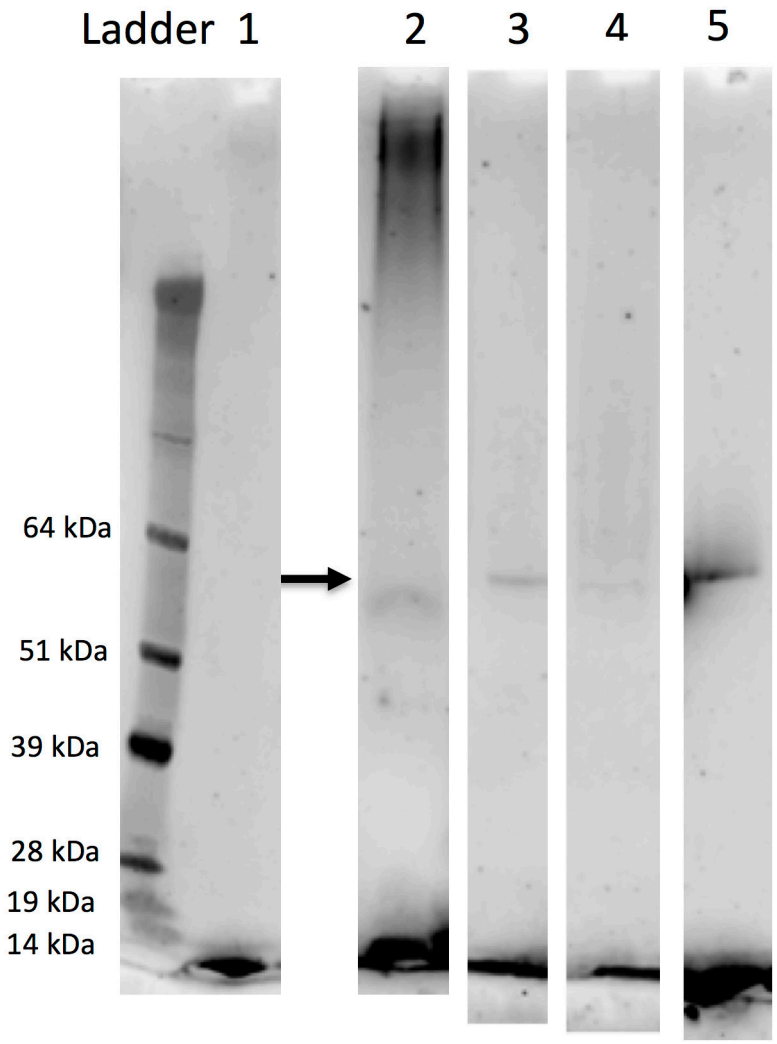

FIGURE 2 | Western blot performed using a *Drosophila melanogaster* α-amylase-specific antibody toward the purified α-amylases from the oral secretions of larvae of *Chilo partellus* (1), *Busseola fusca* (2), *Sesamia calamistis* (3), and *Sesamia nonagrioides* (4). Ladder: molecular weight markers (pre-stained SeeBlue Plus2, Thermo Fischer). 5: α-amylase from *Drosophila melanogaster*.

high diversity of the *Cotesia* spp., particularly among *Busseola* spp. and *Chilo* spp., which also revealed a strong host-parasitoid specificity. This suggests that the chemical(s) involved in host recognition and acceptance by these parasitoids must be specific to the host species involved, as verified in the present study. However, the response of parasitoid females to α-amylase is not binomial (yes or no), and becomes more intense with the α-amylase of its natural host. Some behavioral responses still occurred with α-amylases of unsuitable hosts, nonetheless. The probability of an encounter between *B. fusca* with *C. flavipes* and *Cs*-coast, as well as between *C. partellus* with *Cs*-Inland, is very low, however, due to the different geographical distribution of their respective hosts: *B. fusca* is mostly present in the highlands, whereas *C. partellus* is mostly found in the lowlands (Mailafiya et al., 2010; Mwalusepo et al., 2015). Such ecological patterns of the host-parasitoid associations suggest that their preference for the α-amylase of their host results from adaptation (even recent adaptation, e.g., for *C. flavipes* toward *S. calamistis*) to local hosts, as shown for the virulence function for *C. sesamiae* populations (Dupas et al., 2008; Gauthier et al., 2018).

α-Amylases are among the important classes of digestive enzymes used by the insects to hydrolyse starch in various plant tissues to oligosaccharides. Thus, they play a critical role in insect survival by providing energy (Franco et al., 2000). They have also been identified in most insect orders, such as Orthoptera, Hemiptera, Heteroptera, Hymenoptera, Diptera, Lepidoptera, and Coleoptera (Kaur et al., 2014). In Lepidoptera, several α-amylase genes commonly occur (e.g., Özgür et al., 2009; Pytelkova et al., 2009; Da Lage et al., 2011). In our study the same enzyme had different isoforms in electrophoresis that exhibited species-specific migration patterns. Since isoform migration distance depends on the molecule electric charge, it is

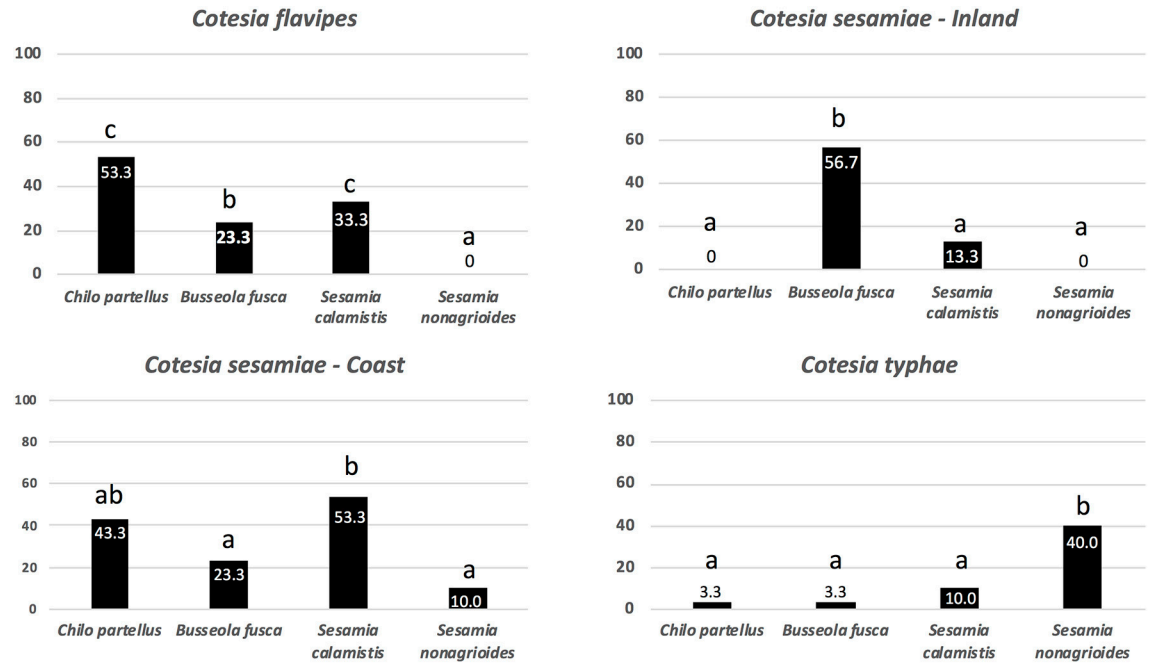

FIGURE 3 | Response of *Cotesia flavipes*, *Cotesia sesamiae*-Inland, *Cotesia sesamiae*-Coast, and *Cotesia typhae* females to purified α-amylase from different host species. The percentages of females ($n = 30$) that exhibited antennation and stinging attempts are given for each bar. After Pearson's Chi-squared test, bars headed with a different letter are significant at a 5% level according to the Marascuilo procedure (multiple proportions comparison).

not obvious whether different bands represent allelic variation or if they duplicate gene copies. However, in species showing well-separated groups of bands, such as the two species of *Sesamia*, it is likely that at least those groups reflect different gene copies. It can be hypothesized that within these species, individuals can express different isoforms of the α-amylase. To confirm this hypothesis, it would be necessary to look at the α-amylase expression in each individual. Up to now, only one α-amylase gene sequence has been identified in *S. nonagrioides* (actually a cDNA; Da Lage J.-L., unpublished study), but given that most Lepidoptera with published genomes harbor several α-amylase genes (Da Lage, 2018 for a review), it is quite likely that this is the case in *S. nonagrioides*. Several α-amylase gene copies are expressed in a species close to *C. partellus*, *Chilo suppressalis*; and three α-amylase gene copies in *Ephestia kuhniella* (Pytelkova et al., 2009). Nevertheless, all these studies indicated that the insects express multiple α-amylase at the same time; suggesting that no individual variation in α-amylase genes expression might occur within the same species. Therefore, the α-amylase gene expression is species-specific.

The two *Sesamia* species have different ranges of host plants (Le Ru et al., 2006a,b), so genes coding for digestive enzymes like α-amylase may have evolved under different selective pressures. Tri-dimensional amylase structures may vary according to the species or even to the isoform if significant sequence differences exist, such as presence or absence of some disulfide bonds, or particular loops (Da Lage et al., 2002). Those structural differences might be discriminated by the sensory equipment of the parasitoid wasp.

For *C. flavipes* it was shown that it is the conformation of the α-amylase rather than its catalytic activity that induces the parasitoid responses (i.e., antennation + stinging attempts; Bichang'a et al., 2018). Therefore, the existence of different α-amylase isoforms specific to each stem borer species as shown in **Figure 1** corroborates the variable behavioral responses obtained in relation to the host-parasitoid association.

The question arises of how the parasitoids access host α-amylase in nature. Lepidopteran stemborer larvae spend their lives and feed inside plant stems. Before entering the feeding tunnel of the host larvae, the wasp first contacts the fecal pellets left by the larvae pushed outside of the stem. These pellets act as a marker of the status of the larva inside the stem tunnel as being host or non-host (Obonyo et al., 2010b), and shows whether they are actively feeding or not. It is most probable that the fecal pellets already contain the stimulatory compounds, since the pellets induced oviposition (Bichang'a et al., 2018). However, the parasitoid is able to definitely recognize the host and accept to oviposit in it only when it is in contact with the host body (Obonyo et al., 2010a,b). We hypothesized that it is during this final step that the parasitoid can confirm the identity of the host larva by detecting the same stimulatory compounds found in the previous fecal pellets and also present on the body of the larva deposited by its feeding activity. These stimulatory compounds need to give quick and appropriate information to the parasitoid on the suitability of the larva (both host and health status) because host larvae often bite the attacking wasps inside the tunnel created by the borer, causing a 50% mortality risk

(Takasu and Overholt, 1997). The high selection pressure due to the high mortality at oviposition should favor wasps that can recognize hosts with a minimal risk of injury (Ward, 1992). In this context, the parasitoid response to α-amylase needs to be specific to the host involved. In addition, this supposes that the parasitoids can perceive the α-amylase through their sensorial equipment.

Obonyo et al. (2010a) observed that female parasitoids use the tip of their antennae to recognize and accept their host larvae for oviposition. They identified the presence of specific sensilla known to have gustatory functions in insects on the last antennal segment (Obonyo et al., 2011). Mailhan (2016) showed that these sensilla chaetica are able to detect the α-amylase. However, this result was not confirmed until recently by Tolassy (2018), who suggested that other sensilla from other sensorial organs, such as from the tarsi, might be involved.

There is no physiological evidence that the parasitoid can detect the α-amylase, since gustation in insects is known to be influenced generally by small compounds such as sugars, free amino acids and water-soluble alkaloids (Thiéry et al., 2013 for review). Nevertheless, it is well-known that hymenopterans are able to detect large molecules such as long chain cuticular hydrocarbons of more than 60 carbons (Cvacka et al., 2006; Blomquist and Bagnères, 2010) and that non-volatile long-chain hydrocarbons can been detected by olfactory sensilla (Ozaki et al., 2005, 2012). We cannot therefore rule out the detection of α-amylase by sensilla specialized in olfaction on *Cotesia* spp. antennae.

In conclusion, this study shows that α-amylase is a key protein for host acceptance and oviposition by species of the *C. flavipes* complex, and that its variation is involved in the specificity of host-parasitoid association. These findings open new routes for the investigation of evolutionary processes at play in Lepidoptera stem borers-*Cotesia* and their interactions.

In addition, these findings highlight some issues in biological control perspectives. The ecosystem service provided by biological control relies to a large extent on the natural adaptive abilities of biological control agents. Pest resistance is less frequent in biological control than in chemical control (Holt and Hochberg, 1997). One reason advanced for this better protection against host resistance is that biological control agents can co-evolve and adapt to host resistance, whereas chemical control agents cannot. The link between α-amylase isoforms and *Cotesia* species and population in this study gives a strong insight into such adaptive processes of the parasitoid to its host. In the near future the main relevance in agriculture will be to deliver more efficient parasitoid strains against pest insects. The identification of α-amylase's receptors involved in host acceptance mediation will help in targeting the genes of these receptors with the aim of carrying out genetic improvements on them.

AUTHOR CONTRIBUTIONS

P-AC made conception and design of the study. GB and J-LD contributed to purify and isolate the α-amylase. KS performed the bioassays. SM performed the α-amylase purifications. P-AC wrote the first draft of the manuscript. GB, J-LD, BL, and LK wrote sections of the manuscript. EM corrected the English. All authors contributed to manuscript revision, read and approved the submitted version.

ACKNOWLEDGMENTS

The authors wish to thank IRD and ANR ABC PaPoGen (ANR-12-ADAP-0001) and ANR CoteBio for funding the research; DAAD for funding the Ph.D. fellowship under the grant number 91560009, the University of Nairobi and the *icipe* capacity building for hosting the Ph.D. student. Thanks are given to Julius Obonyo and Peter Malusi from *icipe* for rearing the host insects and the parasitoids; and to François Rebaudo from IRD for his statistical help. Thanks are also given to Fritz Schulthess for reviewing the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00228/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 19 February 2019
doi: 10.3389/fevo.2019.00020

Western European Populations of the Ichneumonid Wasp *Hyposoter didymator* Belong to a Single Taxon

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: 09 November 2018
Accepted: 22 January 2019
Published: 19 February 2019

Citation:
Frayssinet M, Audiot P, Cusumano A, Pichon A, Malm LE, Jouan V, Vabre M, Malavieille S, Delalande M, Vargas-Osuna E, Bourguet D and Volkoff A-N (2019) Western European Populations of the Ichneumonid Wasp Hyposoter didymator Belong to a Single Taxon. Front. Ecol. Evol. 7:20. doi: 10.3389/fevo.2019.00020

Hyposoter didymator (Hymenoptera, Ichneumonidae) is a generalist solitary endoparasitoid of noctuid larvae. In the present work, we tested whether populations of *H. didymator* were divided in several genetically distinct taxa as described for many other generalist parasitoid species, and whether differences in *H. didymator* parasitism rates were explained by the insect host species and/or by the plant on which these hosts were feeding on. The genetic analysis of natural populations collected in different regions in France and Spain on seven different insect hosts and seven different host plants (775 individuals) showed that *H. didymator* populations belong to a unique single taxon. However, *H. didymator* seems to be somewhat specialized. Indeed, in the fields it more often parasitized *Helicoverpa armigera* compared to the other host species collected in the present work. Also, *H. didymator* parasitism rates in field conditions and semi-field experimental studies were dependent on the host plants on which *H. armigera* larvae are feeding. Still, *H. didymator* can occur occasionally on non-preferred noctuid species. One hypothesis explaining the ability of *H. didymator* to switch hosts *in natura* could be related to fluctuating densities of the preferred host over the year; this strategy would allow the parasitoid to avoid seasonal population collapses.

Keywords: *Hyposoter didymator*, *Helicoverpa amigera*, population genetic structure, host preferences, western European populations

INTRODUCTION

Endoparasitoids are insect wasps. Free-living when they are adults, they complete their larval development within the body of another insect eventually killing it. Because of their particular lifestyle, they have intricate physiological interactions with their hosts that allow them to optimally exploit host resources (Harvey, 2005). Therefore, many endoparasitoids tend to have narrow host ranges and parasitize one or a few phylogenetically related species, which often share similar biological or ecological characteristics (Godfray, 1994; van Veen et al., 2008). However, there is a continuum in amplitude of host ranges within endoparasitoids, some being highly specialized (e.g., *Hyposoter horticola*; Lei and Hanski, 1998) and others reported as generalists (e.g., *Cotesia marginiventris*; Tamò et al., 2006). Generalists can exploit a broad range of hosts, and because of their ability to shift to another host when one host population is at low density, they should have

a higher overall fitness compared to specialists in case of fluctuating host resources. On the other side, specialized parasitoids are thought to be more efficient compared to generalists in exploiting a particular host because of the trade-off between host range and host-use efficiency (reviewed in Gagic et al., 2016). According to Loxdale et al. (2011), "generalism is unlikely to be maintained in nature through speciation," and for many generalist endoparasitoid species, they are in fact a complex of several specialized taxa reproductively isolated and genetically differentiated.

Host range can vary depending on two main parasitoid features: its behavioral responses to hosts (and host environment) allowing successful host detection, and its ability to develop or not in the host. Development will depend on host suitability (e.g., allowing or inhibiting parasitoid development and survival) and quality (e.g., provision of nutritional resources allowing or not the parasitoid to reach optimal body size and development time) which may vary depending on the species, development stage, etc. (Antolin et al., 2006). It also depends on the ability for the parasitoid to regulate certain aspects of host biology. Hence, many endoparasitoids use a large range of strategies to modulate host physiology for their own benefit (i.e., in a way that increases offspring fitness). Better known strategies are injection of immune depressing venom toxins by the ovipositing female (Poirié et al., 2014) or production in female reproductive tract of mutualist virus particles then delivered into the host (Pichon et al., 2015; Strand and Burke, 2015).

We focused here on the Palearctic species *Hyposoter didymator* (Ichneumonidae; Campopleginae) whose host range includes several closely related moth species within the Noctuidae family. *H. didymator* is an important biological control agent of greenhouse pests in the whole Mediterranean region. It has been reported in France, Spain, Italy, but also in the Near East, central Asia, and north Africa (http://www.catalogueoflife.org/col/details/database/id/68). In Europe, its published natural hosts are *Chrysodeixis chalcites* and *Lacanobia oleracea* (Reudler-Talsma et al., 2007), although *H. didymator* attacks other species, including *Helicoverpa armigera* (Carl, 1978; Bar et al., 1979; Torres-Vila et al., 2000), *Mythimna loreyi* (Sertkaya and Bayram, 2005) or *Spodoptera littoralis*, a species native to Africa but present in Spain (Hatem et al., 2016). *H. didymator* can also successfully reproduce in novel hosts in the laboratory and can be maintained successfully on the Nearctic species *Spodoptera frugiperda*, a non-natural host for this parasitoid (Dorémus et al., 2014). Actually, host suitability and quality for *H. didymator* appear to be more dependent on the phylogenetic relatedness of the hosts than on a previous encounter between *H. didymator* and the host species (Harvey et al., 2012). In *H. didymator*, success of parasitism relies on a mutualist endogenous polydnavirus named *Hyposoter didymator* Ichnovirus (HdIV). The virus replicates in the female's ovaries and viral particles are injected along with the egg into the host larva during oviposition. Infection by HdIV of host tissues leads to modulation of the host physiology and development to render it suitable for the wasp development (Dorémus et al., 2014).

The present work arises from the observation that *H. didymator* is widely distributed and currently known as a generalist endoparasitoid of several noctuid larvae feeding on different plants. Our goal was to know whether or not there is a host-associated differentiation in *H. didymator*. For this purpose, we performed a set of experiments and analyses to test: (i) whether or not populations of *H. didymator* are divided into several genetically distinct taxa, and (ii) whether *H. didymator* parasitism rates were linked to host species and/or to the host plants on which these hosts were feeding.

MATERIALS AND METHODS

Population Genetic Structure

Collections of *H. didymator* Populations

Collections of *H. didymator* were undertaken over a period of 5 years (2011–2016) in France and Spain. Depending of the year, the site and the flights of the insect host species, the sampling was done from late April to November (most commonly from June to October). Sampling was performed on several host plants in more than 30 sites in France and 3 sites in Southern Spain. In those sites and host plants, we collected larvae of several noctuid species, mainly *H. armigera*, *Autographa gamma*, *Spodoptera exigua,* and *S. littoralis*. The characteristics of each sample (geographical location, the insect host from which it emerged and the host plant on which the insect host was collected from) is detailed in **Supplemental Table 1**.

Because *H. didymator* is a solitary parasitoid that develops in young larval stages, only early stages (L1 to L4) of noctuid moth larvae were collected. Upon collection, these noctuid larvae were brought to the quarantine laboratory and maintained on their host plants or on artificial diet until parasitoid cocoon formation and adult emergence. The number of adult parasitoids that emerged was recorded for each sampling site.

When adult parasitoids from species other than *H. didymator* emerged from the noctuid hosts, their genera or species have been roughly identified using COI barcoding (Hebert et al., 2003) when collected for the first time. Briefly, genomic DNA was extracted from adults using Wizard® Genomic DNA Purification Kit (Promega) according to manufacturer instructions. Genomic DNA (gDNA) was used as a template for PCR amplification using COI specific primers (LCO1490: 5′- GGT CAA CAA ATC ATA AAG ATA TTG G−3′ and HC02198: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA−3′, as described in Folmer et al. (1994). The 50 µl PCR reaction typically contained 2 µl of gDNA (200 ng), 0.25 µl of Taq DNA polymerase (Promega), 10 µl of 5X Taq Buffer (Promega), 2 µl of each primer (10 µM), 4 µl of MgCl2 (50 mM), 1 µl of dNTP (1 mM) and ultrapure water, with the following thermal cycle: initial denaturing for 5 min at 95°C; pairing for 30 cycles (30 s at 95°C, 45 s at 58°C, and 1 min at 72°C); final extension 5 min at 72°C. PCR amplification products were sent for sequencing by Eurofins, and annotated subsequent to a Blastn similarity search against NCBI nr database. The sequences are available in **Data Sheet 1**.

Genotyping

From the sampling performed between 2011 and 2016, we obtained a total of 775 *H. didymator* adults (451 females) originated from 8 regions, 7 insect hosts and on 7 host plants

(**Table 1** and **Supplemental Table 1**). DNAs were extracted using the DNeasy Blood & Tissue Kit (Qiagen). Each individual was genotyped at the 14 polymorphic microsatellite loci developed by Audiot et al. (2014). They included 10 loci named HD located within the *H. didymator* genome and 4 loci named Hdiv located within the *H. didymator* ichnovirus genome. Hdiv6 was within the segment Hd23 (GenBank KJ586309) and overlaps with the HdIV gene *Rep1_Hd23*, Hdiv7 was within the segment Hd7 (GenBank KJ586326) and was located close to the gene *U1_Hd7*, Hdiv8 was located at the extremity of the proviral segment Hd4 (GenBank KJ586330) and Hdiv9 was within the segment Hd11 (GenBank KJ586322) and was located between two viral ankyrin genes (*Vank3_Hd11* and *Vank4_Hd11*). The forward primer of each locus was 5′end-labeled with a fluorescent dye: FAM (Eurogentec), VIC, NED or PET (Applied Biosystems) as described in Audiot et al. (2014). The 14 loci were amplified in two multiplex (M1 and M2) PCRs and analyzed in two runs. The PCRs were performed on a Mastercycler ep gradient S (Eppendorf) using the Qiagen Multiplex PCR Kit. They were conducted in 10 μL reaction volume containing the QIAGEN Multiplex PCR Master Mix (1x) (including Taq, dNTPs and a final concentration of 3 mM of MgCl2), 0.2 μM of each primer, and 1 μL of genomic DNA. PCRs started with an initial activation step at 95°C for 15 min, followed by 32 cycles with denaturation at 94°C for 30 s, annealing at 58°C for 1.5 min, extension at 72°C for 1 min and final extension of 60°C for 30 min. The PCR products were diluted 100-fold, then 2 μL of each diluted PCR product were mixed with 18 μL of mix for sequencing (8 μL of GeneScan 500 LIZ Size Standard plus 2 mL of Hi-Di Formamide (Applied Biosystems) prepared for one plate). The samples were separated and detected on an ABI 3130 automated sequencer and analyzed using GeneMapper v.3.7 (Applied Biosystems, USA).

TABLE 1 | Number of total (*N*) and females (*N* females) of *Hyposoter didymator* samples that were genotyped by microsatellite sequencing.

Category		*N*	*N* females
Region	Bretagne	49	20
	Corse	92	52
	Hyères	9	6
	Marciac	1	1
	Montpellier	481	255
	Villereal	75	71
	Canaries	4	4
	Seville	64	42
Host plant	*Apium graveolens*	7	5
	Brassica napus	101	44
	Brassica oleracea	2	2
	Cicer arietinum	11	3
	Medicago sativa	644	390
	Sorghum bicolor	3	1
	Zea mays	3	2
	Unknown	4	4
Insect host	*Autographa gamma*	59	26
	Chrysodeixis chalcites	4	4
	Helicoverpa armigera	604	366
	Spodoptera exigua	11	7
	Spodoptera littoralis	15	12
	Xylena exsoleta, Agrochola lychnidis, or Mamestra oleracea	45	11
	Unknown	37	25

Host Plant Preferences

Experimental Set-Up

This non-choice experiment was conducted with BioQuip Greenhouse Cages (cat#1466E) installed outdoors (within a containment dispositive since *H. armigera* is classified as a quarantine species in France; see description in **Supplemental Figure 1**) to prevent contamination and escapes. The dispositive included eight 2-m-high cages covering a surface area of 9 m2 (3 × 3 m) built with iron frames covered with an insect-proof netting (150 μm). The experiment took place at the DIASCOPE INRA research station (Mauguio, France).

Each cage contained three patches—each patch containing several plants—of either alfalfa (*Medicago sativa*), corn (*Zea mays*) or chickpeas (*Cicer arietinum*), probes allowing temperature and hygrometry monitoring and a drip irrigation system. For each host plant species, we performed five replicates over the summer season.

Laboratory Insect Strains

The *H. armigera* strain originated from adults captured in 2014 in Montpellier region using pheromone and light traps. Adults collected in these traps were brought to the laboratory, placed in cages containing alfalfa (*Medicago sativa*) plants, and allowed for mating and oviposition. Eggs were collected and, after hatching, larvae were kept on alfalfa plants until being used in the experiment.

The *H. didymator* DGIMI's laboratory colony originated from wasps issued from *H. armigera* larvae collected on alfalfa fields in Montpellier region, and maintained on *S. frugiperda* for more than 50 generations. The *Spodoptera frugiperda* DGIMI's laboratory strain (corn variant) originated from insects collected in La Réunion, then reared in the DGIMI's insectarium for more than 10 years on Poitout artificial diet (Poitout et al., 1972) at 25°C.

Experiment

On each cage, during the first day of the experiment (day 1), twenty *H. armigera* larvae (early third instars) were deposited on each patch (leading to a total of 60 larvae per cage). Within each cage, 6 mated 3-days old *H. didymator* females and 2 *H. didymator* males were released 6 h after depositing the *H. armigera* larvae. Six days after, all plants were carefully leafed out to count the number of live, dead or missing host larvae. At the end of the experiment, parasitoids were searched for, and the number of live or dead wasps was recorded. Host larvae which were alive were then brought to the laboratory to calculate the parasitism rate (estimated as the number of formed *H. didymator* cocoons).

Data Analyses

Population Genetic Structure

Genetic clustering

Bayesian clustering analyses were conducted using the software STRUCTURE (Pritchard et al., 2000), version 2.3.2 (Hubisz et al., 2009). This version of STRUCTURE offers the possibility to use samples characteristics (i.e., geographical location, insect host and host plant on which the adult of *H. didymator* was originated from) as a prior and to give more prior weight on clustering outcomes that are correlated with these samples characteristics. Analyses were performed using (1) all microsatellite loci (i.e., using HD and Hdiv loci), (2) all 10 HD loci (i.e., excluding the 4 Hdiv loci) and (3) all 4 Hdiv loci (i.e., excluding the 10 HD loci).

We varied the number of putative genetic clusters (K) between 1 and 4 and performed 20 independent runs for each value of K. Each Markov chain was run for 200,000 steps, after a 50,000-step burn-in period, using the admixture model for correlations of allele frequencies across clusters, with the default value for parameter α and the default prior for Fst. As K is increased the most divergent groups are expected to separate into distinct clusters first (Pritchard et al., 2000; Fontaine et al., 2007). To determine the optimal K value, we compared the plots of Ln P(D) and ΔK (Evanno et al., 2005) using STRUCTURE HARVESTER v0.6.94 (Earl and vonHoldt, 2012). In brief, ΔK is a measure of the second order rate of change in the likelihood of K and its maximum indicates the breakpoint in the slope of the distribution of lnP(D) for the different K values tested.

Genetic differentiation

Across all individuals, the overall genetic diversity at each microsatellite locus was calculated using GENEPOP 4.0 (Rousset, 2008). For each sample of *H. didymator* containing at least 20 females ($n = 7$ samples), we tested for deviation from Hardy Weinberg equilibrium (HWE) for each locus and over all loci using Fisher's exact test as implemented in GENEPOP 4.0. Because males *H. didymator* are haploids, these deviations from HWE were tested using females only.

For each locus, across all loci, across HD loci and across Hdiv loci, we estimated the genetic differentiation—using the Fst values (Weir and Cockerham, 1984)—overall samples and between each pair of samples containing at least 20 individuals ($n = 12$ samples).

We also performed a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992), based on allele frequency information, using the hierfstat package (Goudet, 2005) and the version 3.4.4 of R (R Core Team, 2015). This analysis allowed us to assess the relative effect of the geographical location, host plants and insect hosts and on *H. didymator* population structure with respect to total genetic variation. The AMOVA was performed using all loci, using the HD loci and using the Hdiv loci.

Parasitism Rates

Parasitism rates were analyzed with logistic regression models, i.e., generalized linear models (GLMs) and generalized linear mixed models (GLMMs) with binomial error distribution and logit link function. In field collection survey, we constructed a GLM to test the dependence of parasitism rates achieved by *H. didymator* on the fixed factors host species, host plant species, locality and year of sampling. In the cage experiment, we used a GLMM to test if parasitism rates achieved by *H. didymator* on the host *H. armigera* were significantly affected by the fixed factor host plant (chickpeas, alfalfa and corn) using the cage ID as random factor. When overdispersion was detected in the models, we corrected for this by fitting quasi-binomial distributions in order to have a multiplicative overdispersion factor. Model fit was assessed with residual plots. Significance of each fixed factors in GLMs and GLMMs was determined using likelihood ratio tests (LRTs) comparing the full model with and without the factor in question, starting with higher-order interactions (Crawley, 2007). *Post-hoc* comparisons were carried out using the *glht* function found in the *multcomp* package of the R statistical software (Bretz et al., 2010).

A quantitative food web with the data recorded from the field survey of noctuid species and their larval parasitoids was constructed using the *bipartite* package (Dormann et al., 2016) of the R statistical software. In the food web graphic, the bottom bars represent the insect hosts and the top bars represent parasitoid species. The width of the bars is a proportional representation of the host and parasitoid species abundances.

RESULTS

Population Genetic Structure

Microsatellite markers were used to test whether populations of *H. didymator* attacking noctuid larvae correspond to a single species or are subdivided into several genetically differentiated taxa.

Most loci were at HWE in all tested populations (see **Supplemental Table 2**) suggesting a random mating between individuals and a low frequency of null alleles at these loci. Analyses of genetic disequilibrium between the 14 loci indicate that, except HD59 and HD90, they segregate independently from each other (see **Supplemental Table 3**)—hence, the information given by each locus on the population genetic structure is, with one exception, independent from each other.

The genetic differentiation between the 12 populations with at least 20 individuals (i.e., with enough power to test the occurrence of a genetic differentiation between them), although significantly different from zero, was very low. The Fst value overall populations and overall loci equaled 0.0068 and the pairwise Fst values ranged between -0.0113 and 0.0247 (see **Supplemental Table 4**). The most differentiated population was the population from Corsica (which emerged from *H. armigera* collected on alfalfa). The Fst values between this population and the 11 other populations varied between 0.0073 and 0.0247. The Fst values of all the other pairwise comparison, except one, was < 0.001.

The overall genetic differentiation at the 4 Hdiv loci was higher than at the 10 HD loci (Fst = 0.0176 vs. 0.0031). The pairwise comparisons, showed that the higher differentiation of the Corsican population from the other populations was due to a higher level of differentiation at the Hdiv than at the HD loci. Indeed, overall Hdiv loci, Fst values between this

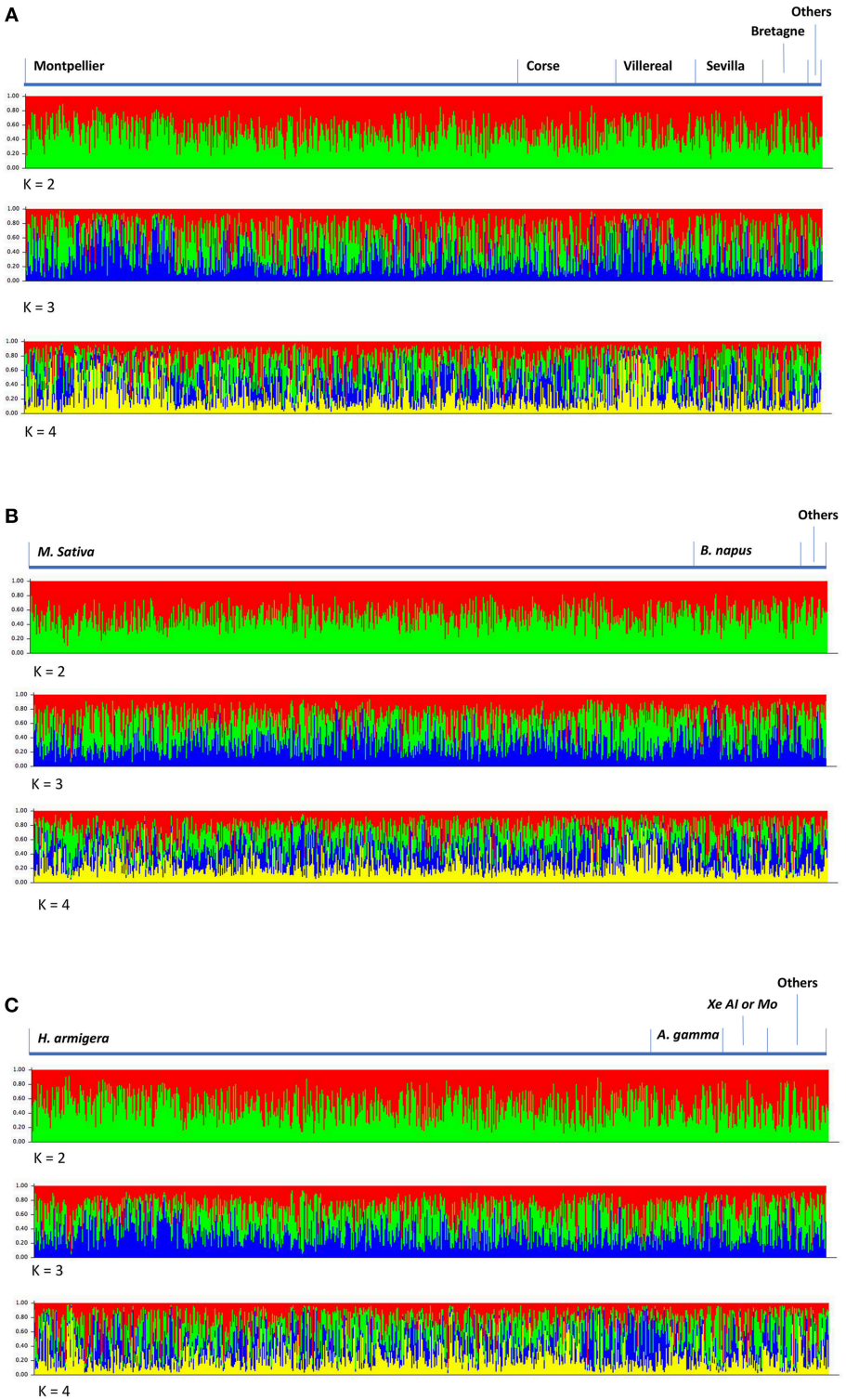


FIGURE 1 | *H. didymator* population structure from Bayesian STRUCTURE analyses using 14 microsatellite loci for different values of *k*. Analyses were carried on depending on wasp geographic origin **(A)**, host plant **(B)** or host species **(C)**.

population and the 11 other populations varied between 0.0376 and 0.0754, whereas the pairwise Fst values overall HD loci were all < 0.0075.

Accordingly, the results of Bayesian population structure analyses did not reveal any significant genetic differentiation, be when considering all loci, HD loci or Hdiv loci. The estimated

logarithm of likelihood for data analyzed with Structure was highest for K = 1 (**Figure 1**). Indeed, the LnP(D) plot only shows a strong drop off in model fit after $K = 2$. For $K > 1$, none of the 775 individuals could be assigned to a given cluster as each individual had a similar probability of belonging to each cluster. The ΔK also suggested only one discernible grouping was present within the data.

Finally, the AMOVA analyses showed that the variation between samples collected on different locations, different host plants and different insect host explained, respectively only 3.3, 0.8, and 0.2% of the total variance while the variation among individuals within samples and within individuals explained 9.8 and 85.9% of the total variation, respectively (**Table 2**). The analyses restricted to HD or Hdiv gave rather similar results (**Table 2**).

Hence, all samples of *H. didymator* collected on different insect hosts and/or on the different host plants appears to belong a single genetic cluster.

TABLE 2 | AMOVA analysis results. HD loci are located within *Hyposoter didymator* genome excluding viral sequences; Hdiv loci are within integrated viral sequences.

Loci	Source	*F*-statistics	% variance	*P*-values
All loci	Between location	0.013	3.3	0.027
	Host plant within location	0.004	0.8	0.957
	Insect host within host plant	0.007	0.2	0.954
	Individuals within insect host	0.156	9.8	<0.0001
	Within individuals	0.294	85.9	<0.0001
HD loci	Between location	0.015	2.8	0.164
	Host plant within location	0.004	0.8	0.965
	Insect host within host plant	0.009	0.4	0.914
	Individuals within insect host	0.145	7.2	<0.0001
	Within individuals	0.352	88.8	<0.0001
Hdiv loci	Between location	0.055	5.4	0.008
	Host plant within location	0.008	0.6	0.876
	Insect host within host plant	0.009	0.6	0.885
	Individuals within insect host	0.237	8.6	<0.0001
	Within individuals	0.278	84.8	<0.0001

Parasitism Rates in Natural Conditions

Parasitism rates by *H. didymator* varied greatly in the different sampling sites (GLM, $\chi^2 = 590.57$, df = 5, $p < 0.001$) and years ($\chi^2 = 32.81$, df = 4, $p < 0.001$), ranging from 0 to almost 60% (**Supplemental Table 1**), and were significantly affected by both the host species ($\chi^2 = 520.39$, df = 4, $p < 0.001$) and the host plant ($\chi^2 = 465.77$, df = 9, $p < 0.001$). The parasitism rate was indeed higher on *H. armigera* (∼10.4%) than on all the other host species collected during this survey (**Figure 2A**). The parasitism rates on *A. gamma* and *S. exigua* were very low (∼1.5%) but significantly higher than on *S. littoralis* (0.2%).

Hyposoter didymator was also the parasitoid species that emerged the most frequently from *H. armigera* parasitized larvae collected in the fields, even though a whole cohort of other parasitoid species (including tachinids) emerged from this species (**Figure 2B**; **Supplemental Table 5**). Conversely, *H. didymator* appeared to be only occasional on *A. gamma*, which was mainly parasitized by braconids from the *Aleiodes* genus. Similarly, *S. littoralis* was mainly parasitized by braconids from the *Cotesia*, *Meteorus* and *Homolobus* genera and only in a few cases by *H. didymator* (**Supplemental Table 5**).

Regarding the host plant, *H. didymator* seemed to preferentially parasitize noctuids feeding on rapeseed than those feeding on other crops, with parasitism rate reaching 37% (**Supplemental Table 6**). Hosts were slightly more often parasitized on alfalfa (∼6%) than on corn and cabbage (1–2%). Finally, noctuid larvae captured on chickpeas displayed the lowest parasitism rates (>0.5%). Regarding the host plant (**Figure 2C**), parasitism rates were higher when caterpillars were feeding on rapeseed (36.8%) than on alfalfa (5.9%) or cabbage and corn (1.5 and 1.7∼2%, respectively).

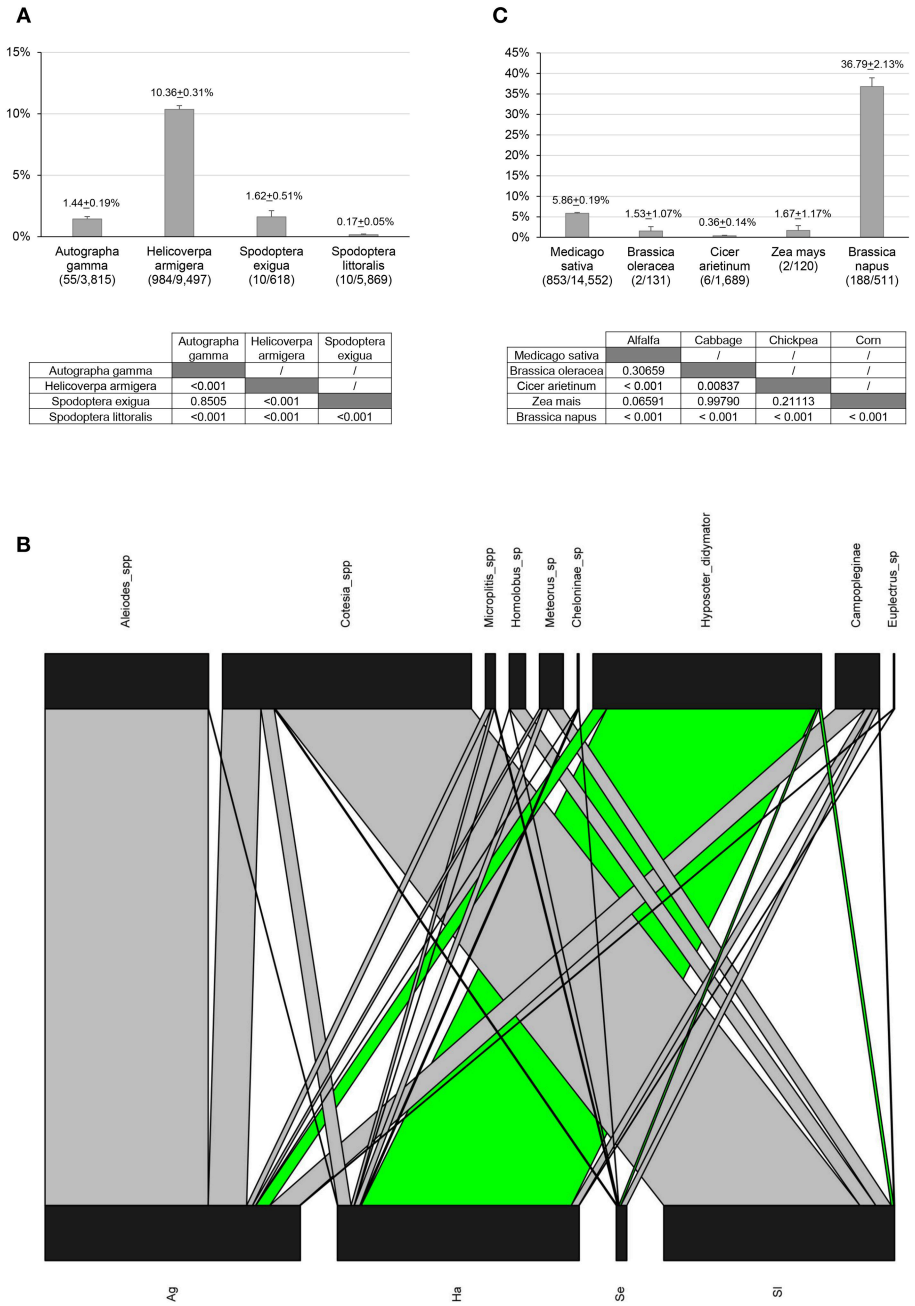
Hence, although all samples of *H. didymator* apparently belong to a single species, this parasitoid probably does not parasitize noctuid larvae at random: indeed, parasitism rates observed in natural conditions strongly suggest host plant and insect host preferences.

Host Plant Preference

Results obtained on plants placed within cages indicate a significant effect of the host plant on parasitism rates (GLMM, $\chi^2 = 67.98$, df = 2, $p < 0.001$). Parasitism rates of *H. armigera* larvae (**Figure 3**; **Supplemental Table 7**) were significantly higher on alfalfa (∼82%) than on corn ($Z = 4.91$, $p < 0.001$) and chickpeas ($Z = 7.87$, $p < 0.001$). *H. armigera* larvae feeding on corn plants were also significantly more parasitized (∼53%) than on chickpeas (∼7%) ($Z = 5.11$, $p < 0.001$). The observed differences in parasitism rates among host plants are in agreement with the *H. didymator* host plant preference observed *in natura*.

DISCUSSION

Hyposoter didymator is currently described in the literature as a generalist parasitoid able to infest the larvae of several noctuid species in nature and to develop indifferently in a range of noctuid hosts in laboratory conditions. In one hand, our results were in line with this characteristic. In addition, we show that, at least in western Europe (France and Spain) and on the 7 insect hosts collected in this work, *H. didymator* populations are not subdivided in several genetically differentiated taxa but rather belong to a unique single taxon. This feature is somewhat different from other examples of insect species feeding on multiple hosts. Indeed, many insect species which were thought to be generalists turned out to actually be a complex of several genetically differentiated taxa more or less specialized on a specific host or set of hosts. This was true in many phytophagous insect genera like the moths *Ostrinia* (Malausa et al., 2007; Bourguet et al., 2014) and *Plutella* (Perry et al., 2018), the flies *Ragholetis* (Feder et al., 1999; Xie et al., 2008) or the aphid *Acyrthosiphon pisum* (Peccoud and Simon, 2010). This feature has also been documented for several species of parasitoid tachninid flies (Smith et al., 2007). In the case of



	Autographa gamma	Helicoverpa armigera	Spodoptera exigua
Autographa gamma		/	/
Helicoverpa armigera	<0.001		/
Spodoptera exigua	0.8505	<0.001	
Spodoptera littoralis	<0.001	<0.001	<0.001

	Alfalfa	Cabbage	Chickpea	Corn
Medicago sativa		/	/	/
Brassica oleracea	0.30659		/	/
Cicer arietinum	< 0.001	0.00837		/
Zea mais	0.06591	0.99790	0.21113	
Brassica napus	< 0.001	< 0.001	< 0.001	< 0.001

FIGURE 2 | Natural parasitism survey. **(A)** Parasitism rates (%±SE) by *H. didymator* for 4 noctuid species. Statistical analyses are indicated below the graph (parasitism rates pairwise comparisons). **(B)** Quantitative food-web network: larval parasitoid communities interacting with *H. didymator*. The bottom bars represent the host species (Ag= *Autographa gamma*, Ha= *Helicoverpa armigera*, Se= *Spodoptera exigua*, Sl= *Spodoptera littoralis*) whereas the top bars represent parasitoid species. The width of the bars is a proportional representation of species abundances. The host-parasitoid interactions involving *H. didymator* are depicted in green. **(C)** Parasitism rates (%±SE) by *H. didymator* for 5 different host plants. Statistical analyses are indicated below the graph (parasitism rates pairwise comparisons). For A and C: the number of parasitized caterpillars relative to the number of caterpillars collected in the fields is indicated in parentheses at the bottom of the graph axis; refer to the **Supplemental Table 1** for details on the collected samples.

Eupelmus vesicularis, a chalcid wasp long considered as one of the most polyphagous species, with more than 200 hosts from 4 insect orders, it was rather a problem of identification due to morphological similarities. Indeed, the species previously named *E. vesicularis* turned out to be actually a group of at least five species (Fusu, 2017). Cryptic species have been also identified among braconid species parasitizing lepidopteran [e.g., *Cotesia melitaearum* and *C. acuminata* (Kankare et al., 2005) and *Cotesia flavipes* complex (Muirhead et al., 2012)] or aphids [e.g., *Aphelinus varipes* species complex (Heraty et al.,

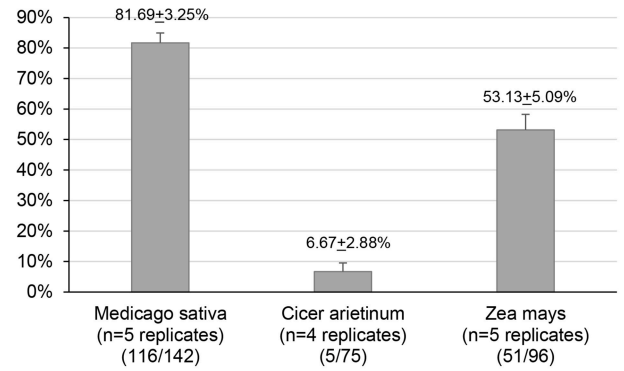


FIGURE 3 | Host plant preference. *Helicoverpa armigera* larvae total parasitism rates (%±SE) by *Hyposoter didymator* depending on the host plant on which *H. armigera* larvae were feeding. The number of parasitized caterpillars relative to the number of caterpillars recovered in the experiment (all replicates merged) is indicated in parentheses at the bottom of the graph axis.

2007) and *Aphidius matricariae* and *A. urticae* (Derocles et al., 2016)]. In the case of parasitoids, specialization is usually related to the host species use and there is often a lack of genetic structure related to host plant species (reviewed in Saskya van Nouhuys, 2016). As molecular studies progresses, it turns out that only a few parasitoid species appear to be true generalists and most parasitoid species have actually a narrower host range than originally assumed. However, some parasitoid species are described as generalists and may be able to switch regularly between different host species. For instance, the same genetic population of the egg parasitoid *Ooencyrtus pityocampae* can alternate seasonally between two hosts belonging to two different orders, the moth *Thaumetopoea wilkinsoni* and the bug *Stenozygum coloratum* (Samra et al., 2015).

Our results indicated that the overall genetic differentiation at the 4 Hdiv polydnavirus loci was higher than at the 10 HD nuclear loci. Polydnavirus proteins are directly subjected to host physiological resistance mechanisms, so polydnaviral genes were hypothesized to be potential good markers of parasitoid specialization (Whitfield et al., 2018). The role of polydnaviruses in parasitoid local adaptation has previously been reported for the braconid *Cotesia sesamiae*. Although *C. sesamiae* was initially considered as a generalist species developing on several lepidopteran hosts, two taxa of *C. sesamiae* were identified in Kenya that vary in their capacity to overcome the resistance of the lepidopteran host, a feature that was linked to allelic variations in viral genes (Branca et al., 2011; Kaiser et al., 2017; Gauthier et al., 2018). Although not differentiated in several genetically differentiated taxa, *H. didymator* probably does not parasitize all noctuid species indifferently and part of this specialization might be due, like *C. sesamiae*, to polydnaviruses, explaining a higher level of genetic differentiation at Hdiv loci.

Based on the results of the parasitism rates recorded in natural populations, we can hypothesize that *H. didymator* actually do have preferences for some insect hosts notably for *H. armigera*. Although not done here, due to problems in maintaining a *H. armigera* strain in the laboratory, further studies on *H. didymator* life history traits on this host species compared to others will be necessary.

According to other studies, *H. didymator* displays some preferences to and has a better development—i.e., a higher fitness—in some noctuid species than others. For instance, the percent of adult emergence was higher in *H. armigera* compared to *S. littoralis* even using *H. didymator* adults emerged from parasitized *S. littoralis* (Hatem et al., 2016). In the same line, Reudler-Talsma et al. (2007) found a better larval development and a bigger adult size when *H. didymator* parasitized *C. chalcites* compared to *S. exigua*. Differences in life history traits and therefore in fitness on different hosts is a classical feature in parasitoid species. Such differences have been reported in several braconid species: *Psytallia cosyrae* (Mohamed et al., 2003), *Cotesia glomerata* (Harvey, 2000), *Bracon hebetor* (Ghimire and Phillips, 2010), or *Aphidius ervi* (Velasco-Hernández et al., 2017). Similarly, the ichneumonid *Venturia canescens* that emerged from *Ephestia kuehniella* were larger compared to those that emerged from *Plodia interpunctella* (Jones et al., 2015). Parasitoids develop inside a single host which provides their exclusive nutritional and physiological environment. Therefore, parasitoid fitness is closely linked to the innate host quality, which depends on the host species, size, developmental stage and on the plant on which it feeds (Ode, 2006).

Overall, our findings are in agreement with previous results suggesting that *H. armigera* is indeed a preferential host of *H. didymator*. For instance, in Spain, although some other parasitoid wasp species were detected, it was the parasitic complex of *H. didymator—Cotesia kazak* that parasitized more than 95% of *H. armigera* larvae in tomato crops (Torres-Vila et al., 2000). Similar results were obtained in Greece and Bulgaria (Carl, 1978). In Israel, *H. didymator* is similarly the most abundant parasitoid of *H. armigera* populations infesting cotton (Bar et al., 1979). *H. didymator* distribution covers Europe, and is not reported in Africa, except for Tunisia (https://cran.r-project.org/web/packages/bipartite/bipartite.pdf). The cotton bollworm *H. armigera* is an indigenous species for Africa and present in a large part of Europe (https://gd.eppo.int), where a single species is described (Colvin et al., 1994) although the population structure is still poorly resolved for this pest (Behere et al., 2007). Our and others' data indicate that *H. armigera* main parasitoid in southern Europe is *H. didymator* whereas in Africa, the bollworm is parasitized by other species (Van den Berg et al., 1988). Supplementary studies will be necessary to evaluate the evolutionary origin of the association between *H. didymator* and *H. armigera* in their common distribution area, and to decipher if *H. didymator* occurred in Europe before *H. armigera* and then switched to this new host after this moth established in European regions.

Finally, parasitism by *H. didymator* is also influenced by the host plant on which their hosts are feeding. In our survey in natural populations, noctuid larvae that fed on rapeseed and alfalfa were more frequently parasitized by *H. didymator* than larvae collected on other host plants (corn, chickpeas and some vegetables). Accordingly, in our controlled experiments performed in outdoor cages, parasitism rates of *H. armigera*

larvae by *H. didymator* were significantly influenced by the host plant on which *H. armigera* were deposited, with higher parasitism rates on alfalfa than on corn or chickpeas. Our results are in agreement with previous studies performed on six host plants (sorghum, sunflower, cotton, soybean, chickpea, and pigeon pea) showing that *H. didymator* parasitism was the lowest on chickpea and the highest on soybean (Murray et al., 1995). Influence of host plant on parasitism is also a classical feature in host-parasitoid interaction. For instance, using a similar cage experiment, there was no parasitism of *H. armigera* larvae by the braconid *Microplitis demolitor* on chickpeas, whereas 60–75% of parasitism was recorded on maize, cotton or soybean (Murray and Rynne, 1994). The host plant complex had also a strong influence on the level of parasitism of *Plutella xylostella* by *Diadegma mollipla* (Hym., Ichneumonidae) with a higher proportion larvae parasitized on peas (2.6%) than on cabbage (0.9%) (Rossbach et al., 2006). In the case of *H. didymator*, the influence of the host plant can be explained by the fact that host larvae are more accessible in alfalfa where they consume the leaves, than in corn, a plant where larvae tend to act as a borer and hide within the stem or the cobs. Alternatively, the results could be explained by differences in odors emitted by the plant species in response to larvae feeding damage. It is known that several parasitoid species use herbivore-induced plant volatiles (HIPVs) to find their hosts (Vinson, 1998; Mumm and Dicke, 2010). In the future, it will be necessary to compare behavior of *H. didymator* to assess if the wasp is more attracted to the alfalfa plant compared to corn or chickpeas and whether wasp attraction can be explained by differences in the blend of HIPVs among plant species. Low parasitism rates on chickpeas have been previously reported (Murray et al., 1995) and could be related to the production of acidic exudates by the leaves which may be detrimental (repulsive) to the parasitoid. Overall, our results nonetheless suggest the existence of some plant-mediated specialization of *H. didymator* at the behavioral host location level.

In conclusion, *H. didymator* does parasitize larvae of several noctuid species feeding on numerous host plants. This polyphagy did not trigger any genetic differentiation at least here in the Western European populations of this wasp. Unlike many species which are in fact a complex of several subdivided populations specialized on different set of hosts, *H. didymator* sampled in natural populations in France and Spain belong to a unique taxon. This does not mean that this parasitoid is truly generalist, which parasitizes with the same level of efficacy all noctuid species. *H. didymator* rather appears to be specialized in some species and to occasionally attack other noctuid species on which it is probably maladapted. This might be explained by the ecology of *H. didymator*. The agricultural areas where *H. didymator* was collected are variable patchwork landscapes with many fields of different crops adjacent to each other and to natural areas, resulting in discrete habitat types. Hence, there may be large variability in host density and availability in *H. didymator* ecological habitat. In southern French regions, there are two main *H. armigera* flights and larvae of this pest are present from May until the end of October. However, in the spring *H. armigera* populations are low, and in alfalfa, they coexist with other a number of other noctuid species (e.g., *A. gamma*). This ability to shift to host species others than *H. armigera*—even if they may be less suitable—may has been selected for as an adaptation to seasonal population collapses.

AUTHOR CONTRIBUTIONS

A-NV and DB managed the project and analyzed the data. MF managed the insect collections with the help of EV-O, VJ, LM, and AP. PA performed the microsat analyses along with AP and VJ. The host plant preference experiment was done by MF, MV, SM, and MD. Statistical analyses were conducted by AC. The manuscript was written by A-NV and DB with the help of AC, LM, and AP.

FUNDING

This work has been provided by ANR (ABC-PaPoGen, ref. ANR-12-ADAP-0001) and by the Département Santé des Plantes et Environnement from INRA. Microsatellite data were produced through molecular genetic analysis technical facilities of the Labex Centre Méditerranéen de l'Environnement et de la Biodiversité.

ACKNOWLEDGMENTS

The authors thanks Romain Reinosa (BTS student), who participated to the outdoor cage experiment, as well as Clotilde Gibard and Gaétan Clabots from the DGIMI insectarium for the insects they have furnished. We thank the quarantine insect platform (PIQ), member of the Vectopole Sud network, for providing the infrastructure needed for pest insect experimentations. Many people helped us to collect noctuids. Special thanks to José Manuel Durán, Laboratorio de Producción y Sanidad Vegetal, Sevilla for his help. We are grateful to the Chambres d'Agriculture de Corse, Bretagne and Var who had informed us on the presence of noctuids *in natura* and helped in finding the plots. Thanks to Ernesto Fuentes and Primitivo Caballero, Universidad Pública de Navarra who send us cocoons from Canarias. And many thanks to all the producers in France and Spain who allowed us to collect in their fields. Funding of the work has been provided by ANR (ABC-PaPoGen, ref. ANR-12-ADAP-0001) and by the Département Santé des Plantes et Environnement from INRA. Microsatellite data were produced through molecular genetic analysis technical facilities of the Labex Center Méditerranéen de l'Environnement et de la Biodiversité.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00020/full#supplementary-material

Supplemental Table 1 | List of samples collected in different localities in France and Spain. For each sampling are indicated the number of noctuid larvae collected, the number of noctuid larvae that survived once brought to the laboratory, the number of obtained parasitized larvae, and the parasitism rate (estimated as the number of parasitized larvae per number of larvae that survived).

Are also indicated the number of *H. didymator* (Hd) that have been used for genotyping using microsatellite markers (total and number of females). Some of the parasitoid genotyped emerged from cocoons found in the field, or were caught in the fields as adults (indicated by the mention "cocoons/adults found). Slash (/) indicates the samplings for which the exact number of noctuid larvae is not known. na, non-applicable.

Supplemental Table 2 | Results showing that all HD and Hdiv microsatellite loci were at Hardy-Weinberg Equilibrium (HWE). Analyses were performed using individuals emerged from *H. armigera* larvae collected on different locations and different host plants.

Supplemental Table 3 | Genetic disequilibrium analyses between the 14 HD and Hdiv microsatellite loci.

Supplemental Table 4 | Pairwise FST values for all, HD and Hdiv loci, considering different locations, host plants and noctuid host species. Xe, Al or Mo: *Xylena exsoleta*, *Agrochola lychnidis,* or *Mamestra oleracea*.

Supplemental Table 5 | Diversity of parasitoids emerged from the different collected noctuid larvae, *Autographa gamma* (Ag), *Helicoverpa armigera* (Ha), *Spodoptera exigua* (Se) and *S. littoralis* (Sl), on different host plants.

Supplemental Table 6 | Parasitism rates in natural populations, depending on the noctuid host species or on their host plants. Compared to the main text, all noctuid host species collected and all the plants prospected are indicated.

Supplemental Table 7 | Plant preference experiments. For each set of experiments for each host plant, are indicated the number of *Hyposoter didymator* (Hd) still alive at the end of the experiment, the number of recovered *Helicoverpa armigera* larvae (Ha), the number of parasitized *H. armigera* larvae and the parasitism percent.

Supplemental Figure 1 | Experimental set-up for host plant preference tests.
Data Sheet 1 | Barcoding of parasitoids emerged from field-collected noctuid larvae. Above: table with the results for Blastn similarity searches against NCBI nr database. Below: list of COI sequences.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

REVIEW
published: 28 March 2019
doi: 10.3389/fevo.2019.00080

Global Climate Change as a Driver of Bottom-Up and Top-Down Factors in Agricultural Landscapes and the Fate of Host-Parasitoid Interactions

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OPEN ACCESS

Edited by:
Nikolaos Papadopoulos, University of Thessaly, Greece

Reviewed by:
Sergio Andrés Estay, Southern University of Chile, Chile
Andrea Sciarretta, University of Molise, Italy

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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *15 November 2018*
Accepted: *04 March 2019*
Published: *28 March 2019*

Citation:
Chidawanyika F, Mudavanhu P and Nyamukondiwa C (2019) Global Climate Change as a Driver of Bottom-Up and Top-Down Factors in Agricultural Landscapes and the Fate of Host-Parasitoid Interactions. Front. Ecol. Evol. 7:80. doi: 10.3389/fevo.2019.00080

The global climate is rapidly changing and the evidence is increasingly manifesting across various biological systems. For arthropods, several studies have demonstrated how changing climates affect their distribution through phenological and physiological responses, largely focusing on various organismal fitness parameters. However, the net-effect of the changing climate among ecological communities may be mediated by the feedback pathways among interacting trophic groups under environmental change. For agroecosystems, the maintenance of the integrity of trophic interactions even under climate variability is a high priority. This is even more important in this era where there is advocacy for sustainable agriculture, with higher emphasis on environmentally benign methods. For this reason, pest management in food production systems using biological control (especially use of parasitoid antagonists) has come to the forefront. In this review, we give an overview of the diversity of physiological responses among host insect and parasitoid populations and how this may influence their interactions. We highlight how climate change may modify bottom-up and top-down factors among agroecosystems with a particular focus on plant-insect host-parasitoid tritrophic interactions. We also outline how habitat management may influence arthropod population dynamics and how it can be manipulated to improve on-farm climate resilience and parasitoid conservation. We wrap-up by highlighting how the application of knowledge of conservation biodiversity, designing of multifunctional resilient landscapes, and evolutionary physiology of arthropods under thermal stress may be used to improve the fitness of mass-reared parasitoids (*in* or *ex situ*) for the improvement in efficacy of parasitoids ecosystem services under thermally stressful environments

Keywords: arthropod assemblages, coevolution, ecosystem responses, ecosystem services, environmental change, habitat loss, tritrophic interactions

INTRODUCTION

The spatiotemporal modification of global biophysical landscapes due to climate change exerts novel challenges to various levels of biological organization. For both managed and natural ecosystems, changes in organismal phenology, and distribution due to altered mean and temperature variability, and precipitation patterns have been widely investigated in recent years (Parmesan, 2006; Lee et al., 2009; Calosi et al., 2010; IPCC, 2014). This is in addition to studies investigating changes in feeding and oviposition preferences of both herbivores (Chidawanyika et al., 2014; Mbande et al., 2019a,b) and natural enemies (Dong et al., 2018) as microcosms of various ecosystems undergoing environmental change. Another important consequence of changing climates is how it influences trophic cascades among food webs with sensitivity varying among different trophic groups (Voigt et al., 2003; Rosenblatt and Schmitz, 2016). Decoupling multitrophic interactions under environmental change is daunting due to the high complexities characteristic of mega biodiverse ecosystems, high resource demands and outright uncertainty in the feasibility of undertaking such studies (Schuldt et al., 2017). Hence, much focus has been placed on feeding preferences, organismal physiology and biogeography (Lee et al., 2009; Calosi et al., 2010; Burrows et al., 2014), and phenological synchronization (Singer and Parmesan, 2010). Significant strides have also been made in investigating thermal energetics underlying consumer-resource trophic interactions where an assumption is made that thermal variability alters resource abundance (Rosenblatt and Schmitz, 2016). The contribution of all these various study approaches to present day understanding of global change ecology is enormous. However, there have been increasing calls for integration of investigative approaches to increase predictive power among higher levels of biological organization under environmental change (Rosenblatt and Schmitz, 2016).

Focal to these integrative approaches is the investigation of how biomass patterns in different food webs (biomass pyramids) will respond to climate drivers (Leroux and Loreau, 2015). Two competing hypotheses have been brought forward to explain the potential outcomes. First is the bottom-up approach or resource-based hypothesis which suggests that resources such as nutrients and primary producers will be key in shaping the biomass pyramids up to higher trophic levels (Leroux and Loreau, 2015). This explanation also accounts for climate factors such as rainfall and solar radiation and their subsequent influence on energy flow among trophic levels. Furthermore, due to the alterations of plant defensive capacity by these climate factors, this standpoint also accounts for changes in trophic dynamics due to alterations in herbivory because of either enhanced or compromised plant defense (Raffa et al., 2013) or poor nutritional value (Leroux and Loreau, 2015). On the other hand, a consumer-based hypothesis has also been brought forward, which posits that the structure of such biomass pyramids will rather be determined by consumers at higher trophic levels (Madrigal et al., 2011; Leroux and Schmitz, 2015). In nature, more so under dynamic systems undergoing environmental change, such bottom-up and top-down factors are likely to interact and also vary along climate/environmental gradients. Nonetheless, climate change will directly influence both bottom-up and top-down factors to varying degrees among different ecosystems and across different trophic levels.

Here we present a synthesis of the impacts of climate change on both bottom-up and top-down factors with a particular focus on herbivorous insect pest host-parasitoid population dynamics and their efficacy in agroecosystems. Current literature is replete with several studies documenting similar effects on herbivorous insects, but little is reported on their antagonists e.g., parasitoids (Vidal and Murphy, 2018). First, we begin by describing how climate change may influence host-parasitoid phenology and subsequent interactions. Second, we address how temperature extremes can act as top-down factors leading to changes in host-parasitoid interactions. Third, we describe the implications of the parasitoid thermal responses to biological control of pests in agriculture. Fourth, due to the persistent exposure to ever transforming environments under climate change, arthropods, like many other organisms, are bound to respond through both transient plastic and long term evolutionary mechanisms (Chidawanyika and Terblanche, 2011; Sih et al., 2011), albeit to varying degrees thereby creating "winners" and "losers" under selection pressure from various climate stressors (Oostra et al., 2018). We therefore explore the potential role of evolutionary adaptive responses to mitigate impacts of climate change on parasitoid populations and provision of their ecological services. Lastly, since agroecosystems typify some of the most highly disturbed ecosystems, almost always succeeded by habitat and biodiversity loss, we outline how such disturbances may also influence both bottom-up and top-down factors for parasitoids. We also discuss how landscape management may be used to mitigate the impact of climate change to ensure stable agroecosystems.

TEMPERATURE EFFECTS ON PARASITOID PHYSIOLOGY AND IMPLICATIONS FOR THE EFFICACY OF BIOCONTROL

Thermal effects on insect performance traits within certain temperature tolerance ranges can be summarized using a thermal performance curve (TPC) (Angilletta, 2009; Furlong and Zalucki, 2017). TPCs tend to take a general generic shape, with performance typically increasing proportionally with temperature, reaching maximum at optimum temperature (Topt), beyond which any increase in temperature causes performance decline. In consequence, TPCs exhibit the effects of temperature on organismal fitness (Schulte et al., 2011). This often varies across taxa, ontogeny, metrics tested and with magnitude of climate variability (Deutsch et al., 2008; Kingsolver et al., 2013; Thompson et al., 2013; Clavijo-Baquet et al., 2014; Vasseur et al., 2014). For interacting species, e.g., herbivorous host-parasitoid interactions, this is highly critical as differential responses in TPCs may lead to decoupled phenological cycles (e.g., Hance et al., 2007; Furlong and Zalucki, 2017; Machekano et al., 2018), with resultant loss of parasitoid essential ecosystem services. Recent studies have documented

that the estimated optimum temperatures for various parasitoids were consistently lower compared to their hosts (Furlong and Zalucki, 2017), suggesting that parasitoids maybe more vulnerable to climate warming compared to their hosts. In agro-ecosystems, this may mean an asymmetrical host-parasitoid interaction and reduced efficacy of parasitoids biological control with warming temperatures.

Parasitoids are ectotherms and thus their development, activity and survival is intimately correlated with ambient temperature (Hance et al., 2007). They can be classified as endoparasitoids and ectoparasitoids in reference to their development within or outside a host, respectively (Godfray, 1994). They are further classified as either koinobiont or idiobiont parasitoids. For koinobiont parasitoids, host development continues following parasitisation and host is only killed following completion of parasitoids development. However, idiobiont parasitoids kill their hosts immediately or shortly thereafter following parasite host entry (Hance et al., 2007). Temperature changes at this stage may differentially affect each trophic level, leading to a system decoupling (Van der Putten et al., 2004). Moreover, temperature variability is likely more significant for higher than lower trophic levels since the former depends on the latter to adapt to changing ambient temperatures. As such, parasitoids and hyperparasitoids (third and fourth trophic levels, respectively) may be the most vulnerable (Hance et al., 2007). Indeed, efficacy of biological control using parasitoids depends largely on (1) habitat location, (2) host location, (3) parasitoids' potential to effectively evade or manipulate host immune system, and (4) ability to constantly track changing host environment. All these attributes are highly temperature dependent, and thus unraveling temperature effects on parasitoids is critical for modeling pest management programmes using parasitoids (Harrington et al., 2001).

The consequences of subjection of parasitoids to temperature extremes are well-documented (Hance et al., 2007). Effects can manifest as lethal or sublethal but both contribute significantly to shaping parasitoid life history traits and efficacy of parasitisation in agricultural landscapes. Parasitoid exposure to extreme high and low temperature for example can result in mortality (Chown and Nicolson, 2004). This may be due to the irreversible damage to the cells, or in the case of extreme low temperatures, change in physical structures due to extra- or intracellular ice formation. Freezing may also be associated with disruption of metabolism and may manifest as osmotic or oxygen stress (Turnock and Fields, 2005). Depending on their cold hardiness, some parasitoids may also suffer lethal effects at temperatures above freezing points (Bale and Walters, 2001), and this mortality may decouple host-parasitoid interactions and the ecological services provided by the later.

Stressful temperature extremes can also have sub-lethal effects on parasitoids that may manifest as failed biological control efficacy. For example, low temperatures are associated with constrained degree day accumulation and longer generation times. For other species, the damage caused by exposure to stressful low temperatures has often been followed by an increase in the degree days needed to complete development (Lysyk, 2004). Moreover, low temperature extremes also change the number of larval developmental instars (see Denlinger and Lee, 1998), which may offset synchrony with host phenology and efficacy of parasitoids in biological control. Parasitoids that developed from lower temperatures generally develop bigger body size, following the temperature-size rule (see Angiletta and Dunham, 2003). Nevertheless, *Trichogramma carverae* reared at low temperatures developed smaller body size, while *Sarcophaga bullata* prematurely pupated at low temperatures. Such anomalies represent negative fitness consequences for parasitoids as biocontrol agents and suggests that parasitoids reared at low temperature may allocate resources to metabolism (for the maintenance of temperature), at the expense of body size (Rundle et al., 2004).

For parasitoids to be effective in regulating pest numbers, they should be highly fecund, have good host searching and finding abilities and have high longevity. However, extremes of temperature may offset these attributes, with negative consequences on biological control. Exposure of parasitoids to extremes of temperature e.g., low temperature has been reported to decrease adult longevity (Pandey and Johnson, 2005; Foerster and Doetzer, 2006) and fecundity (Levie et al., 2005; Pandey and Johnson, 2005) and hence their ecological services. Moreover, temperature stress during development also interferes with sex allocation, causing an adult sex bias toward males (Denlinger and Lee, 1998). It also decreases the mobility of either sex and therefore decreases their efficacy in mate and host finding (Denlinger and Lee, 1998). Parasitoids also possess endosymbiont bacteria, necessary for their function, for example *Wolbachia* and *Buchnera* species. These endosymbiont bacteria may be negatively affected, or in worst cases killed by extreme temperatures (Ohtaka and Ishikawa, 1991; Thomas and Blanford, 2003), affecting parasitoids fitness and thus activity. It is also increasingly becoming apparent that temperature stress may increase abnormal morphological deformations in insects. Low temperature impacts directly on insect differentiating tissues, affects hormonal balance and may cause deformities (Sibly and Atkinson, 1994). Indeed, a positive correlation has been reported between duration of temperature stress, and the magnitude of birth deformities (Tezze and Botto, 2004).

Climate change has also brought increased incidence of heat waves and cold snaps that have negative consequences on parasitoids behavior and activity. Insects exposed to sub-lethal low and high temperature may enter cold and heat stupor, respectively. During this period, activity, which may be anything from flying, mating, feeding, or host finding is decreased (Boivin et al., 2006). Moreover, these extreme temperatures also interfere with habitat, host finding, and evaluation (Herard et al., 1988). The failure to locate a host and parasitise it may be result from (1) failed parasitoids recruitment by host plant secondary metabolites, or (2) directly through temperature effects on the natural enemy. Most plants produce synomones in response to herbivore attack (Micha et al., 2000), which in turn attracts natural enemies of the herbivores. Increased temperatures associated with climate change have been reported to negatively affect the synomone blends, and thus failed parasitoid recruitment. Furthermore, most parasitoids optimally perceive synomones at narrow temperature ranges, e.g., *Cotesia*

plutellae responds optimally between 30 – 35°C (Reddy et al., 2002). Thus, temperature extremes, and increased variability may limit parasitoids' potential to perceive habitat and host cues and consequently offset their ecological services. Similarly, it has also been shown in many studies that for insects, the cost of living is extremely high at stressful high temperatures (>35°C). For example, the efficiency of the mitrochondria in converting carbohydrate substrates into energy has been shown to drop significantly at stressful high temperatures in *Manduca sexta* (Martinez et al., 2017). This reduction in mitochondrial capacity is linked with reduction in juvenile stages e.g., larval growth rates, whereby in the case of parasitoids, this may affect their phenology, abundance, and efficacy of host parasitisation.

EVOLUTIONARY IMPACT AND DECOUPLING OF PARASITOID-HOST THERMAL PREFERENCE

Prediction of parasitoid-host responses to climate change is highly complex (Harrington et al., 1999; Thomas and Blanford, 2003), but association between the two, and any probable climate induced deviations may be unraveled by comparing thermal windows between the two systems (Brooks and Hoberg, 2007; Agosta et al., 2018). Generally, if parasitoids and hosts exhibit similar thermal tolerance, then, temperature variability associated with climate change may not decouple the long evolved relationships and hence efficacy of parasitoids ecological services. However, if parasitoids and hosts differ in their thermal preference, this may mean decoupled long co-evolved relationships with climate change and impacts on parasitoids-host population phenologies and abundance (Hance et al., 2007; Machekano et al., 2018; Mutamiswa et al., 2018). Furthermore, thermal preference is also highly subtle and varies with species, age and ontogeny (Bowler and Terblanche, 2008), thus adding complexity into predicting the effects of climate change on parasitoids-host population dynamics. Hance et al. (2007) documented the negative impacts of temperature differential effects on parasitoids and their herbivorous hosts. If TPCs do not directly superimpose, this may imply a negative effect on parasitism in the face of thermal variability. What worsens the situation is that parasitoids are generally reported to have lower temperature tolerance relative to their hosts, as such, they may likely be more affected critically by changes in their ambient environment (Karban, 1998). Moreover, for parasitoids to be efficient in host parasitisation, they should overcome, or take control of their host immune system. However, higher temperature and variability have been reported to improve host immune resistance, while the capacity of the host to overcome parasitism also increases at higher temperatures (Thomas and Blanford, 2003). For example, host *Spodoptera litolaris* has been reported to be more resistant to its parasitoids *Microplitis rufiventris* at higher temperatures (reviewed in Hance et al., 2007). This means that temperature increases associated with climate change decrease probability of parasitoid immatures to survive in herbivorous hosts and thus decreases efficacy of parasitoids.

CLIMATE CHANGE IMPACTS ON BIOGEOGRAPHY AND PARASITOID-HOST INTERACTIONS

As climate is key in defining the geographic range of insects, another important consequence of climate change is the change in their distribution (Parmesan, 1996, 2007). There is ample evidence of some insect taxa shifting their range to higher altitudes and latitudes, in response to particularly warming, followed by diminishing abundance in the unsuitable areas (Parmesan and Yohe, 2003; Parmesan, 2006). Such changes in distribution patterns have been widely reported in Lepidoptera (Parmesan et al., 1999; Battisti et al., 2005, 2006; Wilson et al., 2005, 2007; Franco et al., 2006). Other examples include the northward spread of the mountain pine beetle *Dendroctonus ponderosae* (Coleoptera: Curculionidae) (Weed et al., 2015; Burke et al., 2017) and *Dendroctronus frontalis* (Coleoptera: Scolytidae) (Ungerer et al., 1999), all in response to winter warming. Such evidence in parasitoids is scant. However, Delava et al. (2014) reported a northward range shift in parasitoids. Hence, in all likelihood, most parasitoid may have such climate-dependent shifts in geographic range depending on their physiology and dispersal propensity. For example, Bale et al. (2002) argued that, under climate warming, non-diapausing insects with rapid development are more likely to expand their geographic range compared to the diapausing and slow developing ones that require low temperatures for diapause induction. Other factors that may mediate the range shifts include the availability of resources, photoperiods, predation by natural enemies, and intra- and inter-specific competition (Walther et al., 2002; Gutierrez et al., 2010).

Whatever the mode and cause of changes in distribution patterns, and indeed for interacting food webs, populations ought to adjust to biogeographic shifts through a suite of mechanisms including demographic patterns, physiological and phenotypic plastic adjustments as well as natural selection (Webster et al., 2016) with consequences on parasitism (Feldman et al., 2017). Two hypothetical scenarios may occur among agroecosystems. First, the reduction in parasitoid diversity due to the migration of species to more suitable habitats may result in increased pest pressure in the cases where pests do not share similar range expansion patterns with their parasitoids. Similarly, such mismatches may lead to reduced population growth and ultimately extinction in the case of specialist parasitoids due to limited hosts. Second, changes in distribution patterns may be beneficial to agroecosystems where the introduction of new parasitoid species may increase parasitisation of pests. Migration of pests from agroecosystems with unsuitable climates may also lead to increase in yield due to reduced pressure. However, this is highly unlikely as some dormant species may become more prevalent due to reduced competition. Hence, the consequences of climate-induced biogeography among parasitoids and pests are multi-faceted and may have differential impacts on crop productivity and biodiversity conservation.

CLIMATE CHANGE EFFECTS ON HOST-PARASITOID PHENOLOGICAL RESPONSES

The modification of trophic interactions at both a spatial and temporal scale is another major consequence of climate change. Due to their higher position in the food web, the fate of parasitoids under changing climates is also very much dependent on the bottom-up factors in the form of responses of the organisms at the lower trophic levels (Jeffs and Lewis, 2013; Rosenblatt and Schmitz, 2016). Several studies have reported how abiotic stressors such as warming, elevated CO_2 and drought can mediate the interaction between parasitoids and their hosts (Buchori et al., 2008; Walther, 2010; Evans et al., 2013; Jeffs and Lewis, 2013). For example, climate change can lead to phenological asynchrony between parasitoids and their hosts in cases where the phenology of the interacting species respond differently to a climate-related cue or where one of the species does not rely on a climate-related cue (Walther, 2010; Jeffs and Lewis, 2013).

Climate warming has been associated with rapid rates of development and multivoltinism (i.e., the completion of several ($\geq$3 generations) per year). Indeed, evidence of multivoltinism has been reported in several agricultural insect pests of economic importance including the maize stemborer *Chilo partellus* (Mwalusepo et al., 2015), bark beetle *Ips typographus* (Jönsson et al., 2009), and mealybugs *Phenacoccus solenopsis* (Fand et al., 2014). For interacting hosts and parasitoids, temporal phenological asynchrony may also occur if one of the interacting species has an obligate seasonal diapause or rapidly develops in response to warming (Forrest, 2016). Such asynchronies can lead to the escape from parasitism pressure by insect pests in the cases where climate change leads to earlier development among host insect pest populations. Theoretically, temporal synchronies stabilize the host parasitoid interactions as complete synchrony may lead to depletion of host populations with subsequent extinction of the parasitoids (Godfray et al., 1994). Thus, even though the initial parasitism pressure is a classical top-down factor, the consequent extinction of the hosts exerts bottom-up effects that lead to extinction of parasitoid populations especially in the case of specialists (Jeffs and Lewis, 2013). Despite scant empirical evidence, some studies focusing on these interactions have reported such asynchronies following even minute climate variability. This is the case with the emerald ash borer *Agrilus planipennis* and its parasitoid *Oobius agrili* where small changes in severity and extreme climate events phenologically excluded emerging parasitoids from host eggs (Wetherington et al., 2017). In the Glanville fritillary butterfly *Melitaea cinxia* larvae, behavioral plasticity such as movement for basking in warm sunny spots enables temporal relief from parasitoids through rapid development to the insusceptible instar stages during spring. This will cost its parasitoid *Cotesia melitaearum*, which will be immobile during that season. However, such rapid development in insects may lead to small body size at maturity and reduced fecundity (Kingsolver and Huey, 2008) thereby impeding the positive demographic effects of shorter generations (Forrest, 2016). On the other hand, such behavioral plasticity in the warm season may not be beneficial to the hosts as there may be more synchronization with the parasitoids (Van Nouhuys and Lei, 2004). In this case, climate warming will, in all likelihood, result in increased parasitisation of *M. cinxia*. Interestingly, warming can also result in mismatches due to parasitoid advanced development relative to the host as is the case with cereal leaf beetles *Oulema melanopus* and its parasitoid *Tetrastichus julis* where warmer years result in phenological asynchrony and reduced parasitism (Evans et al., 2013). Hence, predicting the consequences of parasitoid-host relationships is complex partly due to non-climatic factors that may act as cues for phenological change and the potential disproportionate adaptive evolutionary responses that may occur among interacting species.

IMPACT OF PLANT NUTRITIONAL QUALITY AND FOOD WEB DYNAMICS

Apart from plant diversity and abundance, nutritional quality is also highly responsive to climate variability with cascading effects among higher trophic groups. Greenhouse-based studies have shown how elevated CO_2 and temperature are closely linked with a simultaneous increase in foliar non-structural carbohydrates and a decline in protein concentration among various plant functional groups (Rothman et al., 2015). This is also in addition to changes in plant chemistry owing to alterations in biogeochemical cycles due to land-use change. Other studies of tropical trees along a rainfall and temperature gradient attributed the decrease in foliar nitrogen content and nitrogen-to-fiber ratios to increased precipitation and temperature (Schuur and Matson, 2001; Weih and Karlsson, 2001; Santiago et al., 2004; Craine et al., 2010). Whilst the impact of nutritional variability on insect herbivores is widely documented (e.g., Mody et al., 2009; Gutbrodt et al., 2012; Mbande et al., 2019a,b), information of its impact on parasitoids remains scant (Safraz et al., 2009). Much of the current knowledge on the impact of nutritional gradients on food webs has been generated from phytoplankton-based model systems. Even though stark contrasts exist between the ecology of terrestrial and phytoplankton systems, what is apparent from these studies is that the ecological efficiency and energy transfer to higher trophic levels depends on food quality. For herbivores, stoichiometric constraints exist through the proportion of carbon and nutrients relative to respiratory demands, in addition to assimilation efficiency with potential carryover effects to carnivores (Dickman et al., 2008).

Prey (herbivore) diversity is another aspect that has been previously linked with plant nutritional quality (Marzetz et al., 2017). In the study, Martinez et al. (2017) postulated three hypothetical scenarios of herbivore response to nutritional quality. First, the growth of the herbivore populations is promoted in diverse communities by co-occurrence of species with complementary nutritional traits. Hence, a positive correlation between herbivore performance and food diversity would exist. Second, a single or a few species may possess superior nutritional attributes that enable herbivore growth which in turn

may transform the relationship between producer diversity and consumer growth. In such a scenario, a positive correlation between consumer performance and food diversity would be promoted in a more diverse community due to increased likelihood of having the high quality species. Third, high diversity may also mask the relative contribution of the high quality species if they are not competitive. The net interactive effect of the above processes could result in both null and negative correlations (Marzetz et al., 2017). How such plankton-based herbivore responses are transferrable to terrestrial systems and higher trophic groups is debatable. However, Nitschke et al. (2017) reported contrasting responses in the abundance of parasitoids and herbivores in response to plant diversity. Assemblage of the herbivorous *Chaetorellia jaceae* decreased with increasing plant species and functional diversity whilst parasitism of the chalcid wasps *Eurytoma compressa* and *Pteromalus albipennis* increased with increased plant functional diversity. In another study, Safraz et al. (2009) reported improved performance in several fitness correlates in koinobiont parasitoids in response to increased nitrogen, phosphorous and potassium among host plants. These examples demonstrate how nutritional quality mediates the performance of parasitoids through both herbivore population abundance or nutritional value of the host as mediated by the host plants. Therefore, a plant's nutritive status may not only affect its suitability for herbivorous insects, but fitness parameters of organisms at higher trophic levels such as parasitoids (Olson et al., 2009; Chen et al., 2010; Han, 2014).

Overall, density dependent interactions in response to nutritional gradients caused by global change will play a central role in food webs. Factors that reduce parasitism efficiency weaken the top-down forces (Power, 1992). For example, intraguild predation among parasitoids and hyperparasitism, which may increase when herbivore populations are low, may result in reduced parasitoid abundance and diversity (Rosenheim et al., 1993) thereby counteracting conservation efforts (Snyder and Wise, 2001; Symondson et al., 2002). Host plant quality may also mediate the interaction between parasitoids and hosts through its influence on herbivore body size. For example Chen et al. (2010) reported that development time of immature parasitoids is positively related to host sizes due to the close link between host body size and nutritive value leading a compromise of size dependent individual and population level parameters, as earlier stated (Thompson, 1999). This further highlights the critical role of plant nutritive value on parasitoid population dynamics, demographics and efficacy of their ecological services (Han, 2014). Hence, climate variability induced nutritional gradients may, in all likelihood, affect both the herbivorous hosts and their antagonists e.g., parasitoids through both bottom-up and top-down effects.

LANDSCAPE MANAGEMENT AND PARASITOID RESPONSES TO HABITAT COMPLEXITY AND CONNECTIVITY

Agricultural intensification is typically characterized by a high rate of disturbances resulting in fragmented habitats, significant biodiversity loss and poor ecosystem function due to modification of bottom-up and top-down processes (Crowder and Jabbour, 2014). Perhaps the most direct impact of disturbances is the loss of habitats, which leads to a reduction in their population carrying capacity of various species thereby limiting the provision of ecosystem services (Cronin and Reeve, 2005; Holzschuch et al., 2010; Crowder and Jabbour, 2014). The consequent existence of smaller populations on small but fewer suitable patches makes them highly vulnerable to genetic, demographic and environmental perturbations such as climate change (Baguette et al., 2013). Indeed, several studies have reported a high extinction risk among small parasitoid populations occupying small patches (Bennett and Gratton, 2012). Parasitism and inbreeding depression are some of the demographic factors that limit population persistence, the extent, effect, and manifestation/expression of which are magnified in small populations on small fragmented patches (Coudrain et al., 2014; Start and Gilbert, 2016). Apart from this, adaptive evolutionary responses to environmental stressors in such smaller populations are known to be highly limited (Bay et al., 2017). Hence, climate change will further increase the pressure exerted by demographic parameters leading to potential extinction. Moreover, climate warming among interacting trophic levels has already been reported as a catalyst for extinction in species at higher trophic levels like parasitoids (Jones, 2008; Northfield and Ives, 2013; Mellard et al., 2015) with population sizes mediating the evolutionary dynamics (Oostra et al., 2018).

Habitat complexity is widely reported as a conduit for parasitoid assemblages together with other pest natural enemies (Langellotto and Denno, 2004; Buchori et al., 2008; Holzschuch et al., 2010; Pierre and Kovalenko, 2014) thereby maximizing the provision of their ecosystem services (Fiedler et al., 2008). Indeed, emperical evidence has shown how landscape complexity can aid conservation biological control through improved provision of resources to pest natural enemies (Jonsson et al., 2012). This is because, for many species, highly complex habitats give more resources which form broad niches that reduce niche-overlap thereby promoting species coexistence (Smith et al., 2014). However, such diversity has occasionally been cited as a disadvantage for parasitoids. For example, some studies have reported a decrease in their foraging efficiency under complex habitats (Gols et al., 2005; Kruidhof et al., 2015), even though this may be ameliorated by their high associative learning capacity of the emitted herbivore induced plant volatiles (HIPVs) (Meiners et al., 2003; Kruidhof et al., 2015). Such capacity for associative learning is of high ecological importance and contributes immensely to the evolutionary fitness of parasitoids in cases where conditions allow for rapid learning (Dukas and Duan, 2000). Kruidhof et al. (2015) attributed a 28% increase in foraging efficiency in *Cotesia glomerata* (Hymenoptera: Braconidae) to associative learning under controlled outdoor experiments. Furthermore, another study reported differential responses to polycultures (regarded here as complex habitats) in the foraging capacity of generalists and specialist parasitoids. Naïve generalist parasitoids had poor foraging efficiency under complex habitats compared to specialists (Perfecto and Vet, 2003). However,

this poor performance among generalists was nullified when they had an opportunity for associative learning (Perfecto and Vet, 2003), thus underlying the ecological importance of such behavioral plasticity. Therefore, poor capacity for associative learning of odors may result in fitness costs including longer foraging durations and increased exposure to predation (Dukas and Duan, 2000). It is however likely that where odor cues may not be sufficient during associative learning, other cues such as visual may be employed (e.g., Desouhant et al., 2010). Likewise, push-pull strategies, a stimulo-deterrent cropping tactic consisting of intercropping cereals with legumes and surrounded by grasses, can also be incorporated for repulsion and attraction of stem borer pests and parasitoids, respectively (Cook et al., 2007; Kebede et al., 2018).

Another important challenge posed by disturbances and climate change in agricultural landscapes is poor habitat connectivity, at both spatial and temporal scales. Indeed, climate stressors among fragmented landscapes exacerbate the pressure on biodiversity due to the limitations they exert on metapopulation and biogeographical responses (Opdam and Wascher, 2004). Ecological landscape processes such as herbivory, dispersal, and gene flow are highly dependent on the connectivity of habitats with both geographic isolation and seasonal quality or availability of resources all being important for species persistence (Cronin and Reeve, 2005; Baguette et al., 2013; Smith et al., 2014; Maguire et al., 2015). For parasitoids, the functional connectivity of natural and semi-natural habitats with cropping systems ensures a continuum of suitable habitats where acquisition of critical resources such as nectar, pollen and sap is made possible with short-term improvement in crop yield through increased parasitism of pests (Gurr et al., 2003; Wilkinson and Landis, 2005; Cook et al., 2007). Such connectivity minimizes foraging time thereby reducing risk of predation (Weisser et al., 1994) or environmental stress, which becomes more frequent under changing climates (Mutamiswa et al., 2018).

By promoting the conservation and activity of natural enemies of insect pests, habitat connectivity is availed thereby contributing positively to agricultural landscapes (Jonsson et al., 2014). This includes the assemblage of outbreak herbivorous insect species, which contribute toward ecosystem services such as nutrient cycling, soil formation, and carbon sequestration (Isaacs et al., 2009; Maguire et al., 2015). However, it can also be detrimental in cases where connectivity aids the spread of crop pests and diseases (Margosian et al., 2009; Maguire et al., 2015). For example, increased connectivity is beneficial for the establishment and spread of the mountain pine beetle *Dendroctonous ponderosae* (Coleoptera: Curculionidae), an insect pest of the boreal forests of North America (Maguire et al., 2015). This connectivity has been reported as interactive with climate warming resulting in major pest outbreaks (Aukema et al., 2008; Raffa et al., 2008; Safranyik et al., 2010; James et al., 2011; Bone et al., 2013). Conversely, connectivity in other parts of that region results in the suppression of the forest tent caterpillar *Malacosoma disstria* (Lepidoptera: Lasiocampidae) due to increased predation pressure by parasitoids (Cooke and Roland, 2000; Maguire et al., 2015). Factors such as habitat fragmentation were cited as key for disrupting parasitoid assemblages in such cases (Cooke and Roland, 2000). Apart from affecting intra-population dynamics like abundance, low connectivity resulted in poor diversity of tachinid parasitoids in 18 different grasslands in agricultural landscapes (Inclán et al., 2014). This underlies the profound role that habitat complexity and connectivity plays in the community assembly of parasitoids. Other arguments against maintaining connectivity in agricultural landscapes is their potential for providing pathways for dispersal of invasive species and noxious weeds (Pringle, 2003). However, this is highly debatable considering invasive plants can be more successful in highly disturbed areas where succession easily occurs in the absence of competition from native plants (Theoharides and Dukes, 2007).

From the foregoing, it is apparent and widely documented that habitat complexity and connectivity provide both ecosystem services and "disservices" in agricultural landscapes (Zhang et al., 2007). These dissensions however need to be evaluated in a landscape context taking into consideration the ecological attributes and the desired ecosystem services (Maguire et al., 2015; Landis, 2017). Several conceptual frameworks for incorporating the provision of various ecosystem services in landscape planning and design of agroecosystems have been posed (e.g., Buchori et al., 2008; Maguire et al., 2015). Landis (2017) points out the need to merge fundamental and applied ecological principals with agroecosystem concepts. These calls are not new and have resulted in the birth of what is now described as "agroecology" with emphasis on biodiversity conservation and sustainable agricultural production systems (Jonsson et al., 2014; Altieri et al., 2015; Gliessman, 2017). When meticulously planned, incorporation of agroecology principles that maintain plant diversity and connectivity will also ensure the resilience of agroecosystems under changing climates through buffering of biodiversity against climate shocks (Altieri et al., 2015). This is in addition to other pro-climate resilient ecosystem services such as carbon sequestration, soil formation, and moisture conservation (Altieri et al., 2015), albeit the possibility of trade-offs due to competition in water usage between crops and non-crop plants (Zhang et al., 2007). This is common for agroecosystems with high tree abundance that can reduce the replenishment of aquifers important for irrigation (Zhang et al., 2007) and also increase water loss through evapotranspiration from streams and dams within agroecosystems (Zavaleta, 2000). Hence, landscape planning and design based on an in depth understanding of the ecological processes at both on-farm and area-wide level is required to enhance ecosystem services whilst minimizing trade-offs (Maguire et al., 2015). For parasitoids, landscape design should enhance the drivers for parasitoid assemblage and movement or dispersal at both farm and the entire landscape level (Mazzi and Dorn, 2011; Macfadyen and Muller, 2013). All these interventions can account for the metapopulation theory with improved resilience against climate change.

PROSPECTS FOR IMPROVING THE EFFICACY OF BIOCONTROL EFFICACY

It is increasingly documented that temperature fluctuations associated with climate change are shifting parasitoid-host phenologies and population dynamics (Agosta et al., 2018). As such, there is increasing interest on experiments elucidating effects of different trophic interactions (Machekano et al., 2018; Mutamiswa et al., 2018), and the most convenient indices to be employed (Agosta et al., 2018). A variety of simple matrices have been proposed, including warming tolerance (Hoffmann et al., 2013) and thermal safety margins (Kingsolver et al., 2013). Though diverse indices point to a potential asynchrony of interacting trophic levels with climate change (Hance et al., 2007), few studies have looked at the second to fourth trophic levels (but see Agosta et al., 2018).

Outside this, parasitoids may also adapt to changes in their thermal environment in order to conserve those co-evolved trophic relationships. Evolutionary physiology may potentially be used to enhance the efficacy of biological control in the face of climate change (Sgrò et al., 2010; Hoffmann and Sgrò, 2011). Hence, apart from improvement of *in situ* genetic diversity or conservation of *in situ* evolutionary adaptation focusing on physiological traits may be key (Pörtner and Farrell, 2008; Chidawanyika et al., 2012). For example, during extreme temperature stress, and depending on environmental predictability, parasitoids may undergo dormancy or quiescence (Tauber et al., 1986), and this may manifest at any stage of parasitoids development. When environmental thermal variability is predictability low, and temperature stresses are short and transient, parasitoids often use behavioral adjustments to cope with stress, e.g., insects may go into chill coma (Mutamiswa et al., 2018). Another form of behavioral host manipulation by parasitoids has been reported for koinobiont parasitoids (Lagos et al., 2001). Koinobiont parasitoids often induce behavioral changes in their hosts, so they move to habitats that ensure maximum survival chances. Induction of this behavior has been reported for parasitoids *Aphidius ervi*, (Lagos et al., 2001) *A. nigripes* (Brodeur and McNeil, 1989), and *Eucelatoria bryani* (Reitz and Nettles, 1994). Such behavioral adaptations form the first line of stress defense because they are energetically less costly, and are adaptive in the face of changing environments.

When faced with freezing low temperatures, parasitoids have also evolved freeze tolerance as a survival strategy (Vernon and Vannier, 2002). While freeze tolerance is rare in parasitoids, it means they will be vulnerable to freezing temperatures if their host bodily fluids freeze, for freeze tolerant hosts. However, a few parasitoid genera are reported to be freeze tolerant e.g., endoparasitoids *Ichneutes* (Braconidae) and *Syndipnus* (Ichneumonidae). These have evolved freeze tolerance to survive freezing when living within the freeze tolerant host larvae under freezing Arctic conditions (Humble and Ring, 1985). For freeze intolerant parasitoids (see Vernon and Vannier, 2002), parasitoids have often evolved manipulation of their hosts to avoid freezing. Parasitoids do this through physiological manipulation of the host following parasitisation. For example, unparasitised host *Diuraphis noxia* has a supercooling point (SCP) of $\sim -25^{\circ}$C. However, physiological manipulations of this host by parasitoids *Aphelinus asychis, A. albidopus,* and *Diaeretiella rapae* (Hymenoptera: Braconidae) have been reported to depress host SCP to temperatures below -30°C (Nowierski and Fitzgerald, 2002). This is also consistent with reports on other insect taxa (Parish and Bale, 1990; see Hance and Boivin, 1993), and such physiological host manipulations by parasitoids are adaptive and may conserve ecological services in the face of changing climates. Moreover, color also plays a significant adaptive mechanism for surviving stressful temperatures in parasitoids and indeed parasitoid color morphs have been reported (Schlinger and Hall, 1960; Langer and Hance, 2000; Legrand et al., 2004), which may compensate for thermally stressful environments.

Investigating the effects of environmental heterogeneity on parasite-host interactions and predicting consequences on ecological services is complex, but very significant in biology. We conclude that basic thermal physiology comparative experiments across interaction species (e.g., Agosta et al., 2018; Machekano et al., 2018; Mutamiswa et al., 2018) may be the first step in elucidating some of the complex drivers. Nevertheless, it is generally agreed that climate change may decouple long co-evolutionary relationships across interacting species (Hance et al., 2007). Such divergence between parasitoid-host phenologies may disrupt ecological equilibrium and may lead to rapid insect pest outbreaks consequent of a climate change induced failure in biological control.

CONCLUDING REMARKS

Climate change presents new challenges and limits in agriculture. Concerted efforts will be required to ensure that the integrity of trophic interactions are maintained *in situ*. Since the factors associated with poor parasitoid assemblages and performance are largely attributed to high disturbances in agricultural landscapes, management practices should take an integral role in order to maintain resilient farming systems, with emphasis on those that incorporate evolutionary capacity in landscape organization in order to maintain parasitoids genetic heterogeneity. Whilst such disturbances vary across spatial and temporal scales, improved landscape planning at both local and area-wide levels will be key in order to improve parasitoid effectiveness. For example, development of multifunctional landscapes that promote biodiversity whilst maintaining essential ecological services must be encouraged. This landscape planning will require robust ecological indicators for both evaluation and determination of interventions. Research should therefore potentially aim at identifying parasitoids that are winners under changing climates, in particular those using adaptive evolutionary potential. These adaptive processes should be

incorporated in biocontrol strategies aimed at maintaining interacting species and their essential ecological services.

AUTHOR CONTRIBUTIONS

FC and CN conceptualized the scope of the paper. FC, PM, and CN contributed equally to writing and editing of the manuscript.

ACKNOWLEDGMENTS

We are grateful for funding and institutional support from the University of the Free State to FC, Agricultural Research Council to PM, and Botswana International University of Science and Technology to CN. We also appreciate comments from two reviewers which greatly improved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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PERSPECTIVE
published: 28 March 2019
doi: 10.3389/fevo.2019.00084

Integrating Parasitoid Olfactory Conditioning in Augmentative Biological Control: Potential Impact, Possibilities, and Challenges

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Despite the vast body of theoretical and empirical literature dealing with parasitoid learning, this knowledge has thus far rarely been exploited for manipulating the efficacy of augmentative biological pest control. This may be due to the fact that most studies on learning behavior were performed under laboratory conditions, whereas field trials remain scarce. However, the few studies that did investigate parasitoid foraging success under (semi-)field conditions show strong learning effects. Using so-called "parasitoid olfactory conditioning" (POC), parasitoids can be trained to become more efficient in the different phases involved in the process of host searching and host acceptance. POC can thus result in a "foraging efficacy gain", defined as the difference between the number of naive and conditioned parasitoids that need to be released to reach a certain parasitization level of the target-pest in the crop environment. This "gain" increases with an improved parasitoid learning ability and depends on the interplay between the parasitoid, crop, target-pest species and parasitoid rearing method. Moreover, the "foraging efficacy gain" depends on the technical implementation of POC, as this will determine the strength, duration and stability of the learning-induced behavioral change. In this perspective paper we will discuss (a) the conditions that can enhance the "foraging efficacy gain," (b) the possible approaches to implementation of POC and their costs and benefits, and (c) a stepwise approach to develop appropriate POC methods for the optimization of biological pest control.

Keywords: associative learning, foraging behavior, natural enemies, parasitoids, searching efficacy

OPEN ACCESS

Edited by:
Paul-André Calatayud, Institut de Recherche Pour le Développement (IRD), France

Reviewed by:
Sylvia Anton, Institut National de la Recherche Agronomique (INRA), France
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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *19 November 2018*
Accepted: *05 March 2019*
Published: *28 March 2019*

Citation:
Kruidhof HM, Kostenko O, Smid HM and Vet LEM (2019) Integrating Parasitoid Olfactory Conditioning in Augmentative Biological Control: Potential Impact, Possibilities, and Challenges. Front. Ecol. Evol. 7:84. doi: 10.3389/fevo.2019.00084

INTRODUCTION

Augmentative biocontrol using predatory arthropods or parasitoid wasps has become a major way to reduce insecticide use, especially in protected horticulture (Pilkington et al., 2010; van Lenteren et al., 2018). The efficacy of parasitoids to find and eliminate pest insects, however, may be constrained by an initially weak response to foraging cues emanating from the crop environment, and/or an innate tendency for dispersal upon release. Learning can greatly enhance the efficacy of parasitoids to locate hosts under field or greenhouse conditions (Gross et al., 1975; Lewis and Martin, 1990; Papaj and Vet, 1990; Hare et al., 1997; White and Andow, 2007; Wilson and Woods, 2016). Using so-called "parasitoid olfactory conditioning" (POC from here on; **Box 1**)

Box 1 | Definition of terms used in this paper.

Parasitoid olfactory conditioning (POC): defined here as training parasitoids to respond more strongly and/or specifically to odors involved in target-pest-finding or target-pest-acceptance by making use of one or more of the learning mechanisms outlined below.

Associative learning: involves the establishment, through experience, of an association between two stimuli (classical conditioning) or between a stimulus and a response (operant conditioning). Typically, the association is formed through the close temporal and spatial pairing of the stimuli or the stimulus and response (Papaj and Prokopy, 1989). In the context of this paper, target pest-derived odors serve as rewards in the associative learning of better detectable, but less reliable target-pest induced crop volatiles.

Sensitization: A form of non-associative learning, characterized by a gradual increase in response to a stimulus with (repeated) exposure to that stimulus even when it has not been paired with any other stimulus. Sensitization is often considered as the counterpart of habituation (Papaj and Prokopy, 1989).

Priming: A form of non-associative learning characterized by a general increase in responsiveness to foraging cues after a certain experience (Turlings et al., 1993). Conversely, associative learning, sensitization and habituation are characterized by a change in responsiveness to specific stimuli that the parasitoid encounters during the experience.

NB: It is important to note that the different types of learning outlined above are not necessarily independent of one another (e.g., mechanisms of sensitization can be involved in associative learning and any learning procedure is likely to involve priming).

Foraging efficacy gain: defined here as the difference between the number of naive and conditioned parasitoids that need to be released to reach a certain parasitization level of the target-pest in the crop environment.

Conditioned parasitoid: defined here as a parasitoid that has had experience with odors that are characteristic for the target-pest or for the crop in which it has been released. This experience could have been gained during its development (pre-adult learning), during eclosion from the pupal case (early-adult learning) or during the adult stage (adult learning).

Naive parasitoid: defined here as a parasitoid that during its development, during eclosion from the pupal case or during the adult stage has had no experience with odors that are characteristic for the target-pest or for the crop in which it has been released.

parasitoids can be trained to enhance their searching activity, their ability to locate target-pest infested plants and target-pest insects, as well as their acceptance of the target-pest for egg-laying. The aims of this perspective paper are to discuss (a) the conditions under which the impact of POC on biological pest control will be highest, (b) the costs and benefits of possible approaches to POC implementation, and (c) a stepwise approach for the development of POC methods for the optimization of biological pest control. For a detailed overview of current knowledge on parasitoid learning and memory we refer to the recent reviews of Hoedjes et al. (2011), Giunti et al. (2015), Smid and Vet (2016), and Nieberding et al. (2018).

THE ROLE OF LEARNING IN PARASITOID FORAGING BEHAVIOR

Parasitoids need to complete three host-searching phases, namely (a) the initiation of host-searching behavior, (b) the detection of a host-infested plant, and (c) the detection and acceptance of the host itself (Vinson, 1976). Because pests have generally evolved toward becoming as inconspicuous as possible to their natural enemies, parasitoids usually need to resort to indirect cues, such as herbivore-induced plant volatiles (HIPVs) (Vet and Dicke, 1992; McCormick et al., 2012; Wajnberg and Colazza, 2013). HIPVs, which are emitted by the plant in response to herbivore feeding, are generally better detectable than host-derived stimuli but are less reliable as predictors of host presence. Parasitoids can use associative learning (**Box 1**) to solve this so-called "reliability-detectability" dilemma, by associating the less reliable HIPVs to host presence (Vet et al., 1991). This can cause parasitoids to increase their response level toward the learned HIPVs, possibly resulting in a preference shift and temporary specialization. Moreover, associative learning of HIPVs can cause parasitoids to move in a more directed manner toward a target-pest infested plant (Vet and Groenewold, 1990; Vet and Papaj, 1992; Ishii and Shimada, 2010). Apart from "associative learning," also other learning processes, including, "sensitization," "habituation" and "priming" may play a role in the host location process (**Box 1**). Moreover, parasitoid learning may take place at different stages of parasitoid ontogenesis. Here, we distinguish between "adult learning," "early adult learning" and "pre-adult learning" (see the definition of "conditioned parasitoid" in **Box 1**).

EFFECT OF POC ON PARASITOID HOST SEARCHING EFFICACY IN THE FIELD

Despite overwhelming laboratory-based evidence that parasitoids can learn, few attempts have been made to demonstrate the benefit of learning for optimizing host searching efficacy under (semi)field conditions (but see Gross et al., 1975; Lewis and Martin, 1990; Papaj and Vet, 1990; Hare et al., 1997; White and Andow, 2007; Wilson and Woods, 2016; Kruidhof et al., unpublished results). Papaj and Vet (1990) showed that *Leptopilina heterotoma* females that had experienced the host microhabitat odor in the presence of *Drosophila* host larvae had a 3–4 times higher chance of finding a host microhabitat than inexperienced females. Moreover, Kruidhof et al. (unpublished results) found *Cotesia glomerata* females released in a semi-natural environment one day after associative POC to have a twice as high host finding rate compared to inexperienced females. Other studies have found that the early adult exposure or pre-release exposure of parasitoid females to host-related stimuli (such as feces, scales, or synthetic kairomones) in the absence of the host microhabitat odor can also enhance host finding and parasitization efficacy under semi-field conditions (Gross et al., 1975; Lewis and Martin, 1990; Hare and Morgan, 1997). The results of Gross et al. (1975) suggest that parasitoids with a strong innate tendency to disperse away from the crop environment without initiating host-searching behavior may be more affected by conditioning compared to parasitoids that disperse at short distances to search for hosts.

THE POTENTIAL IMPACT OF PARASITOID CONDITIONING APPROACHES ON PARASITOID FORAGING EFFICACY

To facilitate thinking about the conditions under which parasitoid training through POC may provide a benefit for biological pest control, we first introduce the term "foraging efficacy gain," defined as the difference between the number of naive and conditioned parasitoids that need to be released to reach a certain parasitization level of the target-pest in the crop environment. This "gain" increases with an improved parasitoid learning ability and depends on the interplay between the parasitoid, crop, target-pest species, and parasitoid rearing method. This can be depicted as a tetrahedron, with each side of this tetrahedron corresponding to a different stage in the host searching process (**Figure 1A**).

The initiation of host searching behavior depends on the interplay between the parasitoid, the crop and the parasitoid rearing method (**Figure 1C1**). When the parasitoid has a high innate tendency for dispersal upon release, and/or a low innate preference for the crop odor, POC is expected to enhance the initiation of host searching behavior most (**Figure 1B1**). Integrating the crop odor into the rearing host diet or impregnating the parasitoid pupal case with the crop odor (**Figure 1E1**) may improve parasitoid retention in the crop and their motivation to search for hosts upon release through pre-adult or early-adult associative learning or sensitization. Moreover, pre-release exposure of adult parasitoids to target-pest (derived stimuli), either in the presence (**Figure 1E2**) or absence (**Figure 1E3**) of (target-pest infested) crop volatiles may also enhance the initiation of host searching behavior through priming.

Location of target-pest infested crop plants within the crop environment depends on the interplay between the parasitoid, the target-pest and the crop (**Figure 1C2**), and can be enhanced by adult pre-release exposure to target-pest (derived stimuli) in the presence of target-pest infested crop volatiles (**Figure 1E2**). Only when the target-pest induces a change in the volatile blend emitted by the crop plant that is detectable by the parasitoid, the parasitoid can use HIPVs to locate a target-pest infested plant. Moreover, the "foraging efficacy gain" increases when naive parasitoids do not have an innate preference for the target-pest induced crop odor over the non-induced crop odor. Finally, the chance of locating a host-infested plant through directed search resulting from associative learning will increase compared to random search when pest densities become lower (**Figure 1B2**).

Lastly, the location of the target-pest itself after the arrival of the parasitoid on the target-pest infested plant, as well as the acceptance of the target-pest for egg-laying, will depend on the interplay between the parasitoid species, the parasitoid rearing method and the target-pest species (**Figure 1B3**). When the target-pest represents a non-preferred host (**Figure 1B3**), pre-release exposure of adult parasitoids to the target-pest (or target-pest derived stimuli), either or not in the presence of (target-pest infested) crop volatiles, may increase target-pest location and acceptance through sensitization (**Figures 1E2,3**). Changing the host used in the rearing method for the non-preferred target-host may also enhance target-pest location and acceptance through early-adult sensitization (**Figure 1E3**).

Thus, the host location phase that contributes most to the foraging target-pest gain should co-determine the POC approach. Moreover, the POC approach as well as its technical implementation will determine the strength, duration and stability of the learning-induced behavioral change, which is directly related to the memory type that is formed. Memory formation following a learning event can be divided into different types or stages (Hoedjes et al., 2011). So-called short-term memory (STM) and mid-term memory (MTM) serve to store information temporally. Depending on the subsequent occurrence of conflicting or confirming information these memory forms usually wane within minutes to hours (STM), or hours to days (MTM). Long-term memory (LTM) is the most durable and resistant to extinction. Especially when parasitoids are released into a crop environment with low target-pest density, memory durability, and resistance to extinction in the face of unrewarding experiences may be very important for successful location of a target-pest infested plant. Finally, the four main factors (parasitoid, crop, target-pest species, and parasitoid rearing method) may all be influenced by multiple other factors but this goes beyond the scope of this perspective paper.

THE TECHNICAL IMPLEMENTATION OF POC – COSTS AND BENEFITS

In addition to the potential impact of POC on parasitoid foraging efficacy, the costs and benefits of POC should be weighed when deciding on the POC approach and its technical implementation. There may be direct costs (labor, ingredients and spatial requirements) and indirect costs (potential risks) involved in the POC method. These potential risks are context-dependent. For example, when POC takes place at the release site, using living pest insects as a reward in the conditioning procedure can constitute a contamination risk. When POC takes place at the manufacturer's site, economic risks may arise from an increased product diversification in relation to difficult-to-predict market demands. For parasitoid species that are expensive to rear, the benefits of POC will more quickly outweigh its costs. Furthermore, POC approaches can be applicable at all scales of augmentative biological control (greenhouses, small, and large fields). Below we discuss the different approaches to POC in relation to their technical implementation, potential impact, and direct and indirect costs along the lines of "*what*," "*how*," "*where*" and "*when*."

With "*what*" we mean the characteristics of the parasitoids that will undergo POC. The learning rate of the parasitoid, i.e., the number of experiences that is needed to form LTM, is an important determinant of a successful implementation of POC. Depending on the natural circumstances in which parasitoid learning has evolved, a higher or a lower learning rate may be most adaptive (Stephens, 1993; Dukas and Duan, 2000; Dukas, 2008; Hoedjes et al., 2011). This has resulted in, often strong,

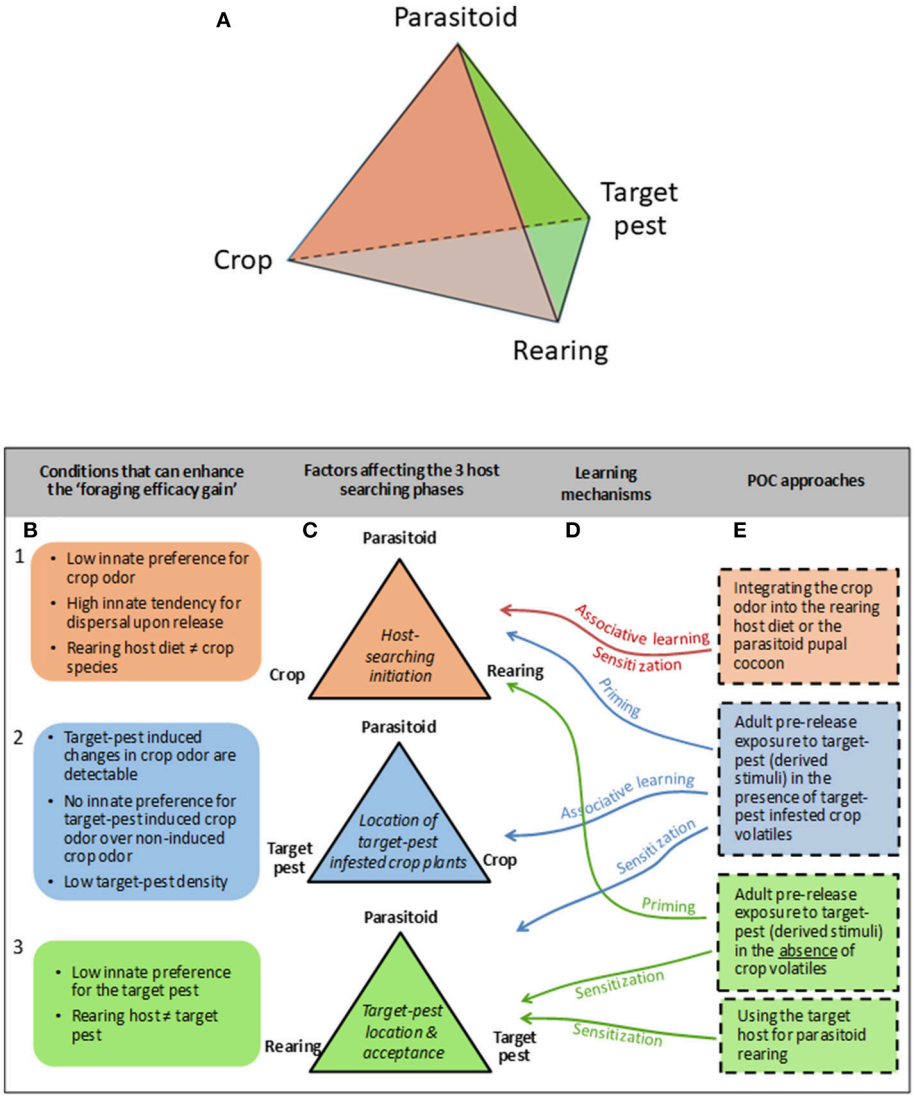

FIGURE 1 | Tetrahedron depicturing the interplay between the four main factors affecting parasitoid efficacy for target-pest control **(A)**. Each side of this tetrahedron corresponds to a different host-searching phase depicted by different colors **(C)**. In particular, the "host-searching initiation" phase is marked by the orange color; the "location of target pest infested crop plants" phase is marked by the blue color that is at the back of the tetrahedron and is not visible on the **(A)**; and the "target-pest location and acceptance" phase is marked in green. Each host-searching phase can be associated with specific conditions that can enhance the "foraging efficacy gain" **(B)**, and can potentially be affected by different parasitoid olfactory conditioning (POC) approaches **(E)** mediated by associative and/or non-associative learning mechanisms **(D)**.

between-species (e.g. Smid et al., 2007) as well as within-species (van den Berg et al., 2011; Koppik et al., 2015) variation in learning rate. Selection for a higher learning rate within a single parasitoid species is possible (van den Berg et al., 2011; Liefting et al., 2018) and its feasibility for improving the efficacy of POC should be further explored. Apart from genetic characteristics, the parasitoid developmental stage, age and physiological state can also affect learning rate. Parasitoid developmental stage will dictate the possible approaches to POC (**Figure 1E**). The relationship between parasitoid adult age and learning ability may depend on the learning mechanism involved. For example, *Cotesia congregata* females are only receptive to sensitization for plant odors for a few hours after emergence (Kester and Barbosa, 1991), whereas Honda and Kainoh (1998) found the associative learning ability of female *Ascogaster reticulatus* to be much lower during the first day after emergence when these parasitoids are not yet able to oviposit. Feeding status can also impact parasitoid responses to conditioned odors indicative of host-presence (Takasu and Lewis, 1993), but less is known about the influence of parasitoid satiation level during POC on subsequent memory formation for odors indicative of host presence and/or—identity (Siekmann et al., 2004; Tertuliano et al., 2004).

With "*how*" we mean the technical implementation of POC, covering aspects such as the conditioning approach, the duration

and number of conditioning trials, the number of parasitoids that are conditioned in unison, as well as the characteristics of the rewarding stimulus and the conditioned stimulus. The amount of labor involved is tightly linked to the number of parasitoids that can be conditioned simultaneously, as well as to the complexity of the conditioning procedure, which in turn determines the number and time duration of the manual operations as well as the possibilities for automation.

Of all the POCapproaches outlined in **Figure 1E**, parasitoid pre-release exposure to the target-pest (derived stimuli) in the presence of target-pest infested crop volatiles is expected to have the highest impact on parasitoid foraging efficacy and target-pest control, because it may enhance all phases of the host searching process, and because it may induce LTM. At the same time, it may also be the most difficult to implement POC method. Especially when implemented at the grower's site, it will often be preferable to avoid the use of living target-pests. POC will then require both an adequate alternative reward as well as a stimulus that resembles the target-pest induced crop volatiles. Pest-derived stimuli such as artificial fecal pellets containing the reinforcing host recognition kairomone (Lewis and Martin, 1990), honeydew or scales may be used as an alternative reward, but are expected to act as a weaker reinforcer compared to an actual oviposition experience into a host-insect (Takasu and Lewis, 2003; Schurmann et al., 2012; Koppik et al., 2015). This potential drawback emphasizes the importance of selecting for parasitoids with a high learning rate. Artificially providing the correct HIPV blend during POC may pose a potentially more difficult challenge. One option to mimic HIPVs may be the addition of those volatile compounds that are enhanced by target-pest feeding to an intact crop plant. Another option may be the addition of elicitors of plant defensive pathways (Guo et al., 2013; Mack et al., 2013; e.g., Dinh et al., 2013; such as the plant hormones jasmonic acid or salicylic acid and/or enzymes from host saliva/regurgitant), either or not in combination with mechanically damaging a crop plant. It may not be necessary to mimic the complete volatile blend, as parasitoids are expected to generalize among non-functional differences in volatile blend composition resulting both from plant developmental and from environmental factors (Vet et al., 1998). When the composition of the volatile blend used in POC will more closely resemble the volatile composition of a target-pest infested plant than the volatile composition of a non-infested plant, parasitoids could be given a "head-start," enabling them to further refine their odor preferences during foraging (Geervliet et al., 1998; Vet et al., 1998).

With "*where*" we mean the site where POC is carried out (i.e. at the manufacturer's site or at the release site), and with "*when*" we mean the time in between POC and release into the crop environment. Whenever POC takes place at the manufacturer's site, the effect of the conditions the parasitoids experience during shipment (such as the duration of transportation, abiotic conditions, chance of physical damage etc.) on memory retention should be assessed. Thermal or physical stress experienced during shipment may have a negative effect on memory retention (Margulies et al., 2005; van den Berg et al., 2011; Abram et al., 2015). Moreover, if shipment takes a long time it would be desirable that parasitoids form LTM that will not vanish during transportation (Hoedjes et al., 2011). When parasitoids are shipped as pupae, POC of adults can only take place at the release site. This has the benefit of the target crop already being present, which is more difficult and costly to realize at the manufacturer's site, especially when the parasitoids can be used in many different cropping systems. When the target-pest is already present in the crop, adapting the release strategy by confining the parasitoids for some time on target-pest infested plants before allowing them to disperse further may be sufficient to enhance their efficacy. However, when released as a preventative strategy, it will be preferable to resort to alternative rewards and artificial ways of mimicking the target-pest induced crop odor. In those cases, the manufacturer can provide the end-user not only with the parasitoid pupae, but also with a "POC package" that contains an alternative reward as well as HIPV containing pellets or HIPV elicitors.

RESEARCH APPROACHES FOR THE DEVELOPMENT OF POC METHODS

When facing the challenge of developing an optimal POC method for a specific combination of parasitoid species, target-pest and crop environment, it may be worthwhile to structure different types of experiments along a few clear research lines. Here we propose a structure of three main research lines, with a first research line focusing on the behavior of naive parasitoids, a second research line assessing—and possibly improving—parasitoid learning capability, and a third research line focusing on the technical implementation of POC.

The aim of the first research line would be to identify the potential for improving parasitoid efficacy during each of the three host searching phases by studying the behavior of naive parasitoids. Experiments can be carried out to determine: (a) the degree to which the parasitoid innately responds toward the crop odor as well as the parasitoids' innate tendency for dispersal upon release, (b) whether the parasitoid innately prefers the odor of the target-pest induced crop over non-induced crop odor and whether the parasitoid can distinguish between these odors at different target-pest densities, and (c) the extent to which the parasitoid is innately attracted toward target-pest derived cues and accepts target-pests for parasitization.

The main aim of the second research line would be to assess the impact of different types of learning (associative learning, priming, and sensitization) in combination with the parasitoids' developmental stage on the strength and duration of the learned response, and – in the case of associative learning – to determine the number of conditioning trials required for the establishment of long-term memory. The results from the first research line can be used to determine the focus of this second research line. If multiple conditioning trials result in a significant enhancement of the strength and duration of the learned response, it may be worthwhile to further investigate the possibilities for improvement of the learning rate through an artificial selection program.

The main aim of the third research line would be to identify a POC method that leads to the highest possible parasitoid

efficacy gain at the lowest direct and indirect costs. Depending on the POC approach chosen based on the results of the other research lines, it may be important to assess the effects of alternative rewards and/or artificial ways of mimicking target-pest induced crop volatiles in POC on the strength and duration of the parasitoids' learned response. Moreover, determining the maximum number of parasitoids that can be conditioned in unison, as well as the best timing for conditioning in relation to parasitoid release are important pieces of information for the optimization of a POC method. In case the parasitoids are conditioned at the manufacturer's site, it will also be important to assess the impact of shipment conditions on memory retention.

CONCLUSIONS

The ability of parasitoids to optimize their foraging behavior through learning has been widely demonstrated in controlled laboratory conditions. This offers a great potential for optimizing the use of parasitoids in augmentative biological pest control. Especially when a parasitoid species is expensive to rear, POC may quickly pay off. We will therefore need more studies that systematically assess the potential impact of POC on parasitoid efficacy for biological pest control, as well as the possibilities for optimizing parasitoid learning rate and POC implementation for a series of commercially important parasitoid-pest-crop combinations.

AUTHOR CONTRIBUTIONS

HK and LV conceived the original idea. HK and OK developed the main conceptual ideas and wrote the first draft of the manuscript. All authors provided critical feedback and contributed to the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 29 May 2019
doi: 10.3389/fevo.2019.00187

The Parasitic Wasp, *Cotesia congregata* (Say), Consists of Two Incipient Species Isolated by Asymmetric Reproductive Incompatibility and Hybrid Inability to Overcome Host Defenses

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OPEN ACCESS

Edited by:
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Reviewed by:
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Freerk Molleman, Adam Mickiewicz University in Poznan, Poland

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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *09 November 2018*
Accepted: *08 May 2019*
Published: *29 May 2019*

Citation:
Bredlau JP, Kuhar D, Gundersen-Rindal DE and Kester KM (2019) The Parasitic Wasp, Cotesia congregata (Say), Consists of Two Incipient Species Isolated by Asymmetric Reproductive Incompatibility and Hybrid Inability to Overcome Host Defenses. Front. Ecol. Evol. 7:187. doi: 10.3389/fevo.2019.00187

Parasitic wasps are highly diverse and play a major role in suppression of herbivorous insect pest populations. Several previously identified species of parasitic wasps have been found to be complexes of cryptic species resulting from adaptations to specific hosts or host foodplants. *Cotesia congregata* (Say) (Hymenoptera: Braconidae), which has long served as a model system for host-parasitoid interactions, can be used for investigating the process of diversification among sympatric populations that differ in host and host foodplant usage. Two incipient species of *C. congregata* have been identified in the USA mid-Atlantic region, "MsT wasps" originate from *Manduca sexta* (L.) (Lepidoptera: Sphingidae) on tobacco and "CcC wasps" originate from *Ceratomia catalpae* (Boisduval) (Lepidoptera: Sphingidae) on catalpa. Both wasp sources can develop in either host species. Hybrids resulting from MsT♂xCcC♀ crosses are fertile, whereas hybrids from CcC♂xMsT♀ crosses are typically sterile. In this study, we compared relative expression *in vivo* of seven *C. congregata* bracovirus (CcBV) genes among MsT and CcC parental and hybrid crosses. Also, we established hybrid crosses between MsT and CcC wasps and four additional host foodplant sources of *C. congregata*. Patterns of relative expression *in vivo* of MsT and CcC CcBV genes differed; a few were not expressed in hosts parasitized by CcC wasps. Overall, relative expression of CcBV genes from MsT and CcC wasps did not differ with respect to the host species parasitized. Low or absent expression of CcBV genes was found in hosts parasitized by sterile hybrids. For the most part, the other four host-foodplant wasp sources were reproductively compatible with either MsT or CcC wasps and hybrid crosses with the alternative wasp source were asymmetrically sterile. Crosses involving CcC males or MsT females produced sterile hybrids that lacked mature ovaries. Cumulatively, results indicate that *C. congregata* is composed of two sympatric incipient species that can utilize multiple host species rather than several host-associated races or cryptic species.

Keywords: polydnavirus, bracovirus, hybrid dysgenesis, host expression, host-associated differentiation, reproductive isolation, speciation, virulence

INTRODUCTION

Parasitic wasps are among the most speciose of all terrestrial animals and represent 20% of all described insect species (LaSalle and Gauld, 1991). This diversity is likely to be grossly underestimated because most phytophagous insect species are attacked by one or more host-specific parasitic wasps, most of which are currently undescribed (Forbes et al., 2018). For example, detailed genetic and ecological analyses of parasitic wasps, previously identified as generalist species on the basis of morphology, are now known to consist of closely related cryptic species that specialize on different hosts (Kankare et al., 2005a,b; Smith et al., 2013). The processes leading to this remarkable diversity are not well understood. Although the importance of ecological speciation for phytophagous insects is debated (e.g., Matsubayashi et al., 2010; Nyman et al., 2010), it is likely an important process in the diversification of parasitic wasps (e.g., Feder and Forbes, 2010). In particular, parasitic wasps that develop inside larval hosts (koinobiont endoparasitoids) tend to be highly host specific (Askew and Shaw, 1986). Because they use plant cues to locate hosts and are exposed to plant chemicals during development, they also must adapt to the foodplants of their hosts (Kester and Barbosa, 1991a, 1994). As for all parasites, endoparasitoids also must adapt to the host physiology and immune defenses that limit their development and reproduction (Godfray, 1994).

Many endoparasitic wasps possess symbiotic polydnaviruses (PDVs) that suppress the immune response and development of their, primarily lepidopteran, hosts. The biology and history of PDVs has been extensively reviewed (Beckage and Drezen, 2012; Gundersen-Rindal et al., 2013; Herniou et al., 2013; Strand and Burke, 2015). Briefly, PDV genomes are integrated into parasitic wasp genomes and transmitted vertically from parents to offspring. Virions are produced in specialized calyx cells of the wasp ovaries where DNA circles encoding virulence genes are encapsidated and then injected into the host during oviposition. When expressed in the host tissues, PDV virulence genes manipulate host physiology, behavior, and cellular immune responses that benefit the developing wasp. Polydnaviruses have evolved independently in the Braconidae and the Ichneumonidae and those associated with the Braconidae are referred to as bracoviruses (BVs). Bracovirus-associated wasps derived ∼100 Mya (Murphy et al., 2008) from the stable integration of a large DNA virus in the family Nudiviridae (Bézier et al., 2009) and define the highly diverse Microgastrinae, which includes 17,000-46,000+ species (Rodriguez et al., 2013; Whitfield et al., 2018). Within the Microgastrinae, the genus *Cotesia* includes many important biological control agents of globally important lepidopteran pests (Van Driesche, 2008; Furlong et al., 2013; Aya et al., 2017). Due to their role in suppression of host immune responses, BVs have potential applications as biopesticides (Beckage and Gelman, 2004; Gill et al., 2006; Pennacchio et al., 2012; Gundersen-Rindal et al., 2013).

The role of BVs in reproductive isolation and host-adaptation has been extensively studied in *Cotesia sesamiae* (Cameron) (Hymenoptera: Braconidae), an important parasitoid and biocontrol agent of noctuid stemborers in Africa (Kaiser et al., 2017). *Cotesia sesamiae* has at least two allopatric biotypes that differ in host usage and are not reproductively compatible. Inland populations of *C. sesamiae* normally develop in the host *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) common in mountainous regions, whereas the lowland coastal populations, where *B. fusca* is uncommon, cannot develop in this host species because eggs are encapsulated (Ngi-Song et al., 1998). Both populations develop in the widespread host *Sesamiae calamistis* Hampson (Lepidoptera: Noctuidae). Encapsulation of lowland *C. sesamiae* eggs in *B. fusca* is due to differences in PDV virulence (Mochiah et al., 2002a). Encapsulation occurs when the well-characterized virulence gene, CrV1, is not expressed in *B. fusca* (Gitau et al., 2007); differences in this gene correlate with host range (Dupas et al., 2008; Branca et al., 2011). The CrV1 gene encodes a glycoprotein responsible for actin cytoskeleton interference in host hemocytes, preventing adhesion (Asgari et al., 1996, 1997). Several additional BV orthologs among populations have been shown to have sites under positive selection (Jancek et al., 2013). Moreover, only the coastal strain is infected by the endocellular bacteria, *Wolbachia*, which leads to unidirectional incompatibility in *C. sesamiae*—hybrid females are not produced in crosses between inland females and coastal males (Ngi-Song et al., 1998; Mochiah et al., 2002b). *Wolbachia* is responsible for similar hybrid inviability in many other insects (Werren et al., 2008). Subsequent studies on both pre- and post-zygotic reproductive barriers among multiple host-associated lineages indicate that at least one lineage specializing on *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae), is a cryptic species (Kaiser et al., 2015). Reproductive incompatibility has been reported for other parasitic wasps (Breeuwer and Werren, 1995; Stouthamer et al., 1996), but only a few have been examined in such widespread ecological and genetic detail across multiple host species (e.g., *Cotesia flavipes* by Muirhead et al., 2012).

The congeneric, *Cotesia congregata* (Say), offers a complementary system for the study of host-associated divergence and BV differentiation among parasitic wasps. Importantly, *C. congregata* is a major model system for BV genomics. The genomic organization of BV provirus in *C. congregata* as a macrolocus of proviral segments with other segments dispersed in the wasp genome has been analyzed (Bézier et al., 2013), as have host expression patterns of 88 *C. congregata* BV (CcBV) genes, 24 hours post-parasitism (Chevignon et al., 2014). In addition, *C. congregata* has served as a model system for host-parasite interactions and immunology (Beckage, 1998, 2008; Harwood et al., 1998), tri-trophic interactions (Kester and Barbosa, 1991a, 1994; Kester et al., 2002), and insect learning (Kester and Barbosa, 1991b; Lentz and Kester, 2008; Lentz-Ronning and Kester, 2013). In contrast to *C. sesamiae*, *C. congregata* includes at least two host-associated lineages that are not geographically isolated and can develop within both host species, yet they have significant reproductive incompatibility.

Two sympatric "host-foodplant races" of *C. congregata* have been described: "MsT wasps" originate from *Manduca sexta* (L.) (Lepidoptera: Spingidae) on tobacco (*Nicotiana tabaccum* L.) and "CcC wasps" originate from *Ceratomia catalpae* (Boisduval) (Lepidoptera: Spingidae) on catalpa (*Catalpa speciosa* Warder).

Manduca sexta is a specialist on solanaceous plants, including cultivated tobacco and tomato, and is a common pest on garden tomatoes, even in urban areas. A one-acre tobacco field can support hundreds of hosts and thousands of wasps. Likewise, a single mature catalpa tree can support hundreds of *C. catalpae* often leading to complete defoliation (Lampert et al., 2010, and personal observations). Both *M. sexta* and *C. catalpae* support multiple generations of *C. congregata* each year and parasitism rates in September and early October often exceed 90%. Wasps from these two host sources are genetically differentiated and likely represent incipient or nascent species with limited gene flow (Kester et al., 2015). Both MsT and CcC males are ∼30% less likely to respond to female pheromone produced by the reciprocal source and their courtship songs differ somewhat (Bredlau and Kester, 2015). These two host-foodplant sources of *C. congregata* will mate and produce hybrids when paired in enclosed vials; however, ∼90% of hybrid females produced from CcC male x MsT female crosses are sterile whereas females from MsT male x CcC female crosses are fertile (Bredlau and Kester, 2015). Caterpillars of either host species parasitized by the sterile hybrids develop and pupate normally but dissections of parasitized hosts reveal melanized spots, typically in the fat bodies, indicating encapsulation of wasp eggs. The precise cause for this asymmetric sterility is unknown. One explanation is the incompatibility of BV genes or inhibition of BV particle production in the sterile hybrids.

In addition to *M. sexta* and *C. catalpae*, *C. congregata* is reported to parasitize at least 13 other sphingid species (Krombein et al., 1979), representing 12 genera, most of which are plant family specialists (Tietz, 1972). All reported host species occur in the USA mid-Atlantic region. Several, including *Eumorpha pandorus* (Hübner) and *Darapsa myron* (Cramer), are in the subfamily Macroglossinae and others, including *M. sexta*, *C. catalpae, Dolba hyloeus* (Drury) and *Sphinx kalmiae* Smith, are in the Sphinginae (Kawahara et al., 2009). Given the genetic and reproductive divergence of MsT and CcC wasps and the reported host range, which includes more phylogenetically distant host species, *C. congregata* may consist of an array of host-foodplant associated "races" or incipient species. Alternatively, *C. congregata* may consist of two primary lineages (MsT and CcC) that can utilize some or all of the less common and more dispersed host species. To test these alternative hypotheses, we evaluated the reproductive compatibility of MsT and CcC wasps with other host-foodplant sources of *C. congregata* and compared *in vivo* host expression and sequences of selected BV genes of MsT and CcC wasps and their hybrids.

The objectives of this study were to: (1) determine the pattern of reproductive compatibility among additional sympatric host-foodplant sources with MsT and CcC wasps, and (2) evaluate relative expression *in vivo* of CcBV genes known to be virulence factors (including CrV1) from MsT and CcC wasps and their hybrids. Crosses between MsT and CcC with four additional host-foodplant sources of wasps were established to evaluate fertility of resulting hybrids. Relative expression of seven CcBV genes from MsT and CcC wasps in both *M. sexta* and *C. catalpae*, and from MsT and CcC hybrids in *M. sexta* were compared. We hypothesized that *C. congregata* either consists of multiple races or incipient species with hybrid sterility or two incipient species that may utilize multiple hosts. Moreover, we hypothesized that the observed pattern of hybrid sterility in which parasitized hosts encapsulated wasp eggs and developed normally would correspond to a reduction or absence in CcBV gene expression in hosts parasitized by hybrids, and likely differences in hosts parasitized by MsT and CcC wasps.

MATERIALS AND METHODS

Parasitoids

Parasitoids were collected from sites in Virginia, USA over a 3-year period (**Table 1**). "MsT wasps" were from a laboratory colony originating from *M. sexta* feeding on cultivated tobacco (*Nicotiana tabaccum* L.) and supplemented annually from the same site. "CcC wasps" were collected from *C. catalpae* feeding on mature catalpa trees (*Catalpa speciosa* Warder). Wasps from these two sites were used in prior genetic and behavioral studies (Bredlau and Kester, 2015; Kester et al., 2015), and multiple generations were sampled each year. Other hosts of *C. congregata* were collected during extensive searches in both wild and cultivated habitats. Searching effort was focused on the foodplants of the most commonly reported sphingid hosts in the region. These plants included: grape (*Vitis spp.*) and Virginia creeper [*Parthenocissus quinquefolia* (L.) Planch.] for *D. myron* and *E. pandorus*, privet (*Ligustrum spp.*) for *S. kalmiae*, pawpaw [*Asimina triloba* (L.) Dunal] for *D. hyloeus*, honeysuckle (*Lonicera spp.*) for *Hemaris diffinis* (Boisduval), trumpet vine [*Campsis radicans* (L.) Seem.] for *Paratrea plebeja* (Fabricius), and pine (*Pinus*) for *Lapara coniferarum* (J. E. Smith). Collected caterpillars were kept in individual plastic containers with leaves from their respective plant under ambient laboratory conditions (22 ± 2°C; 30–50% RH) until egression of parasitoid larvae. Parasitoid cocoons were removed 3 days after formation and placed individually into clear gel capsules (size 00). Resulting adults were sexed under a dissecting microscope for use in reciprocal crosses.

Reciprocal Crosses

To determine patterns of hybrid sterility, reciprocal crosses were established between MsT and CcC wasps and wasps from additional host-foodplant complex sources. Sib-crosses were performed as controls. Because only MsT and CcC wasps were consistently available, crosses were not established among wasps from other host sources which were rarely collected at the same time. Reciprocal crosses of MsT and CcC wasps were established for comparison with MsT and CcC crosses with wasps from four additional host-foodplant sources and also, for subsequent bracovirus gene expression assays. Upon host egression, wasps were sorted and males were placed in sets of three into a series of glass vials (7 × 2 cm diameter) with a water soaked cotton ball and plugged with a cotton ball with honey as a food source. Because mating success between MsT and CcC wasps is only ∼40% (Bredlau and Kester, 2015), we used a technique to increase mating success adapted from forced contact mating (see Kitthawee, 2008 for another parasitoid). Females, held in

TABLE 1 | *Cotesia congregata* host-foodplant complex (H-FPC) sources collected and used in this study with lepidopteran host names, foodplant, collection locations (county/city, state), coordinates (lat, long, datum: WGS84), and number and letter designations of wasp broods collected at each site.

H-FPC	Host species	Host foodplant	Location	Coordinates		*N*	
MsT	*Manduca sexta* (L.)	Tobacco	Nottoway Co., VA	37.095	−77.963	–	
CcC	*Ceratomia catalpae* (Boisduval)	Catalpa	Cumberland Co., VA	37.7127	−78.1639	–	
DhP	*Dolba hyloeus* (Drury)	Pawpaw	Hanover Co., VA	37.731	−77.713	1	
DmV	*Darapsa myron* (Cramer)	Grape	Gloucester Co., VA	37.304	−76.498	1	A
		Virginia creeper	Gloucester Co., VA	37.2572	−76.4525	2	BC
		Virginia creeper	Richmond, VA	37.530	−77.450	1	D
EpV	*Eumorpha pandorus* (Hübner)	Virginia creeper	Gloucester Co., VA	37.2572	−76.4525	2	AB
		Virginia creeper	Richmond, VA	37.530	−77.450	1	C
		Virginia creeper	Henrico Co., VA	37.586	−77.543	1	D
		Virginia creeper	Richmond, VA	37.5498	−77.4574	1	E
SkP	*Sphinx kalmiae* Smith	Privet	Charles City Co., VA	37.331	−77.210	1	

Wasps from M. sexta and C. catalpae were collected in large quantities (over 100 broods each) as needed for all crosses.
Other sphingids collected that did not yield C. congregata included Hemaris diffinis (usually parasitized by a tachinid) and Paratraea plebeja.

individual gel capsules, were chilled in a −10°C freezer for 6–10 min. During this time, male courtship behavior was initiated by presenting males with a recently dead or immobilized female from the same source. Each chilled female was carefully removed from the capsule with fine-point forceps and positioned in front of one to three courting males; the female used for courtship elicitation was removed immediately. Cold treating females did not affect male copulation behavior. After copulation, the female was carefully placed into a separate vial with food and water for recovery. Most females recovered completely within 1–2 min and this method ensured a greater mating success rate when using wasps from different populations, as compared to freely mating in vials as performed by Bredlau and Kester (2015). Wasps were provided fresh water and honey every 3 days until death. Females from each wild brood were paired with different males. As many pairings as feasible given the number of available wasps were prepared (3–8 successful matings for each collected brood for each cross type) to generate hybrids.

Mated females were presented with an early 4th instar laboratory-reared *M. sexta* two, three, and 4 days after mating. If any host died, a replacement was parasitized if the female wasp was still alive and hosts were available. Parasitized caterpillars were placed into separate plastic cups (4 × 7 cm diameter) with a block (approximately 2 × 2 × 1 cm) of semi-synthetic laboratory diet modified from Yamamoto et al. (1969), that was replaced every 2 days. Resulting wasp cocoons were placed in individual capsules 3 days after larval egression. Note that because males are haploid only females are hybrids in the F_1 generation. Emergent adults were sexed and up to eight females from each brood were transferred to individual vials with honey and water. F_1 hybrid and control line females were presented *M. sexta* for parasitism which were reared as above. All F_2 wasps that were produced were counted. Parasitized hosts that developed normally (as if unparasitized) had a subset dissected in their wandering stage (the others pupated) to record any egg encapsulation. Hybrids were considered sterile if parasitized hosts failed to produce wasp larvae. To record ovarian development, hybrid females were dissected in a petri dish with 70% EtOH using ultra-fine point forceps under a dissecting microscope. F_2 wasps generated from pure line controls were released into separate, acrylic colony boxes with honey and water sources and maintained on *M. sexta* for at least five generations. Voucher specimens were stored in 95% EtOH at −20°C.

Fisher's exact test was used to compare proportions of sterile hybrids produced between reciprocal crosses. When multiple wild broods of the same host-foodplant complex were collected, the Cochran-Mantel-Haenszel test was also used, with each initial brood treated as a block. Statistics were performed using R statistical software (R Core Team, 2017) and JMP Pro v11 (SAS Institute Inc, 2014).

CcBV Gene Expression *in vivo*

In-host expression of CcBV genes was compared between MsT and CcC wasps on both hosts and their hybrids only on *M. sexta* (N = 8–14 biological replicates for each group). A more limited sampling was performed for the additional host-foodplant sources. Wild and hybrid wasps placed in vials were randomly selected from each group. Females parasitized an early 4th instar *M. sexta* from a laboratory strain or a 3rd instar *C. catalpae* reared from field collected eggs. Parasitized caterpillars were held in plastic cups on laboratory diet blocks or catalpa leaves, respectively, for 24 h and then stored in RNA*later* solution (Ambion) at −80°C. Samples were later thawed and homogenized using a FastPrep benchtop homogenizer (MP Biomedicals) in lysis buffer (Ambion) equaling 10x of caterpillar mass with ceramic beads. RNA extraction was performed using *mir*Vana RNA isolation kit with phenol (Ambion) following manufacturer's protocol for animal tissue. RNA concentrations were quantified using an Epoch microplate spectrophotometer (BioTek). Extracted RNA was converted to cDNA using SuperScript II Reverse Transcriptase kit (Invitrogen) following manufacturer's protocol.

Corresponding primer sequences to known virulent CcBV gene factors (**Supplementary Material 1**) were selected from Chevignon et al. (2014) and verified for amplification and proper sequence annealing site identity. Real-time PCR was

performed in 96-well optical reaction plates with Power SYBR Green PCR Master Mix (Applied Biosystems). The cDNA was reverse transcribed from 5 μg of total RNA and amplified in a volume of 25 μL containing 12.5 μL of SYBR Green solution and 0.5 μL of each primer (20 pM). All samples were run in triplicate. *Manduca* 18S rRNA and lepidopteran 18S rRNA diluted 1:1500 were used as homologous controls for hosts *M. sexta* and *C. catalpae*, respectively. PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems) with the following thermal profile: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. A melting point curve was determined using the following conditions: 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s.

DataAssist v3.01 software (Applied Biosystems) was used to normalize data to homologous controls and calculate ΔCt values for each sample. Expression was calculated using the $2^{-\Delta Ct}$ method. Relative expression of BV genes was examined for *M. sexta* and *C. catalpae* parasitized by MsT and CcC wasps and their two reciprocal hybrids using non-parametric Mann-Whitney-Wilcoxon test for two comparisons or Kruskal-Wallis test followed by Dunn's *post-hoc* test for multiple comparisons with R statistical software (R Core Team, 2017). A Bonferroni correction was used to adjust reported *p*-values for the number of genes sampled.

Several BV genes were sequenced using newly designed primer pairs (**Supplementary Material 1**). In some cases these were located up- and down-stream of real-time PCR primer loci to yield larger gene amplicons. The resultant BV gene amplicons were PEG precipitated and sequenced on an ABI 3130XL (Applied Biosystems) with the following thermal profile: 35 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Sequences were analyzed using DNASTAR's Seqman Pro software (DNASTAR) and aligned using DNASTAR's MegAlign software (DNASTAR).

RESULTS

Parasitoids

All wasps used in this study originated from parasitized hosts collected in central or eastern Virginia, USA, and in some cases within the same vicinity (**Table 1**). By far, MsT and CcC wasps were the most common and easily available wasp sources; often, hundreds of parasitized caterpillars were found within close proximity. Additional wasp sources were collected in small numbers. Despite the many plants searched, only a few caterpillars of *S. kalmiae* on privet and *D. hyloeus* on pawpaw were found, and only one of each species was parasitized by *C. congregata*. Four of nine caterpillars of *D. myron* on Virginia creeper were parasitized by *C. congregata*; however, adult wasps tended to be relatively weak, males did not mate, and mated females produced only a few hybrid broods. Several caterpillars collected were parasitized by either a solitary ichneumonid or tachinids.

Eumorpha pandorus produced the largest broods (one had a record of 502 wasp cocoons), although only five of nine caterpillars were found parasitized by *C. congregata* during this study in two regions of Virginia. The two regions were ∼ 95 km apart: two broods from Virginia creeper growing along a fence in Gloucester Co., VA (two other *E. pandorus* already had wasps emerged and were not collected; *D. myron* were collected at same site) and three broods in the Richmond, VA area, 2.3 or 8.5 km from the nearest collection site. These sites were within fragmented urban and suburban areas. In one case, a collected *E. pandorus* that had pupated produced 18 tachinids and no wasps. Despite the small sampling of host-foodplant complex sources of wasps, hybrids were generated in sufficient quantities to discern patterns of hybrid sterility.

Reciprocal Crosses

Hybrid crosses were established between MsT and CcC and four additional host-foodplant complex sources of wasps. All crosses produced hybrid F_1 females with apparently normal appearance and behavior. MsT♂xCcC♀ F_1 hybrid females (28/29 hybrids produced from 9 initial matings) produced F_2 progeny, whereas CcC♂xMsT♀ F_1 hybrid females were all sterile (108 hybrids produced from 21 initial matings). The MsT and CcC control lines from both wasp populations developed normally in both hosts over multiple generations. Asymmetric patterns of hybrid sterility were observed for crosses between the four other host-foodplant complex sources (SkP, DhP, EpV, and DmV) mated with MsT or CcC wasps (**Table 1**; **Figures 1**, **2**). Pure lines of these additional wasp host-foodplant sources were subsequently maintained as separate colonies for at least five generations using host *M. sexta*.

Crosses involving wasps from a single brood of *S. kalmiae* on privet (SkP) and a single brood of *D. hyloeus* on pawpaw (DhP) displayed different patterns of reproductive compatibility with MsT or CcC wasps. SkP♂xMsT♀ crosses produced only 21% fertile F_1 hybrids, whereas MsT♂xSkP♀ and both reciprocal crosses between SkP and CcC wasps were ∼100% fertile ($p < 0.0001$; **Figure 1A**). Likewise, CcC♂xDhP♀ crosses produced only 45% fertile F_1 hybrids, whereas DhP♂xCcC♀ and both reciprocal crosses between DhP and MsT were ∼100% fertile ($p < 0.0001$; **Figure 1B**). These results indicate that at least for these single broods, SkP wasps are fully compatible with CcC wasps and DhP wasps are fully compatible with MsT wasps.

Results of hybrid crosses between wasps collected from *E. pandorus* on Virginia creeper (EpV) and MsT or CcC wasps varied by brood (**Figure 2**). Most F_1 hybrids produced with MsT wasps were fertile, whereas most F_1 hybrids produced from CcC♂xEpV♀ crosses were sterile (Cochran-Mantel-Haenszel test: $X^2 = 128$, $p < 0.0001$); however, one brood originating from Belle Isle in Richmond (brood C) showed a reciprocal pattern. F_1 hybrids produced from this EpV♂xMsT♀ cross were typically sterile, whereas the other crosses produced fertile F_1 hybrids (Fisher's exact: $p < 0.0001$; **Figure 2**). In almost all cases, wasps from additional host sources displayed a pattern of asymmetric sterility whereby F_1 hybrids with MsT♂ *and* CcC♀ parents were fertile; hybrids from CcC♂ *or* MsT♀ parents were sterile and hybrids from the reciprocal cross was fertile. Both patterns can exist from the same host source, as observed with EpV wasps. Sterile crosses that did produce progeny usually had smaller brood sizes.

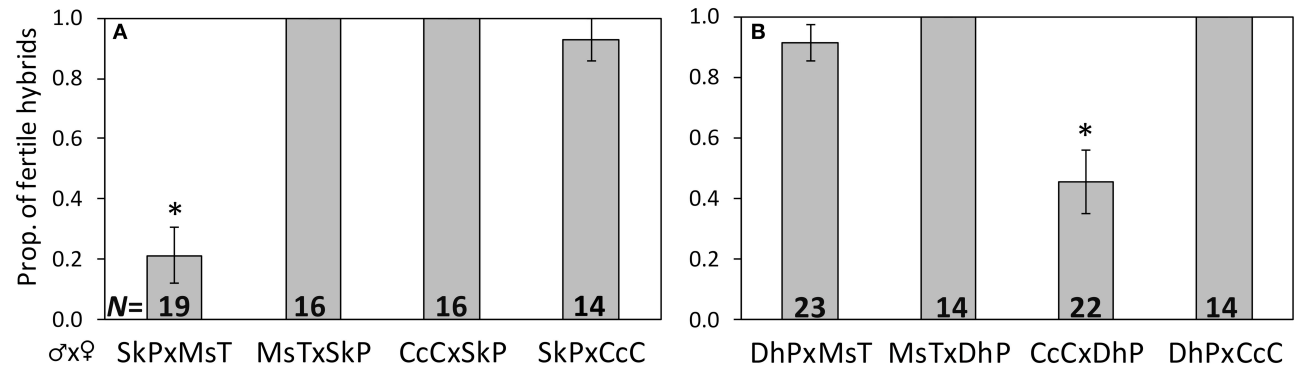

FIGURE 1 | Proportion of F_1 hybrids resulting from crosses between host-foodplant complex sources of *Cotesia congregata* that produced progeny. Three letter abbreviations denote wasp host species and host-foodplant. All F_1 broods were reared on *Manduca sexta.* Uncommon wasp sources **(A)** SkP (*Sphinx kalmiae* on privet) and **(B)** DhP (*Dolba hyloeus* on pawpaw) were crossed with two abundant incipient species, MsT (*Manduca sexta* on tobacco) and CcC (*Ceratomia catalpae* on catalpa). Crosses with a significant reduction in fertile hybrids are marked with *(Fisher's exact test: $p < 0.001$); sample sizes are at the base.

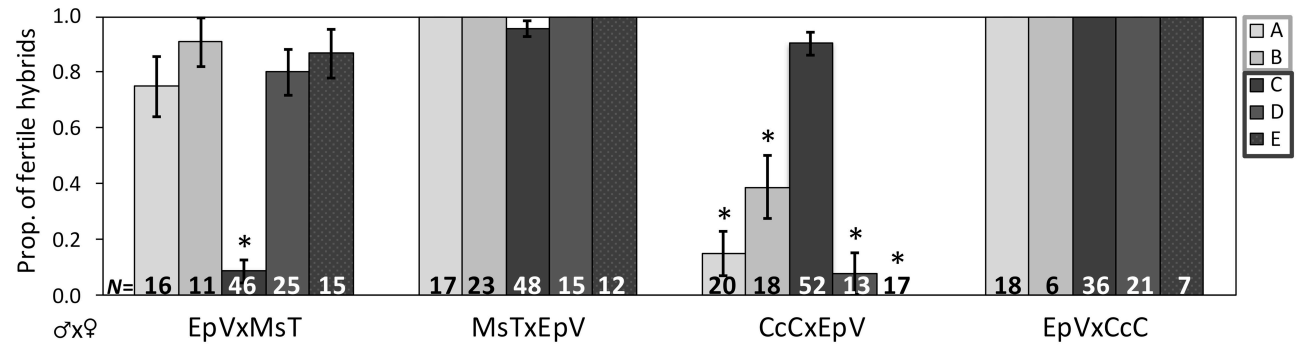

FIGURE 2 | Proportion of F_1 hybrids resulting from crosses between host-foodplant complex sources of *Cotesia congregata* that produced progeny. Three letter abbreviations denote wasp host species and host-foodplant. Five initial broods of EpV (*Eumorpha pandorus* on Virginia creeper) collected at four different locations were crossed with MsT (*Manduca sexta* on tobacco) and CcC (*Ceratomia catalpae* on catalpa) to produce F_1 hybrids. All F_1 broods were reared on *Manduca sexta.* Light gray bars (A,B) indicate broods collected in Gloucester Co., VA and dark gray bars (C–E) indicate broods collected in/near Richmond, VA, ∼95 km apart. Crosses with a significant reduction in fertile hybrids are marked with *(Fisher's exact test: $p < 0.0001$); sample sizes of hybrids are at the base. Note the reversal in the pattern for brood C, suggesting that EpV wasps do not cluster exclusively with either MsT or CcC wasps.

Wasps produced from *D. myron* on grape or Virginia creeper generally showed the same pattern of asymmetric reproductive compatibility; however all cross types could not be established so data are incomplete. Broods from *D. myron* either lacked females (brood A) or produced relatively weak males that would not mate successfully (broods B and C). The DmV♂xMsT♀ cross from brood A produced all sterile F_1 hybrids (17/17); the other crosses could not be established. The CcC♂xDmV♀ cross from broods B and C (from the same site as EpV A and B) produced sterile F_1 hybrids (13/15). The MsT♂xDmV♀ cross produced all fertile F_1 hybrids (31/31). The crosses from DmV brood D produced fertile F_1 hybrids with both MsT and CcC (9–23 hybrids tested for each cross), which was not observed in any other set of crosses. Despite the lack of all reciprocal comparable crosses, the pattern of asymmetric reproductive compatibility (by brood) corresponds to that observed for other wasp sources with MsT or CcC wasps. For example, the MsT♂xDmV♀ and DmV♂xCcC♀ hybrids were always fertile, whereas either the DmV♂xMsT♀ *or* the CcC♂xDmV♀ hybrids were sterile, depending on the original DmV brood.

Dissections of female wasps (986 total) revealed that F_1 hybrids that failed to produce progeny in the sterile crosses had severely reduced or absent ovaries and calyx (**Figure 3**). These hybrid wasps otherwise appeared normal and exhibited typical parasitism behavior. Although failed parasitisms by fertile wasps with normal ovaries (e.g., control lines and MsT♂xCcC♀ F_1 hybrids) did occur, parasitism success rates were typically above 90%. Also, caterpillars that failed to produce emerged wasps did not contain wasp larvae upon dissection and had small spots of melanization from failure to suppress the host immune response (as described in Bredlau and Kester, 2015).

BV Gene Expression *in vivo*

Relative expression *in vivo* of certain CcBV virulence genes differed between *M. sexta* caterpillars parasitized by MsT and CcC wasps (**Figure 4**; **Supplementary Material 2**). Ankyrin 4, CcV3-like, and CcPTP-L transcripts from MsT and CcC wasps were expressed consistently in both host species ($p > 0.3$). Expression of two genes from CcC wasps was not detected or had relatively low expression in either host: CrV1 and cystatin 1 in

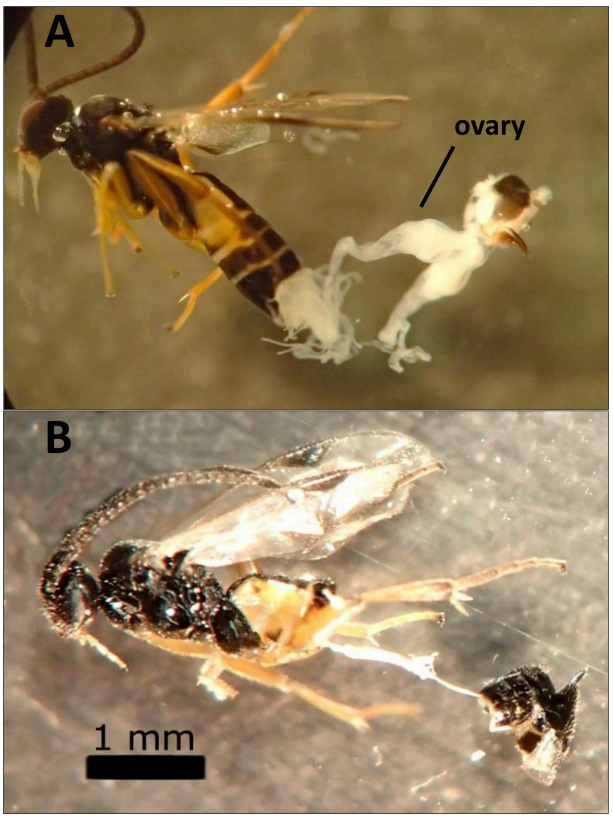

FIGURE 3 | Images of dissected hybrid females produced from crosses between two incipient species of *Cotesia congregata* (MsT = *Manduca sexta* on tobacco and CcC = *Ceratomia catalpae* on catalpa). **(A)** MsT♂xCcC♀ hybrid female with normal ovaries. **(B)** Sterile CcC♂xMsT♀ hybrid female that lacks developed ovaries (alimentary tract visible).

M. sexta ($W = 0$, $p < 0.001$; $W = 0$, $p < 0.001$, respectively) and *C. catalpae* ($W = 4$, $p = 0.013$; $W = 0$, $p = 0.001$). Expression of CrV1 did not differ with respect to the two different CrV1 primer sets. The CcBV 13-2 gene was highly expressed in *M. sexta* parasitized by CcC in comparison to MsT ($W = 75$, $p = 0.006$), but did not differ significantly in *C. catalpae* ($W = 45$, $p > 0.5$). In contrast, Duffy-like was marginally higher in *C. catalpae* parasitized by CcC ($W = 56$, $p = 0.07$), but not significantly in *M. sexta* ($W = 57$, $p > 0.5$). Note that although we used the same quantity of RNA for RT-qPCR, the amount of BV injected into the host by individual wasps could not be controlled and thus, is representative of naturally occurring variation among parasitization events.

Relative expression *in vivo* of Cystatin 1, CrV1, CcBV 13-2, and Duffy-like from the MsT and CcC hybrids in *M. sexta* varied with respect to directionality of the cross (Kruskal-Wallis with Dunn's test: $df = 3$, $p < 0.05$; **Figure 4**). Relative expression of BV genes from the MsT♂xCcC♀ hybrids (produce fertile offspring) was generally intermediate between the MsT and CcC parental lines but did not differ significantly from MsT parental lines ($p > 0.5$). In contrast, relative expression of six CcBV genes from the CcC♂xMsT♀ hybrids (sterile) was generally absent or greatly reduced in comparison to MsT parental genes: Ankryn 4 ($p = 0.004$), CcPTP-L ($p = 0.014$), CcV3-like ($p = 0.012$), CrV1 ($p < 0.001$), Cystatin 1 ($p = 0.002$), and Duffy-like ($p = 0.003$). The exception was for CcBV 13-2 which had relatively low expression in hosts parasitized by MsT wasps ($p = 0.28$). CcC♂xMsT♀ crosses did not differ significantly from CcC for unexpressed CrV1 and Cystatin 1 ($p > 0.5$), and CcPTP-L ($p = 0.31$), but was significantly lower compared to CcC for Ankryn 4 ($p = 0.016$), CcBV 13-2 ($p < 0.001$), CcV3-like ($p < 0.001$), and Duffy-like ($p < 0.001$). In two samples of CcC♂xMsT♀ hybrids, BV genes, including CrV1 and Cystatin 1, had intermediate levels of expression *in vivo* ($N = 14$). Similar results were found among limited sampling of the other host-foodplant complex sources in which sterile hybrid BV genes were not expressed in parasitized hosts.

Several BV virulence gene sequences were examined among pure and hybrid crosses of MsT and CcC incipient species (**Figure 5**), including crosses with wasps from other host-foodplant complex sources. A greater number of sequence differences in hybrid crosses were seen for certain virulence genes, for example PTP-p, than for other CcBV gene sequences under positive selection (data not shown). The MsT or CcC wasps crossed with SkP (*S. kalmiae* on privet) wasps displayed the greatest ambiguity. The corresponding transcripts expressed *in vivo* post-parasitization by F_1 hybrid crosses were also obtained by sequencing cDNAs (**Figure 5C**) and favored the male parental allele.

DISCUSSION

We hypothesized that *C. congregata* consists of either multiple host-associated races or incipient species, or two incipient species (MsT and CcC) that utilize multiple hosts. Sterility of CcC♂xMsT♀ hybrids was predicted to correspond to a reduction or absence in CcBV gene expression in hosts parasitized by hybrids, and differences in hosts parasitized by MsT and CcC wasps. We examined reproductive compatibility of MsT and CcC wasps with four other host-foodplant sources of *C. congregata* (DhP, DmV, EpV, and SkP; see **Table 1**). Additionally, we measured relative expression *in vivo* of seven BV genes from MsT and CcC wasps in both *M. sexta* and *C. catalpae* and relative expression of these BV genes from MsT and CcC reciprocal hybrids in *M. sexta*. In general, wasps from these four host-foodplant sources were reproductively incompatible with either MsT or CcC wasps with some crosses producing hybrid females that lack fully developed ovaries and functional BVs (**Figures 1**, **2**). Differences in relative expression of BV genes from MsT and CcC wasps were predicted due to differences in host utilization and the absence of functional BVs in sterile CcC♂xMsT♀ hybrids. Bracovirus gene expression *in vivo* was highly variable with respect to MsT or CcC wasp source (**Figure 4**). Bracovirus genes from typically sterile CcC♂xMsT♀ hybrids had lower or absent expression relative to MsT♂xCcC♀ hybrids. Cumulatively, our results demonstrate that *C. congregata* likely consists of two primary incipient species (MsT and CcC) that can utilize multiple host species rather than a series of host-specific cryptic

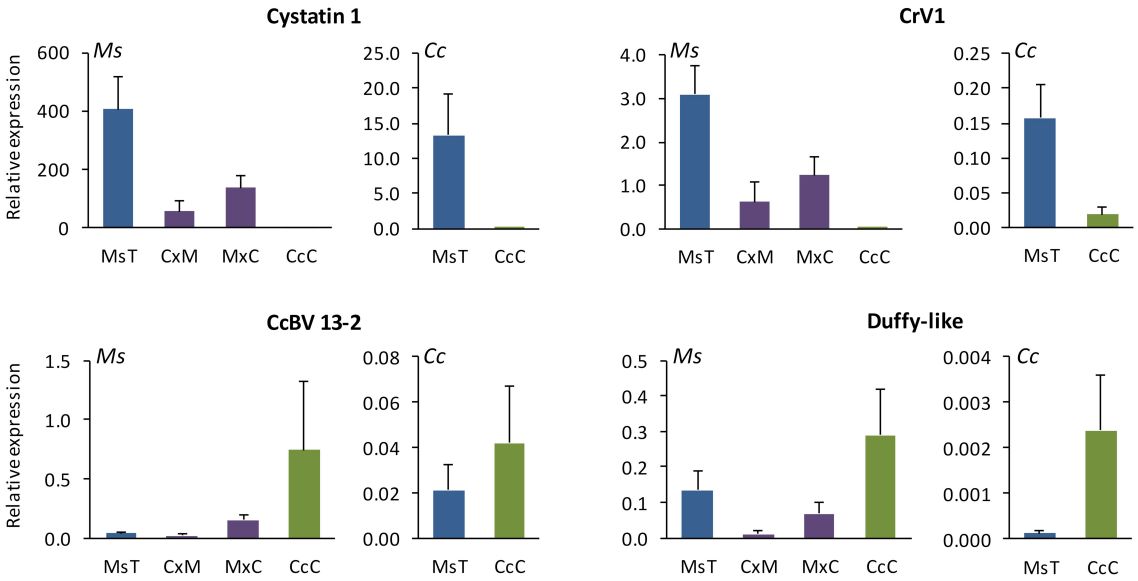

FIGURE 4 | Relative expression *in vivo* (mean ± SE; $N = 14$ for CxM hybrid and $N = 8$ for all other groups) of four virulent bracovirus (BV) genes that differ in hosts *Manduca sexta* (*Ms*; left plot for each gene) and *Ceratomia catalpae* (*Cc*; right plot for each gene) parasitized by two incipient species of *Cotesia congregata* [MsT = *M. sexta* on tobacco (blue) and CcC = *C. catalpae* on catalpa (green) and their hybrids (C×M = CcC♂xMsT♀ and MxC = MsT♂xCcC♀ (purple)] on *M. sexta*. Note that Cystatin 1 and CrV1 had low or absent expression in hosts parasitized by CcC wasps. CcBV 13-2 and Duffy-like genes from CcC wasps were highly expressed relative to those from MsT wasps, depending on the host species parasitized. All seven genes from CcC♂xMsT♀ hybrids had low or absent expression in 12/14 samples; gene expression in two samples was similar to that for MsT♂xCcC♀ samples. Relative expression of three other BV genes, Ankyrin 4, CcPTP-L, and CcV3-like, did not differ significantly between MsT and CcC parental lines.

species or a mixture of host-races and species at different stages of divergence.

All six host species included in the present study have overlapping ranges; however, *M. sexta* and *C. catalpae* were far more abundant than the other four. The other sphingids, *D. myron*, *E. pandorus, D. hyloeus*, and *S. kalmiae*, were widely dispersed both spatially and temporally and occurred in small numbers. Also, they had relatively low rates of parasitism; extensive searching yielded only a few caterpillars parasitized by *C. congregata* over 3 years. Only *D. myron* and *E. pandorus* were found in small groups and were rarely collected on the same plant within the same year. Thus, *M. sexta* and *C. catalpae* appear to be the major hosts of *C. congregata* in Virginia.

Overall, the additional host-foodplant sources of *C. congregata*, SkP and DhP (Sphinginae), and EpV and DmV (Macroglossinae) displayed similar patterns of partial reproductive compatibility with MsT or CcC wasps (both Sphinginae). F_1 hybrids resulting from crosses between these wasp sources and MsT males and CcC females were fertile, whereas crosses with CcC males or MsT females were sterile. This overall asymmetric pattern of hybrid sterility is not explained by the degree of host relatedness or phylogenetic signal (Forister and Feldman, 2011). A possible explanation is that as large populations of MsT and CcC wasps become host limited and disperse, they may utilize other proximate sphingid hosts on other plants (Kester and Barbosa, 1991a). Complete reproductive isolation of other host-foodplant sources of wasps not included in this study cannot be ruled out; however, the pattern of asymmetric sterility among the sympatric host-foodplant sources sampled suggests that this is unlikely. Production of fertile hybrids with both MsT and CcC wasps is possible (e.g., DmV brood D), but this appears to be exceptional. Additional studies are necessary to determine the population structure among different host-associated populations at the landscape level.

Both MsT and CcC wasps can utilize *M. sexta* and *C. catalpae* as hosts; however, differences in relative expression *in vivo* of BV genes illustrate the divergence of these incipient species (**Figure 4**). Relative to MsT, two genes from CcC wasps, CcBV 13-2 and Duffy-like, tended to be more highly expressed, dependent on the host species parasitized. In contrast, two other BV genes from CcC wasps, Cystatin I and CrV1, were either not expressed or had relatively low expression. The best studied BV gene, CrV1, is involved in the inactivation of host haemocytes and has been implicated as an important virulence factor during parasitism in several species including *C. congregata* (Whitfield, 2000; Amaya et al., 2005). Both Cystatin I and CrV1 have been shown to be under strong positive selective pressure (Dupas et al., 2008; Serbielle et al., 2008; Jancek et al., 2013). Similar to sympatric MsT and CcC wasps, allopatric host-associated biotypes of *C. sesamiae* also differ in CrV1 expression and sequences (Gitau et al., 2007).

In the current study, three BV genes did not differ in relative expression *in vivo* between MsT and CcC wasps (Ankyrin 4, CcV3-like, and PTP-L). Studies to date indicate that CcBV PTP genes are generally not under positive selection (Serbielle et al.,

FIGURE 5 | Variation among *Cotesia congregata* PTP-p gene sequences **(A)** MsT and CcC pure lines, **(B)** F_1 hybrid crosses, including hybrid crosses between MsT and CcC and other field collected host-foodplant complex sources of *C. congregata* that produced progeny (see **Table 1**) and **(C)** *in vivo* expressed transcripts from host post-parasitization by F_1 hybrids in **(B)**.

2012; Jancek et al., 2013). In global transcriptomic analyses, Chevignon et al. (2014) found that PTP-p is expressed in hemocytes but not the fat body of *M. sexta* parasitized by *C. congregata*. Sequence differences for PTP-p among hybrid *C. congregata* from different host-plant food sources (**Figure 5**) suggest flexibility that may reflect active modification to counter host immunity.

Relative expression of BV genes in *M. sexta* caterpillars parasitized by MsT and CcC hybrids differed (**Figure 4**). Expression of all seven BV genes was detected in hosts parasitized by MsT♂xCcC♀ hybrids and was generally intermediate relative to MsT and CcC parental lines. In comparison, BV gene expression was not observed or was very low in 12/14 caterpillars parasitized by sterile CcC♂xMsT♀ hybrids and in the remaining two caterpillars, was similar to those parasitized by MsT♂xCcC♀ hybrids. The low number of parasitized hosts with detectable BV expression corresponds with the low percent (∼10%) of CcC♂xMsT♀ hybrids that produced progeny in an earlier study (Bredlau and Kester, 2015). Dissections of sterile CcC♂xMsT♀ hybrids, as well as sterile hybrids resulting from the other crosses, revealed that females had either greatly reduced or absent ovaries. This would result in reduced or absent production of CcBV particles in the calyx cells (Beckage, 1998), and thus explains the decreased or absent CcBV gene expression in hosts parasitized by sterile hybrids. The ∼10% of CcC♂xMsT♀ hybrids that did produce progeny also had drastically reduced brood sizes (Bredlau and Kester, 2015). Future studies should include a larger number of BV genes across a broader array of host-foodplant sources of *C. congregata*. Further, comparative genome-scale sequencing studies, similar to those conducted by Jancek et al. (2013) and Gauthier et al. (2018) for *C. sesamiae* and other *Cotesia* species isolates, as well as analysis of population structure, are required to understand the genetic relationships among sympatric host-foodplant sources of *C. congregata*.

We are currently investigating genetic mechanisms that may be responsible for the pattern of asymmetric hybrid sterility. Although *Wolbachia* occurs in other *Cotesia* species (Mochiah et al., 2002a; Rincon et al., 2006), it typically results in failure of F_1 hybrid females to develop, which we did not observe in our study. *Wolbachia* was not detected in a survey of MsT and CcC parent and reciprocal hybrid

crosses (Gundersen-Rindal and Kester, unpublished data); however, we do not know if *Wolbachia* infection occurs in other populations of *C. congregata*. The unidirectional lack of normal ovary development in F_1 hybrids is similar to hybrid dysgenesis induced by transposable elements in *Drosophila* (Kidwell et al., 1977; Engels and Preston, 1979; Bingham et al., 1982; Kidwell, 1983). Other factors, including nuclear and mitochondrial genome incompatibilities may also be involved (Burton et al., 2013).

In summary, our results indicate that *C. congregata* consists of two incipient species that can utilize multiple hosts, despite differences in host expression of some BV genes. MsT and CcC wasps are unidirectionally incompatible, resulting in sterile hybrids that fail to develop both fully functional ovaries and BV particles necessary to suppress the host immune system. *Cotesia congregata* from other host-foodplant sources are compatible with either MsT or CcC wasps, and rarely both. Incipient species of endoparasitic wasps that are primarily specialized for locally abundant hosts can maintain reproductive isolation while also utilizing less abundant host species.

AUTHOR CONTRIBUTIONS

JB, DG-R, and KK designed the study. JB conducted the hybrid crosses and performed the statistical analyses. JB and DK conducted the molecular component of the study, including analyses. All authors participated in interpretation of the data. JB prepared the initial draft and figures with contributions from all other authors. JB and KK wrote the final version of the manuscript.

FUNDING

This project was supported in part by a USDA-ARS Cooperative Agreement (Project No. 8042-22000-291-04-S) to KK and a Virginia Academy of Science Small Project Grant to KK and JB.

ACKNOWLEDGMENTS

Many undergraduate laboratory interns assisted with rearing caterpillars and wasps. Jessica Bray, Megan Ayers, Marina Williams, Wan-Ling Chiu, Kathryn Bredlau, and Erin Wall found parasitized caterpillars. Buster Tyson and Paul Semtner provided access to catalpa and tobacco collection sites. We thank all of the property owners and managers who granted permission to search for caterpillars on their land.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00187/full#supplementary-material

Supplementary Material 1 | Primer set. Primers for bracovirus genes and homologous controls used in RT-qPCR to examine differences in relative expression among host-foodplant sources of *Cotesia congregata* (designed by Chevignon et al., 2014); and bracovirus gene primers designed for amplification and sequencing.

Supplementary Material 2 | Dataset. Host expression *in vivo* of seven bracovirus genes in *Manduca sexta* and *Ceratomia catalpae* parasitized by MsT and CcC host-foodplant complexes of *Cotesia congregata* and their F_1 hybrids. Data are normalized to 18S rRNA and transformed using the $2^{-\Delta Ct}$ method. Each sample represents an individual caterpillar parasitized by one wasp (mean of three technical qPCR replicates).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 27 August 2019
doi: 10.3389/fevo.2019.00309

Relative Influence of Host, *Wolbachia*, Geography and Climate on the Genetic Structure of the Sub-saharan Parasitic Wasp *Cotesia sesamiae*

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Population and Evolutionary Dynamics, a section of the journal Frontiers in Ecology and Evolution

Received: *27 July 2018*
Accepted: *31 July 2019*
Published: *27 August 2019*

Citation:
Branca A, Le Ru B, Calatayud P-A, Obonyo J, Musyoka B, Capdevielle-Dulac C, Kaiser-Arnauld L, Silvain J-F, Gauthier J, Paillusson C, Gayral P, Herniou EA and Dupas S (2019) Relative Influence of Host, Wolbachia, Geography and Climate on the Genetic Structure of the Sub-saharan Parasitic Wasp Cotesia sesamiae. Front. Ecol. Evol. 7:309. doi: 10.3389/fevo.2019.00309

The parasitoid lifestyle represents one of the most diversified life history strategies on earth. There are however very few studies on the variables associated with intraspecific diversity of parasitoid insects, especially regarding the relationship with spatial, biotic and abiotic ecological factors. *Cotesia sesamiae* is a Sub-Saharan stenophagous parasitic wasp that parasitizes several African stemborer species with variable developmental success. The different host-specialized populations are infected with different strains of *Wolbachia*, an endosymbiotic bacterium widespread in arthropods that is known for impacting life history traits, notably reproduction, and consequently species distribution. In this study, first we analyzed the genetic structure of *C. sesamiae* across Sub-Saharan Africa, using 8 microsatellite markers. We identified five major population clusters across Sub-Saharan Africa, which probably originated in the East African Rift region and expanded throughout Africa in relation to host genus and abiotic factors, such as Köppen-Geiger climate classification. Using laboratory lines, we estimated the incompatibility between the different strains of *Wolbachia* infecting *C. sesamiae*. We observed that incompatibility between *Wolbachia* strains was asymmetric, expressed in one direction only. Based on these results, we assessed the relationships between the direction of gene flow and *Wolbachia* infections in the genetic clusters. We found that host specialization was more influential on genetic structure than *Wolbachia*-induced reproductive incompatibility, which in turn was more influential than geography and current climatic conditions. These results are discussed in the context of African biogeography, and co-evolution between *Wolbachia*, virus parasitoid and host, in the perspective of improving biological control efficiency through a better knowledge of biological control agents' evolutionary ecology.

Keywords: *Cotesia sesamiae*, parasitoid, *Wolbachia*, genetic structure, host specialization

INTRODUCTION

Understanding the extraordinary biodiversity of insects requires both analyzing large-scale beta diversity patterns (Heino et al., 2015) and unraveling mechanisms of genetic differentiation among populations, including geographic, abiotic or biotic interactions (Roderick, 1996). Parasitoid wasps are one of the most diverse groups of insects (Grimaldi, 2005). Coevolutionary interactions are likely major diversifying forces in host–parasitoid systems due to the strength of reciprocal selection pressures (Van Valen, 1973; Henry et al., 2008). As strong insect antagonists, they are the most used agents for biological control programs, which provide one of the best alternatives to chemical control of insect pests (Harvey, 2011). There are theoretical expectations that host parasitoid coevolution generates diversity because several traits related to host specificity, such as specific virulence and host recognition, are mechanistically linked to reproductive isolation, especially when the parasitoid mates on the host just after emergence (Dupas et al., 2008; Hoskin and Higgie, 2010). Other biotic interactions, particularly those involving microorganisms affecting reproduction, such as *Wolbachia* sp., are expected to drive the diversification of parasitoids (Bordenstein et al., 2001; Branca et al., 2009). To distinguish between the different ecological factors responsible for population structure, a combination of, on the one hand, laboratory data on reproductive incompatibility and, on the other hand, field data on the geographic structure of ecological drivers and population differentiation are needed.

Cotesia sesamiae Cameron (Hymenoptera: Braconidae) is a parasitoid wasp widespread in Sub-Saharan Africa that has been used in biological control of *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), a major stemborer pest of maize and sorghum crops (Kfir, 1995; Kfir et al., 2002). *Cotesia sesamiae* is a stenophagous parasitoid that successfully parasitizes diverse host species (Ngi-Song et al., 1995; Branca et al., 2011). However, a variation in parasitism success on different hosts has been shown among populations of parasitoids (Mochiah et al., 2002a; Gitau et al., 2010). In contrast to the *C. sesamiae* population from Mombasa in coastal Kenya (avirulent toward *B. fusca*), the *C. sesamiae* population from Kitale in inland Kenya (virulent toward *B. fusca*) is able to develop in *B. fusca*, but both develop in *Sesamia calamistis* Hampson (Lepidoptera: Noctuidae), the main host of *C. sesamiae* in coastal Kenya (Ngi-Song et al., 1995). These differences in host acceptance and development have been linked to the observed polymorphism of a candidate gene, CrV1, located on the bracovirus locus (Gitau et al., 2007; Dupas et al., 2008; Branca et al., 2011). Bracoviruses are symbiotic polydnaviruses integrated into the genome of braconid wasps and contributing to their adaptive radiations (Whitfield, 2002; Dupuy et al., 2006). The viruses constitute the major components of the calyx fluid of the wasp and are expressed in parasitoid host cells, regulating its physiology, development and immunology (Beckage, 1998). In particular, the CrV1 gene, has been shown to contribute to immune suppression by active de-structuration of the cytoskeleton of host immune cells (Asgari et al., 1997). A comparative genomics study of the virus between *Cotesia* species and *C. sesamiae* populations, virulent and avirulent against *B. fusca*, showed patterns suggesting an important role for positive selection, gene duplication and recombination among viral genes in the adaptive diversification process (Jancek et al., 2013). Whilst host resistance likely puts a strong selective pressure on the local adaptation of the wasp, other ecological and geographic factors must be considered and analyzed for the development of the scenario of *C. sesamiae* response to environmental changes. Climatic differences or geographical barriers might weaken the capacity of some *C. sesamiae* populations to colonize areas recently invaded by a host that is suitable for parasitoid larval development, even if parasitic wasps have been shown to disperse quite efficiently, sometimes beyond the capacity of their associated host (Antolin and Strong, 1987; Ode et al., 1998; Van Nouhuys and Hanski, 2002; Assefa et al., 2008; Santos and Quicke, 2011). Other factors, such as *Wolbachia*, might act as a barrier to gene flow through reproductive incompatibility (Werren, 1997; Jaenike et al., 2006), which can be especially problematic in the context of biological control of invasive species by preventing crosses between ecological or geographic populations along the expanding range. *Wolbachia* is a widespread bacterium infecting the majority of insect species that can induce reproductive incompatibilities (Werren, 1997; Hilgenboecker et al., 2008). Several *Wolbachia* strains have been identified in *C. sesamiae* expressing cytoplasmic incompatibilities (CI) between populations of parasitoids (Mochiah et al., 2002b). The different populations of *C. sesamiae*, virulent and avirulent against *B. fusca*, are infected with different strains of *Wolbachia* (Branca et al., 2011). Reproductive isolation can prevent adapted parasitoid populations from expanding across their host range, a phenomenon that could be particularly relevant in biological control programs. In this study, our objective is to analyze the relative importance of neutral geographic factors and major selective forces—biotic (i.e., host species and *Wolbachia* strain) and abiotic (i.e., climate)—shaping the distribution of the populations of *Cotesia sesamiae* parasitoid across Sub-Saharan Africa. First, the genetic structure was assessed using 8 microsatellites markers with several genetic clustering approaches, each using different pertinent hypotheses in an effort to reach the broadest picture of the structure. Second, we tested the cross-incompatibility between differentially *Wolbachia*-infected *C. sesamiae* populations to infer their potential influence on limiting gene flow. Third, we estimated the amount and direction of gene flow in between genetic clusters of selected *C. sesamiae* populations to see if *Wolbachia* infection can affect the parasitoid metapopulation dynamics. Finally, we interpreted geographic patterns of *C. sesamiae* genetic structure in the context of African climate, *Wolbachia* infection and host occurrence.

MATERIALS AND METHODS

Insect Field Collection

Infected stemborer larvae were collected in 142 localities in 9 sub-Saharan African countries. GPS positions were recorded at each locality. Stemborer larvae were identified using a larval picture library (corresponding to adult moth identifications), and according to the host plant, as most stemborers are host

plant specific (Le Ru et al., 2006). Larvae collected from the field were reared on an artificial diet (Onyango and Ochieng'-Odero, 1994) until pupation or emergence of parasitoid larvae. After the emergence of cocoons, adult parasitoids were kept in absolute ethanol. Insect collection is summarized in **Table S1**.

Morphological identification of parasitoids was based on genitalia shape, following the method of Kimani-Njogu et al. (1997). Total genomic DNA of one female per progeny was extracted using the DNeasy Tissue Kit (QIAGEN). If the progeny contained only males, then a male was extracted. Because wasps are haplodiploids, the haploid genotype of males was converted to homozygous diploids for analyses to avoid discarding data. Although we acknowledged that this procedure strongly deviates the genotype frequencies from the Hardy Weinberg equilibrium, this should not strongly bias the results because of a very low level of heterozygosity due to very high inbreeding. In any case, the methods we used that are not based on Hardy Weinberg equilibrium and only a low number of males was kept at the end. Total genotyped individuals were 590 females and 47 males, discarding individuals with too many missing genotypes (more than 2 over 8 loci).

Insect Rearing

For crossing experiments, females of both virulent and avirulent *C. sesamiae* strains against *B. fusca* were obtained from laboratory-reared colonies. The virulent, thereafter named Kitale *C. sesamiae* strain, was obtained from *B. fusca* larvae collected from maize fields in Kitale, Western Kenya, in 2006, while the avirulent *C. sesamiae* strain thereafter named Mombasa, was obtained from *S. calamistis* larvae collected from maize fields in coastal Kenya in 2007. The two lines have a different *Wolbachia* infection status: the Kitale line is infected with *Wolbachia* WCsesB1 strain, while the Mombasa line is infected with two strains of *Wolbachia* WCsesA and WCsesB2 (**Table 1**). Twice a year, both colonies were realimented by field-collected parasitoids. The wasps of both strains were continuously reared on larvae of *S. calamistis,* as previously described by Overholt et al. (1994). Parasitoid cocoons were kept in Perspex cages (30 × 30 × 30 cm) until emergence.

Adults were fed a 20% honey–water solution imbibed in a cotton wool pad and kept under artificial light for 24 h to mate. In all experiments, only 1-day-old females, putatively mated and unexperienced to oviposition, were used. The experiments were carried out at 25 ± 2°C, 50–80% RH, and a 12:12 h (L:D) photoperiod.

The stemborer species, *B. fusca* and *S. calamistis*, were reared on an artificial diet, as in Onyango and Ochieng'-Odero (1994). This method consists in rearing the stemborer larvae in glass vials, half-filled by an artificial diet constituted by a mixture of brewer's yeast, vitamins, sucrose, maize leaf powder and seeds of bean (*Phaseolus vulgaris*) powder, suspended in Agar. The adults (moths) are placed in a cage for mating. They lay eggs on an oviposition substrate which consists of a wax paper cut rectangularly (15 × 6 cm) and rolled helicoidally from top to bottom to form a cylindrical surrogate stem (Khan and Saxena, 1997). The eggs are then collected from the wax paper for hatching. For each species, three times a year, several stemborer larvae were added to rejuvenate the colonies. Before parasitism experiments, fourth larval instars were fed for 48 h on pieces of maize stem in 10 × 20 cm jars to produce frass that facilitates host acceptance by the parasitoid wasps.

Genetic Marker Sequencing and Genotyping

Eleven microsatellite markers were genotyped (Jensen et al., 2002; Abercrombie et al., 2009). Amplifications were performed in 10 μL with approximately 5 ng of genomic DNA, 1 × HotStarTaq PCR buffer, 2 μL Q-Solution 5× (QIAGEN), 1.6 mM of dGTC, dTTP, and dCTP, 50 μM dATP, 5 pM of each primer, 0.25 U Taq polymerase (HotStarTaq, QIAGEN) and 0.01 U of [33P]-dATP. The "touchdown" PCR (Mellersh and Sampson, 1993) was used as follows: initial activation step at 95°C for 15 min, 18 cycles at 94°C for 30 s, 60 to 51°C for 30 s (-0.5°C/cycle), 72°C for 30 s, 29 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 30 s and a final elongation step at 72°C for 10 min. Results were visualized using an ABI 310 and a ABI 3130 sequencer with fluorescent size standard (GeneScan 600 Liz, Applied Biosystem). Amplifications were made following conditions previously described using fluorescent labeling (Pet, Vic, Ned or 6Fam) of the forward primer.

Peaks identifying fragment sizes were assessed using GeneMapper 4.0 software. Locus B1.42 presented peaks that were difficult to analyze with multiple bumps preventing any accurate measure of fragment size and was therefore discarded. Loci B1.155 and B5.126 were also not considered in the analyses because they presented a high percentage of missing genotypes (respectively 14.6 and 27.0%), probably reflecting the occurrence of null alleles. Eight loci were then genotyped per individual.

Wolbachia infection status was checked using the protocol developed in Branca et al. (2011).

Cross-Mating Experiment

To obtain *Wolbachia*-free parasitoid colonies (named cured lines), the gravid females of each aforementioned *C. sesamiae* Mombasa (Mbsa) and Kitale (Kit) parasitoid line were reared for three generations on larvae of *S. calamistis* previously fed on an artificial diet based on Onyango and Ochieng'-Odero (1994) and enriched with 2,000 mg/L rifampicine (Dedeine et al., 2001).

Cross experiment tests were conducted between both Mbsa and Kit *C. sesamiae* lines to assess mating incompatibilities due to the presence of different *Wolbachia* types. Individual parasitoids were allowed to emerge singly by separating single cocoons from each cocoon mass. Individual male and female parasitoids from each colony (i.e., cured and uncured Kit *C. sesamiae,* as well as cured and uncured Mbsa) were used for cross-mating experiments. Sixteen possible cross-mating combinations were investigated. Each cross-mating combination was repeated at least 20 times.

After mating, females were presented 4th instar larvae of *S. calamistis* for oviposition using the method of Overholt et al. (1994). The larvae were reared and observed daily for mortality or parasitoid emergence. The developmental time of the progeny (egg to adult), the brood size, sex ratio and mortality outside and inside the host were recorded.

TABLE 1 | Status of the Kitale and Mombasa strains (Mochiah et al., 2002a).

Strain	Localization	Major host association in the locality	*Wolbachia* status	Devt. rate on *B. fusca*	Instruct cluster
Kitale (Kit)	Inland Kenya	*Busseola fusca* (resistant)	wCsesB1	100%	5
Mombasa (Mbsa)	Coastal Kenya	*Chilo partellus* and *Sesamia calamistis* (susceptible)	WcsesA-wCsesB2	0%	2

The presence of *Wolbachia* infections in all *C. sesamiae* populations used in the cross-mating experiments was tested using PCR techniques on *ftsZ* and *wsp* genes, as described by Ngi-Song and Mochiah (2001). DNA was extracted from about 50 individuals (a mixture of males and females) from each population previously stored in 99% ethanol.

To test the effect of mating direction on each reproductive trait, a non-parametric Kruskal-Wallis test was applied with crosses as explanatory factor and each trait as variable. Because none of the data were normally distributed nor had homoscedastic variance, ANOVA was not used. Following the Kruskal-Wallis test, a pairwise Wilcoxon's rank sum test was conducted with false discovery rate (FDR) correction for multiple testing. Data were split into four groups for statistical analyses: crosses between Kit wasps, crosses between Mbsa wasps and crosses between populations in both directions. For all crosses, CI is expected between infected males and uninfected or differentially infected females. CI should lead to a reduction in female production either by female mortality (FM phenotype, diminution of the size of the progeny and the number of females) or male development (MD phenotype, only diminution of the proportion of females) (Vavre et al., 2000).

Statistical analyses for *Wolbachia* crosses experiments were performed in R 3.2 (R Core Team, 2013).

Genetic Structure Inference

To infer population structure from genetic data, we used three different Bayesian methods for population partitioning: Instruct, which does not assume Hardy-Weinberg equilibrium within loci and accounts for inbreeding (Gao et al., 2007); TESS3, taking into account spatial autocorrelation based on tessellation (Caye et al., 2016), and DAPC, a statistical partitioning method based on PCA (Jombart and Ahmed, 2011). Instruct software was used with the Adaptive Independence Sample algorithm using the inbreeding coefficient at population level as a prior model (mode 4, option v) (Gao et al., 2007), since *C. sesamiae* is known to have a highly inbred reproductive system (Ullyett, 1935; Arakaki and Ganaha, 1986). The number of clusters corresponding to the strongest genetic structure was determined using the method of Evanno et al. (2005) (**Figure S1**). Each inference had a total number of 200,000 iterations with a burn-in period of 100,000 iterations. Other parameters were kept as a default value except for the significance level of the posterior distribution of parameters, which was set to 0.95. The posterior probability of assignation of each individual was re-calculated over 10 MCMC runs using the CLUMPP software (Jakobsson and Rosenberg, 2007) with a greedy algorithm and 10,000 random permutations. TESS3 was run using an admixture with the BYM model (Durand et al., 2009). To identify the strongest structure, the model was run with K ranging from 2 to 9 using 100,000 sweeps with a 10,000 burning period. The degree of trend was assessed by running the algorithm with a varying value from 0 to 3 by 0.5 steps. The degree of trend showing the best DIC was kept. Genetic structure was then assessed for $K = 5$, the best K, and $T = 1.5$, the best degree of trend, with a MCMC chain run for 1,000,000 sweeps with a 100,000 burn-in period. DAPC was run in R package *adegenet* (Jombart and Ahmed, 2011), which is hypothesis-free, since it only clusters individuals to maximize the explained genetic variance within the data.

The correlation structure between abiotic and *Wolbachia* infection variables and their explanatory power for the other variables was represented using multiple correspondence analysis (MCA, package FactoMineR). The relative effect of each of these variables on genetic structure was assessed using distance and permutation-based multivariate analysis with the *adonis* function in vegan R package (Oksanen et al., 2013). This multivariate analysis corresponds to an extension of AMOVA (Excoffier et al., 1992) for crossed correlated factors and in a non-hierarchical pattern (McArdle and Anderson, 2001). The factors considered were: host genus, *Wolbachia* infection status, spatial cluster of samples and the Köppen-Geiger climate type (Kottek et al., 2006). The Köppen-Geiger climate type has been developed by botanists. It is based on temperature and precipitation data, and is still the most commonly used climate classification in climate-change studies. The distribution of climates in Sub-Saharan Africa is represented in **Figure S2**. As the sampling was not done randomly across Sub-Saharan Africa, we tested the effect of spatial structuration by defining spatial cluster grouping localities close to each other. The spatial cluster of samples was obtained with hierarchical clustering from latitude and longitude data (*Mclust* function) (Fraley and Raftery, 2002; Fraley et al., 2012). Genetic distance between individuals was generated using Smouse & Peakall's formula (Smouse and Peakall, 1999) in GenoDive (Meirmans and Van Tienderen, 2004). To avoid overfitting, we removed host genus from which we sampled only one cocoon mass.

Finally, we wanted to know whether cytoplasmic incompatibilities observed between differentially *Wolbachia*-infected parasitoid lines were influencing the geneflow between the genetic clusters. So, we used the Bayesian method implemented in the Migrate software to estimate the effective population sizes and reciprocal migration rates between the different genetic clusters of *C. sesamiae* (Beerli and Palczewski, 2010). Individuals were assigned to their major cluster, and only individuals with a maximum admixture rate above 0.7 were kept. Only individuals from Kenya were used to avoid to geographic dispersion of data Indeed, Kenya is the most sampled country, and is a region of contact between genetic clusters. Migrate-n

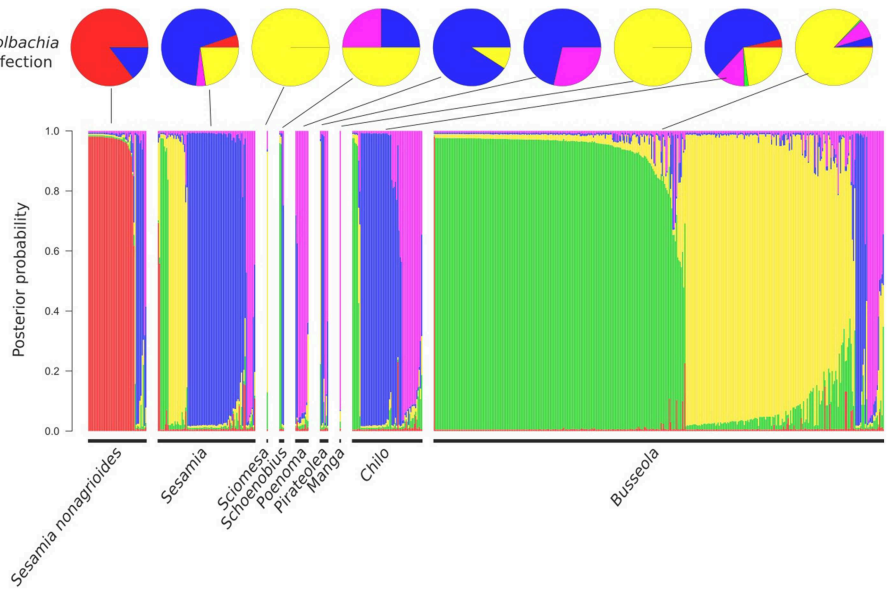

FIGURE 1 | Top: Pie charts representing the proportion of each infection found on each host genus (WCses A in red; WCses B1 in yellow; WCses B2 in green; WCses A/ WCses B2 in blue; Absence in magenta). Bottom: Posterior probability of admixture proportion of each individual of *Cotesia sesamiae* wasps to each of the 5 Instruct clusters and post-processed with CLUMPP (Cluster 1 in red, cluster 2 in yellow, cluster 3 in green, cluster 4 in blue, cluster 5 in purple). Individuals are grouped by the host genus where they were found. Individuals found on an unidentified host are not represented.

software version 3.6.6 was run using the microsatellite model set to Brownian motion and the gene flow model set to asymmetric. Since asymmetric gene flows can only be estimated pairwise, we ran the software independently for each pairwise cluster comparison. Prior distributions of θ and M were chosen to get posterior distributions that are not truncated. Five chains of with heat level ranging from 1 to 10 were run for 500,000 generations with a burn-in period of 10,000.

RESULTS

Genetic Structure

The three clustering methods, Instruct, DAPC and TESS3 used in this study gave similar results regarding the population structure of *C. sesamiae* populations. Therefore, to simplify interpretation, we only represented the Instruct result as the method that explicitly accounts for inbreeding, which is present in our gregarious species that mates just at cocoon mass emergence (**Figures 1**, **2** and **Table 2**). For each method, the best fit was observed for five clusters (maximum delta-K for Instruct, **Figure S1**, diffNgroups criterion for DAPC and Deviance Information Criterion for TESS3). Regarding the structuration in relation to the host species, cluster 1 of all three methods was found exclusively on *Sesamia nonagrioides* (**Figure 1**, in red), clusters 2 and 3 were found mainly on *Busseola ssp.* (**Figure 1**, in yellow and green, respectively), cluster 4 on *Sesamia* and *Chilo* spp. (**Figure 1**, in blue) and finally cluster 5 was recovered from five host genera (**Figure 1**, in purple). Geographically, the three methods provided a similar picture of genetic structure with some difference in admixture proportion. The cluster 1 population was scattered between central Ethiopia, western Kenya and Northern Tanzania, and even Cameroon (**Figure 2** and **Figure S3** in red). This corresponded to the population found on *S. nonagrioides*. *One* discordance appeared with the DAPC method, which failed to assign one individual from Arusha (Tanzania) into Cluster 1. Cluster 2 extended from Eritrea to Western Kenya in Instruct, but was restricted to Western Kenya in TESS3 and DAPC (**Figure 2** and **Figure S3**, yellow). Conversely, cluster 3 was only present in western Kenya and central Tanzania in the three methods but extended to Eritrea in TESS3 and DAPC (**Figure 2** and **Figure S3**, green). Cluster 4 extended from South Africa to eastern Kenya and Rwanda in the three methods (**Figure 2** and **Figure S3**, blue). In Instruct and TESS3 analyses, a very high posterior probability of cluster 4 was also observed further west on the coast of Congo-Brazzaville. Cluster 5 extended from Tanzania to Cameroon in all three methods but was found much more widely spread in DAPC analysis, as far as South Africa, and to a lesser extent in Instruct (**Figure 2** and **Figure S3**, purple). Overall, there seems to be a clear delimitation between cluster 2 and 3, which extend from Tanzania to Eritrea, and cluster 4 and 5, which were found from Cameroon to South Africa. Delimitation within these two groups of two clusters seemed to be shallower and influenced by the method used.

Wolbachia Strains Distributions

A rather good concordance was observed between genetic structure at the microsatellite level and *Wolbachia* strain distributions (**Figure 3**). Cluster 1 was associated exclusively with

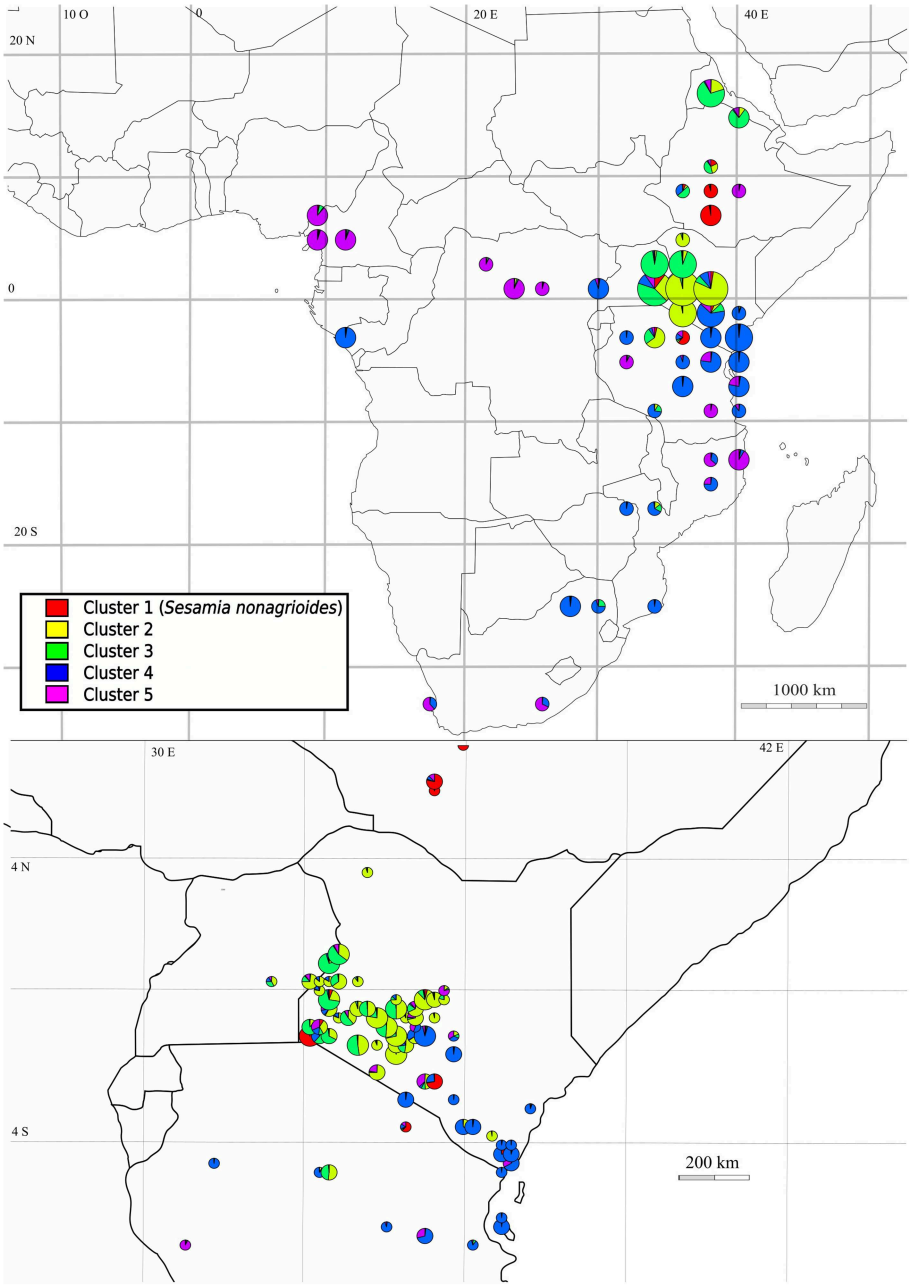

FIGURE 2 | Distribution of genetic clusters of *Cotesia sesamiae* wasps for the Instruct software CLUMPP consensus with $K = 5$. Only individuals with a maximum posterior probability of assignment above 0.5 are represented. Distribution in Sub-Saharan Africa is represented at the top and a zoom in Kenya at the bottom.

the *Wolbachia* wCsesA strain, cluster 2 and 3 with wCsesB1, cluster 4 and 5, with the bi-infection wCsesA/wCsesB2.

Relative Influence of Biotic and Abiotic Factors

The individuals belonging to the cluster found exclusively on *Sesamia nonagrioides* were interpreted as a distinct species by Kaiser et al. (2015, 2017), based on eco-phylogeny analyses and cross-mating experiments, and corresponding to a host and plant-host driven ecological speciation event. As it has now been described as the *Cotesia typhae* species (Kaiser et al., 2017), it was not considered in these analyses to prevent an overestimation of host effect. Multiple correspondence analysis (**Figure 4**) suggested the presence of structure in relation to all the factors considered (spatial cluster, Köppen-Geiger climate classification, *Wolbachia* infection status and host genus). The full models tested with permutational analysis of molecular variance used on the microsatellite distance matrix with the *adonis* function

TABLE 2 | F-statistics for each cluster inferred by Instruct on female individuals.

Cluster	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
Cluster 1 (*C. typhae*)	*0.8337*				
Cluster 2 (Rift Valley)	0.6759	*0.4852*			
Cluster 3 (West Kenya)	0.6023	0.2166	*0.6719*		
Cluster 4 (East Coast)	0.5089	0.5288	0.4337	*0.6725*	
Cluster 5 (Cameroon to East Coast)	0.3683	0.45	0.3534	0.2314	*0.8295*

F_{IS} are represented in the diagonal in italics and FST in the lower diagonal matrix.

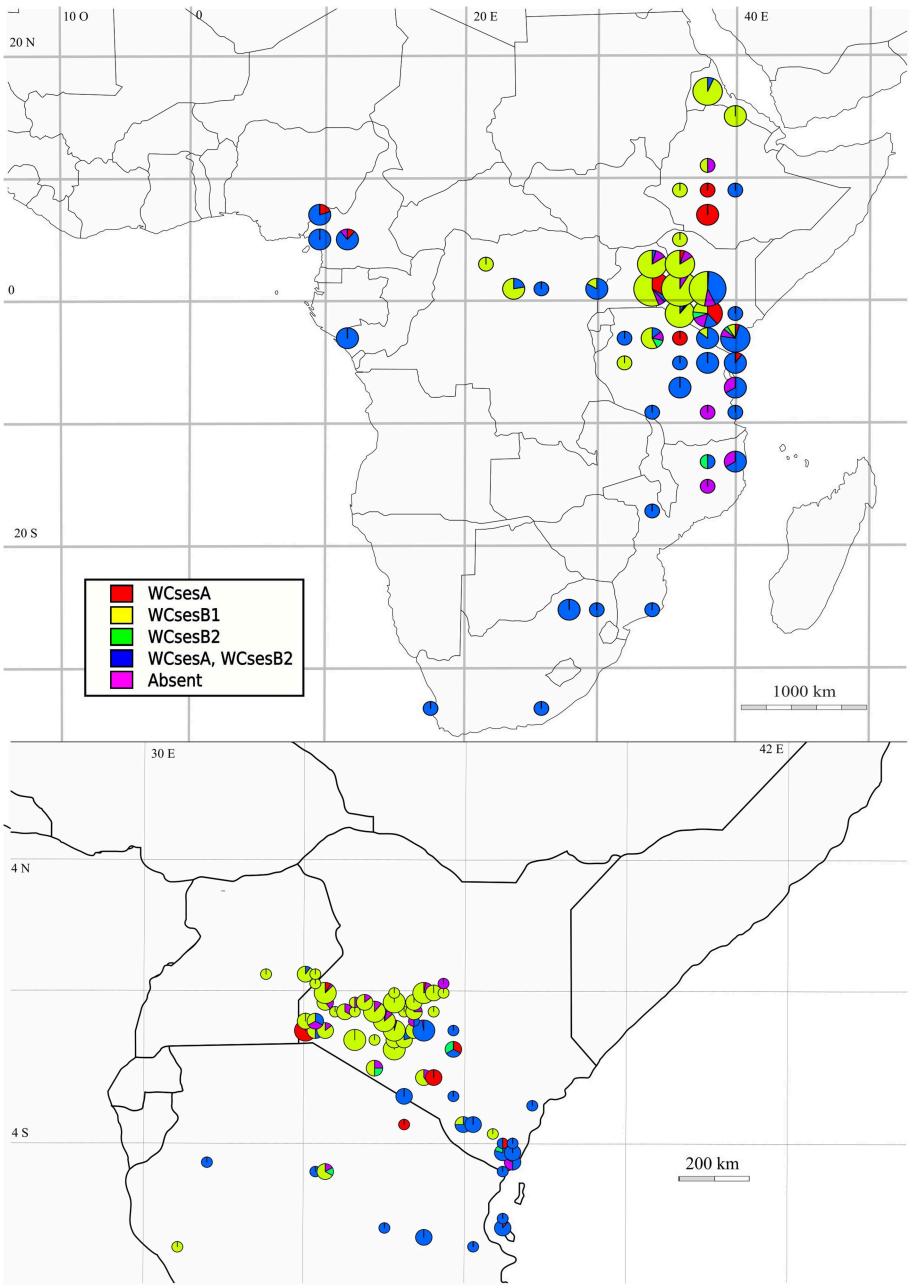

FIGURE 3 | Distribution of *Wolbachia* infection in *Cotesia sesamiae* wasps across Sub-Saharan Africa (top) and Kenya (bottom). WCses A in red; WCses B1 in yellow; WCses B2 in green; WCses A/WCses B2 in blue; Absence in magenta.

(**Table 3**) confirmed that all neutral (geography) and selective forces, both abiotic (climate type and geography) and biotic (host genus, *Wolbachia* infection), contribute significantly to the genetic variance of the microsatellite genotypes. Because the *adonis* method tests factors sequentially, it is important to consider each factor either as a first term or as a marginal (last) term to understand the effect. When added first in the sequence of factors in the *adonis* function, biotic factors had a higher R^2 than the abiotic factors (0.43 and 0.38 for *Wolbachia* and host genus, respectively, and 0.28 and 0.21 for Köppen-Geiger Climate type, and localization, respectively) (**Table 4**). In addition, all the factors had significant marginal effects (**Table 4**). Pairwise interactions between factors were weak ($R^2 < 0.04$), but significant for all the possible interactions (**Table 3**). None of the tripartite interactions was significant.

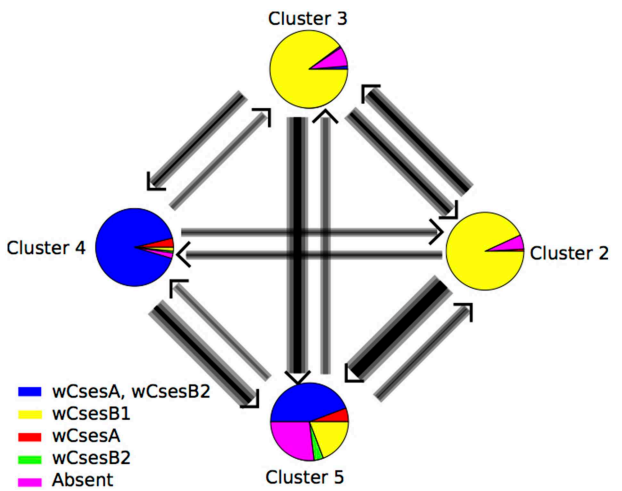

FIGURE 4 | Estimates of gene flow between four geographic genetic clusters of *Cotesia sesamiae* wasps identified by Instruct. Each circle represents the infection status of individuals found assigned to each cluster and the colors correspond to the ones in **Figure 3**. The fifth genetic cluster, found only infecting *Sesamia nonagrioides,* was excluded from the analysis.

Wolbachia Crosses Experiment

For crosses within each population, the brood size dropped in crosses involving infected males and cured females (i.e., Cs Kit x Cs Kit-cured and Cs Mbsa x Cs Mbsa-cured) from 34–36 to 23 for the Kitale population, and from 32–42 to 21 for the Mombasa population (**Table 5**). Although both crosses were potentially incompatible, the sex ratio (or %females) decreased significantly only for the Kitale population, and not for the Mbsa population. The overall number of females was however reduced in both crosses, from 45–62 to 44% and from 57–64 to 55% for the Kitale and Mbsa population, respectively. No significant changes in developmental time and mortalities outside and inside the host through dissection were detected between these incompatible crosses and the other crosses.

In crosses potentially showing bidirectional CI, i.e., crosses involving individuals from different populations and infected with different *Wolbachia* strains (i.e., Cs Kit × Cs Mbsa and Cs Mbsa × Cs Kit), we only found a significant decrease in the percentage of females from 47–67 to 11–0% in the cross involving Mbsa males and Kit females (**Table 5**). In this latter cross, almost no females were recovered despite a normal overall progeny size, suggesting a complete incompatibility with the pure male development (MD) phenotype (Vavre et al., 2000). By contrast, in the cross for Kit males (wCsesB1) with Mbsa females (wCsesA/wCsesB2), CI was expressed only when Mbsa females were cured and only partially, since some females were recovered. No significant changes in developmental time and mortalities outside and inside the host through dissection were detected between these incompatible crosses (i.e., Cs Kit × Cs Mbsa-cured, Cs Kit × Cs Mbsa, Cs Mbsa × Cs Kit-cured and Cs Mbsa × Cs Kit) and the other crosses.

Migration Patterns

For Bayesian analyses of pairwise migration rates, the acceptance rate ranged between 0.20 and 0.56 with an effective MCMC sample size from ∼500 to ∼2,700. Clusters defined by Instruct were used except in Cluster 1 for the main reasons exposed above. Mostly symmetric gene flow was found between Cluster 2 and

TABLE 3 | Analysis of molecular variance using microsatellite distance matrices and a full model containing all terms and interactions.

Factor	Df	Sum of squares	*F*-Model	R^2	Pr(>*F*)
Host genus	8	43,033	86.9617	0.37276	0.001***
Wolbachia	4	13,238	53.5037	0.11467	0.001***
Köppen-Geiger climate	11	4,896	7.1954	0.04241	0.001***
Localization	13	9,926	12.3438	0.08598	0.001***
Host genus * *Wolbachia*	12	3,735	5.0317	0.03235	0.001***
Host genus * Köppen-Geiger climate	20	5,042	4.0756	0.04368	0.001***
Wolbachia * Climate	17	3,261	3.1014	0.02825	0.001***
Host genus * Localization	18	3,071	2.7579	0.02660	0.001***
*Wolbachia** Localization	8	1,603	3.2395	0.01389	0.001***
Köppen-Geiger Climate * Localization	2	111	0.8950	0.00096	0.502
Residuals	445	27,526		0.24927	
Total	547	115,442		1	

****p <0.001.*

TABLE 4 | Sum of squares and partial R^2 of Host genus, *Wolbachia* infection status, Köppen-Geiger climate and localization taken either as marginal effect or as the first term when adding them sequentially.

Factor	Df	Marginal sum of squares	Marginal partial R^2	1st sequential sum of squares	1st sequential partial R^2
Host genus	8	3,709	0.03213	43,033	0.37277
Wolbachia	4	5,711	0.04947	49,576	0.42944
Köppen-Geiger climate	11	2,473	0.02142	32,467	0.28124
Localization	13	9,926	0.08598	34,013	0.29463

TABLE 5 | Brood size, sex ratio, developmental time and mortality outside and inside the host of populations of different crosses on *Sesamia calamistis* (*N* = number of replicates).

								Mortality outside the host		Mortality inside the host
Cross (male x female)	*N*	Brood size (mean ± SE)	*N*	Sex ratio (%female, mean ± SE)	*N*	Developmental time (days, mean ± SE)	*N*	Number of dead cocoons (mean ± SE)	Number of dead larvae not forming cocoons (mean ± SE)	Number of dead larvae (mean ± SE)
Cs Kit cured x Cs Kit cured	28	34.0 ± 3.3b	28	48.8 ± 5.3a	28	18.5 ± 0.5a	28	2.3 ± 0.4a	2.2 ± 0.4b	0.8 ± 0.6a
Cs Kit cured x Cs Kit	25	36.0 ± 4.2ab	25	45.5 ± 4.5a	25	17.8 ± 0.2a	25	1.8 ± 0.4a	0.5 ± 0.2a	0.5 ± 0.2a
Cs Kit x Cs Kit cured	25	23.2 ± 3.0a	24	44.1 ± 5.7a	25	18.8 ± 0.4ab	25	3.8 ± 0.8ab	2.7 ± 0.6bc	1.0 ± 0.3ab
Cs Kit x Cs Kit	22	34.2 ± 3.1b	22	62.7 ± 5.4b	22	20.0 ± 0.4b	22	5.9 ± 0.9b	3.8 ± 0.5c	1.7 ± 0.4b
Cs Mbsa cured x Cs Mbsa cured	20	32.1 ± 3.9b	18	64.2 ± 7.2a	20	21.1 ± 0.4a	20	6.4 ± 1.1c	1.0 ± 0.3a	0.6 ± 0.2a
Cs Mbsa cured x Cs Mbsa	34	41.8 ± 4.3b	34	58.1 ± 4.4a	34	20.2 ± 0.3a	34	5.6 ± 1.1bc	2.6 ± 0.5a	1.0 ± 0.3a
Cs Mbsa x Cs Mbsa cured	19	21.4 ± 3.8a	16	55.1 ± 6.4a	19	21.0 ± 0.3a	19	3.5 ± 0.5a	1.5 ± 0.3a	1.3 ± 0.4a
Cs Mbsa x Cs Mbsa	24	38.9 ± 4.0b	23	57.2 ± 6.7a	24	21.4 ± 1.4a	24	5.4 ± 0.7b	3.5 ± 1.2a	1.0 ± 0.3a
Cs Kit cured x Cs Mbsa cured	25	27.3 ± 4.2a	20	68.1 ± 6.9ab	25	21.7 ± 0.6b	25	7.4 ± 1.0c	6.5 ± 1.4c	8.1 ± 1.4b
Cs Kit cured x Cs Mbsa	19	41.5 ± 3.9a	16	78.7 ± 5.5b	19	20.1 ± 0.2a	19	2.7 ± 0.7a	2.7 ± 0.5b	1.5 ± 0.4a
Cs Kit x Cs Mbsa cured	25	34.1 ± 5.3a	25	52.2 ± 4.8a	25	21.8 ± 0.4b	25	4.4 ± 0.5b	1.6 ± 0.4a	1.5 ± 0.4a
Cs Kit x Cs Mbsa	32	39.0 ± 3.4a	30	73.0 ± 3.5b	32	19.6 ± 0.3a	32	5.1 ± 0.9abc	4.1 ± 0.7bc	2.0 ± 0.6a
Cs Mbsa cured x Cs Kit cured	20	27.5 ± 4.0ab	17	47.8 ± 6.3c	20	19.0 ± 0.2b	20	2.1 ± 0.6a	3.0 ± 0.7ab	1.9 ± 0.6a
Cs Mbsa cured x Cs Kit	25	34.1 ± 4.9b	23	67.4 ± 4.5d	25	21.7 ± 0.4c	25	6.8 ± 0.6b	4.1 ± 0.6b	6.3 ± 1.1b
Cs Mbsa x Cs Kit cured	19	29.9 ± 5.6ab	17	11.6 ± 7.2b	19	18.1 ± 0.2a	19	3.3 ± 1.1a	2.8 ± 0.8ab	0.6 ± 0.2a
Cs Mbsa x Cs Kit	23	20.8 ± 3.3a	23	00.0 ± 0.0a	23	19.7 ± 0.3b	23	2.8 ± 0.7a	1.5 ± 0.3a	1.8 ± 0.6a

Cs Kit, Cotesia sesamiae from the inland Kitale area of Kenya; Cs Mbsa, Cotesia sesamiae from the coastal Mombasa area of Kenya; cured, Wolbachia-free parasitoids colonies (i.e., cured lines); in crosses within each population and between populations, values with different letter are significant (q-value < 0.05; pairwise Wilcoxon's rank sum test, q-value = FDR corrected p-value).

3, which are mainly infected with the same wCsesB1 *Wolbachia* strain (**Figure 5**); they had been sampled mainly on *Busseola,* at least in one contact zone in Central Kenya (**Figure 2**). Otherwise, asymmetric gene flows were found between clusters. All the gene flows involving cluster 5 were oriented toward this cluster. The gene flow between Cluster 4 (found mainly on *Sesamia* and *Chilo*) and Cluster 2 was the lowest despite their geographic contiguity in Kenya (**Figure 2**). The Kit population from the laboratory colony was assigned to Cluster 2 and Mbsa population from the laboratory rearing to cluster 5 as inferred in Instruct clustering (**Table 1**). Therefore, migration was more orientated from the wCsesB1-infected population toward wCsesA/wCsesB2 bi-infected populations, mainly because of an asymmetric gene flow in that particular direction between Cluster 3 and Cluster 4.

DISCUSSION

Geographic, Ecological and Biotic Factors Determining the Genetic Structure of *Cotesia sesamiae*

The five major clusters inferred by the three different genetic clustering methods, TESS, Instruct and DAPC, exhibited a very similar geographic partition. However, TESS3 and Instruct admixture models were more concordant. DAPC results differed through the many geographic areas assigned to just one cluster. The DAPC algorithm optimizes a model without an admixture that assigns individuals and not a portion of their genomes to clusters. It seeks to maximize the discrimination between groups by partitioning the genetic variance into an among and a within group component. Models without admixture are not

robust to the inclusion of admixed individual in the sample. Reciprocally, models with admixture are less able to detect barriers when admixture is limited (François et al., 2010). In the absence of intrinsic biological reproductive barriers between the populations, we would expect the admixture model is the best suited because the five clusters are all represented in Kenya and Tanzania in a geographic continuum. However, the presence of reproductive isolation mechanisms may limit admixture in this geographic continuum. Indeed, the results of the Instruct non-spatial admixture model (**Figure 2**) shows that populations maintained their integrity, with admixture being limited to the hybridization zones despite the ability of *C. sesamiae* to expand throughout Africa. We will discuss below the factors that may limit admixture in this continuum in the light of our results on experimental crosses, *Wolbachia* strain distribution, host ecological specialization, climate, and the biology of *C. sesamiae*.

There are at least three strains of *Wolbachia* infecting *C. sesamiae* populations across Sub-Saharan Africa (Branca et al., 2011). We did not find bidirectional incompatibility between populations as a result of different infections. Only individuals infected with wCsesA and wCsesB2 strains showed incompatibility with cured or wCsesB1 infected Kit individuals. In a previous study, infected wCsesA/wCsesB2 individuals were already found to be highly incompatible with uninfected individuals (Mochiah et al., 2002b). However, cytoplasmic incompatibility was not assessed between wCsesB1-infected and non-infected or differentially infected individuals. The results for *Wolbachia* crosses involving wCsesB1 infected males and cured females does not present the normal CI phenotype because there was no increase in male proportion in the progeny. However, we observed a reduction in progeny size (males and females), leading to a reduced number of females. This result is coherent with *Wolbachia* invasion theory, since *Wolbachia* fitness is linked to the fitness, for which female progeny size is a proxy, of *Wolbachia*-infected females relative to their non-infected counterparts (Werren, 1997). However, the mechanism leading to the high mortality of male eggs in incompatible crosses remains unknown. Possibly, as diploid males are common in *Cotesia* wasps (Zhou et al., 2006; De Boer et al., 2007), part of the male progeny includes diploid males, which are also affected by cytoplasmic incompatibility. A direct effect on development, unrelated to fertilization, could also be considered. In a similar way, surprisingly, no strong incompatibility was observed between Mbsa wCsesA/wCsesB2 cured females and Mbsa infected males, as no biased sex ratio was found in the progeny. However, as in the case of Kit, a reduction in progeny size was observed which probably means that CI is expressed differently between individuals of the same genetic background (Kit or Mbsa) than when incompatible crosses occur between different genetic backgrounds. In the inter-population crosses studied here, an MD phenotype is very likely, as the male-biased sex ratio was not associated with significant progeny size reduction. The consequence of this unidirectional incompatibility will be asymmetric gene flows between differentially infected populations (Jaenike et al., 2006; Telschow et al., 2006). Indeed, CI is an efficient mechanism for *Wolbachia* to spread within populations by giving infected females a higher fitness. We should therefore expect the spread of individuals infected with wCsesA/wCsesB2 across the *C. sesamiae* geographical range, reflected by a higher migration rate from wCsesA/wCsesB2-infected populations toward other populations. However, using microsatellite markers, we observed conversely a lower migration rate from wCsesA/wCsesB2 - infected genetic clusters toward the other clusters (**Figure 5**), except for the migration between cluster 4 and 2. This unexpected result may be explained by local adaptation. Regions where *C. sesamiae* are infected with wCsesA/wCsesB2 are indeed dominated by avirulent parasitoids and susceptible hosts, whereas regions where *C. sesamiae* are infected with wCsesB1 are dominated by virulent parasitoid attacking resistant hosts. Females migrating from bi-infected to wCsesB1 regions are maladapted and killed by encapsulation, but females migrating from wCsesB1 to regions with wCsesA/wCsesB2-infected individuals are able to develop on the host. Yet, males infected with wCsesB1 can reproduce with bi-infected females wCsesA/wCsesB2, which would allow some gene flow from wCsesB1 to wCsesA/wCsesB2. In conclusion, *Wolbachia* incompatibility, even if it does not prove to be a very strong barrier to gene flow between locally adapted populations, may contribute to the expansion of avirulent parasitoid wasps that are not able to survive in some areas. In contrast, the spread of virulent parasitoids may be slowed down in areas where parasitoid populations are dominated by individuals infected with highly incompatible *Wolbachia*. This situation should lead to a stable or slow moving contact zone between populations and current genetic structure.

To disentangle the effects of geography, *Wolbachia* infection, parasitoid hosts, and other ecological factors, a statistical model was optimized using the *adonis* R function. The biotic and abiotic factors, including geography, that were analyzed in our statistical model explained more than 75% of the genetic variance. When looking at the factors most correlated to the genetic structure, our results are consistent with the hypothesis that ecology plays a significant role in reinforcing the *C. sesamiae* population structure across evolutionary time. Indeed, *adonis* analysis showed that the strongest determinant of genetic variance was *Wolbachia* infection, followed by the host species, and the least contributing factors were localization and climate. An illustration of the dominant effect of the host is the particular status of the population represented by cluster 1, consistently collected on *Sesamia nonagrioides*. This population also shows higher Fst when compared to the other populations in every clustering method, confirming that it constitutes a new species, as has recently been proposed (Kaiser et al., 2015, 2017). Another population corresponding to cluster 5 expands from Cameroon to East Africa and Uganda, through the Democratic Republic of Congo (**Table 2**). This region corresponds to the great Equatorial forest of Africa, which is characterized by hot and wet climatic conditions. The cluster 4, located from eastern Kenya to Mozambique along the Coast, is situated in a much drier area than cluster 5. This area is also important regarding hosts, since *B. fusca*, characterized as a resistant host, is rare in these regions (Le Ru et al., 2006; Moolman et al., 2014). The cluster 2 and 3 are both located in north-eastern Sub-Saharan Africa, but their positions

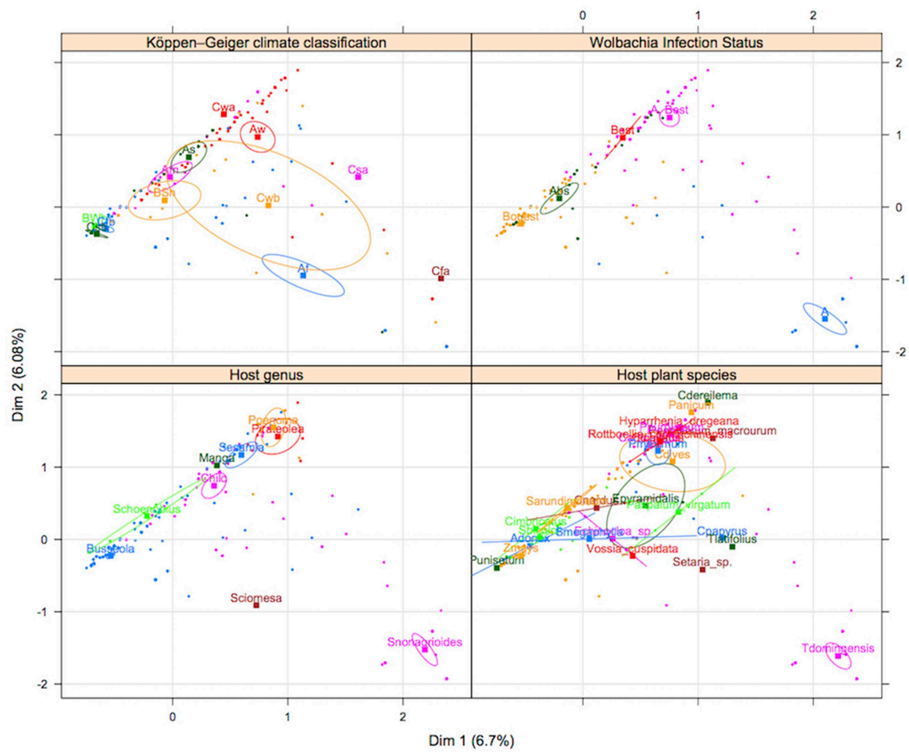

FIGURE 5 | Multiple correspondence analysis between ecological variables. Each point represents a data sample of *Cotesia sesamiae*. Each square represents the barycenter of each index in a categorical variable and the ellipse, the confidence at 0.95 of the barycenter estimates.

differ according to the clustering algorithm (i.e., western Kenya, Ethiopia, and Eritrea). In terms of climatic conditions, these regions are very similar but the observed clusters might reflect two sympatric populations with recurrent gene flows, as they are infected with the same *Wolbachia* strains (**Figure 3**).

These *C. sesamiae* populations show some geographic similarities with the genetic structure observed in the known resistant host *B. fusca* (Dupas et al., 2014), with five clusters observed across Africa, and a strong structure observed in East African Rift Valley regions, contrasting with the reduced structure observed in southern and central African regions. The cluster 3 of *C. sesamiae* located between the Eastern and Western Rift Valley has an overlapping distribution with the "H" cluster of *B. fusca*. The cluster 2 in the east of the Eastern Rift overlaps with the "KE" cluster of *B. fusca*. The cluster 4 of *C. sesamiae* ranges across eastern Africa at lower altitudes, where *B. fusca* is rare or absent (Dupas et al., 2014), and to the south. The clusters 4 and 5 exhibit large distributions that overlap with the "S" cluster of *B. fusca* from southern to eastern and central Africa (**Figure 2**). A fifth population is also present in both species. Cluster 1 of *C. sesamiae* corresponds to parasitic wasps infecting *S. nonagrioides* that have been described as a new species, *C. typhae*. Cluster "W" of *B. fusca* is only present in west Africa and isolated from the other *B. fusca* populations (**Figure 2**). These results suggest that *B. fusca* and *C. sesamiae* share a common phylogeographic history that explains the current genetic structure of both species. For instance, the highest diversity for both species has been found in the East African Rift Valley region. The East African Rift Valley location also explained the differentiation observed between two *C. sesamiae* lineages based on 6 mitochondrial and nuclear markers (Kaiser et al., 2015). One lineage corresponds geographically and ecologically to clusters 2 and 3, and the second one to cluster 4. The East African Rift Valley region has already been observed as a center of diversification for several species (Odee et al., 2012; Habel et al., 2015; Freilich et al., 2016; Mairal et al., 2017). This observed biological diversity has been related to both topological heterogeneity and variable climatic conditions that occurred since the formation of the East African Rift Valley region ca. 20 Mya, with the alternation of arid and wet periods (Sepulchre et al., 2006). Therefore, we could explain this observed pattern either by the colonization of the East African Rift Valley region, followed by diversification, or by the origin of both species lying in the East African East Valley region, which has been followed by a further extension with an admixture across Africa, except in west Africa, where *C. sesamiae* is rare (Gounou et al., 2008) and where *B. fusca* is totally isolated with zero migration observed to date (Sezonlin et al., 2006; Dupas et al., 2014).

Finally, because we found a strong association between the genetic cluster of *C. sesamiae* and the hosts they infect (**Figure 1** and **Tables 2**, **3**), we can hypothesize that genetic differentiations have occurred through host specialization and that the effect of geographic distance might be due to the distribution of hosts rather than isolation by distance.

Wolbachia and Biological Control

It is widely acknowledged that a better understanding of tritrophic interactions between plants, phytophagous insects and associated antagonists can help to develop better pest management strategies by identifying bottom-up and top-down effects in the food chain (Agrawal, 2000). *Wolbachia* can influence the outcome of trophic interactions, but the impact of *Wolbachia* on parasitoid host plant interactions has not received much attention. It was shown that a *Wolbachia* strain invasion temporarily reduces the impact of the parasitoid on its host (Branca and Dupas, 2006). But this reduction of impact can be sustained in the case of stable contact between incompatible strains in "hybrid" zones. Conversely, *Wolbachia* can reinforce adaptive divergence between locally host-adapted populations to the benefit of the parasitoid (Branca et al., 2009). *Cotesia sesamiae* is a good model to test the effect of *Wolbachia* on host parasitoid assemblages, as the four consensus genetic clusters differed for their *Wolbachia* and Lepidoptera host associations. In hybrid area, maladaptive gene flow may be observed but limited by *Wolbachia* incompatibility. This is the case between coastal (Mbsa) and inland (Kit) populations of the parasitoid (Dupas et al., 2008). The maladaptation may be strongest in the AS Köppen Geiger Climate Zone (corresponding to a dry mid-altitude agroclimatic zone) in wet seasons when *B. fusca* represents half of the host community (Ong'amo et al., 2006), whereas avirulent *C. sesamiae* toward *B. fusca* dominates parasitoid populations (Dupas et al., 2008). Strong counter-selection of avirulent alleles is expected in *B. fusca* abundance peaks. *Busseola fusca* is dominant in some seasons in mid-altitude areas where virulent alleles are dominant (Dupas et al., 2008). Although avirulent parasitoids are able to select hosts at contact, which may reduce maladaptation in the field, using contact cues to select hosts is risky because the host can bite and kill the parasitoid before oviposition can be made; 25% of *C. sesamiae* entering the stem tunnel are killed by *S. calamistis* larvae upon contact (Potting et al., 1999). The presence of partially incompatible *Wolbachia* strains in the virulent and avirulent parasitoid populations may favor their cohesiveness in balancing host communities across seasons. Hence, reducing gene flow between locally adapted populations toward their host, in the absence of premating isolation, might reduce maladaptation in hybrid zones, and our study confirms *Wolbachia* can reinforce this process. Nonetheless, *Wolbachia* influence is likely transient and relatively weak compared with selective pressure from the host toward the parasitoid wasp. For instance, very few heterozygous females between virulent and avirulent alleles on the bracovirus CrV1 locus have been found in a previous study, since they are likely maladapted (Branca et al., 2011). Therefore, we would expect a lack of recombination and a strong diversification on genes, particularly at the bracovirus locus, related to host specificity in *C. sesamiae*, a pattern that has yet to be investigated at the genome level.

Thompson (2005), in his seminal book on coevolutionary mosaics, stressed that gene flow had an ambivalent influence on coevolutionary interactions. Gene flow is essentially maladaptive, bringing locally maladapted genes to populations in interaction (Nuismer, 2006), but in the presence of negative frequency-dependent dynamics of coevolutionary interactions, rare new variants originating from other populations may be adapted. Our results show some congruence between *C. sesamiae* and *B. fusca* genetic structure (Dupas et al., 2014). Congruence with host structure is therefore observed at different ecological levels, not only at the level of host genus, as shown by *adonis* analyses, but also at the level of host populations. This may reduce maladaptation of *C. sesamiae* toward *B. fusca* and favor local coevolutionary interactions.

CONCLUSION

Our study presents a unique, comprehensive case for assessing the determinants of genetic structure in a parasitoid species, including multiple interactive biotic and abiotic forces. Like its main host *B. fusca*, the parasitoid is likely diversified across the East African Rift Valleys, where all the genetic clusters are found. Despite their wide distribution across Sub-Saharan Africa, some populations have maintained their integrity, as shown by the non-spatial admixture model. Two important results point toward the strong influence of hosts on parasitoid population dynamics and population genetics on a large geographical scale: (1) although the species genetic clusters appear to have diversified across East African Rift Valleys refuges, host species that are distributed across Africa later became the strongest factor determining genetic structure, rather than climatic selection and geographic isolation; (2) migration rates inferred from Bayesian analysis of microsatellite data suggest a limitation of gene flows due more to host adaptation than to *Wolbachia* infections. The latter result has fundamental importance in the context of a biological control program. As opposed to chemical control agents, biological control agents are expected to be able to cope with host evolution (Holt and Hochberg, 1997), but other interactions may limit this evolutionary sustainability. In our case, parasitoid wasps are able to cope with host evolution despite many additional biotic and abiotic ecological forces, including reproduction manipulators that would be expected to reduce local adaptation to hosts. The insect host dominates the piling up of all these factors and could explain why parasitoids can be very successful biological control agents even when introduced in climatically and geographically distant environments from their native settings (Stiling and Cornelissen, 2005). More generally, this work supports the hypothesis of the higher impact of ecological vs. neutral forces and of host vs. other ecological forces on the diversification of parasitoid—host interactions.

AUTHOR CONTRIBUTIONS

AB wrote the manuscript, designed the study, perform the statistical, and population genetics analyses. BL and BM collected sample on the field. BL also wrote the manuscript. P-AC supervised the crossing experiment and wrote the manuscript. JO performed the crossing experiment. CC-D and CP performed

the DNA extraction and genotyping. EH, JG, and PG wrote the manuscript. J-FS and LK-A wrote the manuscript and supervised the study. SD wrote the manuscript, designed, and supervised the study.

ACKNOWLEDGMENTS

The authors wish to thank IRD and ANR ABC PaPoGen (ANR-12-ADAP-0001) for funding the research.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00309/full#supplementary-material

Figure S1 | Delta-K of Evanno et al. (2005) for the Instruct inference of *Cotesia sesamiae* population genetic structure.

Figure S2 | Köppen-Geiger climate across Sub-Saharan Africa (Af, Tropical Rainforest; Am, Tropical Monsoon; Aw, Tropical Savannah; BSh, Hot Arid Steppe; BSk, Cold Arid Steppe; BWh, Hot Arid Desert; BWk, Cold Arid Desert; Csa, Temperate with Hot and Dry Summer; Csb, Temperate with Warm and Dry Summer; Cwa, Temperate with Dry Winter and Hot Summer; Cwb, Temperate with Dry Winter and Warm Summer; Cfa, Temperate without Dry Season and Hot Summer; Cfb, Temperate without Dry Season and Warm Summer).

Figure S3 | Distribution of genetic clusters of *Cotesia sesamiae* wasps for DAPC with $K = 5$ **(A)**, TESS3 software **(B)** and the Instruct software CLUMPP consensus with $K = 5$ **(C)**. For each clustering method, only individual with posterior probability of assignment above 0.5 are represented for each analysis. Distribution in Sub Saharan Africa is represented at the top and a zoom in Kenya at the bottom.

Table S1 | List of field collected *Cotesia sesamiae* wasp samples and associated geographic and ecological data.

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