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Evolving Reproductive Isolation in the Parasitic Wasp Genus Cotesia

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EVOLVING REPRODUCTIVE ISOLATION IN THE PARASITIC WASP GENUS *COTESIA*

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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All pictures photographed by J. Bredlau

Abstract

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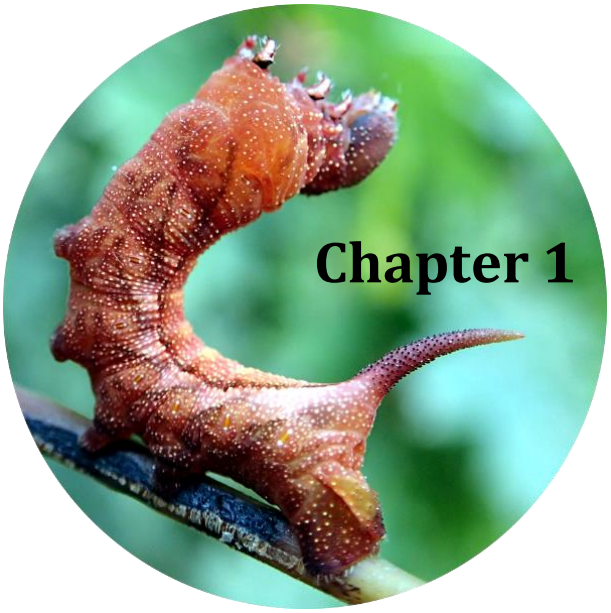
Major Director: Dr. Karen M. Kester, Department of Biology

Parasitic wasps are highly diverse and play a major role in suppression of herbivorous pest populations, but relatively little is known of the mechanisms driving their diversity. Molecular studies indicate that cryptic species complexes resulting from adaptations to specific hosts or host-foodplants may be common. The gregarious endoparasitoid, *Cotesia congregata* (Braconidae), is a model system for understanding parasitic wasp biology. It is reported to attack at least 15 species of sphingid caterpillars, most of which are plant family specialists. Molecular studies have demonstrated genetic differentiation of two host-foodplant complex sources originating from *Manduca sexta* on tobacco (MsT) and *Ceratomia catalpae* on catalpa (CcC). Response to female pheromone and elements of their courtship songs differ. Wasps from both sources mated and produced F_1 hybrid offspring in the laboratory; however, 90% of hybrid females resulting from one of the reciprocal crosses failed to produce offspring. I built on this previous work by evaluating an ecological barrier, the evolution of courtship songs within the genus, and patterns of hybrid sterility among four additional host-foodplant complexes, as well

as differentiation of their symbiotic bracovirus. Tests of developmental tolerance to nicotine demonstrate that MsT wasps are highly adapted to hosts feeding on tobacco, whereas CcC wasps experience high mortality. Acoustic analysis of courtship songs among host-foodplant sources of *C. congregata* and eleven additional species of *Cotesia* demonstrates that songs are species specific and appear to be correlated with genetic relatedness. *Cotesia congregata* from all sources mated and produced F_1 hybrid offspring in the laboratory; however, hybrid females resulting from specific reciprocal crosses failed to produce progeny. Dissections of hybrid females revealed that sterile wasps lacked mature ovaries and functional bracovirus, a symbiotic virus integrated into the wasp genome and necessary to suppress the host immune system. Relative *in vivo* expression of wasp bracovirus genes differs between MsT and CcC host-foodplant complexes. Cumulatively, these behavioral, ecological, and genetic barriers to reproduction indicate that *C. congregata* is diverged into two incipient species with limited gene flow, and provides insight into the role of varied reproductive barriers in speciation of parasitic wasps.



Chapter 1 Introduction

Introduction

Ecological speciation occurs when populations under different selective pressures from their environment evolve reproductive divergence (Rundle and Nosil 2005). When ecologically divergent populations occur in sympatry, limited gene flow may continue (Nosil 2008), leading to gradual transitions between ecological races and non-interbreeding species (Mallet 2008). Populations may even undergo alternating allopatric and sympatric stages during the long speciation process (Xie et al. 2007). However, models suggest that assortative mating must maintain isolation for full speciation (Bolnick and Fitzpatrick 2007). Ecological speciation may require multiple barriers to gene flow, such as divergence of courtship cues and post-zygotic genetic incompatibilities (see Matsubayashi et al., 2010 for review), which would develop during intermediate stages.

Ecological speciation may be initiated by host-plant shifts and then reinforced through host fidelity across generations. Host-plant shifts often provide a temporary selective advantage by reducing resource competition or attacks by specialist predators (Loxdale et al. 2011). Host-plant shifts and subsequent adaption to specific plants leads to divergent selective pressures, particularly among phytophagous insects (Funk et al. 2002). For example, alfalfa and clover host races of pea aphids prefer their natal plant in field experiments (Via et al. 2000). Likewise, hawthorn and apple host races of the fruit fly, *Rhagoletis pomonella*, arose rapidly after the colonization of apple with demonstrated host fidelity and temporal isolation based on diapause duration (Bush 1969; Feder et al. 1994). The *Rhagoletis* parasitoid, *Diachasma alloeum*, on hawthorn and blueberries also are preferentially attracted to the odors and mate on their natal host plant (Stelinski and Liburd 2005). Further, divergent host preference and host performance in a species of ladybird beetle has led to significant genetic divergence without other isolating

barriers, demonstrating the strong effect of habitat isolation (Matsubayashi et al. 2011). Ecologically divergent populations are predicted to exhibit greater reproductive isolation as demonstrated across several disparate taxa (Funk et al. 2006). Besides creating increased diversity by their own speciation, the divergence of phytophagous insects onto different host plants also may create new niches for predators, thereby generating further host-associated differentiation.

Specialist predators may undergo ecological divergence based on differing selective pressure by their preferred prey. Insect parasitoids in particular must adapt not only to a new host, but also their host's foodplant. Therefore, the specialization of phytophagous insects on host plants may lead to cascading host-associated differentiation (Stireman et al. 2006) or sequential radiation (Abrahamson and Blair 2008) of parasitoids in which diversification on one trophic level creates diversification on the next trophic level. For example, the host switch of *R. pomonella* from hawthorn to apple led to the development of ecological barriers to reproduction for its parasitoid, *D. alloeum*, on the two plant species (Forbes et al. 2009). Likewise, genetic evidence supports that the gallmaking moth *Gnorimoschema gallaesolidaginis* and its parasitoid *Copidosoma gelechiae* have undergone host-associated differentiation in multiple populations across their range (Kolaczan et al. 2009). Specialization on certain hosts leading to population divergence is likely dependent on a number of factors such as strong host fidelity, parallel life histories between host and parasitoid, fitness benefits to specialization that contributes to non-random mating, and mate choice correlated with host choice (Feder and Forbes 2010). Testing whether a host-parasitoid complex is undergoing divergence should account for at least these characteristics.

Parasitic wasps may be diversifying due to evolutionary influences from plants, hosts, or a combination of both. For example, parasitoids of leafmining Lepidoptera may evolve faster than their insect hosts, yet remain phylogenetically conserved according to their host-plant (Lopez-Vaamonde et al. 2005), which may be due to reliance on plant volatiles to locate hosts (Dicke and van Loon 2000). Besides volatiles, wasps must also be adapted to toxic plant chemicals sequestered by their hosts which may contribute to specialization (Harvey et al. 2005; Reudler et al. 2011). Determining the major factors contributing to parasitic wasp reproductive isolation remains a problem in understanding the evolution of parasitoids. Differences among potential host species, such as feeding in different environments and exposure to different plant chemicals, present different selective pressures for which the wasps must adapt. Also, differences in immune responses to wasp eggs create a pressure to specialize. Understanding these differing selective pressures can lead to understanding how ecological isolation and speciation occurs.

Diversity of parasitic wasps

Parasitic wasps are highly diverse with over 50,000 described species (LaSalle and Gauld 1991), and are grossly understudied with an estimated two or three times more species than currently described (Dolphin and Quicke 2001). The group displays rapid speciation associated with specific hosts (Kankare et al. 2005a; Stireman et al. 2006) and possibly the foodplants of these hosts. Parasitic hymenopteran genetic divergence is accelerated compared to other parasitic insects such as dipterans (Castro et al. 2002).

Genetic work has revealed a number of possible cryptic species complexes. For example, *Cotesia melitaearum* and *C. acuminata* attacking different checkerspot butterfly species in Spain consist of multiple cryptic species based on mtDNA sequences, microsatellite allele frequencies,

and behavioral experiments (Kankare et al. 2005b). Likewise, genetic barcoding of parasitic wasps in Costa Rica has revealed many assumed generalist species may in fact be a series of specialists of one or a few hosts (Smith et al. 2008, 2013); however, genetic barcoding does not provide information on mode of reproductive isolation or mechanisms of divergence. In one extreme example, genetic data identified 14 species of *Bellopius* parasitic wasps each emerging from one of 14 *Blepharoneura* fly species all existing on only two host-plant flowers in the same range (Condon et al. 2014). This first appeared to be niche overlap but was determined to be extreme niche partitioning since, although wasps would parasitize multiple fly species, most were only able to fully develop in one indicating a strong fly immune response that the wasps could not counter. The authors suggest that this complex mosaic of interactions among wasps and their host flies' immune systems provide support for the "escape and radiate" hypothesis for divergence. This immunological barrier may have contributed to ecological speciation among the 14 wasp species; however, the reproductive mechanisms that first maintained species isolation remain unclear. The process of divergence remains unknown as it appears that these are truly distinct species with no reproductive overlap. Undoubtedly, parasitic wasps are far more diverse than previously thought, but what are the underlying patterns that have led to this remarkable diversity?

Plant toxicity and immigrant inviability

Gauld *et al.* (1992) proposed that toxic secondary compounds in plants sequestered by potential hosts limits utilization by parasitic wasps, and therefore restricts parasitic wasp diversity in the tropics relative to temperate zones. However, parasitoids adapting to specific protected hosts could also be predicted to increase wasp diversity (Quicke 1997). The greater host-specificity of many tropical ichneumonid species compared their temperate close relatives

(Gauld and Janzen 1994), suggests that plant defensive chemicals could facilitate selective adaptation and divergence, even while still limiting potential colonization of new hosts. Even if a population or ecotype adapts to toxic plant chemicals, individual immigrants from other sources without those adaptations may not be able to survive or compete on the new toxic plant. Immigrant inviability thereby acts as a strong ecological reproductive barrier as it prevents populations from utilizing other host systems and restricts potential mating (see Nosil *et al.*, 2005). For example, nicotine in tobacco reduces developmental success of *Cotesia congregata* within their host, *Manduca sexta* (Barbosa et al. 1986, 1991; Thorpe and Barbosa 1986). Although populations of *C. congregata* adapted to *M. sexta* on tobacco have reduced developmental success on hosts with a nicotine diet, many of them still survive. Wasps not adapted to nicotine toxicity, such as those adapted to other host plants, may experience higher mortality when exposed to nicotine. Differences in tolerance to a plant defensive chemical may serve as an ecological isolating mechanism between parasitoid populations originating from hosts on different plants.

Behavioral reproductive barriers and sexual selection

Assortative mating is predicted to be necessary to maintain genetic isolation when gene flow is possible due to overlapping ranges. For example, the walking stick insect, *Timema cristinae*, from different host-plants discriminate in mate selection despite being capable of producing viable hybrids, thereby allowing genetic isolation to develop (Nosil et al. 2003, 2007). Assortative mating is often facilitated by different courtship signals which may be used for species recognition, to initiate a response in the opposite sex, and likely as a display of mate quality (Bradbury and Vehrencamp 2011). Signals may consist of stereotypical visual, chemical, and/or acoustic components which over time may become species specific (e.g. acoustic: Wilkins

et al., 2013, and chemosensory: Smadja & Butlin, 2009). For example, courtship songs among cryptic sibling species of green lacewings are controlled by a simple genetic architecture that permits evolutionarily fast sexual divergence by mate choice (Henry et al. 2002). Likewise, the courtship songs in the *Drosophila willistoni* species group are species specific, evolving differences due to sexual selection (Ritchie and Gleason 1995), with song differentiation developing more rapidly than other sexual or post-mating isolation (Gleason and Ritchie 1998). Similarly, pheromones may act as a species or population specific mechanism to elicit courtship. For example, *Drosophila montana* have population-specific cuticular hydrocarbon profiles that serve in mate choice (Jennings et al. 2014).

Among parasitic wasps, primary identified reproductive signals are both olfactory and acoustic, consisting of female pheromones and male courtship songs. Some male parasitic wasps display searching behavior in response to female pheromones by moving across the substrate and palpating with antennae to locate the source. They then perform rapid wing fanning that likely draws the pheromones over the scent glands and allows the male to orient toward the female (Vinson 1972). Following wing fanning, male parasitoids produce acoustic signals in the form of wing vibrations and pulses transmitted through the substrate (Sivinski and Webb 1989; Field and Keller 1993; Joyce et al. 2008). Acoustics have been described for six species in the Microgastrinae subfamily (Sivinski and Webb 1989; Danci et al. 2010; Joyce et al. 2010b; Bredlau et al. 2013), and each song is structurally distinct at least among these more distantly related species. However, only Joyce *et al.* (2010) have provided support for acoustic divergence among populations within the *Cotesia flavipes/sesamiae* species complex, suggesting that acoustic signals may play a role in reproductive isolation. However, the described differences

were minor and it is possible that wasps will mate despite slight differences in acoustic signals and genetics.

Post-zygotic reproductive barriers and hybrid sterility

According to the widely accepted Biological Species Concept (Mayr 1942), separate species status is determined by whether different populations mate and produce viable offspring capable of further reproduction. Many other species concepts have been proposed, some with overlapping definitions; alternative species concepts have been evaluated elsewhere (Coyne and Orr 2004) and will be avoided here in favor of describing the varied modes of reproductive isolation and genetic differences maintaining a reduction in gene flow. Post-zygotic reproductive barriers are divided into extrinsic and intrinsic isolation. Extrinsic isolation results from intermediate hybrid phenotypes not being well adapted to parental environments or intermediate courtship displays being unattractive to mates. Intrinsic isolation is often a result of incompatibles among genes leading to inviability or sterility. Intrinsic reproductive isolation between crosses of species and races of *Drosophila* has been well characterized (Coyne and Orr 2004; Jennings et al. 2011).

Various studies have demonstrated different degrees of reproductive isolation among parasitic wasps. Gounou *et al.* (2008) found that reciprocal crosses between populations of *C. sesamiae* have a slight reduction in mating although crosses still produced offspring. In contrast, Rincon *et al.* (2006) found mating incompatibility, along with genetic and morphological differences, among some geographically isolated populations of *Cotesia plutellae*. Desneux *et al.* (2009) reported complete reproductive isolation in mating crosses between two geographically isolated populations of the aphid parasitoid, *Binodoxys communis*, demonstrating that they are distinct cryptic species. These studies focused on allopatric wasp populations, and support the

formation of pre-zygotic mating barriers evolving before strong post-zygotic barriers. However, limited work has been conducted on wasps separated by host-plant usage rather than geographic barriers.

Immunological responses and the polydnavirus

Parasitic wasps and their hosts are in an evolutionary arms race against one another. The insect innate immune system is used to recognize, encapsulate, and kill foreign invaders such as parasitoid eggs. Wasps therefore have to adapt to overcome this hostile ovipositional environment. Many parasitic wasps accomplish this through the use of a symbiotic polydnavirus (PDV), which have coevolved with braconids for about 100 million years (Whitfield 2002; Murphy et al. 2008). The polydnavirus is a large double-stranded DNA virus that is integrated into the wasp genome and only replicates within specialized wasp calyx cells (Stoltz 1990; Belle et al. 2002). Virons are injected into the host during parasitization and quickly penetrate cells where viral genes are expressed. Unpackaged polydnavirus genes then disrupt host immune function by preventing haemocyte encapsulation of wasp eggs (for review: Gundersen-Rindal et al. 2013; Herniou et al. 2013c). Besides suppressing immunity, injected PDVs also alter metabolism and development to the benefit of eggs and developing larvae (Beckage et al. 1994).

Wasp eggs washed of calyx fluid containing virons are encapsulated and killed upon injection into a host, demonstrating the importance of PDVs in host immune suppression (Edson et al. 1981). PDVs also are host specific; parasitization of a novel host typically leads to egg encapsulation. For example, gypsy moth larvae (Erebidae) had no response to *C. congregata* PDVs despite close associations with other *Cotesia* (Lovallo et al. 2002). Likewise, *C. congregata* PDV early expressed proteins are produced in lower quantities in refractory Sphingid hosts that encapsulate eggs (Harwood et al. 1998). In *Cotesia sesamiae,* the Kenyan

coastal biotype is encapsulated in stemboring host *Busseola fusca*, while the inland population is not (Mochiah et al. 2002a). However, these populations are geographically isolated, and differences may be due to local adaptation. The host specificity of PDVs may be a driver for speciation as small differences in PDV genes from separate populations may be incompatible during hybridization.

Ecological speciation as a complex system

The relative contribution and rates of evolution of multiple reproductive barriers within any single system is usually uncertain (Matsubayashi et al. 2010). This is partially due to the difficulties of studying a complex system with many interdependent factors contributing to speciation. Difficulties arise when trying to singularly isolate factors contributing to speciation (ecological, molecular, and behavioral). Parasitic wasp speciation is governed by the properties of a complex evolutionary system in which each component level (wasp, host, plants, PDVs) interact with each other to contribute to the formation of reproductive isolation between populations at the wasp level. Isolating the effects of the host from the plant is challenging due to the direct influence plants have on hosts and is complicated by divergence on one level leading to divergence on the next. Likely, it is the interaction of several components that allow for speciation to occur via host-foodplant specificity. Ideally, phenotypic differences must be comprehensively characterized to understand traits contributing to a reduction in gene flow (Shaw and Mullen 2011). Evidence suggests that among insects sexual reproductive isolation is a result of divergence of multiple modes of sexual communication that contribute to assortative mating (see Mullen & Shaw, 2014). Therefore, the build-up of multiple reproductive barriers over time must be considered and studied together. Patterns of reproductive isolation, including acoustics, pheromones, and hybrid breeding success, have perhaps been best characterized for

sibling species in the genus *Drosophila* (e.g. Coyne *et al.*, 2002; Sawamura & Tomaru, 2002; Veltsos *et al.*, 2012). However, the chronological order of reproductive barriers is difficult to discern since current barriers do not strictly imply importance during speciation (Coyne and Orr 2004). Also, examples of incipient species may not necessarily imply that all will inevitably become full species. Many may indefinitely remain ecotypes or host races without completion of speciation.

Studying reproductive barriers in a "species continuum" in which sister taxa are at different stages of reproductive isolation would provide the opportunity to describe barriers that exist at different stages of divergence. For example, a species complex of a phytophagous insect on different host plants likely did not radiate all at once, but instead had a series of host shifts. Some host-plant complexes would therefore be more closely related to each other than to others in the same species complex and be at differing stages of reproductive isolation assuming selective pressures to specialize on their host. By examining both pre- and post-zygotic reproductive barriers, along with differences in hosts that contribute to ecological separation, the order and relative importance of isolating mechanisms may be discerned. Ecological barriers may be first to arise in some systems (Funk et al. 2002), but by examining a series of sister ecotypes and incipient species at different levels of isolation the roles of different reproductive barriers can be discriminated. Hosts shifts have been directly implicated in speciation of only a few insects; there are far more examples of host shifts contributing to speciation (Forbes et al. 2017).

Study System

Cotesia congregata (Say) (Hymenoptera: Braconidae) is an ideal species for investigating both underlying reproductive and ecological isolation mechanisms leading to speciation among

parasitic wasps. This species is common within its range and serves as a model system for host-parasitoid interactions and immunology (Harwood et al. 1998; Beckage 2008), insect learning (Kester and Barbosa 1991b; Lentz and Kester 2008; Lentz-Ronning and Kester 2013), and tri-trophic interactions (Kester and Barbosa 1994; Kester et al. 2002). *Cotesia congregata* belongs to the large and economically important subfamily Microgastrinae which contains over 2,000 described species, but Rodriguez et al. (2013) estimate that the true species richness is likely 17,000 to 46,000+. The genus *Cotesia* contains ~85 described species in the United States and Canada (Whitfield 1995) and an estimated 1,000 species globally (Michel-Salzat and Whitfield 2004), few of which are well studied. Molecular phylogenies include 24 species of *Cotesia* (Michel-Salzat and Whitfield 2004) and the Sphingid hosts of *C. congregata* (Kawahara *et al.*, 2009; Fig. 1), which can be used to make predictions of common or diverged characteristics. Moreover, *Cotesia* contains many important biocontrol agents of herbivorous pests, both native and invasive.

Once an appropriate host is located, an adult female oviposits multiple eggs inside the caterpillar. More than one wasp may parasitize a caterpillar, resulting in broods as high as 500 larvae, although 50-200 is more typical. Brood size is highly variable but data support that mean brood size is related to wasp host origin (the largest broods coming from host *Eumorpha pandorus*, and the smallest from *Darapsa myron* and *Ceratomia catalpae*). The wasp larvae feed on nutrients in the hemolymph inside the host until they egress and spin cocoons on the caterpillar. Six to eight days after larval egression, adult wasps emerge from their cocoons. Because *C. congregata* is haplodiploid, fertilized eggs normally develop into females, and unfertilized eggs develop into males.

Cotesia congregata has characteristics that make it likely to be undergoing host-associated differentiation (see Feder & Forbes 2010). This species is reported to attack caterpillars of several hawkmoth species (Sphingidae) with a range of foodplants but most are plant family specialists (Tietz, 1972; Krombein *et al.*, 1979; Table 1). However, laboratory and field evidence demonstrate that populations of *C. congregata* are adapted to locally abundant host foodplants (Kester and Barbosa 1991a, 1994). At least three generations may occur in a year in Virginia, but reproduction is necessarily parallel to the generation times and abundance of host caterpillars – all of which have at least two generations per year. Typically, newly emerged wasps mate with the cohort on the same plant species from which they emerged before females seek out hosts for oviposition (Kester and Barbosa 1991b), leading to mate choice being directly tied to hosts. Also, post-emergence learning may contribute to assortative mating (Kester and Barbosa 1991b) and allocation of fertilized eggs (Lentz and Kester 2008). Additionally, the toxicity of some host plants induces a fitness benefit to wasps that adapt to the plants and may serve as an isolating mechanism. However, the degree of host fidelity in the wild is uncertain, and laboratory reared wasps will parasitize and properly develop in different available hosts.

Prior Research

Prior work on *C. congregata* has focused on two host-foodplant complexes, wasps originating from tobacco hornworms (*Manduca sexta*) on tobacco (MsT) and catalpa sphinx (*Ceratomia catalpae*) on catalpa (CcC). Molecular studies using the mitochondrial COI locus and seven microsatellite loci indicate that MsT and CcC wasps are genetically distinct regardless of geographic distance or whether they occur in sympatry (Kester et al. 2015). *Cotesia congregata* has been demonstrated to learn plant cues post-emergence and subsequently display

a stronger searching response on that plant which could facilitate pre-zygotic isolation (Kester and Barbosa 1992; Lentz-Ronning and Kester 2013).

Cotesia congregata originating from MsT and CcC host-foodplant complexes have potential reproductive barriers that could lead to speciation. 1) Males respond less to the female pheromone of the other host-foodplant complex, 2) male courtship songs have significant differences but the same overall structure, similar in magnitude to those reported for *Cotesia flavipes/sesamiae* (Joyce et al. 2010b) and 3) wasps mate under confined laboratory conditions, however ~90% of hybrid females from crosses between CcC males x MsT females failed to produce offspring (Bredlau and Kester 2015). These females have their eggs encapsulated within the host possibly due to the non-expression of the polydnavirus, a symbiotic virus used to suppress the host immune response (Beckage 1998; Whitfield and Asgari 2003) and is adapted for a limited range of hosts (Harwood et al. 1998). Differences in encapsulation of wasp eggs have been found in other parasitic wasp species such as *Cotesia sesamiae*, however reproduction is complicated by the presence of *Wolbachia* (Mochiah et al. 2002a), which is not present in *C. congregata*.

Cumulatively, previous work indicates that MsT and CcC host-foodplant complexes of *C. congregata* are incipient species. However, despite a clear post-zygotic isolation mechanism, the potential mating barriers tested were not different enough to prevent mating. The reproductive barriers tested are likely in the early stages of divergence and therefore have not had enough selective pressure to be used as isolating mechanisms in no-choice encounters. The relative development times of reproductive barriers could be determined by comparing host-plant complexes that are likely more distantly related based on hosts, which are predicted to have greater post-zygotic barriers.

Objectives

Two newly identified incipient species of *C. congregata* differ with respect to courtship signals and although they will mate and produce hybrid offspring in the laboratory, ~90% of F_1 hybrids resulting from CcC♂ x MsT♀ matings fail to produce offspring (Bredlau and Kester 2015). However, fourteen other hosts have been reported for *C. congregata*, some of which may be hosts of new incipient or distinct species. I tested the hypothesis that *C. congregata* consists of a cryptic species complex, and examined underlying mechanisms that could explain this diversity. Contrasting across multiple host-plant complexes and *Cotesia* species that utilize more distantly related hosts and host-foodplants provided the opportunity to investigate several major questions:

1. Do MsT and CcC wasps differ in tolerance of toxic plant chemicals (nicotine)? If so, tolerance to plant chemicals may serve as an important ecological isolation mechanism (Chapter 2).
2. What is the pattern of courtship song evolution in the genus *Cotesia*? Can host-foodplant sources of *C. congregata* be differentiated by songs? Courtship songs likely play a role in species recognition but differences may evolve after other reproductive barriers (Chapter 3).
3. Is the currently recognized species, *C. congregata*, actually a "species continuum" that contains multiple races and species at varying stages of genetic isolation based on host-plant complex specialization, discrete clusters of interbreeding groups, or a radiation of partially compatible host-plant complexes? Patterns of reproductive compatibility across host-foodplant complexes would indicate likely drivers of isolation (Chapter 4).

4. Does expression of bracovirus genes differ between MsT and CcC wasps and their hybrids? Differences in expression of bracovirus genes may be related to host usage and failure of certain hybrid lines to develop (Chapter 4).

TABLES AND FIGURES

Table 1.1: Reported hosts of *Cotesia congregata* (Krombein et al. 1979) and their common host-foodplant (Tietz 1972). Most of these caterpillars will feed on other plant species in the same family; the plant species on which they are most likely to be found is listed here.

Host	Common foodplant	Plant family	Plant order	Clade/class*
Manduca sexta	tobacco, tomato	Solanaceae	Solanales	asterid[1]
M. quinquemaculatus	tobacco, tomato	Solanaceae	Solanales	asterid[1]
Agrius cingulata	sweet potato	Convolvulaceae	Solanales	asterid[1]
Ceratomia catalpae	catalpa	Bignoniaceae	Lamiales	asterid[1]
Paratrea plebeja	trumpet vine	Bignoniaceae	Lamiales	asterid[1]
Sphinx kalmiae	privet, ash, lilac, etc.	Oleaceae	Lamiales	asterid[1]
Sphinx chersis	privet, ash, lilac, etc.	Oleaceae	Lamiales	asterid[1]
Hemaris diffinis	honeysuckle	Caprifoliaceae	Dipsacales	asterid[2]
Dolba hyloeus	holly	Aquifoliaceae	Aquifoliales	asterid[2]
-	pawpaw	Annonaceae	Magnoliales	Magnoliids
Darapsa myron	Virginia creeper, grape	Vitaceae	Vitales	rosid
Eumorpha achemon	Virginia creeper, grape	Vitaceae	Vitales	rosid
Eumorpha pandorus	Virginia creeper	Vitaceae	Vitales	rosid
Sphecodina abbottii	grape	Vitaceae	Vitales	rosid
Hyles lineata	several, incl. grape	several	Myrtales, Rosales…	rosid
Lapara coniferarum	pine	Pinacene	Pinales	Pinopsida

*Asterids and rosids are clades within eudicots. Superscript indicates a branch within the clade asterid.

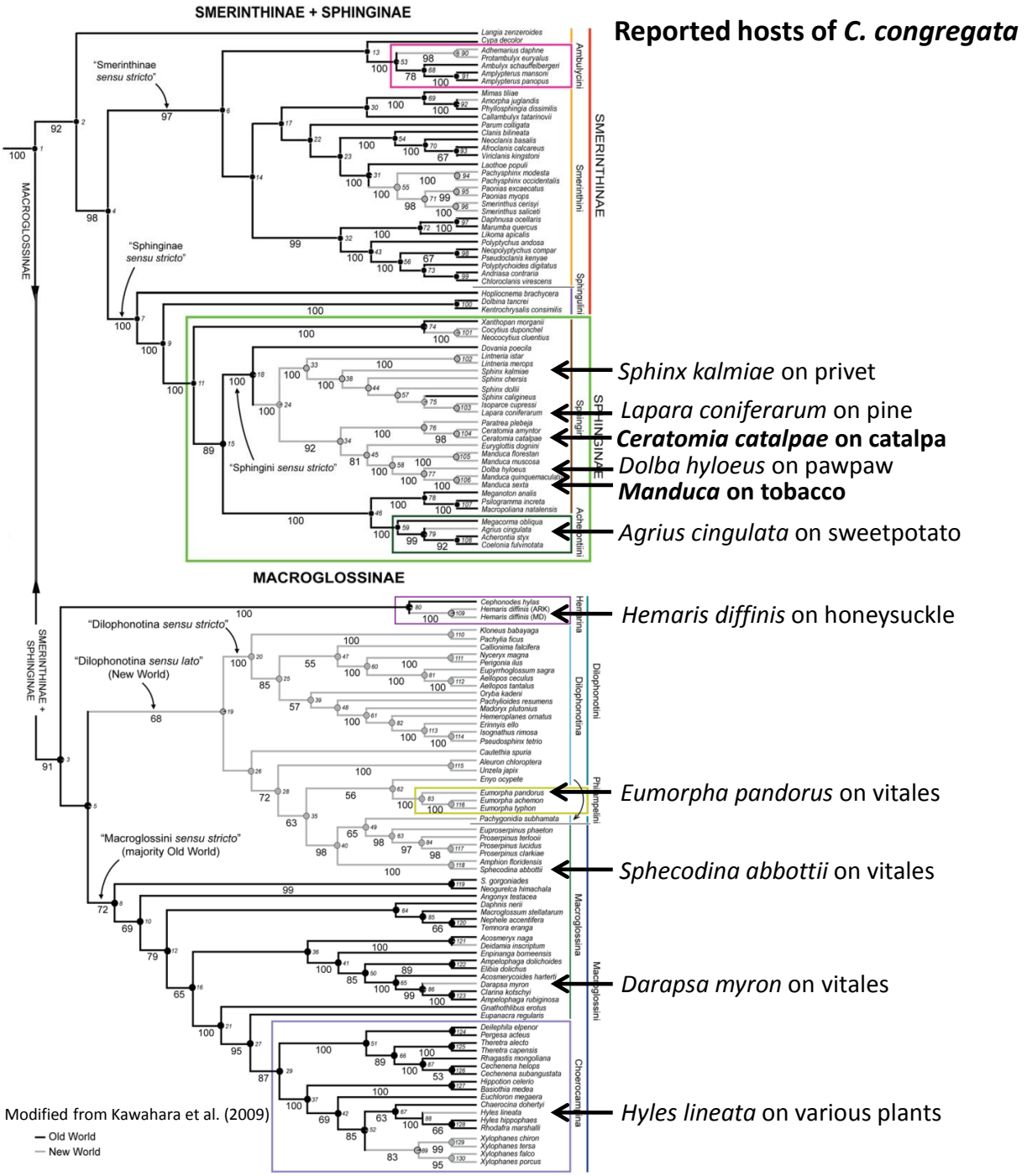
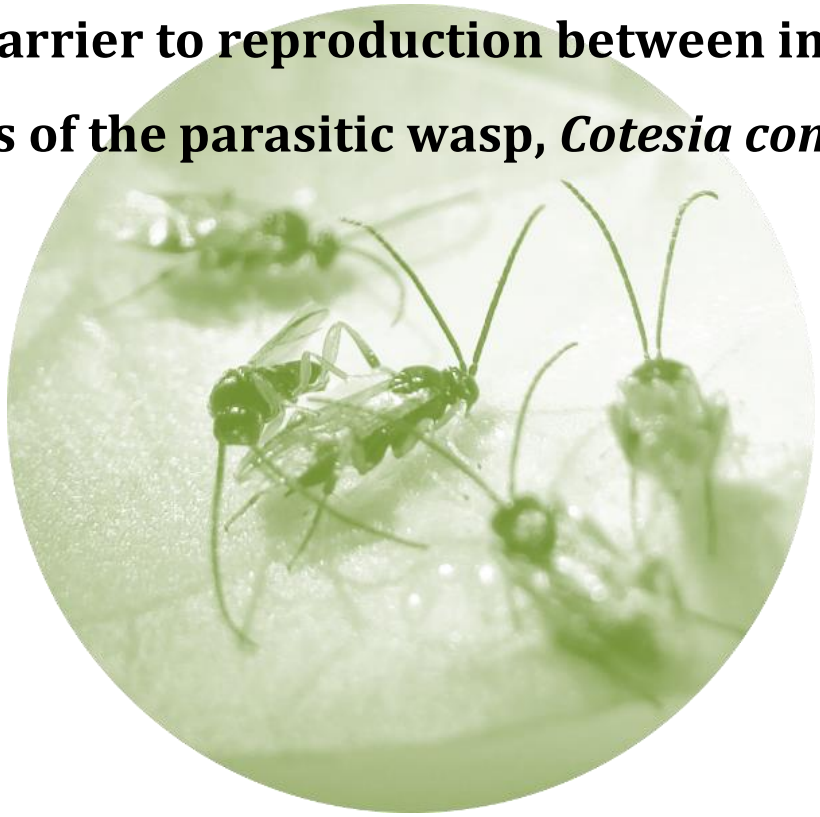


Fig 1.1: Phylogenetic tree of hawkmoths (Lepidoptera: Sphingidae) with arrows indicating targeted hosts and common host-foodplant of *Cotesia congregata.* Wasps from *Manduca sexta* (MsT) and *Ceratomia catalpae* (CcC) host-foodplant complexes have been previously compared (bold), but they also have among the most closely related hosts. Additional hosts were collected and reared, and resulting wasps were cross-bred to create hybrids to test for reproductive isolation. Their songs were recorded for comparison. Figure modified from Kawahara *et al.*, (2009).

Chapter 2 Developmental sensitivity to nicotine as a barrier to reproduction between incipient species of the parasitic wasp, *Cotesia congregata*



Developmental sensitivity to nicotine as a barrier to reproduction between incipient species of the parasitic wasp, *Cotesia congregata*

Justin Bredlau and Karen Kester

Abstract— Nicotine evolved as a plant defense against insect herbivores and in response, tobacco-feeding insects, as well as their endoparasitoids, evolved counter defenses. We tested the hypothesis that two incipient species of the braconid wasp, *Cotesia congregata* (Say), and their reciprocal hybrids differ in developmental responses to nicotine dosage level in the host diet. "MsT wasps" originated from the solanaceous specialist, *Manduca sexta* L. ("tobacco hornworm") on tobacco, and "CcC wasps" from the catalpa specialist, *Ceratomia catalpae* Boisduval ("catalpa sphinx") on catalpa. Reciprocal crosses were established by pairing unmated males and females. Females were permitted a single oviposition into a 4th-instar larva of *M. sexta*. Parasitized caterpillars were fed on a laboratory diet with 0%, 0.1%, or 0.3% wet weight nicotine until wasp larvae egressed from the host; subsequently, the number of egressed and unegressed larvae, and emergent adults by sex from each host were counted. Tobacco-adapted MsT wasps responded to dietary nicotine in a dosage-dependent manner with a significant increase in larval mortality at 0.3% nicotine. In contrast, CcC wasps experienced high mortality even at 0.1% nicotine. Offspring resulting from hybrid crosses differed in responses with respect to the maternal wasp source. CcC mothers produced female-biased sex ratios of emergent adults, whereas MsT mothers produced both haploid males and hybrid females. Female-biased sex ratios indicate differential larval mortality of haploid progeny. Results suggest that nicotine tolerance is a dominant heritable trait, and that host diet can function as an ecological isolating mechanism between MsT and CcC incipient species.

Keywords: ecological speciation, plant chemical defense, *Manduca sexta*, *Ceratomia catalpae*, tritrophic interaction, habitat isolation

Introduction

Plants produce an array of toxic secondary compounds in response to herbivory. These chemicals play an important role in tri-trophic interactions among plants, insect herbivores, and their natural enemies. Specialist herbivores survive on defended plants by diverse adaptations to metabolize or sequester chemicals, which can provide a selective advantage by chemically shielding them from predators and parasitoids (Kant et al. 2015 for review). Parasitoid fitness can be negatively impacted without similar adaptations when using hosts that sequester toxic plant chemicals (Ode 2006). For example, generalist parasitoids have been reported to have impaired development when utilizing hosts on chemically defended plants; in contrast, specialist parasitoids tend to display higher fitness and development within similarly defended hosts (Harvey et al. 2005; Lampert et al. 2011b; Reudler et al. 2011).

Different populations within a species may have differential tolerance to plant chemicals by local adaptation or phenotypic plasticity. For example, populations of the generalist aphid, *Myzus persicae*, have developed chemical tolerance upon host-range expansion to lupine-specific alkaloids in narrow-leafed lupin (Cardoza et al. 2006) and to nicotine in tobacco (Ramsey et al. 2014). Individuals from other non-adapted populations displayed reduced growth or fecundity. Therefore, when a population or ecotype adapts to toxic plant chemicals, individual immigrants from other sources without those adaptations may be unable to survive or compete as effectively. Immigrant inviability thereby can act as an ecological reproductive barrier as it prevents populations from utilizing the same host/plant systems and restricts mating (Nosil et al. 2005; Nosil 2012). These effects may likewise separate populations of parasitoids as they adapt to sequestered chemicals in hosts feeding on different plants.

The gregarious endoparasitoid, *Cotesia congregata* (Say) (Hymenoptera: Braconidae), is an ideal species for investigating the role of plant-mediated ecological divergence and adaptation among parasitic wasps. This species has served as a model system for tri-trophic interactions (Kester and Barbosa 1994; Kester et al. 2002), learning (Kester and Barbosa 1991b; Lentz and Kester 2008; Lentz-Ronning and Kester 2013), and immunology (Harwood et al. 1998; Beckage 2008). *Cotesia congregata* has characteristics that suggest it is undergoing ecological differentiation by host (see Feder & Forbes 2010). This species is reported to attack caterpillars of at least fourteen Sphingid species utilizing a range of foodplants, most of which are plant family specialists (Tietz, 1972; Krombein *et al.*, 1979). Laboratory and field evidence demonstrate that populations of *C. congregata* are adapted to locally abundant host foodplants (Kester and Barbosa 1991a, 1994). Typically, newly emerged wasps mate within its cohort on the same plant species from which they emerged before females seek out hosts for oviposition (Kester and Barbosa 1991b). Therefore mate choice is directly tied to hosts or host-foodplants. Also, post-emergence learning may contribute to assortative mating (Kester and Barbosa 1991b) and sex ratio allocation of fertilized eggs (Lentz and Kester 2008). The degree of host fidelity in the wild is uncertain, but laboratory reared wasps will parasitize and properly develop in different available hosts (Kester, unpublished data).

Our recent work on *C. congregata* has focused on two host-foodplant complex sources: wasps originating from *Manduca sexta* (tobacco hornworm) on tobacco ("MsT wasps") and *Ceratomia catalpae* (catalpa sphinx) on catalpa ("CcC wasps"). Molecular studies using the mitochondrial COI locus and seven microsatellite loci indicate that MsT and CcC wasps are genetically distinct even in sympatry (Kester et al. 2015). The two host-plant complex sources of wasps have a lower response rate to the reciprocal female pheromone, and male courtship songs

differ in amplitude, frequency, and duration of some song components. Wasps mate under confined laboratory conditions, but ~90% of hybrid females from matings between CcC males x MsT females fail to produce offspring (Bredlau and Kester 2015). Therefore MsT and CcC host-foodplant complexes of *C. congregata* are considered as incipient species.

The primary host plants of MsT and CcC wasps have substantial differences in chemical composition. The MsT incipient species develops within *M. sexta* feeding on solanaceous plants such as tobacco and tomato, many of which produce toxic alkaloids. The alkaloid in tobacco, nicotine, serves as a chemical defense against most herbivores. For example, Steppuhn et al. (2004) demonstrated that tobacco modified for reduced nicotine production were more susceptible to insect herbivores, had three times more feeding damage in field plots, and was preferred by *M. sexta* over wild type tobacco. Likewise, nicotine can have direct effects on parasitoids of hosts feeding on nicotine diets. The parasitic wasp, *Hyposoter annulipes*, experiences dose dependent mortality when developing within fall armyworm, *Spodoptera frugiperda*, feeding on diets at different nicotine concentrations (El-Heneidy et al. 1988). *Manduca sexta* is adapted to alkaloids through detoxification and rapid excretion (Snyder et al. 1993, 1994; Wink and Theile 2002). Survival of *M. sexta* and its parasitoid is dose dependent; however, *C. congregata* has a much greater sensitivity than its host (Barbosa et al. 1991). Hornworms feeding on artificial diet containing nicotine that are parasitized by *C. congregata* had significantly decreased parasitoid larval egression (Thurston and Fox 1972; Barbosa et al. 1986). Thorpe and Barbosa (1986) found that fewer parasitoids egress from *M. sexta* on a high-nicotine variety of tobacco compared to a low-nicotine variety. Although nicotine reduces brood sizes even at low concentrations, 77% of wasp larvae still egressed from hosts fed 0.1% nicotine artificial diet (Barbosa et al. 1986). Dietary nicotine even has an effect at an additional trophic

level – the hyperparasitoid *Lysibia nana* has reduced survival when developing within *C. congregata* on 0.5% wet weight nicotine (Harvey et al. 2007).

Instead of alkaloids, catalpa contains iridoid glycosides such as catalpol sequestered by the specialist caterpillar, *C. catalpae* and may accumulate in small quantities in *C. congregata* (Bowers 2003; Lampert et al. 2011a). Catalpol deters feeding by non-adapted insects (Bowers and Puttick 1988) but does not have an apparent impact on the development of *C. congregata* (Lampert et al. 2010). Of the two chemicals, nicotine likely has the most potent effect on insect herbivores and their parasitoids. Prior research has only tested *C. congregata* on the host-plants from it originated, but testing tolerance on other plants or plant chemicals has not occurred.

We tested whether the incipient species, MsT and CcC, differ in their survival on hosts feeding on a nicotine diet. Both incipient species were reared on *M. sexta* fed an artificial diet containing nicotine. Hybrids between MsT and CcC wasps were also tested for nicotine tolerance to determine patterns of inheritance. We predicted that CcC wasps would not be adapted to nicotine consumed by *M. sexta* and therefore would have reduced egression from hosts. This would limit CcC wasps utilizing *M. sexta* as a host and tobacco as a host-plant, and facilitate ecological isolation between these two incipient species.

Materials and Methods

The parasitoids originated from caterpillars at two sites in central Virginia USA. "MsT wasps" came from a laboratory colony established in 2005 from *M. sexta* feeding on cultivated tobacco (*Nicotiana tabaccum* L.) at the Southern Piedmont Agricultural Research and Experimental Station near Blackstone, Nottoway County – "BLACKSTONE" site (37.0817, -77.9755). The laboratory colony is supplemented annually with wild wasps from this site. "CcC wasps" were collected from *C. catalpae* feeding on mature catalpa trees (*Catalpa speciosa*

Warder) at a private property in Cumberland County – "TYSON" site (37.7127, -78.1639). Wasps from these locations were previously used in genetic and behavioral studies (Kester et al. 2015; Bredlau and Kester 2015). Collected caterpillars were kept in plastic containers (28 x 16 x 11 cm; 10-15 caterpillars in each) with leaves from their respective plant. Upon parasitoid larval egression, caterpillars were isolated in separate cups. Parasitoid cocoons were placed individually into clear gel capsules (size 00) three days after formation and resulting emergent adults were sexed under a dissecting microscope.

Wasps were used to establish four types of mating pairs: MsT♂ x MsT♀, MsT♂ x CcC♀, CcC♂ x MsT♀, and CcC♂ x CcC♀ (146 total pairings). Note that since *C. congregata* is haplo-diploid only females are hybrids in the F_1 generation, and males inherit only maternal chromosomes. Two males and one female were kept in a glass vial (7 cm x 2 cm diam.) with a water-soaked piece of cotton ball, a 1 cm^2 piece of catalpa or tomato leaf to promote mating, and plugged with a honey-streaked cotton ball. Mating groups were stored under ambient laboratory conditions (22 ± 2°C; 20-50% RH). Wasps were given two days to mate, and then each female wasp was presented with laboratory-reared early 4^{th} instar *M. sexta* larvae for oviposition on three separate days. The three parasitized caterpillars from each wasp mating group were randomly assigned to one of three diet treatment groups.

Parasitized caterpillars from each cross type and pure line controls were placed on blocks of laboratory diet (approximately 2 x 2 x 1 cm) modified from Yamamoto et al. (1969). Diet was modified to contain 0%, 0.1%, or 0.3% wet weight nicotine (≥99% (GC) (-)-nicotine, N3876, Sigma-Aldrich, St. Louis, MO), which is at or below the range of wild *Nicotiana* (0.3-2% wet mass) and cultivated tobacco (0-1.9% wet mass) (Vandenberg and Matzinger 1970; Sisson and Saunders 1983). Diet blocks were replaced with fresh diet every two days. Individual caterpillars

were reared in separate plastic cups (7 cm diam. x 4 cm) until three days after egression of wasp larvae or the appearance of black spots on the caterpillar from wasp larvae attempting to egress. Wasp cocoons were counted and isolated in gel capsules until wasp emergence. Emerged adults were sexed and counted. Caterpillars were dissected after removal of cocoons or three days after the appearance of black spots if no wasp larvae egressed. All unegressed wasp larvae still within the caterpillars were removed and counted.

Statistical Analysis. "Clutch size" indicates the total number of larvae from all eggs oviposited which includes those that egressed from the host and those that failed to egress. "Brood size" refers to the total number of larvae that egressed from the host. Clutch size was square root transformed (sqrt + 0.5) and compared among cross types and diet types to determine whether nicotine affected the development of larvae or only inhibited their exit from the caterpillar host using a two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test.

Since *C. congregata* is haplodiploid, males have only maternal genes and only females are hybrids in the F_1 generation. Therefore, broods consisting of only males (from females that failed to mate) from the reciprocal crosses were combined with their respective control cross to compare wasp larval survival between MsT and CcC wasps. Brood size of egressed wasps was compared between MsT and CcC wasps across diet treatments using a two-way analysis of covariance (ANCOVA) followed by Tukey's post-hoc test. Clutch size was used as a covariate in the model because it varied by individual cross and source, was not affected by diet type (see *Results*), and may affect brood sizes of emerged larvae. Brood size was square root transformed (sqrt + 0.5) to meet assumptions of ANCOVA.

For the hybrid crosses that produced females, brood size was compared across nicotine diet treatments within each cross using ANOVA followed by Tukey's post-hoc test. Proportion of hybrid females making up egressed wasps were compared within the two types of hybrids and the MsT non-hybrid group across the three nicotine diet treatments using ANOVA followed by Tukey's post-hoc test for each factor. Comparisons within the CcC group were not appropriate due to low survivorship of broods reared on any nicotine diet. Proportion female was arcsine transformed to meet the assumptions of ANOVA. All statistical analyses were performed in SAS and JMP v11 (SAS Institute, Cary, NC).

Results

Clutch Sizes of Incipient Species. Dissections revealed that remaining wasp larvae were fully developed 2nd instars within the host. Clutch size (egressed and uneggressed larvae total) by diet and cross type was significant ($F_{11, 277} = 7.701$, $p < 0.0001$) and did not differ among nicotine levels ($F_{2, 277} = 0.4065$, $p = 0.67$); however, cross type did affect clutch size ($F_{3, 277} = 26.27$, $p < 0.0001$). Clutch size was determined by the female parent (mean ± SE: MsT = 94.0 ± 3.6 larvae, n = 141; CcC = 157.0 ± 5.9 larvae, n = 148; Table 1). Since clutch size differed between MsT and CcC wasps, and could affect the number of egressed larvae, clutch size was used as a covariate in subsequent statistical models testing for differences in number of egressed larvae.

Brood Sizes of Incipient Species. Wasps originating from the tobacco source (MsT) were more tolerant of nicotine than wasps originating from the catalpa source (CcC). Brood size (egressed larvae) was significant by source and diet ($F_{6, 165} = 52.83$, $p < 0.0001$), and there was an interaction between wasp source and diet ($F_{2, 165} = 27.46$, $p < 0.0001$). Brood size of both types of wasps decreased with increasing nicotine (Table 1); MsT broods decreased by ~25% between diet levels, whereas CcC broods decreased by over 80% from the control diet to 0.3%

nicotine (Fig. 1). Hosts parasitized by MsT wasps always produced egressed larvae regardless of nicotine (all 29 at 0.3%). In comparison, 18 of 30 hornworms parasitized by CcC wasps produced no egressed larvae at 0.3% nicotine. These caterpillars developed dark spots on their cuticle where wasp larvae unsuccessfully attempted to chew their way out of the host.

Hybrid Females. Hybrid females resulting from MsT and CcC crosses displayed the same tolerance to nicotine as MsT wasps, regardless of cross type (Fig. 2). Number of egressed larvae and proportion of females from the CcC non-hybrid group could not be appropriately compared since most broods failed to produce wasps or failed to mate, creating a low sample of broods with females. CcC♂ x MsT♀ broods containing females did not have a significant reduction in egressed larvae ($F_{2, 23} = 1.392$, $p = 0.27$). MsT♂ x CcC♀ broods had a ~50% reduction in the number of egressed larvae from 0% nicotine to 0.1% and 0.3% nicotine ($F_{2, 40} = 9.250$, $p = 0.0005$) due to a disproportionate number of haploid males not surviving (Table 2).

Since F_1 males inherit only maternal chromosomes (haplodiploidy), males produced by CcC mothers failed to egress regardless of cross type. Therefore broods were highly female biased (>90% female) from MsT♂ x CcC♀ when their host is fed a nicotine diet ($F_{2, 43} = 4.043$, $p = 0.0246$; Fig. 3). At 0.1% nicotine 11/14 broods were >90% females and 4/14 were 100% female. At 0.3% nicotine 15/17 broods were >90% female and 7/17 were 100% female. In contrast, the 0% nicotine diet group had only 4/13 that were >90% female and none at 100%. The sharp decline in brood sizes between 0% and 0.1% nicotine groups was due to the inability of haploid males to egress from their host (Fig. 2). One brood did yield a normal number of males even at 0.3% nicotine.

CcC♂ x MsT♀ crosses did not have female biased broods with different diets ($F_{2, 26} = 0.104$, $p = 0.90$) since males from their MsT mother tended to survive around the same

proportion as their hybrid sisters (Fig. 3). Of this cross, 1/12 at 0.1% nicotine and 1/8 at 0.3% nicotine were >90% female and both of these broods were small, <10 individuals. The MsT control cross likewise had no difference in sex ratios across diet treatments ($F_{2, 27} = 0.296$, $p = 0.75$) (Table 2).

Discussion

Cotesia congregata utilizes diverse sphingid hosts that specialize on plants in different families. Of these, tobacco likely has the most potent chemical defense. MsT wasps from the host *M. sexta* on tobacco are adapted to nicotine consumed by their host; in contrast, CcC wasps originating from host *C. catalpae* on catalpa are not. Clutch sizes of both incipient species did not differ among nicotine diets and development of wasp larvae was apparently not hindered. Clutch size did differ between the two incipient species with CcC females producing more larvae. The larger clutch size contributed to the larger egressed broods in CcC not exposed to nicotine. However, a greater percentage of MsT egressed from their hosts when not exposed to nicotine. This may be due to the use of *M. sexta* for both MsT and CcC wasps in this experiment, the native host of MsT and not CcC wasps. Bredlau and Kester (2015) reported larger broods for MsT than CcC, with a higher percentage of MsT egressing as well. Wasps in both studies originated from the same sources but in different years. Brood sizes can vary considerable in both wild and laboratory strains of *C. congregata*, which may account for these differences. Most CcC larvae failed to egress when reared on a host feeding on a nicotine diet. Exposure to nicotine during development likely has a physiological effect on CcC wasps that interferes with their ability to chew their way out of the host.

Hybrid females produced from CcC and MsT parents display the same tolerance to nicotine as MsT wasps, indicating that nicotine tolerance is derived from a dominant gene or

suite of genes. Since haploid MsT males and hybrids are able to survive in hosts on a nicotine diet, only one copy of each nicotine tolerance allele is necessary. Several mechanisms of nicotine detoxification have been identified. *Manduca sexta* has alkaloid pumps as part of their excretory (Maddrell and Gardiner 1975) and nervous systems (Murray et al. 1994). Cytochrome P450 detoxifying enzymes are associated with nicotine resistance in at least *M. sexta* (Snyder et al. 1993), the whitefly *Bemisia tabaci* (Kliot et al. 2014), and the aphid *Myzus persicae* (Bass et al. 2013). Gene duplications of P450 are responsible for higher expression that contributes to alkaloid detoxification ability by aphids (Puinean et al. 2010; Bass et al. 2013). P450 genes have been characterized in at least one parasitoid species, *Trichogramma cacoeciae* (Tarès et al. 2000). The mechanisms of nicotine tolerance in *C. congregata* before egression from the host are unidentified but may consist of similar mechanisms. Post-egression, the highest concentration of alkaloids are found in their cocoon silk, with no difference in successful development or adult mortality (Barbosa et al. 1986). Since the evolutionary history of the two incipient species of *C. congregata* is unknown, we cannot speculate whether the MsT lineage independently evolved nicotine tolerance, or whether CcC wasps subsequently lost nicotine tolerance since catalpa contains no alkaloids to maintain selection for this trait.

Nicotine in tobacco may serve as a partial ecological barrier between MsT and CcC wasps. CcC wasps are not adapted to hosts feeding on nicotine and most would die if wasps switched hosts. If CcC males moved into the tobacco system, their hybrid daughters would survive, but most would be unable to reproduce. Most CcC♂xMsT♀ hybrids either fail to produce eggs or any eggs oviposited are encapsulated within their host (Bredlau and Kester 2015). If CcC females were to switch to the tobacco system, their haploid sons would die but their hybrid daughters would survive. Taking into account the asymmetric hybrid sterility, the

only method for CcC genes to regularly flow into the tobacco system is through CcC females hybridizing with MsT males. In contrast, MsT wasps can utilize *C. catalpae* feeding on catalpa without an apparent loss in fitness (Kester, unpublished data). Although *C. catalpae* sequesters the iridoid glycosides from catalpa, they have little harmful effect on *C. congregata* (Lampert et al. 2010, 2011a). Therefore, gene flow could occur from the MsT system to the CcC system, but plant chemical defenses may act as a partial barrier for CcC wasps that have dispersed to tobacco. Since one CcC female produced more males than the others, some population variation to nicotine tolerance may exist. Kester et al. (2015) found some possible crossover between wasps on adjacent tobacco and catalpa, but this was limited to only a few individuals. Moreover, sib-mating on the natal plant is likely common (Kester and Barbosa 1991b). Overall, gene flow between these two incipient species is likely limited by the chemical defenses of tobacco and may result in speciation when combined with other pre- and post-zygotic barriers.

Differences in nicotine tolerance among host-plant associated populations have been identified in other insect species that feed on tobacco. For example, the whitefly, *Bemisia tabaci*, is a broad generalist, but populations differ in honeydew production and expression of genes related to chemical detoxification when fed a diet containing nicotine (Kliot et al. 2014). Likewise, populations of the generalist aphid, *Myzus persicae,* that expanded their host range to tobacco, were able to survive and reproduce on artificial nicotine diet lethal to non-adapted lineages; further genes encoding detoxifying enzymes were up-regulated (Ramsey et al. 2014). Unlike these generalist species, *C. congregata* has additional reproductive barriers that may facilitate differentiation, including differences in pheromones, courtship songs, and hybrid sterility (Bredlau and Kester 2015). However, continued work is required to determine relative

importance of reproductive barriers developing during speciation (Matsubayashi et al. 2010; Nosil 2012).

This study demonstrates that two incipient species of a parasitic wasp differ in the adaptation to plant defensive chemicals, which may restrict host and plant utilization in one incipient species. Given the genetic difference (Kester et al. 2015) and asymmetric sterility among hybrids (Bredlau and Kester 2015), we predicted additional differences based on host-plant usage. Gene flow to the tobacco system by CcC wasps is likely limited in nature, but tolerance to nicotine is probably just one of several isolating mechanisms. The difference in host and hostplant utilization is likely reinforced by natal learning of plant chemical cues (Kester and Barbosa 1991b; Lentz and Kester 2008; Lentz-Ronning and Kester 2013). Plant specialization via adaptation to defensive chemicals consumed by the host may serve as an initial or early barrier to reproduction among parasitic wasps and permit the evolution of additional reproductive barriers leading to speciation.

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TABLES AND FIGURES

Table 2.1: Clutch size (sum of egressed and unegressed larvae), brood size (number of egressed larvae that spun cocoons) (mean ± SE), and number of egressed larvae adjusted by clutch size as a covariate used in statistical analysis (ANCOVA: least-square mean ± SE) of *Cotesia congregata* originating from *Manduca sexta* on tobacco (MsT) and *Ceratomia catalpae* on catalpa (CcC), for three nicotine levels (% wet weight) in their host diet. "Male only broods" from the crosses were combined with control broods by female parent source.

Wasp source	Diet	*N*	Clutch size	Brood size (unadjusted)	Brood size (adjusted for covariate)
MsT	0	29	89.1 ± 8.4	60.7 ± 7.5	72.7 ± 5.4
	0.1	30	97.8 ± 8.0	46.5 ± 5.0	56.0 ± 5.3
	0.3	29	93.3 ± 7.0	33.4 ± 4.1	40.1 ± 5.2
CcC	0	26	158.9 ± 11.4	85.3 ± 9.4	78.7 ± 5.5
	0.1	33	163.9 ± 11.9	13.9 ± 3.8	6.3 ± 4.9
	0.3	31	146.9 ± 15.1	5.7 ± 2.2	1.7 ± 5.0

Table 2.2: Mean (± SE) clutch size (total larvae), brood size (egressed larvae), percent egressed, and percent female in broods that produced females between different crosses of *Cotesia congregata* (♂ x ♀) originating from *Manduca sexta* on tobacco and *Ceratomia catalpae* on catalpa, and nicotine (% wet weight) diet treatments. Only MsT♂xCcC♀ matings using hosts on a nicotine diet produced broods that were 100% female. Broods that did not egress from the host and broods containing only males resulting from wasps failing to mate were not included in this summary. Note that CcC♂xCcC♀ contains low sample sizes due to most of these broods failing to survive on a nicotine diet or failing to produce females.

Wasp cross	Diet	*N*	Clutch size	№ egressed larvae	Percent egressed	Percent female
MsT♂xMsT♀	0	10	109.7 ± 12.2	90.6 ± 9.7	83.5 ± 2.4	60.9 ± 7.7
	0.1	12	79.6 ± 14.4	57.3 ± 10.9	75.3 ± 4.2	62.6 ± 6.1
	0.3	8	103.8 ± 15.7	51.9 ± 12.2	47.2 ± 6.3	67.9 ± 5.4
CcC♂xMsT♀	0	10	95.5 ± 12.8	82.8 ± 10.3	87.0 ± 2.3	74.1 ± 4.7
	0.1	10	100.7 ± 11.3	67.0 ± 7.4	68.7 ± 5.4	68.7 ± 3.9
	0.3	7	91.9 ± 22.1	39.1 ± 13.2	45.7 ± 10.6	68.0 ± 8.5
MsT♂xCcC♀	0	13	169.5 ± 11.5	114.4 ± 11.3	68.1 ± 5.4	72.3 ± 9.1
	0.1	14	150.8 ± 14.7	57.5 ± 9.9	38.1 ± 5.0	91.8 ± 3.2
	0.3	17	141.2 ± 16.6	48.5 ± 7.9	34.9 ± 5.3	88.4 ± 6.0
CcC♂xCcC♀	0	5	174.0 ± 70.0	120.2 ± 55.8	63.2 ± 6.8	75.7 ± 5.7
	0.1	3	152.0 ± 51.1	56.7 ± 49.7	31.8 ± 21.0	65.3 ± 19.3
	0.3	2	226.0 ± 88.0	42.5 ± 13.5	19.4 ± 1.6	91.3 ± 8.7

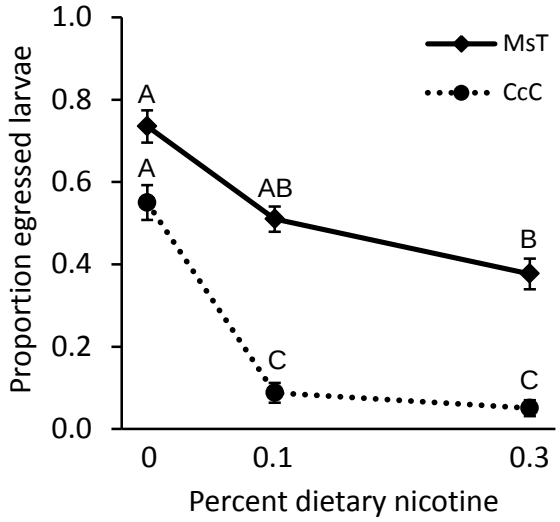

Fig. 2.1: Proportion of wasp larvae (mean ± SE) from MsT (from host *M. sexta* on tobacco) and CcC (from host *C. catalpae* on catalpa) lineages of *Cotesia congregata* egressing from host *M. sexta* fed three different nicotine diet treatments. Letters indicate significant differences in brood size when controlled for clutch size (ANCOVA, $p < 0.05$).

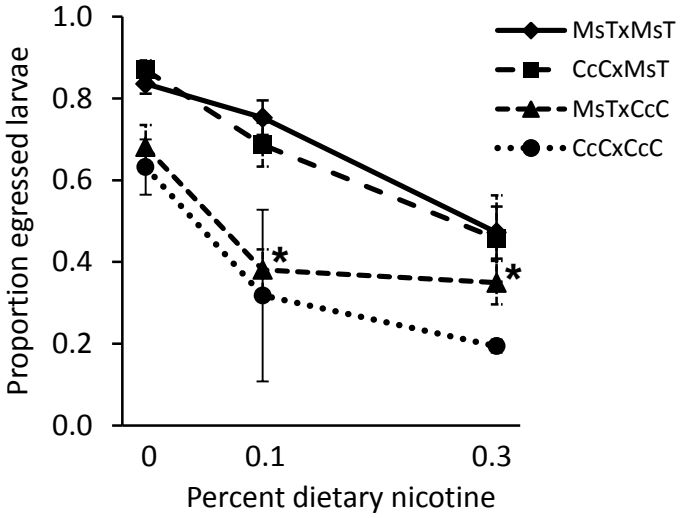

Fig 2.2: Proportion (mean ± SE) of egressed larvae among broods containing females from crosses (♂ x ♀) between MsT (from host *M. sexta* on tobacco) and CcC (from host *C. catalpae* on catalpa) lineages of *Cotesia congregata* developing in host *M. sexta* fed three different nicotine diet treatments. Broods that failed to produce any egressed larvae from the host or contained all males are not included. Asterisks indicate a significant reduction in brood size in the MsT♂xCcC♀ cross due to haploid male mortality ($p < 0.05$). CcC♂xCcC♀ has small sample sizes from high mortality from nicotine or failing to produce females.

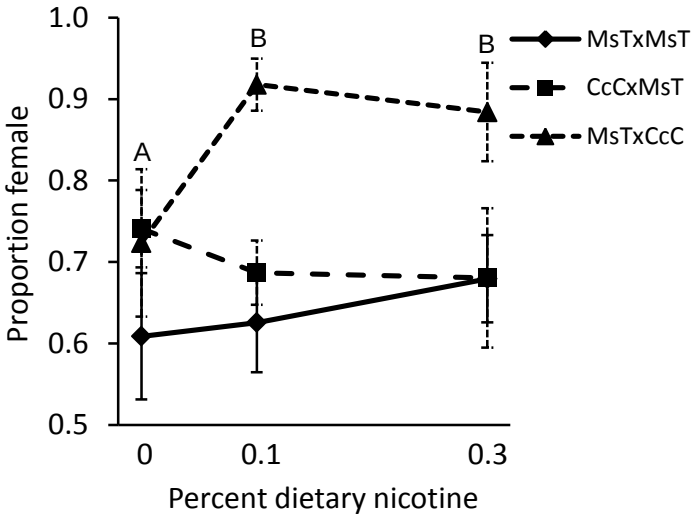

Fig 2.3: Proportion of females (mean ± SE) of broods in crosses (♂ x ♀) between MsT (from host *M. sexta* on tobacco) and CcC (from host *C. catalpae* on catalpa) lineages of *Cotesia congregata* egressing from *M. sexta* hosts across three nicotine diet treatments. CcC♂xCcC♀ is not included due to small samples sizes from high mortality from nicotine or failing to produce females. Only MsT♂xCcC♀ broods were female biased on nicotine diet due to haploid male mortality (letters denote $p < 0.05$).

Chapter 3 Evolutionary relationships of courtship songs in the parasitic wasp genus, *Cotesia* (Hymenoptera: Braconidae)

Evolutionary relationships of courtship songs in the parasitic wasp genus, *Cotesia* (Hymenoptera: Braconidae)

Justin Bredlau and Karen Kester

Abstract–- Acoustic signals play an important role in premating isolation based on sexual selection within many taxa. Parasitic male braconid wasps produce a characteristic courtship song used by females in mate selection. In *Cotesia* (Microgastrinae), courtship songs are generated by wing fanning and repetitive pulses in stereotypical patterns. Our objectives were to determine if male courtship songs within *Cotesia* are species-specific and the underlying patterns of differentiation and evolution. We compared songs among 12 of ca. 80 described *Cotesia* species in North America, including ten species that have not been recorded previously. For *C. congregata*, we compared songs of wasps originating from six different host-foodplant sources, two of which are considered incipient species. Songs of emergent males from wild caterpillar hosts in five different families were recorded, and pattern, frequency, and duration of song elements analyzed. Principal component analysis converted the seven elements characterized into four uncorrelated components used in a hierarchical cluster analysis and grouped species by similarity of song structure. Species songs varied significantly in duration of repeating pulse and buzz elements and in fundamental frequency. Cluster analysis resolved similar species groups in agreement with the most recent molecular phylogeny for *Cotesia* spp., indicating the potential for using courtship songs as a predictor of genetic relatedness. Limitations and exceptions are discussed. Courtship song analysis may aid in identifying closely related cryptic species that overlap spatially, and provide insight into the evolution of this highly diverse and agriculturally important taxon.

Keywords: bioacoustics, courtship songs, reproductive isolation, wing fanning, Microgastrinae

Introduction

Acoustic signals are used by diverse groups of insects for species recognition, fitness displays, and courtship elicitation. Songs used during insect courtship are generally stereotypical within a species and likely play a role reproductive isolation. Moreover, courtship songs may be a useful identifying character, especially among cryptic or closely related species (Sueur 2006). For example, songs of *Drosophila* species groups are species-specific and have been studied for evolutionary patterns (Ritchie and Gleason 1995; Gleason and Ritchie 1998; Oliveira et al. 2013). In highly diverse taxa of parasitic wasps, courtship songs may play a significant role in species differentiation and reproductive isolation.

Parasitic wasps respond to female pheromones by wing fanning, which draws air over olfactory organs for orientation to the female (Vinson 1972) and likely acts as a display of male fitness. Wing fanning at different amplitudes and velocities generates sound patterns with sound frequency corresponding to wing beat frequency (Bredlau et al. 2013). Wing fanning generates substrate vibrations detected by nearby wasps, and substrate type effects mating success (Field and Keller 1993; Joyce et al. 2008, 2014). Male wing fanning is a necessary precursor for successful mating in many species. For example, in the aphid parasitoid, *Lysiphlebus testaceipes*, females only mate after wing fanning and are more likely to mate with males producing high frequency and high amplitude wing movement (Benelli et al. 2016).

Wing fanning in parasitic wasps usually produces patterns of repeating pulses or buzzes. For example, five genera of the dipteran parasitoids in the subfamily Opiinae (Braconidae) produce songs with short repeating pulses of 40-200 ms and a frequency of 128-190 Hz (Sivinski and Webb 1989; Joyce et al. 2010a; Canale et al. 2013). Likewise, the aphid parasitoid, *Aphidius ervi* (Braconidae: Aphidiinae), produces repeating pulses lasting ~200 ms at 180 Hz with an

equivalent ~200 ms pause between pulses (Villagra et al. 2011). Within the largest subfamily, Microgastrinae, parasitoids of lepidopteran larvae, courtship songs are formed from a combination of low-amplitude and high-amplitude elements that correspond to changes in frequency (Sivinski and Webb 1989). For example, *Glyptapanteles flavicoxis* produces songs that consist of low-amplitude "percussion clicks" from wing vibrations followed by higher amplitude wingbeats increasing in frequency before transitioning back (Danci et al. 2010). Considering estimates of 17,000-48,000+ species in this subfamily (Rodriguez et al. 2013), the diversity of song patterns and their evolution is almost entirely unknown. Investigating multiple courtship songs within one diverse genus that includes closely related cryptic species, phylogenetic data, and well-characterized models would provide insight into the general patterns, diversity, and evolution of wasp courtship songs.

The large Microgastrinae genus *Cotesia* contains several species that have served as globally-important biocontrol agents of agricultural pests and as model systems for understanding host-parasitoid interactions. For example, *C. sesamiae* is a major biocontrol agent of maize stemborers in Africa, and long-term studies have revealed patterns driving host-associated specialization and co-evolution of virulent bracovirus genes (Kaiser et al. 2017). *Cotesia rubecula* and *C. glomerata*, both successfully introduced to parts of North America to control the imported cabbageworm, *Pieris rapae* (e.g., Van Driesche 2008) and have served as models for parasitoid behavior (e.g. Field and Keller 1993; Bezemer et al. 2010). *Cotesia congregata* is a model system for studying tri-trophic interactions (Barbosa et al. 1991; Kester and Barbosa 1994), insect learning (Lentz and Kester 2008; Lentz-Ronning and Kester 2013), insect immunology (Beckage et al. 1994; Amaya et al. 2005), and the genomics of symbiotic bracoviruses (Bézier et al. 2009; Chevignon et al. 2014). Courtship behavior of some *Cotesia*

species has been studied to improve mass rearing in biological control programs (e.g. Avila et al. 2017). However, most species remain undescribed and limited information is available beyond descriptions, host usage, and ranges for the majority of described species.

Courtship songs have been characterized in detail for four species of *Cotesia* (Sivinski and Webb 1989; Joyce et al. 2010b; Bredlau et al. 2013); however, no comparisons have been made among distantly related species, and the songs of most species clusters remain unknown. The current phylogeny of *Cotesia* based on four genes contains nineteen species, several of which are common and well-studied (Michel-Salzat and Whitfield 2004). This phylogeny provides a basic evolutionary framework for comparing courtship songs in *Cotesia.* Moreover, *C. congregata*, which is reported to parasitize at least fourteen sphingid species feeding on different plant families (Tietz 1972; Krombein et al. 1979), offers an opportunity to compare courtship songs among multiple host-foodplant complex sources. Two of these host-foodplant complex sources, wasps from *Manduca sexta* on tobacco ("MsT") and *Ceratomia catalpae* on catalpa ("CcC"), have diverged genetically (Kester et al. 2015) and likely represent incipient species (Bredlau and Kester 2015). They display a lower male response rate to the female pheromones of the reciprocal source, slight differences in duration and frequency of some song elements, and typically produce sterile hybrid females resulting from CcC♂xMsT♀ crosses. In this study we describe the courtship songs of ten additional species of *Cotesia*, and use clustering to explore the evolutionary relationships and patterns among songs. Further, we identify song differences among select host-associated populations and incipient species of *C. congregata*. Courtship songs allow us to supplement and expand the most recent phylogeny.

Materials and Methods

Parasitic wasp collection

Cotesia spp were collected from wild caterpillar hosts at multiple sites in the United States although some *C.* nr. *phobetri* came from an ongoing laboratory colony. Caterpillars known to be hosts of *Cotesia* were targeted for collection, particularly *Cotesia* that have had genes already sequenced. When possible, wasps from different sites were collected to enable wider population sampling. In most cases, each wasp species came from a single host species. In contrast, *C. congregata* were collected from six different sphingid host species feeding on different plant families (Table 1). Caterpillars, usually collected before parasitization status was known, were reared on their host plant in plastic containers in the laboratory until parasitoid egression or pupation. Individual loose *Cotesia* cocoons were placed in clear gel capsules (size 00) 2-4 days after egression. *Cotesia* species forming a connected cocoon mass were chilled upon adult emergence and placed in individual capsules or vials. Adults were sexed under a dissecting microscope. Wasp songs were recorded within 24 hours of emergence. Voucher samples of each species were both point pinned and stored in 95% EtOH at -20°C. The song of one species included in analysis (*Cotesia marginiventris*) was obtained from a USDA-ARS sound library (https://www.ars.usda.gov/ARSUserFiles/3559/soundlibrary.html) and originally described by Sivinski and Webb (1989).

Audio recordings

Males in capsules were haphazardly selected from each brood for recording. Individuals were placed in an open paper arena with a drop of honey as a food source to encourage them to stay in the arena. Courtship songs were induced by exposing individual males to a female of the same species. Songs were recorded using a miniature omnidirectional microphone (model 4060,

DPA, Longmont, CO; 20-20,000 Hz) positioned 5-7 mm above the male and a high resolution digital audio recorder (model 702, Sound Devices, Reedsburg, WI; 48 kHz sampling rate, 24 bit resolution) in a sound isolation booth (Industrial Acoustics, Bronx, NY) at 23 ± 1.5°C and 40-55% RH. Generally, one recording per brood was analyzed; however, recordings of different individuals were analyzed for species with fewer than four collected broods. Additional individuals of *C. congregata* were recorded to test for relatively small differences among host-foodplant sources. Additional *C.* nr. *phobetri* were recorded from each brood because initially they could not be identified to a known species. Duration of song elements and fundamental frequency were quantified using Raven Pro v1.3 (Charif et al. 2008). Waveforms were high-pass filtered at 100 Hz to reduce background noise.

Songs were divided into multiple elements based on acoustic characteristics shared across species (Figure 1). "Pauses" occur between other song elements when wings are held motionless above the body and do not generate sound. "Pulses" are high amplitude elements comprising the greatest range of wing movement, referred to as "boings" in *C. congregata* (Bredlau et al. 2013). "Terminal buzzes" immediately follow pulses and consist of continuous lower-amplitude sounds. "Pre-pulse buzzes" are steady lower amplitude sounds that precede a pulse with no pause in between. For "pulse-buzz units" we measured the duration of the pulse and buzz together. "Interpulse interval" (or "pulse repetition time") is the time from the start of one high-amplitude pulse to the start of the next. Pulses, buzzes, and pauses make up the pulse-buzz units and interpulse interval, which were included because they make discrete units that may be important in species recognition. Song amplitude was not considered because distance from the microphone varied slightly among some species. Quantification of song elements started with the second complete pulse-buzz cycle and continued for six complete pulse-buzz cycles.

Spectrograms of the entire song were produced using a short-time Fourier transform (Hann window, size = 2,000 samples, 50% overlap). Frequency spectra for song sections were calculated using fast Fourier transforms (Hann window, size = 1,000 samples, 50% overlap). Frequency of the first harmonic (fundamental frequency) was used in all comparisons.

Comparisons and analysis

Several statistical procedures were used to determine differences in courtship songs, consolidate elements, and group songs by structure. The considerable differences among some songs present a challenge for direct comparisons using standard statistical tests. For example, some elements were drastically reduced or absent in some species. Moreover, songs with elements of long duration tend to have a greater variance in those elements. In many cases, no statistical test is required to determine that the song of a species is different from the others. For statistical tests, the repeating song elements were averaged so that each individual wasp was treated as an *N* of 1. Where appropriate in similar species, duration and fundamental frequency were compared using analysis of variance (ANOVA) followed by Tukey's post-hoc test. Song element durations were log transformed to meet the assumptions of ANOVA. Pulse and terminal buzz frequency were compared between adjacent elements of each song for each species and all species together using linear regression.

Principal components analysis (PCA) for each individual wasp was used to condense dimensionality of the data into dimensionless principal components (PCs) based on communitive explained variance. Hierarchical cluster analysis (Ward method) of mean principal components of each species was used to group wasps. The resulting dendrogram was compared to the most recent molecular phylogeny of *Cotesia* (Michel-Salzat and Whitfield 2004) to determine if the

same groups were resolved (see Table 2 for genes used and GenBank ascension numbers of species included in this study).

Subsequent PCAs were performed on six different host-foodplant complex sources of *C. congregata,* on the MsT and CcC incipient species, and the two geographically isolated sources of *C.* nr. *phobetri*. Sources that displayed separation in the PCAs were followed by a Welch's unequal variance t-test to compare duration and frequency of song elements. The feasibility of matching songs back to their species or population based on song elements and PCs was tested using linear discriminant analysis. All statistical and multivariate analyses were performed with JMP v11 (SAS Institute, Cary, NC).

Results

Description of wasp songs

Eleven species of *Cotesia* were collected at different sites in the United States (Table 1); one additional species was from a published recording (*C. marginiventris*). Some target host species collected did not yield any *Cotesia.* Multiple broods were collected of each species except for the uncommon *C. teleae*, which came from a single *Antheraea polyphemus* larva that produced only one living male concurrently with females. *Cotesia congregata* were collected from six different sphingid host species. *Cotesia* nr. *phobetri*, (currently undescribed) were supplied from a laboratory colony originating from host *Grammia incorrupta* (formerly *G. geneura*) that feed on forbs in grassland habitat and found in Reddington Pass, Pima County, AZ (Stireman and Singer 2002). Initially unknown specimens found as cocoon clusters independent of hosts in a recently mowed horse pasture in Gloucester County, VA, were later identified to be either *C.* nr. *phobetri* or a closely related sister species based on morphology, cocoon structure, similarity of habitat, and song structure (see Discussion). Although these cocoons were not

attached, *Grammia virgo* were found in an adjacent field and are the most likely host. All other collected *Cotesia* species were each collected from single host species.

All twelve species have songs generated from wing fanning and consisting of repeating high-amplitude pulses and, in most species, lower amplitude buzzes (Fig. 2). Pulses were accompanied by abdominal movements. All males continued to produce songs until copulation or the female moved away; therefore number of pulses was not considered as a factor. Songs vary in duration of pulse, buzz, and pause elements (Table 3). Some songs were different enough from the others as to not require statistical comparisons of individual elements. Moreover, songs with considerably longer element durations had greater variance than in other species, failing the assumptions of ANOVA. Instead of comparing all songs together in one ANOVA model, subsets of highly similar species were compared. Among all species, fundamental frequency ranged from 328 Hz in *C. euchaetis* to 176 Hz in *C. phobetri* (Table 4). All songs produced harmonics up to 4-5 kHz for pulses and 1-2 kHz for buzzes (Fig. 2). Analysis of individual elements was useful for comparing similar or sister species but of limited use for comparisons across the entire genus. Courtship songs were divided into groups using a combination of element duration and frequency, and general patterns rather than focusing on individual song elements.

The most common courtship song structure includes pause-pulse-buzz elements repeating ca 2-3 times a second. A subset of four species with similar pause-pulse-buzz patterns was used to determine differences. The songs differed in either duration of interpulse intervals and component elements (ANOVA: $F_{3, 37} = 8.03$, $p = 0.0003$; Fig. 3A) or fundamental frequency (ANOVA: $F_{3, 37} = 46.08$, $p < 0.0001$; Fig. 3B). In *Cotesia congregata*, the courtship songs consist of an initial buzz followed by repeating pulse-buzz elements with a short (20-26 ms) pause followed by a pulse ("boing") that decays into a buzz (Bredlau et al. 2013). *Cotesia*

phobetri and *C. euchaetis* have similar patterns although terminal buzzes after pulses are longer than in *C. congregata*. *Cotesia* nr. *phobetri* have songs similar to those of *C. euchaetis* but with shorter terminal buzzes. *Cotesia marginiventris* have pulse-buzzes that vary more in duration, producing a warble sound (Sivinski and Webb 1989; Joyce et al. 2008). The song of *C. marginiventris* does not contain the discrete high-amplitude pulses present in *C. congregata*, *C. phobetri*, and *C. euchaetis*, but otherwise follows a similar pattern. *Cotesia glomerata* is similar but lacks discrete pauses between pulse-buzz elements and has a quick power spike at the start of each pulse that is not observed in other species.

Three species have courtship songs that consist of rapid repeating pulses without long terminal buzzes. *Cotesia empretiae* produces a pulse train of repeating pulses of different durations lasting about two seconds and then repeats. *Cotesia diacrisiae* has rapid short pulses at a rate of four per second. *Cotesia orobenae* also has rapid repeating pulses which are shorter in duration with a longer pause compared to *C. diacrisiae*. These three species can be readily distinguished by their waveforms (Fig. 2).

Three species have songs with long pauses between pulses. *Cotesia rubecula* has pulses similar in duration and pacing to those of *C. phobetri* or *C. congregata* but lacks a terminal buzz. *Cotesia teleae* has a pulse-buzz element with pauses that are about five times longer than those of the *C. congregata* group. *Cotesia flaviconchae* courtship song is substantially different from other known *Cotesia* songs. It is the only species with a buzz before the high-amplitude pulse ("pre-pulse buzz") lasting 486 ± 32 ms at 286 ± 2 Hz. The buzz-pulse repeats every 4-9 seconds, while all other songs repeat in less than a second.

Phylogeny and evolution of all species

Songs from the different *Cotesia* species were grouped using PCA and cluster analysis. Frequency of adjacent pulse and terminal buzz elements could not be accurately calculated in all sections of some species due to short durations of one of these elements (e.g., *C. empretiae*); however, adjacent pulses and buzzes were correlated in song sections containing sufficiently long durations of both elements ($r^2 = 0.65$, d.f. = 770, $p < 0.0001$; Pulse = -31.0 + 1.1*TBuzz; Fig. 4). Therefore, frequency was consolidated into one term by using the "pulse-buzz unit" frequency in the PCA. The PCA using the seven song elements resulted in four PCs explaining 91.7% of total variance. PC1 was best represented by duration of the pulse-buzz unit, interpulse interval, and pre-pulse buzz, PC2 by frequency, PC3 almost entirely by pause duration, and PC4 by frequency, pause duration, and pulse duration (Table 5). The factor scores of the first four PCs differed significantly among some species (ANOVA: $F_{10,61} = 83.7, 32.7, 17.1, 27.1$ respectively; $p < 0.0001$).

Differences in one or more PCs among species indicated groups with most species forming close clusters with some overlap (Fig. 5). Species separated from the main cluster and containing relatively long duration elements had greater variance in PCs (e.g., *C. flaviconchae* and *C. rubecula*). Hierarchical cluster analysis of species using the first four PC mean factor scores resolved four main groups (Fig. 6). Group 1 consists of wasps with short rapid pulses, group 2 with pulses and terminal buzzes, group 3 with long pauses between pulses, and group 4 of only *C. flavichonchae* with a thus far unique song pattern. The courtship songs for each species were mapped onto a genetic phylogeny produced from four genes available (Michel-Salzat and Whitfield 2004) to determine evolutionary trends (Fig. 7). Groupings from the cluster

analysis generally reflect genetic groups, with the exception of *C. rubecula* which lacks the terminal buzz found in all other related species in the "*rubecula*" group.

Differentiation of C. congregata *host-foodplant complex sources*

Songs from the different *C. congregata* host-foodplant complex sources could not be distinguished by courtship songs alone. The PCA using the six song elements present in *C. congregata* resulted in three PCs explaining 88.8% of total variance and four PCs explaining 99.9% of total variance. PC1 was most represented by pulse-buzz unit duration, interpulse interval, and terminal buzz duration, PC2 by pause duration and frequency, PC3 by pulse duration, and PC4 by frequency (Table 6). A PCA of the two geographic sources of MsT and the one source of CcC host-foodplant complexes produced a similar component matrix (Table 7). High overlap of every PC prevents discrimination of host-plant complex sources (Fig. 8) and geographically separated populations (Fig. 9), even if means of some elements and the first threes PCs differ significantly between some groups (ANOVA: $p < 0.001$).

Differentiation of C. *nr.* phobetri *by location*

Songs from the two sources of *C.* nr. *phobetri* could be clearly distinguished by courtship songs. The PCA using the six song elements present in *C.* nr. *phobetri* resulted in three PCs explaining 94.5% of total variance and the fourth PC explaining the remaining variance. PC1 was most represented by duration of pulse and buzz components, PC2 by pause duration and frequency, PC3 by pause duration, and PC4 by pulse duration (Table 8). The *C.* nr. *phobetri* populations from Virginia and Arizona can be reliably distinguished by PC1 (pulse and buzz durations) but not the other PCs (pause duration and frequency) (Fig. 10). Linear discriminant analysis sorts wasps by population with 100% accuracy. Mean duration of the pulse and buzz components were longer in songs of wasps originating in Arizona than Virginia (unequal

variance t-test, $p < 0.001$), with mean pulse-buzz unit duration 0.13 s longer ($t_{13} = -8.6$, $p < 0.0001$). Overall song pattern and structure remained the same in wasps from both populations and were more similar to each other than to the other wasp species analyzed (Fig. 4, 5).

Discussion

Courtship songs generated from wing movement are common among parasitic wasps and likely universal among *Cotesia* and perhaps all Microgastrinae. Courtship songs of similar sympatric species are predicted to be divergent if they are used in mate selection and conspecific identification. For example, courtship songs of 10 species of chalcidoids were distinct even within the same genus, although in this case some females were able to respond to allospecific males (van den Assem and Putters 1980). Song differentiation of reproductively isolated species may also change over time via genetic drift in the absence of strong sexual selection, producing slight changes in elements among closely related species. In this scenario, songs may seem arbitrarily different among species with relatively small changes, yet still be conserved within species. Likely, sexual selection over time influences primary signal elements, such as interpulse interval and amplitude, while other elements shift over time via genetic drift. The differences in song characteristics over time, regardless of mechanism, can be a useful diagnostic for taxonomic groupings and species identification.

Courtship songs of the twelve species of *Cotesia* presented in this study are unique to each species and can be readily distinguished. Songs were quantitatively characterized by dividing songs into elements that were shared across most *Cotesia* species. Principal components analysis was used to reduce dimensionality of song elements that may be correlated. Species grouped using hierarchical cluster analysis corresponded to groups in the genetic phylogeny (Michel-Salzat and Whitfield 2004), with one exception. Some species that have not been

sequenced can be provisionally placed into these pre-identified groups based on courtship song characteristics. Likewise, the general pattern of courtship songs may be predicted for species that have been placed on a phylogeny but not found and recorded in this study. However, the relationships among the groups do not correspond strictly to the genetic phylogeny and the large differences among groups make their placement difficult.

Phylogenetics and taxonomy of the Microgastrinae are active areas of study (Whitfield et al. 2018). Most work has been on the subfamily or genus level (Whitfield et al. 2002; Banks and Whitfield 2006; Murphy et al. 2008; Smith et al. 2013), on closely related species clusters or cryptic species (Kankare and Shaw 2004; Kankare et al. 2005a; Muirhead et al. 2012; Kester et al. 2015), or on determining the evolutionary relationship with symbiotic viruses (Whitfield 2000, 2002; Dupas et al. 2008; Herniou et al. 2013a). Considering the diversity of *Cotesia* and the difficulty of producing high-resolution phylogenies, not all of the same genes or all common species have yet been sequenced. Therefore, some species in this study are not reliably placed in current phylogenies (e.g., *C. phobetri, C. teleae*). Eight of the species can be placed on the most recent genetic phylogeny for *Cotesia*. The phylogeny produced by Michel-Salzat and Whitfield (2004) contains four identifiable groups, three of which are represented in this study and the fourth, "*melanoscela*" group, in other studies (Joyce et al. 2010b, 2014). With the exception of *C. rubecula,* species phylogenetically grouped together had similar courtship songs. Several species not placed on the genetic phylogeny can be putatively placed within a group based on song characteristics (e.g. *C. phobetri*). The most basal genetic group containing *C. empretiae* and *C. diacrisiae* had songs that consisted of rapid repeated pulses although the placement of these species has low nodal support. Most songs of derived groups consisted of pulses and longer terminal buzzes.

The apparent phylogenetic signal of courtship songs allows for predictions of song structure before recording. For example, most species in the "*rubecula*" group have a pause-pulse-buzz pattern of similar duration. Species such as *C. schizurae* and *C. electrae* likely have songs similar in pattern to *C. congregata* and not more different than the more distantly related *C. euchaetis* or *C. marginiventris*. In both the genetic phylogeny and acoustic dendrogram, *C. glomerata* was placed as a sister group to most of the "*rubecula*" group, with its main distinction being the pre-pulse power spike. In the "*kariyai*" group, *C. cyaniridis* is expected to have a song with a buzz that leads directly into a pulse with a long interpulse duration. The song may differ in details of timing and frequency, but otherwise should sound similar to the closely related *C. flaviconchae* (Fig. 7). These predictions were supported by the recent recording of two male *C. schizurae* (host *Schizura unicornis*; 37.7549, -77.3458). This species, closely related to *C. congregata*, was targeted for collection but not found. The courtship song of *C. schizurae* is similar enough in structure and duration of pulse, buzzes, and pauses to *C. congregata* to be firmly placed as a closely related species. The ability to place species into clades by courtship songs will be valuable for the systematics of this very large genus.

Cotesia rubecula is the only species that deviates from expectation. It is genetically placed with species with pulse-terminal buzz patterns (e.g., *C. congregata*) but lacks a discrete terminal buzz (Fig. 2). The most parsimonious explanation is that *C. rubecula* secondarily lost the long terminal buzz and replaced it with a long pause. The time between pulses is similar to those within its genetic group. Alternatively, *C. rubecula* may not belong in this group, although high nodal support for its inclusion makes this possibility less likely (Fig. 7). Notably, *C. rubecula* has other characteristics that differentiate it from most species recorded in the "*rubecula*" and "*glomerata*" groups – it is solitary and the largest *Cotesia* species recorded.

Moreover, *C. rubecula* and *C. glomerata* are the only species collected that utilize the same host, *Pieris rapae* on Brassicaceae. Their songs may display greater divergence in part due to character displacement, which has been demonstrated for the songs of few insects (Marshall and Cooley 2000); however, extensive surveys suggests competitive exclusion over most of the range in the United States (Herlihy et al. 2012).

Cotesia teleae has a song that challenges direct placement into a group. The pattern of the pulse-buzz unit is similar in many ways to those of the "*rubecula*" group; however, it has a short pulse with a high energy terminal buzz that loses amplitude at the end (Fig. 2). Most distinctly, there are long pauses between pulses. Possibly, it belongs with the "*rubecula*" group – the cluster analysis supports a relation with *C. rubecula* – but its placement remains less certain than for other species without either genetic information or another species with a similar song pattern. The song of *C. teleae* is also the only one analyzed using a single male. A single parasitized polyphemus caterpillar yielded few adult wasps. The brood began egression in October with most wasp larvae going into diapause, which was not broken in the lab and yielded only a single male concurrently with females. Considering the courtship songs are conserved within species and this male was healthy, the recorded song is presumably a reliable representation of this species. A second male emerged without a living conspecific female present and would not respond to other species. Attempts to find a second brood over multiple years failed. Since additional samples of *C. teleae* are improbable, it was included in this study.

The "*melanoscela*" group, containing *C. sesamiae* and *C. flavipes*, is the only major group not included in this analysis. These two species, widely used as biocontrol agents of stemborer pests, are not native to North America and could not be acquired. These wasps, like all *Cotesia*, have songs with repeating pulse, buzz, and pause elements with a frequency of 222-290

Hz (Joyce et al. 2010b). The overall pattern of higher-amplitude pulse decaying into a longer terminal buzz has some structural similarities to songs of *C. marginiventris* and others in the "*rubecula*" group.

Courtship song analysis can be used to match unidentified wasps, particularly those with similar morphology, to species. Two cases occurred during this study in which parasitoid cocoons were found separated from their host. Unknown *Cotesia* cocoons found on a garden tomato plant were identified as *C. orobenae* upon recording. Presumably, the cross-stripped cabbageworm hosts had decimated nearby cabbages and had then migrated to the tomato before parasitoid egression. The empty hosts were absent, leaving only the wasp cocoons remaining on the leaves. In the second case, several loose bundles of parasitoid cocoons were found with no host in a mowed horse pasture in Virginia, USA. After recording the adults, the species acoustically matched those grouped with *C. congregata*, but not any currently recorded species. Subsequently, cocoons of *C.* nr. *phobetri* were received that originated from Arizona. The courtship songs of these wasps very closely resembled those of the unknown Virginia wasps, in addition to similarities in morphology, cocoon structure, and habitat. This is the first known record of this species outside of Arizona. Considering that this species utilizes the common caterpillar genus, *Grammia*, as hosts in a common habitat type, they may be widespread in the United States.

Courtship song elements may differ even among closely related species or host-associated populations. For example, allopatric populations of *C. sesamiae* and *C. flavipes* utilizing different hosts had courtship songs that differed in element duration and frequency (Joyce et al. 2010b). Likewise, *C. congregata* originating from hosts *M. sexta* on tobacco (MsT) and *C. ceratomia* on catalpa (CcC) differed significantly in pulse and pause durations though the

differences were not enough to reliably distinguish all individuals (Bredlau and Kester 2015). We expanded this earlier finding by using four additional host-foodplant sources of *C. congregata* in Virginia and an additional population of MsT wasps from Indiana (Table 1). The additional sphingid hosts species were phylogenetically diverse and consisted of two subfamilies (Kawahara et al. 2009). Mean song element duration (pulse and pause) and PCs differ among MsT and CcC wasps, however the degree of range overlap with the additional sources prevents reliable discrimination by source (Fig. 8, 9). The slight differences may indicate recent reproductive isolation that over time became discrete differences under sexual selection or genetic drift. Subsequent breeding crosses using these additional sources of *C. congregata* indicates a pattern of asymmetric hybrid female sterility with either MsT or CcC wasps, suggesting only two primary lineages (Chapter 4).

In contrast, geographically separated populations of *C.* nr. *phobetri* differ in song element durations (Fig. 10), even though they are similar enough to be recognized as the same species. We cannot determine whether the Virginia and Arizona populations represent sister species, host-associated races, or isolated populations without additional information on reproductive compatibility and range. These populations are separated by 3,150 km and thus may be expected to have some differences in song elements regardless of species status. Another possibility is that the rearing of the Arizona population in the lab for three years before recording could have altered their song in comparison to the wild Virginia wasps, as reported in other laboratory reared braconids (Joyce et al. 2010a). Collecting wild *C.* nr. *phobetri* at multiple sites would be required to make that assessment. The other geographically separated samples came from *C. glomerata* and *C. rubecula*; however, not enough individuals were recorded to discern acoustic differences within these species.

Relatively small differences in songs among closely related species indicate a phylogenetic signal that may have useful applications for systematics. Sexual selection likely plays a role in the differentiation of some songs. However, within the majority of the "*rubecula*" group, songs consist of similar pause-pulse-buzz patterns more indicative of a slow build-up of differences rather than active sexual selection. Furthermore, song differences in reproductively isolated incipient species of *C. congregata* are slight. In contrast, species clusters of *Drosophila* have been reported to have large differences in courtship songs, suggesting strong sexual selection leading to differentiation before other traits (Ritchie and Gleason 1995; Gleason and Ritchie 1998; Veltsos et al. 2011; Oliveira et al. 2013). Playback experiments using the *D. buzzatii* species cluster demonstrate that females are more likely to accept males with a conspecific song, supporting the role of sexual selection (Iglesias and Hasson 2017). Other factors may play a greater role in parasitic wasp speciation, such as host-plant learning and adaptations to host immune systems, leading to differing rates of song differentiation.

This form of analysis has several limitations. Even the relatively simple songs of parasitic wasps contain multiple acoustic elements and frequencies, often with different degrees of variance depending on song structure. Principal component analysis is useful for reducing the dimensionality of complex datasets to uncorrelated variables, and in identifying elements that contain the greatest variance. Moreover, PCA is a common method of data exploration widely understood by biologists and has been used in the comparison of songs in diverse taxa including birds (e.g., Päckert et al. 2014; Zhao et al. 2017) and insects (Henry et al. 1999; Eberhard and Eberhard 2013; Oliveira et al. 2013; Vigoder et al. 2015). We used PCA as a means to reduce the acoustic data for comparison and included the number of PCs that adequately explained aspects of the courtship songs in the cluster analysis. However, limitations such as uneven scaling of

long vs short elements and time vs frequency were not accounted for because their biological significance is unknown. No one element could adequately capture the differences among songs; furthermore, some elements calculated were intentionally redundant. Also, selecting a different number of PCs to include in a cluster analysis can slightly alter the results. Alternative methods to a cluster analysis that consider the probability of a given acoustic tree among all possible trees should be considered with additional data. Additionally, the sequencing of all sampled wasps or recordings of those already sequenced will permit a more thorough examination of song trait evolution.

This comprehensive study of courtship song diversity within a genus of parasitic wasps, *Cotesia,* implicates a wide diversity of song patterns that can be divided into groups based on duration and frequency of song elements. The basal song most likely consisted of regular pulses generated by high-amplitude wing strokes, as seen in other members of subfamily Microgastrinae (Sivinski and Webb 1989). This song diverged into the several distinct patterns among the major groups of *Cotesia*. Many wasps not yet recorded can likely be placed into these groups based on a combination of song structure and morphology. The unique structure of songs for each species can potentially be used for species recognition and as a reproductive barrier between cryptic species; however, the influence of sexual selection is uncertain. Despite measurable differences among species, the songs among *C. congregata* host-foodplant complexes cannot be reliable distinguished, suggesting that song differentiation does not proceed without other reproductive barriers. In total, fifteen *Cotesia* species have been recorded out of the estimated 1,000 species globally (Whitfield et al. 2018). Considering the size of this genus, other entirely new song patterns may yet be discovered. When combined with additional genetic data, courtship song analysis should prove useful in determining the systematics and evolutionary

history of groups of parasitic wasps, particularly in this highly diverse and agriculturally important taxon.

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TABLES AND FIGURES

Tables

Figures

Table 3.1: *Cotesia* species collected and used in this study with lepidopteran host names, collection locations (county/city, state; LAT, LONG, datum: WGS84), and number of wasp broods collected at each site. *Cotesia congregata* is divided into six host-foodplant complexes abbreviated by host species and plant name (tobacco, catalpa, holly/pawpaw, Virginia creeper, and privet, respectively).

Wasp species	Host species	Location	Coordinates		*N*
C. congregata (Say) MsT	*Manduca sexta* (Linnaeus)	Nottoway Co., VA	37.095	-77.963	8
		West Lafayette, IN	40.287	-86.883	4
CcC	*Ceratomia catalpae* (Boisduval)	Cumberland Co., VA	37.7127	-78.1639	20
DhH	*Dolba hyloeus* (Drury)	Chesterfield Co., VA	37.453	-77.581	1
		Hanover Co., VA	37.731	-77.713	1
DmV	*Darapsa myron* (Cramer)	Gloucester Co., VA	37.304	-76.498	1
		Gloucester Co., VA	37.257	-76.453	2
		Richmond, VA	37.530	-77.450	1
EpV	*Eumorpha pandorus* (Hübner)	Gloucester Co., VA	37.257	-76.453	2
		Richmond, VA	37.530	-77.450	1
		Henrico Co., VA	37.586	-77.543	1
		Richmond, VA	37.5498	-77.4574	1
SkP	*Sphinx kalmiae* Smith	Charles City Co., VA	37.331	-77.210	1
C. diacrisiae (Gahan)	*Estigmene acrea* (Drury)	Goochland Co., VA	37.646	-77.984	3
		Richmond, VA	37.529	-77.453	1
C. empretiae (Viereck)	*Acharia stimulea* (Clemens)	Richmond, VA	37.519	-77.470	3
		Richmond, VA	37.520	-77.466	1
		Richmond, VA	37.528	-77.455	2
C. euchaetis (Ashmead)	*Euchaetes egle* (Drury)	Richmond, VA	37.539	-77.451	6
		Richmond, VA	37.524	-77.469	1
C. flaviconchae (Riley)	*Colias eurytheme* Boisduval	West Lafayette, IN	40.503	-87.013	3
C. glomerata (Linnaeus)	*Pieris rapae* (Linnaeus)	Richmond, VA	37.6072	-77.5078	1
		Henrico Co., VA	37.595	-77.552	1
		Wellington, CO	40.653	-104.99	1
C. nr. *phobetri*	*Grammia incorrupta* (Edwards)	Pima Co., AZ*	32.31	-110.60	2
	Grammia virgo (Linnaeus) †	Gloucester Co., VA	37.304	-76.497	3
C. orobenae (Forbes)	*Evergestis rimosalis* (Guenée)	Goochland Co., VA	37.646	-77.210	23
		Richmond, VA	37.5360	-77.4127	1
		Richmond, VA	37.519	-77.470	2
C. phobetri (Rohwer)	*Halysidota harrisii* (Walsh)	Richmond, VA	37.520	-77.466	2
		Richmond, VA	37.528	-77.455	4
		Richmond, VA	37.518	-77.474	1
		Richmond, VA	37.549	-77.516	1
C. rubecula (Marshall)	*Pieris rapae* (Linnaeus)	West Hampton, MA	42.3	-72.8	1‡
		St. Paul, MN	44.9	-93.1	1‡
C. teleae (Muesebeck)	*Antheraea polyphemus* (Cramer)	Richmond, VA	37.528	-77.454	1

*Original collection site; wasps for this study were supplied by an ongoing laboratory colony (M. Singer Lab).
† Cocoons at this site were separate from any host. This is the presumed host species found in an adjacent field.
‡ Four additional wasps were F_1 hybrids between these two sites. Collection coordinates are approximate.

Table 3.2: Available genes and GenBank ascension numbers of *Cotesia* species used in this study. Not all species have been sequenced using all four genes, and therefore are not included in the phylogeny.

Species	NADH1	mt16S rDNA	n28S rDNA	LW rhodopsin
C. congregata	AF069198	U68157	AJ535936	AJ535980
C. diacrisiae	AJ535959	AJ535917	--	--
C. empretiae	AJ535961	AJ535919	AJ535939	AJ535983
C. euchaetis	AJ535962	AJ535920	AJ535940	AJ535984
C. flaviconchae	AJ535963	AJ535921	AJ535941	AJ535985
C. glomerata	AF110830	U68158	AJ535944	AJ535988
C. marginiventris	AJ535967	AJ535926	AF102730	AJ535991
C. nr phobetri	--	--	--	--
C. orobenae	AJ535970	U68158	--	--
C. phobetri	--	--	--	--
C. rubecula	AF110831	U06959	AJ535949	AJ535994
C. teleae	--	--	--	--

Table 3.3: Duration (ms; mean ± SE) of male courtship song elements among species of *Cotesia*. *Cotesia congregata* is divided by host-foodplant complex. *N* = number of individual wasps analyzed.

Wasp species	Pause	Pulse	Terminal buzz	Pulse-buzz	Interpulse interval	*N*
C. congregata - MsT	23.4 ± 0.3	133 ± 2	206 ± 3	339 ± 3	362 ± 3	12
C. congregata - CcC	23.0 ± 1.1	147 ± 1	209 ± 2	356 ± 3	378 ± 3	20
C. congregata - DhH	25.2 ± 0.6	128 ± 4	216 ± 9	344 ± 11	369 ± 11	3
C. congregata - DmV	20.8 ± 0.3	126 ± 2	202 ± 4	328 ± 4	349 ± 4	19
C. congregata - EpV	23.3 ± 0.3	131 ± 1	229 ± 4	361 ± 4	385 ± 4	31
C. congregata - SkP	20.0 ± 0.7	145 ± 3	205 ± 4	349 ± 5	368 ± 5	1
C. diacrisiae	26.9 ± 0.8	92 ± 13	109 ± 4	168 ± 5	200 ± 5	4
C. empretiae	30.3 ± 1.5	54 ± 5	20 ± 8	66 ± 8	100 ± 7	6
C. euchaetis	37.8 ± 5.8	128 ± 6	281 ± 11	409 ± 14	454 ± 13	5
C. flaviconchae	0.0 ± 0.0	212 ± 27	240 ± 24	916 ± 38	6056 ± 483	4
C. glomerata	2.6 ± 1.0	176 ± 12	520 ± 17	696 ± 19	707 ± 19	4
C. marginiventris	31.2 ± 2.1	98 ± 15	276 ± 45	375 ± 52	405 ± 53	1
C. nr. *phobetri*	34.3 ± 0.5	118 ± 3	205 ± 6	323 ± 8	358 ± 8	16
C. orobenae	38.9 ± 2.3	65 ± 3	30 ± 3	95 ± 2	265 ± 7	6
C. phobetri	34.5 ± 1.2	109 ± 3	315 ± 10	424 ± 11	454 ± 9	7
C. rubecula	197.2 ± 19.3	102 ± 2	96 ± 16	198 ± 17	484 ± 8	6
C. teleae	155.7 ± 22.3	74 ± 2	293 ± 10	368 ± 11	517 ± 15	1

Table 3.4: Fundamental frequency (Hz; mean ± SE) of male courtship song elements among species of *Cotesia*. *Cotesia congregata* is divided by host-foodplant complex. *N* = number of individual wasps analyzed.

Wasp species	Pulse	Terminal buzz	Pulse-Buzz	*N*
C. congregata - MsT	220.9 ± 1.2	230.6 ± 0.9	223.5 ± 0.9	12
C. congregata - CcC	222.2 ± 1.3	239.3 ± 1.2	228.7 ± 1.3	20
C. congregata - DhH	216.9 ± 3.6	231.5 ± 1.3	220.6 ± 2.9	3
C. congregata - DmV	203.8 ± 1.8	220.6 ± 2.1	206.1 ± 1.8	19
C. congregata - EpV	212.9 ± 1.6	229.1 ± 1.0	218.6 ± 1.4	31
C. congregata - SkP	220.9 ± 1.7	230.9 ± 0.6	223.8 ± 1.4	1
C. diacrisiae	258.8 ± 1.3	241.3 ± 1.9	250.1 ± 1.8	4
C. empretiae	254.3 ± 3.1	220.7 ± 4.7	252.8 ± 3.3	6
C. euchaetis	302.2 ± 2.4	271.4 ± 3.1	282.0 ± 5.1	5
C. flaviconchae	265.9 ± 3.1	294.7 ± 3.5	285.7 ± 2.3	4
C. glomerata	214.2 ± 1.4	262.9 ± 2.9	224.6 ± 2.8	4
C. marginiventris	293.3 ± 2.8	247.5 ± 3.5	248.6 ± 4.9	1
C. nr. *phobetri*	303.7 ± 1.4	285.3 ± 1.2	294.0 ± 1.5	16
C. orobenae	277.4 ± 2.0	282.9 ± 2.5	280.1 ± 2.2	6
C. phobetri	260.5 ± 3.0	228.0 ± 4.6	230.4 ± 4.8	7
C. rubecula	235.2 ± 2.8	235.2 ± 4.3	235.7 ± 3.0	6
C. teleae	227.0 ± 1.6	243.9 ± 2.2	231.7 ± 1.8	1

Table 3.5: Component matrix (eiganvectors), eiganvalues, and explained variance resulting from principal components analysis of male courtship songs from twelve species of *Cotesia*. Parameters strongly associated with song elements are bold.

	Principal components (PC)			
	1	2	3	4
Pause duration	-0.215	0.100	**0.810**	**0.503**
Pulse-buzz duration	**0.534**	-0.169	0.060	0.086
Pre-pulse buzz duration	0.453	0.413	0.196	-0.221
Interpulse interval	0.467	0.371	0.221	-0.201
Pulse duration	0.361	-0.289	0.006	0.473
Terminal buzz duration	0.334	-0.522	-0.075	0.153
Frequency	0.054	**0.545**	-0.498	**0.635**
Eigenvalue	3.250	1.157	0.989	0.660
Explained variance (%)	46.43	21.68	14.12	9.43
Cumulative variance (%)	46.43	68.11	82.23	91.66

Table 3.6: Component matrix (eiganvectors), eiganvalues, and explained variance resulting from principal components analysis of male courtship songs from host-foodplant complex sources of *Cotesia congregata.* Parameters strongly associated with song elements are bold.

	Principal components (PC)			
	1	2	3	4
Pause duration	0.146	**0.668**	-0.108	-0.720
Pulse-buzz duration	**0.564**	-0.094	-0.004	0.054
Interpulse interval	**0.567**	-0.048	-0.017	0.016
Pulse duration	0.288	0.048	**0.886**	-0.018
Terminal buzz duration	**0.504**	-0.130	-0.448	0.075
Frequency	0.048	**0.723**	-0.041	**0.688**
Eigenvalue	3.096	1.287	0.943	0.673
Explained variance (%)	51.60	21.45	15.71	11.21
Cumulative variance (%)	51.60	73.05	88.77	99.96

Table 3.7: Component matrix (eiganvectors), eiganvalues, and explained variance resulting from principal components analysis of male courtship songs from MsT and CcC host-foodplant complex sources of *Cotesia congregata.* MsT are divided into wasps originating in Virginia and Indiana. Parameters strongly associated with song elements are bold.

	Principal components (PC)			
	1	2	3	4
Pause duration	0.083	**0.727**	0.036	-0.679
Pulse-buzz duration	**0.584**	-0.008	0.071	0.099
Interpulse interval	**0.586**	0.028	0.055	0.038
Pulse duration	0.233	-0.171	**0.830**	-0.091
Terminal buzz duration	**0.487**	0.103	-0.489	0.175
Frequency	-0.125	**0.657**	0.252	**0.699**
Eigenvalue	2.889	1.224	1.164	0.722
Explained variance (%)	48.14	20.40	19.39	12.03
Cumulative variance (%)	48.14	68.54	87.93	99.96

Table 3.8: Component matrix (eiganvectors), eiganvalues, and explained variance resulting from principal components analysis of male courtship songs from two sources of *Cotesia* nr. *phobetri*: Virginia and Arizona. Parameters strongly associated with song elements are bold.

	Principal components (PC)			
	1	2	3	4
Pause duration	0.117	**0.591**	**0.795**	-0.062
Pulse-buzz duration	**0.520**	-0.032	-0.081	-0.100
Interpulse interval	**0.521**	-0.002	-0.039	-0.112
Pulse duration	**0.447**	-0.183	0.125	**0.809**
Terminal buzz duration	**0.494**	0.041	-0.161	-0.502
Frequency	0.012	**0.784**	-0.565	0.258
Eigenvalue	3.660	1.138	0.873	0.329
Explained variance (%)	61.00	18.96	14.55	5.48
Cumulative variance (%)	61.00	79.96	94.51	99.99

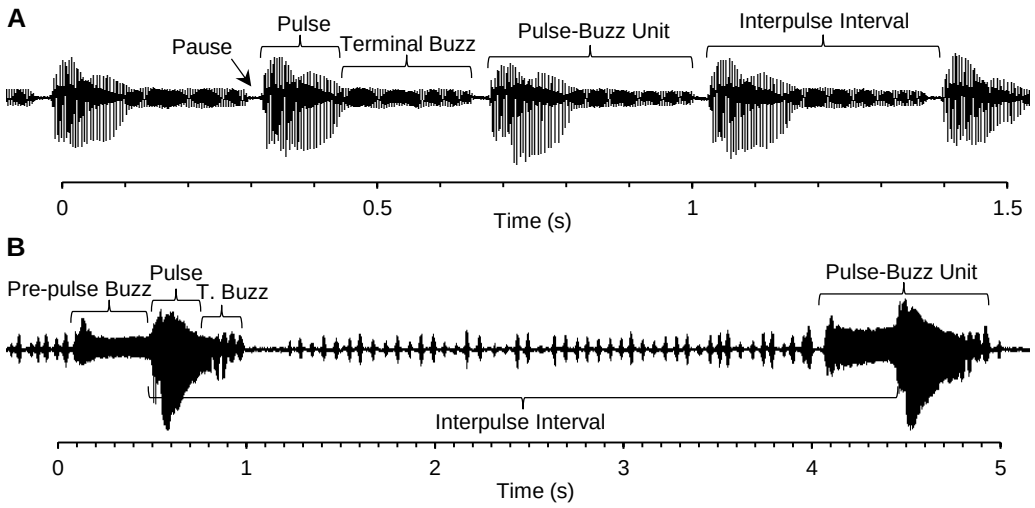

Fig 3.1: Courtship songs of *Cotesia* species were divided into acoustic elements based on relative amplitude and position for use in analysis and comparisons. Represented are typical waveform segments of (A) *C. congregata* and (B) *C. flavichonchae.*

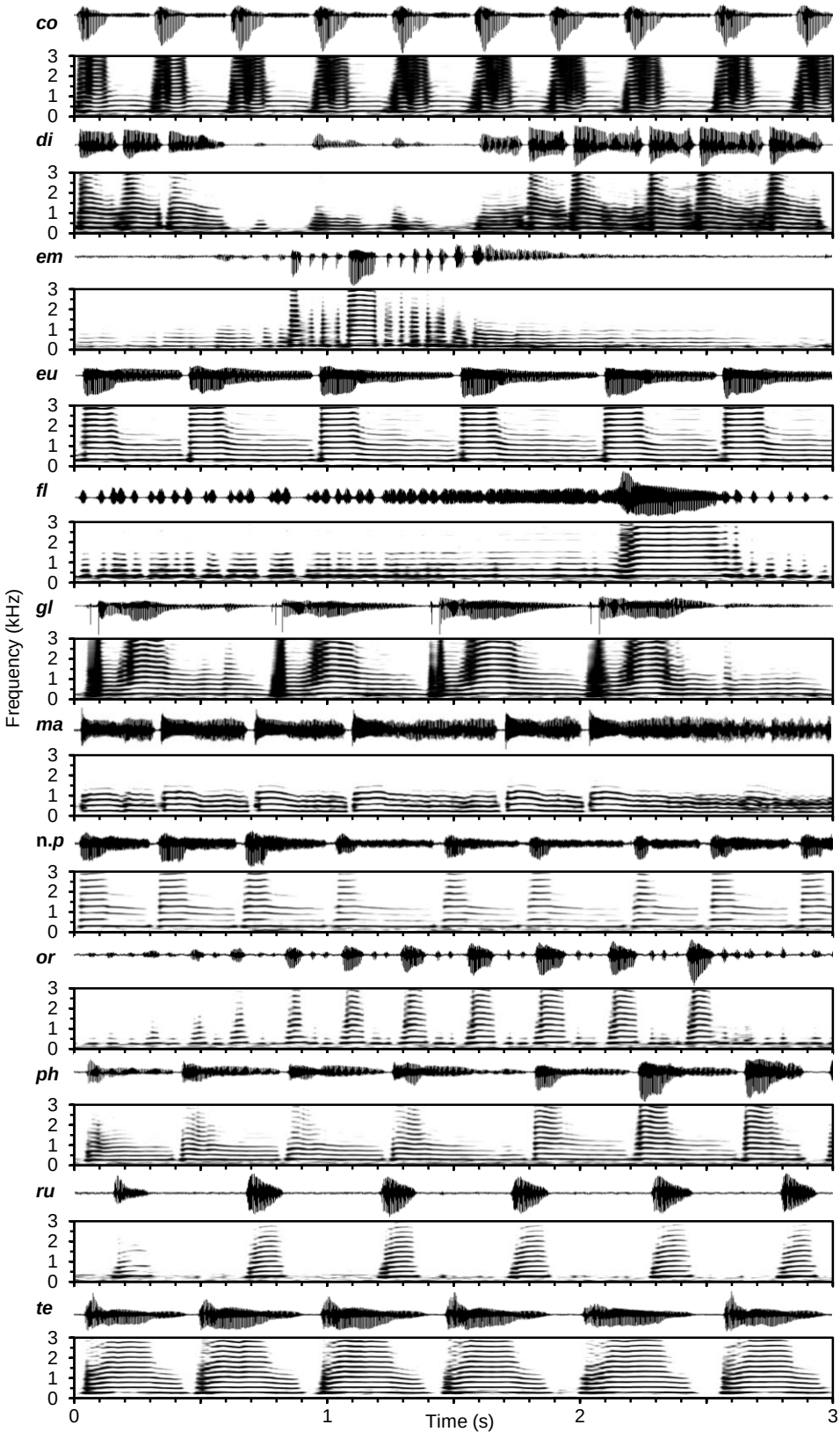

Fig 3.2: Waveforms and spectrograms of typical courtship songs of twelve species of *Cotesia*. Species are labeled by the first two letters of the species name.

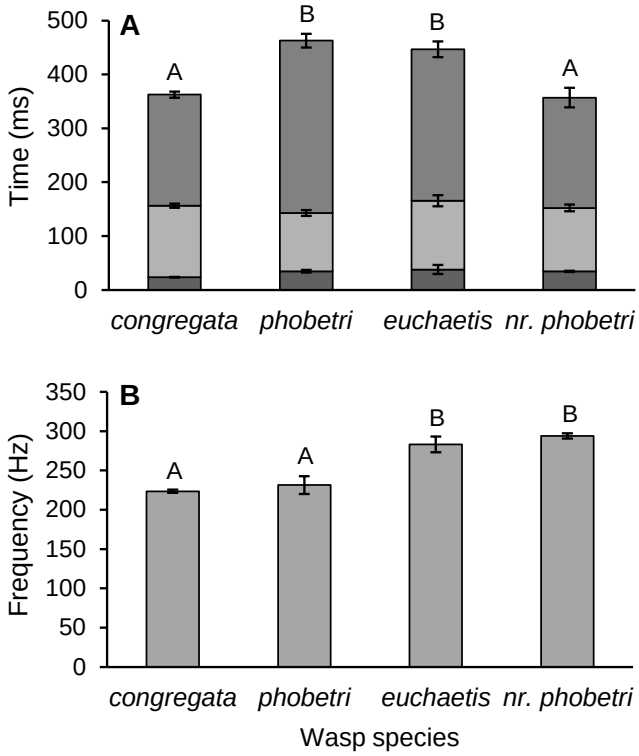

Fig 3.3: A) Mean (± SE) duration of interpulse interval divided into pause (bottom), pulse (middle), and terminal buzz (top) elements and B) fundamental frequency for four *Cotesia* species with similar patterns of song structure. Letters indicate significant differences (ANOVA followed by Tukey's post-hoc test, $p < 0.05$). Species with similar time elements differ in frequency.

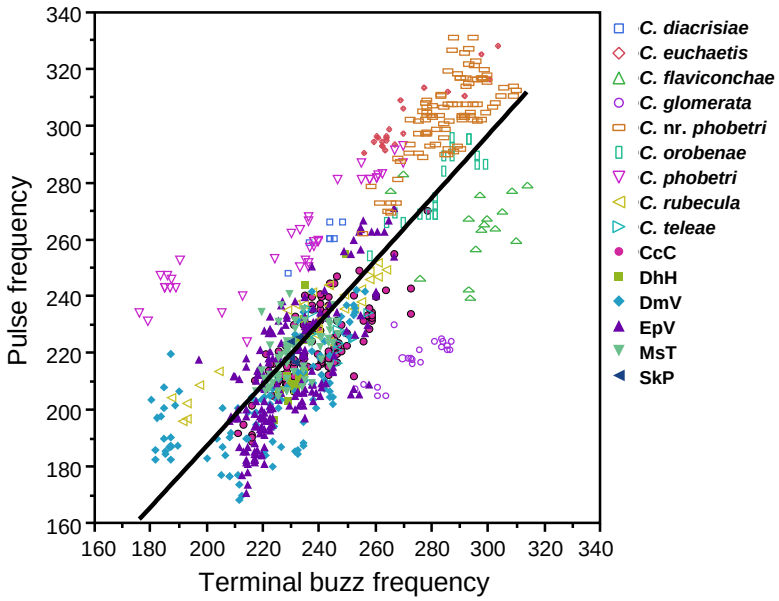

Fig 3.4: Scatterplot of fundamental frequency (Hz) of adjacent pulse and terminal buzz elements in courtship songs across ten *Cotesia* species and six host-foodplant sources of *C. congregata*. Pulses and buzzes were generally correlated in song sections containing both elements (all species, linear regression: $r^2 = 0.65$, $p < 0.0001$; Pulse = -31.0 + 1.1*TBuzz).

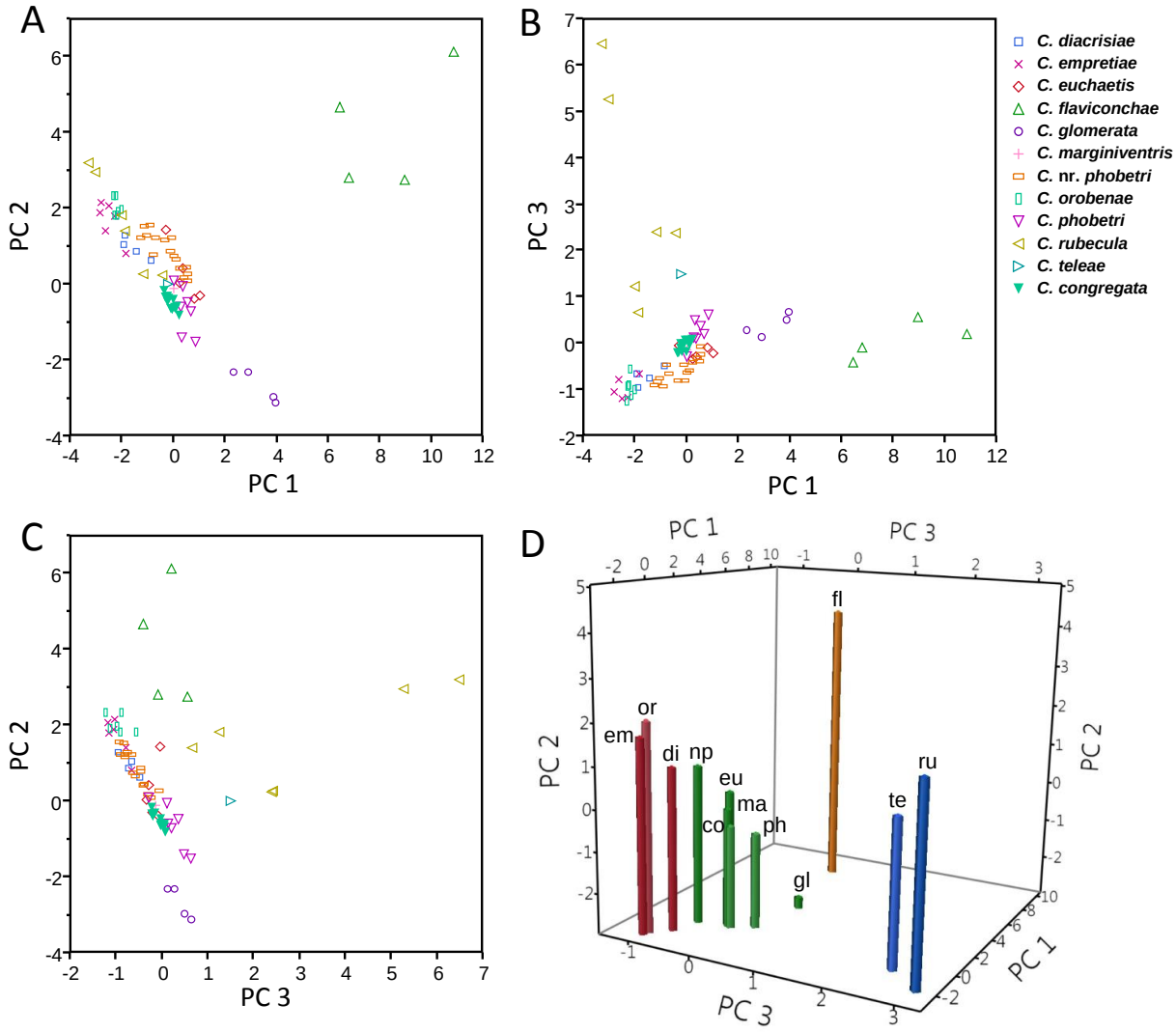

Fig 3.5: Scatterplots of factors of a principal components analysis of seven *Cotesia* courtship song elements: A) PC1 vs. PC2, B) PC1 vs PC3, C) PC3 vs PC2. Each data point represents a single individual coded by species. D) 3-dimensional plot of the mean PC of each species color-coded by dominant courtship song pattern (red – rapid pulses; green – pulse-buzz; blue – long pauses; orange – long buzz-pulse). Two-letter codes indicate species name. *Cotesia congregata* is represented by the MsT host-foodplant complex. Some species are separated from the others by one or more PCs; most species form a close cluster with some overlap.

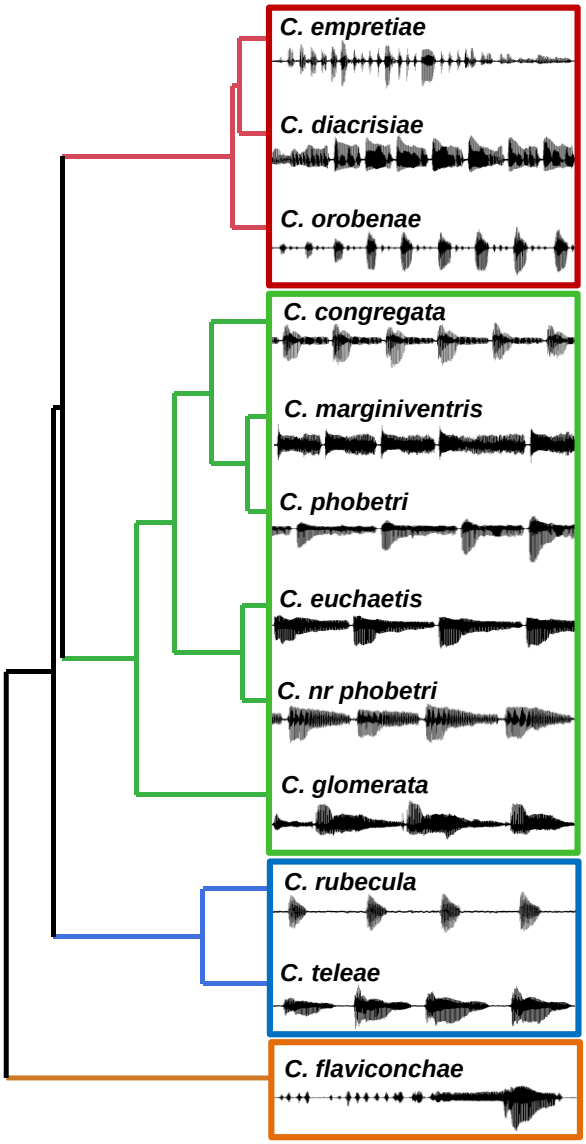

Fig 3.6: Hierarchical clustering using the first four principal components of seven courtship song elements to group *Cotesia* species. Each species is represented by a 2-second waveform section.

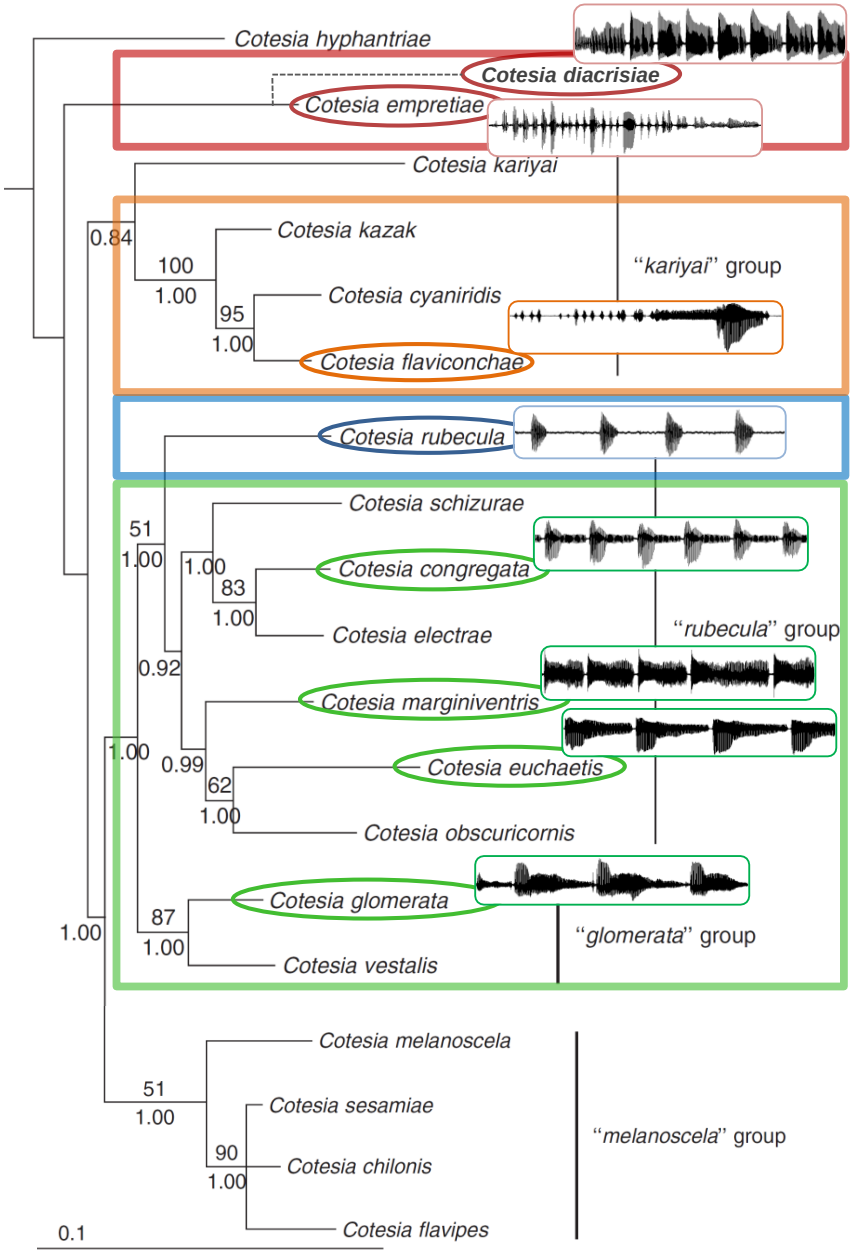

Fig 3.7: *Cotesia* courtship songs of representative species overlaid on a maximum likelihood tree from the analysis of four genes (Michel-Salzat and Whitfield 2004). Recorded species are in ovals and boxed groups were determined by hierarchical cluster analysis using principal components of song elements. *Cotesia diacrisiae* has been added as a sister species of *C. empretiae* based on analysis of the NADH1 gene. Nodes have bootstrap values (above, 100 replicates) and Baysian posterior probabilities (below). Non-*Cotesia* outgroups have been removed for clarity (rooted with *Chelonus inanitus*). Courtship song grouping generally follows the genetic phylogeny with the exception of *C. rubecula*. This may represent a case in which the terminal buzz has been secondarily lost based on the timing and structure of the primary pulses.

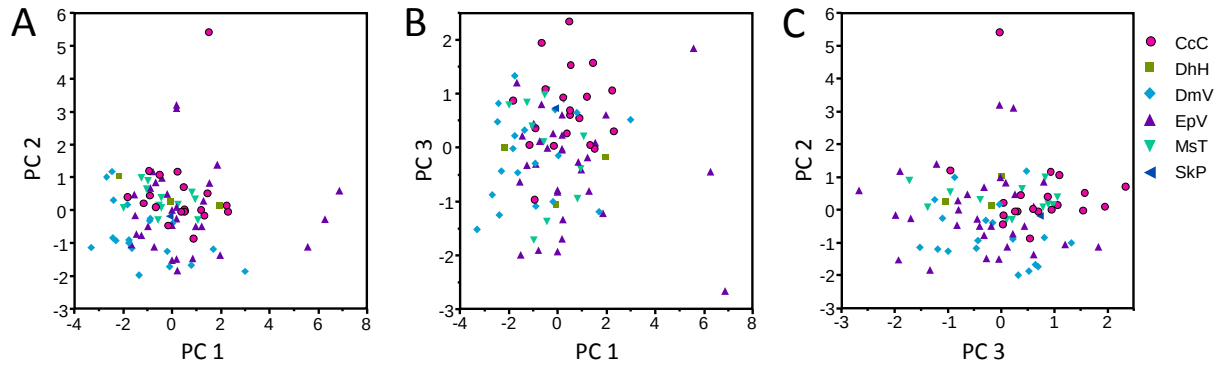

Fig 3.8: Scatterplots of principal components resulting from principal components analysis of courtship song elements produced by six host-foodplant complex sources of *Cotesia congregata*: A) PC1 vs. PC2, B) PC1 vs PC3, C) PC3 vs PC2. Songs from different host-plant complex sources cannot be reliable distinguished from each other.

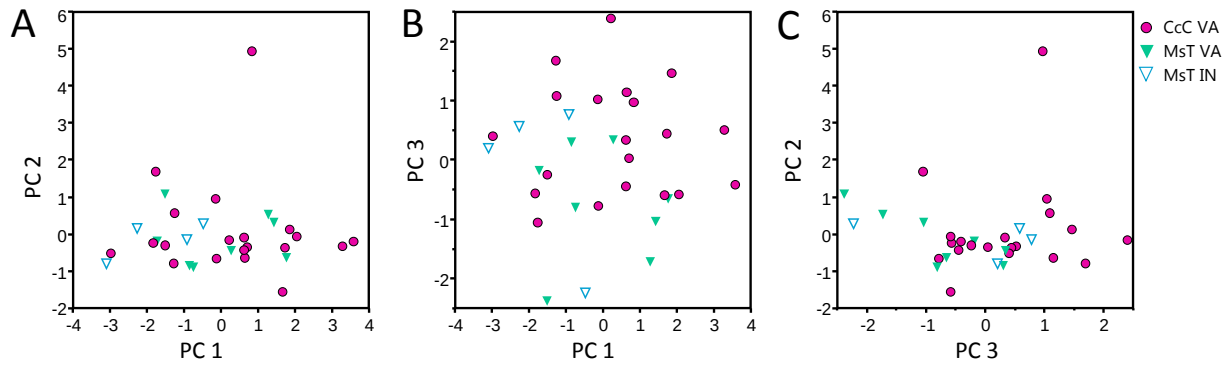

Fig 3.9: Scatterplots of principal components resulting from principal components analysis of courtship song elements produced by MsT and CcC host-foodplant complex sources of *Cotesia congregata*: A) PC1 vs. PC2, B) PC1 vs PC3, C) PC3 vs PC2. MsT wasps originated from two sources in Virginia and Indiana. Songs from different host-plant complex sources or different locations cannot be reliably distinguished from each other.

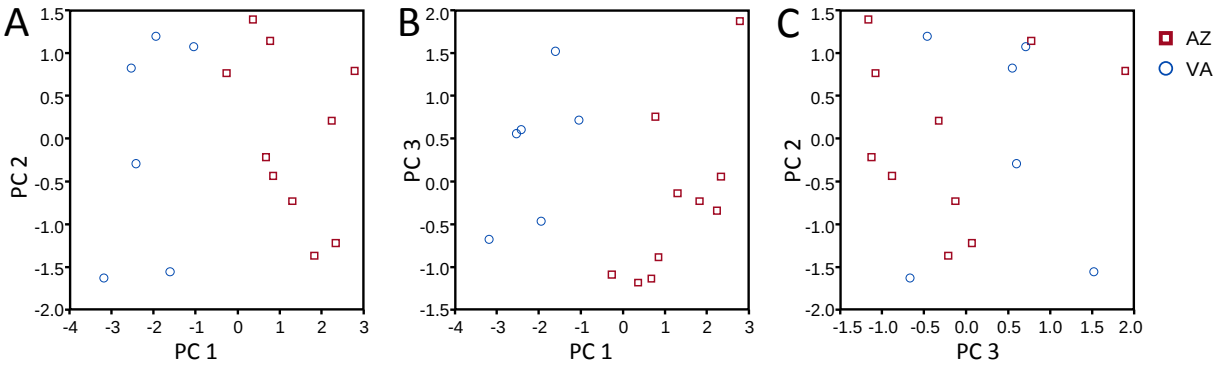

Fig 3.10: Scatterplots of principal components resulting from principal components analysis of courtship song elements produced by *Cotesia* nr. *phobetri* originating in Arizona and Virginia: A) PC1 vs. PC2, B) PC1 vs PC3, C) PC3 vs PC2. Songs from these two locations can be reliably distinguished from each other based on PC1 (active song element durations), but not PC2 or PC3 (frequency and pause duration). Mean pulse-buzz duration is 0.13 s longer in songs of wasps from Arizona vs. Virginia ($t_{13} = -8.6$, $p < 0.0001$).

Chapter 4 Asymmetric hybrid sterility and genetic differentiation among sympatric host-foodplant sources of the parasitic wasp, *Cotesia congregata*

Asymmetric hybrid sterility and genetic differentiation among sympatric host-foodplant sources of the parasitic wasp, *Cotesia congregata*

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Abstract— Parasitic wasps are highly diverse and play a major role in suppression of herbivorous insect pest populations. Previously identified species of some parasitic wasps are actually complexes of cryptic species resulting from adaptations to specific hosts or host foodplants. For example, long-term studies of the African braconid endoparasitoid, *Cotesia sesamiae*, indicate that allopatric populations that utilize different stem-borer host species are genetically and reproductively differentiated, and contain sequence differences in bracovirus (BV) genes that are injected into the host to suppress immune responses. A North American congeneric, *Cotesia congregata*, which has long served as a model system for host-parasitoid interactions, provides a complementary system for investigating host-associated diversification among sympatric populations. Two incipient species of *C. congregata* have been identified in the mid-Atlantic region of the USA, one utilizes *Manduca sexta* on tobacco ("MsT") and the other utilizes *Ceratomia catalpae* on catalpa ("CcC"). Both species can develop in both caterpillar host species; however, hybrids resulting from CcC♂ x MsT♀ crosses are typically sterile and lack mature ovaries. Crosses with *C. congregata* from four additional host sources display a pattern of asymmetric hybrid sterility with either MsT or CcC, indicating only two primary lineages. Relative expression of seven *C. congregata* BV (CcBV) genes in *M. sexta* and *C. catalpae* parasitized by individual MsT or CcC wasps, and in *M. sexta* parasitized by individual MsT and CcC hybrids were compared. These included CrV1, which has been correlated with host range usage in populations of *C. sesamiae*, as well as others involved in parasitism. Patterns of relative

in vivo expression of CcBV genes from MsT and CcC wasps differed; a few genes were not expressed in hosts parasitized by CcC wasps. Patterns of relative expression did not differ with respect to host species parasitized. Parasitization by sterile hybrids resulted in low or absent expression of CcBV genes. Select CcBV genes were sequenced from each wasp source and some varied between incipient species. Differences in BV expression and sequences reflect reproductive isolation in *C. congregata*, and likely represents a step towards host specialization, but do not preclude wasps from utilizing both host species.

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

Introduction

Ecological speciation via adaptation to hosts is probably the primary factor leading to extreme diversity among phytophagous insects and their parasitoids (Feder and Forbes 2010; Matsubayashi et al. 2010). Parasitic wasps in particular are among the most diverse groups of terrestrial animals (LaSalle and Gauld 1991), and tend to have close associations with only one or a few host species. Indeed, genetic analysis has revealed that many parasitoid species assumed to be generalists utilizing various hosts are actually multiple species of specialists (Kankare et al. 2005a,b; Smith et al. 2013). Host shifts initiating speciation have been empirically demonstrated in only a few phytophagous and parasitic insects; host shifts contributing to speciation or the development of reproductive isolation have been documented in far more systems (see Forbes et al., 2017). Additional factors such as host-plant toxicity, associative learning, and genetic incompatibility may lead to or maintain reproductive isolation. Perhaps one of the most important drivers of host-specificity is the evolutionary arms-race with host immune systems.

Many endoparasitic wasps use symbiotic polydnaviruses (PDVs) to suppress the immune response of their lepidopteran hosts. The biology and history of PDVs has been extensively reviewed elsewhere (Beckage and Drezen 2012; Gundersen-Rindal et al. 2013; Herniou et al. 2013b; Strand and Burke 2015). PDV genes are encoded onto the wasp chromosomes and transmitted vertically. Viral capsids are produced only in specialized calyx cells where they incorporate DNA circles containing virulent genes. Upon injection into the host with the wasp eggs, PDVs produce transcripts that interfere with host physiology and cellular immunity to benefit the developing wasp. PDVs have evolved independently in the major parasitic wasp families. PDVs in the Braconidae, primarily in the extremely diverse subfamily Microgastrinae consisting of 17,000-46,000+ species (Rodriguez et al. 2013; Whitfield et al. 2018), are called

bracoviruses (BVs) to distinguish from those in the Ichneumonidae. The genomic organization of BV proviral segments has been obtained (Bezier et al. 2013), as well as the host expression pattern of 88 BV genes 24 hours after parasitism by *Cotesia congregata* (Say) (Chevignon et al. 2014). The association with BVs begun an estimated 100 mya (Murphy et al. 2008) and has very likely played a role in host-associated differentiation of parasitoid lineages.

Reproductive isolation and host-adaptation has been extensively studied in *Cotesia sesamiae* (Cameron), 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* common in mountainous regions, whereas the lowland coastal populations where *B. fusca* is uncommon are encapsulated (Ngi-Song et al. 1998). Both populations develop in the widespread host *Sesamiae calamistis*. Encapsulation of lowland *C. sesamiae* eggs in *B. fusca* is due to differences in PDV virulence (Mochiah et al. 2002a). Encapsulation occurred when the well-characterized virulence gene, CrV1, was not expressed in *B. fusca* (Gitau et al. 2007); differences in this gene partly explain host range (Dupas et al. 2008; Branca et al. 2011). CrV1 encodes a glycoprotein responsible for actin cytoskeleton interference in host hemocytes, preventing adhesion (Asgari et al. 1996, 1997). Several additional bracovirus orthologs among populations have sites under positive selection (Jancek et al. 2013). Moreover, the coastal strain but not the inland strain is infected by the endocellular bacteria *Wolbachia*, which leads to a 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* 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., *C. flavipes* by Muirhead et al., 2012).

The congeneric *C. congregata* offers a complementary system for the study of host-associated divergence and BV differentiation among parasitic wasps. In addition to being used for bracovirus genomics, *C. congregata* serves as a model for tri-trophic interactions (Kester and Barbosa 1994; Kester et al. 2002), insect learning (Kester and Barbosa 1991b; Lentz and Kester 2008; Lentz-Ronning and Kester 2013), and immunology (Harwood et al. 1998; Beckage 2008). Two sympatric incipient species of *C. congregata* have been described: "MsT wasps" originating from *Manduca sexta* (L.) on tobacco (*Nicotiana tabaccum* L.) and "CcC wasps" originating from *Ceratomia catalpae* (Boisduval) on catalpa (*Catalpa speciosa* Warder). These two wasps are diverged genetically, with differences in microsatellite allele frequencies and a ~2% divergence in COI sequences, although some gene flow may still occur (Kester et al. 2015). The two lineages are separated by adaptations to plant allelochemicals; CcC wasps experience near 100% mortality on hosts feeding on nicotine present in tobacco (Bredlau and Kester, *in submission*). MsT and CcC have minor differences in courtship characteristics but will mate and produce hybrids when paired in enclosed vials. However, ~90% of hybrid females produced from crossing CcC males x MsT females are sterile; the reciprocal cross produces normal fertile progeny (Bredlau and Kester 2015). Caterpillars of either host species parasitized by sterile hybrids developed and pupated normally. Dissections of parasitized hosts revealed areas of melanization that were presumed to be encapsulated wasp eggs. The absence of a functional BV

suggested that the genetic mechanisms involved in sterility prohibited the production of BV particles, although the precise cause remained uncertain. Although hybrid incompatibility due to *Wolbachia* infection occurs in other *Cotesia* species (Mochiah et al. 2002a; Rincon et al. 2006), *Wolbachia* has not been found in either population of *C. congregata* (Gundersen-Rindal, unpublished data). Even though a host-shift has contributed to the speciation between MsT and CcC lineages, under what conditions the shift occurred cannot be directly inferred.

Cotesia congregata has been reported from at least fourteen Sphingid species (Krombein et al. 1979), most of which are plant family specialists (Tietz 1972). The foodplants of the other Sphingid hosts are common and have overlapping distributions with MsT and CcC collection sites. Furthermore, the other hosts are phylogenetically diverse, with some species in a separate subfamily from *M. sexta* and *C. catalpae* (Kawahara et al. 2009). Given the divergence of MsT and CcC wasps, the diversity of host utilization suggests the possibility of a larger species complex. Wasps utilizing more phylogenetically distant hosts or plants may be predicted to have even greater reproductive isolation (e.g. sterility in both hybrid crosses), assuming any sort of phylogenetic signal in host adaptation (Feder and Forbes 2010; Forister and Feldman 2011). If not, investigating how wasps from additional sources may group into either MsT or CcC lineage will provide insight into underlying population structure and host-usage. In contrast to *C. sesamiae*, *C. congregata* has diverged host-foodplant sources of wasps that are not currently geographically isolated (although this may have been the case in the past), can develop within both host species, and does not contain *Wolbachia* to create a post-zygotic reproductive barrier.

The objectives of this study were to: (1) determine the pattern of post-zygotic reproductive isolation among additional sympatric host-foodplant sources with MsT and CcC incipient species of *C. congregata*, (2) evaluate differences in host expression of bracovirus

genes among MsT and CcC wasps and their hybrids, and (3) infer potential mechanisms responsible for hybrid sterility. We evaluated post-zygotic barriers and sterility using a series of hybrid crosses between each additional wasp type collected with MsT and CcC wasps. Expression of eight bracovirus genes known to be virulence factors (including CrV1 and CrV1-like) were compared using reverse transcriptase quantitative PCR (RT-qPCR) to determine differences between wasp sources, their hybrids, and host utilized.

Materials and Methods

Parasitoids

Parasitoids were collected from sites in Virginia, USA over three years (Table 1). "MsT wasps" used 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 have been used in prior genetic and behavioral studies (Bredlau and Kester 2015; Kester et al. 2015), and multiple generations were regularly collected each year. Other hosts of *C. congregata* were collected during extensive and routine searches in native and cultivated habitat. Particular effort was placed on sphingids known to be common in the region; however, wasps were collected from only four additional species. 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 parasitoid larval egression. Cocoons were removed three 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

Wasps were used to establish a series of mating pairs to determine patterns of hybrid sterility. Crosses were established between the additional host-foodplant complex sources and both MsT and CcC wasps, with sib-crosses as controls. Because only MsT and CcC wasps were consistently available, crosses were not established among the new sources which were rarely collected at the same time. MsT and CcC reciprocal crosses were established for comparison to the new crosses and 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 cm x 2 cm diam.) with a water soaked cotton ball and plugged with a cotton ball with honey as a food source. Because mating success is approximately 40% between different host-foodplant complex sources in vials (Bredlau and Kester 2015), we instead used a modified methodology to increase mating success similar to forced-contact mating presented in Kitthawee (2008). Females were chilled in their gel capsule in a -10°C freezer for 6-10 minutes. During this time, males were primed by presenting them a recently dead or immobilized female from their same source in order to increase the rate of courtship elicitation (Bredlau and Kester 2015). The chilled female was carefully removed from the capsule with fine-point forceps and positioned in front of courting males; the female used for priming was removed. Cold treating females did not affect male copulation behavior. After copulation was completed, the female was carefully placed into a separate vial with food and water for recovery. Although this process killed some females, most recovered completely within 1-2 minutes and this method ensured a greater mating success rate when using wasps from different populations. 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 hybrid diversity.

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

Hybrids were considered sterile if parasitized hosts failed to produce wasp larvae. Fisher's exact test was used to compare proportions of sterile hybrids produced between reciprocal crosses. When multiple wild broods were collected of the same host-foodplant complex, the Cochran-Mantel-Haenszel test was also used, with each initial brood treated as a

block. Statistics were performed using R statistical software (R Development Core Team) and JMP v11 (SAS Institute, Inc).

Host expression of bracovirus genes

Host expression of bracovirus genes were compared between MsT and CcC wasps on both hosts and their hybrids 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 arbitrarily selected from each group. Females parasitized an early 4th instar *M. sexta* from a laboratory strain or 3rd instar *C. catalpae* from field collected eggs. Parasitized caterpillars were kept in plastic cups on their diet (laboratory diet blocks or catalpa leaves) for 24 hours 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.

Bracovirus genes known to be virulence factors and corresponding primers (Table 2) were selected from Chevignon et al. (2014). Real-time PCR runs were performed in 96-well optical reaction plates with Power SYBR Green PCR Master Mix (Applied Biosystems). An amount of cDNA equivalent to 5 µg of total RNA was 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. RPL3 was used in addition

to 18S in preliminary runs but had more variation. Because different homologous controls had to be used for the different hosts, gene expression cannot be directly compared between these two sets for each gene; however, overall relative patterns can be compared. 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 Δ*Ct* values for each sample. Expression was calculated using the $2^{-\Delta Ct}$ method. Because we were measuring host expression in naturally parasitized hosts, the amount of BV injected could not be controlled and is expected to vary among individual wasps. Relative expression of bracovirus genes were compared between hosts *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 Development Core Team). A Bonferroni correction was used to adjust reported p-values for the number of genes sampled.

Results

Parasitoids

MsT and CcC wasps are common and easily available; primary sites contain hundreds of parasitized caterpillars within close proximity and may produce several thousand wasps in a generation. Additional parasitized sphingids were collected in small numbers. Despite extensive searching over three years on grape (*Vitis spp.*), Virginia creeper (*Parthenocissus quinquefolia*), privet (*Ligustrum spp*.), pawpaw (*Asimina triloba*), honeysuckle (*Lonicera spp*.), and trumpet

vine (*Campus radicans*), and shorter searches on pine (*Pinus*), few parasitized caterpillars were found (Table 1). Several caterpillars of each species were parasitized by other hymenopterans (Ichneumonidae) or dipterans (Tachinidae). For example, almost all collected *Hemeris diffinis* on honeysuckle produced tachinids (*Belvosia spp.*) and no *Cotesia spp.* The trumpet vine sphinxes, *Paratraea plebeja*, found during this study were not parasitized; however, parasitized individuals in other years have been observed. Few *Sphinx kalmiae* on privet and *Dolba hyloeus* on pawpaw were ever found, despite the many plants searched, and only one of each were parasitized by *C. congregata*. A pine barren was searched for *Lapara spp.* (pine sphinx) but none were recovered. Four parasitized *Darapsa myron* on Virginia creeper were found, however the wasps from these broods tended to be relatively weak, males would not successfully mate, and they produced few hybrid broods.

Eumorpha pandorus provided the most wasps (one had a record of 502 wasp cocoons), although only five parasitized broods were found during this study in two regions of Virginia. The two regions were approximately 95 km apart: two broods from Virginia creeper growing along a fence in Gloucester Co., VA and three broods from in and around Richmond, VA which were 2.3 km or 8.5 km from the next closest collection site. These sites were within fragmented urban and suburban areas. Although other groups of *E. pandorus* were encountered, these were rarely parasitized, and more often sites of feeding damage and frass were located after caterpillars had already disappeared or pupated. 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. All broods originated in central or eastern Virginia, USA, and in some cases within the same vicinity.

Reciprocal crosses

Crosses were established between four additional host-foodplant complex sources of wasps with MsT and CcC wasps. All crosses between wild P generation wasps produced hybrid F_1 females that appeared and behaved normally. Hybrid females produced from MsT♂xCcC♀ crosses continued to produce progeny (28/29). Hybrids from CcC♂xMsT♀ crosses were sterile – all 108 hybrids produced from 21 initial matings. Control lines from both wasp populations develop normally in both hosts. These data verify results produced by Bredlau and Kester (2015).

Other host-foodplant complexes produced similar patterns of hybrid sterility with either MsT wasps or CcC wasps, but not both. The wasp brood originating from *S. kalmiae* on privet (SkP) when used in a SkP♂xMsT♀ cross produced only 21% fertile hybrids, significantly lower than all other crosses, including those with CcC which were ~100% fertile ($p < 0.0001$; Fig. 1A). Likewise, the brood from *D. hyloeus* on pawpaw (DhP) when used in a CcC♂xDhP♀ cross produced only 45% fertile hybrids, significantly lower than all other crosses ($p < 0.0001$; Fig. 1B). In these cases, the SkP wasps had a pattern as if they were CcC wasps, and the DhP wasps had a pattern as if they were MsT wasps.

Wasps produced from *E. pandorus* on Virginia creeper followed similar patterns of hybrid sterility (Fig. 2). Most hybrids produced with MsT were fertile, whereas most 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) had a reciprocal pattern. Hybrids produced from this EpV♂xMsT♀ cross were typically sterile, whereas the other crosses were generally fertile (Fisher's exact: $p < 0.0001$; Fig. 2). In almost all cases, wasps from additional host sources produced a pattern of asymmetric sterility whereby any hybrids with

MsT♂ *and* CcC♀ were fertile; hybrids from CcC♂ *or* MsT♀ were sterile and the reciprocal was fertile. Both patterns can exist from the same host source, as seen with EpV wasps. Sterile crosses that did produce progeny usually had smaller brood sizes. All individual control lines continued to produce progeny and were subsequently maintained in separate colonies for at least five generations using host *M. sexta*.

Wasps produced from *D. myron* on grape or Virginia creeper generally followed the same patterns as the others, however all cross types could not be established so data is incomplete. Broods from *D. myron* either lacked females (brood A) or produced relatively weak males that would not successfully mate with the other wasps (broods B-C). The DmV♂xMsT♀ cross from brood A produced all sterile 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 hybrids (13/15). The MsT♂xDmV♀ cross produced all fertile hybrids (31/31). The crosses from DmV brood D produced fertile hybrids with both MsT and CcC (9-23 hybrids tested for each cross), which was not observed in any other set of crosses. Even without the production of all crosses, the general pattern corresponds to that observed in the other wasp sources: either DmV♂xMsT♀ or CcC♂xDmV♀ cross produces sterile hybrids but not the reciprocal crosses.

Dissections of female wasps (986 total) revealed that hybrids that failed to produce progeny in the sterile crosses had severely reduced or absent ovaries and calyx (Fig. 3). These hybrid wasps otherwise appeared and behaved normally with typical parasitism behavior. Failed parasitisms by fertile wasps with normal ovaries do occur, but the success rate is typically above 90%. Caterpillars that did not produce emerged wasps also contained no wasp larvae upon dissection and had small spots of melanization as described in Bredlau and Kester (2015).

Host expression of bracovirus genes

Relative expression of five of eight selected bracovirus genes differed between hosts parasitized by MsT and CcC wasps (Fig. 4). Ankyrin 4, CcV3-like, and PTP-L had no difference in expression by wasp type in either host species ($p > 0.1$). CrV1 and CrV1-like had identical expression across hosts and wasp sources in *M. sexta*; CrV1-like was used in subsequent analysis. CcC wasps produced no detectable expression of two genes in both hosts: CrV1-like and cystatin 1 in *M. sexta* ($W = 0$, $p < 0.001$; $W = 0$, $p < 0.001$, respectively) and *C. catalpae* ($W = 4$, $p = 0.013$; $W = 0$, $p = 0.001$). CcBV 13-2 was overexpressed in *M. sexta* parasitized by CcC in comparison to MsT ($W = 75$, $p = 0.006$), but did not differ significantly in *C. catalpae* ($W = 45$, $p > 0.1$). In contrast, duffy-like was marginally overexpressed in *C. cataplae* parasitized by CcC ($W = 56$, $p = 0.07$), but not significantly in *M. sexta* ($W = 57$, $p > 0.1$). 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 leads to natural variation in each sample.

Hybrids also differed in relative host expression of bracovirus genes (Fig. 4). The MsT♂xCcC♀ hybrids have expression levels similar or between those produced by the parents, and not significantly different from MsT ($p > 0.1$). Sterile hybrids produced by CcC♂xMsT♀ crosses had reduced or absent expression by all genes in comparison to MsT or CcC expressed genes (Kruskal-Wallis with Dunn's test: $df = 3$, $p < 0.05$). Approximately 10% of CcC♂xMsT♀ hybrids do produce progeny (Bredlau and Kester 2015) and two hybrids sampled expressed genes with intermediate expression levels ($N = 14$), including expression of CrV1-like and cystatin 1. Similar results were found among limited sampling of the other host-foodplant complex sources in which sterile hybrids do not produce expressed genes.

Discussion

All host-foodplant complexes examined can be grouped into two incipient species that display hybrid sterility in only one cross type. We tested both reproductive compatibility via crosses and differences in host expression of bracovirus genes. A genetic comparison alone would not have been suitable for determining patterns of reproductive isolation or hybrid sterility. Furthermore, we predicted differences in bracovirus expression between MsT and CcC wasps due to differences in host utilization and the absence of functional BVs in CcC♂xMsT♀ hybrids. We initially hypothesized that differences in BV genes or inheritance created non-functional BVs in certain hybrid lines, i.e. Bateson-Dobzhansky-Muller incompatibilities among alleles (Mack and Nachman 2017). Dissections of sterile hybrids have revealed that in addition to not producing BV particles, these wasps also do not produce developed ovaries. The ~10% of hybrids that do produce progeny in the sterile lines also tend to produce drastically reduced brood sizes (Bredlau and Kester 2015). Given the hybrid sterility found between MsT and CcC incipient species, we predicted that the other host-foodplant sources of wasps would have similar or greater reproductive isolation.

MsT and CcC wasps differ in host and plant usage, are genetically differentiated (Kester et al. 2015), and have subtle differences in courtship behavior (Bredlau and Kester 2015). These are only two of the at least fourteen sphingid species reported as hosts in North America. Moreover, *M. sexta* and *C. catalpae* are closely related compared to most of the other host species; for example, *D. myron* and *E. pandorus* are in a different subfamily from the other three hosts and only *D. hyloeus* is more closely related to *M. sexta* (Kawahara et al. 2009). We expanded earlier findings using MsT and CcC wasps to include additional host sources with overlapping ranges. Considering the consistent asymmetric pattern of hybrid sterility with either

MsT or CcC wasps, there is no evidence for a phylogenetic pattern by either host or host plant. We cannot rule out that wasps from another host source may be completely reproductively isolated and represent a cryptic sibling species; however, the current sampling of sympatric host-foodplant sources makes this possibility less likely.

Despite the ability of wasps from different populations to develop within other sphingid hosts that may be phylogenetically diverse, there is still a clear post-zygotic reproductive barrier between them. The pattern of hybrid sterility indicates only two incipient species rather than a series of host-specific incipient species. Host-fidelity or a preference for certain host-plants via natal or sequential learning has been demonstrated for several parasitoid species (Lewis and Tumlinson 1988; Vet and Groenewold 1990; Turlings et al. 1993; Kaiser and De Jong 1995; Lentz-Ronning and Kester 2013) and may play a role in ecological divergence (König et al. 2015). However, host-fidelity is flexible considering that EpV and DmV broods may be compatible with MsT or CcC even within the same region. A similar condition would likely be found with SkP, DhP, and other populations if enough samples were collected. Production of fertile hybrids with both MsT and CcC is possible (e.g. DmV brood D), but this appears to be exceptional. Why this occurred in only this particular lineage is unknown.

Population numbers of the additional hosts suggests that these are likely incidental hosts. MsT and CcC populations can build up to large numbers in many areas. *Manduca sexta* is a common caterpillar pest on tobacco, tomato, and other solanaceous plants and parasitoids from this species are easily acquired. A one acre tobacco field may support hundreds of hosts and thousands of wasps. Parasitized *M. sexta* are also regularly found on garden tomatoes, even in urban areas (personal observations). Likewise, one catalpa tree may support hundreds of *C. catalpae* leading to tree defoliation (Lampert et al., 2010, and personal observations). These host

populations support multiple generations of *C. congregata* each year and generations in September and early October may parasitize nearly all available hosts at some sites. In contrast, the other potential sphingid hosts are solitary, isolated, and often do not exist in such large numbers. Extensive searching yielded few parasitized individuals over three years. Only *D. myron* and *E. pandorus* were found in groups, however these hosts were rarely observed to reappear on the same plants in the same year.

The pattern of host utilization may be governed by metapopulation dynamics – immigration, establishment of new populations, and local extinction. MsT and CcC are the two primary wasp lineages producing large source populations annually. As these large populations become host limited and disperse, they utilize other nearby sphingids hosts on other plants. These other local host populations in the surrounding landscape may only persist for a few generations or years as host populations are not sustained in adequate numbers. This source-sink model may explain how the presumed host-associated populations are related to either MsT or CcC wasps in the same region. Metapopulations have been well studied in other parasitic wasp species. For example, *C. melitaearum* on the Åland islands in Finland exists in metapopulations on a fragmented landscape limited by the quantity of its host caterpillar, *Melitaea cinxia*, patch isolation, and recolonization rates (Lei and Hanski 1997; van Nouhuys and Hanski 2002; Kankare et al. 2005c). In small isolated patches, *C. melitaearum* may drive their hosts to local extinction. Subsequent recolonization to patches with hosts is limited by the typical dispersal range of ~1 km. For *C. congregata*, additional studies are necessary to determine the community structure among the different host-associated populations on a landscape level.

The cross that produces sterile hybrids is with either a CcC male or MsT female. Caterpillars parasitized by these hybrids typically pupate normally, indicating that no functional

bracovirus is injected into the host. Dissections of sterile wasps revealed reduced reproductive systems, lacking ovaries and a calyx. Bracovirus particles are produced in the calyx and are necessary to suppress the host immune system during development (Beckage 1998). Reduced or absent expression of bracovirus genes in hosts parasitized by sterile hybrids supports that the reduced reproductive system does not produce BV particles. Wasps developing in both host species and the same hybrids failing in both host species further indicates that parasitism success reflect wasp differences more so than immunological effects from the hosts, allowing the utilization of multiple host species..

Differences in bracovirus expression illustrate divergence between MsT and CcC incipient species (Fig. 4). Two genes, CcBV 13-2 and duffy-like, from CcC tended to be overexpressed relative to MsT dependent on host parasitized suggesting differences to host immune response. This response varied by individual wasps and we cannot strictly rule out that this was anomalous. However, CcC wasps certainly do not express three of the sampled genes. Because CrV1, CrV1-like and cystatin 1 are not expressed in either host, the lack of expression is most likely due to CcC wasps not producing transcripts of these genes during BV particle production. PCR and sequencing of the CrV1 gene indicates that it is present in the CcC wasp genome. An undetermined mechanism of gene regulation may be responsible for its lack of transcription or packaging during BV particle production.

CrV1 is the most studied BV gene and is implicated as an important virulence factor during parasitism in several *Cotesia* species including *C. congregata* (Whitfield 2000; Amaya et al. 2005). Gitau et al., (2007) reported differences in CrV1 expression and sequences between two geographically isolated host-associated biotypes of *C. sesamiae*. The lowland (coastal) avirulent biotype experiences egg encapsulation and low or absent expression of CrV1 in the

highland host *B. fusca*. The virulent highland biotype can develop in *B. fusca* and other hosts. Furthermore, positive selection was observed at the CrV1 locus in association with these geographic differences in host usage and immune resistance (Dupas et al. 2008). In contrast, the two *C. congregata* incipient species can develop within either host. Moreover, CrV1 and CrV1-like are not expressed by CcC wasps even though they still suppress host immune responses. Our results suggest that genes included in BV production are more flexible than previously thought and that redundancy in virulent genes may exist. CrV1 and cystatin 1, although likely important in some species, are not necessary for suppression of the host immune system by *C. congregata*. Either other virulent genes are produced with similar functions or the other virulent genes on their own are enough to adequately suppress the host immune response.

The ability to parasitize multiple hosts does not preclude host adaptation. Differences in BV expression without large sequence differences may be an early step in host specialization. For example, *C. catalpae* may be more susceptible to other BV virulent genes than to CrV1. Over time, wasp populations adapting to this host may shift from utilizing one set of BV genes to overexpressing others without losing the underlying genes. These subtle early shifts are then built upon as hosts evolve defenses against wasp BVs until that host is refractory to other wasp populations, as observed in the biotypes of *C. sesamiae* (Mochiah et al. 2002a). In *C. sesamiae*, an analysis of bracovirus genomes between inland and coastal populations suggests that wasps may evolve different pathways to overcome resistance by local hosts (Jancek et al. 2013). The speed in which hosts evolve immunological defenses would dictate wasp BV divergence. Alternatively, these differences may be due to genetic drift between the two incipient species. This assumes enough flexibility in the function of BVs to allow a shift in expression without

reducing fitness. Three genes did not differ in expression between the two incipient species (ankyrin 4, CcV3-like, and PTP-L), and may represent PDV elements that are conserved.

The genetic mechanism responsible for the pattern of asymmetric hybrid sterility is currently under investigation. The characteristics have distinct similarities to P element induced hybrid dysgenesis long-studied in *Drosophila* (Kidwell et al. 1977; Engels and Preston 1979; Bingham et al. 1982; Kidwell 1983). In short, P elements are genomically inherited transposons that are regulated by small non-coding PIWI-interacting RNA (piRNA) which are maternally inherited via the cytoplasm (Brennecke et al. 2008; Jensen et al. 2008). Without the piRNA silencing mechanism, P elements are left unregulated with their transpositions leading to failure of ovarian development. Therefore, crosses between males with P elements and females without P elements and the piRNA repressors generate sterile but often otherwise normal hybrids. In the reciprocal cross, both P elements and the repressors are inherited maternally leading to normal hybrids (for review see: Kelleher, 2016; Luo and Lu, 2017). More recently, P elements have spread throughout the global populations of *Drosophila simulans*, leading to hybrid dysgenesis between some populations in breeding assays (Hill et al. 2016). P element like transposons have also been found in other eukaryotic organisms (see Rio and Majumdar, 2015), but have not been demonstrated to produce hybrid dysgenesis in closely related species outside of *Drosophila*.

In *C. congregata*, CcC wasps would contain the P-like transposons and regulatory small RNA and MsT would lack both. When hybridized, CcC♂xMsT♀ hybrids would inherit the transposons in the nucleus but not the control mechanism in the cytoplasm in the small male sperm. MsT♂xCcC♀ hybrids would inherit both the nuclear transposon and the cytoplasmic control mechanism via the egg. The other wasps from additional host sources fall into either MsT or CcC groups and therefore either contain the transposons or do not. For example, the SkP

wasps collected contain the transposons, whereas the DhP wasps collected do not. Most EpV broods do not contain it, but wasps from one brood do. Interestingly, the lack of ovarian development also hinders the production of BV particles in the calyx. If hybrid sterility is related to transposable elements, full reproductive isolation would occur rapidly between host-associated populations that may already have other ecological barriers in place. We can only speculate on where the CcC wasps acquired a P-like transposon, such as gene transfer from the host; horizontal gene transfer among insects may be more common than previously thought (Drezen et al. 2017a,b). The evolutionary history of the MsT and CcC lineages as well as the geographic pattern under which the initial host-switch occurred is still uncertain.

Parasitic wasps in the genus *Cotesia* are widely used in and have been introduced for several globally important integrated pest management programs (e.g., Aya et al., 2017; Furlong et al., 2013; Van Driesche, 2008). Polydnavirus genes have potential applications as novel biopesticides (Beckage and Gelman 2004; Gill et al. 2006; Pennacchio et al. 2012; Gundersen-Rindal et al. 2013). Understanding the relationship between ecologically differentiated wasp populations and their symbiotic polydnaviruses is necessary for their efficient use as biocontrol agents. Furthermore, the pattern of asymmetric sterility among parasitic wasps has implications for understanding the diversity of this very large and agriculturally important taxon.

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TABLES AND FIGURES

Table 4.1: *Cotesia congregata* host-foodplant complex (H-FPC) sources collected and used in this study with lepidopteran host names, foodplant, collection locations (county/city, state; LAT, LONG, datum: WGS84), and number of wasp broods collected at each site with letter designations. Wasps from *M. sexta* and *C. catalpae* were collected in abundance; the other host populations were less common.

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	

Table 4.2: Primers for bracovirus genes and homologous controls used in RT-qPCR to examine differences in host expression among host-foodplant sources of *Cotesia congregata.* Bracovirus primers were selected from Chevignon et al. (2014).

Gene	Forward Primer	Reverse Primer
Manduca 18S rRNA	5’-CCGGTAACGAACGAGACTCTA-3’	5’-GGGCATCACAGACCTGTTATT-3’
Lep. 18S rRNA	5’-CGGCCAGGACATCTAAGG-3’	5’-ATCACAGACCTGTTATTG-3’
Ankyrin 4	5’-GAAAAATGGTACGCAGCAAAGTTG-3’	5’-GCTAGATGAAGAGCGGTGTTACCTT-3’
CcBV 13-2	5’-TATCTTTATTACGTCAGGAGCAACCAC-3’	5’-TTTCTTGGCCTTGAACATCATCA-3’
CcPTP-L	5’-GCTCCTGGAACAGATGAACT-3’	5’-TTTCTTGGCCTTGAACATCATCA-3’
CcV3-like	5’-TTAAACGAAGAAGGCCACTGGG-3’	5’-TGCTAGAGTTATCCGGTTGTTGATTT-3’
CrV1	5’-CCGTTTGCTGACGTTGCTCGC-3’	5’-GGACCCTTTGGAGGTGCCCA-3’
CrV1-like	5’-CGATCGTTGCAAATGGAATATTTT-3’	5’-TGCTGGTGAGTTCTGGATGTGT-3’
Cystatin 1	5’-CCTCAATCGAAAGAGCAAGCTAGA-3’	5’-CCCAACAATTATAATTTGTTTCCAGTTTT-3’
Duffy-like	5’-TGGATGTTCAACAAACGTTCGA-3’	5’-AAAAACAGGGTATTGATTATTAGGACAAGA-3’
RPL3	5’-AGGCTTTCACTAAAGCCAGCAAG-3’	5’-GATCCTCACCACACTACAGTAGCG-3’

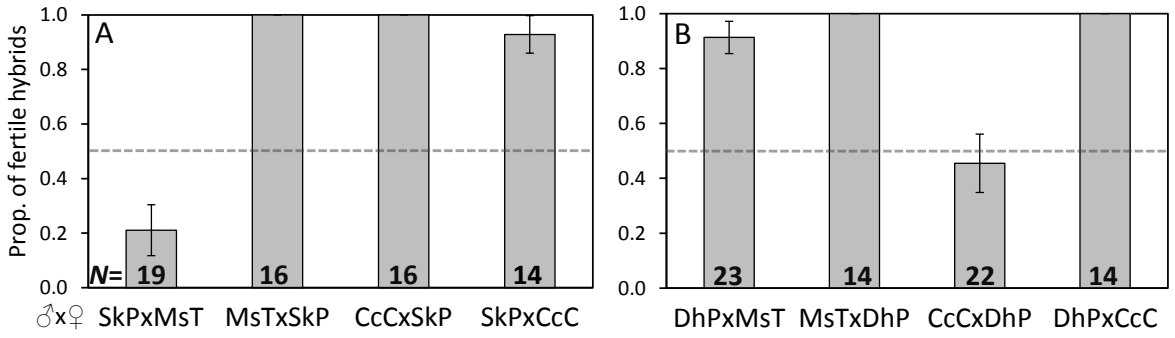

Fig 4.1: Proportion of hybrids resulting from crosses of different host-foodplant complex sources of *Cotesia congregata* that produce progeny. Three letter abbreviations denote wasp host species and host-foodplant. Uncommon wasp sources (A) SkP (*Sphinx kalmiae* on privet) and (B) DhP (*Dolba hyloeus* on pawpaw) were crossed with two known incipient species, MsT (*Manduca sexta* on tobacco) and CcC (*Ceratomia catalpae* on catalpa). Bars below the dotted line are significantly different from bars above (Fisher's exact test: $p < 0.001$) and sample sizes are at the base.

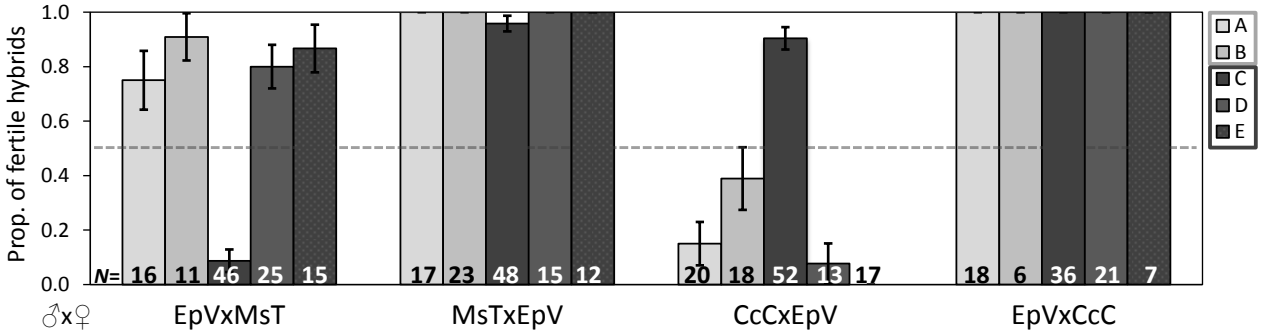

Fig 4.2: Proportion of hybrids resulting from crosses of different host-foodplant complex sources of *Cotesia congregata* that produce progeny. Three letter abbreviations denote wasp host species and host-foodplant. Five initial field-collected broods from different locations of EpV (*Eumorpha pandorus* on Virginia creeper) were crossed with two known incipient species, MsT (*Manduca sexta* on tobacco) and CcC (*Ceratomia catalpae* on catalpa) to produce hybrids. Light gray bars (A-B) indicate broods collected in Gloucester Co., VA and dark gray bars (C-E) indicate broods collected in Richmond, VA, ~95 km apart. Bars below the dotted line are significantly different from bars above (Fisher's exact test: $p < 0.0001$) and sample sizes of hybrids are at the base. Note the reversal in the pattern in brood C, suggesting that EpV wasps do not cluster into only one incipient species group.

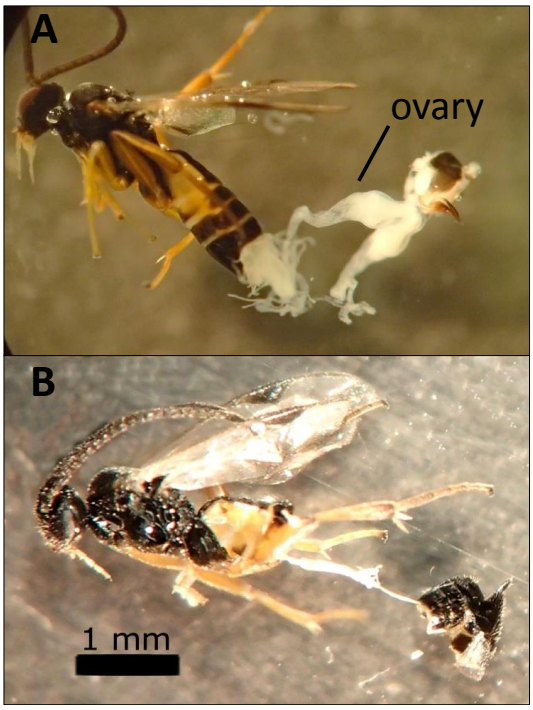

Fig 4.3: Images of dissected hybrid females produced from crosses of two incipient species of *Cotesia congregata* (MsT = *Manduca sexta* on tobacco and CcC = *Ceratomia catalpae* on catalpa). (A) Hybrid female from MsT♂xCcC♀ cross with normal ovaries. (B) Sterile CcC♂xMsT♀ hybrid that does not contain developed ovaries (alimentary tract visible).

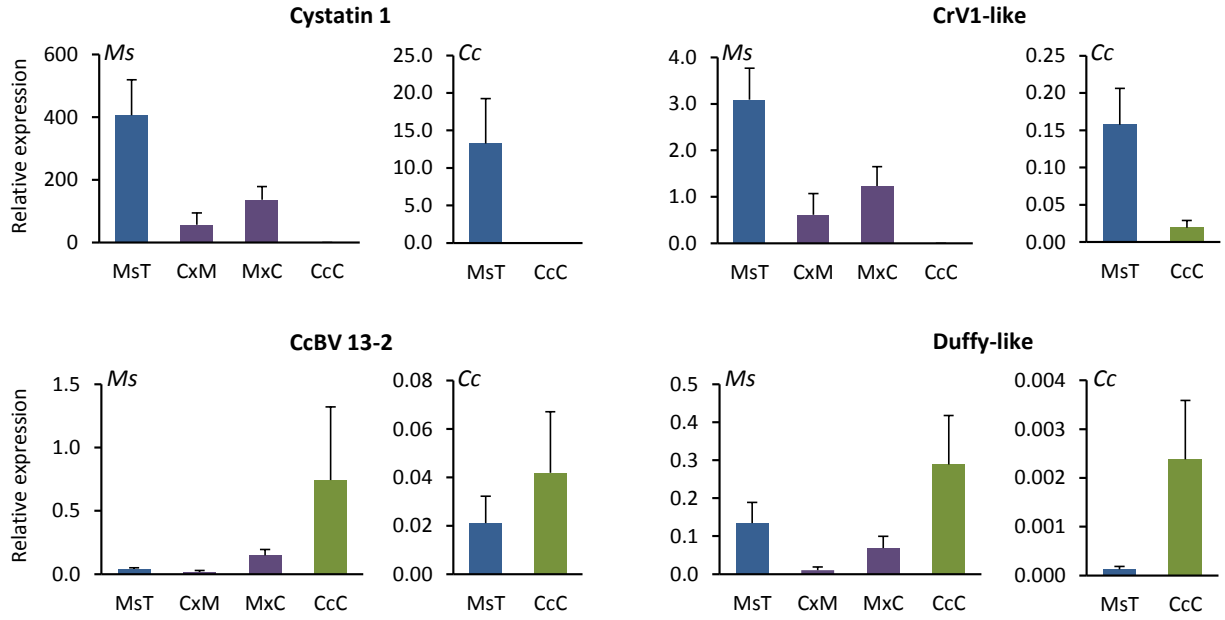

Fig. 4.4: Relative expression (mean ± SE; *N* = 8-14) of four virulent bracovirus genes that differ in hosts *Manduca sexta* (*Ms*) and *Ceratomia catalpae* (*Cc*) parasitized by two incipient species of *Cotesia congregata* (MsT = *M. sexta* on tobacco [blue] and CcC = *C. catalpae* on catalpa [green]) and their hybrids (♂x♀) on *M. sexta* [purple]. Note that cystatin 1 and CrV1-like have low or absent expression in hosts parasitized by CcC wasps. CcBV 13-2 and duffy-like are overexpressed by CcC relative to MsT depending on host parasitized. CcC♂xMsT♀ hybrids had low or absent expression in 12/14 samples; two had expression similar to the reciprocal hybrid. Ankyrin 4, CcPTP-L, and CcV3-like did not differ significantly.

Chapter 5 Conclusion

Conclusion

The rapid speciation of parasitic wasps is associated with their hosts and the plants on which the hosts feed. There are multiple examples of how differential host usage and adaptation facilitates ecological speciation; however, there are few examples where multiple and diverse mechanisms of isolation have been examined (Forbes et al. 2017). Multiple reproductive barriers are acting to reduce gene flow between MsT and CcC incipient species. Here, I place the relative importance of the different evolving isolation mechanisms.

There are two primary direct plant effects: differential tolerance to plant allelochemicals and associative learning of plant cues. In this system, tobacco has a considerably more potent chemical defense than catalpa; CcC wasps experience almost complete mortality of larvae while attempting to egress from hosts feeding on nicotine diet (Fig. 2.1). This would prevent most CcC wasps from utilizing *M. sexta* feeding on tobacco, thereby limiting gene flow unless hybridization occurs. Associative learning by *C. congregata* has been previously demonstrated (Kester and Barbosa 1992; Lentz-Ronning and Kester 2013) and may reinforce natal plant usage but not prevent occasional host shifts. Provisional greenhouse experiments with tobacco and catalpa have not been able to confirm plant fidelity by MsT and CcC wasps. Sib-mating upon emergence limits hybridization and likely plays a role in maintaining the separate lineages.

Assortative mating probably evolves after ecological isolation. Male response rate to female pheromones of other host-foodplant complexes is ~30% lower (Bredlau and Kester 2015), enough that some males need to be tricked into mating with other wasps but not enough to prevent occasional hybridization in no-choice mating assays. Courtship songs are unique to each species; however, the host-plant complexes of *C. congregata* cannot be discerned by song characteristics alone (Fig. 3.8). Moreover, despite a large variety of song structure among

Cotesia, closely related species such as those in the group containing *C. congregata* have similar patterns suggesting some phylogenetic conservation of songs (Fig. 3.6). Songs very likely act as a display of male fitness and can be used by us to identify species, but the degree to which they are used by wasps in species discrimination is still questionable. Songs most likely differentiate after or late in the speciation process.

The strongest evidence for speciation comes from the hybrid crosses and bracovirus differentiation. Rather than being a species continuum or a series of host-specific cryptic species, *C. congregata* consists of two primary incipient species that will utilize multiple hosts. The hybrid crosses all demonstrate directionality of hybrid sterility with either CcC♂ or MsT♀ (Figs. 4.1, 4.2). The hypothesis that this is caused by the presence of a transposable element (TE) in CcC wasps is being tested (see Future Directions). The acquisition and spread of a TE in a population would very quickly lead to reproductive isolation as half of all potential hybrids are sterile. If novel TEs are assimilated after a shift to a new host, this would be a key mechanism driving extremely rapid divergence among endoparasitic wasps. Whether this is a widespread phenomenon is unknown, but similar cases appear to be occurring when formally isolated populations of *Drosophila* hybridize (Hill et al. 2016). In contrast to *Drosophila*, the close association of parasitoids with insect hosts would increase the occurrence of TE acquisition and the combination of other ecological barriers would drive rapid reproductive isolation. Differences in bracovirus expression support host adaption and the considerable differences in expression indicate that shifts in BV gene expression and usage may occur quickly (in evolutionary terms) when under selective pressure.

As with most studies of speciation, the evolutionary past can only be inferred from the present condition. Based on the current data, a population of *C. congregata* shifted to utilizing *C.*

catalpae on catalpa as a host; this is assumed due to the acquisition of a TE in CcC wasps. The abundance of these caterpillars with chemical protection from sequestered iridoid glycosides that do not appear to hinder wasp development would make *C. catalpae* an ideal host. Whether this occurred in geographic isolation or when ranges overlap (as they do today) cannot be easily determined. Although catalpa and solanaceous plants have widespread overlapping ranges in the Eastern United States where this study occurred, it is possible and perhaps likely that these plant and host ranges differed in the past. The debate over whether host shifts can lead to speciation in sympatry is still unsettled, but the discourse has predominantly shifted to a focus on biological processes and ecological factors rather than geographic (Fitzpatrick et al. 2008; Forbes et al. 2017). Nevertheless, the widespread existence of intermediate forms from sympatric biotypes to full species with different levels of reproductive isolation has been argued to confirm that sympatric speciation is relatively common (Drès and Mallet 2002; Mallet 2008). In contrast, others have argued that sympatric speciation, although it could theoretically exist, is a rare form of speciation with a few tentative supporting examples and that ecological races that seem to be in the process of speciation are a result of partial allopatric divergence of populations that later had their ranges overlap (Coyne and Orr 2004; Futuyma 2008). These *C. congregata* populations may have been geographically isolated in the past allowing for initial divergence to develop without gene flow; however, reproductive isolation may continue to gradually develop in the presence of limited gene flow (Nosil 2008). Moreover, reproductive isolation may develop without leading to complete speciation, thereby creating a variety of intermediate forms (Nosil et al. 2009). Regardless, *C. congregata* offers an excellent model for the study of patterns of speciation during intermediate-late stages.

The multiple barriers to reproduction may work in concert to limit gene flow. If CcC females shift to using tobacco their offspring will die. CcC males cannot shift to tobacco because their hybrid progeny with MsT females are sterile. MsT females that shift to catalpa may be successful but produce sterile hybrids if they mate with CcC males. MsT males could shift to catalpa and then hybridize with CcC females—the only scenario in which hybridization could produce viable and fertile progeny. However, hybridization may be limited by differences in female pheromones and rejection of immigrants. Wasps from different origins may encounter each other on the other sphingid hosts, but one can only speculate whether these populations are sustained. Finally, differences in BV expression suggest divergence based on host usage. The differences do not prevent development on novel hosts, but the selective pressures involved in host-parasitoid interactions may propagate new incompatibilities. This collection of evidence indicates that these two incipient species are continuing to evolve reproductive isolation and may one day represent two isolated species.

Future Directions

Several key questions remain unanswered and should be priorities for future research on this system. Namely:

1. What is the genetic foundation of hybrid dysgenesis in *C. congregata*?
2. Why are CrV1 and cystatin 1 (and likely other BV genes) not expressed in hosts parasitized by CcC wasps but are by MsT?
3. What is the underlying population structure explaining host usage on a landscape level?

The genetic mechanism responsible for the observed pattern of hybrid sterility is under investigation. Our current hypothesis is that the CcC populations of *C. congregata* and any host-

foodplant complexes derived from them contain a transposable element (TE) similar to P elements responsible for hybrid dysgenesis in *Drosophila*. P elements have been demonstrated to inhibit ovarian development in hybrids when only the male parent contains P elements. The piRNA that suppress P element transpositions are only inherited maternally. This pattern very closely corresponds to the observed pattern of sterility among the various host-foodplant complexes of *C. congregata.* The mechanism is not identical. P element induced hybrid dysgenesis is influenced by temperature during development, with the most transpositional activity above 24°C (Ronsseray et al. 1984; Gultyaev et al. 2014). In an experiment rearing hybrid wasp larvae in hosts under 27°C and 19°C temperature treatments, ovarian development did not differ (N = 40 hybrid broods, 10 wasp dissections each). More data are necessary to determine why this distinction exists.

Recent sequencing of small RNAs in MsT and CcC wasps lead by Thibaut Josse and Jean Michel-Drezen (IRBI, Université François-Rabelais, Tours, FR) has revealed that CcC wasps contain over four million small RNA sequences not present in MsT wasps (from those that I supplied and in the IRBI colony). These correspond to approximately 500 TEs in CcC. The identification of piRNA sequences is in progress. A comparison of MsT and CcC genomes is necessary to determine the TE responsible for hybrid dysgenesis. The *C. congregata* genome already has been sequenced using wasps derived from an MsT lineage only (IRBI, *unpublished*). Sequencing the CcC genome is planned and will begin in the summer of 2018 after collection of additional wasps with subsequent bioinformatics soon afterwards.

Further work will be required to elucidate the origin of any TEs in *C. congregata.* A likely scenario is that a TE was acquired from a host caterpillar. Drezen *et al.* (2017a, b) suggest that gene transfer among invertebrates, including TEs, may be much more common than

previously assumed. Development within another organism likely increases the probability of horizontal gene transfer and possibly the acquisition of novel TEs. If TEs and corresponding small RNAs are identified in CcC wasps, the next step would be to compare these sequences to the genome of the host *C. catalpae*. The genome for host *M. sexta* is available (Kanost et al. 2016) and would serve as a valuable comparison.

The differences between MsT and CcC bracovirus sequences and expression merits further research. Discrete differences exist in BV gene expression, which may be related to host usage. The lack of CrV1 expression is particularly surprising since this virulent gene has been implicated as an important factor for immune suppression by *C. sesamiae* (Gitau et al. 2007; Dupas et al. 2008). *Cotesia congregata* contains at least 88 virulent BV genes with different levels of expression in host tissue (Chevignon et al. 2014); at least some of these genes likely have redundant functions. Redundancy would provide a very strong selective advantage against hosts evolving immune defenses and against a range of hosts. Considering the similarity of sequences with vastly different expression, mechanisms of gene regulation within the wasps should be tested and would provide insight into the complexity of PDVs.

Cotesia congregata can serve as an instructive model for determining host usage on a landscape scale. I have determined that the additional host-foodplant sources of wasps are likely derived from either MsT or CcC populations; the question remains how these two sources interact across a landscape. I speculated that metapopulation dynamics play an important role (Chapter 4), but I do not know how often these sources encounter each other in a natural landscape. Wasps are commonly found in fragmented urban landscapes and populations that were found within 2.3 km from the same host species (*E. pandorus*) presumably would not be able to produce fertile progeny in both reciprocal crosses (Fig. 4.2). Using population genetic

markers from many samples within a landscape with multiple collection sites (e.g. a city and surrounding areas) would clarify where wasps originate and how they use hosts. A study over multiple years could reveal whether the small isolated populations persist over time or act as population sinks that are experiencing regular local extinction (my prediction). I posit this because my observations over multiple years of collecting caterpillars suggest that certain populations of potential hosts may escape parasitism some years, but be heavily parasitized in subsequent years, whereas the large host populations in catalpa groves and tobacco fields appear always to be parasitized. These predictions can be readily tested, although the project would require an extensive collection effort. Undoubtedly, *C. congregata* will continue to be a valuable model system for testing hypotheses on the mechanisms of speciation and underlying diversity among parasitoids.

Additional fun facts:

1. *Cotesia congregata* has the smallest flagellated sperm of any animal at 6 μm (Uzbekov et al. 2017). An insect of similar size, *Drosophila bifurca*, has the longest at ~6 cm (Joly et al. 1995; Pitnick et al. 1995; Dallai 2014). Why? It may have something to do with different life history strategies related to parasitism vs. non-parasitism. (Paper with C. Bressac in preparation)
2. Females run out of eggs after 21 parasitisms.
3. Bracovirus expression in hosts feeding on different concentrations of nicotine diet parasitized by MsT and CcC wasps does not differ (N = 72 total between MsT and CcC on three diet treatments; seven BV genes tested). I am now quite good at RNA extractions.
4. One *E. pandorus* had 502 cocoons on it and 87 larvae inside. Record?
5. I now routinely have dreams about finding caterpillars after spending so much time searching for them. These have mostly replaced the dreams about finding an ivory-billed woodpecker.

Many *Manduca* died to bring us this information…

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Vita

Justin Paul Bredlau was born on February 4, 1985 in Portsmouth, VA. He lived in Maine and Illinois before moving back to Virginia and graduating from Gloucester County High School in 2003. He graduated from the University of Mary Washington with a B.S. in Biology in 2007. While in college, he had his first field biology job surveying golden-winged and blue-winged warblers in Virginia for the Center for Conservation Biology, College of William and Mary. Wanting more adventure before continuing with school, Justin worked on a variety of other field biology jobs across the country. This included collecting swamp sparrow nestlings in Maryland and Delaware for research at the Smithsonian Migratory Bird Center, searching for the once thought to be extinct ivory-billed woodpecker in Arkansas for Cornell University, collecting and identifying mosquitoes for West Nile virus research in Washington, DC at the Smithsonian Institute, and surveying wading birds from airboats and helicopters on Lake Okeechobee for Florida Atlantic University. Justin joined the Insect Ecology and Behavior Laboratory at VCU to work with Dr. Karen Kester in 2009. He completed his M.S. on pre- and post-zygotic reproductive isolation in *Cotesia congregata* in July 2011. He continued and expanded on this work during his Ph.D. starting in August 2012. Over the course of his graduate education, Justin has taught 24 laboratory sections including Entomology, Invertebrate Zoology, Genetics, and Introductory Biology. He has presented research at nine national scientific meetings and one international invited seminar in Tours, France. He has participated in 28 public outreach education events, primarily with long-term partners Maymont Park, Crestwood Elementary School, and Richmond's PBS Community Idea Stations. For two years he served as treasurer of the Integrative Life Sciences Student Organization. During his time at VCU, he has managed the Kester Lab with help from over a hundred undergraduate laboratory interns.

Publications

Bredlau, JP & KM Kester. 2015. Pre- and postzygotic barriers to reproduction between two host-foodplant complex sources of the parasitic wasp, *Cotesia congregata* [Hymenoptera: Braconidae]. *Annals of the Entomological Society of America* 108: 1026-1036.

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Awards

Honor Society of Phi Kappa Phi Love of Learning Award, 11/2017

Outstanding Biology Ph.D. Student in Evolution, VCU College of Humanities & Sciences, 5/2017

Woolcott Award for Best Student Presentation, Natural History and Biodiversity Section, Virginia Academy of Science, 6/2016

J. Shelton Horsley Research Award, Virginia Academy of Science, 5/2016 (w/KM Kester)

Best Video in Discovery Category, YouTube Your Entomology, Entomological Society of America, 11/2013

Small Project Grant, Virginia Academy of Science, "More diversity than imagined: Characterizing an undescribed parasitic wasp species complex" (KM Kester & JP Bredlau), 5/2013

Outstanding Biology Graduate Student Award, VCU College of Humanities & Sciences, 4/2012

Outstanding Biology Graduate Teaching Assistant Award, VCU College of Humanities & Sciences, 4/2012

1st Place, Student poster competition, Entomological Society of America Eastern Branch, 3/2011