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**INVESTIGATION OF PRE- AND POST-ZYGOTIC REPRODUCTIVE  
BARRIERS BETWEEN TWO HOST-PLANT COMPLEX RACES OF  
THE PARASITIC WASP *COTESIA CONGREGATA* (SAY)  
[HYMENOPTERA: BRACONIDAE]**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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## **Abstract**

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By Justin P. Bredlau

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Director: Karen Kester, Ph.D.  
Associate Professor, Department of Biology

Investigations of host-associated differentiation of parasitoids have largely focused on the degree of molecular genetic differentiation, but a true test of species status must examine the mating patterns of differentiated populations to determine if they can interbreed in the wild and produce viable offspring. We examined possible mechanisms of isolation between two genetically distinct host-plant complex races of the braconid, *Cotesia congregata*, originating from hosts on tobacco and catalpa. We compared male responses

to female pheromones, elements of male acoustic courtship signals, and breeding success between the two races. Males responded to pheromones from both sources and male courtship signals showed only subtle differences, suggesting that factors other than courtship behavior may be involved in isolation of the two races. However, nearly 90% of females from one hybrid cross failed to produce offspring, leading to post-zygotic isolation. Development time, emerged brood size, and sex ratios between the races also differed.

## INTRODUCTION

Sympatric speciation, the division of one species into two within the same geographic area, has remained a problem in evolutionary biology. There have been natural examples, theoretical models, and experimental studies, which are often difficult to replicate (Henriksson et al. 2010). One model in which sympatric speciation may occur consists of populations that diverge when they adapt to different prey items or hosts with different requirements; however, continued mating between populations will prevent any divergence unless there is also reproductive divergence, such as assortment based on courtship signals (Bolnick & Fitzpatrick 2007). This form of speciation may lead to cryptic species complexes within the same geographic region in which species appear morphologically identical, but differ in genetics, behavior, or ecology. Parasitic insects in particular display rapid speciation and high biodiversity associated with their wide variety of hosts.

The genetic radiation of parasitic wasps is possibly linked to the large number of potential host species and the chemical diversity of the plants on which their hosts feed. As their host species diverge to feed on different plants, parasitoids adapt to potentially different plant chemicals and in so doing may undergo sequential radiation (Stireman et al. 2006). Over time, parasitic wasp populations may develop an innate preference for a specific host even in the presence of another host species. Premating isolation such as this facilitates separation of races through the rapid evolution of differentiated courtship behavior (Arbuthnott 2009). Previous studies have found that parasitic wasps show fidelity not to the hosts themselves, but to the plants on which the hosts feed. Thus it is the tritrophic interaction among the plants, the insects that feed on them, and the parasitoid that can lead to parasitoid diversification (Stireman et al.

2006). For example, Stelinski and Liburd (2005) and Forbes et al. (2009) found that the *Rhagoletis* parasitoid, *Diachasma alloeum* (Muesebeck), is preferentially attracted to the plants which are fed upon by the same host species from which they emerged. Further, at least some parasitoid species are able to learn plant cues at emergence and are more strongly attracted to the plant on which their host developed than to alternative host food plants (Kester and Barbosa 1991a, 1992). Postemergence learning also influences sex allocation by females, thereby altering population growth and reproductive potential (Lentz and Kester 2008). Beltman and Metz (2005) argue that learned rather than genetic habitat preference is more likely to lead to disruptive selection. The association with specific plants by parasitoids can further reinforce isolation.

Reinforcement of host or host-plant selection may eventually lead to genetic differentiation. Forbes et al. (2009) report incipient speciation of *D. alloeum* associated with its host *Rhagoletis pomonella* (Walsh) shifting from hawthorn to apple based on differentiation of microsatellite allele frequencies, mitochondrial DNA, and eclosion time. Likewise, Kankare et al. (2005a) found that *Cotesia melitaearum* (Wilkinson) consists of several cryptic species that parasitize different caterpillar species with no gene flow among host-associated groups. When those wasps were offered caterpillars of unfamiliar species, the wasps either did not parasitize the caterpillars or no progeny developed from them even though those same caterpillars are parasitized by other populations of the same wasp species. *Cotesia acuminata* (Reinhard) in Spain was also found to consist of numerous host-specific cryptic species (Kankare et al. 2005b). Furthermore, genetic barcoding of tropical braconids revealed 142 provisional species in addition to the 171 identified by traditional morphology, thereby turning many assumed generalists into specialists (Smith et al. 2008).

Previous studies on parasitoid biodiversity have focused on the genetic differentiation of populations associated with different hosts, often assuming that sufficient genetic distance indicates that the populations are different species. However, isolation mechanisms must exist to prevent gene flow among host-associated populations and to maintain genetic differentiation. A true test of speciation must begin with examining the mating patterns of the populations to determine if they are capable of interbreeding and producing viable offspring in the wild. One approach is to test the response to courtship cues, such as female pheromones and male acoustic signals, which can be used to determine the degree which two host-associated populations are likely to interbreed and provide a sense of their level of speciation.

Courtship signals are used for species recognition and initiate a response in the opposite sex. Like many insects, males of *Cotesia* find mates by the detection of female pheromones. Males display searching behavior in response to female pheromones by moving across the substrate and using antennal palpitations to key in on the source. They then perform rapid wing fanning that likely draws the pheromones over the scent glands and allows the wasp to orient toward the female (Vinson 1972). This behavior has been observed in males only when females are or were recently in proximity. 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). Transmission of acoustic courtship vibrations across the substrate has an effect on the mating success of *Cotesia marginiventris* (Cresson) (Joyce et al 2008). Recently, Joyce et al. (2010) reported that male acoustic signals vary among allopatric populations of the *Cotesia flavipes/sesamiae* (Cameron) complex and suggested that these differences play a role in reproductive isolation. However, it is possible that wasps will mate despite slight differences in acoustic signals and genetics.

Ultimately, the final test of separate species status is whether different populations mate and produce viable offspring. 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 comparison, Rincon et al. (2006) found mating incompatibility, along with genetic and morphological differences, among some geographically isolated populations of *Cotesia plutellae* (Kurdjumov), but no evidence of a post-zygotic isolation barrier. Similarly, Desneux et al. (2009) reported complete reproductive isolation in mating crosses between two geographically isolated populations of the aphid parasitoid, *Binodoxys communis* (Gahan), demonstrating that they are distinct cryptic species. We tested species recognition and mating success in populations of a parasitic wasp that are separated by host-plant complex usage rather than major geographic barriers.

The gregarious endoparasitoid, *Cotesia congregata* (Say) (Hymenoptera: Braconidae) serves as a model system for tri-trophic interactions and biological control. In addition, it is also an important model system for host-parasitoid interactions and insect immunology (Beckage 2008). This species is reported to attack multiple species of sphingid caterpillars, 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 & Barbosa 1991b, 1994). Microsatellite allele frequencies from two host-plant complexes with overlapping geographic ranges, *Manduca sexta* L. (“tobacco hornworm”) on tobacco (“MsT”) and *Ceratomia catalpae* Boisduval (“catalpa sphinx”) on catalpa (“CcC”), differ significantly (Jensen et al. 2002); similarly, a 214 bp fragment from the mtDNA COI region shows a 2% sequence divergence (Karns 2009). Females from these two host-plant complexes also differ in behavioral responses to tobacco and developmental success on nicotine

diets (Crocker 2008). These two distinct genetic lineages may represent incipient or sibling species that do not regularly interbreed in the wild.

Although MsT and CcC host-plant complexes may differ in some aspects, their basic life history is similar. Once an appropriate host is located, an adult female oviposits multiple eggs inside the caterpillar along with segments of polydnavirus (PDV) that disables the host immune system and prevents encapsulation of wasp eggs (Beckage 1998). The wasp larvae grow inside the host until they egress and spin cocoons on the caterpillar. After 6-8 days (temperature dependent) adult wasps emerge from their cocoons. 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 1991a). Because *C. congregata* is haplodiploid, fertilized eggs normally develop into females, and unfertilized eggs develop into males. Only females are genetic hybrids in the F<sub>1</sub> generation whereas haploid males receive only maternal chromosomes.

The objective of this study was to elucidate the species status of *C. congregata* by determining the degree of pre- and post-zygotic isolation between genetically differentiated MsT and CcC host-plant complex “races” and to provide insight into the role of behavioral isolation mechanisms in the speciation of hymenopteran parasitoids. We assayed male response to female pheromones to determine if males recognize females of the reciprocal race, compared male acoustic courtship signals, and determined whether wasps of the different races could mate and produce viable offspring. Development time, brood size, and sex ratios among the crosses were also compared to provide information on the heritability of these characteristics.

## MATERIALS AND METHODS

### *Parasitoid Collection*

Caterpillars were collected from catalpa (*Catalpa speciosa* Warder) and tobacco (*Nicotiana tabaccum* L.) at three sites in Virginia from July to October 2010. Larvae of *C. catalpae* were collected at two private properties with mature catalpa trees in Cumberland County: “Tyson” Site (37.712726, -78.163884) and “Newton” Site (37.672979, -78.219928). Larvae of *M. sexta* from several varieties of tobacco were collected from the Southern Piedmont Agricultural Research and Experimental Station near Blackstone, Nottoway County (37.081707, -77.975566). Caterpillars were stored in plastic containers (28 x 16 x 11 cm; 10-15 larvae in each) with leaves from their respective host-plant and then isolated into cups upon egression of parasitoids. Wasp cocoons were placed into individual clear gel capsules (size 00) 3-4 days after egression and emergent adults were sexed under a dissecting microscope. MsT wasps from a laboratory colony originating from Blackstone in 2005 were used in genetic crosses early in the year due to lack of an adequate number of tobacco hornworms at the field site.

### *Pheromone Assay*

Male responses to female pheromone from the two sources were compared to evaluate species recognition. Live females were chilled and placed in a 1.25 mL vial. Hexane was placed over the females (1 mL per 50 females) and slightly stirred for 10 s. This pheromone-hexane solution was pipetted into a second vial to separate the solution from the wasps and used within 24 hours. For each assay, 70  $\mu$ L of solution was pipetted onto a quarter piece of Whatman #1 (55 mm) filter paper concentrated at the point creating directionality of the signal.

Males were released individually within 2 hours of emergence onto a leaf of their respective host-plant in an open air arena ( $27 \pm 1^\circ\text{C}$ ; 30% RH) and the corner of the filter paper held with fine-point forceps was wafted in front of each individual male to induce a fanning response. Wasps were considered non-responsive if they did not fan within 3 minutes. The test arena was cleaned with 70% EtOH between assays of replicate males. Differences in male response rates between groups were compared using Fisher's chi-square test with R (R Development Group).

### *Male Acoustic Courtship Signals*

Male acoustic courtship signals were recorded to compare elements of signals between wild MsT and CcC wasps. Individual males from multiple cohorts were exposed to an immobilized female of the same host-plant complex source on a piece of leaf from the male's respective host-plant (tobacco or catalpa) in an open plastic dish to induce fanning. Recordings were made in a sound isolation booth (Industrial Acoustics) at  $23 \pm 1.5^\circ\text{C}$  and 40-55% RH using miniature omnidirectional microphones (DPA 4060; 20-20,000 Hz) held 2-3 mm away from the male and a 702 High Resolution Digital Audio Recorder (Sound Devices, LLC; 48 kHz sampling rate, 24 bit resolution). Duration of signal components, fundamental frequency, and root-mean-square (RMS) amplitude was analyzed using Raven Pro v1.3 (Cornell Lab of Ornithology). All waveforms were high-passed filtered at 100 Hz and frequency spectra were created for each signal component (Hann window, 3000 samples, 1.46 Hz grid spacing). The first five distinct sounds, termed "boings," were analyzed for each wasp and component data were averaged and treated as an  $N$  of 1. Over 500 recordings were made of 250 individuals from 126 different

cohorts. Only recordings made during the same time span and from different cohorts were used for comparison between wild MsT (n = 21) and CcC (n = 24) males.

To determine the amplitude of the sounds (absolute pressure in Pascals), the RMS amplitude measured by Raven was multiplied by a calibration constant. The calibration constant is equal to the known amplitude of a test tone (90 dB re: 20  $\mu$ Pa, 500 Hz; produced by a Tektronix CFG250 Function Generator through a Grass AM7 Audio Monitor) divided by the RMS amplitude of the tone as measured by Raven. The sound pressure was converted to decibels by the formula:  $= 20 \log (P/P_0)$ , where P = sample pressure (Pa) and  $P_0$  = reference pressure in air, 20  $\mu$ Pa. Differences in fundamental frequency and amplitude between boing and buzz elements were determined using paired t-tests with R (R Development Group). Significance of differences of male acoustic courtship signal elements of both types was assessed using two sample t-tests with R.

### *Mating Crosses*

Wasps from the two host-plant complex sources were crossed to compare mating success and viability of hybrid offspring, as well as possible differences in larval development time, brood size, and sex ratio of resulting cohorts. Reciprocal crosses (MsT $\text{\textcircled{M}}$  x CcC $\text{\textcircled{F}}$ ; CcC $\text{\textcircled{M}}$  x MsT $\text{\textcircled{F}}$ ) between the two host-plant complex sources were compared to control crosses made within host-plant complexes (CcC $\text{\textcircled{M}}$  x CcC $\text{\textcircled{F}}$ ; MsT $\text{\textcircled{M}}$  x MsT $\text{\textcircled{F}}$ ) used to assess mating success under laboratory conditions. Two males from the same brood and one female from a different brood were placed into a clear glass tube (2 cm diam. x 7 cm) with a 1 cm<sup>2</sup> section of the female's respective host-plant wrapped around a damp piece of cotton ball. The vial was then closed using a cotton ball with honey on the side as a food source. Mating groups were kept

under ambient laboratory conditions ( $22 \pm 1^\circ\text{C}$ ; 30-50% RH) for 4 days. On days 2, 3, and 4 after set-up, a second or third instar larva of *M. sexta* was presented to wasps and removed after parasitism was observed. All caterpillars used for parasitism across treatments were healthy and similar in size ( $0.13 \pm 0.02$  g). After parasitism, caterpillars were placed in individual plastic cups (7 cm diam. x 4 cm) and fed a semi-synthetic laboratory diet modified from Yamamoto (1969) until egression of wasp larvae. Mating success was determined by the presence of female offspring.

Resulting  $F_1$  progeny from cohorts with females were re-crossed with siblings as described above except that hybrid crosses were given pieces of both host-plants. Caterpillars were frozen either after wasp cocoon removal or in their wandering stage near pupation and later dissected to determine parasitization status, encapsulation of eggs, and number of unemerged larvae. All progeny were counted and sexed to determine brood size (number of larvae that egressed and spun cocoons) and sex ratio (proportion of females per brood). Broods observed to be 100% male were not included in sex ratio analysis. Development time was calculated as the difference between the day of parasitism and the day of wasp emergence. Mating and breeding success were compared among cross types with Pearson's chi-square test using JMP v8 (SAS Institute, Inc). Brood size, sex ratio, and development time were transformed to meet normality assumptions and compared with ANOVA using JMP v8 with the multiple parasitizations nested within mating pairs (random factor) and these replicates within cross type (fixed effect). Tukey's test ( $\alpha = 0.05$ ) was used for pairwise comparisons of least square means.

## RESULTS

### *Pheromone Assay*

In tests for species recognition where male wasps were exposed to female pheromone from each host-complex source control pairings had the highest response rates (MsT = 73%; CcC = 60%). However, many males also responded to the female pheromone originating from reciprocal host-complex sources (MsT♂ x CcC♀ = 53%; CcC♂ x MsT♀ = 33%; n = 15 for each group). The greatest difference indicated a trend for reduction in response rate when CcC males were exposed to MsT pheromone compared to the MsT control pairing ( $X^2 = 0.0281$ ,  $p = 0.0656$ ) (Figure 1).

### *Male Acoustic Courtship Signals*

Male courtship signals of both host-plant complexes were characterized by rapid wing fanning followed by higher amplitude boings. During each boing the abdomen raises and then drops while the wings move downward, presumably striking the abdomen, thereby creating the boing. Each boing was followed by a lower amplitude buzz component which consisted of continued fanning of the wings and separated from the next boing by a short gap with no sound produced (Figure 2A). Males continued to produce boings until they attempted mating or the female moved away. Although these three components (boing, buzz, and gap) were apparent in most of the individual signals recorded, details in structure varied among individuals. For most males, there was a clear reduction in amplitude (mean  $\pm$  SE:  $-8.6 \pm 0.4$  dB) between the first and second parts ( $t = 23.18$ , d.f. = 44,  $p < 0.0001$ ) whereas in a few instances there was a more gradual transition between the two components. Boing and buzz duration varied independently ( $r^2 = 0.0089$ ,  $p = 0.5369$ ) (Figure 3). Increasing signal length was more closely correlated with

increasing buzz duration ( $r^2 = 0.7103$ ,  $p < 0.0001$ ) than to boing duration ( $r^2 = 0.1822$ ,  $p = 0.0035$ ), thus boing duration was less dependent on overall signal length. Frequency spectra (Figure 2B-D) of both boings and buzzes consist of a clear harmonic series that decreases in amplitude from a peak of 120 relative dB (uncalibrated) to the background noise level of 70 dB. Energy above background was present to about 7 kHz for boings and 3 kHz for buzzes. The fundamental frequency likely corresponds to individual wing cycles (~ 240 beats per second). Boing fundamental frequency was lower than the following buzz frequency ( $t = 5.33$ , d.f.= 44,  $p < 0.0001$ ), suggesting that wing cycle time slows during boing production.

Elements of the male acoustic courtship signal differed between the two host-complex sources (Table 1). The time from the start of one boing to the start of another was significantly shorter in duration in MsT than CcC males ( $t = 2.38$ , d.f.= 43,  $p = 0.0220$ ). Likewise, boing duration (part 1) was shorter in MsT ( $t = 3.07$ , d.f.= 43,  $p = 0.0037$ ); however, gap time was longer in MsT ( $t = 2.75$ , d.f.= 42,  $p = 0.0088$ ; one MsT outlier removed, 43.4 ms). Buzz (part 2) duration did not differ between the two groups of males ( $p = 0.1960$ ). Fundamental frequency of the overall signal was lower in MsT males ( $t = 2.33$ , d.f.= 43,  $p = 0.0245$ ). However, the fundamental frequency of separate boing ( $p = 0.0845$ ) and buzz ( $p = 0.1191$ ) elements did not differ significantly. Amplitude (re: 20  $\mu$ Pa at 2-3 mm) of both boing and buzz elements were lower in MsT ( $t = 2.08$ , d.f.= 43,  $p = 0.0436$  and  $t = 2.71$ , d.f.= 43,  $p = 0.0096$ ) (Table 1). Acoustic elements did not differ ( $p > 0.1$ ) between CcC wasps used for comparisons (October,  $n = 24$ ) and CcC wasps recorded the previous month (September,  $n = 26$ ).

### *Mating Crosses*

Viability of the F<sub>1</sub> hybrid offspring resulting from the two reciprocal crosses differed from the control crosses, as did mean larval development time, brood size, and sex ratio. Mating success, determined by the presence of female progeny, did not differ between the parental cross types ( $X^2 = 3.29$ ,  $p = 0.3494$ ). However, mating success differed among F<sub>1</sub> crosses ( $X^2 = 7.98$ ,  $p = 0.0185$ ; CcC♂ x MsT♀ crosses not included due to small sample size) with MsT♂ x CcC♀ F<sub>1</sub> hybrids having reduced mating success compared to the control MsT lines ( $X^2 = 8.13$ ,  $p = 0.0044$ ) (Table 2).

Breeding success, the proportion of crosses that produced at least one emerged adult wasp from surviving parasitized caterpillars, differed among F<sub>1</sub> crosses ( $X^2 = 95.62$ ,  $p < 0.0001$ ). Female hybrids from the CcC♂ x MsT♀ crosses produced adult offspring in only 6 of 56 crosses (3 of 15 genetic lines originating from a single parental generation pairing), whereas hybrids from the MsT♂ x CcC♀ crosses produced offspring in 39 of 43 crosses (19 out of 20 genetic lines) (Table 2). CcC♂ x MsT♀ hybrid crosses varied in their ability to produce progeny even within genetic lines, i.e. some hybrids always produced offspring whereas their sisters did not. Among the three genetic lines that produced larvae, two lines had one female breeding successfully out of three ( $n = 9$  and  $6$ , respectively) and one had two females successful out of three ( $n = 3$ ). Unmated F<sub>2</sub> female wasps resulting from sibling matings from MsT♂ x CcC♀ lines continued to produce offspring (3 of 3 females). Dissections of caterpillars with no emerged larvae from both hybrid cross types revealed encapsulation of wasp eggs with melanization, indicating an active immune response to the eggs. Parasitized caterpillars allowed to pupate developed into apparently normal adult moths. Control lines continued to produce offspring in all matings without observable egg encapsulation (Table 2).

Larval development time varied among wasp types (reciprocal transformed; ANOVA:  $F_{3, 136.6} = 8.70$ ,  $p < 0.001$ ; Figure 4A). Progeny from MsT females developed a day faster than those from CcC females. Likewise, larval development time of the  $F_2$  progeny also differed among crosses (reciprocal transformed; ANOVA:  $F_{3, 75.43} = 2.98$ ,  $p = 0.0366$ ), however Tukey's post-hoc test ( $\alpha = 0.05$ ) revealed no significant pairwise comparisons (Figure 4B).

Mean brood size was larger in MsT females than CcC females (square root transformed; ANOVA:  $F_{3, 132.2} = 5.15$ ,  $p = 0.002$ ; Figure 5A).  $F_1$  hybrid females from MsT♂ x CcC♀ matings produced broods similar in size to the MsT control lines whereas the reciprocal CcC♂ x MsT♀ hybrids ( $17.8 \pm 6.6$  larvae; most parasitizations lead to egg encapsulation) had significantly smaller brood sizes (square root transformed; ANOVA:  $F_{3, 79.13} = 20.08$ ,  $p < 0.0001$ ; Figure 5B).  $F_1$  control crosses produced broods not significantly different in size to the previous generation (MsT controls:  $F_{1, 26.45} = 1.08$ ,  $p = 0.3086$  and CcC controls:  $F_{1, 52.26} = 0.05$ ,  $p = 0.8290$ ). Note that only egressed parasitoid larvae were counted for analysis; dissections revealed larvae that failed to egress from most hosts across all cross types (Table 3).

Mean sex ratio (proportion of females produced in each brood) of  $F_1$  broods varied among crosses (arcsine transformed; ANOVA:  $F_{3, 63.31} = 3.60$ ,  $p = 0.0181$ ). Broods produced by MsT females had more balanced sex ratios whereas CcC females produced female-biased broods (Figure 6). Sex ratios of  $F_2$  broods were not compared due to low sample sizes. Day of parasitism had no effect on any of these factors ( $p > 0.05$ ).

## DISCUSSION

Pre-zygotic isolation is often assumed to be a precursor to genetic differentiation and post-zygotic isolation (Mayr 1963). As a measure of pre-zygotic isolation, we tested for the presence of courtship isolation mechanisms between two genetically differentiated host-plant complexes of the parasitic wasp *C. congregata* and established reciprocal crosses to determine mating success and hybrid viability. Although elements of the male acoustic courtship signal differed and CcC males may have a reduced response rate to MsT female pheromones, the two host-complexes mated and produced offspring in no-choice crosses. However, eggs from CcC♂ x MsT♀ hybrids, the same cross type that had a somewhat reduced response in the pheromone assay, were encapsulated within hosts, possibly due to inhibition of the polydnavirus (PDV) that disables the host immune response (Beckage 1998). The presence of this post-zygotic isolation mechanism suggests that a pre-zygotic barrier other than the ones we tested may exist. Additionally, specific differences that were observed between the two host-plant complexes in larval development time, brood size, and sex ratio suggest a high degree of adaption to their respective hosts.

Despite detectable differences in courtship behavior, wasps from the two taxa mated and in most cases produced offspring. CcC males showed a tendency to respond less frequently to MsT female pheromone than MsT males ( $p = 0.0656$ ; Figure 1). Several elements of the male acoustic signal differed significantly ( $p < 0.05$ ; Table 1) but did not prevent mating under confined laboratory conditions (Table 2). Joyce et al. (2010) suggest that courtship acoustics create pre-zygotic isolation within the *C. flavipes* species complex. We found similar differences in acoustic elements; however, our results suggest that these differences do not prevent mating. Further testing is necessary to determine if wasps preferentially mate with the same host-plant

complex under less restrictive conditions or if given a choice, and whether these differences are consistent among geographically distant populations within the same host-plant complex.

Hybrid crosses between CcC males and MsT females established over two field generations had reduced fitness, which indicates a developing post-zygotic isolation mechanism between the two host-plant complex taxa. Eggs from 50 of 56 CcC♂ x MsT♀ hybrid females failed to develop due to encapsulation and melanization within hosts, and those females that did produce offspring had reduced brood sizes (Figure 5B). This finding may be due to either genetic differences in polydnavirus (PDV) variants or differential expression of hybrid PDV with respect to host. Expression of these virus particles is known to inhibit immune defenses in the host, preventing encapsulation of wasp eggs (Beckage 1998, Shelby and Webb 1999). The polydnavirus has coevolved with braconids against host resistance (Dupas et al. 2008) and its genetic components are integrated into the wasp genome (Stoltz 1990, Belle et al. 2002). Le et al. (2003) found that *C. congregata* originating from laboratory *M. sexta* has at least two PDV genes with different mechanisms regulating their expression. Differences in PDV expression exists among populations within some parasitic wasp species. In *C. sesamiae*, eggs from some populations are encapsulated within the normal hosts of other populations (Ngi-Song et al. 1998). Gitau et al. (2007) found differential expression of the PDV CrV1 gene between separate populations of *C. sesamiae* leading to the egg encapsulation within one host and Branca et al. (2011) revealed PDV genotype differences associated with specialization to specific hosts. Unlike the geographically separated populations of *C. sesamiae*, differences in PDVs of *C. congregata* are apparent only in the hybrids and pure lines of CcC females are able to parasitize both *C. catalpae* and *M. sexta*. Ongoing work will determine how the PDVs from the hybrid females are expressed in the host. Alternatively, derived cellular proteins in parasitic wasps may

serve as virulence factors that are species specific (Wetterwald et al. 2010) and some venom peptides are necessary for PDV expression in the host (Zhang et al. 2004). Presenting hybrids with the alternate host (*C. catalpae*) to test for egg encapsulation will elucidate whether hybrid PDVs or other virulence factors are host specific.

Theoretically, post-zygotic reproductive barriers are not under direct selection but are rather byproducts of divergence (Mayr 1963). For example, Coyne and Orr (1989, 1997) demonstrated that pre-zygotic isolation evolves faster than post-zygotic isolation in sympatric populations of *Drosophila*. Likewise, there must be an isolation mechanism associated with the divergence of MsT and CcC host-plant complexes, which can occur in sympatry (Karns 2009). The existence of inviable hybrids should lead to the reinforcement of pre-zygotic differences to prevent a loss in fitness to the wasps. We are currently investigating the role of assortative mating on the host-plant as a pre-zygotic barrier between MST and CcC wasps.

Selection of mating habitat based on learned plant chemical preferences has been proposed as the initial mechanism for sympatric speciation (Bush 1969). Parasitoids display both innate and learned responses to plant chemicals, and associating specific plants with specific hosts or mates may be the first step in restriction of gene flow. For example, *C. congregata* shows innate recognition of tobacco and tomato and searching responses for these plants are enhanced through post-emergence learning (Lentz and Kester 2008; Kester and Barbosa 1991a, 1992). Sibling mating on the natal host plant, typical in *C. congregata*, likely reinforces genetic isolation. Similarly, Forbes et al. (2009) suggest that plant selection based on fruit odor may act as an ecological barrier in *D. alloeum* in the same way as their host fruit fly. Also, Villagra et al. (2008) demonstrate that males of the parasitoid *Aphidius ervi* (Haliday) associate their first copulation with host-plant odors and will continue to search the initial learned plant species for

mates. If individuals seek out and mate on their natal plants, then specific host-plant complexes may evolve (Beltman and Haccou 2005). Additionally, developmental intolerance to plant chemicals within the hosts, such as nicotine in tobacco (Crocker 2008), may prevent females from easily switching over to other host-plants, thereby limiting gene flow.

Further differences in larval development and reproductive parameters between wasp sources are likely to be genetically regulated by multiple loci but also influenced by environmental factors, such as host size and condition. Exactly how such genes may be regulated currently remains unknown. Development time, brood size, and sex ratio in the  $F_1$  generation were determined by the female parent irrespective of cross type (Figures 4A, 5A, 6). This pattern in the  $F_1$  generation was expected since the males, which typically emerge first in broods, have only maternal genes, whereas the female parent determines the number of eggs to be fertilized and oviposited. Development time of the  $F_2$  hybrid larvae followed a different pattern (Figure 4B), possibly due to recombinant chromosomes. In contrast, the emerged brood sizes produced by  $F_1$  hybrid females appear to be similar to those of the parental generation male in  $MsT♂ \times CcC♀$  hybrid crosses but highly reduced in the hybrids from  $CcC♂ \times MsT♀$  crosses (Figure 5B), those that mostly lead to egg encapsulation. Although we measured brood size of larvae that formed cocoons and can only infer the actual number of eggs oviposited, differences exist in brood size regardless of whether it is predominantly determined by the number of eggs oviposited or the proportion of parasitoid larvae that egressed and survived to spin cocoons. Differences between the hybrids may be due to dominance of  $MsT$  alleles with other factors overriding high brood size in  $CcC♂ \times MsT♀$  hybrids. The severe reduction in brood size from hybrid  $CcC♂ \times MsT♀$  may be associated with weakened expression of the polydnavirus. These caterpillars became smaller and sicklier than normal parasitized caterpillars.

Heritability of development time and brood size suggests long-term adaptation of each wasp type to their specific host-plant complex in response to selective pressures created by both the host and plant. Differences in larval development time are difficult to interpret and may be due to CcC larvae not being as adapted to *M. sexta*. Brood size per host of gregarious parasitoids can be adjusted in different situations to maximize fitness, such as laying fewer eggs per host when presented with more hosts (Tagawa 2000; Hasan and Ansari 2010). Given that *M. sexta* is more solitary and larger than the gregarious *C. catalpae*, MsT wasps allocate more eggs to their host, while CcC wasps allocate fewer eggs for their smaller host while also having a greater likelihood of finding other nearby hosts to parasitize. Since caterpillars of equal size and condition were split evenly between cross types our results suggest that wasps are making different decisions based on caterpillar species or there is differential survival of larvae within *M. sexta* between MsT and CcC wasps. It is possible that CcC wasps recognize *M. sexta* as a suboptimal host and therefore allocate fewer eggs to them; however, brood size and sex ratio are comparable to those found in the wild (MsT =  $48 \pm 23\%$  female; CcC =  $72 \pm 17\%$  female; Kester, unpublished data). Similarly, wasps should allocate more females to gregarious rather than solitary hosts since males are more likely to disperse.

Divergence in male acoustic signals, larval development time, reproductive factors, and other traits along with possible differences in female pheromones indicates that these two genetically divergent sources of *C. congregata* also have diverged behaviorally. However, the lack of isolation due to courtship behavior implies that other ecological mechanisms, such as assortative mating by host plant, are preventing these complexes from mating in the wild. The presence of post-zygotic isolation suggests that the polydnavirus can evolve faster than sexual pre-zygotic isolation to exploit hosts and that ecological isolation may be the first step in

speciation. Small differences in courtship signals, initially diverged through genetic drift, may then be selected for to prevent mating between incompatible races and a reduction in fitness from hybrid offspring. Testing across geographically distant populations of these two host-plant complexes can assess whether differences between our two sources hold across all populations within these complexes.

The MsT and CcC host-plant complexes are likely incipient species in that gene flow between these complexes is possible and may occur to a limited extent in nature. However, speciation may still occur despite limited gene flow and without absolute isolation (Nosil 2008). Therefore, given the 2% genetic divergence of mtDNA COI region (Karns 2009), and the decreased fitness observed for one of the hybrid crosses, the MsT and CcC lineages may even be considered sibling species. The identification of parasitoid sibling species has implications for their use in biological control, since crossing incompatible species may inhibit population growth.

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**Table 1** Comparison (mean  $\pm$  SE) of elements of the male courtship acoustic signal between two host-plant complex sources of *Cotesia congregata* (MsT = *Manduca sexta* on tobacco, n = 21; CcC = *Ceratonia catalpae* on catalpa, n = 24). Amplitude re: 20  $\mu$ Pa at 2-3 mm. Two-way t-test between MsT and CcC. Bold are  $p < 0.05$ .

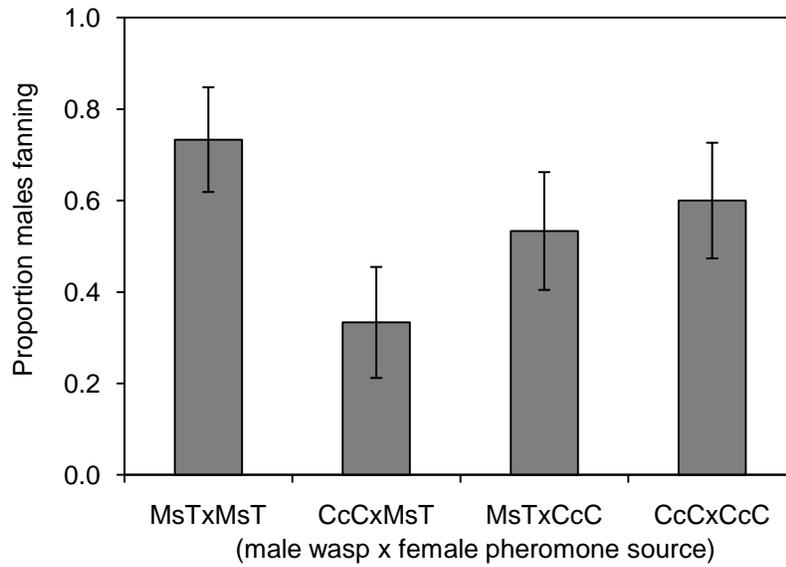
Acoustic element	MsT	CcC	p-value
Boings per second	2.88 $\pm$ 0.05	2.74 $\pm$ 0.04	<b>0.0220</b>
Total boing period (ms)	348.7 $\pm$ 6.2	367.3 $\pm$ 5.4	<b>0.0279</b>
Boing (part 1) time (ms)	121.2 $\pm$ 2.8	133.8 $\pm$ 3.0	<b>0.0037</b>
Buzz (part 2) time (ms)	203.6 $\pm$ 6.4	213.6 $\pm$ 4.4	0.1960
Gap time (ms)	22.8 $\pm$ 0.7	19.9 $\pm$ 0.8	<b>0.0088</b>
Signal Frequency (Hz)	229.4 $\pm$ 3.1	239.2 $\pm$ 2.8	<b>0.0245</b>
Boing frequency (Hz)	229.0 $\pm$ 3.7	237.1 $\pm$ 2.8	<b>0.0845</b>
Buzz frequency (Hz)	239.2 $\pm$ 3.2	245.4 $\pm$ 2.3	0.1191
Boing amplitude (dB)	64.2 $\pm$ 0.8	66.6 $\pm$ 0.9	<b>0.0436</b>
Buzz amplitude (dB)	54.9 $\pm$ 1.1	58.7 $\pm$ 0.9	<b>0.0096</b>

**Table 2** Results of controlled matings producing broods with females, only males, or no larvae in the F<sub>1</sub> (157 crosses) and F<sub>2</sub> (137 crosses from hybrid females x siblings) generations among crosses between two host-plant complex sources of *Cotesia congregata*. Proportion mated is the number of crosses that produced females per total number of matings that produced offspring. Numbers in parenthesis indicate entire lineages originating from single parental generation matings.

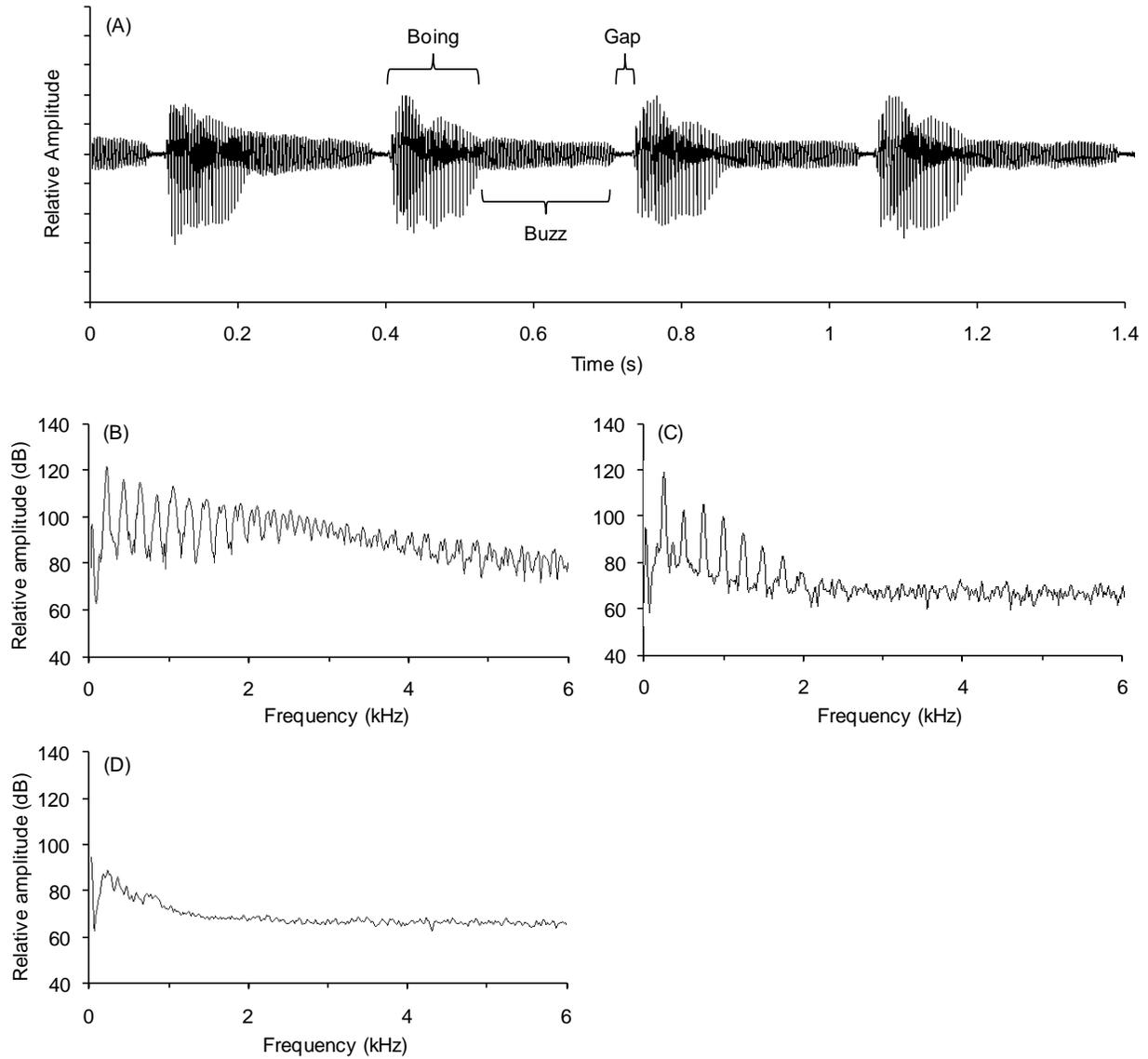
		MsT♂ x MsT♀	CcC♂ x MsT♀	MsT♂ x CcC♀	CcC♂ x CcC♀
F <sub>1</sub>	With females	13	17	29	17
	All male	7	23	35	15
	Prop. mated	0.65	0.43	0.45	0.53
	# w/ no larvae	1	0	0	0
F <sub>2</sub>	With females	7 (4)	3 (2)	5 (3)	6 (5)
	All male	7 (2)	3 (1)	34 (16)	17 (5)
	Prop. mated	0.5	0.5	0.13	0.26
	# w/ no larvae	0 (0)	50 (12)	4 (1)	1 (0)
	Prop. w/ progeny	1.00 (1.00)	0.11 (0.20)	0.91 (0.95)	0.96 (1.00)

**Table 3** Mean percent ( $\pm$  SE) and range of F<sub>2</sub> *Cotesia congregata* larvae that did not egress from individual *M. sexta* hosts among F<sub>1</sub> crosses as determined by host dissections.

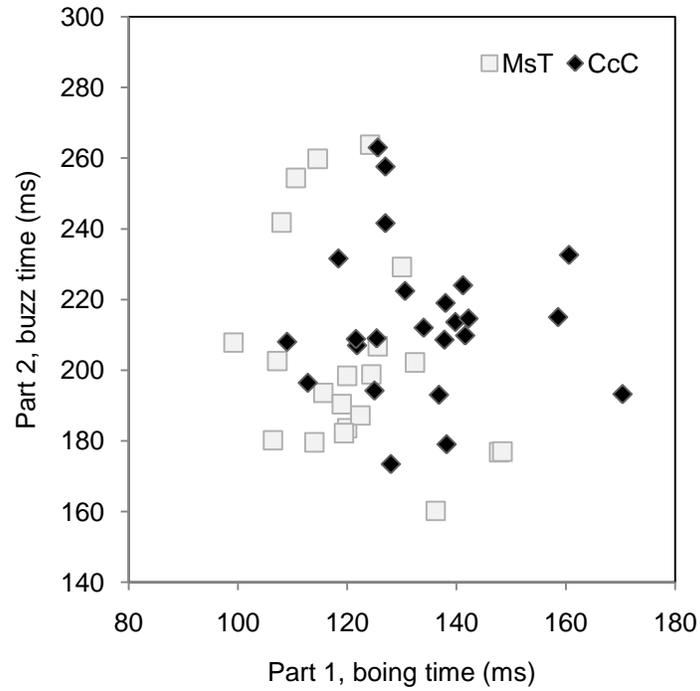
Cross type	n	% larvae in host $\pm$ SE	Range
MsT♂ x MsT♀	5	13 $\pm$ 4%	0-19%
CcC♂ x MsT♀	6	39 $\pm$ 15%	0-88%
MsT♂ x CcC♀	34	26 $\pm$ 2%	4-57%
CcC♂ x CcC♀	7	36 $\pm$ 11%	5-93%



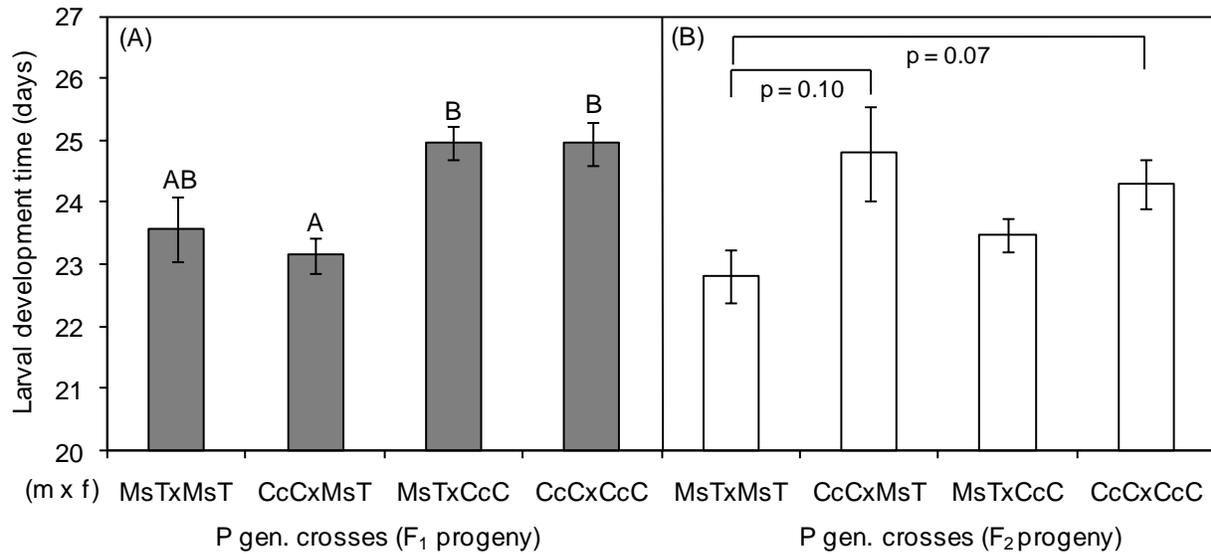
**Figure 1** Proportion of males ( $\pm$  SE) of two host-plant complex sources of *Cotesia congregata* fanning to different female pheromone sources on the male's respective host plant (tobacco or catalpa) (n = 15 each).



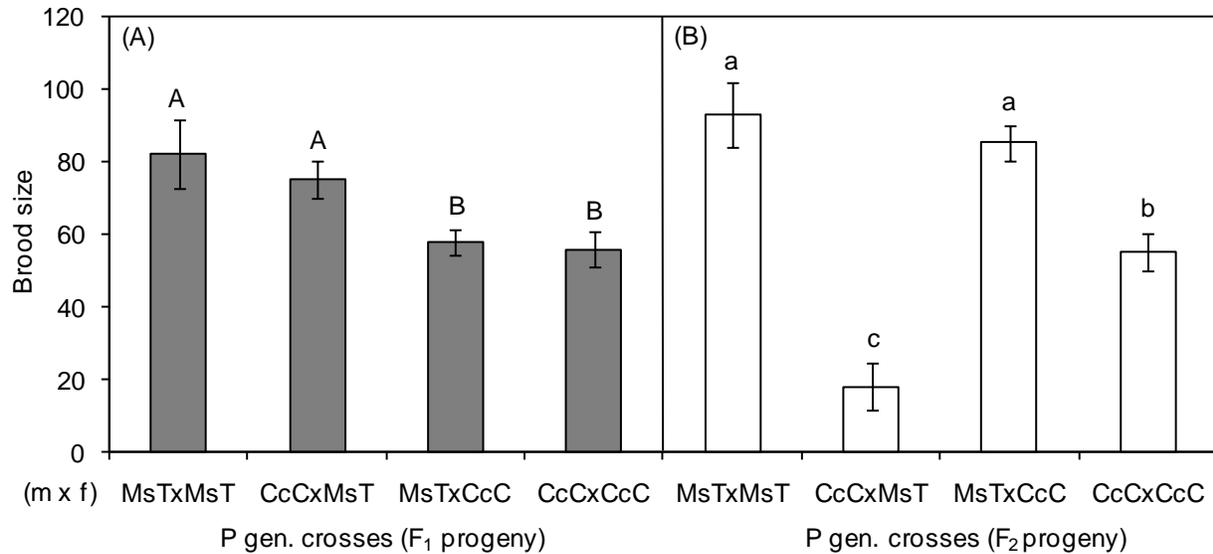
**Figure 2** (A) Typical waveform of the male acoustic courtship signal of *Cotesia congregata*. Each signal contains three components: a high amplitude boing (part 1), a lower amplitude buzz (part 2), and a short gap. Frequency spectra of (B) boing and (C) buzz elements were used to determine fundamental frequency. (D) Frequency spectrum of background noise for comparison.



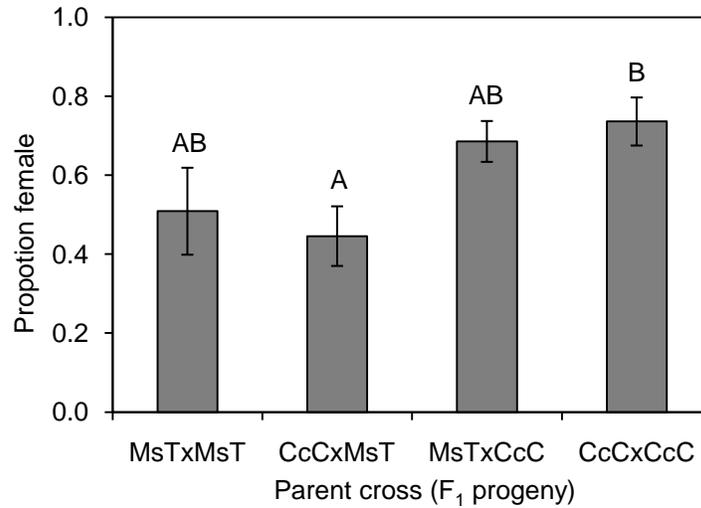
**Figure 3** Scatter plot of duration of boing and buzz acoustic elements of courtship signal from MsT and CcC host-plant complex males of *Cotesia congregata*. The two elements did not correlate ( $r^2 = 0.0089$ ).



**Figure 4** Comparison of larval development time (LS mean  $\pm$  SE) among progeny of control lines and hybrid crosses ( $\sigma \times \rho$ ) produced by (A) parental and (B) F<sub>1</sub> generations of two host-plant complexes of *Cotesia congregata*. Different upper-case letters indicate significant differences among F<sub>1</sub> progeny, while p-values are given for largest differences among F<sub>2</sub> progeny produced by female hybrids crossed with male siblings (ANOVA followed by Tukey's test,  $p < 0.05$ ).



**Figure 5** Comparison of brood size of larvae that egressed and spun cocoons (LS mean  $\pm$  SE) among control lines and hybrid crosses ( $\sigma \times \rho$ ) produced by (A) parental and (B) F<sub>1</sub> generations of two host-plant complexes of *Cotesia congregata*. Different upper-case letters indicate significant differences among F<sub>1</sub> broods, while lower-case letters indicate significant differences among F<sub>2</sub> broods produced by female hybrids crossed with male siblings (ANOVA followed by Tukey's test,  $p < 0.05$ ).



**Figure 6** Comparison of the proportion female (LS mean  $\pm$  SE) produced among control lines and crosses ( $\sigma \times \rho$ ) between two host-plant complexes of *Cotesia congregata*. All male broods were excluded from analysis. Different letters indicate significant differences among crosses (ANOVA followed by Tukey's test,  $p < 0.05$ ).

## 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 variety of other field biology jobs across the country. This included searching for the once thought to be extinct ivory-billed woodpecker in Arkansas for Cornell University, 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. During this time he also made some cross country roadtrips and travelled to several national parks in the west. He joined the Insect Ecology and Evolution Lab in 2009. Justin will remain at VCU for at least another semester to teach the Entomology Labs and continue research on *Cotesia congregata*. He plans to eventually pursue a Ph.D. and continue a career in science.