UNIVERSITY OF CALIFORNIA
RIVERSIDE

Using Isofemale Lines to Investigate the Effect of Mass-Rearing Time on Field Fitness in the Parasitoid Wasp, *Trichogramma pretiosum*

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Entomology

by

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June 2013

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ACKNOWLEDGEMENTS

I would like to thank, first and foremost, my advisor, Dr. Richard Stouthamer. He has been an excellent mentor, and without his guidance, I likely would have floundered through my Master's degree. I must also thank the other members of my committee, Dr. Joseph Morse and Dr. Leonard Nunney. Dr. Nunney was particularly helpful in the statistical analysis of the data I produced. Dr. Paul Rugman-Jones has been unfailingly helpful in the practical aspects of both molecular work and field work, helping to simplify and troubleshoot whenever needed. I must also thank several undergraduate students, namely Brittany Callaham, who created the wasp lines I used, Easther Custodio, who helped with some of my molecular work, and Michael Jones, Kyle Oatman, and Jonathan Estrada, who were instrumental in helping me to complete my field work. Last but certainly not least I would like to thank my husband, Zach Hills, for his love and support as I worked towards my degree.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

The History of *Trichogramma* as a Biological Control Agent

Various species within the family Trichogrammatidae have been recorded attacking eggs of species spanning several orders including Lepidoptera, Hemiptera, Coleoptera, Odonata, Orthoptera, Diptera, Hymenoptera, Thysanoptera, and Neuroptera (Pinto and Stouthamer 1994, Pinto 1998). The genus *Trichogramma* is made up of minute parasitoid wasps, which attack the eggs of various lepidopteran species. Due to this broad host range, *Trichogramma* species have become very popular biological control agents, and as recently as 1998 were reported to be the "most widely used insect natural enemy in the world" (Knutson 1998).

Trichogramma was identified as a potential biological control agent in the early 1900's, and by 1926, a mass-rearing system had been established which allowed widespread international use of the insects. Rearing and release of *Trichogramma* species in the USA can be traced back to the early 20th century, when two Austrian species were imported for the control of the brown-tail moth (*Euproctis chrysorrhoea*) (Parra 2010a). Though the Green Revolution's focus on chemical pesticides diverted US attention away from biological control for decades after mass-rearing became possible, the former Soviet Union, China, and Mexico made, and continue to make, heavy use of *Trichogramma* against lepidopteran pests of several major field crops including corn, rice, sugar cane, and cotton (Li 1994).

Mass rearing of many natural enemies including *Trichogramma* began in earnest around the 1970's, once concerns about chemical overuse became apparent (Parra 2010b).

Due to the ease of rearing and a short generation time (~10 days from egg to adult), several species of *Trichogramma* are now reared and sold commercially in countries across the world. Three species of *Trichogramma* are currently reared and sold by insectaries in the U.S. and Canada; *T. brassicae, T. platneri*, and *T. pretiosum* (Warner and Getz 2008).

Species within the genus occur worldwide, and *T. pretiosum* (Riley) is the most widely distributed American species. This species has been collected as far north as southern Canada and as far south as Argentina, as well as Hawaii, the West Indies, and Australia (Pinto 1998). *T. pretiosum* is released mainly in North and Central American countries (where it is native) against lepidopteran pests of field crops. The most notable and economically important of these pests include *Alabama argillacea* (cotton leafworm), *Anticarsia* spp. (Noctuids including the velvetbean caterpillar), *Diatraea* spp. (Crambids including the sugar cane borer), *Heliothis/Helicoverpa* spp. (Noctuids including tobacco budworm and corn earworm), *Trichoplusia ni* (cabbage looper), *Plodia interpunctella* (Indian meal moth), and *Tuta absoluta* (tomato leafminer) (Li 1994). More exhaustive lists of target species can be found in Thomson et al. (2001), Smith (1996), and Pinto (1998).

Mass Rearing and Release

In general, cultures of *Trichogramma* species are started from parasitized host eggs collected from a local field site. Lines are begun from wasps emerged from the same egg, and once species has been confirmed, these lines are combined to increase genetic diversity (Knutson 1998). In some cases, a population of the desired species is

maintained in a greenhouse to maintain near-natural conditions. Bigler (1986 and 1994) describes a commonly recommended method for rearing *T. brassicae (=T. maidis)* in which wasps are maintained in a greenhouse or field insectary on European corn borer (*Ostrinia nubilalis*) eggs, which are attached to corn plants. Wasps produced in the greenhouse are then collected and reared in a laboratory on an easily produced artificial (factitious) host, usually *Ephestia kuehniella* or *Sitotroga cerealella*. Once sufficient quantities are produced, they are shipped to the grower for release.

Trichogramma can be released by the hundreds of thousands per hectare in two different ways. The wasps are nearly always put into the field as pupae, but these pupae can either be glued to cards and hung at regular intervals on the plants, or distributed as loose eggs and sprinkled by a tractor-sprayer or airplane. Wasps are timed to emerge within hours of placement in the field, to minimize loss to exposure, dehydration, and predation (Li 1994, van Lenteren and Tommasini 2003).

Problems Encountered in Mass Rearing

Mass-rearing of any species of natural enemy is not a simple undertaking. Before colonies can be put into production, insectaries must be certain that they have correctly identified their species, and that their founder stock is free of contaminants, such as hyperparasitoids. Once colonies are established, care must be taken that insectary conditions are synchronized to the insects' natural life cycle. Food and hosts must be of good quality, as artificial diets are not commonly available for natural enemies. Steps must be taken to prevent infection with pathogens, maintain appropriate sex ratios, and keep synchrony with the target pest (van Lenteren 2003). Even when the myriad physical

and mechanical hurdles to successful rearing are overcome, natural enemies may suffer genetic deterioration. This problem is difficult to detect, and harder still to correct.

Several traits in *Trichogramma* have been shown to deteriorate over the course of mass rearing on factitious hosts such as *S. cerealella* or *E. kuehniella*. Pluke and Leibee (2006) found that *T. pretiosum* reared on *E. kuehniella* eggs – which are smaller than those of many wild hosts – would preferentially attack smaller eggs in choice tests, also indicating that mass rearing on factitious hosts may alter host preference and therefore be detrimental to field performance. Bergeijk et al. (1989) found that when populations of *T. brassicae* (=*maidis*) were switched from their native host, *Ostrinia nubilalis*, to a factitious one (in this case *E. kuehniella*) for more than seven generations, the wasps parasitized significantly fewer native hosts in comparison to a population that had only been reared on *E. kuehniella* for one to three generations. However, this finding was contradicted by a similar study conducted by Kölliker-Ott et al. (2003). The results of this more recent study indicated no failure to accept the native host *O. nubilalis* after 27 generations of mass-rearing on *E. kuehniella*. In fact, the authors found that *T. brassicae* reared on *E. kuehniella* had an even higher rate of native host acceptance than a population that had been only recently collected from the field.

Mackauer (1976) noted that size and longevity of laboratory reared insects was often reduced after a number of generations. Increased size of female wasps has been associated with an increased ability to locate hosts, as well as increased fecundity (Kazmer and Luck 1995, Bennett and Hoffman 1998, Bai et al. 1995, Greenberg et al.

1998). As ability to find hosts and high reproductive capacity are very important traits for a biological control agent, a reduction in the size of mass-reared wasps is undesirable.

Other traits shown to be affected by mass rearing include temperature preference (Shchepetilnikova and Kasinskaya 1981), walking mobility, and flight ability (Bigler et al. 1988), all of which have the potential to negatively impact biological control efforts. In light of these observed problems, Bigler (1988) recommends that laboratory stocks be maintained for no more than five or six generations, at which point they should be restarted from greenhouse or preferably wild stock. This recommendation has been followed by insectaries in some European nations (Smith 1996). However, if the demand for insects exceeds the facility's capacity to produce in only a few generations, the stocks may need to be continually propagated for much longer.

Genetic Basis for Mass-Rearing Problems

Multiple reviews recognize that the most important genetic processes associated with problems in mass rearing are the founder effect, genetic drift, inbreeding, and laboratory selection (Mackauer 1972, Chambers 1977, Joslyn 1984, Bigler 1994, Nunney 2003, van Lenteren and Bigler 2010).

The "founder effect" (Mayr 1942) results in laboratory populations that are inherently different from wild populations. Mackauer (1972) and Joslyn (1984) agree that ideally, a founding population of insects for mass-rearing should reflect the genetic variation present in the wild population. This would allow for the most adaptability upon release back into the field. It is generally agreed that the most appropriate number of founders will vary according to the species of insect in question, but some studies make

the general recommendation that at least 1,000 individuals from wild populations be used to initiate a new laboratory stock (Joslyn 1984, Nunney 2003). However, it is not always possible for insectaries to sample this extensively. As a consequence, only a fraction of the genetic diversity present in a species is represented in the founding population. This may give a mass-reared population an automatic disadvantage.

Compounding this problem, genetic drift may cause populations to diverge from the wild stock they originated from. Boller (1972) noted that it is often the case in mass-rearing that populations will actually decline for the first generation or two. This bottleneck only serves to exacerbate problems caused by genetic drift. When sample sizes are very small, genetic change over time tends to be random rather than adaptive. Some alleles will be lost and others fixed, due mainly to chance in small closed systems. After many generations, the laboratory population may have fixed alleles that are detrimental to field performance, possibly causing one or more of the effects discussed in the previous section.

Inbreeding is extremely common in *Trichogramma*, and for most animals this would lead to the expression of detrimental alleles over several generations. However, micro-hymenopterans such as *Trichogramma* are haplodiploid, and studies have shown very little in the way of inbreeding depression when reared in a laboratory. In a study by Sorati et al. (1996), 21 strains of *T.* nr. *brassicae* were inbred for four generations and compared to 11 outbred strains for five characteristics including fecundity and female size measurements associated with field fitness (see Roush and Hopper 1995). No significant inbreeding depression was found. Supporting Sorati's study, Prezotti et al.

(2004) found very little effect in five biological characteristics even in colonies started with only one breeding pair of *T. pretiosum* after 25 generations of inbreeding.

Though inbreeding may not be an issue in *Trichogramma*, laboratory adaptation may very well be a significant problem. When insects are brought in from the field and pooled together, selection occurs for the genotypes that are best suited to the new conditions; typically, controlled temperature and humidity and a constant light/dark cycle. During the mass rearing process, wasps have easy access to food, host eggs, and mates, eliminating the need for much in the way of searching or locomotion ability. In addition, there is no threat of predation, so selection for traits such as flight instincts is relaxed or eliminated (van Lenteren and Bigler 2010). The combination of relaxed selection on traits that are important for field success and selection for new conditions in the laboratory may lead to lowered genetic diversity and poorer field performance.

Joslyn (1984) suggests three ways in which these genetic effects might be mitigated: first, pooling field-collected insects from across a broad portion of the species' range to increase genetic variability in a founding population; second, varying conditions in the laboratory in which insects are mass-reared; and third, regular addition of new field-collected stock to the laboratory populations. These approaches may be somewhat effective – however, van Lenteren and Bigler (2010) point out that any wild wasps added to laboratory colonies will immediately be under the same selective conditions that the previous laboratory stock was, and the benefits of added diversity may not last for very long.

Nunney (2003) summarizes the genetic problems in mass rearing as the "paradox of captive breeding," which means that the closest one can come to an ideal mass-reared population is to find the best compromise between insects which are adapted to the field and those which are adapted to laboratory conditions. Woodworth et al. (2002) found evidence for such a balancing effect between laboratory and 'wild' fitness in *Drosophila melanogaster.* In this study, the authors started new colonies of *D. melanogaster* with varying effective population sizes, and inbred them under captive conditions for 50 generations. Reproductive fitness was then tested in the lab, and, as might be expected, populations begun with higher numbers of flies performed better. Each population was then moved to simulated 'wild' conditions to compete with a control group, and the competing cultures were monitored over an additional at various time points over the next 50 generations. All captive-reared cultures competed poorly compared to the control, but the cultures begun with 100 or 250 flies showed a significantly lower drop in fitness when compared to ones started with 500, 50, or 25 flies. This shows that while a highly diverse captive population is able to adapt and do well in the laboratory, there is a substantial trade-off for performance under wild conditions. A strategy is clearly needed to optimize a balance between quality and quantity of mass-reared insects.

Potential Solution to Loss of Genetic Diversity and Laboratory Adaptation

A solution to the problems of laboratory adaptation and loss of genetic diversity may be to maintain many, much smaller colonies of inbred insects. Roush and Hopper (1995) reason that highly advantageous alleles are likely to be very common even in a smaller founder population, and drift is unlikely to remove them; however, laboratory

conditions may select against these alleles in a large, diverse population. Therefore, the authors suggest fixing these common alleles in inbred, single-family or isofemale lines to assure that they are maintained until insects are released.

Nunney (2003) agrees with this approach, suggesting that colonies of parasitoid wasps be maintained as isofemale lines to maintain a high overall level of genetic diversity. Colonies with so little genetic diversity should have no way to adapt to the laboratory environment if they are isolated immediately after collection from the field, and should remain genetically identical to their parent stock, aside from random mutations. Antolin (1999) found that *T. pretiosum* lines reared as isofemale lines for 6 generations suffered some loss of fecundity. Antolin then crossed females and males from different isofemale lines, and found that in most cases, the offspring of these crosses had recovered the lost reproductive capacity. Therefore, Antolin and Nunney agree, after sufficient numbers of insects have been produced, isofemale colonies should be mixed and allowed to produce a hybrid F1 generation, which would then be released in the field containing approximately the amount of genetic variation as the original founding sample taken from the native population.

The research contained in this thesis tests the hypotheses set forth by Nunney (2003) and by Roush and Hopper (1995). The experiments I conducted were designed to ultimately answer the following question: does maintaining *T. pretiosum* as isofemale lines result in an overall population with higher field fitness than a population that has been mixed for many generations? Based upon the evidence in the literature, I hypothesize that maintaining isofemale lines will prove a more effective way to preserve

field fitness. The results of my experiments will guide efforts to rear a better biological control agent.

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CHAPTER 2

Introduction: Molecular Markers and *Trichogramma pretiosum*

Trichogramma pretiosum (Riley) is among the tiniest of arthropods, measuring barely a millimeter long. Individual wasps are visible to the naked eye, though certainly not conspicuous. Morphological characters are only distinguishable under a microscope, and even under magnification there is very little apparent variation within and between species. Many species of *Trichogramma* are so similar in appearance that an expert must analyze minute differences in male genitalia in order to confirm a species diagnosis (Pinto and Stouthamer 1994).

For an insect as tiny and morphologically indistinct as *Trichogramma*, it is desirable to have populations bearing distinct markers when conducting field experiments. This allows for simultaneous release of wasps from captive populations of the same species that have undergone different treatments, and all in the same field. Many visual marker systems have been developed for insect mark-release-recapture studies, but most of these are designed for large insects and are impractical for tiny parasitoids (reviewed by Hagler and Jackson 2001). Moreover, external markers are not transferred to offspring and cannot be used to track success of releases over multiple generations. Thus, a molecular marker embedded in the wasps' genome is needed. This should be persistent in individual wasps, and also heritable, allowing for identification of offspring.

A genetic marker for tracking populations must be variable within the species and stable, ideally not subject to recombination each generation. Mitochondrial DNA is

passed clonally from mother to offspring without recombination or paternal contribution, allowing for easy tracking of maternal inheritance in subsequent generations. Mitochondrial DNA sequences are variable between and within populations, though often some haplotypes will be more common than others (examples in Szalanski et al. 2008, Althoff and Thompson 1999, Marquez and Krafsur 2002). In addition, mitochondria are found in multiple copies in every cell, making their genes easy to amplify via the polymerase chain reaction (PCR). The cytochrome oxidase I (COI) gene is a popular marker choice. This region is relatively short, allowing for relatively inexpensive sequencing. Also, like other mitochondrial genes, COI lacks introns and allows for easy sequence alignment (Floyd et al. 2010). For field studies to be discussed in subsequent chapters, a COI sequence marker system was established via introgression of three different haplotypes into many populations of *Trichogramma pretiosum*, and then tested for neutrality (ie, checked for conferred advantage in particular haplotypes).

COI sequence variation within a species has been thought to be adaptively neutral. Cytochrome oxidase I has an important role in cell respiration, and without a functioning copy of this gene, organisms would not survive. Therefore, sequence variation in this gene generally results from synonymous substitution, which does not affect the amino acid sequence of the resulting enzyme (Kimura 1983). However, introgressions performed for the studies described introduced not only the COI gene sequence, but the entire mitochondrial genome. Previous studies have shown that in some cases, mitochondrial variants appear to be co-adapted to the nuclear genome, and this may in fact lead to differences in life-history traits when introgressed into new nuclear

backgrounds. Evidence of this effect has been found in naturally introgressed fish populations (Glémet et al. 1998), heteroplasmic mice (Takeda et al. 2000), and even in human cell cultures (King and Attardi 1989).

In insects, the most relevant evidence of co-adaptation comes from experimental manipulations of *Drosophila simulans* performed by Nigro (1994), and later confirmed by more carefully controlled work done by James and Ballard (2003). James and Ballard introgressed three nuclear genotypes into flies with three different mitochondrial haplotypes, resulting in three populations with native genome combinations and six with novel combinations. Tests of development time, male activity level, and adult longevity showed that one haplotype was associated with faster development regardless of nuclear genome. Another haplotype was associated with higher levels of activity in males, but only when associated with its native nuclear genotype.

In contrast, other studies in *Drosophila melanogaster* have shown no effect of mitochondrial type and nuclear genome combination on fitness. Kilpatrick and Rand (1995) designed experiments using two populations of flies, which were crossed to obtain lines with reciprocal combinations of nuclear and mitochondrial genomes. These populations were then set up in cages to compete for resources against flies with the original sets of genotypes. Frequency of mitochondrial types in the cages remained relatively unchanged over ten generations, and changes that did occur were consistent with genetic drift rather than competitive advantage. In this case, flies seemed to do just as well with a new mitochondrial type as with their native one. Very similar experiments

were also done by Kambhampati et al. (1992) in the mosquito *Aedes albopictus*, with similar results.

We established introgressed *T. pretiosum* lines, with the intent of using variation in COI sequences as a neutral marker for subsequent experiments. It is clear that the introgression of nuclear genotypes into various mitochondrial backgrounds has the potential to affect fitness of the resulting organism, although based on previous research this result is not universal. Due to the conflicting evidence, it was necessary to test various life history traits in our marked lines once introgression of the desired marker sequences was completed. Traits chosen for this study include 10-day fecundity, egg-to-adult development time, and longevity.

Materials and Methods

Trichogramma cultures

All colonies of *Trichogramma pretiosum* were initiated from parasitized host eggs (*Manduca sexta*) collected from tomatoes in Irvine, California, over the summer of 2008. Parasitized eggs were placed in separate 12x75 mm glass culture tubes plugged with cotton wool, and stored at 25°C and variable humidity (about 30% RH). Upon emergence, females were allowed to mate with presumed brothers emerging from the same egg, and then isolated. Each female was given honey and host eggs (*Ephestia kuehniella*), and allowed to oviposit. These lines were sib-mated for 9 generations, with each generation using a single pair to initiate the next generation. Thereafter, wasps were allowed to mate randomly within isofemale colonies. Each initial line was assigned a number between 1

and 53 for identification purposes. Some of these lines did not survive in the laboratory, resulting in discontinuous numbering of the remaining 26 lines.

After identification of four distinct mitochondrial haplotypes in the 26 colonies (see the methods in the next section), the three least common haplotypes were arbitrarily designated A, B, and C. The fourth was designated "O," indicating "other." All 26 nuclear genotypes were introgressed into the A, B, and C haplotypes by mating males of the desired nuclear genotype with females bearing the desired mitochondrial type. The daughters of these crosses were then bred again with males from their father's line, and this continued for nine generations to achieve 99.8% nuclear genome introgression. Ultimately this resulted in 78 colonies distinguishable by microsatellite markers for the nuclear genotype and by real-time PCR and a high-resolution melt curve (HRM) for the mitochondrial type.

Culture Identification

Determination of mitochondrial type was done using a method developed by Dr. Richard Stouthamer and Dr. Paul Rugman-Jones (UC Riverside Entomology) (unpublished). DNA was extracted from individual wasps by grinding each in 2 µl of 20 mg/ml proteinase K. 60 µl of 5% Chelex 100 suspension was added to the ground sample, which was then incubated at 55°C for 1 hour. Proteinase K was inactivated by incubating samples at 99°C for 10 minutes. Extracts were stored at −20°C until use.

Real-time PCR was used to amplify a segment of the *Trichogramma pretiosum* COI gene. Reactions were carried out in 20 µl total volume containing 400 nM uracil, 200 nM each adenine, guanine, and cytosine, 1 mM MgCl, 1X ThermoPol™ buffer (New

England Biolabs), 0.25µM forward primer Irvine 311F (5'-TGGAACAGGTACAGGAACAGG-3'), 0.25µM reverse primer HCO (5'TAAACTTCAGGGTGACCAAAAAATCA-3'), 0.5X Evagreen (Biotium), and 1 unit Taq polymerase (New England Biolabs). 2 µl of extracted DNA was added to each reaction tube. All reactions were run on a Rotorgene RG-3000 (Corbett Research). Reactions were held at 95°C for 5 min, and cycled as follows: 95°C for 15 sec, 55°C for 30 sec, and 68°C for 30 sec for 35 total cycles. Immediately following amplification, products were melted starting at 72°C and ramping to 80°C, increasing by 0.2°C each step. The first step was held for 90 sec, and each subsequent step was held for 10 sec. The resulting melt curves showed distinctive patterns for mitochondrial types A, B, and C (figure 2.1).

PCR products for COI sequences A, B, and C were sequenced by Dr. Paul Rugman-Jones (UC Riverside Entomology), and translated to check for synonymy.

Nuclear genotypes were identified by amplifying microsatellite regions designated A9 and A124. Microsatellite primers were developed by Genetic Information Systems (Chatsworth, CA). Reactions were each made up in 25 µL total volume containing 1X ThermoPol™ buffer (New England Biolabs), 400 nM uracil, 200 nM each adenine, cytosine and guanine, 0.8 mM MgCl, 0.8 mg/ml BSA (New England Biolabs), 0.2 µM forward primer A9 (5'-CAGCACAAGTACACGACTGTC-3') or forward primer A124 (5'-CGTATGCGCGTACATTTATG-3'), 0.2 µM reverse primer A9 (5'-AGCGAAGCGTATATTAGCAAG-3') or reverse primer A124 (5'-TACCGGATGAAGTCGCTATG-3'), and one unit Taq polymerase (New England

Biolabs). Reaction conditions were as follows: initial hold for 3 min at 94°C, followed by 38 cycles of 94°C for 45 sec, 51°C for 45 sec, and 72°C for 45 sec. After 38 cycles, reactions were held at 72°C for 5 min and then stored at 4°C. Products were visualized on a 1% agarose gel to confirm amplification. For fragment sizing, reactions were run using the same conditions as above, except that forward primers carried either VIC (for A9) or 6FAM (for A124) fluorescent dye (New England Biolabs). Products for both primer sets using the same DNA template were amplified separately, then pooled. These were then sent to the Genomics Core Facility at UC Riverside for DNA fragmentation analysis. Once sizing reports were generated, peaks were identified and assigned lengths using PeakScanner software (Applied Biosystems).

10-day fecundity experiments

Mated female wasps (between 20 and 30 per line tested) were isolated on the day of emergence, and kept in individual 12 x 75 mm glass tubes plugged with cotton wool. Each was given her own egg card containing an excess of *E. kuehniella* eggs and a droplet of honey. For the first round of experiments (comparing only lines 6A vs. 6B, 9A vs. 9B, and 29A vs. 29B), cards and honey were changed daily for 10 days. Subsequent experiments compared fecundity in nuclear lines 6, 9, 29, and 43 in A, B, and C mitochondrial types. Due to host egg shortages, cards were switched every two days for these experiments. After a card was removed, it was placed into its own tube and kept at 25°C for two weeks, to allow all offspring to emerge. After two weeks, tubes were frozen overnight at -20°C to kill any surviving wasps. Dead wasps in each tube were then sexed and counted. Cards were also checked for any un-emerged pupae.

Egg-to-adult development time

When wasps from the lines to be studied began to emerge, 5 fresh egg cards were dropped into culture tubes for each line. Egg cards were left to be parasitized for 30 minutes, at which time they were removed from the culture tubes. Each was examined under a dissecting microscope to ensure that no wasps were still ovipositing. Any live wasps found lingering on the cards were brushed off and killed. Cards were placed in individual 12 x 75 mm glass tubes plugged with cotton wool, and kept at 25°C, 16:8 L:D, and uncontrolled humidity (approx. 30% RH). Cards were checked daily, and no emergence was seen before day 9. Beginning on day 9, tubes were checked hourly starting between 6:30 and 7am. At each check, emerged wasps were collected and moved to empty tubes. Checks continued through each day until approximately 4:45pm, and started over the next day. Total daytime emergence was pooled for analysis. This was repeated for 4 days, covering the range of 9-12 days after initial oviposition. After collection, emerged wasps were killed by freezing at -20°C, and later sexed and counted.

Adult longevity

Wasps were isolated as pupae, and allowed to emerge in individual 12 x 75 mm glass vials. These were sexed upon emergence, and 40 males and 40 females from each of the 12 lines were kept for testing. Wasps were housed singly for the duration of the experiment. Half of the males and half of the females from each line (20 each) were starved (given no honey or hosts). The remaining wasps were each given an egg card containing an excess of irradiated *E. kuehniella* eggs and a droplet of honey. Egg cards and honey were changed every 7 days, to avoid emergence of new wasps in the tubes

containing females. All wasps were checked daily in the early afternoon until all wasps had died.

Antibiotic treatment

As fecundity experiments on lines 6A, 6B and 6C yielded unexpected results (see data next section), these lines were examined further to determine the cause. In order to eliminate the possibility of bacterial contamination, all three lines underwent antibiotic treatment. Immediately after culture emergence, cultures were given egg cards containing a surplus of host eggs and a droplet of honey containing 0.5 mg/mL Rifampicin. These egg cards were switched daily for three days, and offspring from the fourth day onwards were kept to produce the next generation. This treatment procedure was repeated for a second generation. 10-day fecundity of wasps from these "cured" lines was then tested as described above.

6 x 6A x 6B crossing experiment

Microsatellite and COI markers were checked and confirmed to be correct for lines 6, 6A, 6B, and 6C. Wasps from lines 6 (haplotype O), 6A and 6B were crossed to determine whether hybridizing the nuclear genome of 6A wasps would restore some fecundity, indicating a previously undetected aberration in the 6A nuclear genome. Line 6C was not included due to time constraints – this would have necessitated too many wasps for one person to mate in one day. Wasps from each line were isolated as pupae, and upon emergence were sexed and given honey. Crosses were done in all 9 possible combinations. Each mating was done by introducing a male of the desired type into the tube of a female of the desired type, and leaving her overnight. The next morning, egg

cards were added to each tube. Ten days later, wasps emerged, and females mated with their brothers. These females were then isolated and 10-day fecundity experiments were conducted as previously described. 20 female offspring per cross were used, with the exception of cross 6Ax6A (10 females used), 6A mother x 6 father (16 females used), and cross 6Bx6B (10 females used). These lower numbers were necessitated due to low female emergence from these crosses.

6A x 29A crossing experiment

Another possible explanation for the anomalous fecundity results in line 6A was an interaction between the '6' nuclear genome and the 'A' mitochondrial type. To test for this, 6A wasps were crossed with 29A wasps. Wasps from each line were isolated and crossed in both possible combinations as in the previous experiment, except that the F1 daughters were isolated and mated with males with the same genotype as their fathers. Twenty-three daughters of each cross were tested for 10-day fecundity as described previously. If the nuclear/mitochondrial interaction was causing the lowered fecundity, both combinations of hybrid offspring were expected to perform similarly. Hybrid nuclear genomes were the same in F1 daughters in both combinations, but one inherited the 'A' mitochondrial type from 29A and the other inherited the 'A' type from 6A.

Female hind tibia length measurements

This experiment was done to determine whether or not a significant size difference existed between female wasps of the 6A, 6B and 6C lines. Fifty newly-emerged female wasps each from 6A, 6B and 6C cultures were killed in ethanol. These were then mounted in a droplet of water on a glass slide, covered with a glass coverslip,

and examined under a light microscope at 40X magnification, following the method of Hohmann et al. (1988). The hind tibia of each wasp was measured to the nearest 0.01 mm.

Statistical analysis

Experiments investigating only one nuclear genotype at a time (antibiotic treatment, backcrossing experiments, 6A x 29A crossing experiment, and female hind tibia measurements) were analyzed using a one-way ANOVA. Experiments investigating multiple nuclear and mitochondrial genotypes simultaneously (10-day fecundity, egg-to-adult development time, and adult longevity) were analyzed across genotypes using a factorial ANOVA to examine possible interactions between nuclear and mitochondrial genotypes, followed by Tukey's LSD to determine significance groups. All calculations were done in Minitab 16 (Minitab, Inc.).

Results

Culture Identification

All introgressed lines were consistently distinguishable by mitochondrial and nuclear genotype. As shown in figure 2.1, HRM following real-time PCR distinguished melting patterns characteristic of the A, B, and C mitochondrial types. Translation of the sequenced PCR product revealed that the sequence variations were synonymous.

Nuclear genotypes were distinguishable by the lengths of two microsatellite regions designated A9 and A124. Fragment lengths for both regions are given for all genotypes in table 2.1.

10-day fecundity experiments

Because fecundity results were not statistically different between the same lines in the initial and second round of fecundity tests, data from both experiments were pooled for analysis. Following a factorial ANOVA, significant effects of both nuclear and mitochondrial genotype were found ($p<0.001$ and $p=0.002$, respectively). A significant interaction between nuclear and mitochondrial genotype was also seen ($p<0.001$). ANOVA results are shown in table 2.2, while average offspring production with significance groups (calculated by Tukey's LSD) is shown in figure 2.2. As illustrated in figure 2.2, nuclear genotypes were generally quite different from one another, with line 6 producing the fewest offspring and line 29 producing the most. Significant variation did occur between mitochondrial genotypes within the same nuclear genotype, reflecting the effect of mitochondrial type and interaction given by the factorial ANOVA.

Egg-to-adult development time

Average development time for wasps in lines 6, 9, 29 and 43 A, B and C are illustrated in figure 2.3 and tabulated in table 2.3. A 4x3x2 ANOVA was done to examine differences between nuclear genotypes, mitochondrial genotypes, the two sexes, and the interaction between nuclear and mitochondrial genotype. This calculation revealed significant effects both mitochondrial and nuclear genotype ($p=0.014$ and $p<0.001$, respectively) as well as an effect of sex ($p<0.001$) and a significant interaction between nuclear and mitochondrial genotype ($p<0.001$). Additionally, significant interactions were seen between nuclear genotype and sex ($p=0.001$) and mitochondrial genotype and sex ($p=0.004$).

As seen in figure 2.3, males always developed faster than females. In general, lines 9 and 29 developed faster than lines 6 or 43, though significance groups calculated via Tukey's LSD show that these differences are not always significant. In males, the A mitochondrial type always developed the fastest regardless of nuclear genotype, while in females no such pattern was seen. Within nuclear genotypes, both sexes showed variation between mitochondrial genotypes.

Adult longevity

A 4x3x2x2 factorial ANOVA was conducted to analyze variation between nuclear genotypes, mitochondrial genotypes, sex, and feeding status (results in table 2.4). No significant interactions were seen between mitochondrial genotype and sex ($p=0.563$) or between mitochondrial genotype and feeding status ($p=0.688$). These interactions were excluded from the analysis and the test was run again to give the remaining values. This analysis found a significant effect of nuclear genotype ($p=0.001$), but no significant effect of mitochondrial genotype ($p=0.416$). Sex and feeding status were both highly significant, with $p<0.001$ for each. Significant interactions were seen between nuclear and mitochondrial genotype ($p=0.016$), nuclear genotype and sex ($p=0.013$), nuclear genotype and feeding status ($p<0.001$), and between sex and feeding status ($p<0.001$).

Average longevity is shown separately for each treatment and sex in figure 2.4. Significance groups were determined using Tukey's LSD. Starved wasps never lived longer than 3 days, regardless of feeding status or sex. When fed, male wasps lived only slightly longer than their starved counterparts, while females survived up to an average of about 16 days depending on genotype. The longest lifespan was seen in fed females of

type 43C, with the last survivor living 22 days. Very little variation was seen between lines in starved treatments. In fed males, line 9B lived significantly longer than 9A or 9C, though no other line showed significant differences between mitochondrial types. In females, no significant differences were seen between mitochondrial types in the same nuclear genotype. Overall, mitochondrial genotype had varying effects on longevity depending on nuclear genotype.

Antibiotic treatment

Average fecundity of 6A, 6B and 6C wasps following Rifampicin antibiotic treatment is shown in figure 2.5. Data were analyzed via one-way ANOVA, and results are shown in table 2.5. Fecundity of females from line 6A remained significantly lower than with females from the other lines ($p=0.001$). Offspring production was still not significantly different between lines 6B and 6C.

6 x 6A x 6B crossing experiment

Results of fecundity tests following back-crosses in all 9 combinations are tabulated in table 2.6. Average production of daughters over 10 days was compared and analyzed via 3x3 factorial ANOVA, testing the impact of maternal vs. paternal genetic contribution as well as the interaction of the two. The genotype of the father of the wasp being tested had no significant effect on fecundity ($p=0.652$), and no significant interaction was found between maternal and paternal contribution ($p=0.266$). However, the genotype of the mother had a dramatic effect on fecundity ($p<0.001$). As seen in figure 2.6, any female that had a 6A mother (regardless of paternal type) produced far fewer daughters than combinations with a non-introgressed type 6 mother or a 6B mother.

Females with non-introgressed mothers did not produce significantly different numbers of daughters when compared to females with 6B mothers.

6A x 29A crossing experiment

Average fecundity of hybrid wasps with a 6A mother and 29A father or the reciprocal combination is shown in figure 2.7. Results were analyzed via one-way ANOVA using Minitab 16, and data are shown in table 2.7. Maternal genotype was found to have a significant effect ($p<0.001$), with female wasps produced from 6A mothers having far lower fecundity than wasps produced from 29A mothers, even though hybrid nuclear genotypes were the same.

Female hind tibia length measurements

Average HTL for 50 female wasps each of type 6A, 6B, and 6C is shown in figure 2.8. Averages were compared via one-way ANOVA, and these numbers are shown in table 2.8. No significant size difference was found between the three populations ($p=0.165$).

Discussion

The experiments reported in this study were designed to investigate whether or not introgression of four nuclear genotypes into three new mitochondrial genotypes had a significant effect on life history traits; namely, fecundity, egg-to-adult development time, and adult longevity. Literature reviewed in the introduction indicate that differences may or may not be caused by such genetic changes, and if these introgressions are to be used

as markers for further field studies, it was necessary to determine if any significant changes were apparent.

Fecundity differences were immediately apparent. Nuclear genotype was the best predictor of fecundity. Consistently, all mitochondrial genotypes in line 6 produced the fewest offspring, while all types in lines 29 and 43 produced on average much higher numbers over the same time period. Line 9 hovered somewhere between, being closer to line 6 when tested in mitochondrial types A and C and closer to lines 29 and 43 in mitochondrial type B. Mitochondrial type alone was also a significant contributor to fecundity, and there was a highly significant interaction between mitochondrial and nuclear genotypes.

Due to dramatic and repeated differences in fecundity between mitochondrial genotypes in line 6, additional experiments were designed to pinpoint a cause. Antibiotic treatment had no effect, indicating that bacterial pathogens were not to blame for lowered fecundity. Measurements of the hind tibia of female wasps indicated that body size was also not a factor. To determine whether there was a problem with the nuclear genotype in line 6A, this line was crossed with its non-introgressed and type B counterparts. If a nuclear flaw had been present, we expected that hybridizing with the same (undamaged) nuclear genotype would restore fecundity. However, this proved not to be the case. Hybrids resulting from crosses with a 6A mother maintained a much lower reproductive capacity compared with the reciprocal crosses (6A father and either a non-introgressed 6 or 6B mother), indicating that the trait was maternally inherited and not solely nuclear in nature. To see if line 6A's lowered fecundity was due to a damaged mitochondrial

genome, it was crossed with line 29A. This resulted in two groups of offspring, both with identical hybrid nuclear genomes and presumably identical type A mitochondrial genotypes. Both groups were tested for reproductive capability, and a significant difference lingered. Female wasps with a 6A mother still produced dramatically lower numbers of offspring than those with a 29A mother. This indicates that there was likely a defect in the 'A' mitochondrial genome in line 6, which may have occurred during the introgression process.

Within all nuclear genotypes, female wasps developed at significantly different rates between mitochondrial types. However, no mitochondrial type seemed to have a consistent effect; different mitochondrial types had different effects across nuclear genotypes. For example, both male and female wasps in line 6A developed faster than wasps from lines 6B or 6C, but this effect was reversed in females of nuclear genotype 9. Only two of the four nuclear genotypes also showed differences in males; in both cases, type A developed faster. There was a significant interaction between nuclear and mitochondrial genotype.

An examination of longevity showed no significant interactions between nuclear and mitochondrial genotypes, and overall less variability was seen in this trait than in others measured. Again, no single mitochondrial type had a consistent effect on longevity.

From these data, it is clear that mitochondrial and nuclear genotypes can and do interact to affect life-history traits in *Trichogramma pretiosum*. All traits tested (except longevity) showed an effect of mitochondrial type in at least one nuclear genotype, and some showed a significant interaction between the two. Because the original intent of

these introgressions was to use the mitochondrial sequence as a neutral marker, this is not an ideal situation. There is no predictable effect of mitochondrial genotype on any of the traits tested here.

It is possible that the observed interactions are due to mutation, and that the mitochondrial genomes are not actually identical between nuclear genotypes. Though our marker COI sequence was tested and confirmed to be unchanged and identifiable after introgressions were complete, there may be differences in other regions of each mitochondrial genome that contribute to fitness differences. This seems to be the case in line 6, and may be the cause of line 6A's low fecundity and relatively fast development time compared to lines 6B and 6C. Further crossing experiments in other lines or development of additional molecular markers would be necessary to confirm this. If mutation is the cause of the differences, the utility of the marker system may be salvageable by re-introgressing the lines.

As the introgressions stand, mitochondrial type cannot be assumed to be a neutral factor in performance. However, because there is no clear advantage for any one mitochondrial type across nuclear genotypes, this may not be a problem when using the marker system for some types of experiments. Experiments in the next chapter pooled nuclear types in each mitochondrial type, on the reasoning that mixing nuclear types may create a net-zero advantage for a mixed 'B' population versus an 'A' or 'C' population.

Table 2.1: Fragment length of microsatellite regions A9 and A124.

Nuclear genotype	A9 (bp)	A124 (bp)
1	267	228
2	310	172
3	298	210
6	245	245
9	320	222
12	224	209
14	305	209
22	290	172
23	226	180
26	299	172
27	246	164
28	300	180
29	225	180
33	217	180
35	246	209
37	290	180
38	263	210
39	269	211
40	299	136
42	230	161
43	264	173
46	225	225
47	276	178
51	246	184
52	300	172
53	265	180

Table 2.2: 10-day fecundity factorial ANOVA

Fecundity 4x3 ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Nuclear genotype	3	204188	58014	63.19	<0.001
Mitochondrial genotype	2	11139	5585	6.08	0.002
Nuclear/ Mitochondrial interaction	6	24640	4107	4.47	<0.001
Error	444	407609	918		

Table 2.3: Development time factorial ANOVA

Development Time 4x3x2 ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Nuclear genotype	3	130.33	33.06	71.72	<0.001
Mitochondrial genotype	2	9.96	1.97	4.29	0.014
Sex	1	30.46	40.88	88.71	<0.001
Nuclear/ Mitochondrial interaction	6	25.49	4.35	9.44	<0.001
Nuclear/Sex interaction	3	8.90	2.58	5.60	0.001
Mitochondrial/Sex interaction	2	5.04	2.52	5.47	0.004
Error	6517	3003.58	0.461		

Table 2.4: Longevity factorial ANOVA

Longevity 4x3x2x2 ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Nuclear genotype	3	114.1	38.0	5.88	0.001
Mitochondrial genotype	2	11.3	5.7	0.88	0.416
Sex	1	6432.5	6432.5	995.19	<0.001
Feeding status	1	8301.4	8301.4	1284.32	<0.001
Nuclear/ Mitochondrial interaction	6	101.5	16.9	2.62	0.016
Nuclear/Sex interaction	3	70.4	23.5	3.63	0.013
Nuclear/Feeding status interaction	3	195.3	65.1	10.07	<0.001
Sex/Feeding status interaction	1	6832.0	6832.0	1056.99	<0.001
Error	939	6069.3	6.5		

Table 2.5: 6 A/B/C post-Rifampicin treatment fecundity ANOVA

6 A/B/C Post-Antibiotic Fecundity ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Mitochondrial genotype	2	18685	9343	7.60	0.001
Error	57	70059	1229		

Table 2.6: 6/A/B backcross factorial ANOVA

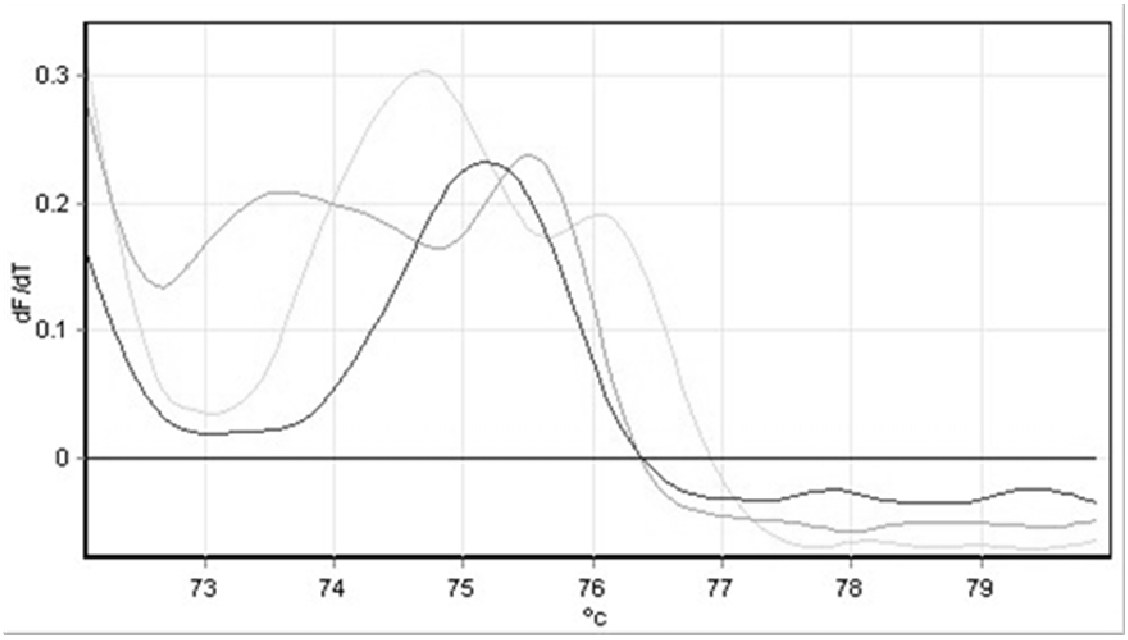
6/A/B Backcross 3x3 ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Maternal genotype	2	101427	48206	37.30	<0.001
Paternal genotype	2	1406	555	0.43	0.652
Maternal/paternal interaction	4	6809	1702	1.32	0.266
Error	147	189962	1292		

Table 2.7: 6Ax29A hybrid fecundity ANOVA

6A x 29A Hybrid 10-day Fecundity ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Maternal genotype	1	100489	100489	133.49	<0.001
Error	44	33123	753		

Table 2.8: Average female hind tibia length ANOVA

6A/B/C HTL ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Mitochondrial genotype	2	0.0003	0.0002	1.83	0.165
Error	147	0.0132	0.0001		



= Type A
= Type B
= Type C

Figure 2.1: High-resolution melt curves for mitochondrial types A, B and C. Each type was consistently identifiable by number and position of peaks. Height of peaks indicates relative fluorescence read by the RotorGene machine, while the temperature reading on the x axis indicates the temperature at the time that each reading was taken.

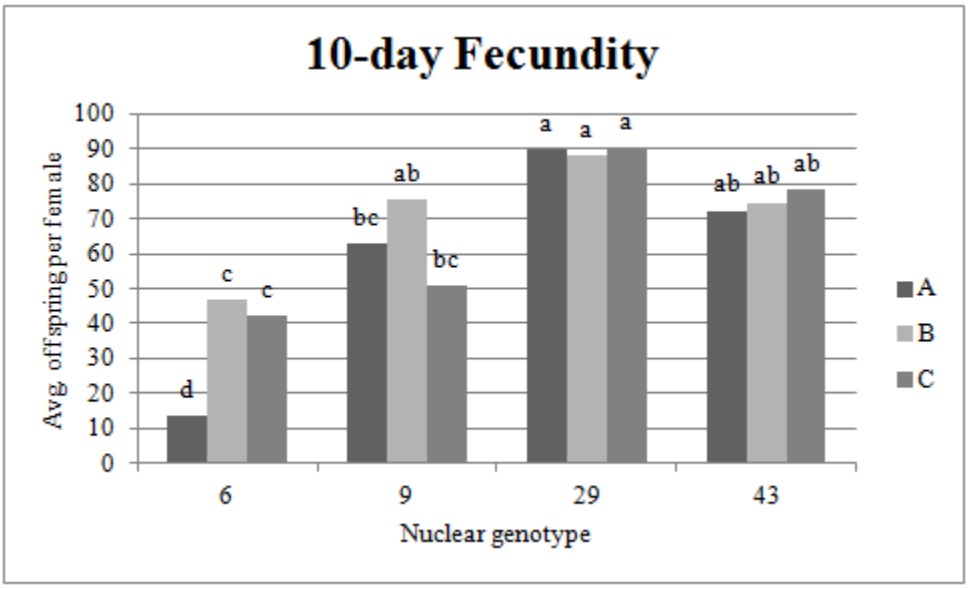


Figure 2.2: Average 10-day fecundity. Letters above each bar represent significance groups calculated via Tukey's LSD.

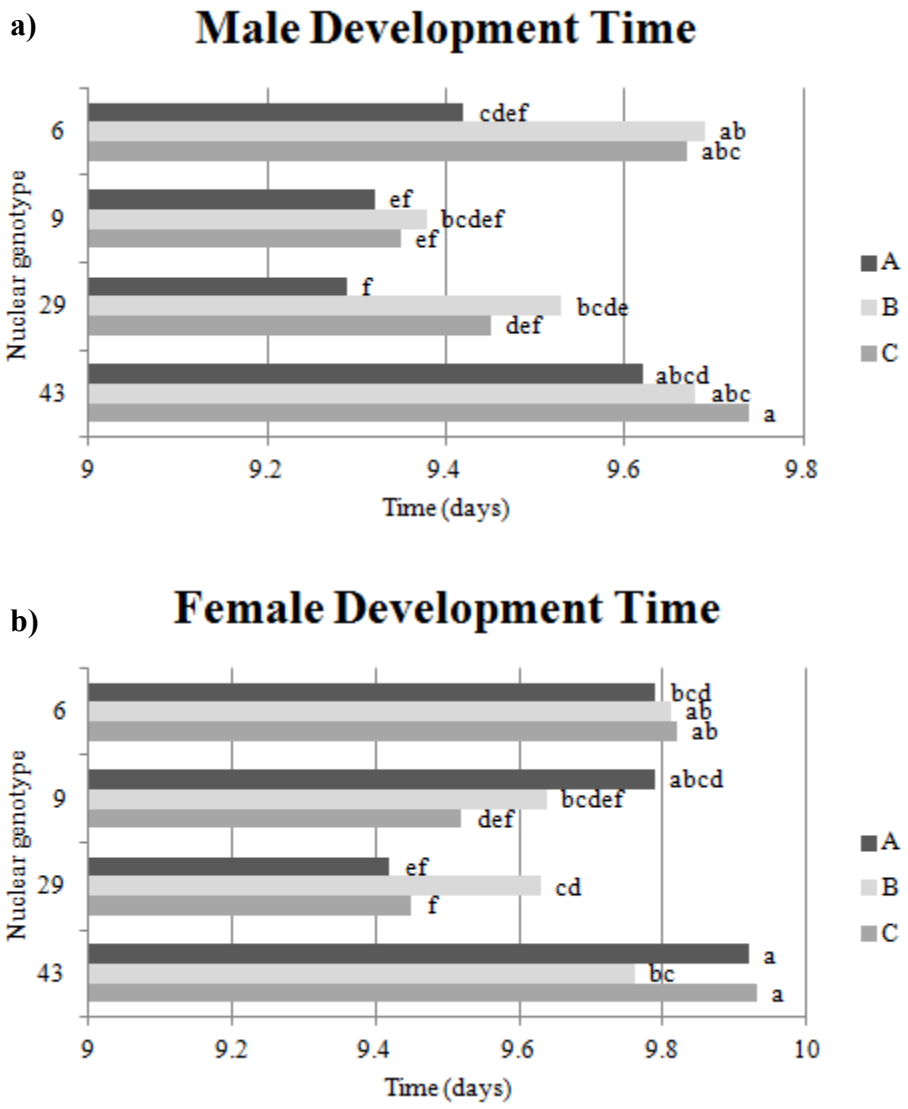


Figure 2.3: Average egg-to-adult development time. Graph (a) displays data for male wasps, while graph (b) displays female data. Letters indicate significance groups given by Tukey's LSD.

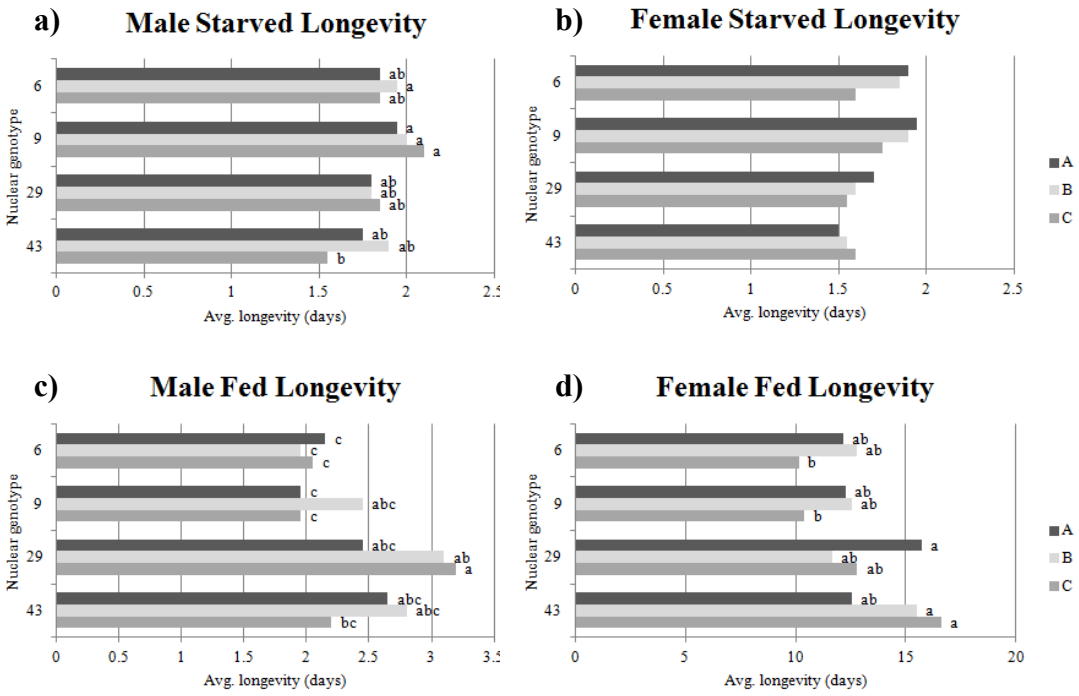


Figure 2.4: Average longevity. Data for male starved wasps are in graph (a), female starved wasps in graph (b), male fed wasps in graph (c), and female fed wasps in graph (d). Letters indicate significance groups given by Tukey's LSD.

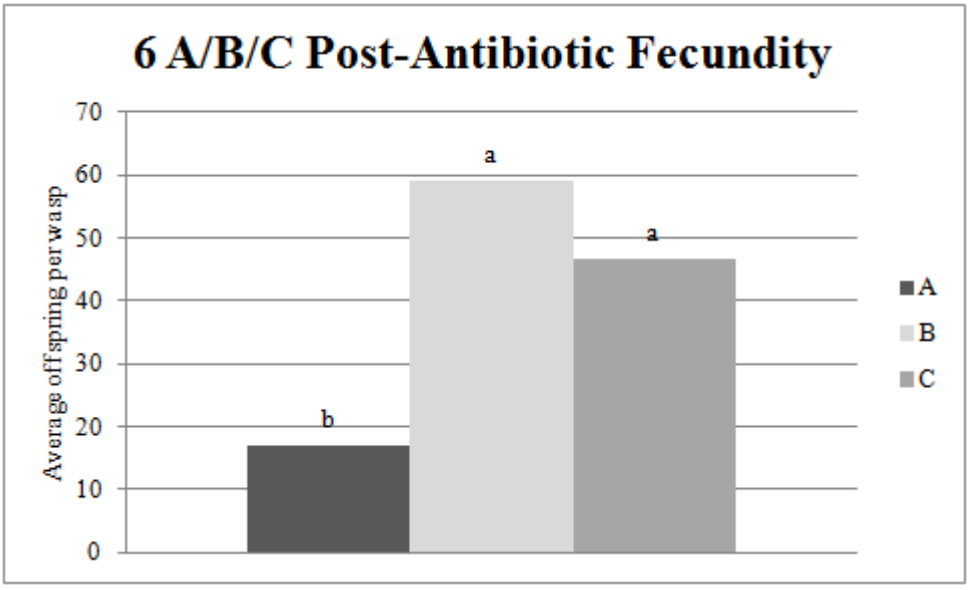


Figure 2.5: Relative fecundity of genotypes 6A, 6B and 6C after treatment with Rifampicin. Letters indicate significance groups determined by Tukey's LSD.

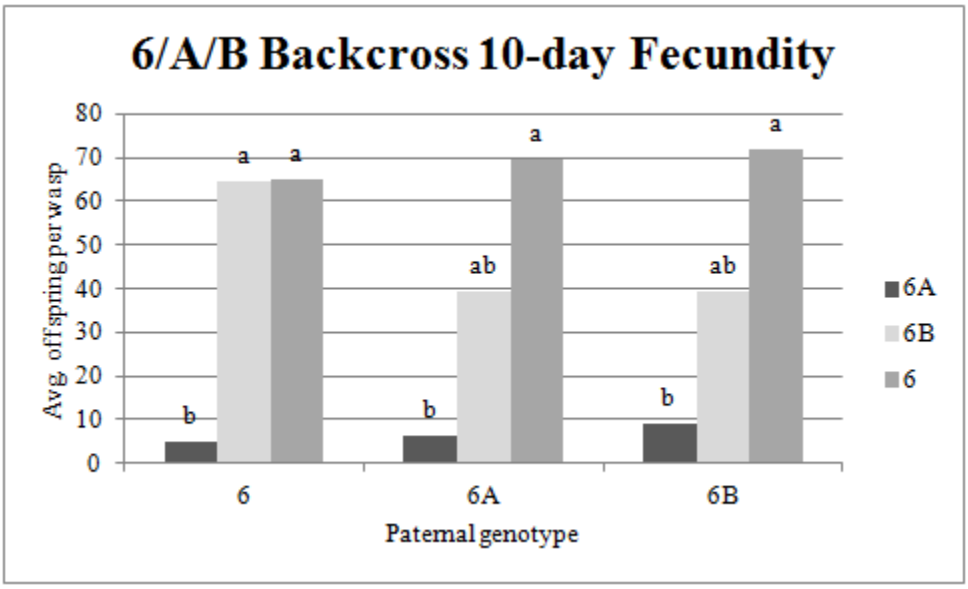


Figure 2.6: 6/A/B backcross fecundity by parental genotypes. The fecundity of female wasps tested is grouped by the genotype of her father along the horizontal axis, and bar color indicates the genotype of her mother. Letters indicate significance groups determined by Tukey's LSD.

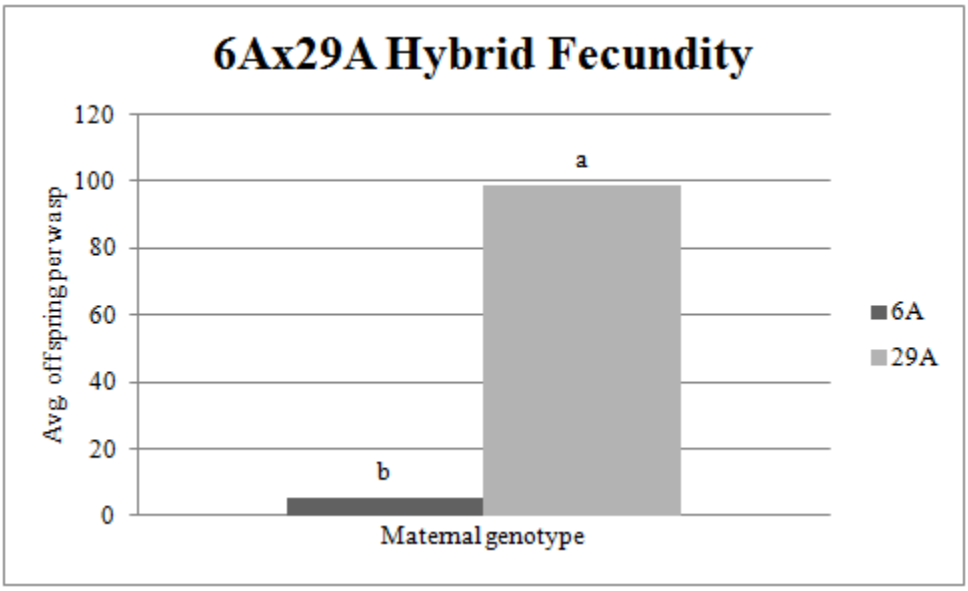


Figure 2.7: 6Ax29A hybrid fecundity. Average 10-day production of female offspring per hybrid wasp is shown. Bars indicate the genotype of the mother of each wasp; the father's genotype is the opposite. Letters indicate significance groups given by Tukey's LSD.

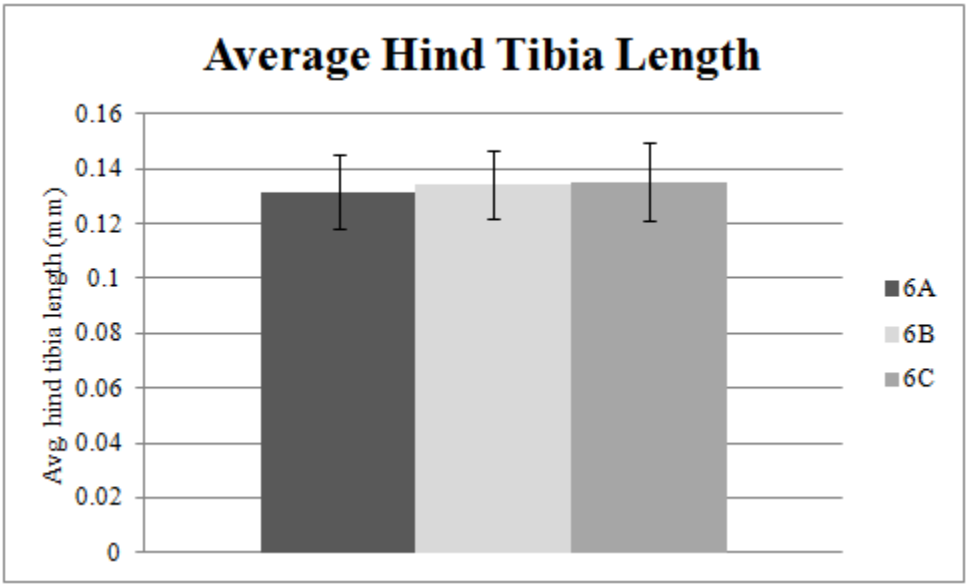


Figure 2.8: Average hind tibia length. Average length of hind tibiae from 50 adult female wasps from genotypes 6A, 6B and 6C are shown in millimeters. Error bars represent standard error of the mean.

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CHAPTER 3

Introduction: Testing Effect of Mass-Rearing Time on Field Performance

Mass-rearing of natural enemies encounters problems when insects are kept in artificial conditions for many generations. As discussed in the general introduction of this thesis, many studies have noted declines in multiple desirable traits over the course of laboratory rearing, including changes in host preference (Bergeijk et al. 1989, Pluke and Leibee 2006), adult wasp size, longevity (Mackauer 1976), temperature preference (Shchepetilnikova and Kasinskaya 1981), walking mobility, and flight ability (Bigler et al. 1988). Several processes are thought to be responsible for these declines, including basic genetic processes such as the founder effect and genetic drift, and selection for laboratory conditions.

In *Trichogramma pretiosum*, genetic bottlenecks and laboratory selection are of particular concern. It is generally considered desirable to start mass-rearing stocks with as much genetic diversity as possible, in order to represent and retain all of the genetic advantages present in a natural population (Boller 1972, Joslyn 1984, Knutson 1998, Nunney 2003, Bigler 1994). However, laboratory cultures show an unfortunate trend of population drop-offs in the first few generations of mass rearing (Boller 1972). This automatically eliminates some of the variation present, and leaves only genotypes which are able to adjust to laboratory conditions. These genotypes may not be the types best suited to field conditions upon release.

Generations of inbreeding continue to eliminate genetic diversity, and mass-reared populations continue to adapt to conditions that are not reflective of the field environment they are meant to be released in (crowding, no need for searching for hosts or mates, constant light/dark and temperature/humidity cycles, etc.). It is not really known how much of an effect this has on the performance of *Trichogramma* wasps once they are released back into the field.

Solutions to this problem have been posed, but many of them require intensive testing of many lines of *Trichogramma* prior to beginning mass rearing. Bigler (1994) suggests detailed studies on several traits to select the most desirable lines to begin a founding colony. In addition, he recommends additional testing on a yearly, seasonal, and even per-release basis to maintain high levels of quality control. These protocols involve a significant undertaking, particularly in the initial stages, and may not be feasible for smaller insectaries, which are common in the biological control industry (Parra 2010).

Several studies suggest maintaining mass-reared colonies for only a short amount of time – five or six generations at the most – before purging them and re-starting from greenhouse or wild stock (Bigler 1986, Knutson 1998, Smith 1996, Nunney 2003). This may prevent laboratory adaptation from becoming too egregious, but also requires facilities to maintain a greenhouse stock or constant access to wild populations. Seasonal changes in insect availability pose problems with this strategy.

Joslyn (1984) recommends adding insects from wild stock every few generations to increase genetic diversity of laboratory populations. However, this also becomes problematic during parts of the year when wild populations are not readily available.

There is also no guarantee that the added diversity would last very long, as these new genotypes would immediately be under the same selective conditions as the existing cultures.

Roush and Hopper (1995) and later Nunney (2003) have posed a solution which forms the basis of the experiments performed for this chapter. Both studies assert that maintaining colonies with a high level of diversity may be a hindrance rather than a help. Variation provides a substrate for selection to act upon; in this case, causing an entire population to lose the genotypes that are not competitive in the laboratory. In contrast, inbred populations lack this variation, and are unable to adapt to change. In the field, this lack of variation would be a disadvantage. However, in a mass-rearing facility, this may be the best way to preserve as many genotypes as possible.

Both studies cited above suggest keeping field-collected *Trichogramma* as single-family or isofemale lines, rather than mixing them all together during mass-rearing. In most animals, this would seem counter-productive; in diploid organisms, this would cause the expression of detrimental recessive alleles result in inbreeding depression that can make the maintenance of inbred lines problematic. However, being hymenopterans, *Trichogramma* are haplodiploid, and do not appear to suffer much from this problem (Sorati et al. 1996, Prezotti et al. 2004). Detrimental alleles are exposed to selection every generation in the male line, rather than being masked in heterozygotes and maintained. Maintenance of many smaller, inbred lines would mitigate loss of genetic diversity to selection. Hybridization immediately prior to release would the restore the original variation and perhaps add back fecundity due to hybrid vigor (Antolin 1999).

In order to test this method, I began with 26 isofemale lines of *T. pretiosum* that have been maintained under inbreeding for over 3 years (over 100 generations). I mixed these lines at three different time points prior to release; after 20 generations, 10 generations, and 2 generations of isofemale line rearing. I then released the populations simultaneously and monitored parasitism rates of each mixed line type over three days. I seek to demonstrate whether or not maintaining *T. pretiosum* as isofemale lines rather than mixed colonies prior to release has any impact on performance in the field upon release, and, if so, whether we can estimate at what generation after mixing we can expect a significant drop-off in performance.

Materials and Methods

Trichogramma cultures

For all field experiments, the 26 isofemale *Trichogramma pretiosum* cultures were the same as those generated for experiments in the previous chapter. Twenty generations prior to the start of planned release experiments, small pieces of egg cards bearing approximately equal numbers of irradiated *E. kuehniella* eggs were added to all 'C' cultures. After one week of parasitism, the two most highly parasitized pieces of egg card from each line were selected and removed from the culture tubes. All of the removed pieces were pooled into a large (18 x 150 mm) glass tube, which was plugged with cotton and incubated at 25°C, 16:8 L:D, and uncontrolled (~30%) humidity. Upon emergence, all wasps were allowed to mate randomly. A large egg card containing an excess of eggs and droplets of honey was placed in the tube. Once eggs were blackened (meaning wasps had pupated) and most adult wasps were dead (day 8 after emergence), the large egg card

was removed and placed in a clean tube. Thereafter, aside from being maintained in a larger tube, the culture was maintained in the same way as the previously described isofemale lines. This culture was designated C-2011, and was later split into three large tubes to allow more space for mass rearing. Four generations later, mixed cultures of A, B, and C were initiated in the same fashion. These were designated A Long, B Long, and C Long. Eleven generations after the 'Long' cultures, mixed cultures were initiated again and this time designated 'Mid.' Seven generations after the initiation of 'Mid' cultures, 'Short' cultures were generated, again in the same fashion. These mixtures at various time points resulted in 10 isolated populations, each initiated with identical levels of genetic diversity but allowed to interbreed for differing numbers of generations.

Field experiments

Two types of field experiments were conducted. The goal of the first was to determine whether or not the mitochondrial marker system established in the laboratory caused a difference in field performance between mixed populations of the same rearing time. The goal of the second set of experiments was to determine the impact of rearing time on field performance of *T. pretiosum*, using the marker system to differentiate between released populations. Release strategies were very similar, so for the sake of simplicity I will describe the general protocol and then the points on which the experiments differed.

Field Site and Plot Design, 2011

In the summer of 2011, experiments were conducted to determine whether or not mitochondrial type has an effect on field fitness. All field experiments were conducted at

the UC South Coast Research Station in Irvine, California. Four plots of untreated "Bobcat" tomatoes were made available courtesy of the laboratory of Dr. John Trumble (UC Riverside Entomology). Three of these plots were made up of three rows of tomatoes, forming plots 3.05 m wide by 12.19 m long. The fourth (designated "white") was made up of five rows of tomatoes, and measured 6.10 m wide by 12.19 m long. All plots were separated by at least 30' of tomatoes that were being used for the Trumble lab's pesticide trials (see figure 3.1).

Release protocols, 2011

Three release experiments were conducted, each experiment running over the course of five days (Monday – Friday). In each release plot, wasps of one nuclear genotype in all three mitochondrial genotypes were released. Lines 6, 9, 29, and 43 were chosen for releases, because these were the same lines that had been examined in laboratory studies.

On Monday of each release experiment, trap cards were placed in the field to monitor for parasitism by native insects. This first set of cards is hereafter referred to as "sentinel cards," and cards placed on later days simply as "trap cards." Sentinel cards and trap cards were constructed identically. Each card was made of a small strip of white cardstock bearing an identification number and a small amount of irradiated *E. kuehniella* eggs, which was then taped to a metal curler clip. These cards were then clipped to the underside of tomato leaves (fig. 3.2a). 20 cards per row were deployed, with 10 cards on each side of the row, evenly spaced down the length of the row (fig. 3.1). The next day (Tuesday), these cards were collected and any insects found on the cards were removed

before sealing them in plastic bags. A new batch of cards was placed in each plot in the same density and arrangement as the sentinel cards. Upon returning to the lab, sentinel cards were removed from the clips and placed into individual 12x75mm glass tubes, plugged with cotton wool, and stored at 25°C, 16:8 L:D, and ~30% RH. These were checked daily for egg blackening. If no blackening had occurred after 5 days in the laboratory, tubes were discarded.

On Tuesday mornings, immediately following trap card replacement, release vials were placed amidst the tomato plants in the center of each plot at approximately 9am. For the first release (7/26/2011), releases were made from 40 dram plastic vials. Wooden skewers were cut to roughly 20 cm long, and three skewers were hot-glued to the outside of each vial to act as stilts, preventing the vials from sitting directly on the ground. Parasitized egg cards were cut into sections approx. 5 cm long, bearing roughly equal numbers of pupae that had been timed to emerge on the day of release. Pupae to be released were added to each vial and the sides of the vial were streaked with honey. The tops of the vials were then covered with small nylon mesh, in an attempt to exclude ants and other predators (fig. 3.2). The two subsequent releases this season (8/23/2011 and 9/2/2011) used the same plastic vials with a smaller mesh, in an attempt to optimize wasp release and predator exclusion (12 holes per cm = 144 holes per cm^2). Release vials were covered with Parafilm™ M (Pechiney Plastic Packaging Co.) to prevent any emerged wasps from escaping before they were placed in the field. A portion of each card was retained in the laboratory to estimate sex ratio. These retained portions were allowed to

emerge in the laboratory and then frozen at -20°C on Tuesday evening, and later counted and sexed.

Three vials of wasps were placed in the center of each plot. For each experiment conducted in 2011, we tested relative performance of three mitochondrial types within several introgressed nuclear genotypes. Each plot was identified by a color (red, white, yellow, or blue), and in each plot, one nuclear type was released (6, 9, 29, or 43 – see fig. 3.1 and table 3.1). Each of three vials contained wasps of the designated nuclear type bearing one of the three different mitochondrial variants (A, B, and C). Upon placement in the field, the Parafilm™ was removed to allow wasps to escape.

On Wednesday, release vials were picked up, re-sealed, and put on ice to discourage further wasp emergence until they could be returned to the laboratory. Trap cards were replaced with a fresh set in the same arrangement, Tuesday's cards were checked for insects and returned to the lab, and these were treated in the same fashion as the sentinel cards. After returning to the lab, release vials were frozen at -20°C overnight and later examined under a dissecting microscope to estimate emergence.

On Thursday, trap cards were again replaced and treated as previously described. On the final day of the experiment (Friday), trap cards were collected and not replaced. These were treated in the same fashion as all previous cards.

All trap cards were allowed 14 days to fully emerge, at which time all glass tubes were frozen at -20°C for at least 24 hours. Wasps were eventually moved to 70% ethanol, and stored at room temperature before being tested for mitochondrial type.

Field Site and Plot Design, 2012

In the summer of 2012, several release experiments were conducted. Some of these were designed to test for an effect of mitochondrial type on field fitness, as in 2011. Others tested rearing times against one another, and yet others tested the fitness of single isofemale lines against mixed lines.

Experiments were again conducted at the UC South Coast Research Station. Wasps were again released in field tomatoes, this time of variety "Ace 55". Ten rows of untreated tomatoes were used measuring 73.15 m down the length of the rows and 12.8 m across all 10 rows. The field was split into 22 equal plots measuring 6.4x6.4 m, and various combinations of these plots were assigned for releases or buffer zones, depending on the experiment. The field was surrounded on three sides by dirt roadway (at least 3 m across), and on the fourth side by additional tomatoes of the same variety being used for a pesticide trial (fig. 3.2). Due to problems with ant predation in the previous season, ant bait was placed in the field 7 days before the first release. Ant bait was a solution of 25% sucrose containing 0.003% thiamethoxam. Bait was dispensed in approximately 10 mL volumes into 42 15 mL conical Falcon tubes, which were evenly spaced at 4.57 m intervals down the 3rd, 6th, and 9th rows. Tubes were left open in the field for 7 days, then capped and removed.

Release protocols, 2012

Release setups differed from 2011 experiments in a few aspects. Plots were grouped into either two blocks of 6 or 7 plots or three blocks of five plots, depending on the experiment (see figures 3.3, 3.4, 3.5). For experiments testing fitness of mitochondrial

types, two blocks of 7 plots at either end of the field were used. For experiments testing mass rearing times against one another, three blocks of 5 were used; one at either end, and one halfway between the two. A gap of at least one plot wide was left between blocks as a buffer zone.

All sentinel and trap cards were constructed using green cardstock, to simulate a leaf surface better than the white cards used the previous year. Only five sentinel cards were deployed in each plot prior to release. This choice was made largely due to the much higher number of plots being used and the time and effort involved in making the sentinel cards. The five sentinels were placed in the centers of the five rows in each plot.

Trap cards were placed at a density of 10 per row for a total of 50 cards per plot per day, rather than 60 cards per plot per day as in 2011 experiments. This decision was made partially due to the altered field design; plots all contained 5 rows but rows were only about half as long as the previous year. The decision was also made for time, effort, and cost considerations. Cards were spaced evenly along each row, approximately 0.6 m apart. This distance varied slightly if gaps in the foliage were present. Trap cards were also placed in buffer zones where no wasps were released, to detect potential movement between blocks.

Releases were made from 50 mL conical plastic tubes (Falcon) with screw-caps. The centers of the screw-caps were drilled out, and a round piece of nylon mesh was inserted underneath the cap when the tubes were closed. The outsides of the tubes were covered with duct tape to promote phototaxis towards the top of the tube, and encourage wasps to leave the release vials. Three tubes per plot were placed as in 2011, and these

were set inside of 473.2 mL glass mason jars to keep them upright. Double-sided Scotch™ tape was adhered to the outside of the jar, in two rows running around the circumference, in order to deter walking predators from climbing up the jar. The release vials were inserted into holes in a green index card, which provided a platform for wasps to disperse from rather than falling into the jar through spaces between the vials. Once assembled, jars were placed on the ground in the center of the center row of each plot (see fig. 3.7).

Just as in 2011, parasitized egg cards were placed in each release tube and timed to emerge the day of release. Kimwipes™ fixed over the tubes with rubber bands prevented emerging wasps from leaving the tubes until after they were placed in the field. All releases were made between 8am and 9am on Tuesdays. Deployment of sentinel and trap cards occurred in the same sequence as the previous year's experiments, and cards and release vials were treated the same way upon return to the laboratory. Sex ratio and emergence were also estimated in the same way as in 2011.

Molecular analysis of recovered field samples

For all experiments conducted in 2011, Chelex DNA extraction (see methods in the previous chapter) was performed on up to 10 individual wasps per trap card. If fewer than 10 wasps had emerged from a particular card, all were used. Between field seasons, Dr. Paul Rugman-Jones (Stouthamer laboratory) discovered that the extraction step was not necessary, and so for 2012 samples, whole wasps were added to real-time PCR tubes rather than using samples of extracted DNA. As in the previous season, up to 10 wasps per card were tested for mitochondrial type in 2012.

Real-time PCR was conducted on each individual wasp or DNA sample, and a high-resolution melt was used to assign a mitochondrial type to each sample. Reaction conditions were the same as described in Chapter 2 of this thesis, with one exception. Due to a PCR contamination problem that arose between field seasons, all 2012 reactions were run with 1 unit of UDG (New England Biolabs) to eliminate any PCR product present in the reaction tube prior to amplification. The addition of this enzyme necessitated a 10-minute incubation at 37°C immediately prior to amplification.

For 2011 field experiments, because isofemale lines were not mixed prior to release, a small sub-sample of recovered wasps were checked to confirm that their nuclear genotype was the same as that released in the plot they were recovered in. This was done to confirm that wasps were not able to travel between plots over the course of each experiment. Nuclear genotypes were checked for 2 wasps per parasitized card recovered, using the microsatellite PCR protocol described in Chapter 2. For 2012, lines were mixed in nearly all experiments, eliminating the possibility of using these markers to check for travel between plots.

Statistical Analysis

Each trap card parasitized in the field had the opportunity to be attacked by more than one female wasp. The number of females finding each card could not be directly determined, because we could not distinguish between an event in which one female of one mitochondrial type found a trap card and parasitized multiple eggs, and an event in which multiple females of the same type found parasitized the same card. Therefore, rather than calculating a Chi-square value using a standard “observed” term, data from

field experiments were analyzed using a modified Chi-square test devised with the help of Dr. Leonard Nunney. The modified "observed" term was found by subtracting the number of cards in each plot that were found by a particular mitochondrial type from the total number of cards deployed in that plot. We then calculated the expected number of trap cards not found by each particular type, based on the numbers of female wasps of each type released and assuming that all mitochondrial types had the same chance (p) of not finding a trap card. We assumed that the number of wasps finding each card approximated a Poisson distribution, and proceeded to calculate the expected zero class (cards not found by each type) for each plot and block.

Example:

In a particular block, 420 female wasps of mitochondrial type A were released. 250 cards were placed in this block (50 cards/plot for a block of 5 plots). The expected zero class is:

$$\text{Exp}(0)=e^{-420p} \times 250$$

Recalling, p is the probability of a single parasitism event. If 480 females of type B and 540 of type C were released in the same block, we calculate for those types:

$$\text{Exp}(0)=e^{-480p} \times 250$$

$$\text{Exp}(0)=e^{-540p} \times 250$$

To find the expected fraction of cards not parasitized by any type, we must multiply these terms:

$$e^{-420p} \times e^{-480p} \times e^{-540p} = e^{-(420+480+540)p}$$

If 173 of 250 cards placed in this block were actually not parasitized by anything, then we can use this information to solve for p:

$e^{-1440p} = 173/250$

$-1440p = \ln(173/250)$

$p = (\ln(173/250))/-1440 = 2.56x10^{-4}$

Now we have an estimate for p, using the null hypothesis that the probability of any one wasp finding and parasitizing a trap card is equal regardless of mitochondrial type. We can put this value back into the original equations for each type, to calculate an expected zero class (Exp[0]), and therefore use this "expected" term along with our "observed" term to run a Chi-square test.

Chi-square values and p-values were calculated using Microsoft Excel.

An additional analysis was conducted on field data from both experiments done in 2012. The square root of each value obtained by calculating "(observed-expected)^2/expected" was taken, and assigned a positive or negative sign depending on whether the observed value was greater (+) or less than (-) the expected value. These transformed values were then used to do a factorial ANOVA to examine the overall effect of either marker type (7/17/12 experiment) or rearing time (7/31/12 experiment), as well as any potential effect of day, block, or interactions between the variables analyzed. After ANOVA, a Tukey's LSD was conducted to examine significance groups where appropriate.

Results

Field Experiments 2011

Two of the three experiments done the summer of 2011 suffered problems severe enough to preclude meaningful data analysis. In the first experiment, Argentine ants

invaded release vials and carried off nearly all of the *T. pretiosum* pupae. The experiment was continued, and some trap cards showed parasitism; however, the ant predation meant that no estimate of release numbers could be made, and therefore no meaningful statistics could be done. *T. pretiosum* pupae placed in the third experiment largely failed to emerge on the scheduled day of release. This was likely due to a synchronization error; the pupae had been held at 15° C for three days prior to release. The chilling period was meant to delay emergence until the scheduled start of the experiment, but was likely done too far in advance and slowed the wasps' development too much. Parasitism in this trial was minimal, and data were not analyzed.

The second experiment, done in the week of 8/23/2011, yielded results sufficient to analyze. No native parasitism was detected on sentinel cards. Modified Chi-square tests were run in two ways; first with all data points included, and again with data points immediately adjacent to the release point removed. Results and p-values are listed in tables 3.1 and 3.2. No significant differences were found between observed and expected parasitism rates in any plot on any day, regardless of whether or not center points were included in the analysis.

Field Experiments 2012

Seven release experiments were run over the course of the 2012 field season. Due to time and budget constraints, only two of the seven could be analyzed for this thesis. Diagrams of the two experiments that were analyzed are shown in figures 3.4 and 3.5, while the remaining five are described in table 3.3.

For the first analyzed experiment (release date 7/17/12), A, B, and C mixed colonies were tested against each other within rearing times. No native parasitism was detected on sentinel cards. Wasps released in the plots of block 1 were all from the 'Mid' rearing time, which at the time of release had been mixed for 10 generations. Wasps released in the 7 plots of block 2 were all from the 'Long' rearing time, which had been mixed for 21 generations. Data were analyzed using the modified Chi-square test and subsequent ANOVA as previously described. After the Chi-square, no significant differences between expected and observed parasitism rates were seen in any plot in either block on the first day after release. On the second day, one plot in each block showed significant differences. In the 'Mid' block, wasps of type C parasitized a greater number of cards than expected in plot 1. In the 'Long' block, all three types found higher numbers of cards than expected in plot 8. However, on the third day after release, these differences disappeared, and no plots in either block showed significant differences between mitochondrial types. As in 2011, the modified Chi-square test was run again with points immediately adjacent to the release vials omitted. After this change, no plot showed any significant difference on any day. Results and p-values are shown in tables 3.4 and 3.5.

After conducting a factorial ANOVA on transformed Chi-square data, a significant effect of both mitochondrial type and day were seen, regardless of whether center points were included in the analysis (see ANOVA results in tables 3.6 and 3.7). The effect of day was not unexpected, as different numbers of cards were parasitized each day. The effect of mitochondrial type was not expected, given the results of the

previous summer's experiment. No significant interactions were seen between mitochondrial type and day or between block and day, though a significant interaction between mitochondrial type and block was found. A Tukey's LSD indicated that although type C parasitized significantly higher numbers of cards than type A or B in block 1 (mid rearing time), type C did not find significantly higher or lower numbers of cards than A or B in block 2 (long rearing time) (data not shown).

The second analyzed experiment was conducted the week of 7/31/2012. Releases were done in 3 blocks of 5 plots each, as illustrated in figure 3.4. Each block tested the relative performance of long, mid and short rearing times, and mitochondrial markers were rotated between blocks to account for any fitness effects caused by introgressions. No pre-existing *T. pretiosum* population was detected via sentinel cards. After Chi-square analysis, no significant differences in parasitism rates were seen in blocks 1 or 2, in any plot, or on any day. Significantly different parasitism rates were seen in a few plots in block 3, where wasps were released from colonies A Short, B Long and C Mid. On day 1, wasps from the A Short colonies parasitized more cards than predicted by a Chi-square test in plots 9, 19, and 20. This effect persisted in plot 9 over the next two days, though it did not reappear in plots 19 and 20. When center points were removed and modified Chi-square tests were repeated, most of these significant results disappeared; however, wasps from the A Short population still performed significantly better than expected in plot 19 on the first day. Results of both modified Chi-square analyses and the associated p-values are listed in tables 3.8 (with center points) and 3.9 (center points removed).

Factorial ANOVA analysis revealed a significant effect of mass rearing time and of day, as well as a significant interaction of block and day (table 3.10). Overall, the short rearing time found significantly higher numbers of cards than either the mid or long groups, which were not significantly different from each other according to Tukey's LSD significance groupings. Groupings also showed that in block 3, significantly higher parasitism rates were found on day 3 than day 2, though no other block/day combinations were significantly different from one another (data not shown). When center points were removed from the analysis, significant effects of rearing time, block, and day were found. Additionally, a highly significant interaction between type and block was found (table 3.11). Tukey's LSD significance groups showed that day 1 had significantly higher overall parasitism than day 3, with day 2 being not significantly different from either. Block 3 had higher overall parasitism than either blocks 1 or 2. Parasitism by wasps from the Short rearing time remained significantly higher than either the Mid or Long populations. Tukey's LSD groupings for the interaction between block and type show that the Short rearing time parasitized a greater proportion of cards than the Mid or Long in only block 3, while no significant differences were seen in any other block (data not shown). This supports the data found by the Chi-square test alone.

Discussion

Field experiments designed to test for fitness differences in A, B and C mitochondrial types failed to detect a significant advantage for any strain in isofemale releases (2011). This is in contrast to results reported in the previous chapter, wherein laboratory experiments showed that significantly different results occurred with wasps

with different mitochondrial types in the same nuclear genotype across three different life history traits. Though statistically significant, it may be that the effects seen in the laboratory are not dramatic enough to cause differences in performance in single isofemale lines.

Some significant differences in field performance were seen between mitochondrial types in mixed lines (2012). A higher rate of parasitism by type C in one plot on one day was sufficient to show a significant effect in an ANOVA. This appeared in the Mid (10 generation) rearing time, but not in the block where Long (21 generation) populations were released. This may indicate an advantage for the C type, though the effect disappears over time. Future analysis of the additional experiment testing this effect in 2012 may either support or contradict this finding. Nonetheless, to account for this possible fitness effect, marker types were rotated in field experiments testing rearing time.

Tests of fitness between rearing times also yielded largely non-significant results, although three plots in one block did show a significant pattern. In all three, wasps bearing the 'A' mitochondrial type parasitized significantly higher numbers of cards than their 'B' or 'C' counterparts. In this block, the wasps with the 'A' marker had only been maintained as a mixed culture for 4 generations, as opposed to 11 generations for the 'C' population and 22 generations for the 'B' population. Unlike the previous experiment, no advantage was seen in the C mitochondrial type, regardless of the rearing time that it was associated with. This pattern suggests that a shorter mass-rearing time may in fact prevent the lowered field performance associated with genetic deterioration in *T. pretiosum*.

The absence of any apparent effect of rearing time in the 12 other plots used in the same experiment is a bit confusing, but travel of wasps between blocks may offer a partial explanation. Trap cards were placed between blocks to monitor for just such movement. Parasitism was found on some of these cards in the experiment testing rearing times against one another. One of ten cards was found between blocks 1 and 2 on the day of release, and four of the ten cards placed between blocks 2 and 3 were parasitized on the third day. This shows that *T. pretiosum* was able to disperse outside the bounds of the plots the wasps were released in within a relatively short amount of time. Based on the data collected, it is not possible to say which direction wasps were moving in, or exactly how many wasps were moving between blocks. A large amount of movement could have washed out significant results, especially in plots towards the center of the field. However, this likely does not affect the remaining significant results. The plots where significant results were found were located towards the end of the field, where they were less likely to be impacted by wasps traveling in from other blocks. It is possible that had blocks been spaced further apart, other plots would also have shown populations from the shortest rearing times out-performing populations that had been mixed for longer times.

No significant differences in field performances were seen between populations that had been mixed for 11 or 22 generations. This suggests that by the 11^{th} generation, any laboratory adaptation happening in the mixed cultures had become fixed, and became no worse after an additional 11 generations. A difference was still apparent in the population that had been mixed for 4 generations, indicating that some loss of fitness occurred between generations 4 and 11. It is likely that a population which had been

mixed for even fewer generations could have shown an even more dramatic field advantage.

Although additional work is needed to confirm the pattern seen in this field study, data reported here suggest that insectaries may be able to maintain the quality of their colonies for a longer amount of time by maintaining many isofemale lines, and mixing them only immediately prior to release. All of the populations used in this study were maintained as isofemale lines in a laboratory for over 3 years prior to release, which is far longer than recommended by either Bigler (1986) or Knutson (1998). Successful adoption of this strategy would eliminate the need to purge and re-start colonies due to genetic deterioration.

The work presented here also supports Bigler's recommendation to replace mixed colonies after a maximum of six generations (Bigler 1986). Though work in the current study did not show to what extent colonies might degrade over the first four generations after mixing, it did indicate a significant drop-off in field performance between generations 4 and 11, which is in keeping with Bigler's findings.

In time, the additional experiments done over the summer of 2012 may yield interesting data. These experiments included two further tests of short vs. medium vs. long mass-rearing times, as well as two experiments testing relative performance of mixed cultures against releases of single isofemale lines. The isofemale approach to mass rearing shows promise so far, and may yet prove to be a highly effective way to preserve quality and genetic diversity in mass-reared parasitoids.

Table 3.1: Chi-square results from field experiment 8/23/2011. Calculated probabilities (described under statistical analysis) used for Chi-square for the Yellow plot were 5.11×10^{-4}, 7.23×10^{-4}, and 3.57×10^{-4} for days 1, 2, and 3. For the Blue plot, values were 9.23×10^{-5}, 1.32×10^{-4}, and 7.32×10^{-5}. For the Red plot, values were 6.11×10^{-4}, 4.14×10^{-4}, and 1.46×10^{-4}. For the White plot, values were 1.02×10^{-4}, 1.02×10^{-4}, and 2.75×10^{-5}.

Field Results 2011 (8/23/2011)															
Plot (Line)	Females released			Cards found (A/B/C)			Empty cards			Chi-square			Significance		
	A	B	C	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3
Yellow (43)	375	90	280	15/2/15	20/5/9	7/4/5	41	35	46	1.37	1.95	0.45	0.51	0.38	0.80
Blue (9)	45	104	794	0/0/5	0/0/7	9/8/10	55	53	56	0.02	0.14	0.15	0.99	0.93	0.93
Red (6)	316	365	298	3/14/21	4/4/14	1/1/6	33	40	52	3.66	2.51	4.67	0.16	0.28	0.10
White (29)	490	720	272	6/6/2	6/7/1	1/3/1	86	86	96	0.03	0.04	0.37	0.98	0.98	0.83

Table 3.2: Chi-square results from field experiment 8/23/2011, no center points. Calculated probabilities (described under statistical analysis) used for Chi-square for the Yellow plot were $4.57x10^{-4}$, $1.07x10^{-4}$, and $3.52x10^{-4}$ for days 1, 2, and 3. For the Blue plot, values were $6.30x10^{-5}$, $4.16x10^{-5}$, and $6.30x10^{-5}$. For the Red plot, values were $6.32x10^{-4}$, $4.04x10^{-4}$, and $1.48x10^{-4}$. For the White plot, values were $1.03x10^{-4}$, $8.59x10^{-5}$, and $3.00x10^{-5}$.

Field Results 2011 (8/23/2011) – center points excluded															
Plot (Line)	Females released			Cards found (A/B/C)			Empty cards			Chi-square			Significance		
	A	B	C	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3
Yellow (43)	375	90	280	11/1/13	17/2/7	6/4/4	37	31	40	1.20	1.79	0.29	0.55	0.41	0.86
Blue (9)	45	104	794	0/0/3	0/0/2	1/0/2	49	50	49	0.01	0.01	0.02	0.99	0.99	0.99
Red (6)	316	365	298	2/12/18	2/4/13	1/1/5	28	35	46	3.24	2.77	4.31	0.20	0.25	0.12
White (29)	490	720	272	1/6/2	4/6/1	1/3/1	79	81	88	0.15	0.03	0.32	0.93	0.98	0.85

Table 3.3: Additional experiments conducted over the summer of 2012

2012 Experiments Not Analyzed			
Release date	Field layout	Block	Genotypes released
7/24/12	Same as Fig. 3.3	1	A/B/C Short
		2	A/B/C Long
8/7/12	Same as Fig. 3.4	1	A Long/ B Short/C Mid
		2	A Mid/ B Long/C Short
		3	A Short/B Mid/ C Long
8/21/12	Same as Fig. 3.4	1	43A/35B/C Long
		2	1A/47B/C Long
		3	29A/28B/C Long
8/28/12	See Fig. 3.5	1	A Long/ B Short/C-2011
		2	A Short/ B Long/C-2011
9/11/12	Same as Fig. 3.4	1	51A/B Long/33C
		2	9A/B Long/12C
		3	38A/B Long/14C

Table 3.4: Chi-square results from field experiment 7/17/2012. Calculated probabilities (described under statistical analysis) used for Chi-square for Block 1 were $1.58x10^{-5}$, $1.83x10^{-4}$, and $1.41x10^{-4}$ for days 1, 2, and 3. For Block 2, values were $1.12x10^{-5}$, $1.43x10^{-4}$, and $9.30x10^{-5}$.

Field Results 2012 (7/17/2012)																	
Block	Plot	Females released			Cards found (A/B/C)			Empty cards			Chi-square			Significance			
		A Mid	B Mid	C Mid	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
1	1	38	161	61	0/0/2	10/3/6	6/4/9	48	34	35	0.08	7.25	2.62	0.96	0.03	0.27	
	2	83	129	114	1/1/1	5/7/12	3/1/8	48	33	40	0.02	4.28	1.44	0.99	0.12	0.49	
	3	18	116	75	0/1/0	2/8/8	3/9/9	49	34	32	0.02	2.59	3.37	0.99	0.27	0.19	
	4	94	9	108	0/0/2	6/5/10	3/5/5	48	33	38	0.07	3.17	1.15	0.96	0.20	0.56	
	12	30	42	23	1/0/0	4/2/7	8/2/3	49	38	40	0.02	1.37	1.53	0.99	0.50	0.47	
	13	10	206	88	0/0/0	0/2/4	0/1/5	50	45	44	<0.01	0.38	0.50	0.99	0.83	0.78	
	14	189	164	82	0/1/2	4/4/13	1/2/5	47	34	31	0.09	3.45	0.57	0.96	0.18	0.75	
		A Long	B Long	C Long	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
2	7	175	83	70	0/1/0	6/5/4	5/5/5	49	40	37	0.02	1.50	1.47	0.99	0.47	0.48	
	8	157	141	115	1/1/1	11/11/12	5/10/3	47	25	36	0.05	7.63	2.63	0.97	0.02	0.27	
	9	181	159	86	0/0/0	9/2/5	1/0/5	50	36	43	<0.01	2.15	0.51	0.99	0.34	0.78	
	10	140	108	74	2/2/1	7/4/6	7/2/4	46	34	35	0.17	1.98	1.35	0.92	0.37	0.51	
	17	36	94	41	0/0/0	3/6/8	0/0/0	50	33	50	<0.01	2.16	<0.01	~1	0.34	~1	
	18	105	82	101	0/0/0	7/5/6	7/2/4	50	35	38	<0.01	2.16	1.35	0.99	0.34	0.51	
	19	145	141	90	0/0/1	0/0/2	5/0/1	49	48	43	0.02	0.08	0.50	0.99	0.96	0.78	

Table 3.5: Chi-square results from field experiment 7/17/2012 – no center points. Calculated probabilities (described under statistical analysis) used for Chi-square for Block 1 were 1.61×10^{-5}, 1.73×10^{-4}, and 1.29×10^{-4} for days 1, 2, and 3. For Block 2, values were 1.13×10^{-5}, 1.33×10^{-4}, and 8.63×10^{-5}.

Field Results 2012 (7/17/2012) – center points excluded																	
Block	Plot	Females released			Cards found (A/B/C)			Empty cards			Chi-square			Significance			
		A Mid	B Mid	C Mid	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
1	1	38	161	61	0/0/2	8/2/6	5/3/8	42	30	31	0.08	7.25	2.62	0.96	0.03	0.27	
	2	83	129	114	0/1/0	4/7/11	2/1/7	43	28	35	0.02	4.28	1.44	0.99	0.12	0.49	
	3	18	116	75	0/0/0	2/8/6	2/6/6	44	30	31	0.02	2.59	3.37	0.99	0.27	0.19	
	4	94	9	108	0/0/2	4/4/9	2/4/3	42	30	36	0.07	3.17	1.15	0.96	0.20	0.56	
	12	30	42	23	1/0/0	3/1/5	6/2/3	43	36	35	0.02	1.37	1.53	0.99	0.50	0.47	
	13	10	206	88	0/0/0	0/1/2	0/1/5	44	41	38	<0.01	0.38	0.50	0.99	0.83	0.78	
	14	189	164	82	0/1/2	4/4/11	1/2/4	41	29	37	0.09	3.45	0.57	0.96	0.18	0.75	
		A Long	B Long	C Long	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
2	7	175	83	70	0/1/0	6/4/3	4/5/3	43	35	35	0.02	1.50	1.47	0.99	0.47	0.48	
	8	157	141	115	1/1/1	8/9/8	4/8/2	41	23	33	0.05	7.63	2.63	0.97	0.02	0.27	
	9	181	159	86	0/0/0	7/1/5	1/0/4	44	33	38	<0.01	2.15	0.51	0.99	0.34	0.78	
	10	140	108	74	2/2/1	6/3/5	6/2/4	40	31	30	0.17	1.98	1.35	0.92	0.37	0.51	
	17	36	94	41	0/0/0	3/4/6	0/0/0	44	30	44	<0.01	2.16	<0.01	~1	0.34	~1	
	18	105	82	101	0/0/0	6/3/4	7/1/4	44	32	33	<0.01	2.16	1.35	0.99	0.34	0.51	
	19	145	141	90	0/0/1	0/0/2	3/0/1	43	42	39	0.02	0.08	0.50	0.99	0.96	0.78	

Table 3.6: ANOVA results from field experiment 7/17/2012.

7/17/12 Chi-square to ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Mitochondrial type	2	1.60	0.80	6.09	0.0031
Block	1	0.44	0.44	3.37	0.0692
Day	2	11.24	5.62	42.69	<0.0001
Type x Block	2	1.18	0.59	4.48	0.0136
Type x Day	4	0.90	0.22	1.72	0.151
Block x Day	2	0.23	0.11	0.86	0.4261
Error	106	14.74	0.13		

Table 3.7: ANOVA results from field experiment 7/17/2012 – no center points.

7/17/12 Chi-square to ANOVA (centers excluded)					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Mitochondrial type	2	0.99	0.49	4.86	0.0096
Block	1	0.06	0.06	0.61	0.4365
Day	2	6.60	3.30	32.55	<0.0001
Type x Block	2	0.70	0.35	3.47	0.0347
Type x Day	4	0.43	0.11	1.06	0.3801
Block x Day	2	0.05	0.02	0.23	0.7949
Error	106	11.35	0.10		

Table 3.8: Chi-square results from field experiment 7/31/2012. Calculated probabilities (described under statistical analysis) used for Chi-square for Block 1 were $2.56x10^{-4}$, $2.53x10^{-4}$, and $1.90x10^{-4}$ for days 1, 2, and 3. For Block 2, values were $2.75x10^{-4}$, $2.14x10^{-4}$, and $1.80x10^{-4}$. For Block 3, values were $4.47x10^{-4}$, $2.54x10^{-4}$, and $3.40x10^{-4}$.

Field Results 2012 (7/31/2012)																	
Block	Plot	Females released			Cards found (A/B/C)			Empty cards			Chi-square			Significance			
		A Long	B Mid	C Short	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
1	1	71	95	129	5/7/9	3/4/5	2/3/3	30	34	42	2.59	0.64	0.64	0.23	0.72	0.72	
	2	91	150	124	10/1/6	6/4/9	6/4/5	35	30	35	3.57	2.93	2.60	0.17	0.23	0.27	
	11	109	85	67	2/4/9	2/6/7	4/4/4	35	34	38	2.43	2.95	2.95	0.30	0.23	0.23	
	12	81	56	126	5/2/4	2/3/5	4/1/5	38	40	40	0.88	0.36	0.36	0.64	0.83	0.83	
	13	65	92	93	7/4/4	8/5/5	5/5/6	35	30	33	1.51	2.88	2.88	0.47	0.24	0.24	
		A Mid	B Short	C Long	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
2	4	47	176	87	1/9/4	4/9/4	4/4/2	36	34	41	1.38	2.10	0.30	0.50	0.35	0.86	
	5	95	70	133	1/4/8	3/2/7	3/3/9	37	32	35	1.27	0.71	1.32	0.53	0.70	0.52	
	6	63	127	130	3/10/7	5/6/6	3/5/3	29	32	39	3.22	1.54	0.60	0.20	0.46	0.74	
	15	95	81	79	4/6/8	5/5/2	6/4/4	33	38	37	1.58	1.45	0.94	0.45	0.48	0.63	
	16	71	78	170	7/2/10	4/5/6	1/4/8	30	38	37	3.05	1.12	1.05	0.22	0.57	0.59	
		A Short	B Long	C Mid	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
3	9	175	56	64	18/4/3	18/9/4	22/7/2	24	18	22	6.43	6.59	12.38	0.04	0.04	<0.01	
	10	143	93	115	8/8/10	13/6/7	13/1/3	26	25	34	5.26	4.48	2.15	0.07	0.11	0.34	
	18	119	78	71	10/2/3	6/9/5	11/2/2	35	30	35	2.48	2.57	1.61	0.29	0.28	0.45	
	19	159	98	48	22/4/2	15/4/1	17/2/2	23	23	31	10.29	3.11	2.15	0.01	0.21	0.34	
	20	71	66	64	15/3/10	11/2/7	8/4/6	23	30	32	7.92	4.83	2.49	0.02	0.09	0.29	

Table 3.9: Chi-square results from field experiment 7/31/2012 – no center points. Calculated probabilities (described under statistical analysis) used for Chi-square for Block 1 were 3.94×10^{-4}, 2.62×10^{-4}, and 1.80×10^{-4} for days 1, 2, and 3. For Block 2, values were 2.54×10^{-4}, 2.28×10^{-4}, and 1.63×10^{-4}. For Block 3, values were 3.91×10^{-4}, 4.37×10^{-4}, and 3.07×10^{-4}.

Field Results 2012 (7/31/2012) – center points excluded																	
Block	Plot	Females released			Cards found (A/B/C)			Empty cards			Chi-square			Significance			
		A Long	B Mid	C Short	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
1	1	71	95	129	5/7/6	3/3/5	2/3/2	28	29	37	1.36	0.31	0.06	0.51	0.86	0.97	
	2	91	150	124	8/2/7	5/3/6	4/3/5	34	28	32	1.56	0.65	0.35	0.46	0.72	0.84	
	11	109	85	67	2/5/8	2/5/5	2/4/3	31	31	36	1.39	0.64	0.23	0.50	0.72	0.89	
	12	81	56	126	6/2/2	2/2/5	4/1/3	34	35	36	0.53	0.23	0.18	0.77	0.89	0.91	
	13	65	92	93	6/4/3	7/4/4	4/4/5	32	28	30	0.74	1.08	0.61	0.69	0.58	0.74	
		A Mid	B Short	C Long	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
2	4	47	176	87	1/8/4	4/7/4	3/2/2	32	30	38	1.09	1.10	0.16	0.58	0.58	0.92	
	5	95	70	133	1/4/7	3/2/6	3/3/6	33	29	32	0.96	0.74	0.69	0.62	0.74	0.71	
	6	63	127	130	4/8/6	4/6/5	3/3/3	31	28	35	1.76	1.04	0.24	0.41	0.59	0.89	
	15	95	81	79	3/7/7	4/4/1	5/4/3	30	35	32	1.82	0.42	0.69	0.40	0.81	0.71	
	16	71	78	170	5/3/8	3/4/5	1/1/7	28	34	35	1.41	0.57	0.63	0.49	0.75	0.73	
		A Short	B Long	C Mid	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day1	Day 2	Day 3	
3	9	175	56	64	17/3/3	14/7/4	17/6/2	22	24	22	5.01	4.03	5.44	0.08	0.13	0.07	
	10	143	93	115	10/7/10	11/5/6	12/0/2	25	29	31	3.63	2.45	2.27	0.16	0.29	0.32	
	18	119	78	71	7/3/4	5/5/5	7/2/2	34	29	33	0.84	0.86	0.62	0.66	0.65	0.73	
	19	159	98	48	18/4/5	14/3/1	14/2/1	23	21	27	6.22	3.15	3.11	0.04	0.21	0.21	
	20	71	66	64	15/3/9	10/1/7	8/3/4	22	27	29	1.36	0.31	0.06	0.51	0.86	0.97	

Table 3.10: ANOVA results from field experiment 7/31/2012

7/31/12 Chi-square to ANOVA					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Mass rearing time	2	2.67	1.33	4.53	0.0129
Block	2	1.31	0.66	2.23	0.1125
Day	2	3.82	1.91	6.50	0.0022
Rearing time x Block	4	2.07	0.52	1.76	0.1423
Rearing time x Day	4	0.24	0.06	0.20	0.9379
Block x Day	4	8.72	2.18	7.41	<0.0001
Error	107	34.14	0.29		

Table 3.11: ANOVA results from field experiment 7/31/2012 – no center points

7/31/12 Chi-square to ANOVA (centers excluded)					
Source of variation	**DF**	**SS**	**MS**	**F**	**Significance**
Mass rearing time	2	5.37	2.68	23.96	<0.0001
Block	2	4.08	2.04	18.21	<0.0001
Day	2	1.08	0.54	4.83	0.0098
Rearing time x Block	4	7.87	1.98	17.57	<0.0001
Rearing time x Day	4	0.07	0.02	0.16	0.9580
Block x Day	4	0.21	0.05	0.48	0.7503
Error	107	12.99	0.11		

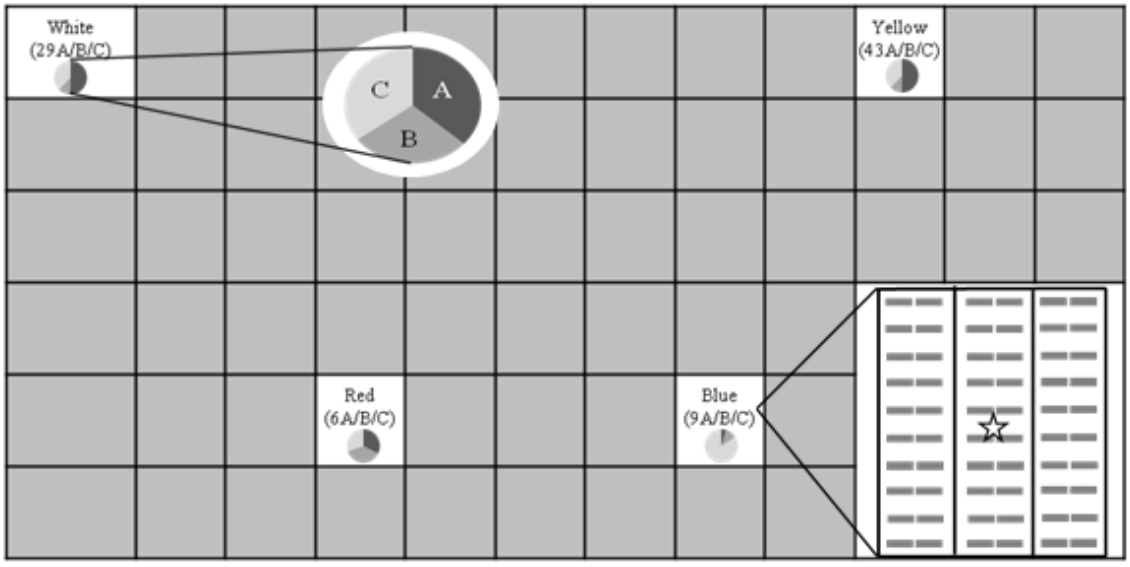

Figure 3.1: Field layout 2011. Shaded boxes indicate plots of tomatoes not used for releases, and text in white boxes indicates which lines were released in each plot. Circle diagrams in each white box indicate the proportion of each mitochondrial type released in each plot. The field was surrounded on all sides by a dirt road.

Figure 3.2: Wasp release strategy 2011. White cards bearing host eggs were clipped to undersides of tomato leaves (a and b). Release vials containing *T. pretiosum* pupae were placed into the soil on wooden skewers to elevate them into the leaves.

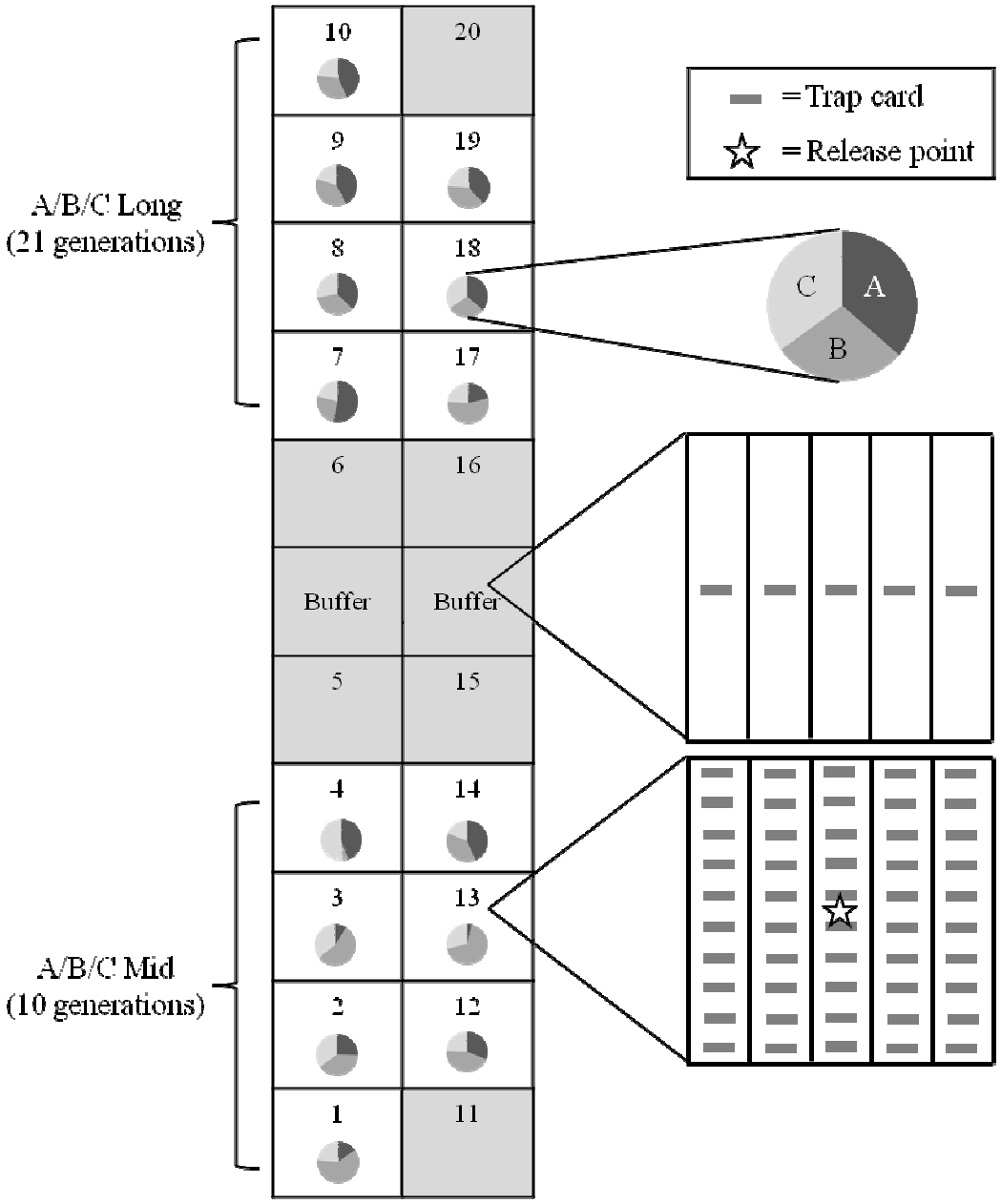

Figure 3.3: Field layout 7/17/2012. Shaded boxes indicate plots of tomatoes not used for releases. Circle diagrams in each white box indicate the proportion of each mitochondrial type released in each plot. The field was surrounded on 3 sides by a dirt road. To the right of the field were additional tomatoes which were part of a pesticide trial.

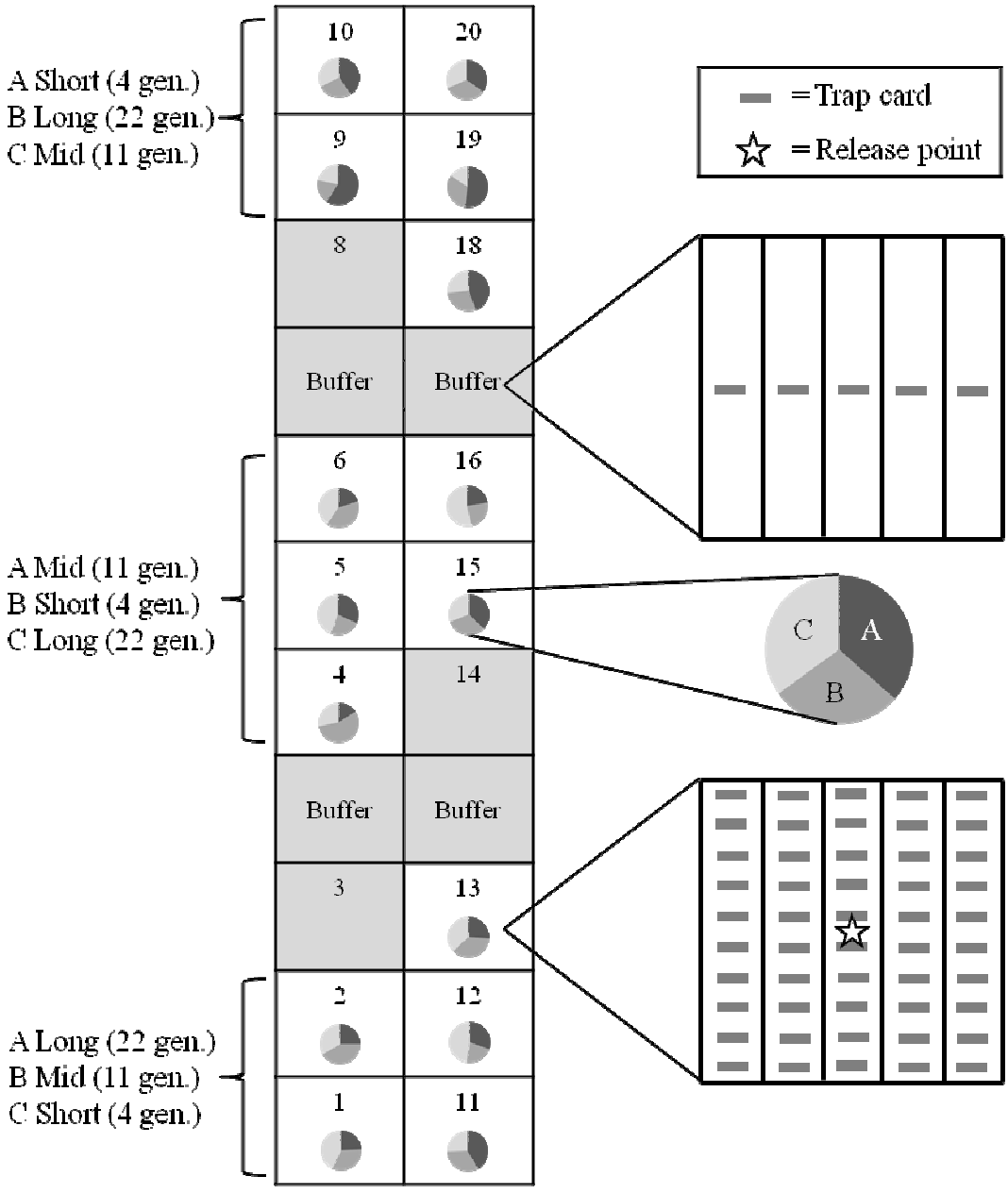

Figure 3.4: Field layout 7/31/2012. Shaded boxes indicate plots of tomatoes not used for releases. Circle diagrams in each white box indicate the proportion of each mitochondrial type released in each plot. The field was surrounded on 3 sides by a dirt road. To the right of the field were additional tomatoes which were part of a pesticide trial.

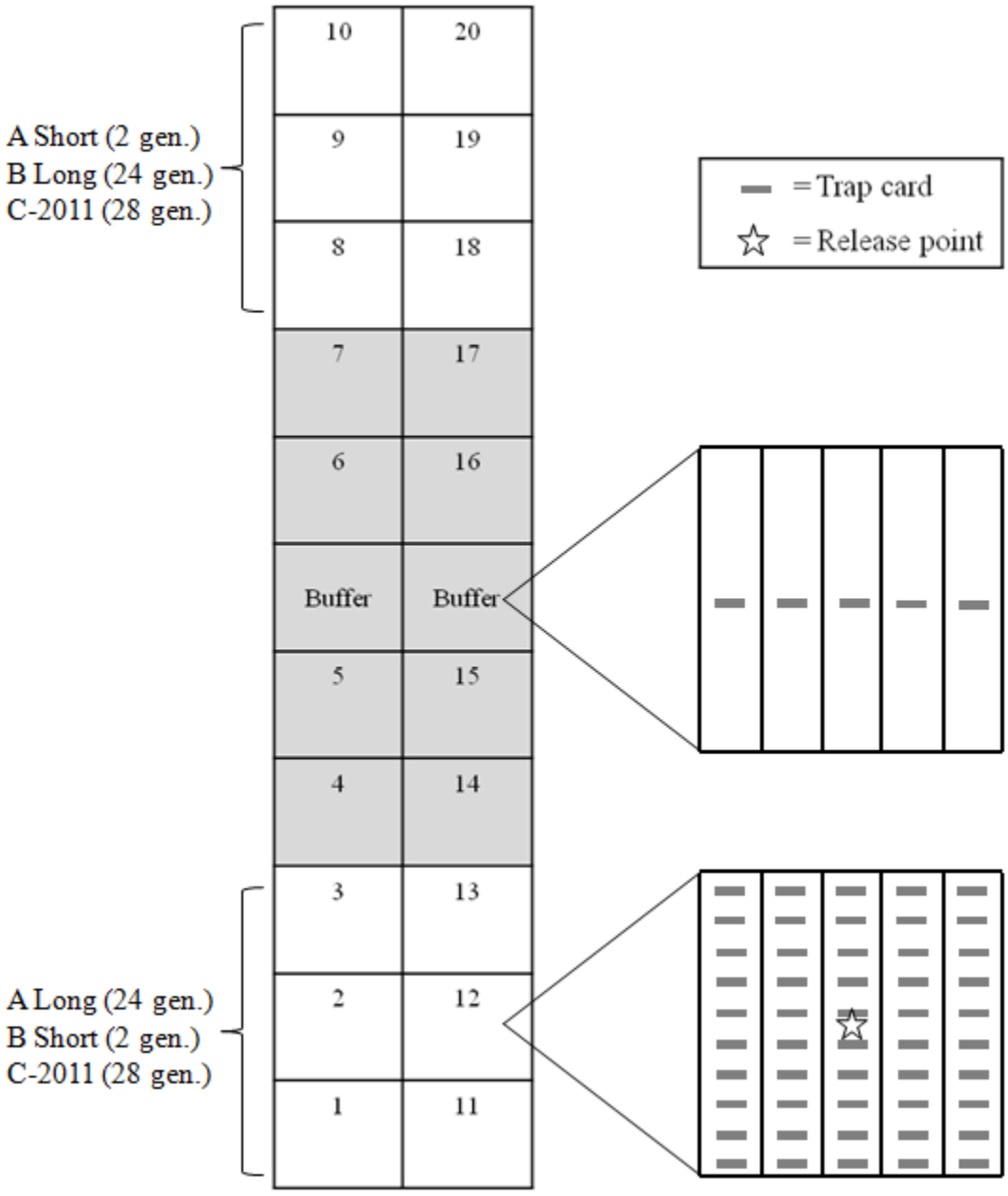

Figure 3.5: Field layout 8/28/2012. Shaded boxes indicate plots of tomatoes not used for releases. The field was surrounded on 3 sides by a dirt road. To the right of the field were additional tomatoes which were part of a pesticide trial. Due to time constraints, data from this experiment could not be analyzed for this thesis.

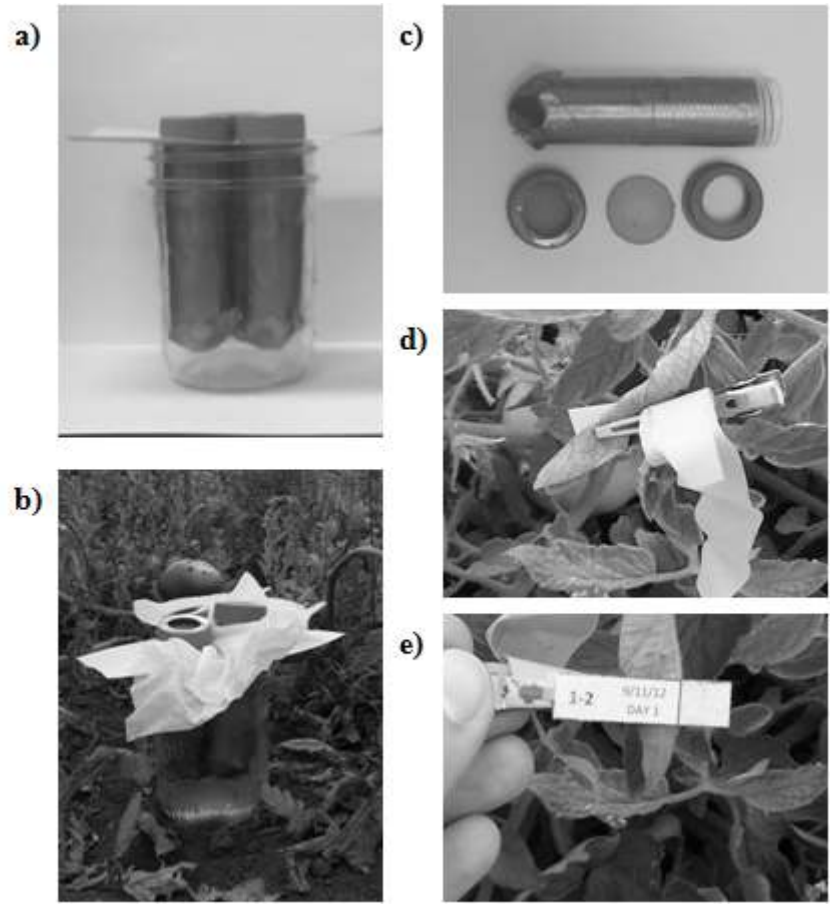

Figure 3.6: Wasp release strategy 2012. Green cards bearing host eggs were clipped to the undersides of tomato leaves (d and e). *T. pretiosum* pupae were placed into conical tubes with mesh lid inserts (c), which were covered with duct tape to encourage phototaxis. Tubes were set into glass jars, which were placed within the tomato plants (a and b).

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CHAPTER 4

Conclusions

Mass rearing of biological control agents is plagued by potential problems. In addition to the physical and environmental challenges of producing insects, genetic problems can arise and potentially decrease the field performance of captive-reared populations. As discussed in the introduction of this thesis, one of the main problems recognized by experts is laboratory adaptation. This adaptation is made possible whenever populations containing a high level of genetic diversity are reared under artificial conditions and inbreeding. Over time, the genotypes best adapted to laboratory settings are selected for, and those with less laboratory fitness are lost. This can select for insects with a high reproductive capacity, but selection on traits associated with searching ability or general mobility is relaxed. This may lead to populations that, though highly fertile, are not as able to disperse and find hosts as the wild populations they were originally initiated from.

In this thesis, I tested the hypothesis that maintaining genetic diversity in isofemale lines of *Trichogramma pretiosum* would result in better preservation of field fitness than allowing genetic diversity to be lost through inbreeding and laboratory adaptation in mixed cultures. I tested this by mixing genetically marked isofemale lines of wasps at various time points before releasing them in the field, and determined their relative field performance based on the relative numbers of egg cards (containing host eggs) that each mixed type found. If my hypothesis was correct, the populations that had

been mixed for the shortest amount of time would perform the best, because they would have had the least opportunity to adapt to the laboratory and lose genetic variation.

After finding some initial problems with the assumption of neutrality in my genetic marker system, I decided to rotate each of the markers between mixed populations. This way, I could test all combinations and account for any inherent advantage of a particular marker type. Though most release plots did not show any significant advantage of any particular rearing time, the few significantly different plots did indeed show an advantage for populations associated with the shortest rearing time (in this case 4 generations, as opposed to 11 or 22).

Work remains to be done in this area. I conducted several additional field experiments that could not be analyzed due to time constraints, and it may be that once these are analyzed, my hypothesis will have even greater support.

Biological control is an important industry, especially considering the growing interest in organic and pesticide-free food crops. Therefore, it is critical to ensure that natural enemies being produced for commercial use are not only plentiful, but of the highest possible quality. The work presented in this thesis suggests that one way to increase quality in mass-reared parasitoids may be to maintain field-collected populations as inbred, single-family lines until just before release, rather than maintaining large mixed colonies for many generations.