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Quality Control and Captive Rearing Genetics of the Biological Control Agent *Trichogramma pretiosum*

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Jaime Gonzalez-Cabrera

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Dissertation Committee:
Dr. Richard Stouthamer, Chairperson
Dr. Joseph Morse
Dr. Subir Ghosh

The Dissertation of Jaime Gonzalez-Cabrera is approved:

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

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Jaime Gonzalez-Cabrera

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Dr. Richard Stouthamer, Chairperson

In these studies, we determined the quality of *Trichogramma* parasitoids reared in several Mexican insectaries, evaluated the clonal variability found in *Trichogramma pretiosum* populations where the infection has gone to fixation, and determined the potential for genetic changes taking place during mass rearing of *Trichogramma* spp. The results showed that there is substantial potential for adaptation to mass rearing conditions when mass reared populations are initiated with genetically variable populations. In addition, we determined that inbreeding depression is possible even in species such as *Trichogramma pretiosum* where their sex determination and normal sibmating mating system would predict a minimal effect of inbreeding. With respect to the quality of mass reared *Trichogramma*, we found discrepancy between the reported and the reared specie, presence of unnoticed species replacement, and we found that the reared species barely met the minimum standards suggested by the international organization for biological control/ European community. From the PI-*Wolbachia* infected population, we found a surprisingly large amount of variability among the

different clonal lines when life history measurements were made. In this dissertation, we also outlined several guidelines for insectaries regarding how to maintain high "field quality" of the natural enemies they produce. Finally, our findings of the high diversity of clones in entirely asexual populations may result in a more rational future use of asexual wasps in biological control.

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General introduction

Trichogramma wasps are tiny egg parasitoids, less than 1 mm long and they have been used successfully as biological control agents since 1920s (Hinds and Spencer 1928, Flanders 1929, Hinds and Spencer 1929). *Trichogramma* parasitoids are mass reared and released worldwide (Hassan 1993, Li 1994) in field crops, orchards, greenhouses and production forests against a wide range of butterfly and moth pests (Smith 1994). The rate and frequency of release of *Trichogramma* parasitoids varies depending upon the crop, the pest, and density of the pest (Guyot 1977, Smith 1996), and can vary from 67, 000, to 2,113,000 females per hectare (Oatman and Platner 1978, Andow et al. 1995, Suh et al. 2000). The frequency of release can be weekly or as few as two releases per season (Guyot 1977, Andow et al. 1995).

Mass reared insects, such as *Trichogramma* parasitoids, are produced by the millions in large rearings either as the only pest control tool or in support of IPM projects (Webb 1984, Postali 2010). Mass rearings can suffer from several problems that may affect their field performance including: physical problems (overcrowding, high humidity, negative air pressure, etc.), pathogens (several kinds of fungi or bacteria), and genetic problems, such as genetic drift, lab adaptation or inbreeding depression (Cohen 2004, Schneider 2009). In mass reared colonies, the negative effect of genetic drift is countered by a large population size, but these large colonies can suffer genetic deterioration caused by lab adaptation and inbreeding depression (Woodworth et al.

2002). Due to the large size of mass reared colonies and if genetic variability is present, the relatively stable insectary conditions will favor the selection of phenotypes adapted to the local insectary conditions (Hoekstra 2003, Nunney 2003); also mass reared colonies being closed populations, due to constant mating among relatives, can suffer inbreeding depression.

Adaptation to captive breeding conditions and inbreeding depression are considered insidious genetic problems because they can go unnoticed, they can lead to the demise of the mass rearing cultures, and if insects suffering these genetic problems are field released, they will show poor field performance. Adapted insects have a high risk of extinction because they adapt to the benign insectary conditions but this can be at the expense of losing alleles that may be important under other conditions (Mackauer 1976), for example, alleles that contribute to fighting bacterial infections. Also, inbred colonies have reduced fitness (Hedrick 1983, Wang et al. 1999, Charlesworth and Willis 2009) because their lack of genetic variability impedes the regulation of their metabolic functions (Oldroyd and Fewell 2007, Kellermann et al. 2009). The dying out of insectary colonies is not a rare event (Nunney 2003); sometimes these failures can be attributed to specific factors, such as failure of cooling systems or the presence of pathogens, but sometimes there is no clear explanation (Cook 1993). Probably some of these inexplicable failures could have been caused by unnoticed genetic problems.

The main concern in biocontrol using mass reared insects is their field performance, and genetic problems can affect field efficacy (Woodworth et al. 2002, Nunney 2003). In insects used as biological control agents, relatively few studies are available on their field efficacy (Van Lenteren and Bueno 2003). Most studies showing that mass reared insects had deteriorated with respect to field performance come from insects reared for sterile insect technique applications. In *Trichogramma* parasitoids, there is some indirect evidence that points towards low field performance being caused by poor quality of the mass reared wasps. First, visible signs of poor quality of the released insects, for example the presence of deformed bodies (Keller and Lewis 1985, Orr and Suh 2000, Suh et al. 2000); and second, there are several reports in which the released insects failed to control the target pest (King et al. 1985, Suh et al. 2000, Lundgren and Heimpel 2002, Ulrichs and Mewis 2004, Vejar-Cota et al. 2005). The low field performance of *Trichogramma* parasitoids can be attributed to external causes such as suboptimal environment conditions (Ashley et al. 1973), residual effect or drifting of insecticides (King et al. 1985, King et al. 1986), lack of food sources in the field, dispersal away from the release site (Kuske et al. 2003) or poor quality of the reared *Trichogramma* (Kazmer and Luck 1991b). Therefore, to determine if poor field performance is caused by poor quality of mass reared *Trichogramma*, it is necessary to implement quality control tests of the reared *Trichogramma* before they are released.

Under lab conditions, we can test the quality of the reared *Trichogramma*, monitoring important traits that have been directly correlated with field performance, such as sex ratio, body size and embryonic mortality, and also we can test for the presence of lab adaptation and inbreeding depression. In the worst scenario, if a problem with the reared insects is detected, several remedial practices can be implemented to restore the colony's vigor, for example, by introducing wild adults to rejuvenate the colony (Van Lenteren and Bigler 2010), exchanging material with other insectaries (Frankham 2008), fluctuating the environmental conditions under which the parasitoids are reared (Guyot 1977, Bigler 1986), keeping colonies under conditions similar to those encountered in the field (Woodworth et al. 2002), limiting the number of generations of laboratory propagation (Guyot 1977) or promoting intra specific competition (Bigler 1994).

Quality control of *Trichogramma* parasitoids can be useful for farmers, pest advisors or insectary personnel for implementing managerial decisions (Bigler 1994, Liu and Smith 2000, Van Lenteren et al. 2003). These quality control tests should be done as frequently as possible, probably every month (Laing and Bigler 1991, Garcia-Gonzalez et al. 2005). Monitoring the quality of mass-reared insects is a requirement to achieve consistent pest control (Van Lenteren 1991, Bigler 1994, Grenier and De Clercq 2003), and as consequence, earn product recognition and customer loyalty. The appearance and disappearance of bioproducers has been a constant trend during the last 40 years

(Van Lenteren and Bigler 2010), which could indicate the loss of customer loyalty due to poor quality of the product on occasion.

In the field, asexual forms of *Trichogramma* parasitoids are known (Stouthamer and Luck 1993, Stouthamer and Kazmer 1994, Stouthamer et al. 2001), and in many cases, the asexuality in these wasps is caused by infection with a bacterial symbiont called *Wolbachia* (Rousset et al. 1992, Stouthamer et al. 1993, Stouthamer 1997). Because these *Wolbachia*-infected populations can reproduce as unmated females and they produce mainly female offspring, it is expected that the *Wolbachia* infected populations will have a higher rate of population increase, and that over time, they will outcompete the sexual populations. Strangely, in the field, *Wolbachia*-infected populations have not displaced sexual populations in all cases, but infected individuals coexist with uninfected conspecifics. The infection frequency among females in these populations is often low (<11%). In *Trichogramma* species, several populations are known where the infection has gone to fixation (Vargas and Cabello 1985, Stouthamer et al. 1990b, Pintureau et al. 2002, Russell and Stouthamer 2011). Understanding the changes that have occurred in these *Wolbachia*-infected fixed populations may have implications for their potential use as biological control agents. Parthenogenetic lines (*Wolbachia*-infected) are considered better insectary lines than sexual lines because they have a higher reproductive rate, they are easier to rear and entire colonies can be initiated with just one female (Slobodchikoff and Howell 1971 , Silva et al. 2000).

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Chapter 1. Inbreeding depression and heterosis in selected fitness traits of the egg parasitoid *Trichogramma pretiosum*

1.1 Abstract. *Trichogramma* egg parasitoids (Hymenoptera, *Trichogramma*tidae) should suffer little or no inbreeding depression because of their haplo-diploid sex determination system. However, there is some evidence that inbreeding depression may occur in *Trichogramma.* There are several reports in which field released *Trichogramma* parasitoids failed to control the target pest, and the presence of inbreeding depression in *Trichogramma* colonies could have contributed to these field failures. We investigated whether lab colonies of *T. pretiosum* suffer inbreeding depression. To detect inbreeding depression, we inbred six lines over nine generations using sib-mating; then, we crossed all of the lines in a 6x6 full diallel design. We measured the total fecundity and sex ratio of females that were the offspring of the diallel crosses. We compared the performance of all outbred females versus all inbred females, the performance within each dam line (all the crosses in each diallel row) of inbred females versus outbred females, and the heterosis of all outbred females. We found evidence of inbreeding depression with respect to total fecundity, i.e. outbred females showed significant heterosis (16.2% ± 2.1). Inbreeding status, however, did not significantly affect offspring sex ratios.

1.2. Introduction

The use of insects as biological control agents of agricultural pests has gained popularity over the years, largely because traditional methods of chemical pest control have shown several drawbacks, such as insecticide resistance, adverse effects on human health, contamination of ground water, etc. (Carson 1962). Hunter (1997) listed 144 species of beneficial insects reared in insectaries; the list of insects covers a great spectrum of insects such as predators and various herbivores used in biological control of weeds. Additionally, some pestiferous species are mass reared and field released in sterile insect programs to disrupt reproduction of the same species, for example the medfly (*Ceratitis capitata*) (Briceno and Eberhard 1998) or the cotton boll weevil (*Anthonomus grandis*) (Villavaso 1981, McKibben et al. 1988). Insect colonies, being closed populations kept under controlled conditions, exhibit mating among close relatives, and can suffer inbreeding depression (Van Lenteren 1991). Inbreeding depression is a decline in the fitness of an organism and can be due to the accumulation of recessive deleterious alleles or to an increased homozygosity of the organism's genome (Mitton 1993, Halliburton 2004, Charlesworth and Willis 2009).

Inbreeding depression is an insidious problem in the mass rearing of insects because in its early stages, it can go unnoticed, but in more advance stages of inbreeding, the constant breeding among relatives reduces the performance of the population (Hedrick 1983, Wang et al. 1999, Charlesworth and Willis 2009). Insectary colonies dying out over

time is not a rare event. Sometimes, factors can be identified as the cause, such as the failure of cooling systems or the presence of pathogens, but sometimes there is not a clear explanation (Cook 1993); also some of the studies detecting inbreeding depression in insects were instigated because anomalies were found in the colonies (Henter and Fenster 2003). The presence of inbreeding depression does not only jeopardizes the integrity of the colony, but also, if these inbred organisms are released in the field, they will show poor field performance (Nunney 2002, Woodworth et al. 2002). Most studies showing that insectary colonies had suffered changes that reduced the fitness in the wild came from insects reared for sterile insect control. Changes detected include: shorter courtship behavior of the medfly, *Ceratitis capitata* (Briceno and Eberhard 1998), earlier mating in the tobacco budworm, *Heliothis virescens* (Raulston et al. 1976), low sexual competitiveness of the melon fly, *Dacus* (Zeugodacus) *cucurbitae* (Iwahashi et al. 1983), low pheromone detection by gypsy moth males, *Lymantria dispar* (Lance et al. 1988), and low mating rate in the cotton boll weevil, *Anthonomus grandis* (Villavaso and Earle 1976, Villavaso 1981).

Using diallel designs (Antolin 1999), we can test for the presence of inbreeding depression by measuring biological or morphological traits, or any trait of interest to the breeder, such as flight capacity, fecundity, sex ratio, etc. Inbreeding depression is detected if the outbred individuals have a higher fitness than inbred individuals. Inbred individuals are the offspring of a mating between a female and a male from the same

line, and outbred individuals are the offspring of a mating between a female and a male from different lines. The extent of the difference in performance between outbred and inbred individuals is called heterosis (William and Pollak 1985, Stalder and Saxton 2004, Charlesworth and Willis 2009). If inbreeding depression is detected, several remedial practices can be implemented. These include: maintaining large population sizes (Hoekstra 2003), introducing adults from the wild (William and Pollak 1985, Hoekstra 2003, Van Lenteren 2003c, Rao et al. 2005, Van Lenteren and Bigler 2010), fluctuating the environmental conditions under which the wasps are reared (Guyot 1977, Bigler 1986), limiting the number of generations of laboratory propagation after field collection (Guyot 1977, Jones et al. 1978), promoting intraspecific competition (Bigler 1994), exchanging genetic material with other insectaries (Frankham 2008), or keeping the original wild population as small separate lines, and periodically mixing all of the lines in order to restore colony vigor (Delpuech et al. 1993, Roush and Hopper 1995, Nunney 2002, Nunney 2003, Frankham 2008, Caprio 2009). Some of these remedial measures carry risks, such as the introduction of pathogens (Van Lenteren 1991) or inefficient invigoration of the colony due to mating incompatibilities among colony and introduced insects (Van Lenteren 1991, Nunney 2002, Van Lenteren 2003c); therefore, it is recommended to first test for inbreeding depression before implementing some of the riskier remedial measures.

While colonies of diplo-diploid insects can suffer substantial inbreeding depression, haplo-diploid insects, such as parasitoid wasps, may not suffer inbreeding depression at all, or at least their inbreeding depression should be less (Waage and Ming 1984, Hopper et al. 1993, Grenier and De Clercq 2003). A meta-analysis of 45 studies by Henter and Fenster (2003) confirmed that haplo-diploid organisms suffer less inbreeding depression than diplo-diploids. There are two reasons why the haplo-diploid insects may not suffer strong inbreeding depression. First, through their haploid males, they will purge recessive deleterious alleles for traits expressed both in males and females and for male limited traits (Waage et al. 1985, Henter and Fenster 2003). Female limited traits are not affected by this purging and consequently in these traits, haplo-diploids are expected to have an inbreeding depression similar to that in diplo-diploid organisms (King and King 1995, Antolin 1999, Jeong and Stouthamer 2005). Second, in those species that practice regular sibmating, female limited traits are also purged; therefore, in these species, no or a very low inbreeding depression is expected (Werren 1993).

The effects of inbreeding have been studied several times in haplo-diploid egg parasitoids of the genus *Trichogramma*, and conflicting results have been found. No inbreeding depression was found in *Trichogramma pretiosum* by several authors (Ashley et al. 1973, Prezotti et al. 2004), or in *T.* nr. *brassicae (*Sorati et al. 1996*),* yet Antolin (1999) found evidence for inbreeding depression in *T. pretiosum* with respect to total fecundity and sex ratio. These conflicting findings led us to investigate the effects of

inbreeding in *T. pretiosum*. Using a diallel design (Antolin 1999), we measured the performance of inbred and outbred females with respect to two traits: total fecundity and sex ratio. Total fecundity is a complex trait that integrates several characters, such as the number of eggs oviposited, egg hatching rate, and larval and adult survival. Such a complex trait may be a valid proxy for adult *Trichogramma* fitness. And presumably, sex ratio is influenced by the inbreeding status of *T. pretiosum* wasps, i.e. inbred and outbred females should show different sex ratios (Antolin 1999).

Trichogramma pretiosum is the most widely mass reared egg parasitoid in the Americas (Hunter 1997, Garcia-Gonzalez et al. 2005, Postali 2010), and with the exception of the work of Antolin (1999), the presence of inbreeding depression had not been reported in *T. pretiosum* colonies. The success rate of *Trichogramma* applications in biological control varies. Sometimes successes are reported (Guyot 1977, Oatman and Platner 1978, Bigler 1986, Yu and Byers 1994, Nagarkatti et al. 2003, Zhang et al. 2010) but in other cases, released wasps fail to control the pest (King et al. 1985, Mills et al. 2000, Suh et al. 2000, Lundgren and Heimpel 2002, Ulrichs and Mewis 2004, Vejar-Cota et al. 2005). How important inbreeding depression is in the reported failures remains to be established. Here we determine if inbreeding depression may be a problem in *Trichogramma* species. If this is established, remedies can be implemented to avoid the negative effects of inbreeding on the field performance of these biological control agents.

1.3 Materials and methods

Study population

T. pretiosum is a minute parasitoid whose body length is less than 1 mm. Here we used six arrhenotokous *T. pretiosum* lines (3, 28, 33, 35, 38, and 40). Each of these lines was initiated with a mated female that had emerged together with at least one male and several other females from a single *Manduca sexta* egg collected from tomato plants at the University of California South Coast Field Station in Irvine, CA, in August or September 2008. Each isofemale line was initiated using a single field-collected *Manduca sexta* egg. We inbred these lines by initiating each subsequent generation by mating a female with her brother for the first nine generations; each inbred line was then kept as a population of approximately 300 individuals per generation.

Diallel crosses

We performed the experiments over two consecutive generations. In the first generation, we did all possible crosses between all six inbred lines, including crosses between individuals belonging to the same line, i.e. using a full 6x6 diallel design, in which each diallel row had virgin females of the same line, and each diallel column had virgin males of the same line. We formed two separate couples per diallel cell. Each diallel couple was allowed to oviposit for 24 h on a host egg card containing approximately 300 host eggs (*Ephestia kuehniella*), which were attached to the card using double sided sticky tape. After 10 days of embryonic development, at the onset of

the photophase, the adults of the second generation emerged. Females and males of the same brood (the offspring of each diallel couple) were allowed to mate randomly with their siblings over three hours. To test for the effects of inbreeding, we measured the total fecundity and sex ratio of four females per brood, as follows: over six days, each female was allowed to oviposit on a host egg card containing approximately 300 host eggs; we replaced the host egg cards every other day. Fifteen days after oviposition, we determined the number of male and female offspring. Total fecundity was measured as the total number of males and females, and sex ratio was calculated as the number of males divided by total fecundity. Males and females were identified using morphological characteristics of the antennae (Pinto and Stouthamer 1994).

In addition, from adults of the first generation in the six inbred lines, we determined their total fecundity and their sex ratio by individually exposing 15 mated females per line to a host egg card for a 6-day period; every other day the egg card was replaced with a fresh one. All experiments were done in an incubator precision 818 L.T.C.® (Thermo Fisher Scientific, Inc., Pittsburgh, PA) set at 25 °C and 16:8 h L: D photoperiod. This incubator model did not allow control for humidity.

Statistical analysis

Using the LSMEANS statement (estimate and contrast), we analyzed the results of the diallel crosses as follows: first, the total fecundity and sex ratio of all outbred females versus all inbred females; second, the performance of each dam line, i.e. all the crosses in each diallel row, in relation with each other; third, the performance, within each dam line, of inbred females versus outbred females; and fourth, the heterosis of all outbred females. This heterosis statistic was calculated as the average performance of the outbred females divided by the average performance of the inbred parents (William and Pollak 1985, Stalder and Saxton 2004). The statistical model used in the diallel experiment was $Y_{ij} = \mu + a_i + b_j + (a*b)_{ij} + \varepsilon_{ij}$; where Y_{ijk} is the total offspring (males and females) of a female i mated to a male j, μ is the overall population mean for this variable, a_i is the effect of the female i, b_j is the effect of the male j, $(a*b)_{ij}$ is the interaction effect between female and male, and ε_{ij} is the random residual error for each measurement (Cockerham and Weir 1977). All experimental data conformed to ANOVA assumptions: random selection of individuals, equality of variances, and normality of the residuals. To increase the normality of the sex ratio, we transformed raw data to the arcsine of the square root of the variable. In all tests, our criterion for statistical significance was an alpha value of 0.05. All statistical analyses were performed using SAS® V.9.2 statistical software.

We repeated the diallel experiment three times, but we analyzed the three data sets as a whole because some diallel cells did not contain data, i.e. some females did not produce offspring or produced only males. Across all three diallel replications, we failed to obtain data (no females) from female mating line 33 mated with male line 33; therefore, we used data from adults of the first generation, specifically data (total fecundity and sex ratio) from inbred line 33.

1.4 Results

Total fecundity of inbred and outbred females

After nine generations of inbreeding, based on the formula described for sex-linked genes (Li 1955), p. 202-203, which also applies for mating systems under haplo-diploidy, the six *T. pretiosum* lines used in this study had an inbreeding coefficient of at least 0.8593. As an overall result from all tested females, we found that inbred females (21.76±0.61) had lower total fecundity than outbred females (24.96±0.27) ($F = 22.58$, $df=35$, $P= <0.0001$). Comparing entire dam lines, we found that line 40 had the highest total fecundity (27±0.57), and lines 28, 33, 35, and 3 showed an intermediate performance, 25.90±0.62, 25.72 ± 0.60, 24.68±0.62, and 23.51±0.63, respectively, with line 38 showing the lowest performance (19.82±0.59) ($F=10.15$, $df=5$, $P=<0.001$); additionally, there were no differences among the six lines when compared as sire lines: 25.65±0.61, 24.61±0.60, 24.57±0.59, 24.32±0.62, 23.99±0.55, and 23.58±0.66 for lines 35, 40, 28, 3, 33, and 38, respectively ($F=197$, $df=5$, $P=<0.0807$). Compared within each dam line, we found that inbred females always had the lower performance (fig. 1). Crosses among different lines showed considerable heterosis (fig. 2); and all the outbred females combined showed a mean heterosis of 16.2±2.1%. Two diallel crosses had the highest heterosis values, females from line 38 mated with males from line 35 (34.8±6.8%) and females from line 38 mated with males from line 3 (25.09±7.22%); however, one diallel cross had a negative heterosis value, females from line 40 mated with males from line 28 (-5.1±6.0%). In summary, the lower performance of the inbred

females in the overall comparison and within each dam line, along with the positive heterosis of the outbred females, indicates that *T. pretiosum* lines suffered inbreeding depression; that is, total fecundity rates of *T. pretiosum* were significantly influenced by their inbreeding status.

Sex ratio of inbred and outbred females

Evaluating the sex ratio of the six inbred *T. pretiosum* lines, no significant differences were found between all inbred females combined (0.591±0.018) versus outbred females (0.577±0.008) ($F = 0.49$, $df=35$, $P= 0.4858$). When comparisons were made within each dam line, no clear pattern emerged. In some cases (lines 40 and 33), inbred females produced offspring sex ratios that did not differ significantly from that for all outbred females combined, while in other cases, the inbred females (lines 38, 3, and 28) produced sex ratios that were significantly higher that one or more for the outbred female lines. Finally, in line 35, the sex ratio of the inbred females was the same statistically as data from four of the outbred females lines, but significantly lower than data from the other two. Lack of statistical separation between inbred females versus outbred females, in both comparisons (overall and within each entire dam line), indicates that the inbreeding status did not influence sex ratios in a particular direction; presumably, sex ratio was regulated by other factors.

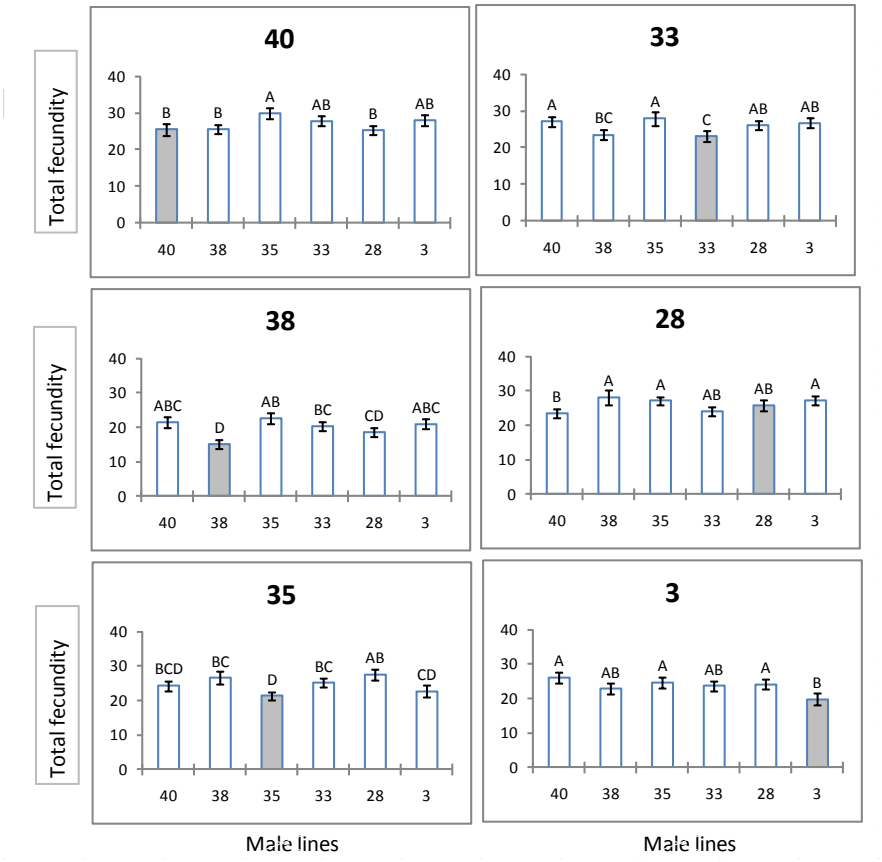


Figure 1.1. Total fecundity of inbred and outbred females, which were the offspring of the diallel crosses (6x6 full diallel design) of six *Trichogramma pretiosum* lines (40, 38, 35, 33, 28, and 3) reared on eggs of *Ephestia kuehniella* eggs over six days. Inbred females (dark bars) were the offspring of the diallel crosses whose parents were from the same line; and outbred females (light bars) were the offspring of the diallel crosses whose parents were from different lines. Female line number is specified at the top of each graph, and male line number at the bottom. Means and errors bars followed by the same letter are not statistically different (Statistical diallel model, $Y_{ij} = \mu + a_i + b_j + (a*b)_{ij} + \varepsilon_{ij}$, with an alpha of 0.05 adjusted for multiple comparisons using Tukey's test).

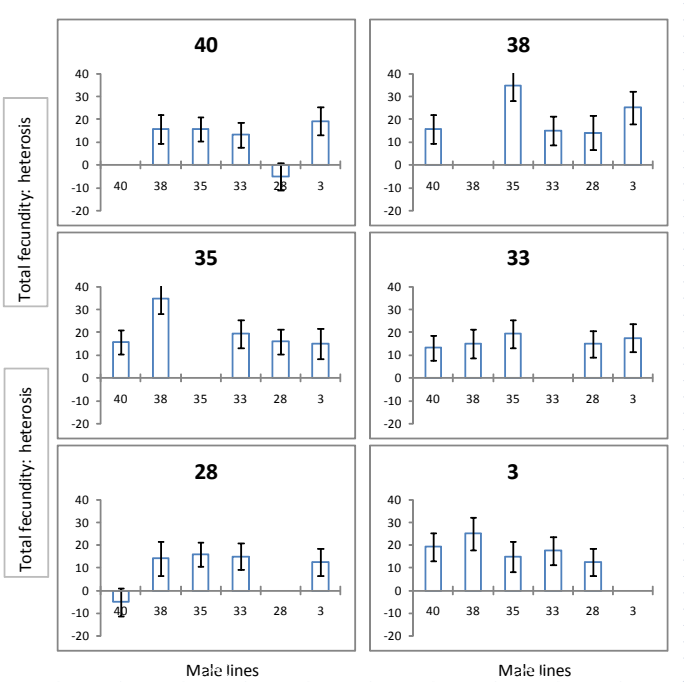


Figure 1.2. Heterosis of total fecundity of inbred and outbred females, which were the offspring of the diallel crosses (6x6 full diallel design), of six *Trichogramma pretiosum* lines (40, 38, 35, 33, 28, and 3) reared on eggs of *Ephestia kuehniella* eggs over six days. Inbred females (baseline bars) were the offspring of the diallel crosses whose parents were from the same line; and outbred females (light bars) were the offspring of the diallel crosses whose parents were from different lines. Heterosis of outbred females was calculated as the average performance of the outbred females over the average performance of the parents, i.e. heterosis is equal to ((AxB)+(BxA))/2 minus ((AxA)+(BxB))/2, divided by ((AxA)+(BxB))/2, and the resultant number is multiplied by 100. In this formula, A is equal to the female line number and B is equal to the male line number. Error bars show means ± S.E. The statistical diallel model used was $Y_{ij} = \mu + a_i +$

$b_j + (a*b)_{ij} + \varepsilon_{ij}$. Female line number is specified at the top of each graph, and male line number at the bottom.

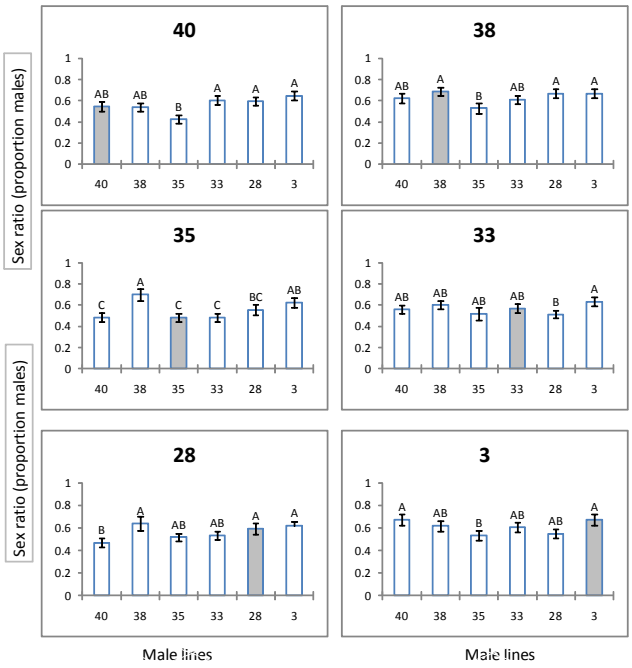
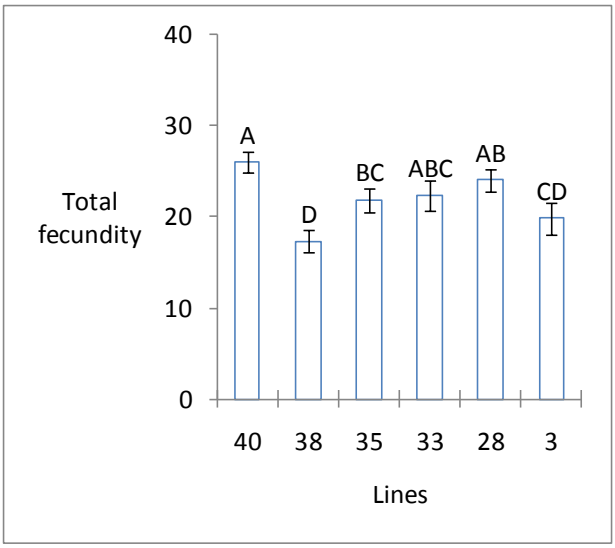


Figure 1.3. Sex ratio (proportion males) of inbred and outbred females, which were the offspring of the diallel crosses (6x6 full diallel design), of six *Trichogramma pretiosum* lines (40, 38, 35, 33, 28, and 3). Inbred females (dark bars) were the offspring of the diallel crosses whose parents were from the same line; and outbred females (light bars) were the offspring of the diallel crosses whose parents were from different lines. Female line number is specified at the top of each graph, and male line number at the bottom. Means and errors bars followed by the same letter are not statistically different (Statistical diallel model, $Y_{ij} = \mu + a_i + b_j + (a*b)_{ij} + \varepsilon_{ij}$, with an alpha of 0.05 adjusted for multiple comparisons using Tukey's test).

We also tested the total fecundity and sex ratio of adults of the first generation in the six inbred lines, i.e. adults that were siblings of the diallel couples. As a group, the six inbred lines had a total fecundity of 22.05±0.57. Within group differences, we found that females of lines 40, 33, and 28 had higher total fecundity; females of line 35 had intermediate total fecundity; and females of lines 38 and 3 had the lowest total fecundity ($F=6.51$, $df=5$, $P= <0.0001$) (fig. 4a). The mean sex ratio of the six lines was 0.5618±0.019; females of lines 38 and 3 had the highest proportion males, whereas females of the other four lines (line 40, 35, 33, and 28) produced a lower sex ratio ($F=3.33$, $df=5$, $P= 0.0064$) (fig. 4b). To determine the stability of these traits over successive generations, we compared five inbred lines (3, 28, 35, 38, and 40) of the first generation with their performance during the next generation (the inbred groups of the diallel design). In these comparisons, we excluded inbred line 33 because we obtained no data from diallel cell 33/33 (female line 33 mated with male line 33). Comparing the two consecutive generations using the Kendall's rank correlation coefficient, we found that the degree of similarity between the two sets of ranks was 80% and 40% for total fecundity and sex ratio, respectively. In addition, comparing the performance of the five inbred lines of the first generation with their performance during the next generation, but as entire dam lines, we found that with respect to total fecundity, they had a 100% Kendall's rank correlation coefficient.



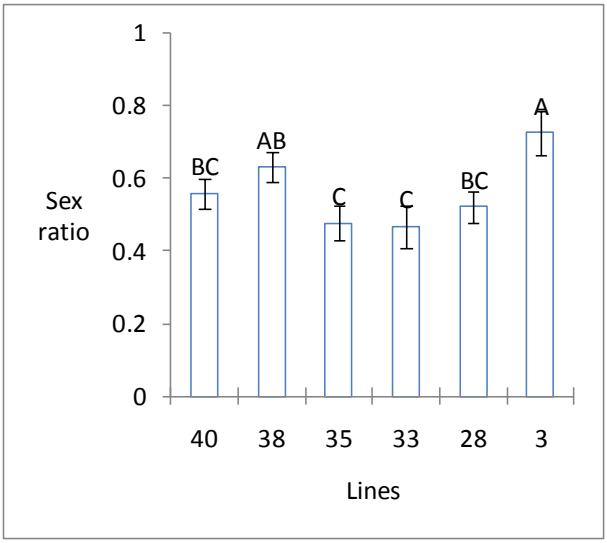


Figure 1.4. (a) Total fecundity and (b) sex ratio (proportion males) of six *Trichogramma pretiosum* lines: 40, 38, 35, 33, 28, and 3; reared on eggs of *Ephestia kuehniella* eggs over six days. Means followed by the same letter are not statistically different (ANOVA model, $Y_i = \mu + a_i + \varepsilon_i$, with an alpha of 0.05 adjusted for multiple comparisons using Tukey's test).

1.5 Discussion

Inbreeding depression has been found in the honey bee *Apis meliffera carnica* (Bruckner 1978), in the parasitic wasps *Muscidifurax raptor* (Fabritius 1984, Antolin 1992*)* and *Uscana semifumipennis* (Henter and Fenster 2003), in the bumblebee *Bombus terrestris (*Gerloff and Schmid-Hempel 2005*),* in the ectoparasitoid *Nasonia vitripennis* (Luna and Hawkins 2004), and in the egg parasitoid *T. pretiosum* (Antolin 1999). In this experiment, consistent with Antolin's (1999) data, we found evidence of inbreeding depression with respect to *T. pretiosum* total fecundity. Antolin (1999) found a general heterosis of 22% in offspring production similar to the value we observed (16.2% ±2.1). We could not compare the heterosis values with the other published studies because they did not report heterosis values. Additionally, Antolin (1999) reported inbreeding depression in the sex ratio of *T. pretiosum*, but we found that sex ratio was not influenced by inbreeding status of the females.

The negative effects of inbreeding can been caused by an accumulation of deleterious alleles or by a reduced heterozygosis of the individual (Mitton 1993, Halliburton 2004, Charlesworth and Willis 2009). However, in a haplo-diploid organism that practices regular sibmating, as is the case of *T. pretiosum*, when it is brought into lab conditions it will experience less inbreeding depression due to the accumulation of deleterious alleles (Werren 1993) for the following reasons. First, due to their haploid males, they have a 75% lower probability of accumulating deleterious alleles than do diploid organisms

(Werren 1993). Second, through their haploid males, they will purge recessive deleterious alleles for traits expressed in both males and females, and for male limited traits. Finally, because of sibmating, female limited deleterious alleles will also be purged (Werren 1993). Most likely, the difference in fitness we detected, between inbred and outbred females was caused by their differences in heterozygosis. The negative effects of reduced heterozygosis are independent of any relationship with recessive deleterious alleles (Beardmore 1983). Heterozygous individuals should have a higher fitness than inbred (homozygous) individuals (Mettler and Gregg 1969, Mitton 1993) because the former can regulate their metabolic functions more closely (Oldroyd and Fewell 2007, Kellermann et al. 2009).

In insect colonies, the negative effects of inbreeding increase as the inbreeding coefficient increases (Falconer and Mackay 1996, Lynch and Walsh 1998), jeopardizing the integrity of the colony. A positive correlation between the intensity of inbreeding and its negative effects has been shown in *D. arizonensis*, *D. mojavensis* (Mettler and Gregg 1969), *D. melanogaster* (Woodworth et al. 2002), and in the cricket *Gryllus firmus* (Roff and Derose 2001). The presence of inbreeding depression in *T. pretiosum* colonies does not only jeopardize the integrity of the insectary colony, but also cast doubts on the effectiveness of the insects once they are released in the field (Woodworth et al. 2002, Nunney 2003). There are numerous examples showing the efficacy of mass releases of *Trichogramma* parasitoids (Guyot 1977, Oatman and

Platner 1978, Bigler 1986, Yu and Byers 1994, Nagarkatti et al. 2003, Zhang et al. 2010), but there are also reports of *Trichogramma* parasitoids failing to control the target pest (King et al. 1985, Mills et al. 2000, Suh et al. 2000, Lundgren and Heimpel 2002, Ulrichs and Mewis 2004, Vejar-Cota et al. 2005). Inadequate field performance of *Trichogramma* parasitoids may be due to multiple causes, such as cold temperatures (Bourchier and Smith 1996), predation on eggs containing developing wasp larvae (Ruberson and Kring 1991), rain, presence of agrochemicals, parasitoid dispersion, or genetic problems with the reared insects (Keller and Lewis 1985, Collier and Van Steenwyk 2004). Therefore, in order to determine that mass reared *Trichogramma* did not fail due to inbreeding, it is necessary to test the insectary colonies for the effects of inbreeding, and if inbreeding depression is detected, the insectary personnel should implement remedial practices.

Based on our results in which almost all hybrids showed a positive heterosis with respect to total fecundity, we recommend maintaining a large lab population separate from several inbred lines, and periodically introducing the inbred lines into the large population, or better yet, completely replacing the large population by merging wasps from the inbred cultures. Each inbred line is intended as a reservoir of different alleles (Cook 1993, Roush and Hopper 1995); due to drift, each small line will preserve a different subset of alleles (Hedrick 1983, Halliburton 2004). Roush and Hopper (1995) suggested that approximately 95% of the genetic diversity observed in field populations

of haplo-diploid organisms would be preserved by initiating isofemale lines with at least 22 mated field collected females. This approach has not been tested in any natural enemy, but it was successful with *D. melanogaster* (Delpuech et al. 1993, Margan et al. 1998, Woodworth et al. 2002). For biological control projects, there is a clear need to test these laboratory results in the field to determine how important inbreeding depression is on the field performance of wasps. If substantial negative effects are found, then practitioners of biological control should take remedial measures. The length of time a colony has been maintained in the lab appears to affect the success rate of biological control programs (Myers and Sabath 1980). The decline in effectiveness of these "older" lab colonies may very well be caused by increased inbreeding.

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Chapter 2. Rapid changes in selected traits during mass-rearing: implications for biological control

2.1 Abstract. There are several reports in which field released *Trichogramma* parasitoids (Hymenoptera, *Trichogramma*tidae) failed to control the target pest, and insectary adaptation of the released insects could explain at least some of these failures. *Trichogramma* parasitoids, because of their haplo-diploid sex determination system, should not suffer much inbreeding depression, but adaptation to mass rearing conditions could take place just like it does in diploid populations. Adaptation to mass rearing conditions is possible if sufficient genetic variation is present in the starting population. Here, we artificially created populations with genetic variation and compared their response to artificial selection versus results with populations lacking genetic variation. We subjected these populations to a selection scheme for early or late emergence of the wasps. At the end of the directional selection, in the population with high genetic variability, we also monitored for concurrent changes related to the imposed selection. Concurrent changes were estimated in sex ratio, embryonic mortality, body size and total fecundity. After 22 weeks of selection, our results showed that the populations with high genetic variation reacted to selection by changing their pattern of adult emergence in a relatively short period of time, and selection for later emergence time results in concurrent negative changes in body size and total fecundity, but not in sex ratio or embryonic mortality. Adaptation to the mass rearing conditions would also be expected to take place in insectary reared populations initiated with large

numbers of field collected wasps. Such adaptations are expected to result in poor field performance.

2.2 Introduction

Releasing beneficial insects for biological control of agricultural and forestry pests is a common practice worldwide (Van Lenteren 2003b). Hunter (1997) listed 144 different species of beneficial insects reared under insectaries conditions; the list ranges from tiny egg parasitoids of the genus *Trichogramma* to large hemipteran predators. There are many potential problems associated with insectary colonies, among these, avoiding diseases is a major concern (Bjornson and Schutte 2003, Inglish and Sikorowski 2009). In addition, genetic problems associated with insectary colonies are assumed to be important, e.g., problems such as genetic drift, inbreeding or adaptation to rearing conditions (Mackauer 1976, Hopper et al. 1993). However, the negative effects of genetic drift and inbreeding (Hopper et al. 1993, Woodworth et al. 2002, Leppla 2003, Halliburton 2004) in insect colonies used for inundative biological control are not considered to be extremely critical (Van Lenteren and Tommasini 2003, Schneider 2009). Generally, the large size of most colonies counteracts genetic drift effects and also reduces the probability of mating between relatives (Mettler and Gregg 1969). But, if the genetic diversity of these large colonies is maintained at a high level by initiating the population with many different founders, the insects will adapt to the mass rearing conditions. The rationale is simple, in a large population (Margan et al. 1998, Woodworth et al. 2002), stable insectary conditions will favor the selection of phenotypes adapted to the local insectary conditions, and every generation, the frequency of these adapted phenotypes will increase (Hoekstra 2003, Nunney 2003).

Adaptation to mass rearing is suspected when recently established colonies do poorly in their initial generations, but during the following generations there is a marked increase in egg and larval survival (Boller 1972, Leppla et al. 1983, Nunney 2002, Leppla 2009). Insectary adaptation is an insidious genetic problem because it can give the false impression that the insectary production is flourishing, producing millions of healthy and strong insects; however, insectary adapted insects are most likely mal-adapted to the field conditions experienced after release (Nunney 2002, Gilligan and Frankham 2003, Frankham 2008). Few studies have been specifically done to determine the effect of mass rearing adaptation on the effectiveness of insectary reared natural enemies. Most studies showing that mass reared insects had suffered changes that reduced wild fitness came from insects reared for sterile insect technique applications including: shorter courtship behavior of the medfly, *Ceratitis capitata* (Briceno and Eberhard 1998), earlier mating in the tobacco budworm, *Heliothis virescens* (Raulston et al. 1976), low sexual competitiveness of the melon fly, *Dacus* (Zeugodacus) *cucurbitae* (Iwahashi et al. 1983), low pheromone detection by gypsy moth males, *Lymantria dispar* (Lance et al. 1988), and low mating rate in the cotton boll weevil, *Anthonomus grandis* (Villavaso and Earle 1976, Villavaso 1981).

While inbreeding depression in parasitoid wasps is generally not considered to be important because of their haplo-diploid sex determination system (Waage and Ming 1984, Hopper et al. 1993, Grenier and De Clercq 2003), adaptation to captive breeding

conditions should take place just like in diploid populations. Inbreeding depression is thought not to be important because males are haploid, and traits expressed both in males and in females will always be under strong selection leading to a low genetic load with respect to these traits. Female limited traits however will not have a lower genetic load than traits in diploid organisms. But, in those parasitic wasps that practice regular sibmating, as is the case with *Trichogramma* parasitoids (Kazmer and Luck 1991c, Luck et al. 2001), the genetic load carried in such traits will also be low. Consequently, in such species, the effects of inbreeding will often not result in a severe declines in fitness. Indeed, the effects of inbreeding depression has been studied in *Trichogramma* parasitoids, and excepting the work of Antolin (1999), inbreeding depression has not been found in *Trichogramma* parasitoids (Ashley et al. 1973, Sorati et al. 1996, Prezotti et al. 2004). Although the effects of inbreeding are not considered important in parasitoid colonies, due to their large size, these colonies will likely become adapted to the mass rearing conditions. The only impediment to such adaptation would be if the genetic diversity of the founding population is low. For instance it has been suggested several times that the negative effects of adaptation to the mass rearing can be avoided by maintaining the initial founders of the population as separate inbred lines (White et al. 1970, Hoy 1979, Hopper et al. 1993, Nunney 2003). For mass rearing of the wasps, these inbred lines can then be mixed and only reared for a limited number of generations to avoid selection for adaptation to the mass rearing conditions.

In *Trichogramma* parasitoids, laboratory adaptation has been studied mainly in *T. pretiosum* and conflicting results have been found. Evidence for laboratory adaptation, that is an increase (over generations) in the productivity of the colony, was found by Pratissoli et al. (2004), but not by Prezotti et al. (2004). Also in *T. pretiosum*, a response to directional selection during several consecutive generations, was not found in heat tolerance, increased locomotion, improved ability to find host eggs (Ashley et al. 1974), improved host acceptance, and increased in fecundity at extreme temperatures (Carriere and Boivin 2001). Studies examining insectary adaptation in other *Trichogramma* species also have shown conflicting results: evidence for insectary adaptation was found in *T. brassicae* (Kolliker-Ott et al. 2003); and response to selection was found in *T. minutum* (Urquijo 1951) and *T. chilonis* (Jalali et al. 2006), but not in *T. ostriniae* (Hoffmann et al. 2001) or *T. australicum* (Abraham and Pradhan 1976). These conflicting results, along with the report that inbreeding depression was found in *T. pretiosum* (Antolin 1999), led us to investigate if laboratory reared *Trichogramma* colonies adapt to captive breeding conditions. In our experiments, we used *T. pretiosum*, which is mass reared and released in the Americas against all kind of lepidopteran pests (Hunter 1997, Garcia-Gonzalez et al. 2005, Postali 2010). To detect insectary adaptation, we subjected our *T. pretiosum* lines to a selection scheme for early or late emergence of the wasps; also at the end of the selection, we monitored for concurrent changes related to the imposed selection. We tested these lines for their sex ratio, embryonic mortality, body size, and fecundity. Insectary adaptation or the lack

thereof has implications for the efficacy of these parasitoids as biological control agents: the main concern with adapted insects is that they may be unsuccessful under field conditions (Hoy 1979, Van Lenteren 1991, Mangan 1992, Bigler 1994, Wajnberg 1994, Nunney 2002, Woodworth et al. 2002, Caprio 2009). In the literature, there are multiple examples of the field efficacy of *Trichogramma* parasitoids, but also there are several reports in which *Trichogramma* parasitoids failed to control the target pest (King et al. 1985, Mills et al. 2000, Suh et al. 2000, Lundgren and Heimpel 2002, Ulrichs and Mewis 2004, Vejar-Cota et al. 2005). Therefore, to ensure that these failures were not caused by insectary adaptation, it is necessary to test the *Trichogramma* colonies for insectary adaptation. If insectary adaptation is detected, there are several remedial practices that can be implemented to counteract this adaptation, e.g., by introducing wild adults into the colony (Van Lenteren and Bigler 2010) or by exchanging genetic material with other insectaries (Frankham 2008).

2.3 Materials and methods

Trichogramma pretiosum lines

The *T. pretiosum* lines used in these experiments were initiated with wasps emerged from *Manduca sexta* eggs collected on tomato plants in August and September 2008 at the University of California's South Coast Field Station in Irvine, CA. Single mated females, each emerged from a different host egg, were used to initiate the 26 inbred lines. Over the next 9 generations, a single sib-mated female was used to initiate the next generation. Based in the formula described for sex-linked genes (Li 1955, p. 202-3), which also applies for mating systems under haplo-diploidy, this inbreeding protocol resulted in highly inbred lines with an inbreeding coefficient of at least of 0.8593. After the ninth generation, we kept these lines as isofemale lines, and every 10 days, we supplied them with an egg card consisting of approximately 300 irradiated *Ephestia kuehniella* host eggs attached to the cardstock with double sided sticky tape Scotch-3M®.

High genetic variation versus low genetic variation populations

Before starting the experiments we formed five populations: four inbred and one high genetic variation population. From the 26 inbred lines (lab stock), we chose randomly four inbred populations (inbred lines 14, 22, 28, and 39) and the high genetic variation population was formed by pooling together approximately 100 pupae from each of the 26 inbred lines. In order to build up the size of the five populations, we allowed these

populations to emerge and mate randomly during two generations. One day before the emergence of the third generation, we divided each of the five populations into five treatments: the four inbred lines were divided into five equal parts, and the high genetic variation population was divided into fifteen equals parts, i.e., in the high genetic variation population we had three replicates of each treatment, and only one replicate of each treatment in the four inbred populations. After we divided each population into five treatments, the number of insects comprising each treatment was 480 and 1,200 pupae per inbred population and high genetic variation population, respectively.

We subjected the five populations to a selection scheme for early or late emergence of the wasps during 22 weeks; for each population, four of the five treatments consisted of supplying the wasps with host eggs at intervals of either 9, 10, 11, or 12 days, and the fifth treatment was to measure the pattern of adult emergence of each population without selection. The populations were maintained in borosilicate glass tube (18x150 mm, Fisher Scientific®), which was closed off with a cotton plug. Each population was given host eggs on two host egg cards (approximately 1,200 host eggs per card). After parasitization, we discarded one of the two egg cards, so that the next generation would not superparasitize the eggs. For each treatment, during the first 17 weeks of directional selection, we allowed all emerged females to oviposit; but afterwards, only adults

emerging on and after the day of selection were allowed to produce the next generation (table 1).

The influence of this selection scheme for early or late emergence of the wasps was tested after 17 and again at 22 weeks by measuring the pattern of adult emergence. Because each treatment was given host egg cards at different time intervals, the number of generations subjected to selection varied for each treatment (table 1). We measured the patterns of adult emergence as follows: one day before the emergence of the adults, we separated 200 pupae, the next day we offered the emerged adults approximately 2,400 host eggs for 30 minutes, from 9:30 to 10:00 (the incubator lights went on at 6:00 AM). At the end of the oviposition period, we withdrew the parasitized host egg cards making sure that no live adults were left on the egg cards. From day 9 to day 12 after oviposition, the egg cards were monitored every hour (from 6:00 am until 4:00 pm) for the number of emerged adults. We also used this same procedure to measure the pattern of adult emergence prior to selection. Statistical analysis of data determined that at the end of 11 days of embryonic development, at least 98% of the adults had already emerged; therefore, to obtain a better display of the pattern of adult emergence, in the results section, we show only the number of emerged adults after 9, 10, and 11 days of embryonic development.

At the end of the selection experiments, we tested the three replicates of each treatment (9, 10, 11, and 12 days) of the high genetic variation population for their total fecundity, embryonic mortality, sex ratio, and body size, by giving 20 females a host egg card (with approximately 300 host eggs) for 24 h during the expected day of selection. From the emerged egg cards, we determined total fecundity as the total number of emerged adults; embryonic mortality as the number of parasitized host eggs (host eggs turn black once parasitoid larvae reach its prepupal stage) minus the number of emerged adults; and sex ratio as the number of females divided by the total number of emerged adults. The sex of each emerged adult was determined using the shape of the antennae (Pinto and Stouthamer 1994). From these 20 females, we also measured their hind tibia length as an index of adult body size (Pavlik 1993, Kazmer and Luck 1995, Van Lenteren and Bigler 2010). The length of the hind tibia was measured as described by Bennett and Hoffmann (1998), with the following modifications: instead of Hoyer's mounting medium we used water, and we read the measurements using an optical micrometer mounted in the 40X-ocular of a phase contrast Axioskop 40 Zeiss® microscope.

All experiments were done in an incubator precision 818 L.T.C.® (Thermo Fisher Scientific, Inc., Pittsburgh, PA) set at 25 °C and 16:8 h L: D photoperiod. This incubator model did not allow control for humidity.

Statistical analysis

We considered that there was a response to selection if per population four conditions were met: 1) the 9-day treatment produced the most adults earlier than the other treatments, 2) the 12-day treatment produced the most adults later than the other treatments, 3) the pattern of adult emergence before selection was similar to the pattern of adult emergence of the 10-day treatment [we expected the pattern of adult emergence prior to selection to match the pattern of emergence in the 10-day treatment because the lines used in this study had been supplied with host eggs every 10 days], and 4) the pattern of adult emergence of 11-day treatment produced an intermediate result between the 10-day and 12-day treatments. To determine statistical significance among the five treatments, we used survival analysis methods (Muenchow 1986) (Logrank test) with strata and replicates. In which the five populations were entered as strata, and the three replications of the five treatments from the high genetic variation population were entered as replicates in the statistical model. The Logrank test makes use of observed versus expected cell counts over the entire data set being analyzed (Kleinbaum and Klein 2005). We adjusted for multiple comparisons among treatments using the Sidak correction (SAS/STAT.9.2 2008). To determine statistical significance with respect to total fecundity, embryonic mortality, sex ratio, and body size among the treatments from the high genetic variation population, we used analysis of variance, adjusting for multiple comparisons using Tukey's test. Also, the three replications of each treatment were treated as replicates in

the statistical model. All experimental data conformed to the ANOVA assumptions: random selection of individuals, equality of variances, and normality of the residuals. To increase the normality of the sex ratio, we transformed raw data using an arcsine square root transformation. In all tests, the criterion for statistical significance was an alpha of 0.05, and all tests were performed using SAS V.9.2.

2.4 Results

Table 2.1. Method and number of generations subjected to selection in the four different treatments of directional selection in five (one high genetic variation and four inbred lines, 14, 22, 28, and 39 respectively) *Trichogramma pretiosum* populations. The high genetic variation line was formed by pooling all 26 inbred lines of *Trichogramma pretiosum*. Each treatment was given host egg cards at different times post-emergence, i.e., at 9, 10, 11, and 12 days, respectively.

Measurement	Method of selection (ovipositing adults)	Weeks of selection	Generations subjected to selection (per treatment)			
			9-days	10-days	11-days	12-days
prior-to-selection	all emerged adults	0	0	0	0	0
first evaluation*	a fraction of adults**	17	13	12	11	10
second evaluation*	a fraction of adults	22	19	18	16	15

* Because each treatment was given host egg cards at different time intervals, during the evaluations, the number of generations subjected to selection varied in each treatment.

** Only adults emerging on or after being given host egg cards were allowed to oviposit.

After 17 weeks of directional selection, we analyzed each of the five populations (one high genetic variation population and four inbred lines) for their response to the different treatments of directional selection, and we did not find a clear change in the pattern of adult emergence in any of the populations (data not shown). Five weeks

later, we again analyzed the five populations for their response to selection, and here, we report the following results.

In population line 14, the treatment interval of 12 days went extinct during the process of selection and the others three treatments (9, 10, and 11 days) did not differ significantly in their pattern of adult emergence ($X^2 \geq 0.32$, $P \geq 0.5342$). In population line 39, the treatment interval of 9 days went extinct and the three remaining treatments (10, 11, and 12 days) did not differ significantly in their pattern of adult emergence ($X^2 \geq 0.107$, $P \geq 0.9999$). In both instances in which the treatments were extinct due to the process of selection, this occurred at generation five, i.e., there were no emerging adults at generation five. In population line 22, each of the treatments had a different pattern of adult emergence ($X^2 \geq 33.56$, $P < 0.0001$); however, the 11-day treatment produced earlier emerging offspring instead of the 9-day treatment. In population line 28, three treatments (9, 10, and 11 days) had the same pattern of adult emergence ($X^2 = \leq 1.31$, $P = 1.0$), and the 12-day treatment had a different pattern of adult emergence compared with wasps exposed to the other three treatments; however, the 12-day treatment produced the earliest emerging adults, instead of producing late emerging adults. In summary for these four inbred populations, two populations (14 and 39) did not show changes due to directional selection, and two populations showed a slight response but not in the expected direction of selection.

In the high genetic variation population, we found a significant response to selection and in contrast with the results with inbred populations, the direction of selection was consistent with expectations. Each of the treatments had a different pattern of adult emergence, i.e., statistically, all of these patterns were different from each other ($X^2 \geq 33.33$, $P < 0.0001$), the 9-day treatment produced the most adults earlier than the other treatments, the 12-day treatment produced the most adults later than the other treatments, and the 10-day and 11-day treatments showed an intermediate pattern of adult emergence between the 9 and 12-day treatments (fig. 1).

Each treatment was given host egg cards at different intervals after emergence, i.e., at 9, 10, 11, and 12 days, respectively. The high genetic variation population was formed by pooling all 26 inbred lines of *Trichogramma pretiosum*. Treatments followed by numbers between parentheses are the number of emerged adults during the experimental trial.

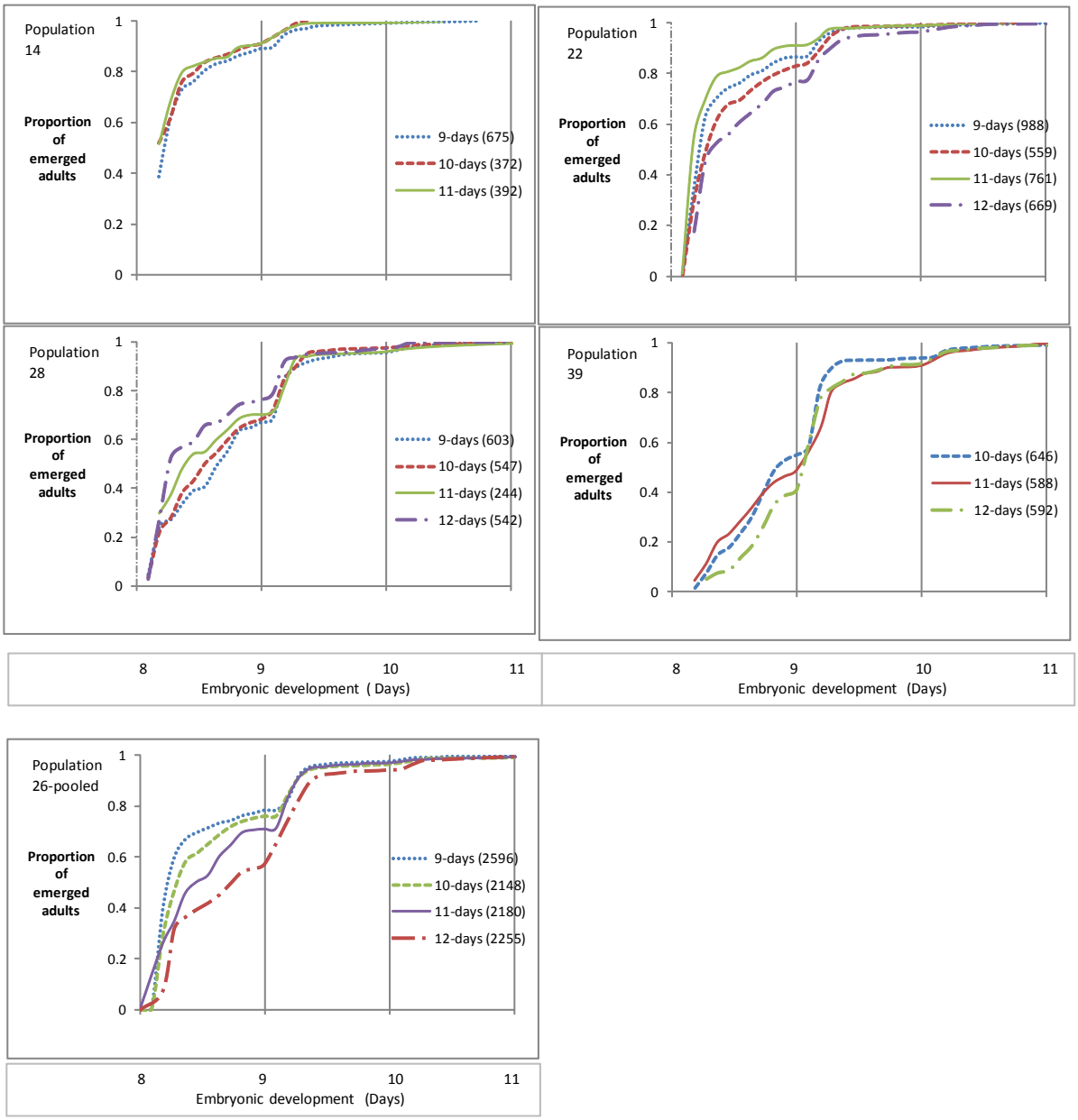

Figure 2.1. Proportion of emerged adults after 9, 10, and 11 days of embryonic development in each of the four treatments of directional selection from the four inbred populations (14, 22, 28, and 39) and from the high genetic variation population of *Trichogramma pretiosum,* after being subjected to directional selection for 22 weeks.

We also compared the pattern of adult emergence prior to selection with the patterns of adult emergence after selection (fig. 1, 2). We found that the pattern prior to selection of population line 28 did not differ from the four patterns of adult emergence after selection ($X^2 \leq 13.46$, $P = \geq 0.0596$). In population lines 14 and line 39, the pattern of adult emergence prior to selection differed from all three patterns of adult emergence after selection ($X^2 \geq 94.32$, $P = <0.0001$), and as previously mentioned, in both populations, one treatment was lost during the selection process. In population line 22, the pattern prior to selection differed with three treatments (9, 10, and 11 days) ($X^2 \geq 22.06$, $P = <0.0001$), but it was not significantly different from the pattern resulting from the 12-day treatment ($X^2 = 0.32$, $P = 1.0$). In summary, in the four inbred populations, there was no response to the imposed method of selection: the pattern of adult emergence before selection was different than the pattern of adult emergence resulting from the 10-day treatment. However, in the high genetic variation population, we found a response due to the imposed method of selection: the pattern of adult emergence prior to selection differed from the pattern of emergence resulting from the 9, 11, and 12-day treatments ($X^2 \geq 15.56$, $P = <0.0200$), but it was not significantly different from the pattern of adult emergence after selection for emergence after 10 days ($X^2 = 0.40$, $P = 1.0$).

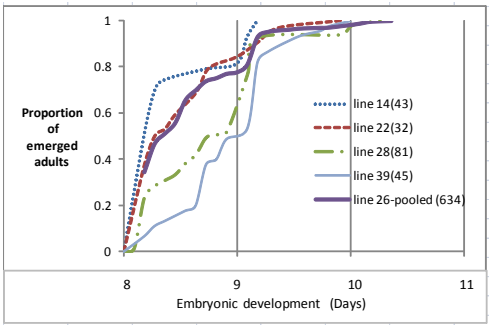

Figure 2.2. Proportion of emerged adults after 9, 10, and 11 days of embryonic development in each of the four inbred populations (lines 14, 22, 28, and 39) and in the high genetic variation population of *Trichogramma pretiosum* prior to selection. Populations followed by numbers between parentheses are the number of emerged adults during the experimental trial. The high genetic variation population was formed by pooling all 26 inbred lines of *T. pretiosum*.

At the end of the experimental trials, for the high genetic variation population, we did not find differences as a function of the selection treatments with respect to sex ratio ($F= 1.82$, $df=3$, $P=0.1451$) and embryonic mortality ($F= 0.54$, $df=3$, $P=0.9397$), but, we did find differences in body size ($F= 24.58$, $df= 3$, $P\leq0.0001$) and total fecundity ($F= 11.70$, $df= 3$, $P\leq0.0001$). With total fecundity, we found that 9 and 10-day treatments resulted in the highest total fecundity (21.29±0.68 and 21.05±0.67) and the other two treatments (11-days and 12-days) resulted in the lowest total fecundity (18.18±0.67 and 15.65±0.87), with a mean for all wasps exposed to the four treatments of 19.42±0.67 (fig. 3a). In body size, we found that the 9 and 10-day treatments resulted in the largest

female body size (hind tibia length of 0.140±0.001 mm and 0.136±0.001 mm), the 11-day treatment had an intermediate value (0.132±0.001 mm), and the 12-day treatment resulted in the smallest female body size (0.126±0.001 mm) (fig. 3b). The overall mean female body size for wasps from the four treatments was 0.132±0.001 mm.

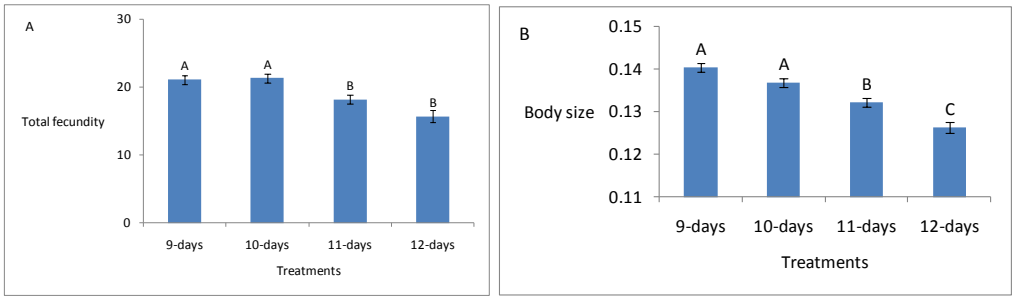

Figure 2.3. Total fecundity (A) and body size (B) for wasps, after being subjected to four treatments of directional selection, from the high genetic variation population of *Trichogramma pretiosum*. Each treatment group was given host egg cards at different time intervals post oviposition, i.e., at 9, 10, 11, and 12 days. The high genetic variation population was formed by pooling 26 inbred lines of *Trichogramma pretiosum*. Means (S.E.'s bars shown) followed by the same letters are not statistically different (ANOVA tests, with an alpha of 0.05 adjusted for multiple comparisons using Tukey's test).

2.5 Discussion

This is not the first successful experiment demonstrating directional selection in a haplo-diploid organism; previously, successful selection was achieved for tolerance to extreme temperatures in *Aphytis lingnanensis* (White et al. 1970), insecticide resistance in *A. melinus* (Rosenheim and Hoy 1988), and temperature preference in *Microplectron fuscipennis* (Wilkes 1942). In *Trichogramma* parasitoids, evidence of lab adaptation has been found in *T. pretiosum (Pratissoli et al. 2004)*, and *T. brassicae* (Kolliker-Ott et al. 2003), and responses to directional selection in *T. minutum* (Urquijo 1951) and *T. chilonis (Jalali et al. 2006)*.

In our experiments we did not find evidence of lab adaptation in inbred populations, which was expected because the success of any artificial selection depends on the initial genetic variation of a population (White et al. 1970, Hoy 1979). Populations with low genetic variation should not suffer laboratory adaptation (Hoy 1979, Nunney 2003, Frankham 2008). However, we could select for traits in the population with high genetic diversity. High initial genetic variation provides the material that can be selected for adaptation to laboratory conditions (Hoekstra 2003, Nunney 2003). However, the increase in frequency of adapted alleles may come at the expense of losing other alleles that could be important under field conditions (Mackauer 1976), such as an enhanced capacity to fight a bacterial attack, response to kairomones, improved flight activity, etc. A reduction in genetic variation (over time) in insects colonies has been shown

using enzymatic markers in several studies, for instance Delpuech et al. (1993) studied 32 isofemale lines of *Drosophila melanogaster*. They found changes in allele frequency for three enzymatic loci from generation 1 until generation 23, and that the number of homozygous lines increased from 4 to 12 for alpha glycerophosphate dehydrogenase, from 4 to 12 for alcohol dehydrogenase, and from 12 to 21 for esterase locus 6, respectively. In two colonies of the screwworm *Cochliomyia hominivorax*, Bush et al. (1976) found an increase in the frequency of the alpha glycerol phosphate dehydrogenase enzyme from 0.03 to 0.55 over 11 months for colony 1, and from 0.03 to 0.60 over 9 months for colony 2. Similarly, in the haplo-diploid organism *Aphidius ervi*, after rearing the parasitoid for 47 generations with a total population size of 150 individuals (100 females and 50 males), Unruh et al. (1983) found that half of the initial genetic diversity was lost.

Several authors have warned about the drawbacks of insectary adaptation in insect populations: insectary adapted colonies can often be successful under insectary conditions, but unsuccessful in the field (Boller 1972, Hoy 1979, Van Lenteren 1991, Mangan 1992, Bigler 1994, Wajnberg 1994, Nunney 2002, Woodworth et al. 2002, Caprio 2009). Most studies showing that adapted insects perform poorly in the field are for insects used in the sterile insect technique. However, in *Trichogramma* parasitoids, there are several studies showing that field released insects showed inadequate field performance. With *T. pretiosum*, Ashley et al. (1973) reported an efficiency of

parasitization of less than 7% in field cages (5,000 females/≈15m^2). Bergeijk et al. (1989) tested the host acceptance of *Ostrinia nubilalis* for *T. brassicae* in field cages. After rearing on *O. nubilalis* or *E. kuehniella* over at least 130 generations, they found that *T. brassicae* decreased its acceptance of *O. nubilalis* with increasing numbers of generations reared on *E. kuehniella*, but the acceptance of *O. nubilalis* by *T. brassicae* reared in *O. nubilalis* did not change. Lundgren et al. (2002) tested for the efficiency of weekly releases of commercially available *T. brassicae* in cabbage plots over two years. Even though the treatments plots had a higher proportion of *Trichoplusia ni* eggs parasitized (first year 0.19 and second year 0.16) in comparison with the control, plant damage did not differ between the *Trichogramma* treated plots and the control. These *Trichogramma* examples showing inadequate field performance can have several causes, for example bad weather conditions, wind dispersal away from the release site, predation on eggs containing developing wasp larvae, lack of natural refuges, and possibly due to poor insectary quality of the released insects (Collier and Van Steenwyk 2004). Therefore, to exclude the possibility that the released insects have a low field performance due to insectary adaptation, insectary personnel should test for insectary adaptation in *Trichogramma* colonies and take action according to the results.

Lab adaptation has been studied in *Trichogramma* parasitoids and conflicting results have been found. These conflicting results could be explained by the fact that only populations with high genetic variability and maintained as large populations will suffer

lab adaptation (as shown here). However, if the colonies were kept under laboratory conditions for an extended period of time with low initial genetic variability, as we did with the inbred populations, no change may be found in their phenotypic means after selection (Hoy 1979, Nunney 2003, Frankham 2008). We analyzed these assumptions on the conflicting results regarding *Trichogramma* parasitoids (table 2), and found that three studies (Kolliker-Ott et al. 2003, Pratissoli et al. 2004, Jalali et al. 2006) that initiated selection with populations of high genetic diversity responded to the imposed selection and one study did not result in a change in phenotypic means (Prezotti et al. 2004). Two studies (Carriere and Boivin 2001, Hoffmann et al. 2001) initiated selection with populations of low genetic variability; that is lab colonies kept under insectary conditions for a certain period of time, did not respond to the imposed selection. And, one study (Urquijo 1951) that was initiated with a population in which the genetic variation was restored by pooling several distinct colonies, responded to selection. In two other studies, no response to selection was found despite the fact that they pooled separate colonies but only up to 6 distinct colonies were used (Ashley et al. 1974, Abraham and Pradhan 1976).

Table 2.2. Literature review of *Trichogramma* parasitoids that have undergone (A) laboratory adaptation or (B) directional selection experiments

Species	Source of material	Duration of the experiment	Traits under observation	Result	Reference
		A. Laboratory adaptation			
T. brassicae	1 field population	27 generations	parasitism rate	yes	Kolliker-Ott et. al, 2003
T. pretiosum	field populations *[1]	23 generations	parasitism rate	yes	Pratissoli et al., 2004
T. pretiosum	field populations *[2]	8 months	parasitism and emergence rate, sex ratio, longevity, deformed adults	no	Prezotti et al., 2004
		B. Directional selection			
T. minutum	several colonies *[3]	4 years	parasitism rate	yes	Urquijo, 1951
T. pretiosum	2 colonies *[4]	7 generations	heat tolerance, locomotion, ability to find host eggs	no	Ashley et al., 1974
T. australicum	6 colonies *[5]	4 generations	resistance to high temperatures	no	Abraham and Pradhan, 1976
T. ostriniae	1 colony *[6]	>10 generations	host preference	no	Hoffmann et. al., 2001
T. pretiosum	1 colony *[7]	15 generations	host acceptance, fecundity	no	Carriere and Boivin, 2001
T. chilonis	4 field populations	341 generations	insecticide resistance	yes	Jalali et al., 2006

*1. Unknown number of populations; *2. Experimental treatments initiated with 1, 5, and 10 pairs of field collected specimens; *3. Unknown number of colonies and unknown period of time under lab conditions; *4. Two colonies kept under lab conditions for 2 and 1 years respectively; *5. Each population was field collected and mixed with laboratory stock; after that each population was inbred through a sibmating procedure for an unknown number of generations; *6. Colony kept under lab conditions for 1.5 years; *7. Colony kept under lab conditions for more than 100 generations.

Our studies have shown that parasitoid wasps can react to selection for specific traits in a relatively short period of time if significant genetic variation is present in the initial population. By extension, selection for adaptation to the lab would also be expected to

take place in mass reared populations initiated with large numbers of field collected wasps. Such laboratory adaptation is expected to result in poor performance in the field. We also showed that selection for traits in the inbred lines was not successful, consistent with expectations, and consequently, adaptation to mass rearing conditions can be avoided by regularly mixing inbred lines to form a new founder population for the mass rearing. Such an approach should result in less laboratory adaptation and more efficient performance of the released wasps in the field.

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Chapter 3. Quality assessment of mass-reared *Trichogramma* egg parasitoids

3.1 Abstract. The accurate identification and quality of mass-reared biological control agents is vital if they are to be used effectively. In the literature, there are several reports that *Trichogramma* spp. (Hymenoptera, *Trichogramma*tidae) failed to control the target pest, and poor quality or inaccurate identification may explain a number of these failures. Previously, unnoticed species replacement and morphological misidentification were reported. The initial goal of this study was to generate a DNA-Multiplex PCR assay, based on differences in ITS2 sequences, to easily identify the *Trichogramma* species that are reared in several Mexican insectaries. The second goal was to investigate if the quality of the reared *Trichogramma* meet the minimum standards suggested by the international organization for biological control/ European community (IOBC/EC). Quality was estimated by investigating three parameters highly correlated with field performance: sex ratio, body size and embryonic mortality. Using our DNA-Multiplex PCR assay, we found discrepancies between the reported and the DNA-determined species identity, and the presence of unnoticed species replacements. In addition, while the sample of all the insectaries together was supposed to contain three species of *Trichogramma* (*T. pretiosum*, *T. exiguum* and *T. platneri*) only two species were present (*T. pretiosum* and *T. fuentesi*). The reared *Trichogramma, T. pretiosum* and *T. fuentesi*, barely fulfilled the minimum quality requirements suggested by the IOBC/EC. For each of these two species, there was substantial uniformity in body

size, sex ratio and embryonic mortality independent of the insectary that produced them.

3.2 Introduction

The use of insects as biological control agents of agricultural pests has gained popularity over the years, largely because traditional chemical methods of pest control have shown several drawbacks, such as insecticide resistance, adverse effects on human health, contamination of ground water, etc. (Carson 1962). One of the most popular insects used as biological control agents are the *Trichogramma* parasitoids, which are mass-reared in insectaries worldwide, and are used for the biological control of lepidopteran pests (Smith 1994, Knutson 1998). There are numerous examples showing the efficacy of mass releases of *Trichogramma* parasitoids (Guyot 1977, Oatman and Platner 1978, Bigler 1986, Yu and Byers 1994, Nagarkatti et al. 2003, Zhang et al. 2010); but also, there are some reports in which *Trichogramma* were not successful in controlling the target pests (King et al. 1985, Mills et al. 2000, Suh et al. 2000, Lundgren and Heimpel 2002, Ulrichs and Mewis 2004, Vejar-Cota et al. 2005). Additionally, there is evidence that mass-reared *Trichogramma,* when compared with recently collected wasps, have a low field performance with respect to survival (Mansfield and Mills 2002), parasitization (Ashley *et al*. 1973) and host acceptance (Bergeijk et al. 1989). Similarly, in lab studies, individuals originating from long term rearings underperformed when compared to wasps recently collected from the field with respect to intrinsic rate of increase (Nagarkatti and Nagaraja 1978), survival rate at extreme temperatures (Nagarkatti 1979), and female sterility (Nagarkatti and Nagaraja 1978). Inadequate field performance of *Trichogramma* parasitoids can have multiple causes: cold temperatures

(Bourchier and Smith 1996), heavy rain, residues of toxic agrochemicals, high parasitoid dispersion or low quality of the reared insects (Keller and Lewis 1985, Collier and Van Steenwyk 2004). Most of these causes are largely beyond the control of insectary personnel; with the exception of monitoring the quality of the reared parasitoids. Quality assessment of mass-reared insects is defined as: monitoring that reared insects conform with previously established set of standards (Chambers 1977). Quality assessment of mass-reared *Trichogramma* parasitoids begins with the correct taxonomic identification of the reared species. Proper identification is important because the field performance of *Trichogramma* wasps varies among species. Some *Trichogramma* species are habitat-specific (Thorpe 1985, Romeis et al. 2005), while others are host-specific (Curl and Burbutis 1978, Yu et al. 1984, Stevens 1995); they also vary in their searching capacity and their climatic tolerance (Hassan 1994). Traditionally, *Trichogramma* parasitoids have been identified using morphological characters, but this has many difficulties. First, the specimens have to be slide-mounted (Pinto 1998), which is a laborious procedure requiring much experience (Platner et al. 1999). Second, some of the morphological characters can be misinterpreted depending on the criteria used by the observer. Some morphological structures are susceptible to changes due to differences in diet or environment. And third, morphological identification requires the presence of males (Pinto 1998), leaving the majority of females unidentifiable. Several populations that consist of only females cannot be identified using morphological keys. In addition to the problems associated with identification, many unnoticed changes can

take place during mass rearing; examples include: inbreeding depression of the insect lines (Antolin 1999), superparasitization of the host eggs or inadvertent replacement of the reared species by another species. Inadvertent replacement has been reported in insectaries from Mexico (Garcia-Gonzalez et al. 2005), the U.S.A. (Lundgren and Heimpel 2002, 2003) and Europe (Sumer et al. 2009). To avoid unnoticed replacement of the reared species, regular identification of insectary colonies should be done as frequently as possible (Laing and Bigler 1991, Garcia-Gonzalez et al. 2005).

An alternative to morphological identification is identification using DNA techniques. Following the methodology of Stouthamer *et al.* (1999), it is possible to generate a molecular key to differentiate among the different *Trichogramma* species present in a geographical area. After the initial development of the molecular key, this methodology can be easily applied by non-specialists (Stouthamer et al. 1999).

The second step in quality assessment of mass-reared parasitoids is to monitor the quality of the reared parasitoids. Bigler (1994) and van Lenteren et al. (2003) recommend measuring traits that are highly correlated with field performance, including: sex ratio, body size and embryonic mortality. Sex ratio (proportion females) is important because the females are the only sex that kills the pest. The body size of *Trichogramma* parasitoids is directly related to their field performance, as larger females are able to oviposit a larger number of eggs (Waage and Ming 1984, Olson and Andow 1998, Silva and Stouthamer 1999), live longer (Waage and Ming 1984, Olson and Andow 1998) and have a higher probability of finding host eggs (Kazmer and Luck

1991a, Bennett and Hoffmann 1998). *Trichogramma* parasitoids are normally shipped to the end user as parasitized host eggs; therefore, the embryonic mortality, or proportion of adults emerging, from these host eggs indicates the real number of wasps that are released into the field.

In Mexico, the use of mass-reared *Trichogramma* parasitoids as biological control agents started in 1962 when the government funded the first center for the study and production of beneficial insects (CREROB). Since then, 22 additional centers have been established, including one national center for biological control. The national center functions as a center for research and development of different beneficial insects, including *Trichogramma* spp. In 1992 the CREROBs were decentralized; now they are directed by a committee formed by federal and state representatives, agricultural organizations and consumer organizations. However, the CREROBs continue to receive technical advice from the national center for biological control. *Trichogramma* parasitoids are the most reared beneficial insect in these CREROBs (Arredondo-Bernal and Sanchez-Gonzalez 2009); Eighteen of the CREROB produce *Trichogramma* as well as 17 private companies (Tamez-Guerra 2001). Four species of *Trichogramma* parasitoids are reared in these companies and CREROB centers: *T. pretiosum, T. exiguum, T. fuentesi and T. pintoi* (Garcia-Gonzalez et al. 2005, Espana-Luna et al. 2008). The CREROBs distribute the *Trichogramma* parasitoids, in the form of prepupal host egg cards, almost free of charge. About 1.5 million hectares are treated with *Trichogramma* spp (Dominguez 1996). In Mexico, *Trichogramma* parasitoids are released against

lepidopteran pests in several crops such as tobacco, sugarcane, apples, etc. (Tamez-Guerra 2001, Arredondo-Bernal and Sanchez-Gonzalez 2009).Not all CREROBs produce *Trichogramma* year-long, some of them produce *Trichogramma* only during the crop season. At the start of the year, these part-year CREROBs buy the initial stock from others CREROBs or from private insectaries.

The objectives of this study were: 1) to identify which *Trichogramma* species are mass-reared from various Mexican insectaries, 2) to develop a molecular identification method to easily identify the reared species, and 3) to monitor the quality of the reared *Trichogramma*. This study was intended to determine if the reared *Trichogramma* meet minimum standards, which were suggested by the international organization for biological control/ European community (IOBC/EC), and that are observed by bio-producers of North America and Europe (Bigler et al. 1991, Van Lenteren 2003a).

3.3 Materials and methods

Trichogramma samples

During early 2009 we asked all Mexican insectaries (CREROBs and private) listed in the catalog "Directorio de laboratorios reproductores y comercializadores de agentes de control biologico, SENASICA-DGSV-CNRF-CNRCB" for samples of their mass-reared *Trichogramma*. In this request we explained that we would use their samples to identify the wasps and to evaluate their quality. Initially, eight insectaries accepted the offer to test their *Trichogramma*, but in the end, only six insectaries (four CREROBs and two private), sent us complete batches of their *Trichogramma* parasitoids. To maintain anonymity of the collaborating insectaries, we only report their geographical location. From April to September 2010, each of these insectaries sent us three samples from all the *Trichogramma* species they reared. The first sample was sent as host egg card in the prepupal stage to Mexicali, Baja California, MX. To prevent the eclosion of the *Trichogramma* parasitoids these samples were shipped in cold storage packages. After their arrival, the prepupal egg cards were allowed to emerge in enclosed containers (Eppendorf tubes of 50 ml capped with a cotton plug). The environmental conditions at the time of adult emergence were fluctuating temperatures from 21°C to 30°C (night/day), 40% ±10 humidity, and approximately 13–14 hrs of light. The second and third samples of *Trichogramma* parasitoids were prepared differently. Each parasitized host egg card was allowed to emerge in enclosed containers at each insectary; after the natural death of the emerged *Trichogramma* adults, these containers were sent to San

Luis Rio Colorado, Sonora MX. All enclosed containers were picked up by personnel of the University of California, Riverside, and brought to the Entomology Department for analysis. We chose to receive the *Trichogramma* samples in Mexico rather than directly in the U.S.A., because as dead insect these samples did not require import permits.

DNA-species identification

To correctly identify the reared species from the different Mexican insectaries, we generated a DNA-molecular identification method. First, we identified the species. Per rearing sample we extracted the DNA of four individuals using the Chelex DNA extraction method (Walsh et al. 1991). Each wasp was ground in 60 ul 5% Chelex-100 (Bio-Rad laboratories, Hercules, CA) and 2 ul proteinase K (20 mg/ml); the mixture was incubated for 60 min at 55°C, followed by 10 min at 99°C. Using the ITS2-forward (5'-TGTGAACTGCAGGACACATG-3') and ITS2-reverse (5'-GTCTTGCCTGCTCTGAG-3') primers the entire ITS2 region of rDNA (Stouthamer et al. 1999) was amplified. PCR was performed in 25 uL reactions containing 2 ul DNA template, 1X PCR-buffer (New England Biolabs, Ipswich, MA), 0.2 mM each dATP, dCTP, dGTP, 0.4 mM dUTP, 1 mM $MgCl_2$, 0.2 mM forward and reverse primer, 1 U Taq polymerase enzyme (NEB), and 13.3 ul sterile distilled water. PCR was performed using a thermocycler Ep gradient S (Eppendorf AG, Hamburg, Germany). The PCR cycling program was 3 min at 95 °C followed by 37 cycles of 45 seconds at 92 °C, 45 seconds at 53 °C and 1 min at 72 °C with 3 min at 72 °C after the last cycle. PCR products were separated on a 1% agarose gel and

stained with ethidium bromide; size ladders were run along with the samples for reference. PCR products and ladders were photographed with a Carestream Molecular Imaging V.5.0.2.3.0 (Carestream Health, Inc., Rochester, NY). PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega Corporation, Madison, WI) and direct-sequenced in both directions at the University of California, Riverside, Genomics Institute, Core Instrumentation Facility using an Applied Biosytems 3730 DNA analyzer with a Big-Dye® V3.1 kit (Applied Biosystems, Foster City, CA). The resultant ITS-2 sequences were manually aligned in BioEdit version 7.0.5.3 (Hall 1999) and matched against sequences present in GenBank® (Benson et al. 2008). Second, we designed species-specific multiplex-PCR primers. Only two species, *T. pretiosum* and *T. fuentesi,* were identified from all our insectary samples (see results)*;* therefore, we designed a Multiplex PCR assay to distinguish specimens of *T. pretiosum* and *T. fuentesi* from each other (see Gariepy et al. 2005, for an overview of the principles of Multiplex PCR). Based on the alignment of ITS2 sequences, three PCR primers were designed using Primer3 v.0.4.0 (Rozen and Skaletsky 2000): a common forward primer *T.pf*-uniF (5'-TCAAACGAAACGCAAGAGAA-3'); and two species-specific reverse primers, *T.fuen*-R (5'-GAGCCTGATCGTGTGCTAAA-3') and *T.pret*-R (5'-GAGCTAGCCAGGCGCTATAA-3') (Rugman-Jones et al., unpublished). The two species-specific primers resulted in PCR products of 173 bp and 250 bp respectively. To corroborate that the *Trichogramma* samples belonged either to *Trichogramma pretiosum* or *T. fuentesi*, we used this multiplex PCR assay on 20 wasps per sample. We used the PCR master mix, the PCR

cycling program, the agarose gel and the UV-photograph equipment, which were previously mentioned, but with the following modifications: PCR master mix, 12.8 ul sterile distilled water, 0.2 mM forward and two reverse primer; PCR program, 30 seconds during the first and second step of the 37 cycles, the first step at 94 °C and the second step at 59 °C; and the product was run in a 1.5% agarose gel.

DNA-*Wolbachia* detection

In addition to identifying the wasps we also tested them for the presence of the bacterial symbiont *Wolbachia*. Parthenogenesis inducing *Wolbachia* have been found in several *Trichogramma* species and such symbionts may influence the fitness of the wasps when used in biocontrol (Tagami et al. 2001, Miura and Tagami 2004). Using the Chelex-method, we extracted DNA from 20 wasps per insectary sample. Evidence of *Wolbachia* infection was sought using *Wolbachia*-specific PCR primers. The primers W-Specf (AGCTTCGAGTGAAACCAATTC) and W-Specr (CATACCTATTCGAAGGGATAG) were used to amplify a 438 bp fragment of *Wolbachia* 16S rDNA (Werren and Windsor 2000). PCR was performed in 25 uL reactions containing 2 ul DNA template, 1X PCR-buffer (NEB), 0.2 mM each dATP, dCTP, dGTP, 0.4 mM dUTP, 0.4 mM BSA, 0.2 mM forward and reverse *W*-spec PCR primers, 1 U Taq polymerase enzyme, and 12.3 ul sterile distilled water. The cycling program was 2 min at 94 °C, 45 cycles of 0.5 min at 94 °C, 45 seconds at 55 °C and 1.5 min at 72 °C, followed by 10 min at 72 °C after the last cycle. To determine that *Wolbachia*-DNA amplified successfully, in each PCR reaction we

included *T. pretiosum*-DNA from a Hawaiian population known to be infected by *Wolbachia*.

Quality of mass-reared *Trichogramma*

To determine if the reared *Trichogramma* parasitoids met the minimum standards of the IOBC/EC (Bigler et al. 1991, Van Lenteren 2003a), we used the suggested minimum standards for four sexual species of *Trichogramma* (*T. brassicae*, *T. dendrolimi*, *T. evanescens* and *T. minutum*): a sex ratio (proportion females) of ≥ 0.5, embryonic mortality ≤ 20% and total fecundity ≥ 40 eggs. To measure the quality of the mass-reared *Trichogramma,* we first determined the sex of 100 adults for each sample using a V8 Zeiss® stereo microscope. The sex of each emerged adult was determined using the shape of the antennae (Pinto and Stouthamer 1994). Sex ratio was estimated as number of females divided by the total number of adults in the sample. Second, we measured embryonic mortality of the parasitized host eggs (host eggs turn black once parasitoid larvae reach its prepupal stage). From each *Trichogramma* sample we checked 100 black eggs for the presence of an emergence hole and dissecting black host eggs that lacked an emergence hole for the presence of unemerged wasps. And third, to estimate total fecundity of the mass-reared *Trichogramma*, we used hind tibia length as a proxy; hind tibia length and total fecundity are reported as highly correlated (Waage and Ming 1984, Olson and Andow 1998, Silva and Stouthamer 1999). We measured the hind tibia for 30 females and 20 males of each insectary sample. The length of the hind tibia was

measured as described by Bennett and Hoffmann (1998), with the following modifications: instead of Hoyer's mounting medium we used water, and we read the measurements using an optical micrometer mounted in the 40X-ocular of a phase contrast microscope Axioskop 40 Zeiss®. We used the hind tibia size classification used by Bai et al. (1992): small (< 0.13 mm), medium (≥ 0.13 to ≤ 0.16 mm) and large adults (> 0.16 mm).

Statistical analysis

From the six different Mexican insectaries, we received three shipments of 10 independent *Trichogramma* rearings. To analyze the quality of the reared *Trichogramma*, we grouped the *Trichogramma* rearings either as *T. pretiosum* or *T. fuentesi*; from each group, we tested the different rearings and the different shipments for statistical differences in body size, sex ratio and embryonic mortality. To test for statistical differences in sex ratio, body size and embryonic mortality, we used a MANOVA model with an alpha of 0.05; the independent variables were shipments and rearings. We adjusted for multiple comparisons using Tukey's test. All our experimental data, including sex ratio, conformed to the MANOVA assumptions: random selection of individuals, equality of variances and normality of the residuals. All the statistical tests were performed using SAS V.9.2. We could not compare the two insectaries that produced *T. fuentesi* statistically because (during the second and third shipment) we had only one *Trichogramma* sample.

3.4 Results

Two of the insectaries reported only one reared species and four insectaries reported two reared species totaling 10 *Trichogramma* rearings (table 1). The initial stock of these 10 rearings had originated in five cases from other Mexican insectaries, from the field in two cases, and from unknown sources in three cases. When the source was unknown the initial stock could have been collected locally from the field or received from other Mexican insectary. In all the rearings (data not shown) the host eggs were eggs of the angoumois grain moth (*Sitotroga cerealella*). According to the insectaries three species of *Trichogramma* (*T. pretiosum*, *T. exiguum*, *T. platneri*) and an unidentified *Trichogramma* species were mass-reared; however, DNA species identification showed that only two species were mass-reared, either *T. pretiosum* or *T. fuentesi* (table 1). Our multiplex PCR identification of approximately 600 specimens always showed the PCR product size consistent with either *T. pretiosum* or *T. fuentesi*. None of the rearing samples consisted of a mixture of these two species. The insectary personnel correctly identified *T. pretiosum* in all four cases; however, four supposedly *T. exiguum* were identified as *T. fuentesi* in two cases and as *T. pretiosum* in the other two cases. Also, the *T. platneri* shipment was identified as *T. pretiosum*, and finally the unidentified *Trichogramma* species also was *T. pretiosum*.

In the first shipment, *T. pretiosum* was produced in eight rearings and *T. fuentesi* in two (table 1). However, during the second and third shipments for rearing 4a, *T. fuentesi*

had been replaced by *T. pretiosum*. We also found that none of the *Trichogramma* samples was infected with *Wolbachia*.

Table 3.1. Species of *Trichogramma* parasitoids which were mass-reared as ten separate rearings during 2010 in the different insectaries from central and northern Mexico. Two *Trichogramma* species were identified: *T. pretiosum* and *T. fuentesi*.

			Origen of the	Reported	Identified species			Wolbachia
Insectary	State	Rearing	stock	species	Ship. 1**	Ship. 2	Ship. 3	infected
1	Baja C.S.	1a	unknown*	*T. pretiosum*	*pretiosum*	*pretiosum*	*pretiosum*	no
2	Coahuila	2a	insectary #3	*T. pretiosum*	*pretiosum*	*pretiosum*	*pretiosum*	no
		2b	native	*T. exiguum*	***fuentesi***	***fuentesi***	***fuentesi***	
3	Colima	3a	native	*T.* spp.	*pretiosum*	*pretiosum*	*pretiosum*	no
		3b	unknown insectary	*T. exiguum*	*pretiosum*	*pretiosum*	*pretiosum*	no
4	Durango	4a	insectary # 2	*T. exiguum*	***fuentesi***	*pretiosum*	*pretiosum*	no
5	Sonora	5a	insectary #1	*T. pretiosum*	*pretiosum*	*pretiosum*	*pretiosum*	no
		5b	insectary #2	*T. exiguum*	*pretiosum*	*pretiosum*	*pretiosum*	no
6	Sonora	6a	unknown	*T. pretiosum*	*pretiosum*	*pretiosum*	*pretiosum*	no
		6b	unknown	*T. platneri*	*pretiosum*	*pretiosum*	*pretiosum*	no

Body size

No significant difference was found in male size of *T. pretiosum* among shipments (MANOVA $F_{2,9}$= 2.67, P=0.0703), but we found differences between rearings (MANOVA, $F_{2,9}$= 2.25, P=0.0226) (table 2). We found that rearing 4a (the last two shipments) had the largest male body size as measured by the hind tibia length (0.14±0.002 mm), and

rearing 1a had the smallest male body size (0.134±0.001 mm). The other seven rearings had intermediate values (table 2). With respect to female body size, we did not find statistical differences among the nine rearings, nor among the three shipments (MANOVA model, F= 1.60, df=10, P=0.1049) (tables 2 and 3). Overall, mean female body size was 0.140 ± 0.001 mm. We measured the body size of males and females of *T. fuentesi* from two rearings. There was no significant difference in male body size among the three shipments, nor between the two rearings (MANOVA Model, F= 2.08, df=3, P=0.1094) (tables 2 and 3). From the two insectaries that mass-reared *T. fuentesi*, male body size was 0.135 ± 0.002 mm. Likewise, female body size did not differ among shipments, nor between rearings (MANOVA model, F=0.63, df=3, P= =0.5993) (tables 2 and 3). Mean female body size was 0.138±0.001 mm.

Sex ratio

For *T. pretiosum*, no significant differences were found in the sex ratios of the three shipments, nor among the nine rearings (MANOVA model, F= 0.78, df= 11, P=0.6460) (tables 2 and 3); the mean sex ratio of all the nine rearings was 0.552 ± 0.038. Regarding the sex ratio of *T. fuentesi*, we could not perform statistical analysis because during the first shipment, only two rearings produced *T. fuentesi*, and during shipment two and three, only one rearing produced *T. fuentesi*. Therefore, we reported, only their average value during the three shipments in rearing number 2b, and the measured value of the first shipment of rearing 4a; the sex ratio in both cases was 0.58 (table 2).

Embryonic mortality

With *T. pretiosum*, we did not find differences in embryonic mortality among the three shipments, nor among the nine rearings (MANOVA model, F= 1.71, df= 10, P=0.1732) (tables 2 and 3); the nine rearings had an embryonic mortality of 21 ± 4.48%. We could conduct statistical analysis of embryonic mortality of *T. fuentesi* due to a lack of samples, as previously described; therefore, we report only the average value for rearing 2a and the measured value of rearing 4a, 31% and 19% embryonic mortality, respectively (table 2).

Table 3.2. Body size, sex ratio and embryonic mortality from mass reared *Trichogramma pretiosum* and T. fuentesi in three shipments from different insectaries (CREROBs and private) located in central and northern Mexico. * - This rearing sample was DNA-identified as *T. fuentesi* during the first shipment, but during the second and third shipment was DNA-identified as *T. pretiosum*. ** - During shipment two and three, only one rearing produced *T. fuentesi*; therefore, we could not perform statistical analysis on sex ratio and embryonic mortality; the values reported here are the average of three shipments for rearing number 2b, and the measured value for place 4a. Means and S.E.'s followed by same letter are not statistical different (MANOVA tests, with an alpha of 0.05 adjusted for multiple comparisons using Tukey's test).

		Body size				**Sex ratio**		**Embryonic mortality**	
		T. pretiosum Mean (S.E.)		*T. fuentesi* Mean (S.E.)		*T. pretiosum* Mean(S.E.)	*T. fuentesi* Mean(S.E.)	*T. pretiosum* Mean(S.E.)	*T. fuentesi* Mean(S.E.)
Insectary		♂	♀	♂	♀				
1	a	0.134(0.001)[b]	0.139(0.001)[a]			57.66(3.81)[a]		23.52(5.62)[a]	
2	a	0.136(0.001)[ab]	0.142(0.001)[a]			56.66(3.81)[a]		18.33(4.48)[a]	
				0.141(0.003)[a]	0.139(0.002)[a]		58**		19**
3	a	0.138(0.001)[ab]	0.141(0.001)[a]			50.66(3.81)[a]		13.66(4.48)[a]	
	b	0.140(0.001)[ab]	0.142(0.001)[a]			50.33(3.81)[a]		14.00(4.48)[a]	
4	a	0.143(0.002)[a]	0.139(0.001)[a]	0.134(0.001)[a]	0.139(0.001)[a]	52.60(4.76)[a]	58**	23.02(5.62)[a]	20**
5	a	0.137(0.001)[ab]	0.141(0.001)[a]			60.66(3.81)[a]		23.00(4.48)[a]	
	b	0.138(0.001)[ab]	0.139(0.001)[a]			54.66(3.81)[a]		22.00(4.48)[a]	
6	a	0.135(0.001)[ab]	0.138(0.001)[a]			56.66(3.81)[a]		31.66(4.48)[a]	
	b	0.136(0.001)[ab]	0.137(0.001)[a]			56.00(3.81)[a]		24.00(4.48)[a]	

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Table 3.3. Mean body size, sex ratio and embryonic mortality of mass reared *Trichogramma pretiosum* and *T. fuentesi* in three shipments from different insectaries (CREROBs and private) located in central and northern Mexico. * - During shipment two and three, only one rearing produced *T. fuentesi*; therefore, we could not perform statistical analysis in sex ratio and embryonic mortality. Means and S.E.'s followed by same letters are not statistical different (MANOVA tests, with an alpha of 0.05 adjusted for multiple comparisons using Tukey's test).

	Body size				**Sex ratio**		**Embryonic mortality**	
	T. pretiosum Mean (S.E.)		*T. fuentesi* Mean (S.E.)		*T. pretiosum* Mean(S.E.)	*T. fuentesi* Mean(S.E.)	*T. pretiosum* Mean(S.E.)	*T. fuentesi* Mean(S.E.)
Shipment	♂	♀	♂	♀				
1	0.139(0.001)^{a}	0.140(0.001)^{a}	0.138(0.001)^{a}	0.140(0.001)^{a}	54.31(2.20)^{a}	*	25.51(3.09)^{a}	*
2	0.136(0.001)^{b}	0.141(0.001)^{a}	0.138(0.001)^{a}	0.138(0.002)^{a}	53.55(2.20)^{a}	*	22.33(2.59)^{a}	*
3	0.136(0.001)^{ab}	0.138(0.001)^{a}	0.136(0.001)^{a}	0.138(0.002)^{a}	57.44(2.20)^{a}	*	16.55(2.59)^{a}	*

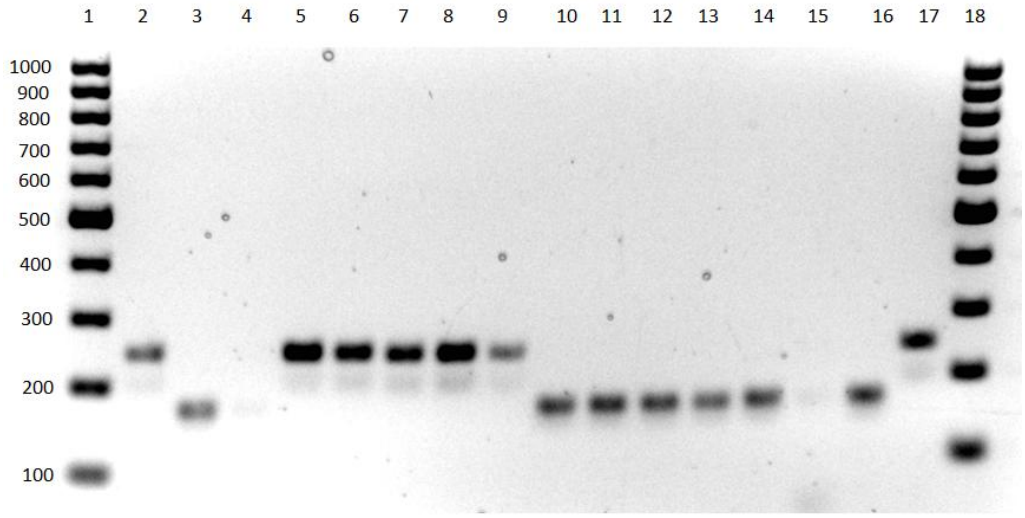

Figure 3.1. Gel showing the species-specific multiplex-PCR for distinguishing *Trichogramma pretiosum* and T. fuentesi. Lane 1, 100-bp ladder size standard (Fermentas). Lane 2, *T. pretiosum* (positive control). Lane 3, *T. fuentesi* (positive control). Lane 4, water (negative control). Lane 5-9, *T. pretiosum* (PCR-products of rearing 5b). Lane 10-14, *T. fuentesi* (PCR-products of rearing 2b). Lane 15, water (negative control). Lane 16, *T. fuentesi* (positive control). Lane 17, *T. pretiosum* (positive control). Lane 18, 100-bp ladder size standard (Fermentas).

3.5 Discussion

The six insectaries reported that they produced four *Trichogramma* species (*T. pretiosum, T. exiguum, T. platneri* and *T*. sp.); however, we identified only two species (*T. pretiosum* and *T*. *fuentesi*). In Mexico, misidentification of *Trichogramma* parasitoids was reported earlier by Garcia-Gonzalez et.al (2005) and Espana-Luna et. al. (2006b); they reported that *T*. *fuentesi* was commonly misidentified as *T*. *exiguum*. Misidentification of *Trichogramma* parasitoids is not unusual because morphological identification of *Trichogramma* parasitoids is difficult (see the introduction for details). Specifically, due to the phenotypic plasticity of the morphological characters, it is easy to confuse *T*. *fuentesi* with *T*. *exiguum* because in one of the last couplets in the morphological key, the broad and short ventral ridge of *T*. *fuentesi* can be interpreted as narrow and long; if this mistake occurs the identified species would be *T*. *exiguum* (Pinto 1998). For the same reason, i.e. misinterpreting the morphological characters, *T*. *pretiosum* can be identified as *T. platneri* and similarly, T. *pretiosum* as *T*. *exiguum* (Pinto 1998).

Due to the complexity of morphological identification of *Trichogramma* parasitoids and the latent risk of misidentification, some researchers have sent their specimens to be identified by taxonomic experts (Rodriguez and Smith 1991, O'Neil et al. 1998, Lundgren and Heimpel 2002, Hohmann and Lovato 2003, Schmidt et al. 2003, Herz et al. 2007) or reported their results as *Trichogramma* sp. (Perales and Arredondo-Bernal 1994, Avila-Rodriguez et al. 2010). The many difficulties of *Trichogramma* identification remains an

obstacle for using uncommon *Trichogramma* species (native species) in biological control projects. In Mexico, the insectaries rear almost exclusively *T. pretiosum* (Garcia-Gonzalez et al. 2005, Espana-Luna et al. 2008); releasing a generalist parasitoid, such as *T. pretiosum* (Pinto 1998, Zucchi et al. 2010) will match the target pest most of the time; however, specificity of *Trichogramma* parasitoids have been reported in habitat (Thorpe 1985, Romeis et al. 2005), host preference (Curl and Burbutis 1978, Yu et al. 1984, Stevens 1995), searching capacity and climate ranges (Hassan 1994). The use of native species opens the possibility of more efficient use of *Trichogramma* parasitoids, because native *Trichogramma* species are likely better adapted to specific habitats or hosts and consequently may result in better pest control than the species currently mass reared (Pinto 1998).

Fortunately, using current techniques of molecular identification (as in the present work, see fig. 1), we can easily and accurately identify *Trichogramma* species. Twenty three native species have been reported from Mexico (Pinto 1998); therefore, we recommended to generate a DNA-molecular identification key for all of these species. This DNA molecular key can be developed by first determining the DNA sequences of the ITS2 region for each of the species. Second, based in the size of each ITS2, roughly subdivide the 23 species in three species-specific multiplex-PCR groups, grouping together the species with similar ITS2 sizes. Third, based on the need for identifying specific species, species-specific primers can be developed (as we did here) for each group based on the areas of maximal difference of the species specific DNA fragments.

After linking the ITS2 DNA sequence for these native species with their name based on morphology, we can use their DNA sequences to verify the identity of field collected specimens, and after being verified, we can test the native parasitoids for their potential habitat or host specificity. In Portugal, a similar approach of molecular identification was developed to distinguish five local species of *Trichogramma* (Silva et al. 1999)*;* also in Mexico, Espana-Luna et al. (2008) developed a dichotomous molecular key to distinguish five *Trichogramma* species of agricultural importance (insectary and field species).

Trichogramma populations when infected for the bacterial symbiont *Wolbachia* reproduce by parthenogenesis (asexual reproduction); in this paper we found that none of the *Trichogramma* samples were infected with *Wolbachia*; therefore, to determine the quality of the reared *Trichogramma*, we used the suggested minimum standards for sexual *Trichogramma*. In the nine rearings that produced *T. pretiosum, w*e found a mean sex ratio of 0.552 ± 0.038 and a mean embryonic mortality of 21 ± 4.48. Therefore, in sex ratio and embryonic mortality, *T. pretiosum* fulfilled the minimum standards suggested by the IOBC/EC. Comparing mass-reared MX *T. pretiosum* with mass-reared U.S.A. *T. pretiosum*, U.S.A. *T. pretiosum* has a lower embryonic mortality (10±4.8) and a higher sex ratio (≥0.87); however, these *Trichogramma* colonies were infected by the bacteria parthenogenesis-inducing *Wolbachia* (Heimpel and Lundgren 2000, Lundgren and Heimpel 2003). As stated in the results section, with sex ratio and embryonic mortality of *T. fuentesi*, we did not perform statistical analysis for lack of replication;

however, the measured values of the two rearings, similar for *T. pretiosum*, fulfilled the minimum quality requirements suggested by the IOBC/EC. In body size, based in the classification made by Bai et al. (1992), we found that the adults (males and females) of *T. pretiosum* and *T. fuentesi* had a medium body size; therefore, we expect that the females of both species (*T. pretiosum* and *T. fuentesi*) will have an intermediate field performance with respect to fecundity (Bai et al. 1992) and the ability to locate a host egg (Kazmer and Luck 1995). To measure the quality of the *Trichogramma* parasitoids, other tests have been suggested, for example walking or flight tests; however, these tests have not been adopted because they have not been correlated with *Trichogramma* field performance. Walking and flight tests need improvement in order to be incorporated in routine quality assessment tests (Van Lenteren et al. 2003). Throughout the different rearings of the Mexican insectaries, we found a great uniformity in body size, sex ratio and embryonic mortality (tables 1, 2 and 3). This uniformity in performance can be explained by three reasons. First, there is one national center of research and development in biological control which transfers technology to the different CREROBs and private producers (Dominguez 1996); therefore, it is expected that technology and procedures should be very similar among the different insectaries, for example, all the insectaries used the angoumois grain moth as host eggs (Garcia-Gonzalez et al. 2005). Second, there is a lot of exchange of material among the insectaries (table 1, and Mexican insectary background, see the introduction for details); therefore, possibly the different rearings have a common genetic background. And

third, some of the insectaries initiated their rearing stocks from field collected *Trichogramma*. *Trichogramma* parasitoids have been released in Mexico on a large scale since 1962 (Arredondo-Bernal and Sanchez-Gonzalez 2009); collecting in areas in which inundative releases of *Trichogramma* have occurred raises the possibility that the collected *Trichogramma* are genetically related to insects previously reared under insectary conditions (Chassain and Bouletreau 1991, Pinto 1998, Kuske et al. 2003). Because of the minute size of *Trichogramma* parasitoids (body size ≤ 1mm) many unnoticed changes can reduce the quality of the reared insects. Changes may included inbreeding depression (Antolin 1999), over-parasitization of the host eggs (Salt 1936) or inadvertent replacement of the reared species by other *Trichogramma* species (in the present work *T. fuentesi* was replaced by *T. pretiosum)*. Such unnoticed changes are likely to result in the release of *Trichogramma* adults that are of low quality. It is assumed that low quality of mass-reared *Trichogramma* parasitoids can be compensated for with massive releases of individuals (Bourchier et al. 1993); however Dutton et al. (1996) showed that high numbers of low quality insects is not directly related with the intensity of field parasitism. In order to avoid releasing low quality *Trichogramma* wasps, quality assessment of mass-reared *Trichogramma* species should be done as frequent as possible, possibly every month (Laing and Bigler 1991, Garcia-Gonzalez et al. 2005). Situations as found in this study and in others (Lundgren and Heimpel 2002, Garcia-Gonzalez et al. 2005, Espana-Luna et al. 2006a, Sumer et al. 2009),

where the wrong species is being delivered for biocontrol purposes do not contribute to the success of biological control and must prevented.

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Chapter 4. Differential clonal performance in a *Trichogramma pretiosum* population in which parthenogenetic inducing *Wolbachia* has gone to fixation

4.1 Abstract. *Trichogramma pretiosum* populations in Kauai, Hawaii consists entirely of females that are infected with Parthenogenesis Inducing *Wolbachia* bacteria (PI-*Wolbachia*). These populations have been completely infected for at least the last thirty years but possibly as long as one hundred years. Here we determined life history traits of 15 different clonal lines, belonging to the two major clonal types found on the island. Upon fixation of PI-*Wolbachia* in a population, several changes are expected in the relationship between the PI-*Wolbachia* and the nuclear genome of their wasp hosts. Before fixation of PI-*Wolbachia* in a population, the conflict between the evolutionary interests of the host and the *Wolbachia* sometimes result in poor transmission of *Wolbachia*, but upon fixation, this conflict is resolved and high *Wolbachia* transmission rates are expected to evolve. Consistent with this hypothesis, we found a very high PI-*Wolbachia* transmission rate of >99.9%. Whereas early in the fixation of the infection in the population, many different genetic clones are expected in the overall population, subsequent clonal competition should result in the rapid elimination of the less fit clones, resulting in a reduction in the number of different clones present. We found that the 15 clones we tested differed in their offspring production with the least productive clone producing approximately 50% of the offspring of the most productive clone. How this large number of different clones can co-exist in the populations is not clear, but

differences in adaptations of the clones to different environmental conditions may contribute to the surprisingly large number of clones present in the population. A factor that may contribute to the clonal co-existence became evident in a clonal competition experiment done in glass vials where we competed two different clones against each other and found that the elimination of one or the other clone depended on the interval at which the populations were given hosts.

4.2 Introduction

In haplo-diploid organisms, such as *Trichogramma* egg parasitoids, the normal mechanism of sex determination is that females are produced from fertilized (biparental) eggs and males are produced from unfertilized (uniparental) eggs. Some of these haplo-diploid populations have been invaded by symbiotic bacteria of the genus *Wolbachia* that induce parthenogenesis (PI-*Wolbachia*) (Rousset et al. 1992, Stouthamer et al. 1993, Stouthamer 1997). PI-*Wolbachia* cause unfertilized eggs to develop into diploid females through gamete duplication (Stouthamer and Kazmer 1994). In PI-*Wolbachia*-infected populations, females will produce almost exclusively female offspring that again can produce daughters without mating. The production of males in some PI-*Wolbachia* infected populations is a rare event. Because the PI-*Wolbachia*-infected population produce almost exclusively females, and they do not need to mate, it is expected that these populations will have a higher rate of increase, and that over time, the infection will spread (Engelstadter 2008).

In mixed field populations, *Wolbachia*-free and PI-*Wolbachia*-infected individuals sometimes coexist (Stouthamer and Luck 1993, Stouthamer and Kazmer 1994, Stouthamer et al. 2001), and contrary to the assumption that PI-*Wolbachia* infection will spread through the whole population, the frequency of PI-*Wolbachia*-infected individuals in the field is sometimes low, e.g., up to 5% in *T. deion* and *T. pretiosum* (Stouthamer 1989) or up to 11% in *T. kaykai* populations from various localities in the

Mojave Desert (Pinto et al. 1997, Stouthamer et al. 2001). How these populations coexist for extended periods of time remains somewhat of a mystery. Several hypotheses have been posed. In *T. kaykai,* the infection remains around 10% over at least a period of 20 years. In this population, one hypothesis is that this frequency remains in equilibrium due to the presence of another sex ratio distorting element that causes the production of males from fertilized eggs. This so called PSR factor reduces female offspring production proportionally more for the infected part of the population, which results in a stable equilibrium with regard to infection frequency (Stouthamer et al. 2001). A second hypothesis was posed by Tagami et al. (2001) and later, Miura et al. (2009) showed that gamete duplication in unfertilized eggs laid by infected females was inefficient and a fraction of these eggs became stuck in the first or second mitotic division and subsequently died. Infected eggs that had been fertilized survived and consequently, a frequency dependent process kept the population from going to infection fixation. Finally, based on modeling, it can be shown that suppressor alleles that either kill or negate the effect of PI-*Wolbachia* can keep a PI-*Wolbachia* infection from reaching fixation. Even though such models predict stable equilibria at rates found in the field, there is no experimental evidence that such suppressor alleles exist (Stouthamer et al. 2001).

The fixation of PI-*Wolbachia* infections in many parasitoid populations is much easier to explain. Two conditions are required for a PI-infection to spread to fixation: 1) perfect

transmission of the PI-*Wolbachia* from mother to offspring and 2) infected females should produce more daughters than uninfected females. It is, however, unlikely that when PI-*Wolbachia* enters a population that it immediately exhibit perfect transmission. In several populations, the transmission of the PI-*Wolbachia* is not perfect at all and under such conditions, an extended equilibrium of infected and uninfected individuals is expected (Stouthamer et al. 2010). This eventually leads to selection for non-fertilization in the population, resulting in the fixation of the infection and a complete lack of sexual reproduction. In several species, patterns consistent with this non-fertilization mutation have been found (Jeong and Stouthamer 2005, Russell and Stouthamer 2011). Upon fixation for PI-*Wolbachia* infection in females, no subsequent sexual reproduction takes place, and a large part of the genome of the wasps will no longer be under selection. All traits involving sexual reproduction in females and all male-limited traits will experience a neutral decay, however female traits that are costly will be selected against if their non-function results in a gain on resources that benefit the female. For instance, if pheromones are no longer produced and the costs associated with their production can be reassigned to other fitness components of the mutant females, the proportion of these mutant females will increase in the population. Upon fixation, the conflict between PI-*Wolbachia* and the nuclear genes over offspring sex ratios will also be resolved (Stouthamer et al. 2010) and mutant alleles that lessen the negative effects of PI-*Wolbachia* on its host and vice versa, will be selected for both in the host genome and in the PI-*Wolbachia* genome. Because no subsequent sexual reproduction is

possible, these changes depend on mutations taking place. In many types of complete parthenogenesis that maintains heterozygosity, the rate at which such mutations are expressed is low, but under the mechanism of gamete duplication, mutations are rendered immediately homozygous and consequently will be expressed. Therefore, upon fixation of the infection and its accompanying lack of sexual reproduction, each clonal lineage becomes independent and different host nuclear genotypes and PI-*Wolbachia* genotypes will compete within the population. While asexual reproduction is often considered to quickly reduce the genetic variation present in a population, under these circumstances, at least initially after the fixation, one can expect the generation of a large number of different clonal types.

Here we studied *Trichogramma pretiosum* found on Kauai, Hawaii. This population consisted of exclusively PI-infected females. *Trichogramma pretiosum* is not native to Hawaii, but was either introduced on purpose or arrived with plant material. This species has been present on Hawaii most likely since at least 1900. It is not known if the species arrived as an asexual strain or initially as a mixed population, but by at least 1979 (Oatman et al. 1982), *Trichogramma pretiosum* was known to reproduce exclusively by parthenogenesis. The generation time for *Trichogramma* is relatively short, i.e. 10-12 days depending on temperature. Consequently, we expect that the wasps have been asexual for approximately 1,000 generations. In this population, we expect that mutations will have accumulated in the part of the genome coding for males

and for traits associated with sexual reproduction in females. Herein we studied several traits of *T. pretiousum* lines collected on Kauai to determine the changes that may have taken place in the population following fixation for PI-*Wolbachia*.

4.3 Materials and Methods

Population of *T. pretiousum* subject to study

In 2005, approximately 655 mostly Monarch butterfly eggs (*Danaus plexippus*) parasitized by *Trichogramma* parasitoids were collected in Kauai, Hawaii. From the parasitized eggs, we initiated 183 isofemale lines. Not all wasps parasitizing Monarch butterfly eggs were *T. pretiosum*, some were parasitized by *T. chilonis* and *T. papilionis.* We identified the wasps to species using ITS2 sequences (Stouthamer et al. 1999).

Female traits examined

To compare the performance of PI-*Wolbachia*-infected with PI-*Wolbachia*-cured females, we tried to cure these lines: we fed several (more than 100) newly emerged PI-*Wolbachia*-infected females with two antibiotics (rifampicin and tetracycline) at 0.5% g active ingredient of both antibiotics/ml honey (i.e. 1.0 g AI in total). Both antibiotics are known to cure the lines from the PI-*Wolbachia* bacteria (Stouthamer et al. 1990a). Females subjected to the antibiotic treatment were not easily mated with their male siblings and when mated, they produced a very male biased sex ratio of about 90%; this low fertilization rate did not allow for the establishment of sexual lines from the PI-*Wolbachia* lines.

PI-*Wolbachia* effect and overall embryonic mortality

PI-*Wolbachia* infected eggs develop into diploid females through gamete duplication (Stouthamer and Kazmer 1994). The meiosis in these wasps is normal and the haploid egg becomes diploid because of a segregation failure of the two sets of chromosomes in the anaphase of the first mitotic division. This process of gamete duplication does not always work with 100 percent efficiency; in some PI-*Wolbachia* infected *Trichogramma,* unfertilized and infected eggs show a higher mortality than fertilized and infected eggs (Tagami et al. 2001, Huigens et al. 2004, Miura and Tagami 2004). This failure of gamete duplication could be a side effect of the genomic conflict between PI-*Wolbachia* and the nuclear genome during the time period when the infection with PI-*Wolbachia* is not fixed in the population. Consequently, we would expect that this failure of gamete duplication would be less in lines where the infection is fixed.

To determine the effect of PI-*Wolbachia* (early embryonic mortality) and the overall embryonic mortality of oviposited eggs in the *T. pretiosum* Hawaiian population, we tested four clonal lines (3, 4, 9 and 10), and as a positive control, we included a *Wolbachia*-free mated population of *T. pretiosum* from Irvine, California (SC-100). We tested these five lines, as follows: we offered an individual wasp a card strip containing 12 to 20 *Ephestia kuehniella* host eggs, which were attached to the card using water as glue. Using a V8 Zeiss® stereoscope, we considered that a wasp oviposited an egg into a host egg if the wasp performed the entire oviposition sequence, whose stages

(drumming, drilling and oviposition) were previously described by Klomp et al. (1980) and Suzuki et al. (1984). After oviposition, we removed the parasitized host egg, and placed it in a separate vial. We repeated this procedure to obtain at least 100 parasitized host eggs per line. Fifteen days later, we checked the vials for the end result of the oviposition, i.e. eggs were classified as showing early embryonic mortality (clear eggs), late embryonic mortality (black eggs) or adult emergence (black eggs with holes). Host eggs turn black once parasitoid larvae reach the prepupal stage (Pintureau et al. 1999). The developmental time from egg to adult in *T. pretiosum* is between 10 and 11 days under our rearing conditions; therefore, checking the vials on day 15 allowed all viable wasps to emerge. To determine the accuracy of our ability to determine that an oviposition by the wasps indeed had taken place, we observed 79 oviposition sequences on *E. kuehniella* host eggs and immediately dissected the host eggs using a phase contrast Axioskop 40 Zeiss® microscope for the presence of wasp eggs. In all cases, we found a *Trichogramma* egg in the parasitized host egg.

PI-*Wolbachia* transmission rate

We measured the PI-*Wolbachia* transmission rate from mother to offspring, using as a proxy the offspring sex ratio of unfertilized infected females, in the four clonal lines (3, 4, 9 and 10) as follows: 30 females per clonal line, during their lifetime, were provided individually with egg cards containing approximately 300 host eggs (*E. kuehniella*), which were attached to a card using double sided sticky tape. Each wasp received a new egg

card every other day and the two day old egg card was transferred to a new vial. After 15 days of embryonic development, we determined the number of male and female offspring emerged from each egg card. Female sex ratio was calculated as the number of females divided by total offspring. Males and females were identified using morphological characteristics of the antennae (Pinto and Stouthamer 1994). We repeated this experiment twice.

Reduction of clonal lines

Stouthamer et al. (2010) suggested that in population in which PI-*Wolbachia* has gone to fixation, interspecific competition among clonal types should lead to a reduction in the number of different clones. Slight differences in fitness should result in the rapid elimination of less fit clones (Engelstadter 2008).

To test for clonal reduction, we competed two clonal lines (3 and 9) as follows: we put together 25 females of each clonal type in borosilicate glass tube (12 x 75 mm, VWR®, West Chester, PA), which was closed off with a cotton plug. To each combined population, during 10 consecutive generations, we offered two host egg cards in one experiment every 10 days (4 replicates) and in a second experiment every 11 days (5 replicates). Each generation, one of the two egg cards was used to continue the clonal competition experiment, and the wasps emerging from the other egg card were frozen and stored in 90% ethanol, to later determine the relative proportion of each clonal

type during each generation. In these competition experiments, we chose clonal line 3 and 9 because from previous results (data from the PI-*Wolbachia* transmission experiment), we found that the two lines did not differ in total fecundity during their second day of oviposition (ANOVA test, F= 0.97, df= 3, P= 0.3289) or during their entire lifetime (ANOVA test, F= 7.56, df= 3, P= 0.4446).

We determined the relative proportion of each clonal type by qPCR and melting curve analysis as follows: the mitochondrial cytochrome oxidase I gene (COI) of both clonal lines was direct-sequenced in both directions at the University of California, Riverside, Genomics Institute, Core Instrumentation Facility using an Applied Biosytems 3730 DNA analyzer with a Big-Dye® V3.1 kit (Applied Biosystems, Foster City, CA). From the resultant COI sequences, we developed primers surrounding a section containing a single nucleotide polymorphism (table 1). The sequence of the *T. pretiosum* forward primer was 5'- ATA CTT CAT TTT TTG ATC-3', and the sequence of the reverse primer was 5'-GAT GCT GAT ATA AAA TAG-3'. Using these primers in quantitative PCR followed by resolution melting analysis, which dissociates (over a temperature gradient) the double strand of DNA, we generated the melting pattern of the amplified *COI* gene. The melting pattern for the two forms differed consistently allowing us to assign a clonal type to each wasp. DNA was extracted, using the Chelex-100 protocol (Walsh et al. 1991), from 23 wasps per generation per competition experiment. Each wasp was ground in 60 µl 5% Chelex-100 (Bio-Rad laboratories, Hercules, CA) and 2 µl proteinase

K (20 mg/ml) and then each mixture was incubated over 60 min at 55°C, followed by 10 min at 99°C. We amplified the DNA using the following PCR master mix: 2 µl DNA template, 10 µl Syber Green master mix (dNTP's, buffer and HotStartTaq Plus DNA polymerase enzyme) (Clontech, Mountain View, CA), 0.4 µl forward and 0.4 reverse primer for *T. pretiosum* CO1 and 7.2 µl sterile distilled water. PCR was performed in 20 µl reaction volumes using a real time PCR Rotor-Gene-300 (Corbett Research, Valencia, CA). The qPCR program was 15 min at 95°C followed by 40 cycles of 10 seconds at 94°C, 20 seconds at 50°C and 10 seconds at 68°C, with one min at 65°C after the last cycle. The melting curve was generated increasing the temperature in 0.2°C intervals from 65-80°C, holding 20 seconds on the first step and 5 seconds on subsequent steps.

Table 4.1. Aligned sequences of the mitochondrial cytochrome oxidase I gene of two clonal lines of *Trichogramma pretiosum* from Hawaii, in which PI-Wolbachia infection has gone to fixation. Dashes (-) indicate identity to the line 3 sequence.

Clonal line	Mitochondrial-cytochrome-oxidase-I-gene sequences
3	ATACTTCATTTTTTGATCCTTCTGGGGGCGGTGATCCTATTTTATATCAGCATC
9	----------T----------

Fitness differences among clonal lines

To measure the overall fitness of the clonal lines, we used as a proxy their total fecundity. We tested the total fecundity of 16 clonal lines following the procedure of the

PI-*Wolbachia* transmission experiment. However, this time, we tested 15 females per clonal type, and we gave a host egg card every two days for a total period of six days. Total fecundity was calculated as the number of males and females produced on all the egg cards during the entire experimental period. To determine the stability of the total fecundity trait over successive generations, 10 generations later, we again tested 15 of the 16 clonal lines for their total fecundity. Due to human error, we did not include clonal line 9.

All experiments were done in an incubator precision 818 L.T.C.® (Thermo Fisher Scientific, Inc., Pittsburgh, PA) set at 25°C and 16:8 h L:D photoperiod. The humidity was not controlled in these experiments. All statistical analyses were performed using SAS® V.9.2 statistical software, and in all tests, our criterion for statistical significance was an alpha value of 0.05. All experimental data conformed to ANOVA assumptions, i.e. random selection of individuals, equality of variances, and normality of the residuals.

4.4 Results

PI-*Wolbachia* effect and overall embryonic mortality

We found differences in the early embryonic mortality (a PI-Wolbachia effect) among the four clonal lines; three clonal lines (3, 4, and 9) suffered high embryonic mortality, and one clonal line (10) suffered the lowest embryonic mortality. But, we did not find differences in the early embryonic mortality between the four clonal lines and the *Wolbachia*-free mated *T. pretiosum* strain (X^2 test with Bonferroni correction, $F \leq 4.2892$, df= 4, $P \geq 0.0384$) (Table 2). With regard to overall embryonic mortality, we also found differences among the four clonal lines. Lines 3 and 4 showed the highest embryonic mortality, line 9 had intermediate embryonic mortality, and line 10 showed the lowest mortality rate. Comparing the overall embryonic mortality between the four clone lines and the *Wolbachia*-free mated *T. pretiosum,* we found that three of the clonal lines had a similar embryonic mortality (3, 4 and 9), but one clone line (10) had a lower embryonic mortality rate than the *Wolbachia*-free population (X^2 test with Bonferroni correction, $F \leq 9.4073$, df= 4, $P \geq 0.0022$) (Table 2).

PI-*Wolbachia* transmission rate

We did not find a difference in the PI-*Wolbachia* transmission rate among the four clonal lines. The four clonal lines showed no significant differences in offspring sex ratios, with a mean of 0.99±0.001 (ANOVA test, F= 1.61, df= 3, P= 0.19). Clonal line 3 produced 1 male in 929 offspring, line 4 produced 1 male in 787 offspring, line 10

produced 5 males in 1,587 offspring, and line 9 produced 7 males in 1,301 offspring (table 3). No males were produced during the first six days of oviposition for any clonal line.

Table 4.2. Early and overall embryonic mortality of the oviposited eggs in four clonal lines (PI-Wolbachia-infected) of *Trichogramma pretiosum* from Hawaii and a sexual (Wolbachia-free) line from California. Early embryonic mortality indicates clear eggs, i.e. eggs that did not develop in their initial stages, and overall embryonic mortality indicates all oviposited eggs that failed to develop as adults. Means followed by the same letter in a row are not statistically different (X^2 test of independence with Bonferroni correction, α =0.05).

		Oviposited	Embryonic mortality (%)	
Population	Lines	eggs	early	overall
asexual	3	169	0.13[a]	0.23[a]
asexual	4	122	0.17[a]	0.24[a]
sexual	SC-100	103	0.09[a b]	0.18[a]
asexual	9	205	0.12[a]	0.17[a b]
asexual	10	198	0.06[b]	0.09[b]

Fitness differences among clonal lines

With regard to both evaluations of the clonal lines, i.e. over time, we found small differences in total fecundity among the clonal lines: the 16 clonal lines at the time of the first measurement fell into three groups, with a 46% difference in performance between the least and the most fecund of the 16 clonal lines; and the 15 clonal lines

Table 4.3. Number of males produced, as a function of total fecundity, per individual female of four clonal lines of *Trichogramma pretiosum* from Hawaii.

	Females: identification number	produced males	first-male produced at day	females produced before first-male produced	Total fecundity
	2	1	9	39	55
line 3	1 (29)*	1			929
	23	1	9	38	39
line 4	1 (29)	1			787
	5	1	9	40	49
	10	1	7	43	53
	15	1	7	44	55
	17	3	7	45	60
	22	1	7	54	68
line 9	5 (29)	7			1301
	2	1	9	68	86
	8	3	7	50	97
	28	1	7	57	65
line 10	3 (30)	5			1587

*.- numbers within parenthesis indicate the total number of females tested per clonal line, and numbers behind parenthesis indicate the number of females per clonal line that produced one or some males.

during the second measurement fell into six groups with a 51% difference in performance between the least and the most productive of the 15 clonal lines. At the time of the second measurement, we did not include line 9 (fig. 1). Comparing the first performance of the 15 clonal lines with their second performance, we found that the degree of similarity between the two data sets was 96% (using Kendall's rank correlation coefficient).

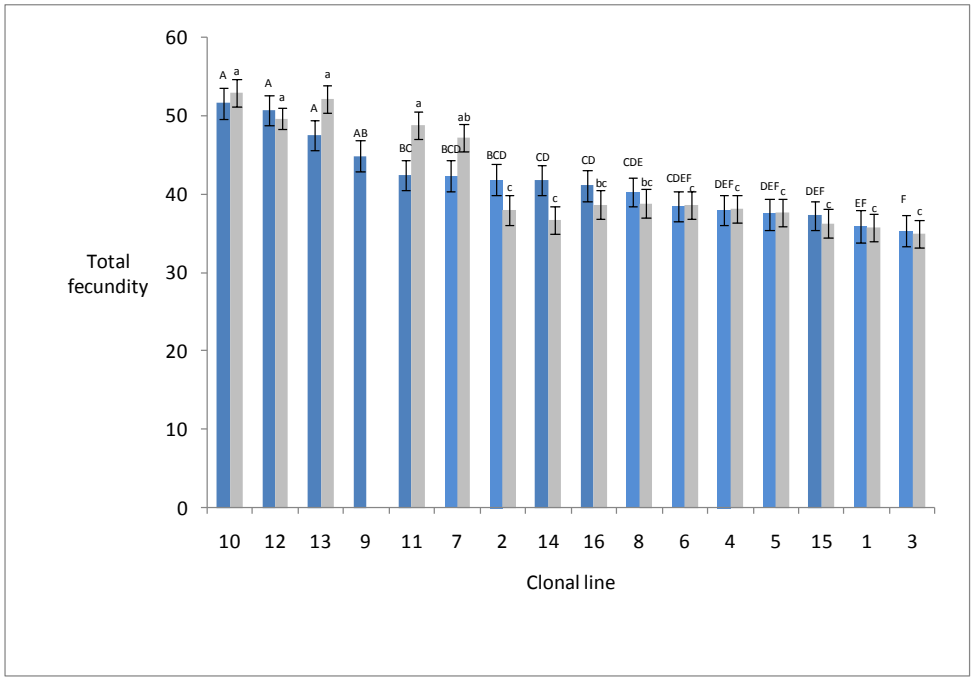
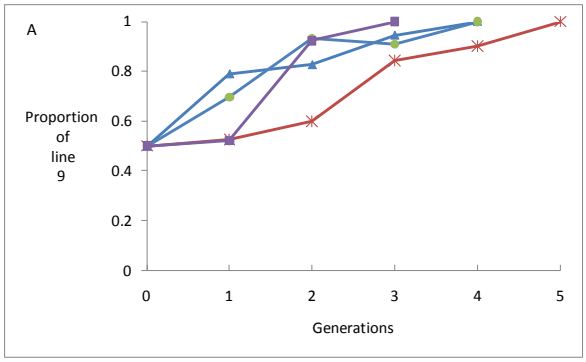


Figure 4. 1. Total fecundity (males and females) during six days in 16 clonal lines of a *Trichogramma pretiosum* population from Hawaii. The first measurement (dark bars) of the clonal lines is arranged from the highest to lowest performance (left to right), and the second measurement (light bars) of the clonal lines follows the pattern of the first measurement. During the second measurement, we measured only 15 clonal lines. Capital letters indicate statistical separation based on the first measurement, and lower cap letters indicate statistical separation during the second measurement. Means and errors bars followed by the same letter (either capital or lower cap) are not statistically different (ANOVA test, with an alpha of 0.05 adjusted for multiple comparisons using Tukey's test).

Clonal competition

In the competition experiments between two clonal lines, we found that when host eggs were provided at an interval of every 10 days, line 9 always outcompeted line 3 consistently over the four replicates and line 9 always went to fixation at generation five (or earlier) (fig. 2a). However, when the host eggs were given every 11 days, both lines coexisted within three of four replicates after 10 generations. Fixation did take place in one case for line 3 and in another case for line 9 (fig. 2b).



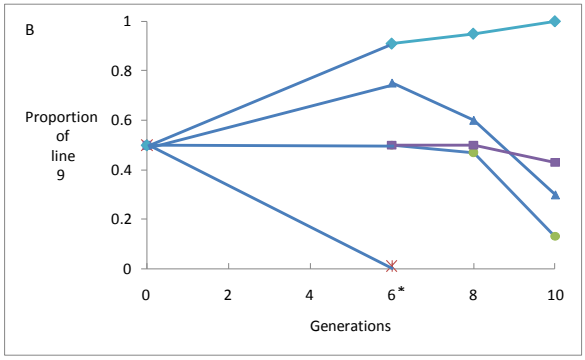


Figure 4.2. Clonal competition between two *Trichogramma pretiosum* clones (clonal line 9 and 3) when host eggs were provided (A) every 10 or (B) every 11 days. *.- In the competition experiment for host eggs provided every 11 days, we did not take data during the first five generations.

4.5 Discussion

Non-fertilizing allele and degeneration of female traits

In this study, we could not restore sexual (PI-*Wolbachia*-cured) lines from the infected lines because these cured females showed a very low mating responsiveness and even when mated, they fertilized only 10% of their eggs (pers. comm. B. Callaham). *Trichogramma pretiosum* from Kauai was previously tested for their egg fertilization rate in wasps collected in 1986. Unfortunately, only a single isofemale line was tested (Stouthamer et al. 1990b) and at that time, the females of this line fertilized 40% of their eggs. We cannot draw strong conclusions from this one data point, but a reduction in the fertilization rate of these females over time would be expected (Stouthamer et al. 2010). According to modeling (Stouthamer et al. 2010), the complete loss of fertilization should be selected for rapidly, under the assumption of some inefficiency in the transmission of the PI- *Wolbachia* from mother to offspring. If, however, the transmission becomes quite efficient, as we find in these populations, the complete loss of sexual function of the infected females should be slowed down substantially.

PI-*Wolbachia* effect

The PI-*Wolbachia* effect, i.e. early embryonic mortality of the oviposited eggs, has been estimated in mixed populations by comparing the early mortality of oviposited eggs, between infected lines versus PI-*Wolbachia*-cured lines. In all of these studies, some negative effect has been found, i.e. the PI-*Wolbachia* infected lines showed higher early

embryonic mortality (Tagami et al. 2001, 2002, Huigens et al. 2004, Miura and Tagami 2004). We compared early embryonic mortality of the oviposited eggs with the control (a sexual *T. pretiosum*), and we found that none of the clonal lines differed significantly from the sexual control in their ability to develop. When PI-*Wolbachia* has gone to fixation, there is selective pressure to reduce the negative effects of PI-*Wolbachia* infection (Douglas 2009) and a more mutualistic relationship should develop over time (Stouthamer 1997). In this study, we found the early embryonic mortality to be the same as that in the control sexual line of *Trichogramma pretiosum*. Unfortunately, we did not have a proper control in the form of either an infected *T. pretiosum* line originating from a mixed population, or a sexual line that we had derived from our Hawaiian clones.

Transmission efficiency

As theorized by Stouthamer et al. (2010), we found that there appears to be a very high *Wolbachia* transmission efficiency consistent with expectations in populations where the infection has gone to fixation. The clonal lines produced almost exclusively female offspring (< 2 males per 100 adults produced). The production of males in PI-*Wolbachia*-infected species has been attributed at the depletion of bacterial titer (Huigens and Stouthamer 2003), i.e. eggs not infected with a high enough titer of the bacteria develop as normal haploid males. However, finding males under field conditions, at least in the Hawaiian population, has not been reported (Oatman et al. 1982,

Stouthamer and Luck 1993). It is obvious from our lab experiment that the males are only produced late during the female's oviposition period. In our experiments, the first males appeared at the earliest in the offspring of the 7th day of oviposition. Possibly, there is lack of males in field populations because few females will survive to that age in the field. Egg parasitoids, such as *Trichogramma*, emerge with approximately 50% of their ovarian eggs already mature (Olson and Andow 1998, Jervis et al. 2001), and under proper conditions, such as stable laboratory temperatures and an abundance of food, they continue producing eggs. Most likely, these later eggs could be more vulnerable to the depletion of the bacterial titer.

Fitness differences between lines

The variation found between the different clones in the number of offspring produced, may be an indication of the large number of different clones that co-exist in the Hawaiian field population. This variation is somewhat surprising because a simple view of clonal reproduction should result in a rapid elimination of clonal types with the lowest fitness. Yet it appears that many different clones co-exist. Apparently, the different clones each have some adaptation that makes them outcompete other clones under particular circumstances. An example of that may be the outcome of our clonal competition experiment in which line 9 in all 4 cases outcompeted line three within 5 generations if host eggs were supplied every 10 days, but these two lines coexisted for much longer when host eggs were supplied every 11 days. Similar results, i.e. variation

in performance among clonal lines, were found in a PI-*Wolbachia*-infected parthenogenetic population (99.5% females) of the salt-marsh parasitoid, *Anagrus sophiae*.

Cronin and Strong (1996) found a 54% difference in fecundity between the least and the most fecund of 41 clonal lines.

The observed clonal variation could have been caused by initial variation present in the population upon fixation, or may have been caused by the subsequent generation of new clonal types by changes in the wasps' or *Wolbachia*'s genome. Changes in the genome of both partners (wasp and symbiont) are expected to take place through mutation. We hypothesize that the large number of different clones is maintained by the differential performance of clones under different environmental circumstances. In relatively simple environments, clones are capable of rapidly eliminating each other as we showed in our clonal competition experiment. The large number of different clones present in a population may be a particular feature of cytogenetic mechanism of parthenogenesis. Under gamete duplication, all mutations are immediately rendered homozygous and will thus be expressed. Under most other mechanisms of parthenogenesis, heterozygosity is either maintained completely or is lost at a slow rate. In those cases, phenotypic changes following mutation in one of the two alleles at a locus will often remain hidden when such mutations are recessive.

From an applied point of view, if PI-*Wolbachia* infected wasps are considered for application in biological control, multiple clones should be collected in the field and maintained separately in the lab before they are released for biocontrol. In that way, the different genetic variants that apparently are present in the field are maintained and can better contribute to the control of pest insects in the release area.

4.6 References

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Concluding remarks

It was long assumed that inbreeding species such as *Trichogramma,* due to their haplo-diploid sex determination system and because of sibmating, would not suffer from inbreeding depression at all, or at least their inbreeding depression should be less than in other species (Waage and Ming 1984, Hopper et al. 1993, Grenier and De Clercq 2003). However, my results showed –as Antolin (1999) showed earlier– that inbreeding depression does take place in *Trichogramma*. In these experiments, we found evidence of inbreeding depression when measuring total fecundity: outbred females showed significant heterosis (16.2% ±2.1) in comparison with inbred females. However, inbreeding status did not influence sex ratios in a particular direction. We also found that lab adaptation can occur in *T. pretiosum* colonies if significant genetic variation is present in the initial population, but if the populations are already inbred, no response to the imposed methods of selection was possible. Previously, lab adaptation was studied in *Trichogramma* parasitoids and conflicting evidence was found (Urquijo 1951, Ashley et al. 1974, Abraham and Pradhan 1976, Carriere and Boivin 2001, Hoffmann et al. 2001, Kolliker-Ott et al. 2003, Pratissoli et al. 2004, Prezotti et al. 2004, Jalali et al. 2006). Based on our results, these conflicting results may be explained by the initial genetic variation present in the starting populations used by these researchers. If experiments were done with colonies that had been kept under lab conditions for several generations and had already lost a large part of their initial genetic variation,

then they could not respond to selection; but if the experiments were initiated with populations with high initial genetic variation, either from field collected individuals or by pooling inbred lines, then these populations did respond to selection. Our results are consistent with the fact that inbred populations with low genetic variability cannot adapt (Hoy 1979, Nunney 2003, Frankham 2008).

Inbreeding depression and adaptation to mass rearing conditions are insidious problems because they can go unnoticed in *Trichogramma* mass rearings, and the main concern with mass reared insects is that they may suffer genetic problems and underperform when they are field released (Woodworth et al. 2002, Nunney 2003). *Trichogramma pretiosum* wasps are mass reared and released by the millions all over the Americas to control numerous lepidopteran pests (Hunter 1997, Garcia-Gonzalez et al. 2005, Postali 2010). There are many cases in which mass-releases of *Trichogramma* parasitoids have been successful in controlling the target pest poulations (Guyot 1977, Oatman and Platner 1978, Bigler 1986, Yu and Byers 1994, Nagarkatti et al. 2003, Zhang et al. 2010); however, there are also several reports in which *Trichogramma* parasitoid failed to control the target species (King et al. 1985, Mills et al. 2000, Suh et al. 2000, Lundgren and Heimpel 2002, Ulrichs and Mewis 2004, Vejar-Cota et al. 2005). Likely, these failures could have been due to the poor quality of the reared *Trichogramma*. Genetic problems in reared colonies can result in the release of natural enemies that were

genetically “handicapped” either by inbreeding or by selection for adaptation to the laboratory.

Field releases of *Trichogramma* can fail due to a myriad of causes (Ashley et al. 1973, King et al. 1985, King et al. 1986, Kazmer and Luck 1991b, Kuske et al. 2003). To exclude poor quality of the reared parasitoid as the cause of failure, it is recommended to test for the quality of the reared parasitoid (Bigler 1994, Van Lenteren et al. 2003). Here, we tested for the quality of reared *Trichogramma* from several Mexican insectaries using the standards set for several parasitoid species by the IOBC/EC (Bigler et al. 1991, Van Lenteren 2003a). We tested for parameters that are thought to be highly correlated with field performance such as sex ratio, embryonic mortality, total fecundity (Waage and Ming 1984, Olson and Andow 1998, Silva and Stouthamer 1999) and species identity. As a result, we found misidentification of the reared species, unnoticed replacement in the mass rearing of one species by another, and that the reared *Trichogramma* barely fulfilled the minimum requirements of adequate quality suggested by the IOBC/EC. Some *Trichogramma* species are considered niche-specific (Curl and Burbutis 1978, Yu et al. 1984, Thorpe 1985, Stevens 1995, Romeis et al. 2005); therefore, it is important to correctly identify the reared *Trichogramma*. In this dissertation, we outlined a DNA-based molecular identification methodology to correctly identify the *Trichogramma* species reared in Mexican insectaries. The use of this DNA-based methodology opens the possibility for a more rational use of native

species, and also can be used to monitor the reared species, avoiding problems such as unnoticed replacement of the reared species by a contaminant *Trichogramma* species. Due to the short life cycle of *Trichogramma* parasitoids (10-12 days), we recommended testing be done for the presence of genetic problems and for the good quality of the reared parasitoids, as frequent as possibly. If resources and personnel are available, these tests should be made every month.

For the last two decades, there have been ongoing discussions on the preference of using sexual versus asexual strains of a species in biological control projects (Aeschlimann 1990, Stouthamer 1993, Stouthamer 2003). While in case of *Trichogramma* many of the studies have focused on asexual lines that originated from populations in which both sexual and asexual individuals coexisted (Stouthamer and Luck 1993, Silva et al. 2000, Miura and Tagami 2004), here we studied a population that consisted entirely of asexual wasps. The ability to reproduce entirely by parthenogenesis in these wasps is caused by an infection with a *Wolbachia* bacterium that induces parthenogenesis (Rousset et al. 1992, Stouthamer et al. 1993, Stouthamer 1997). In those populations, where infected and uninfected individuals co-occur, a genomic conflict between the wasp's nuclear genome and the *Wolbachia* genome may result in reduced fitness of the infected wasps (Hurst 1992, Cook and Butcher 1999); however this evolutionary conflict is resolved upon fixation of the infection in the population (Stouthamer et al. 2010). Subsequent to this fixation, a more mutualistic

relationship will evolve between the wasp's genome and that of the symbiont (Stouthamer 1997). Here, we studied a population of *Trichogramma pretiosum* in which PI-*Wolbachia* infection has gone to fixation (Oatman et al. 1982), i.e. a population consisting entirely of PI-*Wolbachia* infected females. We expected that some of the evolutionary changes in these PI-*Wolbachia* infected clones would result in wasps with traits that would be beneficial for biological control. We found characteristics not found previously in mixed PI-*Wolbachia* populations, such as a nearly perfect transmission rate of PI-*Wolbachia,* and a high diversity of clonal lines. Different clones may perform better under different circumstances in the field and may outcompete other clones. In our experiments we showed that the outcome of clonal competition may depend on factors such as the timing of host availability. The differences we found between the clonal lines is particularly important for future biological control efforts using PI-*Wolbachia* infected wasps that are derived from fixed populations. Such wasps should not be treated as a single clone but should be introduced into the area of release as many different clones that were kept separate during the quarantine and mass rearing phases of the importation.

In this dissertation, we illustrated some of the potential problems that can occur when parasitoids are mass reared, and we also outlined several guidelines for commercial insectaries regarding how to maintain high "field quality" of the natural enemies they

sell. Finally, our findings of the high diversity of clones in entirely asexual populations may result in a more rational future use of asexual wasps in biological control.

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