Stellingen

1. Een betrouwbare *Trichogramma*taxonomie kan pas ontwikkeld worden met behulp van moleculaire technieken.

   Pinto, J.D., 1998. Systematics of the North American species of *Trichogramma* Westwood. Memoires of the Entomological Society of Washington, Washington D.C.

   Stouthamer, R. J. Hu, F.J.P.M. van Kan, G.R. Platner & J.D. Pinto, 1999. The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *BioControl*, 43: 421-440.

   Dit proefschrift.

2. *Trichogramma*vrouwtjes die gepaard hebben zijn minder aantrekkelijk voor mannelijke soortengenoten dan maagdelijke vrouwtjes, waarschijnlijk doordat ze na paring ophouden met het produceren van twee specifieke chemische stoffen.

   Dit proefschrift.

3. Thelytoke *Trichogramma*lijnen zijn, ondanks de lagere fecunditeit van hun vrouwtjes, geschikter voor inundatieve biologische bestrijding van Lepidopteraplagen dan arrhenotoke lijnen van dezelfde soort.

   Dit proefschrift.

4. In tegenstelling tot beweringen van Bjorksten & Hoffmann (1995), zijn gastheer-keuzestudies in het laboratorium nuttig bij de selectie van *Trichogramma*lijnen, ook bij de keuze van lijnen die het minst schadelijk zijn voor niet-doelwit arthropoden.

   Bjorksten & Hoffmann, 1995. Effects of pre-adult and adult experience on host-acceptance in choice and non-choice tests in two strains of *Trichogramma*. *Entomologia Experimentalis et Applicata*, 76: 49-58.

   Dit proefschrift.

5. Bij de selectie van biologische bestrijders van zowel endemische als exotische plaag-soorten, zouden endemische natuurlijke vijanden altijd als eerste kandidaten getoetst moeten worden.

6. Omdat horizontale transmissie van genen plaats vindt tussen soorten (Doolittle, 1998; Ibba *et al.*, 1999), zijn de meeste fylogenetische analyses gebaseerd op een enkel gen onbetrouwbaar.

   Doolittle, W.F., 1998. You are what you eat: a gene transfer ratchet could account for bacterial genes in eukaryotic nuclear genomes. *Trends in Genetics*, 14: 307-311.

   Ibba, M., H.C. Losey, Y. Kawarabayasi, H. Kikuchi, S. Bunjun & D. Söll, 1999. Substrate recognition by class I lysyl-tRNA synthetases: a molecular basis for gene displacement. *Proceedings of the National Academy of Sciences USA*, 96: 418-423.

7. Ondanks hun veronderstelde positieve effect op het milieu (French *et al.*, 1999), blijven genetisch gemanipuleerde tabaksplanten ongewenst.

French, C.E., S.J. Rosser, G.J. Davies, S. Nicklin & N.C. Bruce, 1999. Biodegradation of explosives by transgenic plants expressing pentaerythritol tetranitrate reductase. *Nature Biotechnology*, 17: 491-494.

8. Omdat het nucleair en het cytoplasmatisch genoom van Dolly uit twee niet-verwante individuen komt, is dit schaap onterecht als kloon gekwalificeerd.

Wilmut, I., A.E. Schnieke, J. McWhir, A.J. Kind & K.H.S. Campbell, 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385: 810- 813.

Campbell, K.H.S., A. Colman & I. Wilmut, 1998. Letters: response to Sgaramella, V. & N.D. Zinder, Dolly Confirmation. *Science*, 279: 637-638.

Contra: Pennisi, E. & N. Williams, 1997. Will Dolly send in the clones? *Science*, 275: 1415-1416.
Solter, D., 1998. Dolly is a clone - and no longer alone. *Nature*, 394: 315-316.

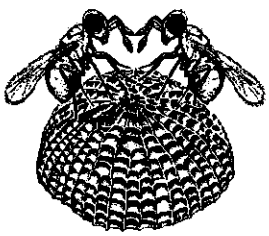
9. Het berechten van (voormalige-)dictators, zelfs als ze oud en ziek zijn, is nuttig als erkenning van de slachtoffers en als voorbeeldfunctie voor latere generaties.

10. In het rijke Nederland is het opmerkelijk hoe vaak er bezuinigd "moet" worden.

11. "Wie geen Pentium heeft werkt met een 386" is een moderne versie van het Portugese spreekwoord "wie geen hond heeft jaagt met een kat".

12. Nederlanders zijn de latinos van het Noorden maar de koude kikkers van het Zuiden.

13. Misverstanden zijn makkelijker te kweken dan insecten.



Stellingen behorend bij het proefschrift: **Identification and evaluation of *Trichogramma* parasitoids for biological pest control.** Isabel M.M.S. Silva.
Wageningen, 17 september 1999.

Identification and evaluation of *Trichogramma* parasitoids for biological pest control

Isabel Maria Martins Santos e Silva

Promotor: dr. J.C. van Lenteren
Hoogleraar in de Entomologie
Co-promotor: dr. ir. R. Stouthamer
Universitair Docent Entomologie

Isabel Maria Martins Santos e Silva

Identification and evaluation of *Trichogramma* parasitoids for biological pest control

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Wageningen Universiteit
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 17 september 1999
des namiddags te vier uur in de Aula

Silva, I.M.M.S. (1999)
Identification and evaluation of *Trichogramma* parasitoids for biological pest control.
Doctoral thesis.

ISBN: 90-5808-074-9
Cover by Piet Kostense

Table of contents

Acknowledgements

I want to express my gratitude to all persons who contributed to this thesis. Firstly, I want to thank Joop van Lenteren, for being my promoter and for his support throughout my research and also during the writing of the thesis. Secondly, I thank my co-promoter and direct supervisor, Richard Stouthamer. His guidance was much appreciated during the outline of the thesis, the practical work and the writing of manuscripts.

I am grateful to everybody at the Department of Entomology, in Wageningen. To the following persons I am especially thankful for their important contribution to this thesis and for the many pleasant hours we spent together, mostly in the laboratory but also in the pub: Jeff Honda, Marnix van Meer and Michel Roskam for all the work we did together in the laboratory and in the greenhouse; Frenk van Kan (my personal molecular biology teacher!), Jianguo Hu and Bertha Koopmanschap for their very helpful collaboration on the molecular biology work; Hanneke van Heest and Astrid Schoenmaker for all the practical help during experiments; Antoon Loomans, Leo Koopman, Marnix van Meer and Menno Schilthuizen always had good suggestions to improve my work, from rearing methods to how to write and present more clearly.

I also much appreciated working together with several MSc students: Alicja Godlewska, Merijn van Tilborg, Jessica Vereijssen and Aldo Hoogenboom. Much of their work is included in this thesis.

It was a very pleasant and fruitful experience to work together with Maarten Posthumus and Teris van Beek, from the Department of Organic Chemistry. To Gerrit Gort, from the Department of Mathematics, I am thankful for his unbelievable patience during the many hours we spent together analysing data behind the computer.

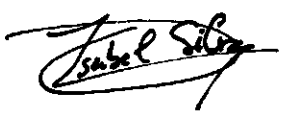
I am grateful to Luis Neto and Bernard Pintureau (INRA, Lyon, France) for the identification of *Trichogramma* specimens and for their work on the esterase analyses, to Guy Boivin (Agricultural Canada Research Station, Quebec) for discussions of my protocols and comments on several manuscripts. A special *obrigado* to Jorge Araújo and Carola Meierrose (Department of Biology of Évora University, Portugal), for encouraging me to pursue research in Wageningen.

I am thankful to the people who contributed to this work from the Secretariat and Administration of the Laboratory of Entomology in Wageningen, the Biology Department of Évora University in Portugal, Koppert B.V., Unifarm, the Photography Department, the Drawing Department – most of the drawings in this thesis were made by Piet Kostense – and the Plant Protection Centre library of the Wageningen University. I

express my gratitude to other people who in some way also contributed to one or more chapters of this thesis: Andre Gidding, Bas Suverkropp, Elsa Polícia, Eric Wajnberg, Ernesto Prado, F. Vanlerberghe-Masutti, Francisca Figo, Frans van Aggelen, Frans van Alebeek, Franz Bigler, Gerard van Leeuwen, Grazia Tommasini, Herman Dijkman, Jocelijn Meijerink, Joop van Loon, Kate Flanagan, Louise Vet, Marielle Witgens, Patricia Garcia, Peter Meisner, Rieta Gols, Roel Potting, S. Greenberg, Sherif Hassan and the PhD students of the laboratory of Entomology. I also want to thank everybody who helped me in learning Dutch, which certainly contributed to my pleasant stay in The Netherlands. Some of my best teachers were Max and my other flatmates of Nobelweg 6, Antoon, Frenk, Hanneke, Hermien, Leo and Marnix.

Finally, I want to thank all the people who contributed to this thesis mainly through their moral support and invaluable friendship. I want to mention some of them by name: my best friend and partner Max Rietkerk, my parents Maria Luísa P.M. Silva and João Pastor S. e Silva, my sister Celeste Silva de Sá and her husband Vicente de Sá, and my good friends Láli de Araújo and Damien Quintius.

The research work presented in this thesis was financially supported by Junta Nacional de Investigação Científica e Tecnológica (BD 2107/92-IE and BD 5620/95), now named Fundação para a Ciência e a Tecnologia, and Fundação Calouste Gulbenkian, Portugal.



Isabel Silva

Chapter 1

General Introduction

Introduction

Trichogramma parasitoids are ubiquitous wasps that are widely used as biological control agents against lepidopterous pests. However, they do not always control the pests at the desired level. Therefore, pre-introductory studies should be performed before application of biological control agents on a large scale to demonstrate their effectiveness (e.g. Ridgway *et al.* 1977, Miller 1983, Ridgway & Morrison 1985, van Lenteren1986 a, b, Pak 1988).

One of the first problems researchers are confronted with when working on *Trichogramma* is the limited knowledge about their taxonomy. These wasps are members of the order Hymenoptera, superfamily Chalcidoidea, family Trichogrammatidae, genus *Trichogramma*. Their taxonomy at the species level is still being developed, and from the ca. 180 species known (Pinto, 1998), about 120 have been described in the last 20 years (Voegelé 1988, Pinto 1998). Many criteria have been developed for the identification of *Trichogramma* species (Nagakartti & Nagaraja 1971, 1977, Pintureau & Babault 1981, 1982, Pinto & Stouthamer 1994), but the minute size of these wasps (ca. 0.5 mm in length and 8μg in weight) and their homogeneous morphology make this task difficult. Not much is known about the phylogeny of *Trichogramma* species, but with the aid of molecular characters, phylogenetic reconstruction of American and of European species is now being performed (Pinto 1998, Stouthamer *et al.*, in press, Stouthamer, pers. com.).

Trichogramma females lay their eggs inside eggs of other insects, mainly of Lepidoptera. They are solitary or gregarious, depending on the size of their hosts. The development of the parasitoid from egg to adult takes place inside the host egg in ca. 12-13 days at 23-25°C (Pinto 1998). In most species, the host egg turns black when the parasitoid inside reaches the pupal stage. When the adult wasps emerge from the host eggs, through openings that they make in the chorion, they are ready to mate and the females are immediately able to parasitize new hosts.

Trichogramma are pro-synovigenic insects, i.e., the females have 30 to 80 mature eggs at the time of emergence (Franz & Voegelé 1974) and may continue to mature new eggs during their life. As in most Hymenoptera, the normal mode of reproduction in *Trichogramma* is arrhenotoky: haploid males arise from unfertilised eggs and diploid females from fertilised eggs. A less common mode of reproduction is thelytoky, in which unfertilised eggs give rise to diploid female offspring. To date, thelytoky has been reported for 14 *Trichogramma* species and it is mostly induced by endosymbionts of the genus *Wolbachia* (α-proteobacteria) (Pinto & Stouthamer 1994, Stouthamer 1997). Thelytokous

Trichogramma can be converted to arrhenotoky by feeding antibiotics to the infected females or by rearing them at high temperatures (Stouthamer *et al.* 1990).

Trichogramma spp. are investigated in more than 50 countries and are applied commercially on several million ha per year. The countries with the largest application of these wasps are China, the ex-Soviet Union and Mexico (Li 1994). From the ca. twenty *Trichogramma* species mass-reared and used in field releases (Li 1994, Smith 1996), only five are frequently used in biological control: *T. evanescens, T. dendrolimi, T. pretiosum, T. brassicae* and *T. nubilale* (Smith 1996). In Western Europe, *T. brassicae* is the only species with large-scale application, against *Ostrinia nubilalis* Hübner (Lepidoptera, Pyralidae) in maize (Suverkropp 1997).

In the last years criteria have been developed for selection and quality control of *Trichogramma* for biological control programs (Pak 1988, Frei & Bigler 1994, Bigler 1994). The general criteria have led to listings of quantifiable traits, such as fecundity, acceptance and suitability of the target host, longevity, sex-ratio, rate of emergence from laboratory hosts, locomotion skills and body size (reviewed by Bigler 1994 and Hassan 1994). The choice of the mode of reproduction of the wasps to be released, i.e., thelytokous *versus* arrhenotokous lines has been proposed as an additional criterion and studies regarding this topic have been performed in the laboratory (Stouthamer 1993, Stouthamer & Luck 1993). In spite of these achievements several questions remain unanswered:

1. How can we identify *Trichogramma* effectively?
2. Can laboratory tests reasonably predict parasitism and dispersal rates of *Trichogramma* in the greenhouse?
3. Which are better for biological control, thelytokous or arrhenotokous *Trichogramma* lines?
4. Do *Trichogramma* species differ in the parasitism of target and non-target hosts?

Research aims

The main aim of the research presented in this thesis was to investigate criteria and methods for identification and selection of *Trichogramma* species/strains for biological control. The present work was started by surveying *Trichogramma* in tomato fields in Portugal, from *Helicoverpa armigera* hosts, where it was known that *Trichogramma* spp. were present. In these fields biological control had not been applied at all or it had been applied at an experimental scale, with native unidentified *Trichogramma* (Meierrose & Araújo 1986, Meierrose 1990). The *Trichogramma* collected in Portugal were studied in the laboratory

and in the greenhouse in Wageningen, The Netherlands. In addition to the five Portuguese species, another European and two American species that reproduce by thelytoky were studied.

Special emphasis was given to 1) the identification of sympatric Portuguese *Trichogramma* species; 2) the determination of dispersal and parasitism of *Trichogramma* lines in the laboratory and in the greenhouse; 3) the biological control potential of *Wolbachia*-infected (thelytokous) *versus* uninfected (arrhenotokous) *Trichogramma* lines and 4) the comparison of *Trichogramma* species regarding the parasitism of target and non-target hosts.

Outline of the thesis

Molecular methods are used in the identification of 5 sympatric Portuguese *Trichogramma* species: 1) electrophoresis of PCR amplified ribosomal DNA internal transcribed spacer 2 (ITS-2), followed by restriction endonuclease digestions, and 2) esterase electrophoresis. Dichotomous keys based on each of the methods are constructed for easy and quick species identification (*chapter 2*).

In addition to the molecular methods, the possibility of creating a new tool for *Trichogramma* species identification is studied, based on "mating-attempts-specificity" of the 5 Portuguese *Trichogramma* species. Two female compounds of one of these species that may be involved in mate recognition are characterised (*chapter 3*).

The fitness of *Trichogramma* females can be influenced by the presence of *Wolbachia* endosymbionts. It is hypothesised that in fixed *Trichogramma* populations (i.e., all females infected) the negative effect of *Wolbachia* on the wasps fitness will be lower than in mixed populations (i.e., infected and uninfected females coexist). The effect of *Wolbachia* on the female fitness (fecundity, longevity, pre-adult mortality, sex-ratio of the progeny) of four mixed and two fixed *Trichogramma* strains is studied in the laboratory (*chapter 4*).

While in the experiments above mentioned females do not need to search for hosts, we aimed also to develop an evaluative tool to monitor *Trichogramma* dispersal in laboratory. Two thelytokous conspecific lines are tested as to their parasitism and dispersal in this laboratorial set-up and in the greenhouse (*chapter 5*). Subsequently, we will compare the potential of *Wolbachia*-infected and uninfected *Trichogramma* as biological control agents. For that purpose, the fecundity and dispersal of thelytokous and arrhenotokous lines

of two *Trichogramma* species are determined in the laboratory and, for the first time, in the greenhouse (*chapter 6*).

When selecting *Trichogramma* strains for biological control it is not only important that they find and parasitise the target host, but also that other beneficials present in the system are preserved. Parasitism of two host species by the five Portuguese *Trichogramma* species is studied in the laboratory (*chapter 7*). The host species are: the bollworm *H. armigera*, an important pest of many crops in the tropics and subtropics, and one of its natural enemies, the lacewing *Chrysoperla carnea*, a predator often used as a biological control agent. In the final chapter the results are discussed in general terms, the main conclusions are given, and future research work is proposed.

Chapter 2

Molecular differentiation of five *Trichogramma* species occurring in Portugal

To be published as Silva, I.M.M.S., J. Honda, F. van Kan, J. Hu, L. Neto, B. Pintureau & R. Stouthamer. Molecular differentiation of five *Trichogramma* species occurring in Portugal. *Biological Control* (accepted).

Abstract

Two molecular methods were developed to distinguish between five *Trichogramma* (Hymenoptera, Trichogrammatidae) species which occur naturally in tomato fields of Portugal: *Trichogramma cordubensis*, *T. evanescens*, *T. turkestanica*, *T. pintoi* and *T. bourarachae*. The methods used were: 1) electrophoresis of PCR amplified ribosomal DNA internal transcribed spacer 2 (ITS-2), followed by restriction endonuclease digestions, and 2) esterase electrophoresis. Dichotomous keys based on each of the methods are provided for easy and quick species differentiation.

Introduction

Egg parasitoids of the genus *Trichogramma* are widely used in biological control against lepidopterous pests, but they are difficult to identify because of their small size and lack of sufficient diagnostic characters (*e.g.* Pinto & Stouthamer 1994). Several methods have been used to clarify *Trichogramma* taxonomy including morphological comparisons of male genitalia (*e.g.* Nagarkatti & Nagaraja 1971, 1977), allozymic analysis (*e.g.* Pinto *et al.* 1992, 1993, Pintureau 1993a, Pintureau & Babault 1980, 1981, 1982, Pintureau & Keita 1989) and reproductive compatibility studies (*e.g.* Nagarkatti & Nagaraja 1968, Pinto *et al.* 1991, Pintureau 1991). However, much controversy still surrounds *Trichogramma* taxonomy and the current methods are usually time consuming and/or difficult to apply by people other than specialists. Allozymic analyses, particularly esterase electrophoresis, have provided good results for species differentiation in *Trichogramma* (*e.g.* Pinto *et al.* 1992, 1993). However, it has been stressed that such results are often difficult to interpret and are often based on a restricted number of samples. Thus, results should be considered preliminary until confirmed by other methods (*e.g.* Pinto & Stouthamer 1994, Richardson *et al.* 1986). Recently, DNA studies, using PCR techniques, have been investigated as a tool for identification and phylogeny (*e.g.* Landry *et al.* 1993, Vanlerberghe-Masutti 1994). Ribosomal DNA has been one important target for these studies (*e.g.* Orrego & Agudelo-Silva 1993, Pinto *et al.* 1997, Sappal *et al.* 1995).

Ribosomal DNA (rDNA) is present in all organisms, and is composed of several regions (genes and spacers) that evolve at different rates. Ribosomal DNA sequences are suitable for phylogenetic studies at many taxonomic levels: from major lineages of life to intraspecific levels (Hillis & Dixon 1991 and references therein). At the species and intraspecific level, the internal transcribed spacer regions (ITS-1 and ITS-2) are often used as a taxonomic tool in many groups such as fungi (*e.g.* Carbone & Kohn 1993), plants (*e.g.* Hsiao *et al.* 1994, but see Buckler IV *et al.* 1997) and animals (*e.g.* Bowles & McManus 1993), including insects (*e.g.* Campbell *et al.* 1993, Hoy 1994; Kuperus & Chapco 1994, but see Vogler & DeSalle 1994). The sequencing and restriction analysis of ITS rDNA have been described as promising and/or effective in distinguishing *Trichogramma* individuals from different populations (Orrego & Agudelo-Silva 1993, Pinto *et al.* 1997, Sappal *et al.* 1995, Schilthuizen & Stouthamer 1997, van Kan *et al.* 1996, 1997).

Our objective was to develop methods that can clearly differentiate between *Trichogramma* species occurring naturally in tomato fields of Portugal, which yield consistent and repeatable results. This study is part of a broader project on the use of molecular methods for the differentiation of European *Trichogramma* species (Stouthamer *et al.*, pers. com.).

Materials and Methods

Cultures

We studied 27 isofemale lines identified by L.N. and B.P. as: *T. turkestanica* Meyer 1940, *T. evanescens* Westwood 1833 (*sensu* Pintureau 1987), *T. pintoi* Voegelé 1982, *T. bourarachae* Pintureau & Babault 1988 and *T. cordubensis* Vargas & Cabello 1985. Voucher specimens are kept by B.P. in INSA-INRA (Villeurbanne, France). All lines, except Tt4 and Tc15, were collected from noctuid eggs in tomato (*Lycopersicon esculentum* Miller) fields in Alentejo province (Southern Portugal). Origin (location and year of collection) of each line studied is mentioned in Table 1. Four of the five species were found in a single 4 hectare tomato field in Divor, Portugal. *Trichogramma turkestanica* was not found in this field but was found within 100km radius. Line Tt4 was collected on wild tomato plants. Cultures were maintained on *Ephestia kuehniella* Zeller (Lep., Pyralidae) eggs (killed by ultraviolet irradiation), at 15-25°C, RH 50 ± 20 %, LD= 16:8.

DNA extraction

DNA was extracted as described by Edwards & Hoy (1993) with modifications described below. One to five previously frozen wasps were crushed with a glass rod in 25 to 100 µl Chelex (5%) (Biorad Corp.) and 2-4 µl proteinase K (20 mg/ml). Mixtures were incubated overnight at 56°C, followed by 10 min at 95-99°C.

PCR amplification and electrophoresis

PCR reactions were performed in 50 µl volumes using a Hybaid thermocycler, 5 µl DNA template, 5 µl (10x) PCR-buffer, 1 µl dNTP's (each in a 10mM concentration), 0.6 µl forward and reverse primer (20 pmol/µl), 0.1 - 0.2 µl SuperTaq polymerase (Sphaero-Q; 5 units/µl) and 38 µl sterile water. The primers used to amplify the ITS-2 region (plus portions of the flanking regions 5.8 S and 28 S genes) were:

Table 1. *Trichogramma* lines studied, analyses performed, identity and origin (locality and year of collection).

Line designation	Studies	Species	Origin	Collection date
Tt1 (MB 39)	1,2	*T. turkestanica*	Mora	1992
Tt2 (MB21)	1,2	*T. turkestanica*	Mora	1992
Tt3 (TS15)	1	*T. turkestanica*	Ferreira do Alentejo	1992
Tt4 (PB)	1,2	*T. turkestanica*	Gambelas (Algarve)	1991
Te5 (MB36)	1,2,3	*T. evanescens*	Mora	1992
Te6 (TS21)	1,2	*T. evanescens*	Ferreira do Alentejo	1992
Te7 (MB34)	1,2,3	*T. evanescens*	Mora	1992
Te8 (RS44)	1,3	*T. evanescens*	Mora	1992
Te9 (35A-1)	1	*T. evanescens*	Divor	1993
Te10 (34B-8)	1	*T. evanescens*	Divor	1993
Te11 (RS20)	1	*T. evanescens*	Mora	1992
Te12 (RS17)	1	*T. evanescens*	Mora	1992
Tc13 (28B143b)	1,2	*T. cordubensis*	Divor	1993
Tc14 (28A63a)	1,2,3	*T. cordubensis*	Divor	1993
Tc15 (Azores)	2	*T. cordubensis*	R.Guilherme (S.Miguel)	1992
Tc16 (MB35)	1	*T. cordubensis*	Mora	1992
Tc17 (MB35grey)	1	*T. cordubensis*	lab mutant from Tc16	1993
Tp18 (29B97)	1,2,3	*T. pintoi*	Divor	1993
Tp19 (Morenos85)	1,2	*T. pintoi*	Ferreira do Alentejo	1985
Tp20 (Mora88)	1,2	*T. pintoi*	Mora	1988
Tp21 (30A126b)	1,3	*T. pintoi*	Divor	1993
Tp22 (29B122a)	1	*T. pintoi*	Divor	1993
Tp23 (Mora 87)	1,2	*T. pintoi*	Mora	1987
Tp24 (34B 7a)	1	*T. pintoi*	Divor	1993
Tp25 (30B[2+3])	1	*T. pintoi*	Divor	1993
Tb26 (28B123b)	1,2,3	*T. bourarachae*	Divor	1993
Tb27 (28B123c)	1,2	*T. bourarachae*	Divor	1993

Between brackets are the original designations of each line. Numbers in second column (studies) refer to type of analysis performed:1. esterase electrophoresis; 2. sequencing; 3. restriction analysis.

5'-TGTGAACTGCAGGACACATG-3' (forward) and 5'-GTCTTGCCTGCTCTGAG-3' (reverse). The PCR cycling program was 3 min. at 94°C followed by 33 cycles of 45 sec. at 92°C, 45 sec. at 53°C and 45 sec. at 72°C, with 3 min. at 72°C after the last cycle. PCR products were electrophoresed on a 1-2% low melting point agarose gel (Sphaero-Q). Gels were stained using ethidium bromide. Molecular weight standards were run along with the samples for reference. Six to fifteen amplifications and respective electrophoreses were performed per isofemale line.

Cloning and sequencing

Following electrophoresis, PCR products were excised from the agarose gel, frozen and freeze-squeezed. The liquid phase was alcohol precipitated, washed and ligated into a T-tailed vector (Amersham Life Science) and amplified in *Escherichia coli* cells. Colonies containing the PCR insert were checked by PCR using the primers mentioned above and sequenced on an Applied Biosystems automatic sequencer. One to four ITS-2 regions were sequenced per isofemale line.

Sequences were aligned manually using the ESEE 3.0s sequence editor (Cabot, 1995). Due to the large differences in several regions of the ITS-2 sequences between two groups (group a: *T. turkestanica*, *T. evanescens* and *T. cordubensis*, and group b: *T. pintoi* and *T. bourarachae*), two distinct alignments were performed.

Restriction analysis

For those PCR products of similar size, a restriction enzyme was selected that would result in species-specific banding patterns. For the selection of such a restriction enzyme we applied the program DNA strider™ 1.0 (Christian Marck) to DNA sequences.

Restriction analysis was performed in a 20 µl volume, using 5-10 µl PCR product, 2 µl (10x) reaction buffer, 2 µl BSA (1.0 mg/ml), 1 µl MnlI (10 units/µl) and 5-10 µl distilled water. The mixture was incubated for 2 hours at 37°C. Digestions were checked by running them on a standard 1.5-2% agarose gel. One to three samples per isofemale line were used for restriction analysis.

Esterase electrophoresis

Electrophoresis was performed on vertical polyacrylamide slab gels (7% pH 8.9) as described by Pintureau (1987) and Pintureau & Babault (1981), with modifications described in Pintureau *et al.* (1991a). Samples of 20 to 30 individuals, progeny of a virgin female, were used. Three repetitions were performed. The determination of the exact

position of esterase bands was performed by means of comparisons with control samples belonging to several European *Trichogramma* species. These samples consisted of material previously analysed in Pintureau (1993a). Esterase electrophoresis on *Trichogramma turkestanica* populations has been previously reported by Neto & Pintureau (1995).

Results

Internal transcribed spacer 2

Based on the size (bp= base pairs) of ITS-2 rDNA PCR products, three groups could be distinguished:

1) *T. turkestanica* (485-489 bp);

2) *T. cordubensis* (529bp) / *T. evanescens* (542-551bp) and

3) *T. pintoi* (668-695bp) / *T. bourarachae* (665-666bp).

These groups could be easily recognised after electrophoresis on agarose gels (Fig.1). Complete ITS-2 sequences (Fig. 2a and 2b) have been deposited in GenBank, accession numbers AF043612-AF042626.

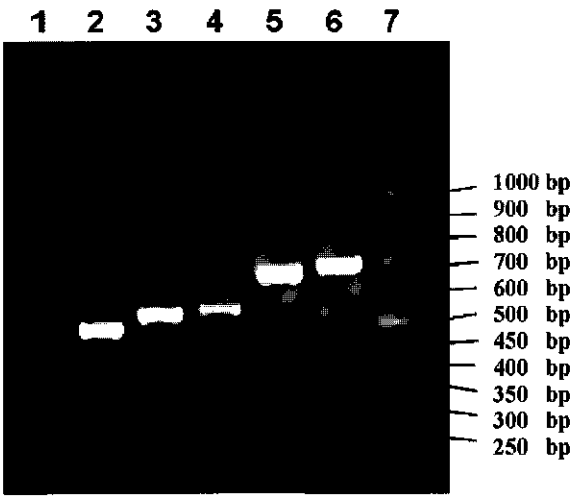


Fig. 1. Gel electrophoresis of ITS-2 PCR products. Lanes 1 and 7: low molecular weight markers; lane 2: *T. turkestanica* (Tt1); lane 3: *T. cordubensis* (Tc14); lane 4: *T. evanescens* (Te12); lane 5: *T. bourarachae* (Tb26); lane 6: *T. pintoi* (Tp18).

```
Tt1     GTTTATAAAAACGAACCCGACTGCTCTCTC----------------GCAA-- 34
Tt2     GTTTATAAAAACGAACCCGACTGCTCTCTC----------------GCAA-- 34
Tt4     GTTTATAAAAACGAACCCGACTGCTCTCTC----------------GCAA-- 34
Te5     GTTTATAAAAACGTACCCGACTGCTCTCTC----------------GCAA-- 34
Te6     GTTTATAAAAACGAACCCGACTGCTCTCTC----------------GCAA-- 34
Te7     GTTTATAAAAACGAACCCGACTGCTCTCTC----------------GCAA-- 34
Tc13    GTTTATAAAAACGAACCCGACTGCTCTCTCTCTCTCTCTCTCTCTTGCAAGA 52
Tc14    GTTTATAAAAACGAACCCGACTGCTCTCTCTCTCTCTCTCTCTCTTGCAAGA 52
Tc15    GTTTATAAAAACGAACCCGACTGCTCTCTCTCTCTCTCTT------GCAAGA 46

Tt1     ------GAGAGAGCGTTGATCTGGGCGCTCG--TCTCTATCTCTTCTCTATA 78
Tt2     ------GAGAGAGCGTTGATCTGGGCGCTCGTGTCTCTATCTCT-CTC--TA 77
Tt4     ------GAGAGAGCGTTGATCTGGGCGCTCGTGTCTCAATCTCT-CTC--AA 77
Te5     ------GAGAGAGCGTTGATCTGGGCGCTCG--TCTCTATCTC-----TAT- 72
Te6     ------GAGAGAGCGTTGATCTGGGCGCTCG--TCTCTATCTC-----TAT- 72
Te7     ------GAGAGAGCGTTGATCTGGGCGCTCG--TCTCTATCTC-----TAT- 72
Tc13    GAGGAGGAGAGAGCGTTGATCTGGGCGCTCG--TCTCTATCTCT------TA 96
Tc14    GAGGAGGAGAGAGCGTTGATCTGGGCGCTCG--TCTCTATCTCT------TA 96
Tc15    GAGGAGGAGAGAGCGTTGATCTGGGCGCTCG--TCTCTATCTCT------TA 90

Tt1     C------------------TCTT-CTTC------------------------ 87
Tt2     C------------------TCTT-CTTC------------------------ 86
Tt4     C------------------TCTT-CTTC------------------------ 86
Te5     -GCGCGCGCGCGCGCT--CTCTTTCTTCTATTCGTAGAGAGAGA--GTGCGC 119
Te6     -GCGCTCGCGCGCGCG--CTCTTTCTTCTATTCGTAGAGAGAGAGAGTGCGC 121
Te7     -GCGCGCGCGCGCGCGCGCTCTTTCTTCTATTCGTAGAGAGAGAGTGTGCGC 123
Tc13    C------------------TCTT-CT-CTC--------------------GC 108
Tc14    C------------------TCTT-CT-CTC--------------------GC 108
Tc15    C------------------TCTT-CT-CTC--------------------GC 102

Tt1     GA-AGTGTA----TAG----CAGTGTGATATGATACGTCGCCTCAAACGGAC 130
Tt2     GA-AGTGTA----TAGAGAGCAGTGTG---TGATACGTCGCCTCAAACGATT 130
Tt4     GA-AGTGTA----TAGAGAGCAGTGTG---TGATACGTCGCCTCAAACGATT 130
Te5     GAGAGTGTGCG--TAG----CAGTG-----TGACACGTCGCCTCAAACGAAA 160
Te6     GAGAGTGTGCG--TAG----CAGTG-----TGACACGTCGCCTCAAACGAAA 162
Te7     GAGAGTGTGCGTGTAG----CAGTG-----TGACACGTCGCCTCAAACGAAA 166
Tc13    GA-AGTG------TAG----CAGTG-----TGATACGTCGCCTCAAACGAAA 144
Tc14    GA-AGTG------TAG----CAGTG-----TGATACGTCGCCTCAAACGAAA 144
Tc15    GA-AGTG------TAG----CAGTG-----TGATACGTCGCCTCAAACGAAA 138

Tt1     AGCAAGACAAAAGATGAATTCGTTCGTCTAGCTGGCGCGCGCGCTTACCGCT 182
Tt2     AGCAAGAAAAAAGACGAATTCGTTCGTCTAGCTGGCGCGCGCGCTTACCGCT 182
Tt4     AGCGTGAAAAAAGACGAAT--GTTCGTCTAGCTGGCGCGCGCGCTTACCGCT 180
Te5     CGCAAGAAAAAAGATGAATTCGTTCGTCTGGCTGGCGCGCGC--TTACCGCT 210
Te6     CGCAAGAAAAAAGATGAATTCGTTCGTCTAGCTGGCGCGCGCGCTTACCGCT 214
Te7     CGCAAGAAAAAAGATGAATTCGTTCGTCTAGCTGGCGCGCGCGCTTACCGCT 218
Tc13    CGCAAGAGAAAAGATGAATTCGTTCGTCTAGCTGGCGTGCGCGCTTACCGCT 196
Tc14    CGCAAGAGAAAAGATGAATTCGTTCGTCTAGCTGGCGTGCGCGCTTACCGCT 196
Tc15    CGCAAGAGAAAAGATGAATTCGTTCGTCTAGCTGGCGTGCGCGCTTACCGCT 190
```

Fig. 2a. ITS-2 sequences of *Trichogramma turkestanica* (Tt), *T. evanescens* (Te) and *T. cordubensis* (Tc). A dash indicates that the nucleotide is absent. MnlI restriction sites for *T. evanescens* and *T. cordubensis* are underlined.

```
Tt1     TGGAGAGTACTCGCTCGC----CCGAGTACTTCCGATCGTTCTGCGTCGAGT 230
Tt2     TGGAGAGTACTCGCTCGC----GCGAGTACTTCCGATCGTTCTGCGTCGAGT 230
Tt4     TGGAGAGTACTCGCTCGC----GCGAGTACTTCCGATCGTTCTGCGTCGAGT 228
Te5     TGGAGAGTAC------------GTCAGTACTTCCGATCGTTCTGCGTCGAGT 250
Te6     TGGAGAGTAC------------GTCAGTACTTCCGATCGTTCTGCGTCGAGT 254
Te7     TGGAGAGTAC------------GTCAGTACTTCCGATCGTTCTGCGTCGAGT 258
Tc13    TGGAGAGTACTCGTGT--AAAAGCGAGTACTTCCGATCGTTCTGCGTCGAGT 246
Tc14    TGGAGAGTACTCGTGT--AAAAGCGAGTACTTCCGATCGTTCTGCGTCGAGT 246
Tc15    TGGAGAGTACTCGTGT--AAAAGCGAATACTTCCGATCGTTCTGCGTCGAGT 240

Tt1     CCCGGAGCTTCCTCGACTCG---TCGAGCAGCGGACTGACGTCTAGCACACG 279
Tt2     CCCGGAGCTTTCTCGACTCG---TCGAGCAGCGGACCGACGTCTAGCACACG 279
Tt4     CCCGGAGCTTTCTCGACTCG---TCGAGCAGCGGACCGACGTCTAGCACACG 277
Te5     CCCGGAGCTTTCTCGACTCG---TCGAGCAGCGGACCGACGTCTAGCACACG 299
Te6     CCCGGAGCTTTCTCGACTCG---TCGAGCAGCGGACCGACGTCTAGCACACG 303
Te7     CCCGGAGCTTTCTCGACTCG---TCGAGCAGCGGACCGACGTCTAGCACACG 307
Tc13    CCCGGAGCTTTCTCGACTACTCGTCGAGCAGCGGACCGACTGCTAGCACACG 298
Tc14    CCCGGAGCTTTCTCGACTACTCGTCGAGCAGCGGACCGACTGCTAGCACACG 298
Tc15    CCCGGAGCTTTCTCGACTACTCGTCGAGCAGCGGAACGACTGCTAGCACACG 292

Tt1     ATCAGGCTCGTCCATGCATCGGTAATTGAACGCGCGCGA-------TGCACT 324
Tt2     ATCAGGCTCGTCCATGCATCGGTCATTGAACGCGCGCGCG------TGCACT 325
Tt4     ATCAGGCTCGTCCATGCATCGGTCATTGAACGCGCGCGCG------TGCACT 323
Te5     ATCAGGCTCGTCCATGCATCGGTCATTGAACGCGCGCGCGCGCTCGTGCTCT 351
Te6     ATCAGGCTCGTCCATGCATCGGTCATTGAACGCGCGCGCGCGCTCGTGCTCT 355
Te7     ATCAGGCTCGTCCATGCATCGGTCATTGAACGCGCGCGCGCGCTCGTGCTCT 359
Tc13    ATCAGGCTCGTCCATGTTTCGGTTATCGAACGCGCGCT--CC---GTG---- 341
Tc14    ATCAGGCTCGTCCATGTTTCGGTTATCGAACGCGCGCT--CC---GTG---- 341
Tc15    ATCAGGCTCGTCCATGTTTCGGTTATCGAACGCCCGCTCTCC---GTG---- 337

Tt1     TTT----------AC----------------ATGGTTAGCTCG-AA--T 344
Tt2     TTT----------ACAC--------------GTGGGGCTAGCTCG-AA--T 349
Tt4     TTT----------ACAC--------------GTGGGGCTAGCTCG-AA--T 347
Te5     CTTTTGTTTTAACGAACGAAAGTAGG--T-AACGACGGCTAGCTCG-AAGCT 399
Te6     CTTTTGTTTTAACGAACGAAAGTAGGAGT-AACGACGGCTAGCTCG-AAGCT 405
Te7     CTTTTGTTTTAACGAACGAAAGTAGGGGT-AACGACGGCTAGCTCG-AAGCT 409
Tc13    --TATGTATATGCGTATATGCGTGTATATATATAATGGCTAGCTCG-AA--T 388
Tc14    --TATGTATATGCGTATATGCGTGTATATATATAATGGCTAGCTCG-AA--T 388
Tc15    --TTTGTTTATGCGTATATGCGTNTATATATA-AAGGGCTANCTCCCAAA-T 385

Tt1     TTTTGC--TGAACGA---GTCTTTTTTCT-CGAT                   372
Tt2     TTTTGC--TGAACGA----TCTTTTTTCT-CGAT                   376
Tt4     TTTTGC--TGAACGA---GNCTTTTTTCTTCGAT                   376
Te5     TTtTGCGCTGAACGA---GTCTTTTTTCT-CGAT                   429
Te6     TTTTGCGCTGAACGA---GTCTTTTTTCT-CGAT                   435
Te7     TTTTG-GCTGAACGA---GTCTTTTTTCT-CGAT                   438
Tc13    TTTT-CG-TGAATGAA---TCTTTTCTCT-CGAT                   416
Tc14    TTTTT-G-TGAATGAA---TCTTTTCTCT-CGAT                   416
Tc15    TTTTT-G-TGGAAANGAAA-CTTTTCTCTCCCAT                   416
```

Fig. 2a (cont.). ITS-2 sequences of *Trichogramma turkestanica* (Tt), *T. evanescens* (Te) and *T. cordubensis* (Tc). A dash indicates that the nucleotide is absent. MnlI restriction sites for *T. evanescens* and *T. cordubensis* are underlined.

```
Tp18        GTTTATAAAAAAAAACCNGACGGCTCGTTTTTTTTTTTTTAAATTAAAAAA 50
Tp23        GTTTATAAAAAATAANCCGACTGCTCTTTTTTTTTTTTTT----GAAAAA 46
Tb27        GTTTATAAAAAATAACCCGACTGCTCTCTTTTACTTTTT-----GTAAAA 45
Tb26-1      GTTNATAAAAAATAACCCGACTGCTCTCTTTTACTTTTT-----GTANAA 45
Tb26-2      GTTTATAAAAAATAACCCGACTGCTCTCTTTTACTTTTT-----GTAAAA 45
Tb26-3      GTTTATAAAAAATAACCCGACTGCTCTCTTTTACTTTTT-----GTAAAA 45

Tp18        AAAGAGCGTNGATCTGG-CGNTCTTCGCGCGNTTNTTCAA---------- 89
Tp23        AA-GAGCGTTGATCTGGGCGCTCGTCGCGCGCTACTTCAA---------- 85
Tb27        A--GAGCGTTGATCTGGGCGCTCGTCGCGCGCTTTTTTGTAAAAGAAAAA 93
Tb26-1      A--GAGCGTTGATCTGGNCGCTCGTCGCNCGCTTTTTTGTAAAAGAAAAA 93
Tb26-2      A--GAGCGTTGATCTGGGCGCTCGTCGCGCGCTTTTTTGTAAAAGAAAAA 93
Tb26-3      A--GAGCGTTGATCTGGGCGCTCGTCGCGCGCTTTTTTGTAAAAGAAAAA 93

Tp18        GCGCGGG--ACGTCGCCTCAAGCAAGACGCAAGAATTATAAGATCGAAAG 137
Tp23        GCGCGCTN-ACTTCGCCTCAAGCAAGCCGCAAGAATTATAAGATCGAAAG 134
Tb27        GCGCGCG--ACGTCGCCTCAAGCAAGACGCAAGAATCACAAGATCGAGAG 141
Tb26-1      GNGNTNTTTTCGTCGCCTCAAGCAAGACGCAAGAATCACAAGATCGAAAG 143
Tb26-2      GCGCGCG--ACGTCGCCTCAAGCAAGACGCAAGAATCACAAGATCGAAAG 141
Tb26-3      GCGCGCG--ACGTCGCCTCAAGCAAGACGCAAGAATCACAAGATCGAAAG 141

Tp18        GATACGGGACAATCGTGAAAAGACGTTCGCTCGTCGAAAGCAAGACGCAA 187
Tp23        GATACGGGACAATCGTGAAAAGACGTTTGCTCGTCGAAAGCA-GACGCAA 183
Tb27        GATACGGGACAATCGTGAAAAGACGTTCGCTCGTCGAAAGCA-GACGCAA 190
Tb26-1      GATACGGGACAATCGTGAAAAGACGTTCGCTCGTCGAAAGCA-GACGCAA 192
Tb26-2      GATACGGGACAATCGTGAAAAGACGTTCGCTCGTCGAAAGCA-GACGCAA 190
Tb26-3      GATACGGGACAATCGTGAAAAGACGTTCGCTCGTCGAAAGCA-GACGCAA 190

Tp18        GT--CT---GTTTTCACACACACGAACTCGCACGATTTTCTCCGATTTCA 232
Tp23        GT--CT---GTTTTCACACACACGAACTCAAACGATTTTCTCCGATTTCA 228
Tb27        GTGTCTCTCG----------CGATTTTCACCGAT--CA 216
Tb26-1      GTGTCTCTCG----------CGATTTTCACCGAT--CA 218
Tb26-2      GTGTCTCTCG----------CGATTTTCACCGAT--CA 216
Tb26-3      GTGTCTCTCG----------CGATTTTCACCGAT--CA 216

Tp18        ATATCCGATCGTCTAGCTGGCGCGCGCG--CGACTCTCTTGGAGAATGAG 280
Tp23        ATATCCGATCGTCTAGCTGGCGCGCGCG--CGACTCACTTGGAGAATGAG 276
Tb27        ATATCCGATCGTCTAGCTGGCGCGCGCGCACGACTCTCTTGGAGAATGAG 266
Tb26-1      ATATCCGATCGTCTAGCTGGCGCGCGCGCACGACTCTCTTGGAGAATGAG 268
Tb26-2      ATATCCGATCGTCTAGCTGGCGCGCGCGCACGACTCTCTTGGAGAATGAG 266
Tb26-3      ATATCCGATCGTCTAGCTGGCGCGCGCGCACGACTCTCTTGGAGAATGAG 266

Tp18        CA-CACACACCGC----------TGTGTGCGAATTCC 306
Tp23        CA-----CACCGC----------TGTGTGCGAATTCC 298
Tb27        CATCATCCAC-GCGCACTTGATCGTGCGTGTGTGTGTGTGTGCGAATTCC 315
Tb26-1      CATCATCCAC-GCGCACTTGATCGTGCGTGTGTGTGTGTGTGCGAATTCC 317
Tb26-2      CATCATCCAC-GCGCACTTGATCGTGCGTGTGTGTGTGTGTGCGAATTCC 315
Tb26-3      CATCATCCAC-GCGCACTTGATCGTGCGTGTGTGTGTGT--GCGAATTCC 313

Tp18        CGATCGTCGTCGTCGAGTCCCGGAGCTCTTTGCGAGCGGACCGACGTCTA 356
Tp23        CGATCGTCGTCGTCGAGTCCCGGAGCTCTTTGCGAGCGGACCGACGTCTA 348
Tb27        CGATCGTCGTCGTCGAGTCCCGGAGCTCTTTGCGAGCGGAACGACGTCTA 365
Tb26-1      CGATCGTCGTCGTCGAGTCCCGGAGCTCTTTGCGAGCGGACCGACGTCTA 367
Tb26-2      CGATCGTCGTCGTCGAGTCCCGGAGCTCTTTGCGAGCGGACCGACGTCTA 365
Tb26-3      CGATTGTCGTCGTCGAGTCCCGGAGCTCTTTGCGAGCGGACCGACGTCTA 363
```

Fig. 2b. ITS-2 sequences of *T. pintoi* (Tp) and *T. bourarachae* (Tb). Tb26-1, Tb26-2 and Tb26-3 represent sequences from different individuals of the same *T. bourarachae* line. A dash indicates that the nucleotide is absent. MnlI restriction sites are underlined.

```
Tp18      GAACGATTGGCTCGTCCAAGAGAGTTTATTTAATAGCGTGCG--ATCGCC 404
Tp23      GAACGATTGGCTCGTCCAAGAGAGTTTTTT-AATAGCGTGCT--ATCGCC 395
Tb27      GAACGAATGGCTCGTCCCAGAATT------GAATAACGC-CGCGAATGCC 408
Tb26-1    GAACGATTGGCTCGTCCAAGAGTT------GATTAGCGCGCGCGAATGCC 411
Tb26-2    GAACGATTGGCTCGTCCAAGAGTT------GATTAGCGCGCGCGA-TGCC 408
Tb26-3    GAACGATTGGCTCGTCCAAGAGTT------GATTAGCGCGCGCGAATGCC 407

Tp18      CGTGTGCTAGCCTCGATCGGTTCGTGTGAAAAATCACGAGTCGTGTGCGT 454
Tp23      CGTGTGCTAGCCTCGATCGGATCGTGTGAAAAATCACGAGTCGTGGTTGT 445
Tb27      TGTGTGCTGGC-TCGATCGGATCGTGTGAATA-TCA----TCGTG----- 447
Tb26-1    TGTGTGCTGGC-TCGATCGGATCGTGTGAATA-TCA----TCGTG----- 450
Tb26-2    TGTGTGCTGGC-TCGATCGGATCGTGTGAATA-TCA----TCGTG----- 447
Tb26-3    TGTGTGCTGGC-TCGATCGGATCGTGTGAATA-TCA----TCGTG----- 446

Tp18      GTTTATACACTTACACT--GACTTGCCGTCGTTTCTGTTGTTGCGTTGTT 502
Tp23      GTTTA--CACTTACACT--GACTTGC-GTCGTTTCTGTTGTTGTGTTGTT 490
Tb27      ----------CACTGCGACTTGC-GTCGTTTCTGTTTGTCGAC---- 479
Tb26-1    ----------CACTGCGACTTGC-GTCGTTTCTGTTTGTCGAC---- 482
Tb26-2    ----------CACTGCGACTTGC-GTCGTTTCTGTTTGTCGAC---- 479
Tb26-3    ----------CACTGCGACTTGC-GTCGTTTCTGTTTGTCGAC---- 478

Tp18      GTTGTCGTTCTTCGACACAGCAGCACAA--------CAACGGCGTCGTTT 544
Tp23      G----------CACAGCAGCACAA--------CAACGGCGT-GTTT 517
Tb27      -----CGTTC------AC-GAA-C----GGGGCGTCAAACGGCGTCGTCT 512
Tb26-1    -----CGTTC------AC-GAA-CA---GGG-CGTCAAACGGCGTCGTCT 515
Tb26-2    -----CGTTC------AC-GAA-C----GGGGCGTCAAACGGCGTCGTCT 512
Tb26-3    -----CGTTC------AC-GAA-C----GGGGCGTCAAACGGCGTCGTCT 511

Tp18      TTTCGTCACGTTT--AAATCCTTTC--TTTTTAATTCTCGAT          582
Tp23      TTTCGTCACGTTT--AAATCCTTAC--TTTTAAATTCTCGAT          555
Tb27      TTTCGTCACGTTTTTAAATCC-AACGATCTTTGATTCTCGAT          553
Tb26-1    TTTCGTCACGTTTTTAAATCC-AACGATCTTTGATTCTCGAT          556
Tb26-2    TTTCGTCACGTTTTTAAATCC-AACGATCTTTGATTCTCGAT          553
Tb26-3    TTTCGTCACGTTTTTAAATCC-AACGATCTTTGATTCTCGAT          552
```

Fig. 2b (cont.). ITS-2 sequences of *T. pintoi* (Tp) and *T. bourarachae* (Tb). Tb26-1, Tb26-2 and Tb26-3 represent sequences from different individuals of the same *T. bourarachae* line. A dash indicates that the nucleotide is absent. MnlI restriction sites are underlined.

Restriction analysis showed differences between the species that had PCR products of similar sizes (Fig. 3). Based on the ITS-2 size differences and the MnlI restriction patterns, we constructed a dichotomous key (Table 2). The 82 bp band corresponds to a restriction site within the forward flanking region of ITS-2 (i.e. the 5.8S gene).

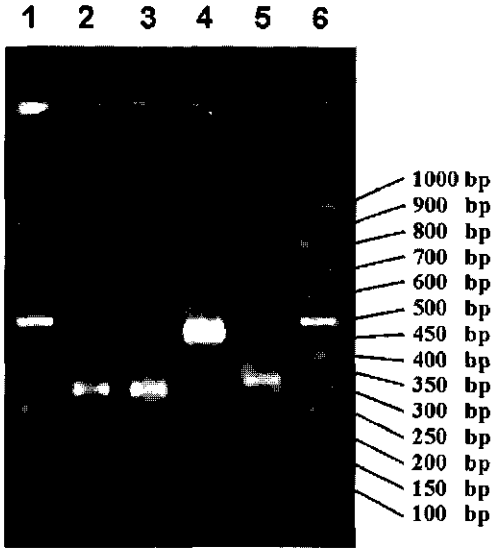


Fig. 3. Gel electrophoresis of restriction digestions by MnlI endonuclease. Lanes 1 and 6: low ladder markers; lane 2: *T. cordubensis* (Tc14); lane 3: *T. evanescens* (Te12); lane 4: *T. bourarachae* (Tb26); lane 5: *T. pintoi* (Tp18).

Table 2. Dichotomous key for differentiation of five Portuguese *Trichogramma* species based on PCR of ITS-2 rDNA, followed by restriction analysis, using the endonuclease MnlI (bp=base pairs).

1.	PCR product < 490 bp	*T. turkestanica*
	PCR product > 520 bp	2
2.	PCR product < 560 bp	3
	PCR product > 660 bp	4
3.	MnlI bands ca. 300 and 82/77 bp	*T. cordubensis*
	MnlI bands ca. 300, 160 and 82 bp	*T. evanescens*
4.	MnlI bands ca. 310, 160 and 104/82 bp	*T. pintoi*
	MnlI bands ca. 450 and 110/82 bp	*T. bourarachae*

Esterase electrophoresis

The Rf values obtained for each line are presented in Table 3. A dichotomous key based on these values is presented in Table 4.

Table 3. Position (R^f values) of observed esterase bands of the five *Trichogramma* species studied.

Locus → Species and lines ↓	EST1	EST2	EST5	EST5'
T. turkestanica				
Tt1	0.07; 0.08	0.24		0.48-0.51
Tt2	0.07; 0.08	0.22		0.45-0.48
Tt3	0.08	0.22		0.45-0.48
Tt4	0.07	0.22		0.45-0.48
T. evanescens				
Te5 and Te12	0.13	0.22; 0.24	0.44	
Te6 and Te7	0.13	0.22	0.42	
Te8	0.10	0.22	0.51	
Te9	0.10	0.24	0.44	
Te10	0.10	0.22	0.44	
Te11	0.13	0.22; 0.24	0.42	
T. cordubensis				
Tc13, Tc14, Tc16 and Tc17	0.13	0.20	0.45	
T. pintoi				
Tp18-Tp24	0.10	0.16		0.46-0.49
Tp25	0.11	0.16		0.46-0.49
T. bourarachae				
Tb26 and Tb27		0.15; 0.16		0.46-0.49

Rf values separated by ";" represent polymorphism within one line; Rf separated by a "-" represent 2 bands for the same locus; no values represent no bands for the locus.

Table 4. Dichotomous key for differentiation of five Portuguese *Trichogramma* species based on esterases electrophoresis ($LOCUS^{Rf}$)

1.	$EST2^{0.15}$ or $EST2^{0.16}$	2
	$EST2^{0.20}$ or $EST2^{0.22}$ or $EST2^{0.24}$	3
2.	$EST1^{0.10}$ or $EST1^{0.11}$	*T. pintoi*
	EST1 no band	*T. bourarachae*
3.	$EST1^{0.07}$ or $EST1^{0.08}$	*T. turkestanica*
	$EST1^{0.10}$ or $EST1^{0.13}$	4
4.	$EST2^{0.20}$	*T. cordubensis*
	$EST2^{0.22}$ or $EST2^{0.24}$	*T. evanescens*

Discussion

Individuals belonging to *T. evanescens*, *T. cordubensis*, *T. turkestanica*, *T. pintoi* and *T. bourarachae* could be distinguished both by ITS-2 PCR product electrophoresis followed by restriction analysis using MnlI endonuclease and by esterase electrophoresis. These methods gave unambiguous and repeatable results, which can be reproduced reliably. The use of the dichotomous keys here presented makes these methods practical to use.

These methods are not intended to replace more traditional morphological methods of species identification. However, they can be very valuable in: 1) differentiating between particular species, when it is known which species occur in a certain area; 2) taxonomy, when used complementary to other methods; 3) checking for potential contamination during laboratory rearings; 4) studies on parasitoid dispersal; 5) evaluation of parasitism following wasp releases. Moreover, these techniques are easy to implement.

PCR amplifications followed by restriction digestions can be performed in laboratories equipped with PCR machines, and DNA used for analysis can be extracted from living, frozen, ethanol-preserved or dried material (Post *et al.* 1993, van Kan *et al.* 1996). Esterase electrophoresis can only be done on fresh or snap frozen material (*e.g.* Richardson *et al.* 1986), but can be less costly than DNA-based analysis.

Esterase 1 and 2 loci were sufficient to differentiate the five species examined. So, for the purpose of differentiation between these species, only these 2 loci need to be tested.

The intraspecific variability both in total ITS-2 length and in restriction fragments length appears to be low. This is corroborated by analyses of ITS-2 sequences of 8 other European *T. evanescens* (Stouthamer, pers. com.), as well as by analyses of 6 American *Trichogramma* species (Stouthamer *et al.*, in press).

Species belonging to the same groups (*T. evanescens* and *T. turkestanica* from *evanescens* group) or to related groups (*T. pintoi* and *T. bourarachae* from *pintoi* group and *perkinsi* group, respectively) are more similar to each other in ITS-2 nucleotide sequence and size than to other species. This supports previous reports indicating that the ITS-2 region has good potential to be used in phylogenetic studies at the species level (*e.g.* Schilthuizen & Stouthamer 1997, van Kan *et al.* 1996, 1997).

Presently, we do not know whether the five species studied are the only *Trichogramma* species occurring in Southern Portugal. We hope that the here described methods will enable us to detect the eventual presence of other species not yet recorded for Portugal.

A good way to confirm species identities would be to perform crossing experiments as described by Pinto *et al.* (1991) and Pintureau (1991). Our results are in concordance with the crossing compatibility studies by van Tilborg *et al.* (1997). From our study, we conclude that rDNA markers and esterase patterns can be used for reliable differentiation between *Trichogramma* species indigenous to tomato fields in Portugal.

Acknowledgements

We are grateful to Guy Boivin, Menno Schilthuizen and Joop van Lenteren for their useful comments on the work, Carola Meierrose for her collaboration during *Trichogramma* field collection, Patricia Garcia for providing *Trichogramma* specimens and Koppert Co. for supplying the host eggs. The research of I.S. and L.N. was financially supported by Junta Nacional de Investigação Científica e Tecnológica (JNICT, Portugal; I.S.: BD 2107 92-IE and BD 5620/95), while J.H. and H.J. were supported by an EU grant AIR-CT94-1433 and F.v.K. by an NWO-STW grant.

Chapter 3

Specificity of courtship behaviour in *Trichogramma* and characterisation of *Trichogramma turkestanica* putative sex pheromone

Partly published (with modifications) as: Silva, I.M.M.S. & R. Stouthamer (1997). To mate or not to mate...can sex pheromones be used as a taxonomic tool in *Trichogramma* spp.? *Proc. Exper. & Appl. Entomol., N.E.V.*, Amsterdam, 8: 41-46.

Abstract

Intra- and interspecific mating attempts and castings were studied in five sympatric *Trichogramma* species. With exception of *Trichogramma cordubensis*, most of the males attempted to mate with conspecific females. However, only *T. pintoi* exhibited complete specificity. For the other four species, interspecific mating attempts were observed. Castings were strongly correlated with mating attempts. Thus, the mating behaviour of *Trichogramma* spp. cannot be universally used as a taxonomic tool for species recognition. For one of the *Trichogramma* species, *T. turkestanica*, two compounds ($C_{17}H_{32}$ and $C_{17}H_{32}O$) were identified as possible sex pheromonal components. These compounds were characterised by their mass spectra, accurate masses and retention indices. Their exact structure is still unknown. To our knowledge these two compounds have not yet been reported for insects.

Introduction

Animals have developed, in the course of evolution, a variety of ways of recognising and interacting with conspecifics of the opposite sex. In general, and for short-living animals in particular, it is important to quickly recognise suitable mates and to avoid sterile attempts of mating with individuals of other species. So, we can expect that many stimuli (chemical, acoustic, tactile and visual) and behaviours involved in mating will be specific and relatively conserved within a species.

Mating behaviour has been studied in a number of parasitoids, for both fundamental and practical reasons (see van den Assem 1996 for a review on mating behaviour of parasitic wasps). Since minute wasps are often difficult to identify at the species level (*e.g.* Pinto & Stouthamer 1994), mating behaviour may be a useful tool in species recognition. Indeed, studies on parasitoid courtship behaviour revealed species-diagnostic characters useful to identify sibling species, otherwise difficult to distinguish from each other (*e.g.* van den Assem & Werren 1994).

The mating behaviour of the egg parasitoid *Trichogramma* (Hymenoptera, Trichogrammatidae) has been described in detail by Hase (1925). His report concerned individuals identified as *T. evanescens*, but his descriptions seem to apply to many other, if not all, *Trichogramma* species. The male walks while rapidly swinging the body to the left and to the right (casting). Next, he grasps the female from the back with his fore- and mid-legs, mounts her (his body does not touch the substrate or only the last pair of legs does, his wings are positioned upwards) and introduces its aedeagus in the female's genitalia. Females frequently walk away from males that persistently follow them. We are not sure whether this behaviour of the females is part of their courtship behaviour, nor do we know whether copulation implies an acceptance of the male by the female or if females can be forced to engage in copulation. Males may also try to copulate with dead females or with other males, particularly soon after emergence. In the laboratory, females may mate more than once. Post-insemination guarding of the female by the first male has not been reported for *Trichogramma* (Pintureau *et al.* 1997).

Visual detection may be involved in *Trichogramma* mating recognition but casting behaviour can be displayed even in absence of females (Pompanon *et al.* 1997), which is evidence of an important role of chemicals in mate recognition. Behavioural evidence for the existence of sex pheromones in hymenopterous parasitoids has been reported in over 35 species of ten families (Swedenborg & Jones 1992). Living wasps, body parts or fluids and/or solvent extracts have been used as sources of attraction (Swedenborg & Jones 1992).

Only a few components of (putative) sex pheromones of hymenopterous parasitoids have been identified. These compounds are esters of fatty acids, monoterpenoids, acetates of unsaturated alcohols, aldehydes, a hydrocarbon, a lactone and a ketone (after review by Quicke 1997), which shows a large diversity of chemical groups involved. They include ethyl palmitoleate [in *Syndipnus rubiginosus* (Ichneumonidae)], neral and geranial [in *Itoplectis conquisitor* (Ichneumonidae)], Z-(4)-tridecenal and (3R*, 5S*,6R*)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydro-pyran-2-one [in *Macrocentrus grandii* (Braconidae)], 6-methyl-5-heptene-2-one [in *Alloxysta victrix* (Alloxystidae)](Eller *et al.* 1984, Robacker & Hendry 1977, Swedenborg *et al.* 1993, Micha *et al.* 1993).

Most of the examples from literature concerning pheromones of parasitic wasps refer to volatile short-range attraction pheromones (few millimetres distance) or contact sex pheromones (*e.g.* Eller *et al.* 1984, Kainoh & Oishi 1993, Pompanon 1995). The scarcity of information on parasitoid long-range sex pheromones may be due to the low number of studies on this subject. A plausible alternative explanation is that these pheromones are less common in haplo-diploid species (in which females can produce descendants without mating) than in other groups (Godfray 1994).

Most of the known parasitoid sex pheromones are produced by females (*e.g.* Lewis *et al.* 1971, Gordh & DeBach 1978, Robacker & Hendry 1977, Kainoh & Oishi 1993, Vinson 1972), but male sex pheromone production is not uncommon (*e.g.* Gonzalez *et al.* 1985, Kimani & Overholt 1995). Males can also be attracted by other males (*e.g.* Lewis *et al.* 1971, Robacker & Hendry 1977), which suggests that sex pheromones can be multifunctional chemicals because the distinction between sex pheromones and aggregation pheromones is not always clear (for a review on semiochemical parsimony in the Arthropoda see Blum 1996).

There is evidence for the production or presence of sex pheromones in a variety of glands or body parts: mandibular glands (Swedenborg *et al.* 1993), a gland at the base of the 2nd valvifer (Tagawa 1977), Dufour's gland (Syvertsen *et al.* 1995), metasoma (abdomen) (Yoshida 1978) and mesosoma (thorax) [tibia (Kainoh & Oishi 1993)]. It has been suggested that in some cases a recognition and/or aphrodisiac pheromone may be produced in and released from the (male) antennae (Pedata *et al.* 1993, Isidoro & Bin 1995) and that some sex pheromonal components may be present all over the surface of the body (Tagawa 1977).

Studies on sex pheromone specificity have been performed for several parasitoids. In some cases attraction appears to be species-specific, as for the ichneumonid *Syndipnus rubiginosus* (Eller *et al.* 1984) and the aphelinids *Aphythis maculicornis* and *A. mytilaspidis*

(Khasimuddin & DeBach 1975). In other cases attraction appears to be restricted to a group of closely related species as in the braconid *Cotesia flavipes* complex (Kimani & Overholt 1995). The characterisation and identification of parasitoid sex pheromones can be useful in systematics (as a chemotaxonomic tool and in phylogenetics), in mating structure characterisation, in population dynamics studies and in predicting parasitism rates in the field.

Little is known about *Trichogramma* sex pheromones. Pintureau & Toonders (1983) have shown evidence for volatile sex pheromones in *T. brassicae*. Pompanon *et al.* (1997) and de Schepper (1993, in Pompanon 1995) have shown evidence for substrate-borne sex pheromones in *T. brassicae* and *T. bourarachae*. Males, whatever their mating status, responded to virgin female extracts and to hexane used to rinse tubes where virgin females had walked. These responses can be recorded as arrestment or display of casting. Males do not respond to extracts of mated females (Pompanon *et al.* 1997 and personal observations) or to male extracts (personal observations). *Trichogramma brassicae* and *T. bourarachae* substrate-borne sex pheromones are not specific, but in a choice situation males choose conspecific females (de Schepper in Pompanon 1995).

In this study we have investigated the specificity of mating attempts of 5 *Trichogramma* species and we characterised a putative female sex pheromone of one of these species. Our purpose was to study the possibility of creating a tool for species identification. Furthermore, we are interested in possible interactions between these sympatric species and in the factors involved in mating behaviour in *Trichogramma* species.

Material and methods

Cultures

Trichogramma lines were started with single females collected in Portugal (Alentejo province) from field-collected *Helicoverpa armigera* Hbn. (Lepidoptera, Noctuidae). We studied five lines identified as: *T. turkestanica* Meyer 1940, *T. evanescens* Westwood 1833, *T. pintoi* Voegele 1982, *T. bourarachae* Pintureau & Babault 1988 and *T. cordubensis* Vargas & Cabello 1985. The collections were performed in two tomato fields: Mora, in 1992 (*T. evanescens* and *T. turkestanica*) and Divor, in 1993 (*T. cordubensis*, *T. pintoi* and *T. bourarachae*). These localities are about 50 km apart. The *T. cordubensis* line was originally thelytokous (uniparental). An arrhenotokous line of *T. cordubensis* was established by feeding thelytokous females antibiotic (Stouthamer *et al.* 1990), about 50

generations prior to the experiments. Cultures were maintained on UV killed *Ephestia kuehniella* Zeller (Lep., Pyralidae) eggs, at 22 ± 2 °C, RH 50 ± 20 %, 16 h light/8h dark.

Mating behaviour experiment

One male and one female (virgin, fed, 2-4 days old) were placed, just before each observation, in a gel capsule [(25x8x8mm), code 000]. Observations were performed until the male mounted the female (named "mating attempt" throughout the text) or until 5 minutes had passed. Time until mating attempt and whether the male performed casting were recorded. Trials were performed at 22 ± 1°C, RH 40 ± 10%. Males of each species were tested against females of each of the five species. All possible 25 combinations were tested on the same morning; the trial was repeated seven times (3 missing values for *Trichogramma cordubensis*, see Table 1).

For each individual observation we registered whether mating attempts and castings had been observed. The proportions of couples where mating attempts were observed from the total number of observations for each female species – male species combination were statistically compared in several ways: a) all conspecific couples vs. all heterospecific couples; b) differences between species in the conspecific couples and c) effect of male species, female species and interaction male * female in the heterospecific couples. The statistical analyses were performed using generalised linear models, fitted with the statistical software SAS (genmod procedure, binomial distribution, and logit as link function).

Identification of volatiles

Biological samples

Individuals of *Trichogramma turkestanica* (line MB39) were used.

Virgin individuals. In order to obtain virgin individuals, parasitised (black) *Ephestia kuehniella* eggs (ca. 500) were individually placed in a glass vial (75 mm length, 10 mm diameter) containing a tiny drop of honey. These eggs were incubated at 23 ± 1°C, RH 50 ± 20%, 16 h light/ 8 h dark. After adult emergence (ca. 13 days after parasitism had occurred), parasitoids 1-3 days old were sexed and, by means of an aspirator, collected into two pipette tips, one for males and the other for females. Individuals in both pipette tips were CO_2 anaesthetised for ca. 2 minutes, and introduced into glass screw vials (2 ml, Phase Separations) containing several tiny drops of honey. The vials were closed by a screw cap fitted with a PTFE-lined rubber septum. Two samples, one of males and one of

females, of 40-80 individuals each, were prepared each time. For the accurate mass measurement, two vials containing ca. 40 virgin females were prepared in the same way as above.

Mixed population. Females and males 1-3 days old, presumably mated, were allowed to walk in an empty glass vial (15 cm length, 1.5 cm diameter). Then, they were anaesthetised and introduced in a new vial, as explained above. Samples consisted of ca. 80 individuals (in 2ml Phase Separations glass screw vial) or ca. 200 individuals (4.5 ml, Phase Sep glass screw vial). In both cases, more than the half of the individuals was females.

Solid phase microextraction (SPME)

Volatiles were collected by SPME needles with 100 μm polydimethylsiloxane coated fibbers (Supelco, Inc.). The needle was inserted through the septum in the vial containing the wasps and remained inside for 20-24 h, at 18-23 °C. Virgin females were sampled five times, males four times (simultaneously with females) and mixed populations were sampled twice.

Gas-Chromatography

A Hewlett Packard 6890 gas chromatograph equipped with a HP autosampler one split/splitless injection system, a 1:1 inlet splitter, two columns and two FI detectors was used. The two columns were of the dimethylsiloxane (J&W 60 m DB-1, 0.25 mm i.d. and 0.25 mm film thickness) and polyethyleneglycol (Restek 60 m Stabilwax DB-Wax, 0.25 mm i.d. and 0.25 mm film thickness) type respectively. Injection was splitless during 1 min. After 1 min a split ratio 1: 50 was maintained, carrier gas H2, initial inlet pressure 20 psi, linear velocity 47 cm/sec; a constant flow was maintained during the entire run; temp. prog. 30° (1 min hold) to 238° (2 min hold) at 4°/min; inj. temp. 220°; det. temp. 260°, N2 as make-up gas. Integration of peaks was carried out by HP Chemstation software.

Gas Chromatography-Mass Spectrometry (GC-MS)

For characterisation of the components, a gas chromatograph-mass spectrometer (Finnigan MAT 95), equipped with a BP5 column, was used. The SPME-needle was introduced into the injection port (splitless, 220 °C) during 1 minute with the column maintained at 30 °C. After removal of the SPME needle and changing to split-mode (24 ml/min), the analysis was started by programming the column-oven temperature from 60°C to 260°C, at 4°/min. The mass spectrometer was operated in the 70 eV EI ionisation mode and scanning from mass 24 to 300 at a speed of 0.7 seconds/decade. For the accurate mass measurements only

virgin females were used. Accurate mass measurements were performed using a primary standard (PFK) and a secondary standard (S_8) at a resolution of ($M/\Delta M$) = 1000.

Results

Mating behaviour experiment

Castings and mating attempts were strongly correlated and occurred intra- and interspecifically. In general, mating attempts occurred more frequently towards conspecifics. However, *T. cordubensis* differed in this respect: no males attempted to mate with *T. cordubensis* females. A high frequency of mating attempts between *T. turkestanica* males and *T. bourarachae* females was found. Only *T. pintoi* males attempted mating with *T. pintoi* females. *T. pintoi* and *T. bourarachae* males attempted to mate only with conspecific females (Table 1). Average time to mate was about 1.5 minutes for all combinations, ranging from 19 seconds to almost 5 minutes.

Table 1 shows clear evidence for different intra- and interspecific mating attempts and casting rates. These rates are higher in couples of conspecifics. A summary of the statistical analysis of the results is presented in Table 2.

Differences between species in intraspecific mating attempts and casting rates are mainly due to the differences between *T. cordubensis* and the other species. Pairwise comparisons between mating rates of conspecifics through the Bonferroni-approach (using deviance tests at significance level 0.05/10=0.005) resulted in significant differences between *T. cordubensis* on one hand and *T. turkestanica*, *T. evanescens* and *T. bourarachae* on the other.

Differences between interspecific mating attempts and casting rates can be explained by male and female species. The comparison between males (or females) of different species for the interspecific matings (and castings) concerned the same female (or male) species. For example, the comparison of *T. turkestanica* and *T. evanescens* males concerned the matings of these males with *T. cordubensis*, *T. bourarachae* and *T. pintoi* females. Pairwise comparisons between male species in matings, resulted in significant differences (Bonferroni approach) between *T. turkestanica* on one hand and *T. evanescens*, *T. bourarachae* and *T. pintoi* on the other, and in castings, between *T. turkestanica* on one hand and the other 4 species on the other. Pairwise comparisons between female species in mating resulted in significant differences (Bonferroni

Table 1. Results of *Trichogramma* spp. mating behaviour experiment, where one male and one female were confined until mating was attempted or until five minutes had passed; Mating = number of couples where mating attempts were observed; Casting = number of males which exhibited casting behaviour. Sample size = 7, except for the combinations: male *T. cordubensis* vs. female *T. evanescens*, male *T. cordubensis* vs. female *T. cordubensis* and male *T. cordubensis* vs. female *T. turkestanica*, for which sample size = 6.

	♀ *T. turkestanica*		♀ *T. evanescens*		♀ *T. cordubensis*		♀ *T. bourarachae*		♀ *T. pintoi*	
	Mating	Casting	Mating	Casting	Mating	Casting	Mating	Casting	Mating	Casting
♂ *T. turkestanica*	7	7	2	4	0	0	5	6	0	0
♂ *T. evanescens*	1	1	5	7	0	0	0	1	0	0
♂ *T. cordubensis*	2	2	1	2	0	0	0	0	0	0
♂ *T. bourarachae*	0	0	0	0	0	0	7	7	0	0
♂ *T. pintoi*	0	0	0	0	0	0	0	0	4	4

approach) between, on one hand *T. turkestanica* and *T. bourarachae* and on the other hand *T.cordubensis* and *T. pintoi*. Similar results were obtained for casting, in which *T. evanescens* joined the *T. bourarachae* and *T. turkestanica* group.

Table 2. Analysis of deviance table of mating attempts and casting proportions in five *Trichogramma* species. Males and females (single pairs) were confined in a gelatine capsule, in a total of 25 combinations. The following comparisons were performed: a) all couples of conspecifics vs. all couples of heterospecifics; b) between species, in the conspecific couples and c) effect of male species, female species and interaction male * female species in the heterospecific couples.

Source	Deviance*		df	*p*-value	
	Mating	Casting		Mating	Casting
Conspecifics versus heterospecifics	51.20	50.31	1	<0.0001	<0.0001
Conspecifics	24.87	29.74	4	<0.0001	<0.0001
Heterospecifics	41.04	56.71	19	<0.0024	0.0001
Males	24.56	31.62	4	<0.0001	<0.0001
Females	19.07	25.58	4	<0.0008	<0.0001
Interaction	3.85	5.25	11	0.99	0.92

*) Deviances of males, females and interaction do not sum up to heterospecific deviance, because deviance for males is corrected for females and deviance for females is corrected for males.

Characterisation of volatiles

Two compounds (A and B) have been detected from virgin females in 5 samples. Two samples resulted from the procedures described in the material and methods (see *virgin individuals* and *SPME*) and 3 were prepared during preliminary samplings. These compounds have not been detected in mixed (females + males) populations (2 samplings) or in virgin males (4 samplings).

Gas chromatograms of *Trichogramma turkestanica* samples and mass spectra of the two compounds are shown in Fig. 1 and 2. The detected amounts of A and B were 0.6 ng and 2.8 ng, respectively.

The accurate mass measurements gave the following elemental compositions: $C_{17}H_{32}$ (observed mass 236.2492 atomic mass units [amu], calculated mass 236.2504 amu) for compound A; and $C_{17}H_{32}O$ (observed mass 252.2458 amu, calculated mass 252.2453 amu) for compound B. Both molecules have an r+d (rings and/or double bonds) value of 2. Retention times for each column were converted into retention indices. The retention index of a substance for a given stationary phase represents 100 times the number of carbon atoms

in a molecule of a hypothetical n-alkane that has the same retention on that phase (Ettre 1964, Pacáckovά & Feltl 1992). The retention indices found are shown in Table 3. The retention index for compound A on all three columns is unusually small for a C_{17} hydrocarbon, indicating that compound A is probably a highly branched acyclic compound. Compound B is probably a closely related structure containing a primary alcohol group (T.A. van Beek and M.A. Posthumus, pers. com.). The exact structure of compounds A and B could not be deduced from our data.

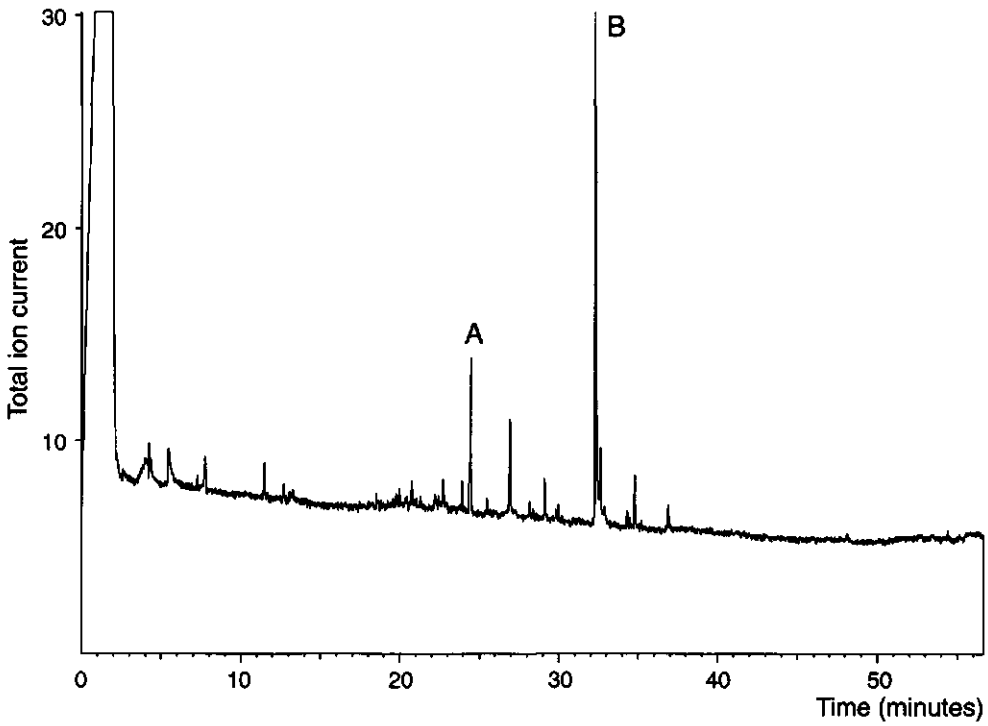


Fig. 1. Gas chromatogram of *Trichogramma turkestanica* virgin females sample.

Table 3. Retention indices of compounds A and B on three different chromatographic columns (DB1 is the most apolar column and DB-Wax the most polar).

Columns	DB1	BP5	DB-Wax
Compound A	1511	1518	1579
Compound B	1766	1791	2317

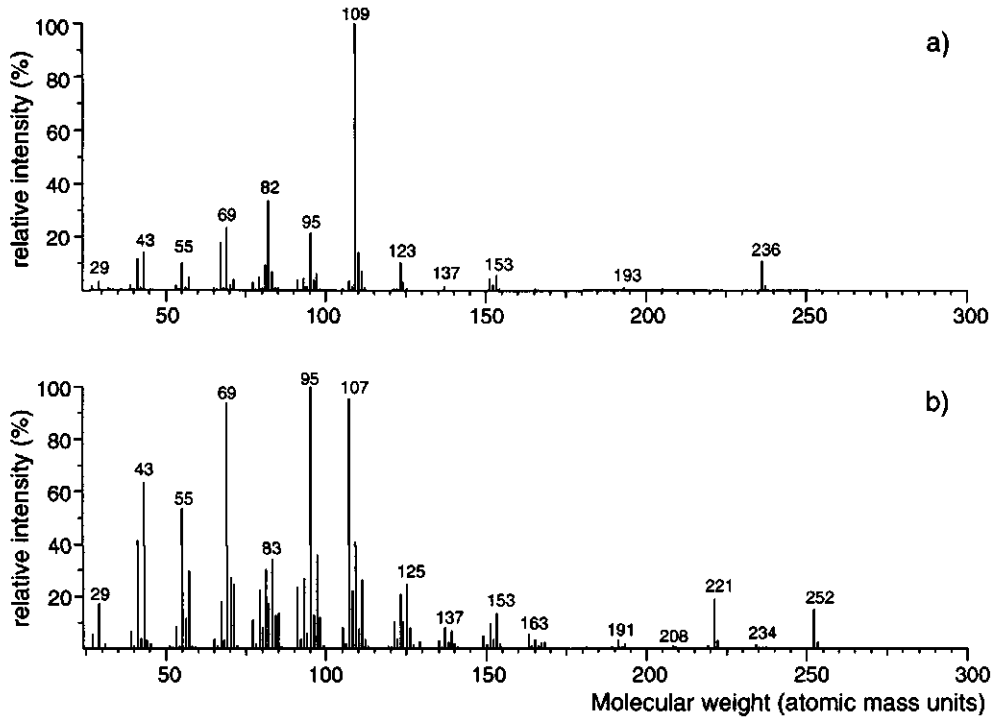


Fig. 2. Mass spectra of a) compound A and b) compound B, detected for *Trichogramma turkestanica* virgin females samples.

Discussion

From the five *Trichogramma* species studied, only *T. pintoi* exhibited complete specificity in mating. In general, more intraspecific mating attempts and castings occurred intraspecifically than interspecifically. Although this difference was significant, revealing that recognition of conspecifics exists, many interspecific mating attempts and castings occurred. Thus, determining mating indices is not a suitable method for taxonomic purposes in *Trichogramma* spp. Nevertheless, males of *T. pintoi* and *T. bourarachae* attempted to mate only with conspecific females, which suggests that this method could be used as a taxonomic tool to distinguish between these two species. However, because not all *T. pintoi* males mounted their conspecific females within 5 minutes, repetitions would have to be performed more often or for a longer period if this method was to be used. Furthermore, more reliable approaches in identification of *Trichogramma* species are now available, *e.g.*

by means of DNA analyses (Chapter 2, van Kan *et al.* 1996, 1997, Stouthamer *et al.*, in press).

Trichogramma evanescens and *T. turkestanica* are closely related species, both belonging to the *evanescens* group. *T. pintoi* and *T. bourarachae* belong to the groups *pintoi* and *perkinsi*, respectively, which are closely related. *T. cordubensis* belongs to the *minutum* group, which is closer to the *evanescens* group than to the *pintoi* and *perkinsi* groups (Pintureau 1987, 1993b). A relationship between the phylogeny of these species and mating attempts (and castings) appears to exist, with females of *T. turkestanica* and *T. evanescens* being courted by *T. turkestanica*, *T. evanescens* and by *T. cordubensis* males and not by males of the two more distant species. However, when one looks at the males performance, *T. turkestanica* has a remarkably low specificity, with a high proportion of males that attempted to mate with females from the distant *T. bourarachae* species. *Trichogramma pintoi* and *T. bourarachae* males were completely species-specific, in contrast to males of the other species. We would expect males to be less specific than females, since for a male the costs of mating with a wrong partner are lower than for a female. In our experiment we could not recognise an acceptance or rejection of the males by the females, nor do we know whether the production of female sex pheromone is dependent of the willingness of the female to mate. It is known that the presence of males is not necessary for virgin females to release sex pheromone (Pompanon *et al.* 1997).

Trichogramma turkestanica and *T. evanescens* are one-way partially compatible: the cross of male *T. turkestanica* with female *T. evanescens* produces female progeny in the laboratory (Neto & Pintureau 1995, van Tilborg *et al.* 1997). So, in this case specificity of mating attempts was not expected. It is unknown, with the exception of these two species, whether interspecific matings result in sperm transfer and fertilisation.

The behaviour of *Trichogramma cordubensis* males was exceptional: they exhibited casting towards *T. turkestanica* and *T. evanescens* females, but not towards *T. cordubensis* females. The lack of this behaviour towards conspecific females has been previously reported for *T. cordubensis* (Silva & Stouthamer 1996) and casting by *T. cordubensis* males towards heterospecifics is, to our knowledge, reported for the first time. Not a single male attempted to mate or performed casting towards *T. cordubensis* females. Possibly, the production of sex pheromone by *T. cordubensis* females is low or completely lacking. This could be expected in fixed thelytokous populations, where costs of sex pheromone production are not compensated by the benefits of sexual reproduction. If all natural *Trichogramma cordubensis* populations are composed of females only (and there is no evidence to the contrary), activities related with mating, either in males or in females, may

not be maintained or may be selected against. It seems that in similar cases the female function is usually lost first, although it is not clear yet why female traits may accumulate mutations more rapidly (for possible explanations see Pijls *et al.* 1996 and Stouthamer 1997). However, we know that successful matings within *T. cordubensis* do occur and we have been able to establish pure sexual lines of this species and to maintain them for several years. Preliminary experiments (Silva, unpublished) suggest that the placement of a group of individuals of both sexes together (as opposed to single pairs) may trigger mating in *Trichogramma cordubensis*. This phenomenon has been previously reported for the eulophid *Chrysocharis larcinellae* (Ratz.), where single pairs would not mate, but individuals readily mated when several pairs were placed together (Quednau 1967 in Gordh & DeBach 1978). Differences in the concentration of sex pheromone present could be responsible for this phenomenon.

The five species studied here are sympatric (specimens of at least four of these species have been found from the same host species in a single 4 ha tomato field, within a few weeks time). We would then expect that mechanisms of avoidance of interspecific matings would have evolved. However, not much is known about the ecology of these species and it is possible that heterospecifics of opposite sexes do not meet often in the field.

Behavioural evidence of the existence of substrate-borne sex pheromones, by observing castings of males as a response to hexane extracts of virgin females, was found for all species studied here, except for *T. cordubensis* (Silva, unpublished). It would be very interesting to find out how the identity of the sex pheromones of these species relate to their phylogeny and mating behaviour, and to study the possibility of using sex pheromone composition as a chemotaxonomic tool.

We found two volatile compounds, A and B, which are present in virgin *T. turkestanica* females but not in males or in mixed populations. These compounds have not been previously reported for insects. We hypothesise that these compounds may constitute the *T. turkestanica*'s equivalent to the substrate-borne sex pheromone mentioned for *T. brassicae* by Pompanon *et al.* (1997). Whether A and B are, in fact, sex pheromonal components remains to be demonstrated.

Acknowledgements

To Teris van Beek and Maarten Posthumus for the analyses of volatiles and discussions; Gerrit Gort for the statistical analyses; Kate Flanagan, Rieta Gols, Hanneke van Heest, Jeff

Honda, Frenk van Kan, Gerard van Leeuwen, Marnix van Meer, Menno Schilthuizen and Jessica Vereijssen for help in isolating eggs; Carola Meierrose for collaboration during field work; Luis Neto and Bernard Pintureau, for *Trichogramma* identifications, and Louise Vet and the Entomology PhD group for discussions. The work of the first author was financially supported by Junta Nacional de Investigação Científica e Tecnológica (JNICT: BD 5620/95) and by Calouste Gulbenkian Foundation (Portugal).

Chapter 4

Female fitness in *Wolbachia*-fixed and *Wolbachia*-mixed *Trichogramma* populations

Slightly modified from Meer, M.M.M. van, I.M.M.S. Silva, M.M. Roskam, G. Gort & R. Stouthamer. Female fitness in *Wolbachia*-fixed and *Wolbachia*-mixed *Trichogramma* populations (submitted).

Abstract

In haplodiploid species, the normal mode of reproduction is arrhenotoky: males arise from unfertilised eggs and females from fertilised eggs. A less common mode of reproduction is thelytoky in which all unfertilised eggs give rise to female offspring. In Hymenoptera, thelytoky can be induced by *Wolbachia*, an α proteobacterium. The infection can be fully established in a population so that only thelytokous females are present (= fixed population). But there are also cases where thelytokous females coexist with arrhenotokous ones (= mixed population). In mixed populations, thelytokous females are still able to mate and produce offspring sexually. In fixed populations, the degree of co-evolution between insect host and symbiont *Wolbachia* will increase and it is theorised that this will result in a reduction of negative impact of the symbiont on its host. This hypothesis was tested with wasps of the genus *Trichogramma* because both mixed and fixed populations occur within this genus. Thelytokous females of two isofemale lines from fixed populations and four isofemale lines from mixed populations were "cured" from *Wolbachia* infection using antibiotics. Several fitness parameters were measured for these *Wolbachia* infected lines and their cured counterparts. Daughter production was significantly higher for the thelytokous lines from fixed populations compared to the arrhenotokous ones. This contrasts with three lines from mixed populations where the daughter production was lower for the infected lines compared to the cured lines. Only a slight fecundity effect of *Wolbachia* in lines from fixed populations was found for total offspring numbers while a severe negative impact of *Wolbachia* was found in the lines from mixed populations. The *Wolbachia* transmission efficiency (% females) was generally higher for the thelytokous lines from fixed populations with the exception of one line of *T. deion* from a mixed population. Finally, negative *Wolbachia* effects were detected for longevity, pupa and embryo mortality for several *Trichogramma* lines. Our results are discussed in the context of a previously published model that describes the dynamics of *Wolbachia*-infected *Trichogramma* wasps in mixed populations.

Introduction

The role of cytoplasmic genes as an important component of hereditary material has long been neglected (Cosmides & Tooby 1981). While nuclear genes are passed from both parents to the offspring, cytoplasmic or extranuclear genes are inherited mainly from the mother. This implies that males constitute a dead end for cytoplasmic genes. Therefore it is advantageous for cytoplasmic factors to bias the offspring sex ratio of their host towards the production of daughters. For the nuclear genes however, reproductive fitness is also gained through males. These differences in evolutionary interests can create a conflict between nuclear and cytoplasmic genes (Cosmides & Tooby 1981).

Several cytoplasmic sex ratio distorters, which interfere with the host mode of reproduction, have been described (for a review see Hurst 1993) and one of the most conspicuous examples is the intracellular symbiont *Wolbachia*. This bacterium is widespread in the phylum Arthropoda, cytoplasmically inherited and can modify the reproductive phenotype of its host in various ways such as through cytoplasmic incompatibility (CI), induction of thelytoky (T) or by feminisation of the male host (for a review see Werren & O'Neill 1997).

Wolbachia-induced thelytoky (T) is a form of parthenogenesis that has presently only been found in various species of the order Hymenoptera (Rousset *et al.* 1992, Stouthamer *et al.* 1993, van Meer *et al.* 1995, Zchori-Fein *et al.* 1995). In this order, the normal mode of reproduction is arrhenotoky, in which unfertilised eggs develop into males, while fertilised diploid eggs develop into females. The presence of *Wolbachia* in eggs of thelytokous females causes a disruption of the chromosome segregation in the first mitotic division (anaphase) of the haploid egg. As a result, haploid eggs become diploid and develop into thelytokous females (Stouthamer & Kazmer 1994). Thelytokous strains can be maintained without the involvement of males. When thelytokous females age, male offspring is sometimes produced probably because the *Wolbachia* titer declines with the mother's age.

The *Wolbachia* infection can be fully established in a population so that only thelytokous females are present (=fixed population) or the infection is only partially present in a population so that it consists of a mixture of infected and uninfected individuals (Stouthamer 1997). In mixed populations, a nuclear-cytoplasmic conflict can exist between thelytoky-inducing *Wolbachia* (T-*Wolbachia*) and their host. In such populations, mating can take place between infected thelytokous females and uninfected males. The mated thelytokous females produce daughter offspring sexually from fertilised

eggs and homozygous females from unfertilised eggs (Stouthamer & Kazmer 1994). When the frequency of infection rises in a population, the population sex ratio becomes increasingly female biased. In this situation, few males can mate with many females. Therefore, increased reproductive fitness can be gained by the nuclear genes that favour production of males. These conflicting interests of nuclear and cytoplasmic genes (*i.e. Wolbachia*) could lead to the evolution of nuclear genes that suppress the effect of *Wolbachia* and thus restore the female's ability to produce male offspring. Suppressor genes have been found against feminising *Wolbachia* in the isopod *Armadillidium vulgare* (Rigaud & Juchault 1992) but have not yet been found for T-*Wolbachia*, although there are suspected cases (Stouthamer 1997). To compensate for the effects of the suppressor genes, T-*Wolbachia* could respond e.g. by overexpression of proteins responsible for thelytoky-induction or by increasing their density in the host. These compensatory effects could lead to a negative fitness effect of T-*Wolbachia* on their hosts. Fecundity experiments have shown that in mixed populations of the egg parasitoids *Trichogramma deion* (Irvine) and *Trichogramma pretiosum* (Nuevo Leon) there is indeed a negative effect of T-*Wolbachia* on daughter and total offspring production (Stouthamer & Luck 1993, Horjus & Stouthamer 1995), which is consistent with the hypothesis of a nuclear-cytoplasmic conflict.

Once the T-*Wolbachia* infection has reached fixation in a population, the nuclear-cytoplasmic conflict disappears because males are no longer present in the population and thus no fitness can be gained for the nuclear genes through the male function. Therefore, a reduction of negative impact of the symbiont ("benevolence") on its host can be expected over time (Ewald 1987, Bull *et al.* 1991, Lipsitch *et al.* 1995). Female-symbiont combinations that are most fit will spread through the population. An additional implication of fixation of the infection is that since sexual recombination is absent, slightly deleterious mutations will accumulate in expressed genes of the host's genome (Muller's ratchet) but accumulate at a much higher rate in those genes which are not expressed any longer. For example, in arrhenotokous populations there is a continuous selection on genes coding for sexual reproduction, i.e. specific male traits (mating behaviour or sperm production) or female traits (mating behaviour, sperm storage, fertilisation, pheromone production). But in fixed populations this selection either disappears, or some costly sexual traits may be selected against (Pijls *et al.* 1996). Therefore, one can expect a decrease in the ability of females and males to successfully mate, the longer a population is fixed. Evidence for this phenomenon has been found for fixed populations of the parasitoids *Muscidifurax uniraptor* and *Encarsia formosa* from

which cured females have lost the ability to mate successfully (van den Assem & Povel, 1973, Zchori-Fein *et al.* 1992). For the fixed populations of *M. uniraptor*, effects of the symbiont on host fecundity were studied and no negative impact of T-*Wolbachia* was found (Horjus & Stouthamer 1995). This supports the hypothesis of a reduced impact of the symbiont on its host after a relatively long time of co-evolution.

The number of studies on T-*Wolbachia* impact on host fitness is limited and in our study we addressed the two following questions: first of all, is the negative impact of *Wolbachia* on their host consistently linked to the infection status (fixed or mixed) of the population? And secondly, can benevolence of T-*Wolbachia* be found in fixed populations that have relatively recently become fixed for infection? To address these issues, we studied fitness effects of T-*Wolbachia* on several host species. Wasps of the genus *Trichogramma* provide an excellent opportunity to address these questions because several mixed and fixed populations exist within this genus. The fixation for *Wolbachia* infection in *Trichogramma* is probably relatively recent in comparison with *E. formosa* and *M. uniraptor* because arrhenotokous *Trichogramma* lines can still be established in contrast to the latter species.

Material and methods

Cultures

Thelytokous lines were started with single females. Wasps were reared in glass vials (150 mm length, 15 mm diameter), on UV irradiated eggs of *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) and were incubated at 23 ± 1° C, L:D = 16:8 and 70 ± 10% RH. *Ephestia kuehniella* eggs were provided by Koppert Biological Systems (Berkel en Rodenrijs). The generation time of *Trichogramma* lines under these conditions was approximately 12-13 days. The *Trichogramma* species, their infection status, geographic origin and abbreviations are listed in Table 1.

Curing of the *Trichogramma* lines

The thelytokous strains OL, AW, LC, JT and SW were cured by feeding the females honey with 0.5% w/v tetracycline for at least 3 generations. After being cured, the wasps were reared for at least 4 generations without antibiotics before they were used in the experiment so that no residual effect of tetracycline treatment would influence the fitness results. The line CO was cured in 1994 with the antibiotic rifampicin (1% w/v). To verify

that lines were cured of their infection, the following tests were performed i) 20 virgin females were isolated and allowed to produce offspring (all male broods indicate complete curing of the infection); ii) PCR with specific *Wolbachia wsp* primers (Braig *et al.* 1998) on the cured and infected strains.

Table 1. *Trichogramma* species and lines used in this study with their geographic origin and their infection status.

Species	Isofemale line code (abbreviation)	Geographic origin	*Wolbachia* infection status	Reference
T. cordubensis	28B143b (CO)	Divor, Portugal, 1993	Fixed	Silva & Stouthamer, 1997
T. oleae	1A (OL)	Yugoslavia	Fixed	Voegelé & Pointel 1979
T. kaykai	AW 7-5 (AW)	Sky valley, Riverside CA, USA	Mixed	Schilthuizen *et al.* 1998
T. kaykai	JT 6-3 (JT)	Joshua Tree, San Bernardino, CA, USA.	Mixed	-
T. kaykai	LC 110 (LC)	El Paso Mountains, CA, USA	Mixed	Schilthuizen *et al.* 1998
T. deion	SW 436-1 (SW)	Mojave desert, CA, USA	Mixed	Schilthuizen & Stouthamer 1997

PCR

Twenty-five *Trichogramma* wasps were homogenised with a sterile pestle in 50 µL STE (100mM NaCl/10mM Tris/1mM EDTA, pH 8.0) (O'Neill *et al.* 1992) and incubated with 1 µL proteinase K (20mg/ml) for one hour at 37°C, followed by 5 min at 95° C. Samples were spun for 2 min in a centrifuge and 1µL of the supernatant was used as template in subsequent PCR reactions. The *Wolbachia* outer membrane protein gene (*wsp*) was amplified using specific primers (Braig *et al.* 1998). For PCR, a temperature profile of 94°C for 3 min (1 cycle); 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (40 cycles) was utilised.

Fitness experiment

Trichogramma females emerging from *Ephestia* eggs are small. Because fitness differences between lines might be more pronounced using larger wasps, we reared *Trichogramma* on the larger *Mamestra brassicae* (Lepidoptera, Noctuidae) host eggs for one generation. *M. brassicae* was reared on cabbage plants (*Brassica oleracea* cv. Icarus)

and incubation conditions were 22°C ± 1°C and a photoregime of L16:8D. The fitness experiment, its preparation and incubation of progeny, were carried out at 23 ± 1° C, L:D = 16:8 and 70 ± 10% RH. Thirty female wasps, less than 24h old and emerging from *Ephestia kuehniella* eggs, were individually placed in glass vials with approximately twenty *Mamestra brassicae* eggs for one day. After 5 days, when the parasitised eggs had turned black, they were isolated and incubated in separate vials. To limit size differences between the females, only adults that emerged from host eggs producing two individuals were selected for the experiment. Per line, forty-two females were used. Due to a shortage of *Mamestra brassicae* eggs, only thirty-two thelytokous females of SW could be set up. Virgin females from the cured lines were allowed to mate with a male. After four hours, the males were removed. In the glass vial, a small droplet of honey was added and the wasps were offered an egg card with approximately 120 *Ephestia kuehniella* eggs. The first five days the egg masses were changed daily, after that they were changed every other day. The removed egg cards were placed in a new vial and incubated. Four days after the first offspring started to emerge, the vials were frozen (-80°C) and offspring was subsequently counted. Per female we determined the total number of host eggs parasitised (i.e. that had turned black) during her lifespan, longevity, the number of daughters and embryo or pre-adult mortality, and the sex ratio of the offspring. Individuals from thelytokous lines, which were partially haploid and diploid (gynandromorphs), were counted as males. The sex ratio is expressed as the percentage daughters of the total offspring. Because greenhouse studies showed that line SW can parasitise hosts for not longer than five days (Chapter 6) we calculated daughter and total offspring for 5 days and their lifespan respectively. Embryo and pupal mortality was determined by opening the parasitised unhatched eggs. For simplicity, all individuals with no body pigmentation were categorised as embryos. Only for line LC, embryo and pupa mortality was not distinguished. Because the size of a female has been shown to correlate with her fecundity (Waage & Ng 1984), females hind tibias were measured, using an optical micrometer mounted in an eyepiece of a compound microscope. All experiments were performed within a 3 month period and the infected and corresponding cured lines were always tested simultaneously. The lines OL and SW were tested in two different time periods.

Statistical analysis

Data were analysed with SAS (version 6.12) statistical software using a generalised linear model with the following distributions: binomial distribution for embryo and pupal

mortality, normal distribution for longevity, Poisson distribution for number of daughters and total offspring production. Tibia length, time period (block factor) and infection status (thelytoky or arrhenotoky) were explanatory variables. No time period effects were detected for OL and SW for any of the parameters studied and therefore the results of the separate time periods are not included in the results shown. Most tests were two-sided. Because the experiments for the different lines were not carried out simultaneously, no comparative statistical tests were performed between the lines with exception of the tibia length.

Results

Curing of the lines

All lines were cured after treatment with tetracycline or rifampicin for three generations. PCR with the *Wolbachia* specific *wsp* primers on infected and cured lines resulted in amplification of the proper product only in the infected lines. In addition, cured virgin females from all lines produced male offspring only (data not shown).

Offspring production and sex ratio

The fitness parameters daughter production and total offspring were calculated for five days and for the whole lifespan (Table 2). It was expected that *Wolbachia* would have a less negative fecundity effect on wasps from fixed populations than on wasps from mixed populations. In general, daughter production was significantly different between the thelytokous and cured conspecific arrhenotokous lines and there was also a clear difference between the fixed and mixed populations. The thelytokous wasps CO and OL, from fixed populations, produced more daughters in the first 5 days than their cured arrhenotokous counterparts. In contrast to the fixed populations, the opposite was found for the lines from the mixed populations AW, JT, LC and SW where thelytokous females produced less daughters than the cured arrhenotokous females. For lifespan daughter production the differences between the thelytokous and the arrhenotokous lines were similar to the ones mentioned for the first five days fecundity, with the exception of line SW where the difference between the arrhenotokous and the thelytokous line was not significant.

For total offspring production we expected to find a similar trend as for daughter production. But in the five first days no significant difference in offspring production was detected for the line CO (Table 2). For line OL an interaction occurred between

Table 2. Mean values of daughter and total offspring of day 1-5 and of whole lifespan with standard deviation (sd) and mean tibia length of the different thelytokous (T) and arrhenotokous (A) *Trichogramma* lines. (n= number of females tested; mT= mean tibia length; m♀♀= mean daughter offspring; m♀♀♂♂ = mean total offspring).

Line		mT (mm)	m♀♀ ± sd Day 1-5	n	*p*	m♀♀ ± sd Day 1-end	N	*p*	m♀♀♂♂ ±sd Day 1-5	n	*p*	m♀♀♂♂ ± sd Day 1-end	n	*p*
CO	T	0.1894	85.7 ± 14.5	40	**	113.9 ± 30.3	36	*	86.9 ± 14.7	40	n.s	123.7 ± 37.0	36	**
	A	0.1899	71.3 ± 18.3	41		98.3 ± 22.9	39		81.2 ± 21.1	41		152.5 ± 45.7	39	
OL	T	0.1767	88.1 ± 11.0	41	***	159.3 ± 20.9	37	***	91.1 ± 9.1	41	**§	236.6 ± 26.5	37	**§
	A	0.1809	61.0 ± 22.7	38		69.1 ± 30.5	34		87.0 ± 16.2	40		223.8 ± 51.7	36	
AW	T	0.1922	48.9 ± 15.2	41	**	56.5 ± 17.9	33	***	54.5 ± 15.6	41	***	87.7 ± 24.9	33	***
	A	0.1960	68.6 ± 20.2	38		98.9 ± 32.7	34		86.4 ± 21.7	39		133.8 ± 31.9	34	
JT	T	0.1918	27.9 ± 6.3	40	***	31.9 ± 8.2	31	***	37.2 ± 7.9	40	***	77.2 ± 23.4	31	***
	A	0.1971	60.2 ± 16.4	39		82.0 ± 28.4	32		82.0 ± 14.1	42		152.2 ± 39.9	36	
LC	T	0.1954	39.7 ± 10.3	37	***	47.5 ± 11.8	33	***	52.2 ± 12.9	37	***	99.9 ± 27.3	33	***
	A	0.1961	73.2 ± 17.3	39		91.2 ± 30.0	32		95.8 ± 14.3	39		173.8 ± 32.8	32	
SW	T	0.1874	50.6 ± 13.8	32	**	101.3 ± 34.6	28	n.s.	53.8 ± 15.2	32	***	114.0 ± 39.4	28	***
	A	0.1899	61.3 ± 9.7	40		108.0 ± 23.5	35		80.1 ± 9.0	40		185.6 ± 43.3	35	

* $p<0.05$ ** $p<0.005$ *** $p<0.0005$ § interaction between tibia length and *Wolbachia* infection

Wolbachia infection and tibia length (Fig. 1) where a positive "*Wolbachia* effect" on total offspring production was detected for wasps with a tibia length smaller than 0.1815 mm (day 1-5) or 0.1827 mm (day 1-end). Approximately 75% of the tested OL wasps meet this criterion. Corresponding to daughter production for the mixed populations, again a negative *Wolbachia* effect was found for the mixed lines AW, JT, LC and SW. For lifespan total offspring production, results were similar to the first 5 days with exception of CO where a negative *Wolbachia* effect was found.

Mean sex ratios of the thelytokous lines, which are a measure for *Wolbachia* transmission, were determined as well because in fixed populations, there is selection towards higher transmission. Indeed, the highest *Wolbachia* transmission was found for the lines CO and OL for the first five days and for the whole lifespan. Of the mixed populations, only line SW showed the same pattern as the fixed populations (Table 3).

Table 3. Mean sex ratio (Ms.r.) with standard deviation (sd) of the different thelytokous (T) and arrhenotokous (A) *Trichogramma* lines (n = number of females tested).

Line	Mode of reproduction	Day 1-5 Ms.r. ± sd	n	Day 1-end Ms.r. ± sd	n
CO	T	1.00 ± 0.00	40	0.97 ± 0.04	41
	A	0.90 ± 0.06	36	0.71 ± 0.18	39
OL	T	1.00 ± 0.00	40	0.74 ± 0.08	37
	A	0.72 ± 0.22	38	0.34 ± 0.16	34
AW	T	0.92 ± 0.07	41	0.68 ± 0.12	34
	A	0.80 ± 0.15	38	0.77 ± 0.16	34
JT	T	0.86 ± 0.06	40	0.51 ± 0.14	31
	A	0.75 ± 0.14	39	0.56 ± 0.16	33
LC	T	0.86 ± 0.06	37	0.60 ± 0.11	33
	A	0.80 ± 0.14	39	0.56 ± 0.19	32
SW	T	0.99 ± 0.01	28	0.95 ± 0.04	28
	A	0.78 ± 0.06	40	0.61 ± 0.09	35

A significant *Trichogramma*-species effect was found for the tibia length and *Wolbachia* infected lines had smaller tibia lengths than the uninfected ones. This could be explained by a negative effect of *Wolbachia* on their host. However, an alternative explanation is that mothers from arrhenotokous lines were derived from *Mamestra* eggs from which, in many cases, one female and one male emerged. The situation is different for the thelytokous lines where only two females emerged from the egg. Because males

are generally smaller than females (Kazmer & Luck 1995), more host resources would be left for females co-residing with a male in a host egg, resulting in larger females.

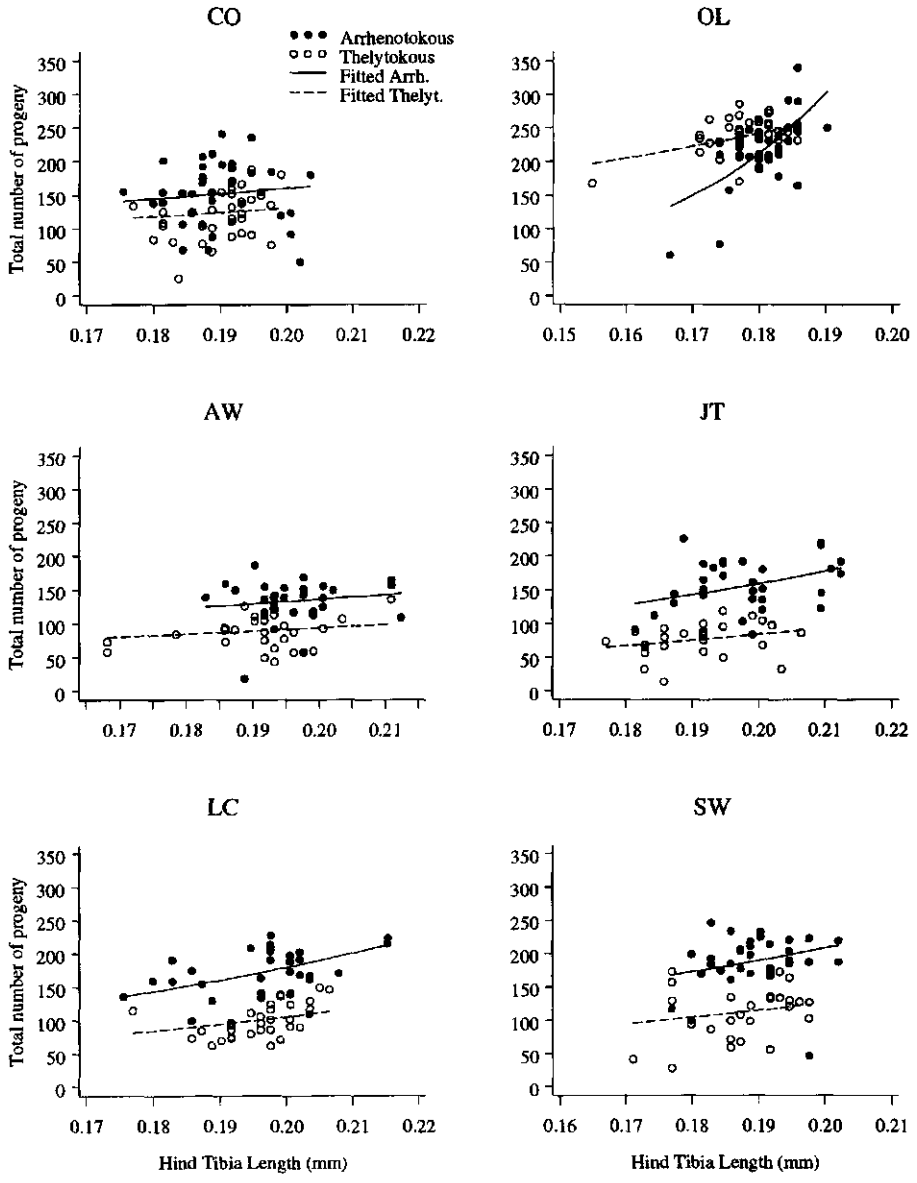


Fig.1. Relationships between size of the mother (measured as hind tibia length) and lifetime total offspring production for thelytokous (i.e., *Wolbachia*-infected) and arrhenotokous (i.e. cured) *Trichogramma* lines. For explanation of line codes abbreviations see Table 1.

Longevity and pupa/embryo mortality

Regarding the females' longevity and embryos/pupae survival, it was expected that *Wolbachia* would have a less negative effect on wasps from fixed populations than on wasps from mixed populations. However, a negative *Wolbachia* effect on longevity was detected for the lines CO and OL (Table 4) but not for the lines from mixed populations. This effect was more pronounced for CO where non-infected females lived eight days longer.

Table 4. Mean female longevity in days (age) with standard deviation (sd) of the different thelytokous (T) and arrhenotokous (A) *Trichogramma* lines (n = number of females tested).

Line	Mode of reproduction	n	Age±sd	*p*
CO	T	36	18.1 ± 6.8	
	A	39	25.6 ± 6.9	***
OL	T	37	26.5 ± 4.0	
	A	36	28.6 ± 5.1	*
AW	T	34	28.7 ± 3.8	
	A	36	26.7 ± 6.2	*n.s.*
JT	T	31	24.5 ± 6.6	
	A	36	25.4 ± 5.0	*n.s.*
LC	T	37	24.7 ± 3.6	
	A	33	25.6 ± 3.8	*n.s.*
SW	T	28	26.8 ± 5.2	
	A	35	26.1 ± 4.5	*n.s.*

*n.s. not significant * $p<0.05$ ** $p<0.005$ *** $p<0.0005$*

A negative *Wolbachia* effect was detected for pupa and embryo mortality in all lines except for AW (pupal mortality) and for CO (embryo mortality) (Table 5). For LC, embryo and pupa mortality was not distinguished but total mortality was significantly higher for the thelytokous line as well. The differences in mortality rates between arrhenotokous and thelytokous lines however, are marginal. Only the thelytokous line OL showed considerably higher embryo mortality (>5 times) in comparison with the arrhenotokous line. In general, mortality rates are initially low, increase after approximately 5 days and fluctuate in the following days (data not shown).

Table 5. Mean pupal and embryo mortality (percentage from total number of parasitised eggs per female) with standard deviation (sd) of the different thelytokous (T) and arrhenotokous (A) *Trichogramma* lines (n = number of females tested).

Line	Mode of reproduction	n	Mean pupal mortality ± sd	*p*	Mean embryo mortality ± sd	*p*
CO	T	39	2.17 ± 2.15	*	2.44 ± 2.10	ns
	A	36	1.62 ± 2.14		2.31 ± 1.88	
OL	T	36	2.36 ± 1.99	**	10.22 ± 4.02	***
	A	36	1.19 ± 0.95		2.03 ± 2.03	
AW	T	35	1.85 ± 1.42	ns	1.35 ± 1.70	***
	A	34	1.74 ± 2.07		0.71 ± 1.10	
JT	T	36	3.16 ± 1.99	***	7.39 ± 4.09	***
	A	31	1.63 ± 1.33		3.11 ± 3.73	
LC [a]	T	33	16.3 ± 9.07	***		
	A	37	8.15 ± 4.94			
SW	T	28	2.46 ± 3.22	***	4.14 ± 2.53	***
	A	35	1.74 ± 1.92		3.09 ± 3.22	

* $p<0.05$ ** $p<0.005$ *** $p<0.0005$ [a] pupae and embryos were not discriminated for this line and numbers indicate the percentage of parasitised eggs per female from which no adults emerged.

Discussion

In this study we determined whether the infection status of a *Trichogramma* population is correlated with the influence *Wolbachia* has on various host life-history characters. We hypothesise that in populations where the infected individuals coexist with uninfected conspecifics and where sexual reproduction of infected females still takes place, a nuclear-cytoplasmic conflict exists over the offspring sex ratio. This conflict may result in an "arms race" between the nuclear genes of the wasp and *Wolbachia*. Effects of this conflict can be an inefficient transmission of *Wolbachia* or a reduced offspring production of the infected wasp. Once a population has gone to fixation for the infection and thus no more sexual reproduction takes place, we expect that "benevolence" will evolve, i.e. those nuclear-cytoplasmic (wasp-*Wolbachia*) combinations that produce the most infected offspring will spread through the population. We determined whether this predicted pattern holds by comparing various female fitness characters of thelytokous lines and cured arrhenotokous lines of two populations where the infection has gone to fixation and of four populations where both infected and uninfected individuals coexist.

The fitness parameters that are important for the spread of *Wolbachia* can be clarified by a model that describes the dynamics of the fraction of infected *Trichogramma* individuals in a mixed population (Stouthamer 1997). According to this model, the main parameters important for the spread of *Wolbachia* in a population are: 1) w = total offspring production of an infected female divided by the total offspring of an uninfected female; α = the frequency of infected daughters in the offspring of infected mothers; x = fertilisation rate of the eggs. In fixed populations, when males are no longer present, α is 1 and we expect selection for increased w in fixed populations compared to w of mixed populations.

Using the parameters α and w, we can distinguish three different groups of *Wolbachia*-host interactions in the populations studied. Group one consists of the thelytokous lines CO and OL from fixed populations, which produced higher numbers of daughters ($w\alpha>x$), and relatively similar offspring numbers compared to its arrhenotokous counterpart (w close to one). The second group consists of the thelytokous line of the mixed population SW which produced equal numbers of females ($w\alpha \approx x$) but showed reduced offspring numbers compared to their cured counterparts ($w < 1$). Finally, the third group consists of the T. *kaykai* lines AW, LC and JT from mixed populations where $w\alpha < x$ and $w < 1$. The lines from mixed populations of *T. pretiosum* (Nuevo Leon) and *T. deion* (Irvine) (Stouthamer & Luck 1993) belong also to the third group while *T. pretiosum* (Hawaii) belongs to the second. From the last species only thelytokous individuals were collected but it is unknown whether this line is fixed (Stouthamer & Luck 1993).

Besides differences in α and w, differences in longevity were also detected between the lines tested. It was expected that *Wolbachia* would have less negative effect on their host in fixed populations than in mixed populations. But the opposite was found: thelytokous CO and OL lived significantly shorter than their arrhenotokous counterparts with only slight differences for OL and a difference of on average eight days for CO. Because the reduced longevity of the thelytokous lines CO and OL is not linked to a severe negative impact on offspring production compared to the cured lines, it remains unclear what causes this difference. It may be that *Wolbachia* change the host's physiology in such a way that it reallocates the host resources resulting in higher offspring numbers but shorter longevity (G.J. Driessen, pers. com.).

Pupa and embryo mortality rates were generally significantly higher for the thelytokous lines compared to the cured arrhenotokous lines, for both mixed and fixed populations. In general, mortality rates were initially low but started to increase when

females were a few days old. In this period, daughter production began to decline and the production of males and of gynandromorphs increased. The cytological processes responsible for the production of gynandromorphs (Stouthamer 1997) may cause the increased mortality. In addition, because mortality varied among the thelytokous lines, host or symbiont genotype may play a role as well.

The differences found in offspring numbers between thelytokous lines of mixed populations and their conspecific arrhenotokous lines do not necessarily reflect the field situation. A previous study of Stouthamer & Luck (1993) showed that despite the negative effect of *Wolbachia* on *T. pretiosum* and *T. deion* (Irvine), the thelytokous females produced more daughters when the number of host eggs available was limited. But so far, it is unclear how many potential hosts *Trichogramma* females encounter in the field. It is therefore difficult to extrapolate our results to the field situation. Because there can be a negative effect of *Wolbachia* on offspring production, *Wolbachia* may also have a negative impact on additional life history parameters such as host searching behaviour. But greenhouse experiments with the same CO and SW lines used in our experiment showed no significant differences in the number of patches parasitised on tomato plants between the thelytokous and arrhenotokous lines. The arrhenotokous lines parasitised more eggs per patch than the thelytokous lines (Chapter 6) which corresponds with our offspring results of line SW, but not of line CO (discussed in chapter 6).

Our results and the results of Stouthamer & Luck (1993) show that fixed *Trichogramma* populations have both a high transmission fidelity ($\alpha \approx 1$) and hardly show any negative impact of the *Wolbachia* infection on the offspring production ($w \approx 1$), while the opposite is true for the mixed populations with the exception of the line SW and the line *T. pretiosum* Hawaii (Stouthamer & Luck 1993), which have a low w but high α. These last two lines may represent examples where the infection is going to fixation or alternatively, a situation in which *Wolbachia* has temporarily overcome the host's countermeasures. The results are consistent with the idea that "benevolence" should evolve when the infection has gone to fixation, whereas the nuclear-cytoplasmic conflict in mixed populations results in a reduced *Wolbachia* transmission and/or a negative impact of the infection on offspring production. However, a less likely explanation may also be given. Infection will go to fixation in those populations that became initially infected with a *Wolbachia* that has hardly any influence on the host offspring production and has a high transmission rate, whereas those *Wolbachia*-host interactions where the transmission efficiency is low and the fitness cost of carrying a *Wolbachia* is high results in, at best, a mixed population. Because physiology can differ substantially between

different arthropod hosts, it is questionable whether primary benevolent symbiont-host combinations exist. The study of Grenier *et al.* (1998) already showed that even within the genus *Trichogramma* not all symbiont-host combinations are initially successful: *Trichogramma dendrolimi*, infected with *Wolbachia* of *Trichogramma pretiosum* only showed extreme low levels of thelytoky. A series of micro-injection experiments of *Wolbachia* strains (from fixed and mixed populations) into *Trichogramma* lines could show whether benevolent *Wolbachia* - novel host combinations exist. A second set of experiments could test the assumption that "benevolence" will evolve once the infection has gone to fixation. This can be done in the laboratory by artificial selection of *Wolbachia* infected lines for increased transmission (α=1). Then, over time, these lines should be tested to determine whether w is increasing.

In conclusion, we can state that our results are consistent with the idea that *Wolbachia* has less negative impact in fixed populations than in mixed populations. The mechanisms responsible for these differences need to be studied next.

Acknowledgements

We thank Hanneke van Heest for rearing the different *Trichogramma* lines, F. Vanlerberghe-Masutti (INRA, Antibes) for providing *T. oleae* and Astrid Schoenmaker for practical assistance with the experiments. We also thank Joop van Lenteren, Menno Schilthuizen, and the Entomology PhD group for critical review of this manuscript. This project was financially supported by the Technology Foundation (STW), grant number 790.42.793, the Netherlands and by Fundação Calouste Gulbenkian, Portugal.

Chapter 5

Laboratory bioassay and greenhouse evaluation of *Trichogramma cordubensis* strains from Portugal

To be published as: Honda, J.Y., I.M.M.S. Silva, J. Vereijssen & R. Stouthamer. Laboratory bioassay and greenhouse evaluation of *Trichogramma cordubensis* (Hymenoptera, Trichogrammatidae) strains from Portugal. *BioControl* (in press).

Abstract

A simple, inexpensive chamber, was developed and tested as an evaluative tool to monitor *Trichogramma cordubensis* Vargas and Cabello (Hymenoptera: Trichogrammatidae) dispersal in the laboratory. The chamber consisted of a continuous, winding channel that was cut into an aluminium block. Wasps were released at one end of the channel and allowed to walk in the channel for 21 h and to parasitize *Mamestra brassicae* L. (Lepidoptera: Noctuidae) eggs placed 3.4 m from the point of wasp introduction. Comparisons between two *T. cordubensis* populations demonstrated that one population (TCM) dispersed more in the chamber and located host eggs more successfully than the other population (TCD). Subsequent greenhouse releases confirmed that the TCM population dispersed more readily and had significantly higher parasitism rates on sentinel *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) eggs on tomato plants. The potential utilisation of this chamber as a tool to evaluate quality of *Trichogramma* populations, mainly dispersal activity, is discussed.

Introduction

Egg parasitoids of the genus *Trichogramma* have been widely used as biological control agents throughout the world on numerous agricultural commodities (Li 1994, Smith 1996). The successful utilisation of this group in biological control systems rests on the proper selection of *Trichogramma* species and populations (Kot 1968, Hassan 1989, 1994, Pak 1988). In addition, the quality of mass produced parasitoids should be maintained (van Lenteren 1986b, Greenberg 1991, Laing & Bigler 1991, Bigler 1994). Laboratory evaluations usually compare different traits between populations, which serve as indices and are (occasionally) followed by field evaluations to assess searching efficiency (Hassan & Guo 1991, Wührer & Hassan 1993, Hassan 1994). Similarly, parasitoid quality is assessed in the laboratory using a set of parameters related to field performance (Greenberg 1991, Cerutti & Bigler 1995). The question often arises as to whether laboratory tests for monitoring parasitoid potential/quality can adequately assess traits important under field conditions (Laing & Bigler 1991, Bigler 1994 and references therein). Searching efficiency (i.e. the ability to find hosts in the field) has long been considered an important attribute in determining suitable *Trichogramma* species, but may be difficult to evaluate (Suverkropp 1997). Locomotion traits (walking speed, turning rate, time spent walking) recorded from individual wasps have been used as possible indicators of searching efficiency (Bigler *et al.* 1988). These authors, working on *Trichogramma maidis* Pintureau & Voegele (= *T. brassicae* Bezdenko), found a strong correlation between travel speed in the laboratory and parasitism in the field.

As Bigler (1994) points out, single trait evaluations will hardly ever predict the overall performance of a biological control agent. Thus, it is important to determine which traits will be useful to determine potential field performance. Prohibitive costs in collecting data may also limit which individual traits are examined. Ideally it would appear that the most useful and practical bioassays would be ones which are inexpensive, quick, and repeatable. The objective of this study is to develop such a bioassay and determine if it is possible to: 1) distinguish differences in dispersal between two *Trichogramma* populations in the laboratory and 2) determine if a relationship exists between our bioassay results and performance in the greenhouse. We chose to evaluate dispersal as a parameter because of its importance for field and greenhouse efficacy and it is still poorly studied in the laboratory.

Material and Methods

Trichogramma stock cultures

Two thelytokous (parthenogenetic) populations of *T. cordubensis* were used for assays. One population was collected in 1992 at Mora, Portugal (TCM) while the other population was collected in 1993 at Divor, Portugal (TCD). Both populations (isofemale lines) were collected on tomato plants (*Lycopersicon esculentum* Miller), as *Helicoverpa (Heliothis) armigera* Hübner (Lepidoptera, Noctuidae) parasitised eggs. Cultures of both populations were maintained on *Ephestia kuehniella* eggs killed by UV radiation, for 50-70 generations, at 15-25 °C, 30-80% RH, L:D=16:8. Five generations prior to the experiment and during the experiment the host eggs (on double-sided tape) were available to the wasps for 24 hrs, at 23 ± 0.5 °C, 45 ± 10% RH, L:D=16:8, in glass vials (15 x 150mm). Upon emergence, wasps were given a fresh egg card with a drop of honey and were allowed to parasitise hosts that were maintained at an approximate host:parasitoid ratio of 10:1.

Bioassay development

Our bioassay chamber was modelled after Greenberg (1991), with the major modifications described below. It consisted of a solid aluminium block (639 x 232mm) containing a continuous winding channel measuring 8m in length which was routed out of the metal. The channel consisted of 40 connected subchannels, each 20cm in length. A hole (15mm diameter) was cut at the origin of the first subchannel to introduce the wasps (Fig. 1). Our objective in using the bioassay was to determine dispersal by walking of *T. cordubensis* strains. However, preliminary studies indicated two inherent problems after the construction of the chamber. First, it was noticed that wasps placed in the chamber travelled a maximum distance of approximately 4 m (i.e. subchannel 20 in the chamber) over a period of 24 hrs.

The second problem was that because wasps were able to move freely within the apparatus, it was possible for very active individuals to work their way back towards the entrance over the testing period. Thus, wasp dispersal estimates in the bioassay could be drastically underestimated. To alleviate these problems, approximately 400 *Mamestra brassicae* eggs were placed at a distance (3.4 m or channel 17) to arrest the wasps (see Kaiser *et al.* 1989a and Noldus 1989). *M. brassicae* eggs were used because they attract and/or arrest *T. cordubensis* females (personal observations).

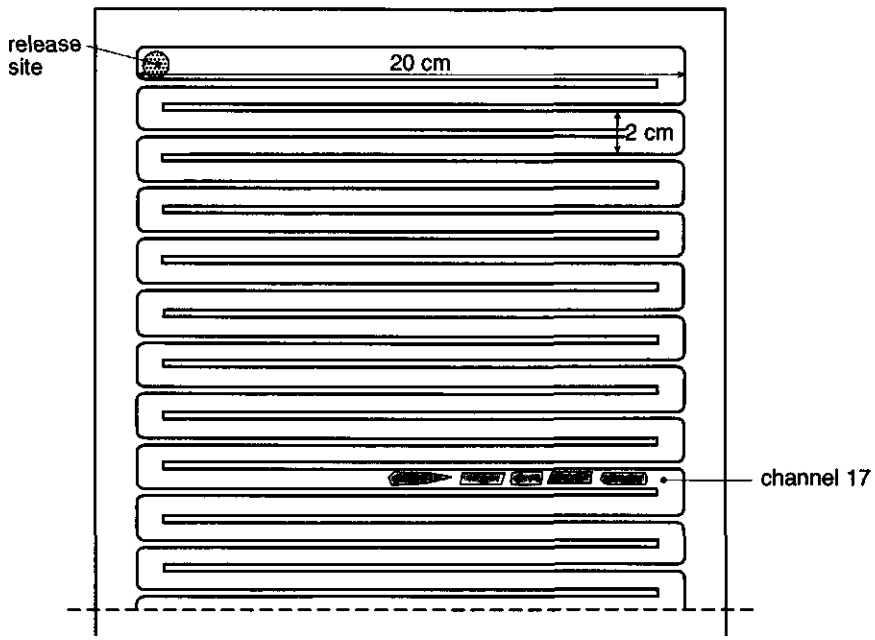


Fig. 1. Section of aluminium block (top view) showing continuous winding channel cut into metal block. Wasps were allowed to walk in the channel for 21 h and to parasitize *Mamestra brassicae* egg masses present in subchannel 17, ca. 3.4 m from the entrance. Total length 8 m (subchannel dimensions 1 cm x 1 cm x 20 cm).

Bioassay trials

Approximately 400 host eggs (*Mamestra brassicae*, 24-48 h old) deposited naturally on filter paper were placed approximately 3.4 m from the entrance hole (channel # 17). A glass plate was placed over the aluminium block and secured with clamps. One-day old, honey-fed *Trichogramma* females were introduced through the hole into the aluminium chamber with a Pasteur pipette. Care was taken to obtain injury-free wasps of uniform age, hence wasp numbers varied between 40-120 per replicate. Wasps were drawn into the pipette using gentle mouth suction. Two pieces of cotton placed at both ends of the pipette eliminated accidental wasp inhalation and escape. The pipette was held in place with foam rubber to prevent slippage and wasp escape. The cotton plug in the top-end of the pipette was removed prior to introduction into the chamber to afford wasps access to the first channel of the chamber. Each replicate began at 13:30 h and ended at 10:30 h the next day, after which the pipette was removed and a gentle stream of CO_2 was introduced to quickly

anaesthetise the wasps. Data were then recorded as numbers of individuals per channel. Ten replicates for each population were run at 22.5 ± 0.5 °C, 75 ± 10% RH, L:D=16:8; 24000 lux. After each experiment the chamber was cleaned with ethanol (96%) and dried. The mean channel distance travelled was calculated by adding the products of number of wasps in each channel with the channel number (release point corresponding to channel 1) and dividing the total by the number of wasps released. Mean channel distance travelled of populations TCM and TCD was compared by means of a Wilcoxon 2-sample two-sided test, with α=0.05. A generalised linear model with Poisson distribution and log link was fitted to the number of parasitised eggs. The expected number of parasitised eggs was assumed to be proportional to the number of females arriving at the egg masses or further. We tested for possible differences between TCM and TCD (α=0.05). Correction was made for possible overdispersion of the response with respect to the Poisson distribution. We used the SAS program version 6.12, procedure Genmod, to fit the model.

Assessment of parasitism in the greenhouse

Measurement of parasitism in a greenhouse was conducted by monitoring sentinel egg cards placed on tomato plants. We chose irradiated *E. kuehniella* eggs, as they were readily available and easy to manipulate and create uniform egg cards. Sentinel egg cards were placed on 13 tomato plants (*Lycopersicon esculentum*, variety Moneymaker) ca. 40 cm in height, with the stems spaced approximately 40 cm apart and arranged in a U-shaped pattern (Fig. 2).

One plant free of eggs was used as release point (Fig. 2: release plant), by placing the vial containing the wasps against the plant stem. Each plant touched adjacent plants and contained 12 egg cards (six cards at the upper surface and six cards at the lower surface of the leaves). Thus a total of 156 egg cards were used per replicate. Each egg card consisted of a circle (6 mm diameter) of double-sided tape containing approximately 130 eggs, which were affixed to the leaf surface. Approximately 700 to 800 1-2-d old female wasps were released and the eggs remained on the plants for 5 days after parasitoid release. Afterwards the egg masses were incubated at 23 ± 0.5 °C, 45 ± 10% RH, L:D=16:8.

Parasitism was quantified by scoring the number of egg masses containing at least one parasitised (black) egg out of the 156 from each greenhouse compartment. The TCD and TCM *Trichogramma cordubensis* populations were released simultaneously in adjacent greenhouse compartments. Nine replicates for each population were performed.

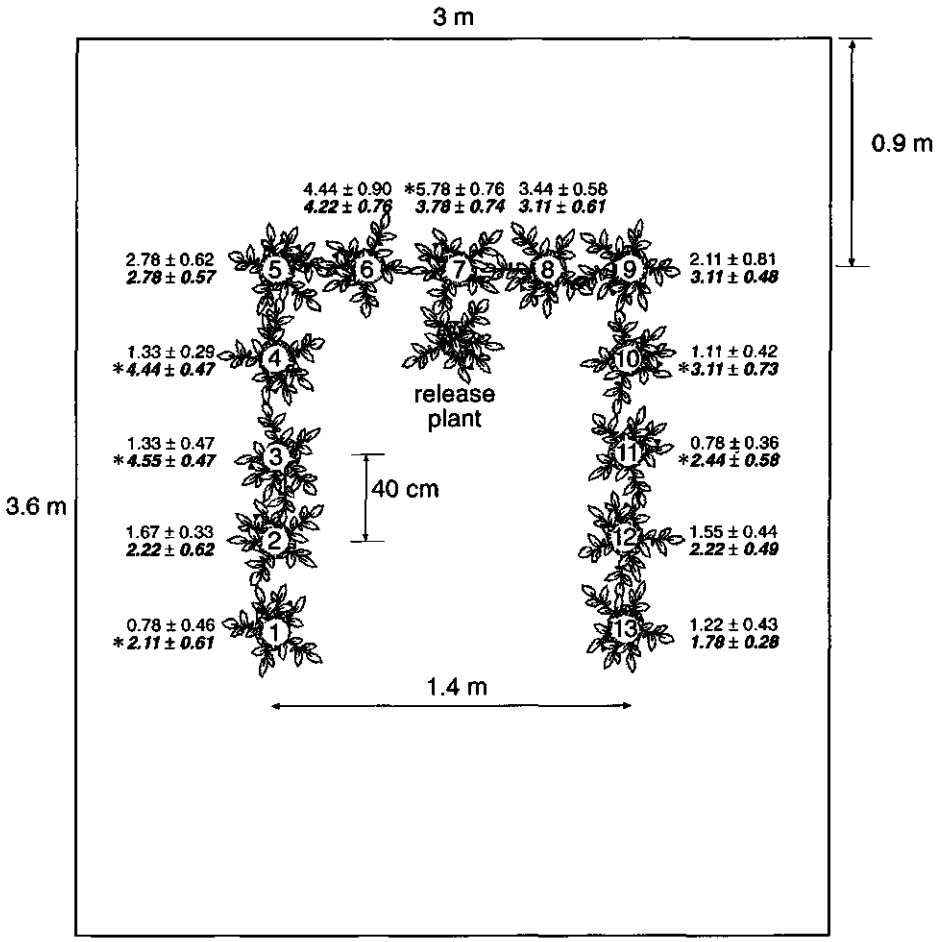


Fig. 2. Position of tomato plants and parasitism patterns of two *Trichogramma cordubensis* populations (TCD and ***TCM***) on 13 tomato plants in nine paired greenhouse releases. Numbers represent mean numbers of sentinel egg masses parasitised (± SEM) per each tomato plant. * indicates significant differences between means for each plant (t-test, $p<0.05$). Approximately 700-800 wasps of each population were released per replicate.

Population releases were alternated between compartments to prevent biases. The release compartments were not artificially illuminated, in contrast with other compartments in the same greenhouse. However, lateral light from adjacent compartments was visible in the release compartments. In the release compartment receiving light from the left, plants at the left side were numbered 1-6, while in the other release compartment (which received light from the right side), numbers 1-6 were attributed to the plants at the right side of the

compartment. Greenhouse conditions were 18 ± 2 °C, 70% ± 15% RH. The paired greenhouse releases covered a span of four months under varying conditions.

We used the statistical software SAS (version 6.12), for the following tests [(explanatory variables: *Trichogramma* population, compartment and release (i.e., repetitions in time)]:

- Proportion of egg masses parasitised of 156 (the total number of egg masses present): proc genmod, binomial distribution, logit as link function, correction for overdispersion using heterogeneity factor (Collett 1991), α=0.05;

- Average distance of parasitised egg masses to release plant (distance was calculated by adding the products of number of parasitised egg masses for each plant with a number corresponding to its distance to the release plant [plant 7: 1, plants 6 and 8: 2,..., plant 1 and 13: 7], and dividing the sum by the total number of egg masses parasitised): proc GLM, using the square root of the total number of egg masses parasitised as weight, α=0.05;

- Proportion of egg masses parasitised of 12 (the number of egg masses present per plant), for each plant: proc genmod, binomial distribution, logit as link function, α=0.05;

- Proportion of the number of egg masses parasitised at plants 1-6 of the total number of egg masses parasitised at plants 1-6 and 8-13, proc genmod, binomial distribution, logit as link function, α=0.05.

Results and Discussion

By means of the bioassay described, we could distinguish between the two *Trichogramma cordubensis* populations. A percentage distribution (percentage of released wasps found per channel) shows the dispersal pattern of females from both populations in the bioassay chamber (Fig 3). Clearly, the TCM population dispersed more than the TCD population. Some females continued to move beyond the channel containing *M. brassicae* eggs, which indicated a degree of behavioural variability among females, with a group of females apparently ignoring host cues. A similar phenomenon has been previously observed for *T. minutum* during flight initiation studies (Forsse *et al.* 1992). Alternatively, the presence of other wasps and subsequent increase in parasitised eggs over time in the channel may have stimulated wasps to disperse, which has been previously postulated (Suverkropp 1997). Overall, the TCM population showed significantly more dispersal while the TCD population remained closer to the release point (Table 1). Previous studies using this bioassay have been performed using *T. deion* which demonstrated similar differences in

population dispersal (Vereijssen *et al.* 1997). The number of eggs parasitised per female found at channel 17 or further did not differ significantly between the *T. cordubensis* populations (Table 1), suggesting that they differ in dispersal but not in fecundity.

We next hypothesised that the population that dispersed the most in the laboratory bioassay also disperses the most under semi-field conditions. This hypothesis was tested by

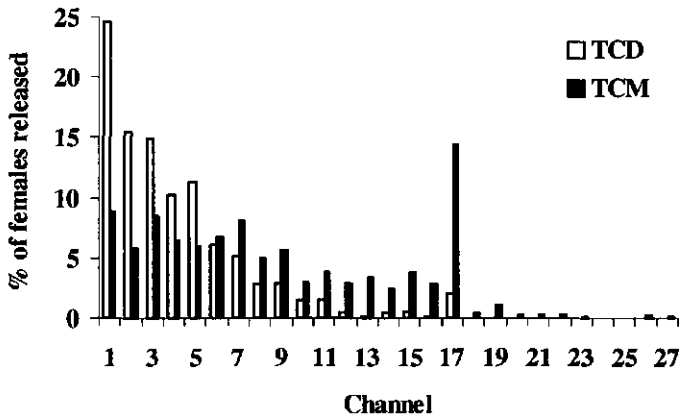


Fig. 3. Percentage of two *Trichogramma cordubensis* populations (TCD and TCM) found per channel in the laboratory chamber after being allowed to walk for 21 h; wasps were released in channel 1. *Mamestra brassicae* eggs were present in channel 17.

Table 1. Dispersal of two *Trichogramma cordubensis* populations in the laboratory chamber: population studied, number of replicates, mean number of wasps released per replicate, mean channel where wasps were found and mean number of eggs parasitised per female found at channel 17 or further. Mean channel was significantly different between populations (p=1.08 x 10^{-5}), as indicated by the means (± SEM) followed by different letters (Wilcoxon 2-sample 2-sided test, α=0.05).

Trichogramma population	Number of replicates	Mean (± SEM) number of wasps released	Mean channel (± SEM)	Mean (± SEM) number of eggs parasitised per female found at channel 17 or further
TCM	10	76.7 ± 9.07	8.8 ± 0.50 **a**	5.6 ± 0.64
TCD	10	69.4 ± 5.69	4.1 ± 0.18 **b**	4.3 ± 0.65

performing greenhouse releases.

In the greenhouse, overall parasitism rate (measured as number of egg masses containing at least one parasitised [black] egg divided by the total number of egg masses present) was significantly higher for TCM compared with TCD (p=0.048). TCM parasitised

in the total of nine releases 359 egg masses and TCD parasitised 255, out of the 1404 egg masses available for each population. Population TCM had a higher dispersal than population TCD, as given by the (significant different, p=0.0179) average distances of parasitised egg masses to the release plant: 3.9 and 3.1, respectively. The mean number of parasitised egg masses for each of the plants was compared between the *Trichogramma* populations. Significant block effects were detected for some plants, indicating differences between repeats. This result is not too surprising as experiments were carried out over a period covering the fall and winter seasons. Temperature varied between 16 and 20°C in the greenhouse, such changes may have profound effects on *Trichogramma* searching behaviour (Boldt 1974, Bigler *et al.* 1988).

Parasitism rates for those plants close to the release plant (plants 5-9) showed only one significant difference as the TCD population had a higher parasitism rate for the plant immediately adjacent to the release plant. In contrast, for plants 1-4 and 10-13 the mean number of parasitised egg masses for each plant was higher for TCM and five of eight means were significantly higher (Fig. 2).

Parasitism at plants 1-6 was significantly higher than parasitism at plants 8-13 (p=0.0089). This is probably due to influence of the light from other compartments. Plants 1-6 were placed between the release point and the light source. *Trichogramma* are phototactic (e.g. van Steenburgh 1934, Noldus *et al.* 1991), so the difference between the two groups of plants is not surprising.

The chamber used in the laboratory bioassay to evaluate *Trichogramma* dispersal possesses a number of advantages. First, the experiments themselves are easy to run, as there is no need for continuous observations. Data may be collected daily and scored within 30 minutes per replicate. Second, the apparatus is inexpensive and has few parts; chamber construction costs less than 100 US dollars and multiple chambers can be made and run simultaneously. Finally, based on the present results, we conclude that this bioassay may serve as a quick first stage sieving process to evaluate *Trichogramma* populations in the laboratory. Poorly performing populations in this chamber can quickly and easily be distinguished from superior populations which may then be more rigorously tested under field or greenhouse conditions. This device may also be useful as a quality control device to monitor wasp dispersal and parasitism. Quality indices could be developed for mass-produced material. The material should then be tested in this device periodically to determine if the established quality standards are maintained. Such management techniques may be used to attain high-quality wasps (Dutton *et al.* 1996). However, we cannot be sure that differences in dispersal between populations are always translated in measurable

differences when strictly using this bioassay and advise that other parasitoid traits (*e.g.* fecundity) be studied concurrently. More studies comparing dispersal of *Trichogramma* populations in laboratory and parasitism rates in the greenhouse have been performed (Chapter 6).

Acknowledgements

We thank S. Greenberg for his assistance and ideas in developing our chamber. We are grateful to: L. Neto and B. Pintureau, for *Trichogramma* identifications; Koppert, for providing *E. kuehniella* eggs; L. Koopman, A. Gidding and F. van Aggelen for providing *M. brassicae* eggs, G. Gort, for help in statistics, F. Bigler, G. Boivin, Joop van Lenteren, M. van Meer, M. Roskam, B. Suverkropp, E. Wajnberg and two anonymous reviewers are acknowledged for their helpful comments on earlier drafts of the manuscript. This research was funded partly through EU grant AIR-CT94-1433 (J.H.) and through JNICT (Junta Nacional de Investigação Científica e Tecnológica), Portugal; BD 2107 92-IE and BD 5620/95)(I.S.).

Chapter 6

Biological control potential of *Wolbachia*-infected *versus* uninfected wasps: laboratory and greenhouse evaluation of *Trichogramma cordubensis* and *T. deion* strains

Slightly modified from: Silva, I.M.M.S., M.M.M. van Meer, M.M. Roskam, A. Hoogenboom, G. Gort & R. Stouthamer. Biological control potential of *Wolbachia*-infected *versus* uninfected wasps: laboratory and greenhouse evaluation of *Trichogramma cordubensis* and *T. deion* strains (submitted).

Abstract

The effects of thelytoky-inducing *Wolbachia* (α-proteobacteria) on *Trichogramma cordubensis* and *T. deion* (Hymenoptera, Trichogrammatidae) were studied in laboratory and greenhouse conditions. One infected (thelytokous, all female) line of each wasp species was compared with its conspecific uninfected (arrhenotokous, sexual) counterpart for several fecundity and dispersal traits. Arrhenotokous lines had a higher fecundity than their thelytokous counterparts, which suggests that *Wolbachia* negatively affect the fecundity of *Trichogramma* females. The arrhenotokous females dispersed more in the laboratory than their thelytokous counterparts. In the greenhouse, the opposite effect or no difference between lines was found, indicating that the laboratory set-up used to measure dispersal is not useful to predict relative dispersal of the females in the greenhouse. Calculations show that by releasing hundred adult wasps of both lines, thus including arrhenotokous males in the sexual line, more eggs are parasitised by the thelytokous wasps. Therefore, in spite of their lower individual female fecundity, thelytokous lines have a better potential for biological control than their arrhenotokous counterparts.

Introduction

Hymenoptera are haplo-diploid insects which are generally arrhenotokous, *i.e.* unfertilised eggs develop into haploid males while fertilised eggs yield diploid females. Thelytokous forms, in which unfertilised eggs give rise to diploid females, are also known in several families of Hymenoptera. Thelytoky is often associated with the presence of endosymbiotic bacteria of the genus *Wolbachia* (α-proteobacteria) (Stouthamer 1997).

Infection with thelytoky-inducing *Wolbachia* (T-*Wolbachia)* leads to a disruption of the chromosome segregation in the first mitotic division (anaphase) of the haploid egg. As a result, the egg becomes diploid and develops into a homozygous thelytokous female (Stouthamer & Kazmer 1994). T-*Wolbachia* can also affect their host's fecundity, longevity and pre-adult mortality, which has been reported for *Trichogramma* spp. (Chapter 4, Stouthamer & Luck 1993).

Trichogramma are minute wasps, which are widely used in biological control programs against lepidopteran pests. A few species (mainly arrhenotokous forms) have been frequently used as biological control agents (Li 1994, Smith 1996). From the ca. 180 species described (Pinto 1998), at least 14 have thelytokous forms (Pinto & Stouthamer 1994, Stouthamer 1997). When *Trichogramma* infected with T-*Wolbachia* are "cured" from the bacterial infection by antibiotic treatment or exposure to high temperatures, they are rendered permanently sexual (Stouthamer *et al.* 1990).

Arrhenotokous and thelytokous forms of the same *Trichogramma* species are often sympatric ("mixed" populations) but in at least two species (*T. cordubensis* and *T. oleae*) only thelytokous forms are known ("fixed" populations) (Stouthamer 1997). The hypothesis that adverse effects of *Wolbachia* are less severe for fixed populations of *Trichogramma* than for mixed ones has been supported by laboratory studies, in which a surplus of hosts was available (Chapter 4). Whether arrhenotokous or thelytokous wasps do better in reducing their host population may also depend on host availability, assuming that both forms are equally effective in finding hosts (Stouthamer 1993, Stouthamer & Luck 1993).

The best choice for the mode of reproduction of parasitoids in biological control has been discussed and advantages of either mode of reproduction in this context have been listed (Aeschlimann 1990, Stouthamer 1993). Thelytokous forms may be preferred because in the asexual lines a low density of mates is not a limiting factor for reproduction, so they may establish better at low host densities. Furthermore, they are cheaper to produce in mass rearings because no resources are "wasted" for the production

of males. On the other hand, arrhenotokous forms may be able to better adapt to new environments, due to genetic recombination, and may have a higher rate of population increase at high host densities.

Infection with T-*Wolbachia* has in some cases a severe negative effect on the host fecundity (Chapter 4, Stouthamer & Luck 1993). Therefore, additional effects on other important biological control traits such as host searching ability may also be expected. Traits considered to be important for the success of parasitoids as biological control agents have been discussed in general (*e.g.* van Lenteren 1986 a,b) and for *Trichogramma* in particular. For *Trichogramma*, these traits are: fecundity, longevity, dispersal, adult size (Kot 1979, Greenberg 1991, Kazmer & Luck 1995), emergence rate of the parasitised eggs, percentage of non-deformed females, fecundity on the factitious host and on the natural host (Greenberg 1991, Cerutti & Bigler 1995). In addition, walking speed and turning rate have been shown to positively correlate with parasitism in the field (Bigler *et al.* 1988).

Assessment of the overall quality of the wasps during pre-release studies is laborious. A less time-consuming approach is to split "quality" into parameters that are then quantified (Cerutti & Bigler 1995). However, a single trait will hardly ever predict the overall performance accurately and therefore, the best combination of a set of laboratory methods must be developed. These measurements should be quick, simple and reliable in order to be applied in a production unit (Bigler 1994).

One such approach is to measure the parasitoids fecundity and dispersal. Fecundity is important for mass production of the wasps and may be relevant in field situations, too. Dispersal is crucial in field (or greenhouse) situations, since the wasps must be able to find eggs situated at some distance from the release sites. To study dispersal, indirect methods, based on the distance of parasitised host eggs to the wasps release site, are frequently used. When these methods are used, not only dispersal is involved but also host-habitat location, host location and host acceptance and suitability (Suverkropp 1997). Differences in dispersal between two *Trichogramma cordubensis* lines in a laboratory chamber corresponded to the relative dispersal of those lines (assessed by parasitism) in the greenhouse (Chapter 5). If such a relation is shown to be a general rule, dispersal in this chamber could be a very useful trait in pre-release studies.

To determine if the presence of *Wolbachia* influences the biological control potential of their hosts, the fecundity and dispersal of thelytokous lines of *Trichogramma deion* (mixed population) and of *T. cordubensis* (fixed population) were compared with those of their arrhenotokous counterparts by testing: a) fecundity in glass vials, when

females are given a surplus of host eggs; b) combined fecundity and dispersal in a laboratory chamber; c) combined fecundity and dispersal in a greenhouse.

Material and Methods

Cultures

Trichogramma thelytokous lines were started with single females from field-collected parasitised lepidopteran eggs. Arrhenotokous lines were obtained by feeding thelytokous lines antibiotics, as described by Stouthamer *et al.* (1990). The procedure used to check whether lines were completely cured is described in Chapter 4. These lines had been cured for at least 4 generations prior to our experiments. *Trichogramma cordubensis* Vargas & Cabello (1985) were collected from parasitised *Helicoverpa armigera* Hübner (Lepidoptera, Noctuidae) eggs, on tomato plants (*Lycopersicon esculentum* Miller), in Divor (Alentejo, Portugal) in 1993. *Trichogramma deion* Pinto & Oatman (SW) were collected as parasitised *Apodemia mormo* Felder & Felder (Lepidoptera, Lycaenidae) eggs, on the desert trumpet *Eriogonum inflatum*, near Barstow (Sidewinder mountains, California, USA) in 1996. Voucher specimens are kept in the collection of J.D. Pinto (Univ. California, Riverside, USA).

Wasps were reared in glass vials (150 mm length, 15 mm diameter), on *Ephestia kuehniella* (Lepidoptera, Pyralidae) UV-killed eggs, at 23±1°C, RH 50±20%, 16h light/8h dark. One-day-old honey-fed wasps were given fresh egg cards. The egg cards were parasitised for at least one day. After ca. 13 days the newly emerged wasps were used for the rearing or for experiments. Thelytokous and arrhenotokous *T. cordubensis* and *T. deion* lines are designated as COT, COA, SWT and SWA, respectively.

Fecundity in glass vials: surplus of hosts and no searching required

Individual one-day-old females were placed in glass vials (75 mm length x 10 mm diameter), with an egg mass (6 mm diameter, ca. 130 eggs) and a drop of honey. The vials were closed with cotton wool. The females of the thelytokous line were unmated. The arrhenotokous females had emerged together with males and had thus been allowed to mate. The egg masses were changed daily, for five days. The experiment was carried out at 23±1°C, 70±10% RH, L:D=16:8, 8 000 lux. After parasitisation, the egg masses were placed in a clean vial and incubated at 23±1°C. We determined the total number of host eggs parasitised (which turned black) per female per day. Sample sizes for COT, COA,

SWT and SWA were 28, 29, 54 and 57, respectively. Conspecific thelytokous and arrhenotokous lines were tested simultaneously. For *T. cordubensis*, the size of the mothers was determined by measuring their hind tibia length. The size of the mothers was not measured for *T. deion*.

Dispersal and parasitism in a laboratory chamber

We used the chamber described in Chapter 5, with the modifications mentioned below (see Fig. 1). It consisted of a solid aluminium block (63.9 x 23.2 cm) containing a continuous winding channel measuring 8 m in length, which was routed out of the metal. The channel consisted of 2 x 20 connected subchannels, each 20 cm in length. To split the chamber in two, a small piece of rubber at the end of channel 20 closed off the connection between adjacent channels. A glass plate, placed over the aluminium block and secured with clamps, closed the chamber. At the both ends of the chamber an entrance hole (15 mm diameter) allowed the release of *Trichogramma* individuals into the chamber. The wasps emerged from two parasitised *E. kuehniella* egg masses in a glass vial (75 mm length x 10 mm diameter).

Just before the release, these egg masses were removed from the vial. The vial opening was then inserted in the entrance hole of the chamber, allowing the 1-3 days old wasps to disperse into the chamber. The experiment was run at 23±1°C, 75±10% RH, L:D=16:8, 24 000 lux. Two releases (one at each side) were performed simultaneously. *Trichogramma* lines were alternated between sides to avoid biases. Three different types of release were performed: a) release of a thelytokous line at one side of the chamber and of its arrhenotokous counterpart at the other side; b) release of the same line at both sides of the chamber to test the chamber for symmetry; c) release of thelytokous females at one side of the chamber and of thelytokous females together with arrhenotokous males at the other side. This last kind of release was only performed with *T. cordubensis*. For arrhenotokous lines, females were always released together with conspecific males that had emerged together with the females. To arrest and eventually attract the wasps to the end of the chamber (see Noldus 1989, Kaiser *et al.* 1989a, Renou *et al.* 1989) and to determine the number of eggs parasitised per female, we placed *Ephestia kuehniella* eggs (eight egg masses, ca. 130 eggs each) in channel 17 of each side. Twenty hours after the release, individuals were anaesthetised with CO_2 and the number of females and males in each channel was recorded. After each release the chamber was cleaned with ethanol and dried. The number of parasitised eggs was recorded for 8 replicates, for both *T. cordubensis* and *T. deion*.

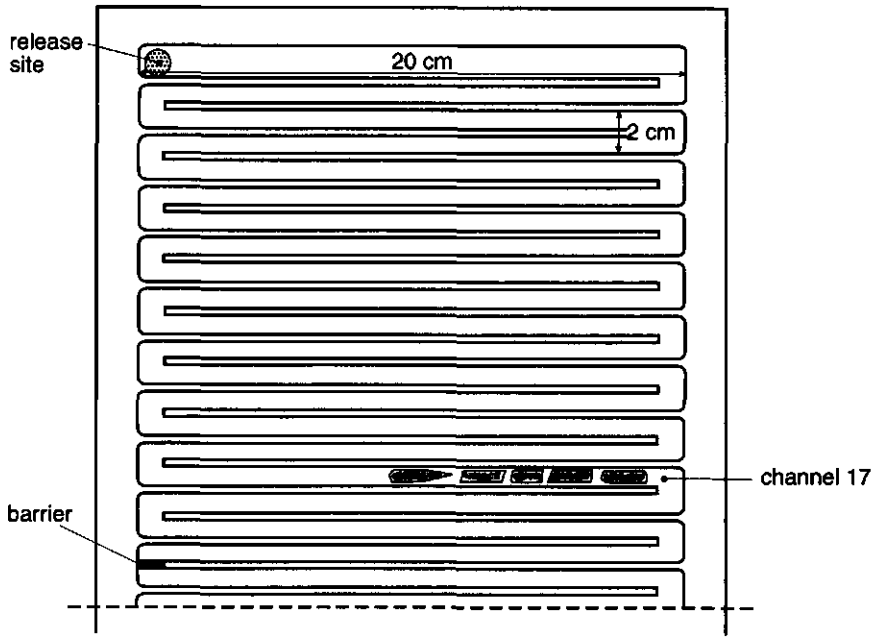


Fig. 1. Section of aluminium block (top view) showing the channel cut into metal block. Only one of the two release sites is shown. Wasps were allowed to walk in the chamber for 20 h and to parasitise *Ephestia kuehniella* eggs present in subchannel 17, ca. 3.4 m from the entrance. Total length 2 x 4 m (subchannel dimensions 1 cm x 1 cm x 20 cm).

Parasitism and dispersal in greenhouse

Set-up

Releases were performed in 2 adjacent compartments of 10.8 m^2 each. In each compartment 13 tomato plants (*Lycopersicon esculentum*) of the cultivar Moneymaker, were placed in a U-shape (Fig. 2 and Fig. 3). Plants were 40-50 cm high, had ca. 15 leaves each and the total leaf area per plant was ca. 1500 cm^2 (Garg & Mandahar 1972). For uniformity, flowers were removed from the plants. Each plant touched the two adjacent plants. Twelve *Ephestia kuehniella* egg masses were placed on each plant, except on the plant at the mid-point of the U, where the wasps were released. An egg mass consisted of eggs fixed on doublesided adhesive tape in a circle (6 mm diameter, ca. 130 eggs each), surrounded by white paper. Two egg masses per leaf were tape-glued on

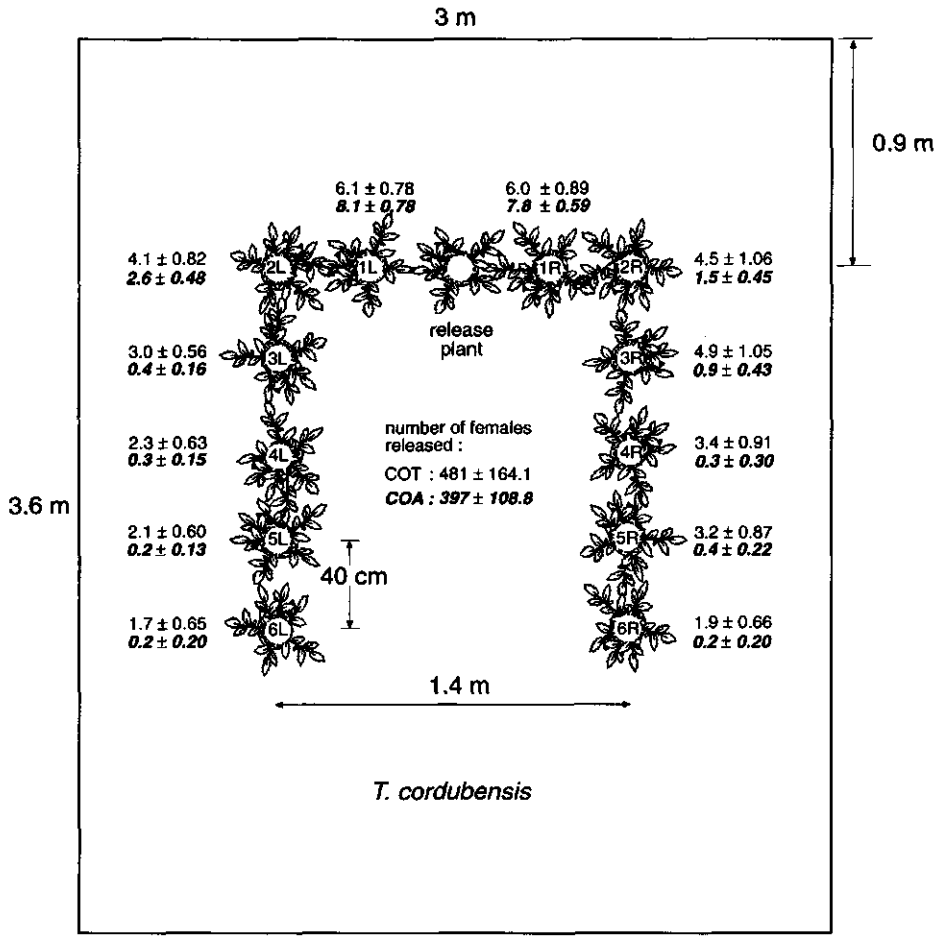


Fig. 2. Position of tomato plants and parasitism patterns of *Trichogramma cordubensis* populations (COT and ***COA***) on 12 tomato plants in paired greenhouse releases (experiment 1). Numbers represent mean numbers of sentinel egg masses parasitised (± SEM) at each tomato plant.

6 leaves of each plant, one egg mass at the uppersurface and the other at the undersurface of the same leaflet.

Releases

Three experiments were performed in which conspecific arrhenotokous and thelytokous *Trichogramma* were released simultaneously. *T. cordubensis* releases (experiment 1) were performed from February to May 1997. *T. deion* releases were performed from June to August 1997 (experiment 2) and in September-October 1997 (experiment 3).

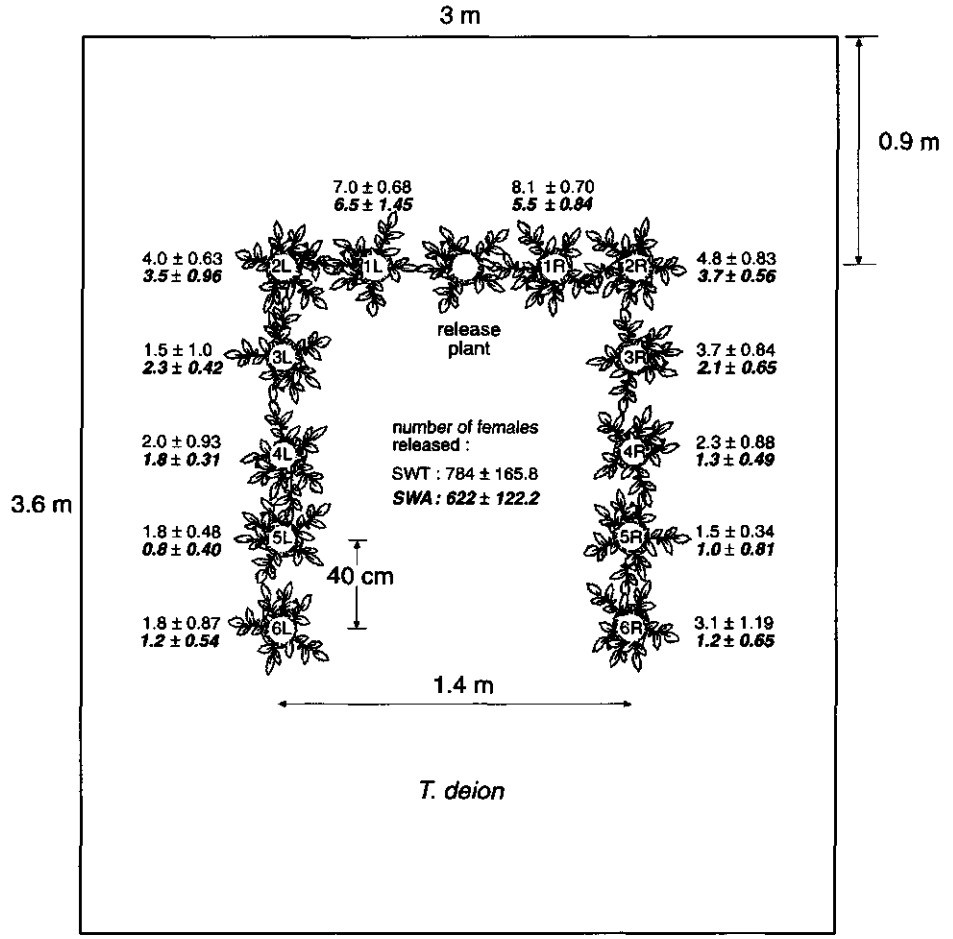


Fig. 3. Position of tomato plants and parasitism patterns of *Trichogramma deion* (SWT and ***SWA***) on 12 tomato plants in paired greenhouse releases (experiment 2). Numbers represent mean numbers of sentinel egg masses parasitised (± SEM) at each tomato plant.

In experiments 1 and 2 the thelytokous and arrhenotokous lines were released in separate, adjacent, compartments. In experiment 3 the arrhenotokous and the thelytokous lines were released together in both compartments.

Fifteen well parasitised egg masses (ca. 80 parasitised eggs each) were selected, from which ten were randomly chosen for the release; the remaining egg masses were placed in the freezer (-80°C) at the time of release, for later determination of the sex ratio. One-three-days old wasps were released from a glass vial (150 mm length, 15 mm diameter), after removing the egg masses from which they had emerged. The release was

performed at the side most distant from the door at the midpoint of the U on the plant free of egg masses. The vial containing the wasps to be released was placed in the soil of the flowerpot, its opening leaning against the stem. The number of females released was estimated *a posteriori*, by counting the number of host eggs from which parasitoids had emerged and by assessing the sex ratio of the non-released subsamples. Mean numbers of females released for experiments 1 and 2 are given in Fig. 2 and Fig. 3, respectively. In experiment 3 an average of 394 ± 60.1 thelytokous and 310 ± 93.0 arrhenotokous females were released. The trials were repeated 10 times, for *T. cordubensis* (experiment 1), 6 times for *T. deion* (experiment 2) and 4 times (x 2 compartments) for *T. deion* (experiment 3).

Climatic conditions were measured during the experiments and varied between: 16-35°C, 55-85% R.H. (experiment 1); 16-50°C, 30-100% R.H. (experiment 2) and 18-52°C, 49-98% R.H. (experiment 3). In all experiments, day temperature usually remained above 20°C and periods above 35°C did not exceed 4 hours a day. Additional meteorological data were obtained from the Department of Meteorology in Wageningen Agricultural University.

Collection of egg masses

The egg masses were collected five days after the release and were incubated at 23±1°C, 60±10% R.H., 16h light/8h dark. If the eggs were to be counted more than 5 days later, the egg masses were subsequently incubated at 15±1°C, 60±10% R.H., 16h light/8h dark. The parasitised (black) eggs were counted for each egg mass, except for replicate one of experiment 3, as mentioned below in this section. Dispersal in the greenhouse was measured indirectly, based on the parasitised eggs.

Between replicates, forty fresh *E. kuehniella* egg masses were placed at the same location where the tomato plants were during the experiment. These egg masses were never found parasitised, indicating that after 5 days no more ovipositing wasps were present. In addition, live wasps were never found during collection of the egg masses.

In experiment 3, in which the thelytokous and the arrhenotokous lines of *T. deion* were released together in both compartments, we determined whether an egg mass had been parasitised by the thelytokous line, the arrhenotokous line, or both. For that purpose, individual females emerging from egg masses parasitised in the greenhouse were given an unparasitised *E. kuehniella* egg mass and the sex ratio of their progeny was determined. When individual greenhouse parasitised eggs had been isolated (done only for a fraction of the eggs in replicate one) we categorised all-female broods as being produced by a

thelytokous mother and all-male broods as having an arrhenotokous mother. However, most wasps emerged from eggs that were not isolated. In these cases, mixed (male and female) progeny was often obtained from arrhenotokous mated mothers. When an egg mass had been parasitised by both wasp lines, the number of parasitised eggs per line was not determined. In all other cases, except in replicate one of experiment 3, the number of parasitised eggs per egg mass was recorded. For 23 out of the 273 parasitised egg masses it was impossible to determine to which wasp line parasitism should be attributed, because only a few eggs were parasitised or the eggs had been eaten by *E. kuehniella* larvae. These egg masses were not included in the statistical analyses.

Statistical analyses

The statistical software SAS (version 6.12) was used in all analyses. In the case that a generalised linear model (*e.g.* Crawley 1993) was appropriate, we used the procedure Genmod. A correction for possible overdispersion with respect to the Poisson distribution was made. Linear models (including the two sample situation) were fitted with Proc GLM, while paired sample t-tests were calculated with the procedure Means. Wilcoxon two-sample tests were performed through the Npar1way procedure. Below, detailed information is given for each experiment. For all analyses an α=0.05 was chosen.

Fecundity in glass vials: surplus of hosts and no searching required

The number of eggs parasitised per female (first day, total of first three days, total of five days) was compared between thelytokous and arrhenotokous lines as follows: for *T. deion*, a Wilcoxon two-sample test was used; for *T. cordubensis*, a generalised linear model was fitted, with Poisson distribution, log link function, and using tibia length as a covariable. A t-test was used to compare hind tibia lengths of mothers between lines.

Dispersal and parasitism in a laboratory chamber

By means of a linear model we compared the two *Trichogramma* lines for the following response variable, correcting for repetition in time and release side, which were removed from the model if not significant:

- Average distance of females to the release site, using channel number as distance unit. Since group sizes differed, we performed a weighted analysis, using the square root of group size as weight. The comparison of average distance to the release site between males and females was calculated as above.

By means of a generalised linear model with Poisson distribution and log link function and the explanatory variables *Trichogramma* line, repetitions in time and release side, we studied the following response variable:

- Number of parasitised eggs, assuming the expected number of parasitised eggs to be proportional to the number of females arriving at channel 17 (where the egg masses were present) or further. Factors release side and repetitions, which showed no significant effect, were subsequently excluded from the model. The number of parasitised eggs per female released was compared between lines by using a Wilcoxon two-sample test.

Parasitism and dispersal in greenhouse

For all three experiments, temperature and relative humidity were initially used as explanatory variables. For experiment 1, air pressure, light intensity and percentage of hours of sunshine per day were tested as well. No significant effects were found for these variables, so they were excluded from the analyses.

For experiments 1 and 2, we compared, by means of a linear model, the two *Trichogramma* lines for the following response variables, correcting for repetitions in time and greenhouse compartment:

1. number of parasitised eggs per female, using the square root of the total number of females released as weight;
2. number of parasitised egg masses per female; for weights see 1;
3. average distance of parasitised eggs to the release plant. Distance was calculated as the average plant number (see Fig. 2 and 3) where parasitised eggs were found. The square root of the total number of parasitised eggs was used in the model as weight.
4. average distance of parasitised egg masses to the release plant; for weights see 3;
5. average height on the plant of parasitised eggs. Height was calculated as the average plant height where parasitised eggs were found. Lowest leaves with eggs were assigned number one; highest leaves with eggs were assigned number 6. The square root of the total number of parasitised eggs was used in the model as weight.
6. average height on the plant of parasitised egg masses; for weights see 5;
7. average number of parasitised eggs per parasitised egg mass;
8. proportion of parasitised eggs at the uppersurface of the leaves of the total number of parasitised eggs: comparison of the wasp lines with each other and of each of the lines with 0.5.

In experiment 3, each released group consisted of wasps from both lines. We therefore have paired observations and the response variable is the difference between the averages of each line. The analyses were performed by means of a paired sample t-test.

Results

Fecundity in glass vials: surplus of hosts and no searching required

In both *Trichogramma* species, the arrhenotokous females parasitised significantly more eggs than their thelytokous counterparts, in all cases (i.e, first day, total first three days and total five days) (Fig. 4). Hind tibia lengths differed significantly between *T. cordubensis* lines (p= 0.015), with the mean values (± sd) of 0.167 mm (± 0.0083) for the arrhenotokous line and 0.162 mm (± 0.0075) for the thelytokous line. Note that a significant difference in number of parasitised eggs was found between *T. cordubensis* lines after correction for tibia length.

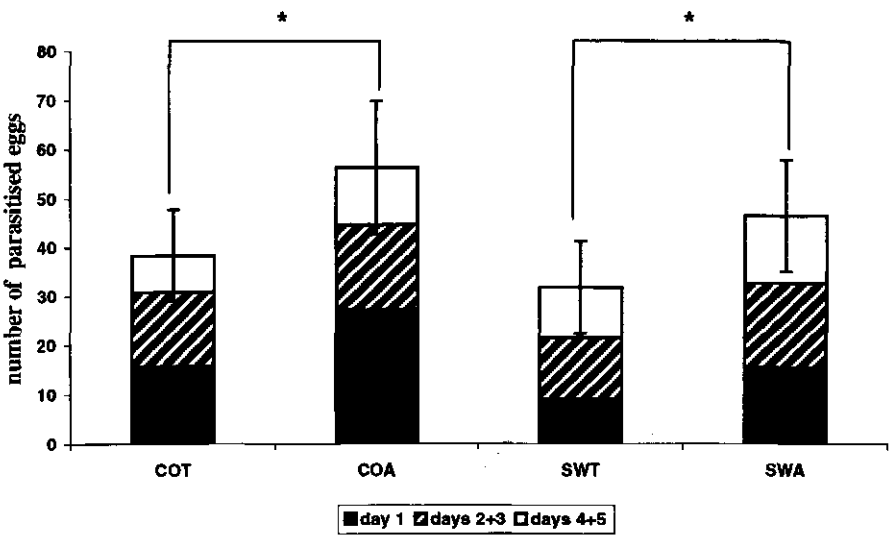


Fig. 4. Parasitism of *Ephestia kuehniella* eggs by thelytokous and arrhenotokous *T. cordubensis* (COT and COA) and *T. deion* (SWT and SWA) in glass vials: surplus of hosts and no search needed. Columns represent mean number of parasitised eggs per *Trichogramma* female (day 1, days 2+3 and days 4+5). Standard deviation bars refer to cumulated number of parasitised eggs (days 1 to 5). For sample sizes see Materials and Methods. *: values for the arrhenotokous line are significantly higher than for their conspecific thelytokous line (p< 0.05).

Dispersal and parasitism in a laboratory chamber

Percentage distributions show the dispersal pattern of females of arrhenotokous and thelytokous forms of *T. cordubensis* and of *T. deion* in the chamber (Fig 5). For both species, arrhenotokous females showed significantly more dispersal (as expressed by average distance to release site) than their thelytokous counterparts and than their conspecific males. Thelytokous *T. cordubensis* females that were released with arrhenotokous males did not differ significantly in dispersal from thelytokous conspecific females that were released alone.

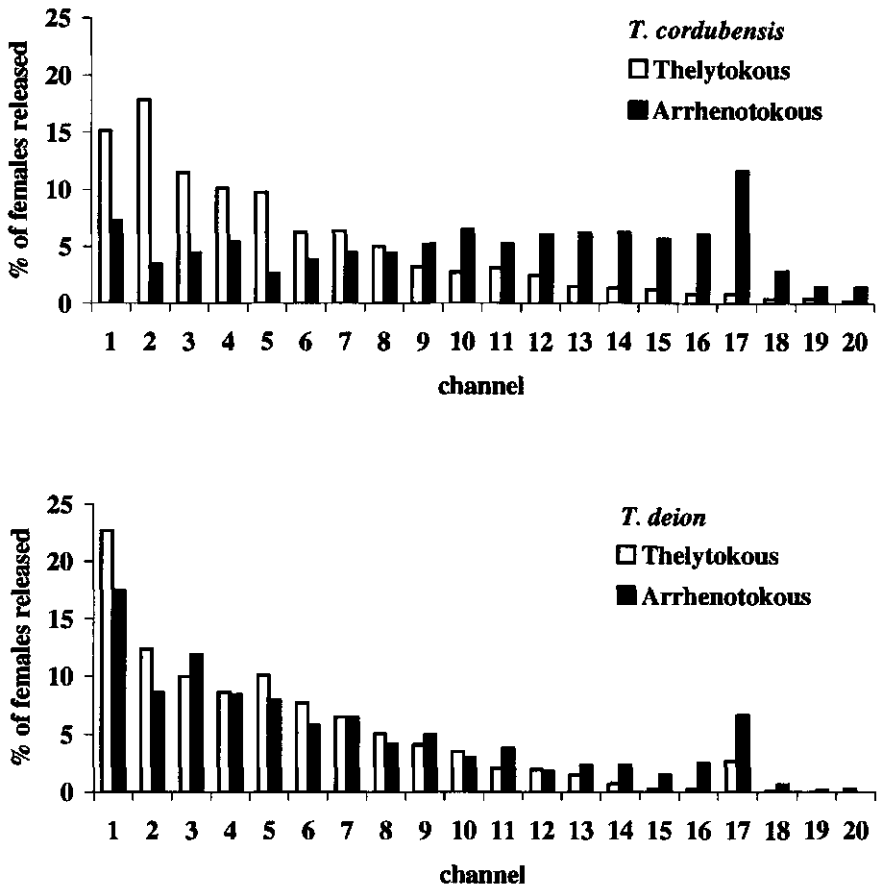


Fig. 5. Percentage of thelytokous (white bars) and arrhenotokous (black bars) females of a) *Trichogramma cordubensis* (COT and COA) and b) *T. deion* (SWT and SWA) found per channel in the laboratory chamber after being allowed to walk for 20 h; wasps were released in channel 1. *Ephestia kuehniella* eggs were present in channel 17.

Table 1. Dispersal and parasitism of thelytokous and arrhenotokous lines of *Trichogramma cordubensis* (COT and COA, respectively) and of *T. deion* (SWT and SWA, respectively) in the laboratory chamber (descriptive statistics).

Gender and wasp line released to which the results refer to	Mean (± sd) wasps released	Released together with	Number of replicates‡	Mean channel (± sd) reached after 20 h	Mean (± sd) number of eggs parasitised per ♀ channel ≥ 17 (n=8)	Mean (± sd) number of eggs parasitised per ♀ (n=8)
♀♀ COT	89.5 ± 26.47	-	17	5.2 ± 1.42	2.2 ± 3.30	0.06 ± 0.09
				*	*	*
♀♀ COA	56.6 ± 23.87	♂♂COA	12	9.3 ± 2.01	21.2 ± 7.60	2.88 ± 1.65
				ns		
				*		
♂♂COA	34.3 ± 15.46	♀♀COA	12	6.1 ± 1.87	-	-
♀♀COT	88.3 ± 8.14	♂♂COA	3	5.3 ± 0.90	-	-
				*		
♂♂ COA	28.3 ± 15.89	♀♀COT	3	7.0 ± 1.30	-	-
♀♀SWT	117.3 ± 34.45	-	10	5.2 ± 0.92	9.1 ± 6.52	0.31 ± 0.26
				*	ns	ns
♀♀SWA	75.9 ± 32.30	♂♂ SWA	8	6.3 ± 0.85	12.7 ± 4.76	0.75 ± .050
				*		
♂♂SWA	51.4 ± 22.97	♀♀SWA	8	4.8 ± 2.04	-	-

Significant differences between conspecific populations are indicated by a * ($p<0.05$); ‡:considering each side of the maze independently; -: not tested or no biological meaning; ns: not significant; n: number of replicates used for number of eggs parasitised.

Table 2. Several greenhouse experiments parameters, regarding thelytokous and arrhenotokous *Trichogramma cordubensis* (COT and COA, experiment 1) and *T. deion* (SWT and SWA) released apart (experiment 2) and released together (experiment 3). For experiments 1 and 2 values are result of GLM model, for experiment 3 data were not transformed. Average distance of eggs and egg masses to the release plant are given in plant numbers units (Fig. 2 and 3). Average height on the plant of parasitised eggs is given in leaf numbers: lowest leaf with eggs is number one, highest leaf with eggs is number six.

	Experiment 1 (n =10)		Experiment 2 (n=6)		Experiment 3 (n=4x2)
Parameters↓ /*Trichogramma* lines →	COT (mean ± SEM)	COA (mean ± SEM)	SWT (mean ± SEM)	SWA (mean ±SEM)	SWT – SWA (diff ±SE)
Number of parasitised eggs/♀	1.9 ± 0.42	2.4 ± 0.43	0.84 ± 0.06	1.12* ± 0.07	-0.46 ± 0.20
Number of parasitised egg masses/♀	0.09 ± 0.01	0.06 ± 0.01	0.06 ± 0.003	0.05 ± 0.003	-0.01 ± 0.009
Eggs distance	2.4*± 0.15	1.3 ± 0.15	2.4 ± 0.11	2.1 ± 0.10	0.12 ± 0.27
Egg masses distance	2.7*± 0.16	1.5 ± 0.18	2.6 ± 0.12	2.4 ± 0.13	0.35*± 0.12
Eggs height on plant	4.4*± 0.13	2.8 ± 0.13	4.1 ± 0.13	4.0 ± 0.13	-0.46 ± 0.38
Egg masses height on plant	4.1*± 0.12	3.2 ± 0.13	4.0 ± 0.07	4.0 ± 0.08	0.12 ± 0.22
Number of eggs/egg mass	20.2 ± 2.15	36.8*± 2.24	15.1 ± 0.77	23.2* ± 0.81	-5.95*± 0.56
Proportion of eggs at upperside of leaf	0.59#± 0.03	0.64#±0.03	0.57 ± 0.03	0.48 ± 0.03	0.12 ± 0.05

*: values for the conspecific thelytokous and the arrhenotokous line within an experiment are significantly different ($p<0.05$); #: values are significantly different from 0.5. ($p<0.05$), no significant differences between lines were detected; n: number of replicates

For *T. cordubensis*, arrhenotokous females, which dispersed more than their thelytokous counterparts, parasitised more eggs per female as well. For *T. deion* the difference in parasitism between lines was not significant (Table 1).

Parasitism and dispersal in greenhouse

In all three experiments, the number of eggs parasitised per female was higher for the arrhenotokous lines, but this difference was found to be significant only in *T. deion* released in separate compartments (experiment 2, Table 2). No significant differences between thelytokous and arrhenotokous lines in the number of egg masses parasitised per female were found.

In all three experiments, the average distance of parasitised eggs and egg masses to the release point was higher for the thelytokous line, with significant differences found for *T. cordubensis* (experiment 1) and *T. deion* lines released together in the same compartments (experiment 3). Regarding the vertical distribution of parasitised eggs, thelytokous *T. cordubensis* parasitised egg masses significantly higher on the plant than their arrhenotokous counterparts, while no such a difference was found for *T. deion.*

In all cases, average number of parasitised eggs per egg mass was significantly higher for the arrhenotokous lines. Significantly more eggs were parasitised by *T. cordubensis* at the uppersurface of leaves than at the undersurface. For *T. deion* no such difference could be found. No significant differences in this last parameter were found between arrhenotokous and thelytokous lines.

Table 3. Parasitism by thelytokous and arrhenotokous *Trichogramma cordubensis* and *T. deion* wasps per hundred individuals released. Mean sex ratio (% females), mean number of parasitised eggs per female and number of eggs parasitised per 100 wasps released, calculated as sex ratio x mean number of parasitised eggs per female x 100.

Species	Line	Mean sex ratio	Mean number of parasitised eggs per female	Number of eggs parasitised per 100 wasps
T. cordubensis	Thelytokous	1	1.9	190
T. cordubensis	Arrhenotokous	0.55	2.4	132
T. deion	Thelytokous	1	0.84	84
T. deion	Arrhenotokous	0.61	1.12	68

Discussion

To determine the effect that an infection with *Wolbachia* may have on the biological control efficiency of *Trichogramma*, we compared fecundity and dispersal of thelytokous (i.e, infected) and arrhenotokous (i.e., uninfected) *T. cordubensis* and *T. deion* wasps in a series of experiments, both in the laboratory and in the greenhouse. We first determined the fecundity of thelytokous and arrhenotokous females, when they were offered daily a surplus of host eggs in glass vials. Arrhenotokous females of *T. deion* had a higher fecundity than their thelytokous counterparts. These results agree with previous findings (Chapter 4, Stouthamer & Luck 1993) for *T. deion*, *T. pretiosum* and *T. kaykai* and are compatible with the hypothesis of a negative effect of *Wolbachia* on female fecundity for mixed populations. On the other hand, we expected that a negative effect of *Wolbachia* would be less or non-existent in *Trichogramma* from fixed populations. However, in our laboratory fecundity experiment, arrhenotokous *T. cordubensis* females parasitised significantly more eggs than their thelytokous conspecifics. Previous work on the same *T. cordubensis* lines had shown a negative effect of *Wolbachia* on lifetime fecundity, but not in five days fecundity (Chapter 4). The main difference between the two fecundity experiments is that the wasps we tested were smaller than the ones tested in Chapter 4. *Mamestra brassicae* L. (Lepidoptera, Noctuidae) was then used as rearing host, instead of *E. kuehniella*. In the experiment of the present chapter, arrhenotokous wasps were, on average, larger than thelytokous wasps. Larger *Trichogramma* wasps often have higher fecundity than small ones (e.g. Waage & Ng 1984). But the size of mothers explains only in part the difference in fecundity obtained between arrhenotokous and thelytokous *T. cordubensis*. A possible explanation for the difference between the results of this chapter and the ones of chapter 4 in relative parasitism of thelytokous and arrhenotokous *T. cordubensis* is that the *Wolbachia* exert a more pronounced negative effect on smaller wasps. More fecundity experiments should be performed using, simultaneously, hosts of different species and sizes, including hosts of different species but with the same size, to clarify the effects of host-species, host-size and infection status on the wasps' size and fecundity.

In the second set of experiments, in a laboratory chamber, arrhenotokous *T. cordubensis* and *T. deion* females dispersed more than their thelytokous counterparts. Also, previous work done on *T. deion* (isofemale lines Seven Pines California and Texas) has shown a higher dispersal of arrhenotokous females in the laboratory chamber, when compared with the thelytokous ones (Vereijssen *et al.* 1997). These authors have

observed that immediately after the release, thelytokous females remained near the release site, while arrhenotokous females dispersed much quicker. We also observed this phenomenon for *T. cordubensis* during our laboratory chamber experiments. Our releases of thelytokous *T. cordubensis* females with conspecific males in the laboratory chamber indicate that the presence of males is not the cause of the differences in dispersal found between females of the two *T. cordubensis* lines. Therefore, the infection with *Wolbachia* could be responsible for a lower dispersal of the females in the laboratory chamber. The difference in number of parasitised eggs per released female was particularly pronounced between the lines of *T. cordubensis*. While in glass vials the *T. cordubensis* arrhenotokous females parasitised ca. 1.5 times more eggs than their thelytokous counterparts during day one, in the chamber the arrhenotokous females parasitised almost 50 times more eggs than their thelytokous counterparts. This difference between lines is partly due to a difference in dispersal (affecting the number of females reaching the eggs) and partly due to a difference in fecundity between the two lines (Table 1). In both wasp species males dispersed less than females, which agrees with findings for other *Trichogramma* species in field conditions (Stern *et al.* 1965, Suverkropp 1997).

In the third type of experiments, *i.e.*, the greenhouse releases, arrhenotokous females of both species parasitised more eggs than their thelytokous counterparts. The number of eggs parasitised per female was not in all cases significantly different between the lines but the number of eggs parasitised per egg mass was always significantly higher for the arrhenotokous lines. This indicates that arrhenotokous lines were more effective in parasitising aggregated host eggs. No differences were found between thelytokous and arrhenotokous lines in the number of egg masses parasitised per female, suggesting that lines do not quantitatively differ in their ability to find host egg masses. When we calculate the number of eggs which would be parasitised when we release one hundred individuals of each line, taking into account the average fecundity per wasp (from our greenhouse results) and the sex ratio (of the wasps we released in the greenhouse), we see that a higher parasitism is achieved by the thelytokous wasps (see Table 3).

The number of females that we released in the greenhouse was high when compared with the general recommendation for heavy host infestations, which is 20 individuals/m^2 (van Lenteren *et al.* 1997). We released, on average, from 29/m^2 (arrhenotokous females, experiment 3), to 73/m^2 (thelytokous females, experiment 2). Yet parasitism was low, particularly at the plants far from the release point. Extreme climatic conditions at times in the greenhouse and absence of nutrition for the released parasitoids could be responsible for the results obtained. Also the plant species and structure, the lepidopteran

host species, the distribution of the eggs on the plants, the presence or absence of moth scales on the leaves and the release method may play an important role on the number of hosts parasitised (Bigler *et al.* 1997). On the other hand, the fact that there were egg masses close to the release site could lead to arrestment behaviour of the wasps and demotivate dispersal.

For *Trichogramma cordubensis*, we found that the thelytokous line dispersed significantly more than the arrhenotokous line. The difference found in the vertical distribution of parasitised eggs between *T. cordubensis* lines may be explained by a larger vertical dispersal of the thelytokous line. Plants touched each other usually at the level of leaf 2 or 3 (counting from the bottom). A mean height of the parasitised eggs at leaf 4 may indicate that, after walking from one plant to another through the leaves, females of the thelytokous line disperse more, by moving up in the plant. For *T. deion*, no significant differences were found in vertical distribution and with exception of egg masses distance in experiment 3, in which dispersal of the thelytokous line was significantly higher, no differences were found in dispersal.

In the chamber, arrhenotokous females of both *T. cordubensis* and *T. deion* dispersed more than their thelytokous counterparts, but the opposite effect or no differences were found in the greenhouse. So, contrary to the findings reported in Chapter 5, the dispersal in the chamber did not give an indication of relative dispersal of arrhenotokous and thelytokous females in the greenhouse. The presence of plants and the climatic conditions in the greenhouse could have provided the necessary stimuli for a relatively high dispersal of the thelytokous *T. cordubensis* wasps. We suggest that flight may play a role in the differences found between lines in the greenhouse experiment. *Trichogramma* individuals could not fly in the chamber but they could fly in the greenhouse. Suverkropp (1997), studying dispersal of *T. brassicae* on maize, found that flight was important in the movement from one plant to another, while walking was the most important way of locomotion within a plant. In a preliminary set of *T. cordubensis* releases in a cage (ca. 200 individuals per release, 5 replicates per line) thelytokous and arrhenotokous females wasps were compared in their ability to fly (Roskam, unpublished). While for the thelytokous line 41-50% of the released females were caught on the sticky trap, which could be reached by flying only, for the arrhenotokous line only 0-3% of the released wasps were trapped. The different mating status of the thelytokous and arrhenotokous females may have played a role in the differences just mentioned. However, in contrast to these results, it has been reported that unmated *Trichogramma minutum* (arrhenotokous) females had less propensity to fly than mated ones (Forsse *et al.*

1992). More experiments are needed to clarify the reasons for differences in dispersal between thelytokous and arrhenotokous lines and the role that mating status, flight and laboratory rearing play in it.

In inoculative biological control, thelytokous females can be advantageous simply because they always produce daughters and do not need males for mating, while for arrhenotokous lines daughter production can decrease drastically in situations of low host density. For inoculative biological control at high host densities, the choice between thelytokous and arrhenotokous wasps should depend on the rate of population increase, adaptation to the environment and production costs.

In inundative biological control, adaptation to environment and rate of population increase play a much less important role. When instead of egg masses, single and scattered eggs of the pest are present, the fecundity disadvantage of the thelytokous females becomes insignificant. We assume that each female is able to locate only a few hosts over her lifetime and that thelytokous and arrhenotokous wasps do not differ in the ability of finding eggs. Under such circumstances, thelytokous females will be able to parasitise equal numbers of hosts as arrhenotokous females. Thelytokous females are advantageous because of their lower production costs (no host eggs "wasted" in males). But also in the case of pests that produce egg masses, despite the lower individual female fecundity of the thelytokous lines, more eggs are parasitised per 100 wasps released. Hence, thelytokous wasps are advantageous because they are relatively more economic biological control agents.

The range of dispersal of the wasps is crucial when a large area is to be controlled and relatively few release points are used. Also in this point the thelytokous line of *T. cordubensis* is advantageous.

In conclusion, arrhenotokous *T. cordubensis* and *T. deion* parasitised more eggs per female than their thelytokous counterparts in glass vials, in the chamber and in the greenhouse. These differences between conspecific lines were significant except for *T. cordubensis* and once for *T. deion* in the greenhouse (experiments 1 and 3) and for *T. deion* in the chamber. These results suggest that a negative effect of *Wolbachia* on the fecundity of their female hosts exists. The effect of *Wolbachia* on *Trichogramma* dispersal appears to be positive or neutral under greenhouse conditions, although it was negative in the laboratory. Thelytokous females seem to be more appropriate to release against scattered isolated eggs because they may disperse more and they produce females only. Even though arrhenotokous females are more efficient in parasitising large egg

masses, calculations show that the use of thelytokous females is more advantageous, because production costs are lower.

The artificial transformation of arrhenotokous strains to thelytokous ones by microinjecting arrhenotokous females with *Wolbachia* (van Meer *et al.* 1996, Grenier *et al.* 1998) may prove to be useful not only to answer evolutionary questions about the relation between *Wolbachia* and their hosts, but it could also have an application in biological control projects. To assess its practical usefulness, more studies are needed on the direct and indirect impact of these thelytokous-made wasps on target and non-target species (Howarth 1991, Simberloff & Stiling 1996a) and on the final costs of producing these wasps.

Acknowledgements

We are thankful to Koppert B.V. for providing hosts, to Astrid Schoenmaker and Hanneke van Heest for practical assistance with the experiments and to Joop van Lenteren and the Entomology PhD group for a review of the manuscript. The work of I.S. was financially supported by Junta Nacional de Investigação Científica e Tecnológica (BD 5620/95) and Calouste Gulbenkian Foundation, Portugal. The work of M.M. and M.R. was financed by Technology Foundation (STW: 790.42.793), The Netherlands, and the work of R.S. was supported by an EU FAIR grant (CT9-1433).

Chapter 7

Do sympatric *Trichogramma* species parasitise the pest insect *Helicoverpa armigera* and the beneficial insect *Chrysoperla carnea* in different proportions?

To be published as Silva, I.M.M.S. & R. Stouthamer. Do sympatric *Trichogramma* species parasitise the pest insect *Helicoverpa armigera* and the beneficial insect *Chrysoperla carnea* in different proportions? *Entomologia experimentalis et applicata* (in press).

Abstract

Parasitism of two host species by five *Trichogramma* species (Hymenoptera, Trichogrammatidae) was studied in the laboratory. The host species were: i) the bollworm *Helicoverpa armigera* Hübner (Lepidoptera, Noctuidae), an important pest of many crops in the tropics and subtropics, and ii) one of its natural enemies, the lacewing *Chrysoperla carnea* Stephens (Neuroptera, Chrysopidae), a predator often used as a biological control agent. The proportion of *H. armigera* eggs parasitised from the total number of parasitised hosts differed between *Trichogramma* species. The average number of parasitised eggs per female in 24 h by *Trichogramma pintoi* and *T. bourarachae* was 10 of *H. armigera* and about 0.5 of *C. carnea*. For the other three *Trichogramma* species (*T. cordubensis*, *T. evanescens* and *T. turkestanica*) these averages varied from 6 to 11 *H. armigera* eggs and from 3 to 4 *C. carnea* eggs. Total adult offspring production, contacts with hosts, secondary clutch size and sex-ratio of each *Trichogramma* species were determined as well. The results show that sympatric *Trichogramma* may parasitise target and non-target species in different proportions. If this difference corresponds to the field situation, simple laboratory tests could be performed to select not only efficient biological control agents, but also species that are the least detrimental to non-target hosts.

Introduction

Biological control is a method used worldwide in pest management. It has been considered as a sustainable, economical and environmental attractive alternative for chemical pest control (*e.g.* Hokkanen & Lynch 1995). Recently, biological control has been criticised for potential side effects when new species are introduced. Although there are few well documented cases of environmental problems due to release of natural enemies, none of which caused by insects, the concern that these might attack beneficial non-target organisms and rare or endangered species is growing (Howarth 1991, Simberloff & Stiling 1996a, b, van Lenteren 1997). To ensure that biological control will continue to be a useful method in integrated pest management, research should be performed to assure that releases of biological control agents are environmentally safe. The need of natural enemies that are harmless to non-target organisms has been repeatedly referred to as an important factor in the selection of biological control agents (*e.g.* van Lenteren 1986b, Samways 1994). However, research in this area has been scarce and biological control agents are still often selected by trial and error. During the past few years biological control researchers have developed guidelines to select and to control the quality of arthropods used as biological control agents (Bigler 1991, Nicoli *et al.* 1994). *Trichogramma* spp. are the most commonly used entomophagous insects in biological control programs, released on a large scale against lepidopterous pests [for a review, see Li (1994) and Smith (1996)]. Several approaches have been discussed and standard procedures have been suggested to select and control the quality of *Trichogramma* strains. Host selection is one of the important factors in a pre-evaluation procedure (Bigler 1994, Hassan 1994) and it can be successfully tested in the laboratory (Hassan 1989). Inter- and intraspecific variation in host preference have been studied by several authors (*e.g.* van Dijken *et al.* 1986, Pak 1988, Hassan & Guo 1991, Wührer & Hassan 1993). In some cases, *Trichogramma* can loose the preference for their natural host. This has been reported for *T. maidis* (=*brassicae*) (van Bergeijk *et al.* 1989) and for *T. ostriniae* (Hassan & Guo 1991), both in relation to *Ostrinia nubilalis* Hbn. (Lep., Pyralidae).

Trichogramma spp. parasitise eggs of over 400 species belonging to at least seven insect orders (Bao & Chen 1989 in Li 1994). All *Trichogramma* species in which parasitism has been studied are capable of parasitising more than one host species (Pintureau 1990). As van Dijken *et al.* (1986) already pointed out, little is known of host preferences and host ranges of different *Trichogramma* strains in the field. Host ranges determined in the laboratory tend to be broader than the ones found in the field. This appears to be particularly

true for *Trichogramma* spp. (Curl & Burbutis 1978). Preference for certain habitats and host plants (Rabb & Bradley 1968, Martin *et al.* 1981, Pinto & Oatman 1988) are probably responsible, at least in part, for such differences. Nevertheless, several field studies report on egg parasitisation of non-target organisms, including beneficial predators of the family Chrysopidae (Johnson & Bin 1982, Kapadia & Puri 1991, Zucchi & Monteiro 1997). In Europe and in Canada, research is performed to assess the effect of *Trichogramma* releases on non-target Lepidoptera and on native parasitoids of the target pests (van Lenteren, pers. com., Smith 1996).

In Portugal, *Trichogramma* species occur naturally in agricultural fields where several host species are present. Among these, we find *Helicoverpa armigera* Hübner (Lepidoptera, Noctuidae), an important pest of many crops in the tropics and subtropics, and *Chrysoperla carnea* (Stephens)(Neuroptera, Chrysopidae), a cosmopolitan polyphagous predator, used in biological control against Homoptera, Coleoptera and Lepidoptera (Meierrose 1990).

The purpose of our research was to study in the laboratory the degree of successful parasitisation by several *Trichogramma* species of *H. armigera* (their natural host) and *C. carnea* (a beneficial insect). To our knowledge this is the first study on selection of *Trichogramma* species which are the least detrimental to a beneficial insect.

Material and methods

Helicoverpa armigera: culture and eggs handling before trials

Pupae were received from F. Figo (Évora University, Portugal) and were collected in Alentejo (Portugal), tomato fields, summer 1994. Individuals were reared in the laboratory (21 ± 1 °C, r.h. 50 ± 20 %, L18:D6) on artificial diet [adapted from Poitout & Bues (1970) and Araújo (in Meierrose 1990)]. The eggs used in the experiment were collected from tomato plants.

A number of authors have demonstrated that *Trichogramma* can successfully develop on host eggs that have been frozen or heated, as long as they remain turgid (Singh 1969, Ashley *et al.* 1974, Monier 1983, Ma 1988, Morrison 1988). However, despite several attempts, we were not able to freeze and thaw *H. armigera* eggs without them losing their turgidity. For this reason, the eggs used in the experiment were not killed.

Due to time and egg availability limitations, not all the eggs used in the experiment were equally fresh. We named "young eggs" the ones not older than 3 days and "old eggs"

the ones older than 3 days. The *H. armigera* eggs were stored within 48 h after oviposition at 4 °C before being used in the experiments.

***Chrysoperla carnea*: culture and eggs handling before trials**

Pupae were received from S. Hassan (Institute for Biological Control, Darmstadt, Germany). The adults, reared at 23 ± 1 °C, r.h. 50 ± 20 %, L18:D6) were reared in the laboratory on artificial diet (recipe obtained from S. Hassan) and oviposited on gauze. *Chrysoperla carnea* eggs, less than 24 h old, were killed by exposing them to -18 °C for about 20 hours. Preliminary trials showed that *Trichogramma* can successfully parasitise *C. carnea* hosts killed this way. By using dead hosts one does not need to isolate each egg at the end of the experiment, to avoid larval cannibalism.

***Trichogramma* spp.: culture and handling before trials**

Trichogramma lines were started with single females from field-collected *Helicoverpa armigera* Hbn. (Lepidoptera, Noctuidae) eggs, in Portugal. We studied six isofemale lines which belong to the five following species: *T. evanescens* Westwood 1833 (Te), *T. cordubensis* Vargas & Cabello 1985 (Tc), *T. turkestanica* Meyer 1940 (Tt), *T. pintoi* Voegelé 1982 (Tp) and *T. bourarachae* Pintureau & Babault 1988 (Tb). Two *T. evanescens* isofemale lines were studied that were collected on the same day, at the same location, same host plant species and insect host species, and that were reared in the laboratory under identical circumstances. Voucher specimens are kept by B. Pintureau in INSA-INRA (Villeurbanne, France). The wasps were collected in two tomato fields: Mora, in 1992 (*T. evanescens, T. cordubensis* and *T. turkestanica*) and Divor, in 1993 (*T. pintoi* and *T. bourarachae*). These localities are about 50 km apart. Cultures were maintained for 30-60 generations on *Ephestia kuehniella* eggs (killed by UV radiation), at 22 ± 2 °C, 50 ± 20 % R.H., L18:D6. The females used in the experiments emerged from isolated eggs, were 1-day old, honey-fed, had no previous oviposition experience and were allowed to mate for 4-6 hours (except *T. cordubensis*, which consisted of females only).

Bioassay

The methodology is based on the work reported by Wührer & Hassan (1993), with the modifications described below. Because the eggs of both host species give rise to, on average, two *Trichogramma* spp. adults (field data, unpubl.), the same number of eggs of each host species was used in the experiment. Sixteen eggs of each host species were attached with honey on a paper card at the corners of a piece of paper (2x2 cm^2), 8 eggs

from the same host at diagonal corners (Fig. 1). *Chrysoperla carnea* eggs were placed directly on the paper (they were not in their natural position, which is on a stalk). The experiment was performed at different times (two blocks).

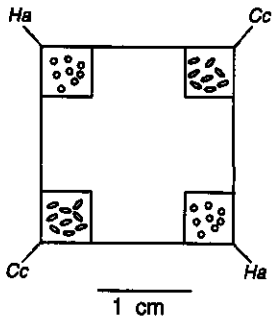


Fig. 1. Schematic drawing showing relative positions of eggs of *H. armigera* (*Ha*) and *C. carnea* (*Cc*) attached to the corners of a piece of paper (2x2 cm^2).

Individual *Trichogramma* females were confined with the host eggs (arranged as mentioned above) in glass tubes (25 x 140 mm), closed off with cotton wool. The females were observed every 15 minutes (block 1) or 30 minutes (block 2), during 4.5 hours and their position was registered (on *H. armigera* eggs, on *C. carnea* eggs, or elsewhere). The proportion of times that a female was observed on a particular host (from the total number of observations of that female on hosts) is referred to as "proportion of contacts". The 32 eggs were available for parasitisation during 24 hours, at 25 ± 1 °C, 60% R.H., L18:D6 and the incubation occurred at 24 ± 1 °C, 60% R.H., L18:D6. After allowing the females to parasitise the eggs, these were isolated in gel capsules (size 000). After 7 days, the parasitised (black) eggs were counted and ca. 10 days later the offspring was sexed. Hind tibia lengths of the mothers were measured, to correct for size-fecundity relationships. For sample sizes see Table 1.

Statistical analyses

We used the statistical software SAS (version 6.12), for the following tests [variables: *Trichogramma* line, repetitions in time, host age, host position (*H. armigera* or *C. carnea* at a specific corner) and mother's hind tibia length]:

- Proportion of *H. armigera* eggs parasitised of the total hosts parasitised: generalised linear model (GLM), proc genmod, binomial distribution, logit as link function; α=0.05. Bonferroni corrections were used in pairwise comparisons. Total number of eggs parasitised

per female was used as an extra explanatory variable, to check whether differences in fecundity could be responsible for differences in host preference.

- Proportion of contacts with *H. armigera* eggs of the total number of contacts with hosts: GLM, proc genmod, binomial distribution, logit as link function; α=0.05. Bonferroni corrections were used in pairwise comparisons.
- Sex ratio: proportion of females of the total offspring: GLM, proc genmod, binomial distribution, logit as link function; α=0.05. Bonferroni corrections were used in pairwise comparisons. *T. cordubensis* was not included in this analysis because it was already known that only females are produced.
- Total offspring produced per female: GLM, proc genmod, Poisson distribution, log as link function; α=0.05, adjustment for multiple comparisons between lines by Bonferroni.
- Secondary clutch size per egg of each host species: GLM, proc genmod, Poisson distribution, log as link function, total number of viable eggs as offset; α=0.05, adjustment for multiple comparisons between lines by Bonferroni.
- Correlation between the "proportion of contacts with *H. armigera* eggs of total number of observed contacts with hosts" and the "proportion of *H. armigera* eggs parasitised of the total hosts parasitised": proc corr, Spearman rank correlation, α=0.05.

Females that did not parasitise any egg were excluded from the statistical analyses.

Results

None of the following variables - repetitions in time, host age and host position - showed a significant effect in any of the analyses performed, so these variables were subsequently excluded from the analyses. No significant differences were found between the two *T. evanescens* isofemale lines in any of the parameters analysed. Therefore, we grouped the data of these lines. The proportion of parasitised *H. armigera* eggs from the total number of parasitised hosts (*H. armigera* + *C. carnea*), per female, was significantly different between *Trichogramma* species. Lines Tp and Tb parasitised a significantly higher proportion of *H. armigera* than line Tc (Fig. 2 and Table 1). In a few cases (5 for line Tt, 4 for line Te and 1 for line Tc) females parasitised 16 eggs or more. The difference between *Trichogramma* lines in the proportion of parasitised *H. armigera* cannot be attributed to differences in the total number of eggs parasitised (p>0.05). Therefore, females which parasitised 16 eggs or more were also included in the analyses. Differences in total offspring production per

Table 1. Performance of *Trichogramma* spp. in parasitism experiments (means ± SEM). Females that did not parasitise have been excluded. For each individual *Trichogramma* female, 16 eggs of each host (*Helicoverpa armigera* and *Chrysoperla carnea*) were available, during 24 hours.

Trichogramma species	*T. turkestanica*	*T. pintoi*	*T. bourarachae*	*T. cordubensis*	*T. evanescens*
Number of ♀♀ tested	14	14	19	14	14
Proportion of *H. armigera* eggs parasitised	$0.76^{b} \pm 0.05$	$0.94^{a} \pm 0.04$	$0.98^{a} \pm 0.01$	$0.65^{b} \pm 0.07$	$0.75^{b} \pm 0.07$
Proportion of contacts with *H. armigera*	$0.84^{ac} \pm 0.05$	$0.95^{ab} \pm 0.03$	$0.99^{b} \pm 0.00$	$0.61^{c} \pm 0.12$	$0.75^{c} \pm 0.08$
% ♀♀ which did not parasitise	0	0	0	21.4	7.1
Numbers of offspring per ♀	$29.0^{b} \pm 1.67$	$19.9^{a} \pm 1.02$	$21.9^{ac} \pm 1.04$	$18.0^{a} \pm 1.69$	$25.3^{bc} \pm 1.74$
Clutch size per *H. armigera* egg	$2.0^{a} \pm 0.10$	$2.1^{a} \pm 0.09$	$2.2^{a} \pm 0.07$	$1.6^{a} \pm 0.12$	$1.9^{a} \pm 0.11$
Clutch size per *C. carnea* egg	$1.9^{a} \pm 0.12$	$1.2^{a} \pm 0.20$	$2.0^{a} \pm 0.00$	$1.5^{a} \pm 0.12$	$1.8^{a} \pm 0.19$
Sex-ratio of offspring (% ♀♀)	$64.5^{a} \pm 0.08$	$70.5^{a} \pm 0.02$	$75.6^{a} \pm 0.05$	100.0	$66.0^{a} \pm 0.06$

Within rows, means followed by different letters are significantly different. Note that the statistical model uses transformed data, so although the means and se refer to the actual results obtained, the significance levels refer to the output of the model.

female were found between lines. Line Tt produced most progeny and Tc the least. Bigger females (size given by hind tibia length) produced more offspring than smaller wasps ($p<0.01$). For both hosts every egg gave rise to about two *Trichogramma* adults (Table 1). For sexual species, sex ratios varied from 64 to 76% females. Overall emergence rate was high: 99% for *H. armigera* eggs and 98% for *C. carnea* eggs. No significant differences between species were found for clutch size, sex ratio or emergence rate. The correlation between the "proportion of contacts with *H. armigera* eggs" and the "proportion of *H. armigera* eggs parasitised" was positive and significant (Spearman rank correlation coefficient = 0.669, p=0.0001).

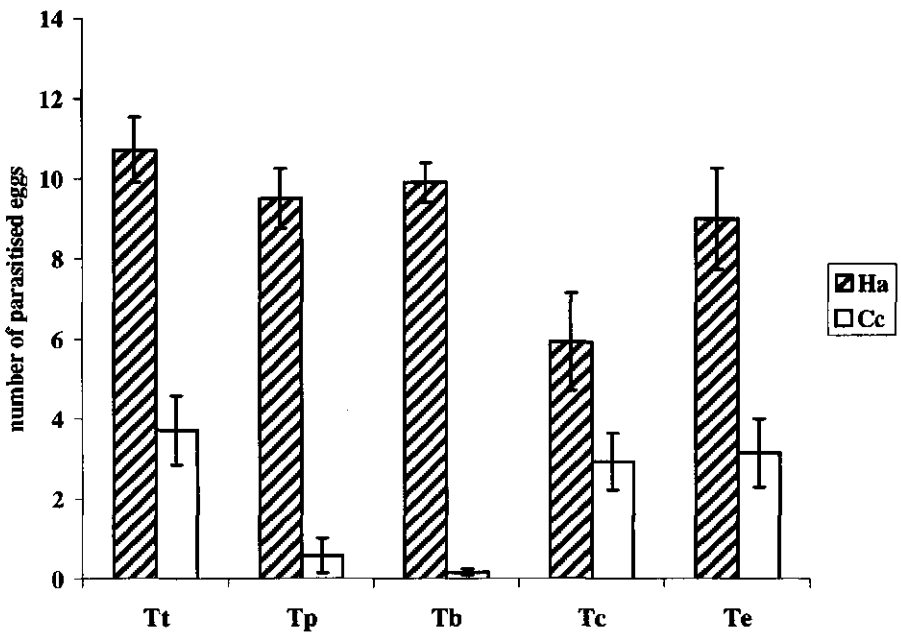


Fig. 2. Average number (mean ± standard error) of parasitised *Helicoverpa armigera* (=Ha) and *Chrysoperla carnea* (=Cc) eggs per female, for each *Trichogramma* line (Tt = *T. turkestanica*, Tp= *T. pintoi* Tb = *T. bourarachae*, Tc= *T. cordubensis* and Te= *T. evanescens*). Females that did not parasitise are included. For sample sizes see Table 1.

Discussion

The laboratory experiment clearly demonstrated that *Trichogramma* species differed in the fraction *H. armigera*/*C. carnea* eggs that they parasitised. *Trichogramma pintoi* and *T. bourarachae* parasitised the lowest and *T. cordubensis* the highest proportion of lacewing eggs. *Trichogramma turkestanica* and *T. evanescens* were, in this respect, intermediate species. Under the conditions studied, all parasitoid species were able to parasitise both host species and all of them parasitised a higher number of *H. armigera* than of *C. carnea* eggs.

Based on this study, *T. pintoi* and *T. bourarachae* appear to be the most promising candidates for the control of *H. armigera* in agroecosystems where Chrysopidae are present. Lines Tp and Tb parasitised not only a smaller proportion of *C. carnea*, but a much lower absolute number as well (Fig. 2), and scored well in number of *H. armigera* parasitised eggs. The ranking order of the parasitoid species by numbers of offspring produced is consistent with a previous study where four *Trichogramma* species (the same as in the present study, except for *T. evanescens*) were given *H. armigera* as single host (Silva & Stouthamer 1998).

The significant positive correlation between "proportion of contacts with *H. armigera* eggs" and "proportion of *H. armigera* eggs parasitised" suggests that the differences found between *Trichogramma* species in the proportion of *H. armigera* eggs parasitised are due to differences in host acceptance and not in host suitability. Variability in host acceptance in *Trichogramma* spp. has been previously reported (*e.g.* van Dijken *et al.* 1986, Pak *et al.* 1990, Wäckers *et al.* 1987).

Several host selection studies on *Trichogramma* have demonstrated distinct preferences based on host differences in size, age, surface odour, colour and contents (de Jong & Pak 1984, van Dijken *et al.* 1986, Pak *et al.* 1986, Pak 1988), but the reason for differences in host preference between *Trichogramma* species and lines is unknown. Differences in host preference could be the process that allows sympatric *Trichogramma* species to coexist. On the other hand, host preference could have changed in the course of evolution in such a way that closely related species exhibit similar preferences. In this study, the species that showed similar proportions of parasitised hosts are also phylogenetically close (Pintureau 1987). We must, however, point out that, in our study, lines belonging to phylogenetically distant species differed as well in origin (see material and methods).

If host preference is genetically determined and intraspecific variability is adequate, genetic selection of the most specific *Trichogramma* lines could be performed in laboratory.

Host preference may also depend on larval experience (Bjorksten & Hoffmann 1995) and (more strongly) on adult learning (Kaiser *et al.* 1989b, Bjorksten & Hoffmann 1995).

Unfortunately, past studies on host selection and results of parasitism in the field are often difficult to evaluate due to the easy misinterpretation of *Trichogramma* species identities and lack of voucher specimens. The taxonomy of this genus is still in development (Pinto & Stouthamer 1994, Pinto 1998).

Choice tests cannot reproduce the field situation where, *e.g.* the hosts are found in other spatial patterns and densities (Bjorksten & Hoffmann 1995). But host-choice experiments in the laboratory can still be useful during a pre-screening evaluation procedure of candidates for biological control.

It would be very interesting to know which *Trichogramma* species parasitise *C. carnea* in the field and in which proportions. In field or semi-field conditions, the (single or in small groups) stalked eggs of *C. carnea* are supposedly more difficult to reach than the (single) eggs of *H. armigera*, which are deposited directly on the leaves or stems. In the laboratory, we have observed that *Trichogramma* females walk up the stalks of *C. carnea* eggs and reach the eggs, which they eventually parasitise.

Although the criticisms directed to biological control are mainly directed to classical biological control, augmentative releases could also affect the equilibrium of already existing populations of beneficials. Larvae of *C. carnea* also prey on Lepidoptera eggs parasitised by *Trichogramma* (Reed 1965, Al Rouechdi & Voegelé 1981). Other species present in the field may interact with the pest and the beneficials, so a more holistic approach (Waage 1990) is recommended for a better understanding of the population dynamics of the species we are interested in. Furthermore, biological control should be harmoniously integrated in a total system approach for crop protection (Lewis *et al.* 1997).

The fact that parasitoids and predators of pests can be each other natural enemies should not demotivate one with the practice of biological control. Instead, it should be an incentive to choose carefully from the still many species available the one(s) to release according to the specific characteristics of the system one is dealing with.

Acknowledgements

We are thankful to Francisca Figo, Elsa Policia, Sherif Hassan, Grazia Tommasini, Peter Meisner, Roel Potting, Marielle Witgens, Leo Koopman and Koppert B.V., for providing hosts and logistics; to Rieta Gols, Hanneke van Heest, Frenk van Kan, Marnix van Meer and Max Rietkerk for helping with the preparation of the experimental set-up; to Gerrit

Gort, for the statistical analyses. We acknowledge Luis Neto and Bernard Pintureau for the identification of *Trichogramma* lines. Carola Meierrose, Guy Boivin and Sherif Hassan are thanked for discussions. Our thanks to Frans van Alebeek, Joop van Lenteren and the Entomology PhD group for a review of earlier versions of the manuscript. The work of I.S. was financially supported by Junta Nacional de Investigação Científica e Tecnológica (JNICT, Portugal; BD 2107 92-IE) and by Fundação Calouste Gulbenkian (Portugal).

Chapter 8

General Discussion

Introduction

The main aim of the research described in this thesis was to investigate criteria and methods for identification and selection of *Trichogramma* species/strains for biological control. The following questions played a central role: 1. How can we identify *Trichogramma* effectively? 2. Can laboratory tests reasonably predict parasitism and dispersal rates of *Trichogramma* in the greenhouse? 3. Which *Trichogramma* are better for biological control, thelytokous or arrhenotokous lines? 4. Do *Trichogramma* species differ in the parasitism of target and non-target hosts? Next, each subject is discussed in a general context and topics for future research are suggested.

Discussion

Distribution and identification of *Trichogramma*

Trichogramma is one of the most ubiquitous genera of Chalcidoidea in the world. Individuals belonging to this genus can be found from below the sea level to places 3000 high and from cold to tropical climates. The distribution breadth of each species is still largely unknown (Pinto 1998). In Table 1 the known distribution of the *Trichogramma* species I studied is given. The distribution of *T. evanescens* may be in fact more restricted, since misidentifications are frequent for this species. On the contrary, for most of the other species the distribution shown is probably incomplete. In many cases the absence of record of *Trichogramma* species for a certain area is due to the fact that no search was performed there. In areas where *Trichogramma* surveys are performed, individuals of several species are frequently found (Pinto 1998). From the five Portuguese species I studied, four were recorded in a four ha tomato field within a six weeks period. Investments should be made in more field surveys and identification of field material, both in agricultural crops and in natural habitats. In that way the native species and their distribution would be known and better choices could be made in the selection of species for biological control. In particular, identification work on *T. evanescens* should be a priority, since this species is reported as being used in biological control (Hassan 1998) but many individuals have been misidentified as belonging to this species (Pinto 1998).

Molecular methods as the ones described in Chapter 2 will be of great importance for the future of *Trichogramma* identification and hence to biological control by means of these wasps: to select them, to check for contamination in the laboratory cultures and to assess parasitism and dispersal. These methods have the advantage of being easily adapted when

new species are recorded for a certain region. For instance, additional or alternative restriction enzymes can be used if needed. The same basic methods can also be applied to other groups of species (Pintureau 1993a, Stouthamer *et al.* in press). Moreover, these methods may complement classical taxonomic methods, such as morphology and crossing compatibility determinations in the characterisation and identification of new species.

Table 1. Distribution of the *Trichogramma* species used in the research described in this thesis.

Trichogramma species	Distribution	References
T. bourarachae	Portugal[1], Morocco[2]	1. Silva & Stouthamer 1997 2. Mimouni 1992
T. cordubensis	Portugal[1] (including Azores[2] and Madeira[3]), Spain[4], Algeria[5], Morocco[6]	1. Silva & Stouthamer 1997 2. Pintureau *et al.* 1991b 3. P. Garcia (pers. com.) 4. Vargas & Cabello 1985 5. Pintureau 1987 6. Bourarach in Pintureau 1987
T. evanescens	Bulgaria[1], ex-Czechoslovakia[1],Denmark[1], France[1], Germany[1], Italy[1], Poland[1], Portugal[1](including Madeira[2]), Spain[1], The Netherlands[1], Romania[1], Turkey[1], United Kingdom[1], ex-URSS [1](Russia, Ukraine, Moldavia, etc), Egypt[1], Iran[1], Israel[1], Philippines[3], China[1], Japan[1]	1. Reviewed by Pintureau 1987 2. P. Garcia (pers. com.) 3. introduced species (Tran *et al.* 1988)
T. pintoi	Bulgaria[1], Germany[1], Greece[1], Poland[1], Portugal[2], Spain[1], ex-URSS[1] (Russia, Ukraine, Moldavia, etc), Israel[1], Tunisia[1], China[1], North America[3]	1. Reviewed by Pintureau 1987 3. Pinto 1998
T. turkestanica	Portugal[1], Uzbekistan[2]	1. Neto & Pintureau 1995 2. Pintureau 1987
T. oleae	France[1], ex-Yugoslavia[1], Tunisia[2]	1. Pintureau 1987 2. Voegelé & Pointel 1979
T. deion	Western North America	Pinto *et al.*1986, Pinto 1998
T. kaykai	Western North America	Pinto *et al.* 1997, Pinto 1998

DNA methods will also greatly contribute to *Trichogramma* phylogenetic reconstruction. It will be very interesting to know whether certain traits, *e.g.* host range or habitat requirements, are related to phylogenetic distance. Other molecular methods, at the population level, *e.g.* microsatellites, are now being investigated to solve ecological and population biology questions (Stouthamer, pers. com.).

Sex pheromones

In addition to DNA and enzymes, other molecules like pheromones may prove to be useful for *Trichogramma* identification. Virgin females of *T. turkestanica* were found to produce two compounds, referred to as A and B: a C17 hydrocarbon and a closely related structure containing a primary alcohol group, respectively (Chapter 3). If virgin females of other species produce other compounds, or A and B but in different proportions, these could be used as a chemotaxonomic tool in *Trichogramma*. The fact that these compounds could not be found in groups of presumably mated males and females suggests a possible function as sex pheromone components.

We do not know how relevant for mating *Trichogramma* sex pheromones are in the field. Sibmating levels are high in these wasps (Kazmer & Luck 1990, 1995). Therefore, one could think that sex pheromones do not play an important role in mate finding in *Trichogramma*. However, the evidence for the existence of such pheromones indicates that they contribute to the wasps' fitness, are multifunctional compounds or by-products.

Many questions related to these compounds remain to be answered. Some of the most important are:

a) are A and B indeed sex pheromonal components? When the identification of these compounds is completed they may be produced synthetically and tested in bioassays;
b) can sex pheromones be used as a taxonomic tool?
c) what is the mechanism that reduces the attractiveness of females after they mate? Is the production of pheromone stopped or they start to produce something else? Our results suggest that, if A and B are indeed sex pheromonal components, that the production stops in mated females;
d) are female sex pheromones multifunctional compounds?
e) can we use sex pheromones to enhance parasitism in the field? This has been previously suggested for the aphid parasitoid *Aphidius nigripes* (Hym, Braconidae) (McNeill & Brodeur 1995).

When returning to the first question mentioned in the introduction: *How can we identify* Trichogramma *effectively*?, we can conclude that:

- in addition to the classical methods of identification of *Trichogramma*, the use of molecular methods allows a reliable and quick identification of *Trichogramma* individuals of different species. An important new molecular method is the determination of the size of ITS-2 ribosomal DNA and of the size of restriction length polymorphisms of the same DNA region (Chapter 2, Sappal *et al.*, 1995, Stouthamer *et al.*, in press);
- the specificity of mating attempts is not adequate as a general taxonomic tool for *Trichogramma*. Sex pheromonal compounds may prove to be useful as a taxonomic tool in *Trichogramma* (Chapter 3).

Quality of parasitoids in the laboratory versus in field or semi-field conditions. Parasitism and dispersal

The overall quality of a biological control agent has been defined as 'the performance in its intended role after release into the field' (van Lenteren 1991). Pre-evaluation studies on potential biological control agents and quality control of mass reared insects, when done, are mainly performed in the laboratory. The relation between laboratory assessments and field or semi-field performance is still largely unknown.

Because of their minute size it is difficult to know how *Trichogramma* behave in the field. Dispersal may occur by walking, flying – though upwind dispersal is usually impossible, Keller *et al.* 1985 –, wind transportation or foresy on moths. The latter has been largely undocumented (Smith 1996). It is thought that *Trichogramma* find hosts within the host habitat by random search (Suverkropp 1997). Therefore, it is supposed that individual *Trichogramma* females encounter few eggs or egg masses in the field (Stouthamer & Luck 1993). Our greenhouse results (Chapters 5 and 6) support this conclusion. Considering that *Ephestia kuehniella* eggs are well accepted and that they are suitable hosts for *Trichogramma* and that once an egg mass was found, parasitism occurred, then less than one egg mass was found per female. The chance of finding an egg depends on numerous factors such as weather conditions, plant surface area and plant structure. The number of eggs that a female finds depends on her longevity, but it is unknown how long females live in the field. A longevity of 5-7 days is often assumed, based on unpublished results (Smith, 1996). The fact that in our experiments no eggs were found to be parasitised in intervals between experiments supports this assumption.

Differences in dispersal and parasitism in the greenhouse between two strains of *Trichogramma cordubensis* have been reported in Chapter 5. The occurrence of genetic variability and possibility to select strains for certain traits should be taken into account when selecting *Trichogramma* for biological control (*e.g.* Wajnberg 1994). However, the desired characteristics of parasitoids for an efficient rearing in the laboratory are different from the desired qualities in the field (*e.g.* Bigler 1994). Moreover, the prolonged rearing in laboratory can cause a decreased quality of the wasps (*e.g.* Noldus 1989, van Lenteren 1991, Bigler 1994). Therefore, the implementation of a quality control system is absolutely necessary. If we know the changes that can be expected, as the loss of genetic variability or the selection of undesirable characteristics, countermeasures or, preferably, preventive measures can be taken to counteract/prevent those changes. A project funded by the International Organisation of Biological Control (IOBC) and the European Union (EU) has led to guidelines for product quality control of three *Trichogramma* species: *T. brassicae*, *T. cacoeciae* and *T. dendrolimi* (van Lenteren 1998). These guidelines describe the parameters to be measured under specific conditions: sex ratio, fecundity and longevity, and parasitism of natural host. Minimum values for each measured parameter are given, so that products with inferior (laboratory) quality are not delivered. In order to summarise the quality of the *Trichogramma*, quality indices based on laboratory parameters have been proposed but their relation with field performance is mostly unknown (*e.g.* Greenberg 1991). A quality index based on emergence rate, sex ratio, ratio of deformed females, longevity, walking speed and fecundity on artificial and target hosts has been developed for *T. brassicae*. Strains with a high quality index performed better in the field than low quality index strains (Dutton *et al.* 1996). Based on results obtained from a simulation model, Suverkropp (1997) suggested that the removal of walking speed from the quality index should be considered. This is remarkable, because walking behaviour had been the only good predictor of field efficiency (Bigler *et al.* 1988). In spite of these reports for *T. brassicae*, I believe that laboratory results are still poor predictors of *Trichogramma* quality in the field, especially when parameters related to searching ability are excluded. The existing guidelines for product quality control that were mentioned above are useful in the sense that low values of the parameters measured indicate a low potential of the wasps. However, high values of those parameters are not sufficient to guarantee a good performance of the wasps in the field or greenhouse.

Many subjects should be object of further research: from factors that are relevant for selection of strains for biological control to field assessments. Some of the most important ones regarding their relevance for selection of species/strains for biological control are:

a) foraging behaviour, taking into account the wasps' genetic composition, phenotypic plasticity and physiological condition (Vet *et al.* 1990, Lewis *et al.* 1990);
b) development of quality indices that relate to field performance;
c) establishment of suitable rearing conditions;
d) determination of numbers of wasps to apply, transportation method, timing and site of release.

When a parameter related to searching ability – dispersal by walking or flying or activity – is found that relates to greenhouse or field performance, it should be incorporated in the guidelines for product quality control of *Trichogramma*. Regarding the second question mentioned in the Introduction of this chapter: *Can laboratory tests reasonably predict parasitism and dispersal rates of* Trichogramma *in the greenhouse*?, we can conclude that:

- single or multiple parameters cannot be used to predict parasitism under unknown environmental conditions but they can be used for assessing potential field or semi-field performance of the wasps (Dutton *et al.* 1996);

- good correlations have been found between fecundity on the factitious host and field performance (Dutton *et al.* 1996). Fecundity in the laboratory may also give an indication on the relative number of eggs parasitised per egg mass in the greenhouse, i.e., strains that have a higher fecundity in the laboratory also parasitise more eggs per egg mass in the greenhouse (Chapter 6);

- walking speed has been shown to correlate with parasitism rates in the field (Bigler *et al.* 1988). The laboratory chamber used to assess dispersal was considered as being promising for pre-evaluation of *Trichogramma* lines (Chapter 5). However, in Chapter 6 this compatibility of laboratory and field results was not found. Therefore, the laboratory chamber gives no general indication of relative dispersal of *Trichogramma* strains in the greenhouse. Whether this chamber can be used under certain conditions is still to be investigated;

- from the indices proposed by several authors, one was found to relate to field performance of *T. brassicae* (Dutton *et al.* 1996). However, a general index for *Trichogramma* evaluation based on laboratory assessments that has proven to be useful for greenhouse or field predictions is still lacking. Until good laboratory predictors are found, semi-field or field studies are needed to assess the ability of different strains to find and parasitise eggs.

Thelytokous or arrhenotokous *Trichogramma* for biological control?

More than 150 hymenopterous parasitoid species that are or have been used in biological control have thelytokous forms (Luck *et al.* 1993). One well-known example is the aphelinid *Encarsia formosa*, used against whiteflies in protected crops (van Lenteren 1995). In contrast with other parasitoids, most *Trichogramma* species that have thelytokous forms (Pinto & Stouthamer 1994, Stouthamer 1997) also have arrhenotokous forms, making the choice of mode of reproduction within one species possible. There are however at least three *Trichogramma* species where only thelytokous forms have been found. These three species, all reported as biological control agents, are *T. cacoeciae*, *T. oleae* and *T. cordubensis*. *Trichogramma cacoeciae* has been used against several lepidopteran species in orchards and vineyards in Europe (Hassan 1992, 1998, Li 1994). In this species thelytoky is not induced by *Wolbachia* and sexual lines are unknown. *Trichogramma oleae* has been used against *Prays oleae*, in olive trees in Tunisia (Li 1994). The use of *T. cordubensis* has been reported against *Mithimna unipuncta* in pastures in the Azores (Hassan 1992), but it was performed at an experimental level only and it is presently not being released in these Atlantic islands (P. Garcia and J. Tavares, pers. com.). A thelytokous form of *T. sibericum* is used against *Rhopobota naevana* (Lep., Tortricidae) in cranberries, in British Columbia (D. Henderson, pers. com.). From the other species that have thelytokous forms and that are or have been used in biological control - *T. pretiosum, T. evanescens* (as *rhenana*), *T. chilonis, T. pintoi, T. embryophagum* and *T. platneri* - I do not know whether only arrhenotokous or also thelytokous forms are used in biological control.

Understanding the effect of *Wolbachia* on its host may help us to choose the most appropriate thelytokous lines of *Trichogramma* for use in biological control. Results of Chapter 4 strongly suggest that negative effects of *Wolbachia* on *Trichogramma* fitness are less pronounced for fixed than for mixed parasitoid populations. However, in Chapter 6 this hypothesis is not supported from the results of *T. cordubensis* fecundity. Although the exact reason for this discrepancy could not be found, we think that host species and host size could have played a role in it (see discussion Chapter 6). If most lepidopteran hosts in the co-evolution of *Trichogramma* and *Wolbachia* were relatively big, it is possible that co-evolution between *Trichogramma* and *Wolbachia* led to a less negative effect of *Wolbachia* on females from fixed populations that develop in large hosts. In that case the results of Chapter 6 in which small hosts were used, would be relevant for biological control purposes but not for evolutionary interpretations.

In addition to more studies on the performance of thelytokous *versus* arrhenotokous lines of other *Trichogramma* species under semi-field and field conditions, important topics for further research are:

a) the effect of *Wolbachia* on its host: is it *Wolbachia*-specific or a consequence of *Wolbachia* type-host interaction? Research to clarify this point is being performed (Stouthamer, pers. com). If the interaction *Trichogramma* genome/*Wolbachia* plays an important role in the *Trichogramma* fitness, a large negative effect of *Wolbachia* could occur in new host-*Wolbachia* combinations. In that case there would be no use of making thelytokous lines by microinjection –which is already technically possible (Grenier *et al.* 1998) - or one would have to wait for benevolence to evolve before using the microinjected *Trichogramma* in biological control;

b) the effect of *Wolbachia* density on *Trichogramma* fitness, the effect of lepidopteran host species and size on the size and fitness of infected and uninfected *Trichogramma* females. These factors may play an important role on the fitness of *Trichogramma* females and hence on their performance as biological control agents.

Based on theoretical considerations and on laboratory results, Stouthamer (1993) discussed the possible advantages and constraints of the use of sexual and asexual *Trichogramma* in biological control. In the laboratory, arrhenotokous females had a higher fecundity than their thelytokous counterparts (Stouthamer & Luck 1993, Chapters 4 and 6). The opposite was found in one case only (Chapter 4). The first greenhouse results regarding this subject show that per released wasp the thelytokous lines outperformed their arrhenotokous counterpart, despite the higher fecundity per female of the latter (Chapter 6). This is caused by the fact that only 50-70% of the released arrhenotokous wasps were females, whereas all thelytokous wasps are females. It appears that both infected and uninfected forms are equally effective in finding hosts, since the number of egg masses parasitised per female released did not differ significantly between lines. Nevertheless, arrhenotokous females may be advantageous in some cases, namely when the target host lays egg masses that are totally parasitised when found by an arrhenotokous female but left partly intact by thelytokous females. Furthermore, arrhenotokous lines could be advantageous for inoculative biological control because recombination allows a rapid adaptation to a changing environment. However, the release of a higher number of thelytokous females could compensate the handicap of a lower female fecundity and, if thelytokous forms can cross with the sexual forms in the field, they can become adapted as well (Stouthamer 1993). The answer to question 3 of the Introduction, *Which* Trichogramma *are better for biological control, thelytokous or arrhenotokous lines*?, is that

thelytokous lines seem to be better candidates for biological control than their arrhenotokous counterparts and more *Trichogramma* thelytokous lines should be tested as biological control candidates.

Risks and prospects of biological control with *Trichogramma* spp.

Biological control is one of the most environmental friendly methods used in plant protection, but it has risks. *Trichogramma* females do attack other insect species in addition to the pest insects to be controlled. Some of the non-target organisms attacked might be endangered species or natural enemies of pests. Moreover, there is a risk of replacement of endemic *Trichogramma* species by exotic ones. Although there is no knowledge of such kind of problems caused by *Trichogramma* releases, the attack of non-target organisms should be a criterion used in the selection of *Trichogramma* for biological control.

All five Portuguese *Trichogramma* species I studied parasitised both the pest insect *Helicoverpa armigera* and the beneficial insect *Chrysoperla carnea* in the laboratory (Chapter 7). Differences in the ratio of the two hosts parasitised were found between *Trichogramma* species. So, we can affirmatively answer the last question of the introduction: *Do* Trichogramma *species differ in the parasitism of target and non-target hosts*? *Trichogramma pintoi* and *T. bourarachae* parasitised the lowest proportion and absolute numbers of the beneficial insect and are thus the most promising species for biological control of Lepidoptera in fields where Chrysopidae form an important part of the natural enemy assemblage. Field surveys should be performed to assess ecological host ranges of *Trichogramma* species. Physiological host ranges can be determined in the laboratory, as well as intraspecific variability and the effect of laboratory rearing and host age on *Trichogramma*'s host acceptance and suitability. The study of ecosystems where potential pests are naturally controlled and never reach the pest status should enable us to better understand the interaction between the different components involved. That knowledge could then be applied to situations where those potential pest species become serious threats to agricultural production.

In this thesis criteria and methods for identification and selection of *Trichogramma* for biological control of Lepidoptera have been analysed. The choice of species/strains for biological control does not imply that only one strain must be chosen for release. The simultaneous release of several *Trichogramma* species should also been considered and has been previously tried with success (Hassan *et al.* 1988). Because the final goal is plant protection, this discussion would not be completed without the reference to the integration of several control methods. This has been suggested by many authours (*e.g.* Stinner 1977,

Smith 1996). *Trichogramma* spp. can be used together with other biological control organisms, including microbes (Oatman *et al.* 1983, Navarro 1988, Wu 1986, Garcia-Roa 1990, Haji 1997), parasitoids (Navarro 1988, Wu *et al.* 1988, Brower & Press 1990,) and predators (Araújo 1990) and together with other control measures (Nagy 1973, Varadhajaran 1976, Trumble & Alvarado-Rodriguez 1993, Haji 1997). The interaction between the different organisms released and the ones already present in the treated area must be taken into account during the selection and release of biological control agents. In particular, studies on the impact of potential biological control agents on non-target organisms should be performed prior to the release of the natural enemies. Selection of the proper organisms to release is only one of the requirements for a successful biological control program. Other requirements are an efficient mass rearing system, appropriate methods for storage, distribution and field release of the parasitoids and reliable methods of assessing *Trichogramma* efficacy (for a recent review see Smith 1996).

Until now I have mostly regarded the use of *Trichogramma* as a therapeutic method of plant protection. Recently, new long term and preventive approaches have been proposed. A new approach of pest management, which should take the place of Integrated Pest Management (IPM), is Ecologically Based Pest Management (EBPM, National Research Council 1996). The main goals of this approach are: safety, profitability and durability. In this approach the augmentation of natural processes is supplemented by the use of biological control organisms and products, narrow spectrum synthetic pesticides and pest resistant plants. An even broader new concept in plant protection is the total system approach (TSM) (Lewis *et al.* 1997). This approach aims to manage ecosystems in such a way that the effect of natural enemies and agricultural methods prevent the development of pests. The TSM approach has the advantage of being a long term one, anticipating problems before they do occur. Within this approach, biological control releases would ideally be needed only as a correction, not as a first tactic. Hopefully, plant protection will soon be approached in such a way that natural enemies are conserved, making possible the implementation of TSM in the near future.

Acknowledgements

I thank Richard Stouthamer and Joop van Lenteren for a review of the manuscript.

References

Aeschlimann, J.P. 1990. Simultaneous ocurrence of thelytoky and bisexuality in Hymenopteran species, and its implications for the biological control of pests. *Entomophaga*, 35: 3-5.

Al Rouechdi, K. & J. Voegelé 1981. Prédation des trichogrammes par les chrysopides. *Agronomie*, 1: 187-190.

Araújo, J. 1990. Integrated protection of agricultural crops and forest trees in southern Portugal. *Investigação Agrária Cabo Verde*, 3: 28-34.

Ashley, T.R., J.C. Allen & D. Gonzalez 1974. Successful parasitization of *Heliothis zea* and *Trichoplusia ni* eggs by *Trichogramma*. *Environ. Entomol.*, 3: 319-322.

Assem, J. van den 1996. Mating behaviour. *In*: M. Jervis & N. Kidd (eds.) *Insect Natural Enemies. Practical approaches to their study and evaluation*, Chapman & Hall, London: 163-292.

Assem, J. van den & G.D.E. Povel 1973. Courtship behaviour of some *Muscidifurax* species: a possible example of a recently evolved ethological isolating mechanism. *Neth. J. Zool.*, 23: 465-487.

Assem, J. van den & J.H. Werren 1994. A comparison of the courtship and mating behaviour of three species of *Nasonia* (Hymenoptera, Pteromalidae). *J. Insect Behav.*, 7: 53-66.

Bergeijk, K.E. van, F. Bigler, N.K. Kaashoek & G.A. Pak 1989. Changes in host acceptance and host suitability as an effect of rearing *Trichogramma maidis* on a factitious host. *Entomol. Exp. Appl.*, 52: 229-238.

Bigler, F. (ed.) 1991. *Proceedings of the fifth workshop of the IOBC global working group Quality Control of Mass-reared Arthropods*, March 1991, Wageningen, The Netherlands, 205 pp..

Bigler, F. 1994. Quality control in *Trichogramma* production. *In*: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids*. CAB International, Wallingford, U.K., pp. 93-111.

Bigler, F., M. Bieri, A. Fritschy & K. Seidel 1988. Variation in locomotion between laboratory strains of *Trichogramma maidis* and its impact on parasitism of eggs of *Ostrinia nubilalis* in the field. *Entomol. Exp. Appl.*, 49: 283-290.

Bigler, F., B.P. Suverkropp & F. Cerutti 1997. Host searching by *Trichogramma* and its implications for quality control and release techniques. *In:* D.A. Andow, D.W. Ragsdale & R.F. Nyvall (eds.), *Ecological Interactions and Biological Control* Westview Press, Oxford, pp. 241-253.

Bjorksten, T.A. & A.A. Hoffmann 1995. Effects of pre-adult and adult experience on host-acceptance in choice and non-choice tests in two strains of *Trichogramma*. *Entomol. Exp. Appl.*, 76: 49-58.

Blum, M.S. 1996. Semiochemical parsimony in the arthropoda. *Annu. Rev. Entomol.*, 41: 353-374.

Boldt, P.E. 1974. Temperature, humidity and host: effect on rate of search of *Trichogramma evanescens* and *T. minutum* auctt. (not Riley, 1871), *Ann. Entomol. Soc. Am.*, 67: 706-708.

Bowles, J. & D. P. McManus 1993. Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method. *Mol. Biochem. Parasitol.*, 57: 231-240.

Braig, H.R., W. Zhou, S. Dobson & S.L. O'Neill 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol.*, 180: 2373-2378.

Brower, J.H. & J.W. Press 1990. Interaction of *Bracon hebetor* (Hym., Braconidae) and *Trichogramma pretiosum* (Hym., Trichogrammatidae) in supressing stored-product moth populations in small inshell peanut storages. *J. Econ. Entomol.*, 83: 1096-1101.

Buckler IV, E.S., A. Ippolito & T.P. Holtsford 1997. The evolution of ribosomal DNA: divergent paralogues and phylogenetic implications. *Genetics*, 145: 821-832.

Bull, J.J., I.J. Molineux & W.R. Rice 1991. Selection of benevolence in a host-parasite system. *Evolution*, 45: 875-882.

Cabot, E.L. 1995. *The Eyeball Sequence Editor (ESEE)*, version 3.0s. Dept. Biology, Univ. Rochester, Rochester, NY, 14627.

Campbell, B.C., J.D. Steffen-Campbell & J.H. Werren 1993. Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer (ITS2) and 28S rDNA sequences. *Insect Mol. Biol.*, 2: 225-237.

Carbone, I. & L. Kohn 1993. Ribosomal DNA sequence divergence within transcribed spacer 1 of the Sclerotiniaceae. *Mycologia*, 85: 15-427.

Cerutti, F. & F. Bigler 1995. Quality assessment of *Trichogramma brassicae* in the laboratory. *Entomol. Exp. Appl.*, 75: 19-26.

Collett, D. 1991. *Modelling binary data*. Chapman & Hall, London, UK.

Cosmides, L.M. & J. Tooby 1981. Cytoplasmic inheritance and intragenomic conflict. *J. Theor. Biol.* 89,: 83-129.

Crawley, M.J. 1993. *GLIM for ecologists*. Blackwell, Oxford.

Curl, G.D. & P.P. Burbutis 1978. Host preference studies with *Trichogramma nubilale*. *Environ. Entomol.*, 7: 541-553.

Dijken, M.J. van, M. Kole, J.C. van Lenteren & A.M. Brand 1986. Host preference studies with *Trichogramma evanescens* Westwood (Hym.,Trichogrammatidae) for *Mamestra brassicae*, *Pieris brassicae* and *Pieris rapae*. *J. Appl. Ent.*, 101: 64-85.

Dutton, A., F. Cerutti & F. Bigler 1996. Quality and environmental factors affecting *Trichogramma brassicae* efficiency under field conditions. *Entomol. Exp. Appl.*, 81: 71-79.

Edwards, O.R. & M.A. Hoy 1993. Polymorphism in two parasitoids detected using random amplified polymorfic DNA polymerase chain reaction. *Biol. Control*, 3: 243-257.

Eller, F.J., R.J. Bartelt, R.L. Jones & H.M. Kulman 1984. Ethyl (Z)-9-hexadecenoate, a sex pheromone of *Syndipnus rubiginosus*, a sawfly parasitoid. *J. Chem. Ecol.*, 10: 291-300.

Ettre, L.S. 1964. The Kovats retention index system. *Analytical Chemistry*, 36: 31A-35A.

Ewald, P.W. 1987. Transmission modes and evolution of the parasitism-mutualism continuum. *Ann. N.Y. Acad. Sci.*, 503: 295-306.

Forsse, E., S.M. Smith & R.S. Bourchier 1992. Flight initiation in the egg parasitoid *Trichogramma minutum*: effect of ambient temperature, mates, food, and host eggs. *Entomol. Exp. Appl.*, 62: 147-154.

Franz, J.M. & J. Voegele 1974. Les Trichogrammes en vergers. Les auxiliaires en verger de pommiers. *Bull. OILB/SROP*, 3: 201-210.

Frei, G. & F. Bigler 1994. Fecundity and host acceptance tests for quality control of *Trichogramma brassicae*. *In*: Nicoli, G., M. Benuzzi & N.C. Leppla (eds.), *Proceedings of the seventh workshop of the IOBC global working group Quality Control of Mass-reared Arthropods*, September 13-16, 1993, Rimini, Italy, 81-95.

Garcia-Roa, F. 1990. Effectiveness of *Trichogramma* spp. in biological control programs in the Cauca Valley, Colombia. *Les Colloques de l'INRA*, 56: 197-199.

Garg, I.D. & C.L. Mandahar 1972. Note on the measurement of leaf area of tomato (*Lycopersicon esculentum* Mill.) plants from linear parameters. *Indian Journal of Agricultural Science*, 42: 958-959.

Godfray, H.C. 1994. *Parasitoids. Behavioral and Evolutionary Ecology*. Princeton University Press, Princeton, New Jersey, 473 pp.

Gonzalez, J.M., R.W. Matthews & J.R. Matthews 1985. A sex pheromone in males of *Melittobia australica* and *M. femorata* (Hym., Eulophidae). *Fla. Entomol.*, 68: 279-286.

Gordh, G. & P. DeBach 1978. Courtship behavior in the *Aphytis lingnanensis* group, its potential usefulness in taxonomy, and a review of sexual behavior in the parasitic hymenoptera (Chalcidoidea: Aphelinidae). *Hilgardia*, 46 (2): 37-75.

Greenberg, S.M. 1991. Evaluation techniques for *Trichogramma* quality. *In*: F. Bigler (ed.), *Proceedings 5th IOBC Workshop on Quality Control of Mass Reared Arthropods*, 25-28 March 1991, Wageningen, The Netherlands, pp. 138-145.

Grenier S., B. Pintureau, A. Heddi, F. Lassablière, C. Jager, C. Louis & C. Khatchadourian 1998. Successful horizontal transfer of *Wolbachia* symbionts between *Trichogramma* wasps. *Proc. R. Soc. Lond. B*, 265: 1441-1445.

Haji, F.N.P. 1997. Controle biológico da traça do tomateiro com *Trichogramma* no Nordeste do Brasil. *In*: J.R.P. Parra & R.A. Zucchi (eds.), Trichogramma *e o Controlo biológico Aplicado*, FEALQ, Piracicaba, pp. 319-324.

Hase, A. 1925. Beiträge zur Lebensgeschichte der Schlupfwespe *Trichogramma evanescens* Westwood. *Arbeiten aus der Biologischen Reichsanstalt für Land- und Forstwirtschaft*, 14: 171-224.

Hassan, S.A. 1989. Selection of suitable *Trichogramma* strains to control the codling moth *Cydia pomonella* and the two summer fruit tortrix moths *Adoxophyes orana, Pandemis heparana* (Lep.: Torticidae). *Entomophaga*, 34: 19-27.

Hassan, S.A. (ed.) 1992. Trichogramma *News*. IOBC, 6: 46 pp.

Hassan, S.A. 1994. Strategies to select *Trichogramma* species for use in biological control. *In*: E. Wajnberg & S. A. Hassan (eds), *Biological Control with Egg Parasitoids*. CAB International, Wallingford, U.K., pp. 55-72.

Hassan, S.A. (ed.) 1998. *Egg Parasitoid News*, IOBC, 10: 53 pp.

Hassan, S.A. & M.F. Guo 1991. Selection of effective strains of egg parasites of the genus *Trichogramma* (Hym., Trichogrammatidae) to control the European corn borer *Ostrinia nubilalis* Hb. (Lep., Pyralidae). *J. Appl. Ent.*, 111: 335-341.

Hassan, S.A., E. Kohler & W.M. Rost 1988. Mass production and utilization of *Trichogramma*: 10. Control of the codling moth *Cydia pomonella* and the summer fruit tortrix moth *Adoxophyes orana* (Lep.: Tortricidae). *Entomophaga*, 33: 413-420.

Hillis, D.M. & M.T. Dixon 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.*, 66: 411-453.

Hokkanen, H.M.T. & J.M. Lynch (eds.) 1995. *Biological Control: Benefits and Risks*. Cambridge University Press, Cambridge.

Horjus, M. & R. Stouthamer 1995. Does infection with thelytoky-causing *Wolbachia* in the pre-adult and adult life stages influence the adult fecundity of *Trichogramma deion* and *Muscidifurax uniraptor*. *Proc. Exper. & Appl. Entomol. N.E.V.* 6: 35-40.

Howarth, F.G. 1991. Environmental impacts of classical biological control. *Annu. Rev. Entomol.*, 36: 485-509.

Hoy, M.A. 1994. *Insect molecular genetics. An introduction to principles and applications*. Academic Press, Inc. San Diego, California, 546 pp.

Hsiao, C., N.J. Chatterton, K.H. Asay & K.B. Jensen 1994. Phylogenetic relationships of 10 grass species: an assessment of phylogenetic utility of the internal transcribed spacer region in nuclear ribosomal DNA in monocots. *Genome*, 37: 112-120.

Hurst, L. 1993. The incidences, mechanisms and evolution of cytoplasmic sex ratio distorters in animals. *Biol. Rev.*, 68: 121-193.

Isidoro, N. & F. Bin 1995. Male antennal gland of *Amitus spiniferus* (Brethes) (Hymenoptera: Platygastridae), likely involved in courtship behavior. *Int. J. Insect Morphol. & Embryolo.*, 24: 365-373.

Johnson, N.F. & F. Bin 1982. Species of *Telenomus* (Hymenoptera, Scelionidae) parasitoids of stalked eggs of Neuroptera (Chrysopidae and Berothidae). *Redia*, 65: 189-206.

Jong, E.J. de & G.A. Pak 1984. Factors determining differential host-egg recognition of two host species by different *Trichogramma* spp. *Mededelingen van de Faculteit Landbouwwetenschappen Rijksuniversiteit Gent*, 49: 815-825.

Kainoh, Y. & Y. Oishi 1993. Source of sex pheromone of the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera, Braconidae). *J. Chem. Ecol.*, 19: 963-969.

Kaiser, L., M.H. Pham-Delegue, E. Bakchine & C. Masson 1989a. Olfactory responses of *Trichogramma maidis*: effects of chemical cues and behavioural plasticity. *J. Insect Behav.*, 2: 701-712.

Kaiser, L., M.H. Pham-Delegue & C. Masson 1989b. Behavioural study of plasticity in host preferences of *Trichogramma maidis*. *Physiol. Entomol.*, 14: 53-60.

Kan, F.J.P.M. van, I.M.M.S. Silva, M. Schilthuizen, J.D. Pinto & R. Stouthamer 1996. Use of DNA-based methods for the identification of minute wasps of the genus *Trichogramma*. *Proc. Exper. & Appl. Entomol.*, N.E.V., 7: 233-238.

Kan, F.J.P.M. van, J. Honda, J.D. Pinto & R. Stouthamer 1997. Molecular based techniques for *Trichogramma* identification. *Proc. Exper. & Appl. Entomol.*, N.E.V., 8: 59-62.

Kapadia, M.N. & S.N. Puri 1991. Parasitization of *Chrysoperla carnea* (Stephens) in cotton field under Parbhani conditions. *Journal of Maharashtra Agricultural Universities*, 16: 453-454.

Kazmer, D.J. & R.F. Luck 1990. The genetic-mating structure of natural and agricultural populations of *Trichogramma*. *Les Colloques de l'INRA*, 56: 161-165.

Kazmer, D.J. & R.F. Luck 1995. Field tests of the size-fitness hypothesis in the egg parasitoid *Trichogramma pretiosum*. *Ecology*, 76: 412-425.

Keller, M.A., W.J. Lewis & R.E. Stinner 1985. Biological and practical significance of movement by *Trichogramma* species: a review. *Southwest. Entomol.*, 8: 138-155.

Khasimuddin, S. & P. DeBach 1975. Mating behavior and evidence of a male sex pheromone in species of the genus *Aphytis*. *Ann. Entomol. Soc. Am.*, 68: 893-896.

Kimani, S.W. & W.A. Overholt 1995. Biosystematics of the *Cotesia flavipes* complex (Hymenoptera: Braconidae): interspecific hybridization, sex pheromone and mating behaviour studies. *Bull. Entomol. Res.*, 85: 379-386.

Kot, J. 1968. Factors affecting the efficiency of *Trichogramma* introduction. *In*: *Proceedings of the XIII International Congress of Entomology* 2, Moscow, pp. 155-158.

Kot, J. 1979. Analysis of factors affecting the phytophage reduction by *Trichogramma* Westw. species. *Polish Ecological Studies*, 5: 5-59.

Kuperus, W.R. & W. Chapco 1994. Usefulness of internal transcribed spacer regions of ribosomal DNA in Melanopline (Orthoptera, Acrididae) systematics. *Ann. Entomol. Soc. Am.*, 87: 751-754.

Laing, J. E. & F. Bigler 1991. Quality control of mass produced *Trichogramma* species. *In*: F. Bigler (ed.), *Proceedings 5th IOBC Workshop on Quality Control of Mass Reared Arthropods*, 25-28 March 1991, Wageningen, The Netherlands, pp. 111-118.

Landry, B.S., L. Dextraze & G. Boivin 1993. Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability assessment of minute parasitic wasp species (Hymenoptera,: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. *Genome*, 36: 580-587.

Lenteren, J.C. van 1986a. Parasitoids in the greenhouse: successes with seasonal inoculative release systems. In: J.K. Waage and D.J. Greathead (eds.), *Insect Parasitoids*. Acad. Press, London, pp. 341-374.

Lenteren, J.C. van 1986b. Evaluation, mass production, quality control and release of entomophagous insects. In: J.J. Franz (ed.) *Biological Plant and Health Protection*. Fisher Verlag, Stuttgart: 31-56.

Lenteren, J.C. van 1991. Quality control of natural enemies: hope or illusion? *In*: F. Bigler (ed.) *Proceedings of the Fifth Workshop of the IOBC Global Working Group Quality control of mass reared arthropods*, Wageningen, The Netherlands, pp. 1-14.

Lenteren, J.C. van 1995. Integrated pest management in protected crops. *In*: D. Dent (ed.). *Integrated Pest Management*, Chapman & Hall, London, 311-343.

Lenteren, J.C. van 1997. Benefits and risks of introducing exotic macro-biological control agents into Europe. *Bull. OEPP/EPPO*, 27: 15-27.

Lenteren, J.C. van 1998. *Sting. Newsletter on Biological Control in Greenhouses*, 18: 27-32

Lenteren, J.C. van, M.M. Roskam & R. Timmer 1997. Commercial mass production and pricing of organisms for biological control of pests in Europe. *Biol. Control*, 10: 143-149.

Lewis, W.J., J.W. Snow & R.L. Jones 1971. A pheromone trap for studying populations of *Cardiochiles nigriceps*, a parasite of *Heliothis virescens*. *J. Econ. Entomol.*, 64: 1417-1421.

Lewis, W.J., L.E.M. Vet, J.H. Tumlinson, J.C. van Lenteren & D.R. Papaj 1990. Variations in parasitoid foraging behavior: essential element of a sound biological control theory. *Environ. Entomol.*, 19: 1183-1193.

Lewis, W.J., J.C. van Lenteren, S.C. Phatak & J.H. Tumlinson III 1997. A total system approach to sustainable pest management. *Proc. Nat. Acad. Sci. USA*, 94: 12243-12248.

Li, L-Y. 1994. Worldwide use of *Trichogramma* for biological control on different crops: a survey. *In*: E. Wajnberg & S. A. Hassan (eds.), *Biological Control with Egg Parasitoids.* CAB International, Wallingford, U.K., pp. 37-53.

Lipsitch M., M.A. Nowak, D. Ebert & R.M. May 1995. The population dynamics of vertically and horizontally transmitted parasites. *Proc. R. Soc. Lond. B*, 260: 321-327.

Luck, R.F., R. Stouthamer & L.P. Nunney 1993. Sex determination and sex-ratio patterns in parasitic Hymenoptera. *In*: D.L. Wrensch & M.A. Ebbert (eds.), *Evolution and diversity of sex-ratio in insect and mites.* Chapman and Hall, Inc., New York, 442-476.

Ma, H-Y. 1988. Studies on long-term storage of hosts for propagating *Trichogramma. Trichogramma* and other egg parasitoids. *Les Colloques de l'INRA*, Paris, 43: 369-371.

Martin, P.B., P.D. Lindgren, G.L. Greene & E.E. Grissel 1981. The parasitoid complex of three noctuids (Lep.) in a northern Florida cropping system: seasonal occurrence, parasitization, alternate hosts and influence of host-habitat. *Entomophaga*, 26: 401-419.

McNeil, J.N. & J. Brodeur 1995. Pheromone-mediated mating in the aphid parasitoid *Aphidius nigripes* (Hymenoptera, Aphididae). *J. Chem. Ecol.*, 21: 959-972.

Meer, M.M.M. van, F.J.M.P. van Kan, J.A.J. Breeuwer & R. Stouthamer 1995. Identification of symbionts associated with parthenogenesis in *Encarsia formosa* and *Diplolepis rosae. Proc. Exper. & Appl. Entomol. N.E.V.*, 6: 81-86.

Meer, M.M.M. van, F.J.M.P van Kan & R. Stouthamer 1996. Can *Wolbachia* micro-injections induce parthenogenesis in sexual insects? *Proc. Exper. & Appl. Entomol., N.E.V.*, 7: 43-44.

Meierrose, C. 1990. *Luta biológica contra* Heliothis armigera *no ecossistema agrícola "tomate para indústria". Interacções Cultura-Fitófagos-Antagonistas.* Doctoral dissertation, Évora, Portugal.

Meierrose, C. & J. Araújo 1986. Natural egg parasitism on *Helicoverpa (Heliothis) armigera* Hbn. (Lepidoptera, Noctuidae) on tomato in south Portugal. *J. Appl. Ent.*, 101: 11-18.

Micha, S.G., J. Stammel & C. Höller 1993. 6-methyl-5-heptene-2-one, a putative sex and spacing pheromone of the aphid hyperparasitoid, *Alloxysta victrix* (Hymenoptera, Alloxystidae). *Eur. J. Entomol.*, 90: 439-442.

Miller, J.C. 1983. Ecological relationships among parasites and the practice of biological control. *Environ. Entomol.*, 12: 620-624.

Mimouni, F. 1992. Genetic variations in host infestation efficacy in two *Trichogramma* species from Morocco. *Redia*, 124: 393-400.

Monier, C. 1983. *Inventaire des Trichogrammes parasites de Scotia segetum Schiff et* Mamestra brassicae *L. (Lepidoptera, Noctuidae) dans la Basse Vallée du Rhône et contribution à l'étude des relations hotes-parasites.* Thèse pour obtenir le Grade de Docteur de 3ème cycle en Parasitologie, Pathologie et relations écophysiologiques. Académie de Montpellier, Université des Sciences et Techniques du Languedoc.

Morrison, R.K. 1988. Methods for the long term storage and utilization of eggs of *Sitotroga cerealella* (Olivier) for production of *Trichogramma pretiosum* Riley. *Trichogramma* and other egg parasitoids. *Les Colloques de l'INRA*, Paris, 43: 373-377.

Nagarkatti, S. & H. Nagaraja 1968. Biosystematic studies on *Trichogramma* species. I. Experimental hybridization between *Trichogramma australicum* Girault, *T. evanescens* Westw. and *T. minutum* Riley. *Tech. Bull. Com. Inst. Biol. Contr.*, 10: 81-96.

Nagarkatti, S. & H. Nagaraja 1971. Redescription of some known species of *Trichogramma* (Hym., Trichogrammatidae), showing the importance of the male genitalia as a diagnostic character. *Bull. Ent. Res.*, 61:13-21.

Nagarkatti, S. & H. Nagaraja 1977. Bioystematics of *Trichogramma* and *Trichogrammatoidea* species. *Annu. Rev. Entomol.*, 22: 157-176.

Nagy, B. 1973. The possible role of entomophagous insects in the genetic control of the codling moth, with special reference to *Trichogramma. Entomophaga*, 18: 185-191.

National Research Council 1996. Ecologically Based Pest Management. New solutions for a new century. National Academy Press, Washington D.C., 144 pp.

Navarro, A.M. 1988. Biological control of *Scrobipalpa* by *Trichogramma* sp. in the tomato. *Trichogramma* and other egg parasitoids. *Les Colloques de l'INRA*, Paris, 43: 453-458.

Neto, L. & B. Pintureau 1995. Taxonomic study of a population of *Trichogramma turkestanica* discovered in Southern Portugal (Hymenoptera: Trichogrammatidae). *Ann. Soc. Entomol. Fr. (NS)*, 31: 21-30.

Nicoli, G., M. Benuzzi & N.C. Leppla (eds.) 1994. *Proceedings of the seventh workshop of the IOBC global working group Quality Control of Mass-reared Arthropods*, September 13-16, 1993, Rimini, Italy, 238 pp.

Noldus, L.P.J.J. 1989. *Chemical espionage by parasitic wasps. How* Trichogramma *species exploit moth sex pheromone systems.* Doctoral dissertation, Wageningen Agricultural University, The Netherlands, 252 pp.

Noldus, L.P.J.J., J.C. Van Lenteren & W.J. Lewis 1991. How *Trichogramma* parasitoids use moth sex pheromones as kairomones: orientation behaviour in a wind tunnel. *Physiol. Entomol.*, 16: 313-327.

O'Neill, S.L., R. Giordano, A.M.E. Colbert, T.L. Karr & H.M. Robertson 1992. 16S RNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmatic incompatibility in insects. *Proc. Nat. Acad. Sci. USA*, 89: 2699-2702.

Oatman, E.R., J.A. Wyman, R.A. van Steenwyk & M.W. Johnson 1983. Integrated control of the tomato fruitworm (Lep., Noctuidae) and other lepidopterous pests on fresh-market tomatoes in southern California. *J. Econ. Entomol.*, 76: 1363-1369.

Orrego, C. & F. Agudelo-Silva 1993. Genetic variation in the parasitoid wasp *Trichogramma* (Hymenoptera, Trichogrammatidae) revealed by DNA amplification of a section of the nuclear ribosomal repeat. *Fla. Entomol.*, 76: 519-524.

Pacáková, V. & L. Feltl 1992. *Chromatographic retention indices.* Ellis Horwood, Chichester, pp.19-21.

Pak, G.A. 1988. *Selection of* Trichogramma *for inundative biological control.* Doctoral dissertation, Wageningen Agricultural University, The Netherlands, 224 pp..

Pak, G.A., H.C.E.M. Buis, I.C.C. Heck & M.L.G. Hermans 1986. Behavioral variations among strains of *Trichogramma* spp.: host-age selection. *Entomol. Exp. Appl.*, 40: 247-258.

Pak, G.A., J.W.M. Kaskens & E.J. de Jong 1990. Behavioural variation among strains of *Trichogramma* spp.:host-species selection. *Entomol. Exp. & Appl.*, 56: 91-102.

Pedata, P.A., N. Isidoro & G. Viggiani 1993. Evidence of male-sex glands on the antennae of *Encarsia asterobemisiae* Viggiani et Mazzone (Hymenoptera: Aphelinidae). *Boll. Lab. Ent. Agr. Filippo Silvestri*, 50: 271-280.

Pijls, J.W.A.M., J.J. van Steenberg & J.J.M. van Alphen 1996. Asexuality cured: the relations and differences between sexual and asexual *Apoanagyrus diversicornis. Heredity*, 76: 506-513.

Pinto, J.D. 1998. *Systematics of the North American species of* Trichogramma *Westwood (Hymenoptera, Trichogrammatidae).* Memoires of the Entomological Society of Washington 22, The Entomological Society of Washington, Washington D.C., 287 pp.

Pinto, J.D. & E.R. Oatman 1988. *Trichogramma* species in a chaparral comunity of southern California, with a description of a new species (Hym.: Trichogrammatidae). *Pan-Pacific Entomologist*, 64: 391-402.

Pinto, J. & R. Stouthamer 1994. Systematics of the Trichogrammatidae with emphasis on *Trichogramma. In*: E. Wajnberg & S.A. Hassan (eds.), *Biological Control with Egg Parasitoids*, CAB International, Wallingford, pp. 1-36.

Pinto, J.D., E.R. Oatman & G.R. Platner 1986. *Trichogramma pretiosum* and a new cryptic species occurring sympatrically in southwestern North America (Hymenoptera, Trichogrammatidae). *Ann. Entomol. Soc. Am.*, 79: 1019-1028.

Pinto, J.D., R. Stouthamer, G.R. Platner & E.R. Oatman 1991. Variation in reproductive compatibility in *Trichogramma* and its taxonomic significance (Hymenoptera, Trichogrammatidae). *Ann. Entomol. Soc. Am.*, 84: 37-46.

Pinto, J.D., D.J. Kazmer, G.R. Platner & C.A. Sassaman 1992. Taxonomy of the *Trichogramma minutum* complex (Hymenoptera: Trichogrammatidae): allozymic variation and its relationship to reproductive and geographic data. *Ann. Entomol. Soc. Am.*, 85: 413-422.

Pinto, J.D., G.R. Platner & C.A. Sassaman 1993. Electrophoretic study of two closely related species of North American *Trichogramma*, *T. pretiosum* and *T. deion* (Hymenoptera: Trichogrammatidae). *Ann. Entomol. Soc. Am.*, 86: 702-709.

Pinto, J.D., R. Stouthamer & G.R. Platner 1997. A new cryptic species of *Trichogramma* (Hymenoptera: Trichogrammatidae) from the Mojave desert of California as determined by morphological, reproductive and molecular data. *Proc. Entomol. Soc. Wash.*, 99: 238-247.

Pintureau, B. 1987. *Systématique évolutive du genre* Trichogramma *Westwood (Hymenoptera, Trichogrammatidae) en Europe* - Thèse Doctorat d'État, Univ. Paris VII, France, 311 pp.

Pintureau, B. 1990. Polymorphisme, biogéographie et spècificité parasitaire des Trichogrammes européens. *Bull. Soc. Entomol. Fr.*, 95: 17-38.

Pintureau, B. 1991. Indices d'isolement reproductif entre espèces proches de Trichogrammes (Hym.: Trichogrammatidae). *Ann. Soc. Entomol. Fr. (NS)*, 27: 379-392.

Pintureau, B. 1993a. Enzymatic analysis of the genus *Trichogramma* (Hym.: Trichogrammatidae) in Europe. *Entomophaga*, 38: 411-431.

Pintureau, B. 1993b. Morphometric analysis of the genus *Trichogramma* Westwood (Hymenoptera, Trichogrammatidae) in Europe. *Can. Entomol.*, 125: 367-378.

Pintureau, B. & M. Babault 1980. Comparaison des estérases chez 19 souches de *Trichogramma* (Hym., Trichogrammatidae) appartenant au groupe d'espèces evanescens. *Arch. Zool. Exp. Gén.* ,121: 249-260.

Pintureau, B. & M. Babault 1981. Caractérisation enzymatique de *Trichogramma evanescens* et de *T. maidis* (Hym., Trichogrammatidae); étude des hybrides. *Entomophaga*, 26: 11-22.

Pintureau, B. & M. Babault 1982. Comparaison des enzymes chez 10 souches de *Trichogramma* (Hym., Trichogrammatidae). Les Trichogrammes. *Les Colloques de l'INRA*, 9:31-44.

Pintureau, B. & F.B. Keita 1989. Nouvelles données sur les estérases des Trichogrammes (Hym., Trichogrammatidae). *Biochem. Syst. Ecol.*, 17: 603-608.

Pintureau, B. & T. Toonders 1983. Quelques resultats concernant l'etude de l'attraction des males par les femmeles vierges chez *Trichogramma maidis* (Hym., Trichogrammatidae). *Bull. Soc. Linneenne Lyon*, 3: 81-87.

Pintureau, B., A. Grenier & P. Nardon 1991a. Polymorphism of esterases in three species of *Sitophilus* (Coleoptera: Curculionidae). *J. Stored Prod. Res.*, 27: 141-151.

Pintureau, B., L. Oliveira & L. Anunciada 1991b. Contribution to the study of the egg parasitic Hymenoptera of the Azores islands. *Colloques de l'INRA*, 56: 115-118.

Pintureau, B., M.P.I. Calvin & S. Grenier 1997. Effectiveness of the second mating in a bisexual *Trichogramma* species and the first mating in a thelytokous *Trichogramma* species. *Can. Entomol.*, 129: 35-41.

Poitout, S. & R. Bues 1970. Élevage de plusieurs espèces de lépidoptères Noctuidae sur milieu artificiel riche et sur milieu artificiel simplifié. *Ann. Zool. Écol. Anim.*, 6: 434-431.

Pompanon, F. 1995. *L'activité locomotrice chez les Trichogrammes*. These pour l'obtention du diplome de doctorat, Villeurbanne, 176 pp.

Pompanon, F., B. de Schepper, Y. Mourer, P. Fouillet & M. Bouletreau 1997. Evidence for a substrate-borne sex pheromone in the parasitoid wasp *Trichogramma brassicae*. *J. Chem. Ecol.*, 23: 1349-1360.

Post, R.J., P.K. Flook & A.L. Millest 1993. Methods for the preservation of insects for DNA studies. *Biochem. Syst. Ecol.*, 21: 85-92.

Quicke, D.L.J. 1997. *Parasitic wasps*. Chapman & Hall, London, UK, 470 pp.

Rabb, R.L. & J.R. Bradley 1968. The influence of host plants on parasitism of eggs of the tobacco hornworm. *J. Econ. Entomol.*, 61: 1249-1252.

Reed, W. 1965. *Heliothis armigera* (Noctuidae) in western Tanganyka. II. Ecology and natural and chemical control. *Bull. Entomol. Res.*, 56: 127-140.

Renou, M., N. Hawlitzky, A., Berthier, C. Malosse & F. Ramiandrasoa 1989. Mise en évidence d'une activité kairomonale des oeufs de la Pyrale du maïs sur les femelles de *Trichogramma maidis*. *Entomophaga*, 34: 569-580.

Richardson, B.J., P.R. Baverstock & M. Adams 1986. *Allozyme Electrophoresis. A handbook for animal systematics and population studies*. Academic Press Australia, Sydney, 410 pp.

Ridgway, R.L. & R.K. Morrison 1985. Worldwide perspective on practical utilization of *Trichogramma* with special reference to control of *Heliothis* on cotton. *Southw. Entomol. Suppl.*, 8: 190-198.

Ridgway, R.L., E.G. King & J.L. Carrilo 1977. Augmentation of natural enemies for control of plant pests in the Western Hemisfere. *In*: R.L. Ridgway & S.B. Vinson (eds.), *Biological Control by Augmentation of Natural Enemies*. Plenum, New York, pp. 379-416.

Rigaud, T. & P. Juchault 1992. Genetic control of the vertical transmission of a cytoplasmic sex factor in *Armadillidium vulgare* Latr. Crustacea, Oniscidae. *Heredity*, 68: 47-52.

Robacker, D.C. & L.B. Hendry 1977. Neral and geranial: components of the sex pheromone of the parasitic wasp, *Itoplectis conquisitor*. *J. Chem. Ecol.*, 3: 563-577.

Rousset F., D. Bouchon, B. Pintureau, P. Juchault & M. Solignac 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. Lond. B*, 250: 91-98.

Samways, M.J. 1994. *Insect Conservation Biology*. Chapman & Hall, London.

Sappal, N.P., R.S. Jeng, M. Hubbes & F. Liu 1995. Restriction fragment length polymorphisms in polymerase chain reaction amplified ribosomal DNAs of three *Trichogramma* (Hymenoptera, Trichogrammatidae) species. *Genome*, 38: 419-425.

Schilthuizen, M. & R. Stouthamer 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proc. R. Soc. Lond. B*, 264: 361-366.

Schilthuizen, M., J. Honda & R. Stouthamer 1998. Parthenogenesis-Inducing *Wolbachia* in *Trichogramma kaykai* (Hymenoptera: Trichogrammatidae) originates from a single infection. *Ann. Entomol. Soc. Am.*, 91: 410-414.

Silva, I.M.M.S. & R. Stouthamer 1996. Can the parthenogenesis-*Wolbachia* lead to unusual courtship behaviour in *Trichogramma*? *Proc. Exper. & Appl. Entomol.*, N.E.V., 7: 27-31.

Silva, I.M.M.S. & R. Stouthamer 1997. To mate or not to mate...can sex pheromones be used as a taxonomic tool in *Trichogramma* spp.? *Proc. Exper. & Appl. Entomol. N.E.V.*, 8: 41-46.

Silva, I.M.M.S. & R. Stouthamer 1998. Indirect assessment of host acceptance and suitability of four *Trichogramma* species. *Proc. Exper. & Appl. Entomol. N.E.V*, 9: 105-110.

Simberloff, D. & P. Stiling 1996a. How risky is biological control? *Ecology*, 77: 1965-1974.

Simberloff, D. & P. Stiling 1996b. Risks of species introduced for biological control. *Biological Conservation*, 78: 185-192.

Singh, R.P. 1969. A simple technique for rendering host eggs inviable for the laboratory rearing of *Trichogramma* spp. *Indian Journal of Entomology*, 31: 83-84.

Smith, S.M. 1996. Biological control with *Trichogramma*: advances, successes, and potential of their use. *Annu. Rev. Entomol.*, 41: 375-406.

Steenburgh, W.E. van 1934. *Trichogramma minutum* Riley as a parasite of the oriental fruit moth (*Laspeyresia molesta* Busck.) in Ontario. *Canadian Journal of Research*, 10: 287-314.

Stern, V.M., E.I. Schlinger & W.R. Bowen 1965. Dispersal studies of *Trichogramma semifumatum* (Hymenoptera, Trichogrammatidae) tagged with radioactive phosphorus. *Ann. Entomol. Soc. Am.*, 58: 234-243.

Stinner, R.E. 1977. Efficacy of inundative releases. *Annu. Rev. Entomol.*, 32: 49-70.

Stouthamer, R. 1993. The use of sexual versus asexual wasps in biological control. *Entomophaga* 38, 3-6.

Stouthamer, R. 1997. *Wolbachia*-induced parthenogenesis. *In*: S.L. O'Neill, J.H. Werren & A.A. Hoffmann (eds.), *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press, New York, pp. 102-124.

Stouthamer, R. & D.J. Kazmer 1994. Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity*, 73: 317-327.

Stouthamer, R. & R.F. Luck 1993. Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. *Entomol. Exp. Appl.*, 67: 183-192.

Stouthamer, R., R.F. Luck & W.D. Hamilton 1990. Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA*, 87: 2424-2427.

Stouthamer, R., J.A.J. Breeuwer, R.F. Luck & J.H. Werren 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature*, 361: 66-68.

Stouthamer, R., J. Hu, F.J.P.M van Kan, G.R. Platner & J.D. Pinto. The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *BioControl* (in press).

Suverkropp, B. 1997. *Host-finding behaviour of* Trichogramma brassicae *in maize*. Doctoral dissertation, Wageningen Agricultural University, The Netherlands, 249 pp..

Swedenborg, P.D. & R.L. Jones 1992. Multicomponent sex pheromone in *Macrocentrus grandii* Goidanich (Hymenoptera, Braconidae). *J. Chem. Ecol.*, 18: 1901-1912.

Swedenborg, P.D., R.L. Jones, H-W. Liu & T.P. Krick 1993. (3R*, 5S*,6R*)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one, a third sex pheromone component for *Macrocentrus grandii* (Goidanich) (Hymenoptera: Braconidae) and evidence for its utility at eclosion. *J. Chem. Ecol.*, 19: 485-503.

Syvertsen, T.C., L.L. Jackson, G.J. Blomquist & S.B. Vinson 1995. Alkadienes mediating courtship in the parasitoid *Cardiochiles nigriceps* (Hymenoptera, Braconidae). *J. Chem. Ecol.*, 21: 1971-1989.

Tagawa, J. 1977. Localization and histology of the female sex pheromone-producing gland in the parasitic wasp *Apanteles glomeratus*. *J. Insect Physiol.*, 23: 49-56.

Tilborg, M. van, I.M.M.S. Silva & R. Stouthamer 1997. Crossings-compatibility of Portuguese *Trichogramma* lines (Hym., Trichogrammatidae). *Proc. Exper. & Appl. Entomol., N.E.V.*, 8: 53-58.

Tran, L.C., R. Bustamante & S.A. Hassan 1988. Release and recovery of *Trichogramma evanescens* Westw. in corn fields in the Phillipines. *Les Colloques de l'INRA*, 43: 597-607.

Trumble, J.T. & B. Alvarado-Rodriguez 1993. Development and economic evaluation of an IPM program for fresh market tomato production in Mexico. *Agriculture, Ecosystems and Environment*, 43: 267-284.

Vanlerberghe-Masutti, F. 1994. Molecular identification and phylogeny of parasitic wasp species (Hymenoptera: Trichogrammatidae) by mitochondrial DNA RFLP and RAPD markers. *Insect Mol. Biol.*, 3: 229-237.

Varadhajaran, G. 1976. Studies on the utilisation of *Trichogramma australicum* Gr. for the control of sugarcane internode borer. *Madras agric. J.*, 63: 569-570.

Vargas, P. & T. Cabello 1985. A new species of *Trichogramma* (*T. cordubensis* n.sp.) parasitoid of *Heliothis* eggs in cotton crops of Spain. *Entomophaga*, 30: 225-230.

Vereijssen, J., I. Silva, J. Honda & R. Stouthamer 1997. Development of a method to predict the biological control quality of *Trichogramma* strains. *Proc. Exper. & Appl. Entomol. N.E.V.*, 8: 145-149.

Vet, L.E.M., W.J. Lewis, D.R. Papaj & J.C. van Lenteren 1990. A variable-response model for parasitoid foraging behavior. *J. Insect Behav.*, 3: 471-490.

Vinson, S.B. 1972. Courtship behaviour and evidence for a sex pheromone in the parasitoid *Campoletis sonorensis* (Hym., Ichneumonidae). *Environ. Entomol.*, 1: 409-414.

Voegelé, J. 1988. Reflections upon the last ten years of research concerning *Trichogramma* (Hym.,Trichogrammatidae). *Colloques de l'INRA*, 43: 17-29.

Voegelé, J. & J. Pointel 1979. *Trichogramma oleae*, n.sp., espèce de *Trichogramma evanescens* Westwood [Hym., *Trichogrammatidae*]. *Ann. Soc. Ent. Fr. (N.S.)*, 15: 643-648.

Vogler, A.P. & R. De Salle 1994. Evolution and phylogenetic information content of the ITS-1 region in the tiger beetle *Cincidela dorsalis*. *Mol. Biol. Evol.*, 11: 393-405.

Waage, J. 1990. Ecological theory and the selection of biological control agents. *In*: M. Mackauer, L.E. Ehler & J. Roland (eds), *Critical Issues in Biological Control*, Intercept LTD, Andover, UK, pp. 135-157.

Waage, J.K. & S.M. Ng 1984. The reproductive strategy of a parasitic wasp I. Optimal progeny and sex allocation in *Trichogramma evanescens*. *J. Anim. Ecol.*, 53: 401-415.

Wäckers, F.L., I.J.M. de Groot, L.P.J.J. Noldus & S.A. Hassan 1987. Measuring host preference of *Trichogramma* parasites: an evaluation of direct and indirect methods. *Mededelingen van de Faculteit der Landbouwwetenschappen, Rijksuniversiteit Gent*, 52: 339-348.

Wajnberg, E. 1994. Intrapopulation genetic variation in *Trichogramma*. *In*: E. Wajnberg and S.A. Hassan (eds.), *Biological Control with Egg Parasitoids*, CAB International, Wallingford, pp. 245-271.

Werren, J.H. & S.L. O'Neill 1997. The evolution of heritable symbionts. *In*: S.L. O'Neill, J.H. Werren & A.A. Hoffmann (eds.), *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press, New York, pp. 1-41.

Wu, J-W., H-L. Fang, M-D.Yang & Y-Y. Lian 1988. Rearing technology of *Trichogramma dendrolimi* Matsumura for controlling pine caterpillar (*Dendrolimus punctatus* Walker) and effect of releasing *Trichogramma* to control insect pest on a large scale for sixteen successive years in Zhejiang Province. *Les Colloques de l'INRA*, Paris, 43: 621-628.

Wu, Q.L. 1986. Investigation on the fluctuations of dominant natural enemy populations in different cotton habitats and integrated application with biological agents to control cotton pests. *Natural enemies of insects*, 8: 29-34.

Wührer, B. & S.A. Hassan 1993. Selection of effective species/strains of *Trichogramma* (Hym., Trichogrammatidae) to control the diamondback moth *Plutella xylostella* L. (Lep., Plutellidae). *J. Appl. Entomol.*, 116: 80-89.

Yoshida, S. 1978. Behaviour of males in relation to the female sex pheromone in the parasitoid wasp *Anisopteromalus calandrae*. *Entomol. Exp. Appl.*, 23: 152-162.

Zchori-Fein E., R.T Roush & M.S. Hunter 1992. Male production by antibiotic treatment in *Encarsia formosa* (Hymenoptera: Aphelinidae), an asexual species. *Experientia* (Basel), 48: 102-105.

Zchori-Fein, E., O. Faktor, M. Zeidan, Y. Gottlieb, H. Cosnek & D. Rosen 1995. Parthenogenesis-inducing micro-organisms in *Aphytis*. *Insect Mol. Biol.*, 4: 173-178.

Zucchi, R.A. & R.C. Monteiro 1997. O género *Trichogramma* na América do Sul. *In*: J.R.P. Parra & R.A. Zucchi (eds.), Trichogramma *e o Controlo Biológico Aplicado*, FEALQ, Piracicaba, pp. 41-66.

Summary

Egg parasitoids of the genus *Trichogramma* are used as biological control agents against lepidopterous pests. From the 180 species described world-wide, only 5 have large scale application. The development of better methods to select other *Trichogramma* species/strains is necessary for a more effective use of these wasps against target pests. The main aim of this thesis is to investigate criteria and methods for identification and selection of *Trichogramma* species/strains for biological control. In **chapter 1,** I give a short review on biology, research and use of *Trichogramma*, state the research aims and present the outline of the thesis.

One of the first problems researchers are confronted with when working on *Trichogramma* is their identity. Along with the classical methods, simple and practical methodologies of species identification are needed. The use of molecular methods to characterise and identify five Portuguese *Trichogramma* species is described in **chapter 2.** These methods are: 1) electrophoresis of PCR amplified DNA internal transcribed spacer 2 (ITS-2), followed by digestions by means of restriction enzymes and 2) esterase electrophoresis. Five *Trichogramma* species occurring in Portugal, *T. bourarachae, T. cordubensis, T. evanescens, T. pintoi* and *T. turkestanica* can be easily distinguished from each other by means of dichotomous keys that were constructed based on the two methods. The two methods may be used together with other identification methods for field specimens or be used on its own for detection of possible laboratory contaminations. The specificity of mating attempts was investigated as an additional tool to quickly distinguish between *Trichogramma* species (**chapter 3**). Although mating attempts occurred more often between conspecifics, they were not always species-specific. Two compounds that might be involved in mate-specific recognition in *Trichogramma* were characterised. These two compounds are produced by virgin females and were not found in mixed groups of males and presumably mated females or in groups of males only. We concluded that the specificity of mating attempts cannot be used as a general taxonomic tool for *Trichogramma*.

From the point of view of biological control only the females are useful, because they kill the pest eggs. We know of several *Trichogramma* species in which virgin females produce female offspring (thelytoky). In most of these cases thelytoky is related to the presence of *Wolbachia*, an endosymbiotic bacterium. In some cases all female wasps in a population are infected with the bacteria (fixed population), while in other cases infected and uninfected females coexist (mixed population). The hypothesis that in fixed populations

the effect of the bacteria on *Trichogramma* female fitness is less negative than in mixed populations was tested in **chapter 4**. In these experiments two fixed *Trichogramma* lines of *T. cordubensis* and *T. oleae* and four mixed lines, one of *T. deion* and three of *T. kaykai*, were tested for several female fitness parameters. Each of these original thelytokous lines was compared with a laboratory-generated sexual (=arrhenotokous) line, which had been produced by killing the *Wolbachia* bacteria using antibiotics. Daughter production was higher for the thelytokous fixed lines than for their sexual counterparts, while the opposite was found for three of the four mixed lines studied. As expected, the negative impact of *Wolbachia* on total offspring numbers was much more severe for the mixed lines than for the fixed ones.

In addition to the infection status of the wasp populations, fixed *versus* mixed, there are other factors that can influence the reproductive success of the wasps. One of them is the females' ability to search for eggs to parasitise. The dispersal of two thelytokous lines of *T. cordubensis* was assessed in a laboratory chamber and in a greenhouse (**chapter 5**). This chamber was especially designed and tested as a tool for *Trichogramma* screening. The *Trichogramma* line which dispersed more in the chamber also dispersed more in the greenhouse, suggesting that the chamber might be a useful tool for a pre-evaluation of *Trichogramma* strains. Next, the biological control potential of *Wolbachia*-infected and of uninfected *Trichogramma* was investigated (**chapter 6**). Fecundity and dispersal of *T. cordubensis* and *T. deion* were measured in the laboratory and in the greenhouse. Sexual lines had a higher fecundity than their thelytokous counterparts. The arrhenotokous lines dispersed more in the laboratory chamber but the opposite effect or no difference was found in the greenhouse. Calculations show that by releasing hundred adult wasps of both lines, thus including arrhenotokous males in the sexual line, more eggs are parasitised by the thelytokous wasps. Therefore, in spite of their lower individual female fecundity, thelytokous lines have a better potential for biological control than their sexual counterparts.

Trichogramma females do not only parasitise lepidopteran eggs but also parasitise eggs of some beneficial insects. To determine which *Trichogramma* species is the least detrimental to the beneficial insect *Chrysoperla carnea*, the degree of successful parasitisation of *Helicoverpa armigera* (a pest insect) and *Chrysoperla carnea*, by the five Portuguese *Trichogramma* species was studied in the laboratory (**chapter 7**). The fraction *H. armigera/C. carnea* eggs parasitised differed between *Trichogramma* species. The average number of parasitised eggs per female in 24 h by *T. pintoi* and *T. bourarachae* was 10 of *H. armigera* and about 0.5 of *C. carnea*. For the other three *Trichogramma* species (*T. cordubensis*, *T. evanescens* and *T. turkestanica*) these averages varied from 6 to 11 *H.*

armigera eggs and from 3 to 4 *C. carnea* eggs. Total adult offspring production, contacts with hosts, secondary clutch size and sex ratio of each *Trichogramma* line were determined as well. The results obtained show that sympatric *Trichogramma* may parasitise target and non-target species in different proportions. If this difference corresponds to the field situation, simple laboratory tests could be performed to select not only effective biological control agents, but also species that are the least detrimental to non-target hosts. *Trichogramma bourarachae* and *T. pintoi* are the most promising candidates for the control of *H. armigera* in agroecosystems where Chrysopidae form an important part of the natural enemy assemblage. In **chapter 8**, the most important research findings are discussed in a broader context.

Samenvatting

Sluipwespen van het geslacht *Trichogramma* worden gebruikt als biologische bestrijders van Lepidoptera (vlinders). Van de wereldwijd 180 beschreven *Trichogramma*soorten worden slechts vijf soorten op grote schaal gebruikt. Voor een effectiever gebruik van deze sluipwespen tegen plagen zijn betere methoden nodig om *Trichogramma* soorten/lijnen te selecteren. Het hoofddoel van dit proefschrift is om criteria en methoden voor identificatie en selectie van *Trichogramma* soorten/lijnen voor biologische bestrijding te onderzoeken. In **hoofdstuk 1** wordt een kort overzicht van de biologie, het lopende onderzoek en het gebruik van *Trichogramma* gegeven en worden de onderzoeksdoelen en de opbouw van de proefschrift gepresenteerd. Eén van de eerste problemen die onderzoekers hebben wanneer ze met *Trichogramma* soorten werken is hun taxonomische identiteit. Naast de klassieke identificatiemethoden, zijn simpeler en praktischer methoden hard nodig. Het gebruik van moleculaire methoden om vijf Portugese *Trichogramma*soorten te karakteriseren en identificeren is beschreven in **hoofdstuk 2**. Deze methoden zijn: 1) electroforese van de geamplificeerde – met behulp van de polymerase kettingreactie (PCR) – "internal transcribed spacer 2" van ribosomaal DNA (rDNA-ITS2), gevolgd door digesties - met behulp van restrictie enzymen - en 2) esterases electroforese. De vijf *Trichogramma*soorten uit Portugal, *T. bourarachae, T. cordubensis, T. evanescens, T. pintoi* en *T. turkestanica*, kunnen makkelijk van elkaar onderscheiden worden met behulp van determinatiesleutels die gemaakt zijn op basis van de twee moleculaire methoden. De twee methoden kunnen, samen met de andere determinatiemethoden, gebruikt worden voor identificatie van veldexemplaren, of kunnen op zichzelf gebruikt worden voor detectie van mogelijke laboratoriumcontaminaties.

De poging tot paren tussen soortgenoten en niet-soortgenoten is onderzocht als extra "gereedschap" om individuen van verschillende soorten te onderscheiden (**hoofdstuk 3**). Paringspogingen vonden vaker plaats tussen individuen van dezelfde soort, maar kwamen ook voor tussen individuen van verschillende soorten. De specificiteit van paringspogingen kan dus niet worden gebruikt als een algemeen taxonomisch "gereedschap" in *Trichogramma*. Twee chemische stoffen die mogelijk een rol spelen in de herkenning van paringspartners in *Trichogramma* werden gekarakteriseerd. Deze twee stoffen worden geproduceerd door maagdelijke vrouwtjes en werden niet aangetroffen in groepjes waar mannetjes en vrouwtjes samen voorkomen, noch in groepjes van mannetjes alleen.

Vanuit het standpunt van biologische bestrijding beredeneerd, zijn alleen de *Trichogramma*vrouwtjes nuttig, omdat ze de eieren van het plaaginsect parasiteren. Er zijn *Trichogramma*soorten waar maagdelijke vrouwtjes alleen vrouwlijke nakomelingen produceren (thelytokie). In de meeste gevallen is thelytokie gerelateerd aan de aanwezigheid van *Wolbachia*, een endosymbiotische bacterie. In sommige gevallen zijn alle vrouwtjes in een populatie geinfecteerd met deze bacterie (gefixeerde populaties) en in andere gevallen komen geinfecteerde en niet-geinfecteerde vrouwtjes samen voor (niet-gefixeerde populaties). In **hoofdstuk 4** wordt de hypothese getoetst dat in gefixeerde populaties het effect van de bacterie op de "fitness" van vrouwtjes minder negatief is dan in niet-gefixeerde populaties. In de experimenten worden verschillende parameters van vrouwelijke "fitness" van twee gefixeerde *Trichogramma*lijnen van *T. cordubensis* en *T. oleae* vergeleken met vier niet-gefixeerde lijnen, één van *T. deion* en drie van *T. kaykai*. Iedere oorspronkelijke thelytoke lijn werd vergeleken met een sexuele – *i.e.*, arrhenotoke – lijn, gegenereerd in het laboratorium door de *Wolbachia* te doden met behulp van antibiotica. De productie van dochters was hoger voor de gefixeerde lijnen dan voor hun corresponderende genezen arrhenotoke lijnen. Het tegenovergestelde werd gevonden voor drie van de vier niet-gefixeerde lijnen.

Behalve de infectiestatus van de sluipwesppopulaties (gefixeerde versus niet-gefixeerde populaties), zijn er andere factoren die het reproductiesucces van de sluipwespen kunnen beinvloeden. Eén van hen is de capaciteit van vrouwtjes om de eieren van de gastheer te zoeken. Het zich verspreiden van twee thelytoke lijnen van *T. cordubensis* werd gemeten in een laboratoriumopstelling en in de kas (**hoofdstuk 5**). De laboratoriumopstelling werd speciaal gemaakt en getoetst als "gereedschap" voor beoordeling van *Trichogramma*. De *Trichogramma*lijn die zich meer verspreidde in de laboratoriumopstelling, verspreidde zich ook meer in de kas. Dit suggereert dat de laboratoriumopstelling een goed stuk gereedschap kan zijn voor een pre-evaluatie van *Trichogramma*lijnen. Verder is de geschiktheid voor biologische bestrijding van door *Wolbachia* geinfecteerde *Trichogramma*populaties en die van niet-geinfecteerde populaties onderzocht (**hoofdstuk 6**). De productie van nakomelingen en verspreiding van *T. cordubensis* en *T. deion* werden gemeten in het laboratorium en in de kas. De sexuele lijnen hadden een hogere productie van nakomelingen dan de corresponderende thelytoke lijnen. De arrhenotoke lijnen verspreidden zich beter in de laboratoriumopstelling, maar het tegenovergestelde of geen verschil werd gevonden in de kas. Berekeningen laten zien dat, wanneer honderd volwassen sluipwespen van beide lijnen zouden worden losgelaten (inclusief arrhenotokous mannetjes in de sexuele lijn),

er meer eieren geparasiteerd zouden worden door de thelytoke sluipwespen. Dus, ondanks hun lagere productie van nakomelingen per vrouwtje, lijken de thelytoke lijnen beter geschikt voor biologische bestrijding dan de corresponderende sexuele lijnen.

*Trichogramma*vrouwtjes parasiteren niet alleen eieren van *Lepidoptera*, maar ook die van sommige natuurlijke vijanden van plagen. Om te bepalen welke *Trichogramma*soort het minst schadelijk is voor de natuurlijke vijand *Chrysoperla carnea*, is het parasiteringssucces van *Helicoverpa armigera* (een plaaginsect) en *Chrysoperla carnea* door de vijf Portugese *Trichogramma*soorten in het laboratorium bestudeerd (**hoofdstuk 7**). De fractie *H. armigera*/*C. carnea* geparasiteerde eieren was verschillend voor de *Trichogramma*soorten. Het gemiddelde aantal geparasiteerde eieren per vrouwtje in 24 uur door *T. pintoi* en *T. bourarachae* was 10 voor *H. armigera* en ongeveer 0.5 voor *Chrysoperla carnea*. Voor de andere drie *Trichogramma*soorten (*T. cordubensis*, *T. evanescens* en *T. turkestanica*) varieren deze gemiddelden van 6 tot 11 *H. armigera* eieren en van 3 tot 4 *C. carnea* eieren. De totale productie van nakomelingen, de frequentie dat een *Trichogramma*vrouwtje bij een gastheerei was gevonden, het aantal volwassen wespen per gastheerei, en de sex-ratio van elke *Trichogramma*lijn werden ook bepaald. De resultaten laten zien dat verschillende sympatrische *Trichogramma*soorten, plaaginsecten en natuurlijke vijanden in verschillende proporties kunnen parasiteren. Als dit verschil correspondeert met de veldsituatie, zouden eenvoudige laboratoriumproeven gedaan kunnen worden om niet alleen biologische bestrijders effectief te selecteren, maar ook die soorten te selecteren die het minst schadelijk zijn voor andere natuurlijke vijanden en voor andere organismen die geen plaag zijn. *Trichogramma bourarachae* en *T. pintoi* lijken de geschikste kandidaten voor de bestrijding van *H. armigera* in agroecosystemen waar *Chrysopidae* ook een belangrijk deel uitmaakt van het complex van natuurlijke vijanden. In **hoofdstuk 8** worden de belangrijkste onderzoeksresultaten in een bredere context bediscussieerd.

Resumo

Os parasitoides oófagos do género *Trichogramma* são usados como agentes de luta biológica contra pragas de lepidópteros. Das 180 espécies de *Trichogramma* descritas, apenas cinco têm aplicação a larga escala. O desenvolvimento de métodos mais adequados para seleccionar outras estirpes/espécies de *Trichogramma* é necessário para um melhor uso destes parasitoides contra pragas de lepidópteros. O principal objectivo desta tese é investigar critérios e métodos para identificação e selecção de estirpes/espécies de *Trichogramma* para luta biológica. No **capítulo 1**, apresento uma curta revisão da biologia, investigação e uso de *Trichogramma*, menciono os objectivos da investigação e apresento a estrutura da tese.

Um dos primeiros problemas com que os investigadores que trabalham com *Trichogramma* são confrontados é a sua identificação. Conjuntamente com os métodos clássicos, são necessárias metodologias simples e práticas de identificação de espécies. O uso de métodos moleculares para caracterizar e identificar cinco espécies portuguesas de *Trichogramma* é descrito no **capítulo 2**. Estes métodos foram: 1) electroforese de "internal transcribed 2 spacer" (ITS-2) amplificado por "polymerase chain reaction" (PCR), seguida por digestões por enzimas de restrição, e 2) electroforese de esterases. As cinco espécies portuguesas, *T. bourarachae, T. cordubensis, T. evanescens, T. pintoi* e *T. turkestanica* podem distinguir-se facilmente umas das outras através de chaves dicotómicas que foram construídas com base nos métodos acima mencionados. Estes dois métodos podem ser usados conjuntamente com outros métodos de identificação para exemplares colhidos no campo ou podem ser usados independentemente para detecção de eventuais contaminações no laboratório. A especificidade de tentativas de cópula foi investigada como um método adicional para identificação de espécies de *Trichogramma* (**capítulo 3**). Embora as tentativas de cópula tenham ocorrido em maior frequência entre indivíduos da mesma espécie, elas também ocorreram entre indivíduos de espécies diferentes. Foram caracterizados dois compostos que estão possivelmente envolvidos no reconhecimento de parceiros sexuais em *Trichogramma*. Esses dois compostos são produzidos por fêmeas virgens e não foram encontrados em grupos de machos e fêmeas presumivelmente fecundadas nem em grupos de machos. Conclui-se que a especificidade de tentativas de cópula não pode ser usada como um método taxonómico geral para *Trichogramma*.

Do ponto de vista de luta biológica, só as fêmeas são úteis, porque elas matam os ovos hospedeiros. Nalgumas espécies de *Trichogramma* existem fêmeas que quando

virgens produzem progenia feminina (telitoquia). Na maioria destes casos a telitoquia está relacionada com a presença de *Wolbachia*, uma bactéria endosimbionte. Nalgumas populações de *Trichogramma* todas as fêmeas estão infectadas com a bactéria (população fixa), enquanto que noutras populações se verifica a coexistência de fêmeas infectadas e não-infectadas (população mista). A hipótese de que em populações fixas o efeito da bactéria na "fitness" de fêmeas de *Trichogramma* é menos negativo do que em populações mistas foi testada no **capítulo 4**. Nestas experiências duas estirpes fixas de *T. cordubensis* e *T. oleae* e quatro estirpes mistas, uma de *T. deion* e três de *T. kaykai*, foram testadas relativamente a diversos parâmetros da "fitness" das fêmeas. As estirpes arrenotocas foram geradas a partir das estirpes telítocas em laboratório, através do uso de antibióticos que, causando a morte das bactérias do género *Wolbachia*, induz o início da arrenotoquia. A produção de fêmeas foi mais elevada nas estirpes telítocas fixas do que nas correspondentes estirpes sexuais, enquanto que o oposto ocorreu para três das quatro estirpes mistas estudadas. Como esperado, o impacto negativo da *Wolbachia* no número total de progenia produzida por fêmea foi muito mais severo para as estirpes mistas do que para as estirpes fixas.

Para além do tipo de população dos parasitoides relativamente à infecção por *Wolbachia*, fixa versus mista, existem outros factores que podem influenciar o sucesso reprodutivo dos parasitoides. Um deles é a capacidade de pesquisa das fêmeas. A dispersão de duas estirpes telítocas de *T. cordubensis* foi analisada numa câmara laboratorial e em estufa (**capítulo 5**). Esta câmara foi construída e testada especialmente para avaliação de *Trichogramma*. A estirpe de *Trichogramma* que se dispersou mais na câmara também se dispersou mais na estufa, sugerindo que a câmara pode ser um instrumento útil para uma pre-avaliação de estirpes de *Trichogramma*. Seguidamente, foi estudado o potencial para luta biológica de estirpes de *Trichogramma* infectadas e não infectadas por *Wolbachia* (**capítulo 6**). A fecundidade e a dispersão de *T. cordubensis* e de *T. deion* foram quantificadas em laboratório e em estufa. A fecundidade das estirpes sexuais foi mais elevada do que a das correspondentes estirpes telítocas. As estirpes arrenotocas dispersaram-se mais na câmara laboratorial enquanto na estufa se constatou o oposto ou ausência de diferenças. Cálculos evidenciam que a libertação de cem parasitoides adultos de ambos os tipos de estirpes, incluindo portanto os machos nas estirpes sexuais, resulta num número mais elevado de ovos parasitados pelas estirpes telítocas. Assim, embora as fêmeas de estirpes arrenotocas possuam uma fecundidade individual mais elevada, as estirpes telítocas parecem possuir um maior potencial como agentes de luta biológica.

As fêmeas de *Trichogramma* parasitam não só ovos de lepidópteros nocivos, mas também parasitam ovos de alguns insectos auxiliares. Com o objectivo de determinar quais as espécies de *Trichogramma* que são menos nocivas para os insectos auxiliares da espécie *Chrysoperla carnea*, foi estudado em laboratório o parasitismo efectuado por cinco espécies de *Trichogramma* relativamente aos hospedeiros *Helicoverpa armigera* (um insecto nocivo) e *Chrysoperla carnea* (**capítulo 7**). A fracção *H. armigera/C. carnea* de ovos parasitados por fêmea de *T. pintoi* e de *T. bourarachae* em 24 horas foi de 10 de *H. armigera* e de cerca de 0.5 de *C. carnea*. Para as outras três espécies de *Trichogramma* (*T. evanescens*, *T. cordubensis* e *T. turkestanica*) estas médias variaram de 6 a 11 ovos de *H. armigera* e de 3 a 4 ovos de *C. carnea*. A produção total de progenia, contactos com os hospedeiros, número de adultos emergidos por ovo parasitado e a razão dos sexos (sex-ratio) foram também determinados. Os resultados obtidos evidenciam que *Trichogramma* simpátricos de espécies diversas podem parasitar insectos nocivos e auxiliares em proporções diferentes. Se esta diferença corresponde à situação no campo, poderiam ser efectuados testes simples em laboratório com vista a seleccionar não apenas agentes eficientes de luta biológica, mas também as espécies que são menos nocivas para insectos auxiliares. *Trichogramma bourarachae* e *T. pintoi* são os candidatos mais prometedores para o uso contra *H. armigera* em agroecossistemas onde insectos da família Chrysopidae formam uma parte importante do complexo de inimigos naturais. O **capítulo 8** contém uma discussão dos resultados principais num contexto mais amplo.

List of publications

Some chapters of this thesis are or will be published (with modifications) as:

Silva, I.M.M.S., J. Honda, F. van Kan, J. Hu, L. Neto, B. Pintureau & R. Stouthamer. Molecular differentiation of five *Trichogramma* species occurring in Portugal. *Biological Control (accepted)* - **chapter 2.**

Silva, I.M.M.S. & R. Stouthamer 1997. To mate or not to mate...can sex pheromones be used as a taxonomic tool in *Trichogramma* spp.? *Proc. Exper. & Appl. Entomol., N.E.V.*, Amsterdam, 8: 41-46 - **chapter 3.**

Meer, M.M.M. van, I.M.M.S. Silva, M.M. Roskam, G. Gort & R. Stouthamer. Female fitness in *Wolbachia*-fixed and *Wolbachia*-mixed *Trichogramma* populations (*submitted)* - **chapter 4.**

Honda, J.Y., I.M.M.S. Silva, J. Vereijssen & R. Stouthamer. Laboratory bioassay and greenhouse evaluation of *Trichogramma cordubensis* (Hymenoptera, Trichogrammatidae) strains from Portugal. *BioControl (in press)* - **chapter 5.**

Silva, I.M.M.S., M.M.M. Van Meer, M.M. Roskam, A. Hoogenboom, G. Gort & R. Stouthamer. Biological control potential of *Wolbachia*-infected *versus* uninfected wasps: laboratory and greenhouse evaluation of *Trichogramma cordubensis* and *T. deion* strains (*submitted)* - **chapter 6.**

Silva, I.M.M.S. & R. Stouthamer. Do sympatric *Trichogramma* species parasitise the pest insect *Helicoverpa armigera* and the beneficial insect *Chrysoperla carnea* in different proportions? *Entomologia experimentalis et applicata (in press)* - **chapter 7.**

Other publications:

Hoogenboom, A., I.M.M.S. Silva, M.M.M. van Meer, M.M. Roskam & R. Stouthamer 1998. Quality assessments of *Wolbachia* infected versus non infected lines of *Trichogramma deion*. *Proc. Exper. & Appl. Entomol., N.E.V.*, 9: 99 104.

Kan, J.P.M. van, I.M.M.S. Silva, M. Schilthuizen, J.D. Pinto & R. Stouthamer. 1996. Use of DNA-based methods for the identification of minute wasps of the genus *Trichogramma*. *Proc. Exper. & Appl. Entomol., N.E.V.*, 7: 233-237.

Loomans, A.J.M., I. Silva & J.C. Van Lenteren 1992. *Ceranisus menes* (Hymenoptera, Eulophidae), a potential biological control agent of *Frankliniella occidentalis* (Thysanoptera, Thripidae). *Proc. Exper. & Appl. Entomol., N.E.V.*, 3: 40-45.

Meierrose, C., I. Silva & J. Araújo 1991. Egg parasitoids from *Heliothis armigera* on tomato in Southern Portugal. I. Field studies with natural occurring *Trichogramma* spp. and *Telenomus* spp. *Les Colloques de l'INRA*, 56: 221 - 226.

Meierrose, C., I. Silva & J. Araújo 1992. Luta Biológica contra Lepidópteros. Biotecnologia de produção de *Trichogramma* spp. (Hymenoptera, Trichogrammatidae. *Actas do 1° Encontro Nacional de Protecção Integrada*, Évora (Portugal), 1991, 2: 413-420.

Silva, I.M.M.S. & R. Stouthamer 1996. Can the parthenogenesis - *Wolbachia* lead to unusual courtship behaviour in *Trichogramma*? *Proc. Exper. & Appl. Entomol., N.E.V.*, 7: 27-31.

Silva, I.M.M.S. & R. Stouthamer 1998. Indirect assessment of host acceptance and suitability of four *Trichogramma* species. *Proc. Exper. & Appl. Entomol., N.E.V.*, 9: 105-110.

Silva, I., C. Meierrose & J. Araújo 1991. Egg parasitoids from *Heliothis armigera* on tomato in Southern Portugal. II. Biological characterization of several *Trichogramma* strains occurring naturally. *Les Colloques de l'INRA*, 56: 227-229.

Silva, I., C. Meierrose & J. Araújo 1992. Caracterização Biológica de *Trichogramma* spp. (Hymenoptera, Trichogrammatidae) Provenientes de *Heliothis armigera* Hbn. (Lepidoptera, Noctuidae). Primeiros resultados. *Actas do 1° Encontro Nacional de Protecção Integrada*, Évora (Portugal), 2: 379-386.

Silva, I.M.M.S., J.P.N.F. van Heest, F.J.P.M. van Kan, P. Strippentow, K. Takacs & R. Stouthamer 1995. Phylogeny of egg parasitoids *Trichogramma* spp. (Hymenoptera, Trichogrammatidae). *Proc. Exper. & Appl. Entomol., N.E.V.*, 6: 195-196.

Silva, I.M.M.S., F.J.P.M. van Kan, J.C. van Lenteren & R. Stouthamer 1995. Analysis of Portuguese *Trichogramma* spp. (Hymenoptera, Trichogrammatidae) using ITS ribosomal genes and RAPDs. *Les Colloques de l'INRA*, 73: 37-39.

Tilborg, M. van, I.M.M.S. Silva & R. Stouthamer 1997. Crossings-compatibility of Portuguese *Trichogramma* lines (Hym., Trichogrammatidae). *Proc. Exper. & Appl. Entomol., N.E.V*, 8: 53-58.

Vereijssen, J., I. Silva, J. Honda & R. Stouthamer 1997. Development of a method to predict the biological control quality of *Trichogramma* strains. *Proc. Exper. & Appl. Entomol., N.E.V.*, 8: 145-150.

Curriculum vitae

Isabel Maria Martins Santos e Silva was born on July 11, 1964 in Lisbon, Portugal. In 1982 she started a university course in Biology, at the Faculty of Sciences of the Classical University of Lisbon, Portugal. From 1986 to 1989 she performed research on life-history of *Trichogramma* spp., for her graduation thesis, at the Department of Biology of the University of Évora, Portugal. She graduated ("licenciatura", equivalent to MSc) in 1989, with special reference to Fauna Resources and Environment, at the Classical University of Lisbon. During 1988-89 she also taught Biology and Natural Sciences at a secondary school in Évora.

In 1990, she started a postgraduate course on Integrated Pest Management at the Agricultural Faculty (ISA) of the Technical University of Lisbon, Portugal. In 1991-92 she performed a 10-month research on thrips parasitoids for her post-graduation thesis at the Department of Entomology of Wageningen Agricultural University, The Netherlands. In 1993 she graduated ("mestrado" degree) at the Technical University of Lisbon. From 1993 to 1999 she carried out research at Wageningen Agricultural University, Department of Entomology, resulting in this doctoral thesis.