Whitefly control potential of *Eretmocerus* parasitoids with different reproductive modes

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Contents

General introduction

Hymenopteran parasitoids are a group of wasps that lay their eggs in, or on, other species of insect. Parasitoid larvae develop by feeding on the host, eventually causing the death of the host. To date, parasitoid wasps are used to control undesirable insects: "pests". The research discussed in this thesis aims to evaluate parasitoid species of the genus "*Eretmocerus"* to control a key, worldwide pest: "whitefly". In this chapter I will first summarize damage, biology and control of whitefly. Subsequently, attention is paid to biological control agents of whiteflies, in particular parasitoid. Next, the aim of my thesis project and the outline of the thesis are presented.

1. Whiteflies

Whiteflies (Homoptera; Aleyrodidae) are amongst the key pests of vegetable, ornamental, and agronomic crops throughout the world (van Lenteren and Noldus, 1990; Gerling and Mayer, 1996). The two species that most seriously damage crops are the silverleaf whitefly, *Bemisia tabaci* (Gennadius) and the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (Gerling, 1990; Kirk *et al.*, 2000). Some factors that result in whiteflies to be so widespread and cause a lot of damage are: (1) a wide host range, (2) resistance to many insecticides, (3) a high reproductive capacity, and (4) capability of transmitting viruses.

According to Byrne *et al.* (1990), the life cycle of whiteflies consists of an egg stage, four nymphal stages, and adults (both sexes). Eggs are deposited on the underside of leaves of the host plants. The first nymphal stages (crawler) are able to move a short distance. The other three nymphal stages, however, settle down and feed of the leaves before adults emerge. Approximately four to five days before adult emergence, the red eye-spots of the developing adults are visible, the phase called the "pupal stage". An adult female whitefly can start laying eggs one to three days after emerging. They can oviposit up to 800 eggs and live about 25 to 30 days, depending on environmental conditions. More detailed information on the biology and bionomics of whiteflies is presented by e.g. Byrne *et al.* (1990), van Lenteren and Noldus (1990), and Gerling and Mayer (1996).

2. Control of whiteflies

Control of whiteflies includes the four cornerstones of an integrated pest management (IPM) program: cultural and physical control, host-plant resistance, chemical control, and biological control (Hilje *et al.*, 2001).

2.1 Cultural and physical control

Cultural and physical control can play a significant role in IPM systems targeting whiteflies, because of their preventive nature. However, it may be problematic to adopt cultural and physical control such as living barriers, high planting densities,

floating row covers, mulches, and trap crops, that require significant changes in conventional cropping practices (Hilje *et al.*, 2001). Several colors of sticky traps are also used to attract adult whiteflies in greenhouses as physical control. However, sometimes these methods might interfere with other methods, e.g. inadvertently capturing biological control agents by sticky traps (Simmons, 2003).

2.2. Host-plant resistance

Resistance to whiteflies is rare in cultivated plants. In many cases the range of germplasm evaluated is too limited to get an insight in the diversity of whitefly resistance genes that happened may be available in a given crop species (Bellotti and Arias, 2001). In most cases, resistance cultivars were not specifically developed, but they were cultivars or breeding lines that to show resistance and were selected during field or greenhouse trials (review in Bellotti and Arias, 2001).

2.3. Chemical control

Chemical control has played a significant role in managing agricultural insect pests. However, whiteflies adults, eggs and nymphs are located on the underside of leaves where they are protected from overtop applications of insecticides. Therefore, chemical control of whiteflies is expensive and not always effective. In addition, control of whiteflies with chemical pesticides is often problematic because of the wide occurrence of resistance in whiteflies to these pesticides (e.g. Palumbo *et al.*, 2001).

2.4. Biological control

Because of failing and expensive chemical control, much research was directed at developing biological control by searching for efficient natural enemies of whiteflies (for overviews, see Gerling, 1990; Gerling and Mayer, 1996; Gerling *et al.*, 2001; van Lenteren and Martin, 1999). Biological control is a cost effective method to protect agricultural products and to reduce the use of pesticides, which are applied to control whiteflies (Hoddle, 2003). Therefore, in this thesis I concentrate on biological control as the most promising form of controlling whiteflies.

2.4.1. Entomopathogens

Of the different pathogens of insects, only fungi have been reported to exist on whiteflies (Meekes *et al.*, 2002). Entomopathogenic fungi of the genus *Aschersonia* are specific for whitefly and scale insects and can be used as biological control agents against *B. tabaci and T. vaporariorum* (Meekes *et al.*, 2002). *Beauveria bassiana* and *Paecilomyces fumosoroseus* also show strong potential for microbial control of nymphal whiteflies infesting cucurbit crops (Poprawski and Jones, 2001). However, mycopathogens are not mobile agents; thus, their efficacy might be influenced by the method and frequency of their application, and may result in high application costs. Also, often specific climatic conditions – high humidities – are needed for successful infestation.

2.4.2. Predators

Various researches have been conducted on biological aspects of whitefly predators (Gerling *et al.,* 2001). *Delphastus pusillus* LeConte (Coleoptera: Coccinellidae) is a predatory ladybird beetle that can feed on all stages of whiteflies and seems to have good capabilities as biological control agent (Heinz and Zalom, 1996). Several predaceous mirids (e.g. *Cyrtopelti*s, *Dicyphus, Macrolophus* and *Deraeocoris*) have also been recognized as efficient predators of whiteflies (Kapadia and Puri, 1991). Nevertheless, those predators are often unable to maintain the damage of whiteflies below a critical threshold level (Heinz, 1996). Moreover, they might prey on each other. Mass production requires special rearing conditions to minimize cannibalism.

Recently, two phytoseiids, *Euseius scutalis* (Athias-Henriot) and *Typhlodromips swirskii* (Athias-Henriot), were reported as natural enemies of *B. tabaci* (Nomikou *et al.*, 2003). They do not only feed on herbivorous mites and insects but they also use a variety of non-prey food items, such as pollen and nectar. Therefore, they can survive at low host densities. However, they might cause some damage while using plant tissue as food source as well (Nomikou *et al.*, 2003).

2.4.3. Parasitoids

Commercial biological programs aimed at controlling greenhouse whiteflies, have often used parasitoids (van Lenteren and Martin, 1999). To date a list of 34 species of *Encarsia*, 12 species of *Eretmocerus*, two species of *Amitus*, and one species each of *Signiphora* and *Methycus* have been reported as whitefly parasitoids (Gerling *et al.,* 2001). *Amitus fuscipennis* is a potentially good biological control agent of *T. vaporariorum* in environments that are not overly dry or warm (Manzano *et al.*, 2000, De Vis, 2001). The other *Amitus* species, *A. hesperidium*, along with *En. opulenta* were released in the Caribbean island of Dominica to control citrus blackfly (Martin, 1999).

Biological control of *T. vaporariorum* with the parasitoid, *En. formosa* (Gahan) was used with success between the 1920s and 1940s in England, Australia, New Zealand and Canada (van Lenteren and Woets, 1988). The biology of *En. formosa* and *T. vaporariorum* has been studied extensively by many authors (for reviews, see e.g. van Lenteren *et al.*, 1980; van Lenteren and Noldus 1990; Noldus and van Lenteren 1990; van Roermund and van Lenteren, 1994; van Lenteren and Martin, 1999).

Control of *T. vaporariorum* with *En. formosa* is successful. However, this parasitoid is only a favorable candidate for *Bemisia* control at low temperature (less than 20°C) conditions (Qiu *et al.*, 2004). In addition, *B. tabaci* is an unsuitable host for *En. formosa*, because parasitoid development is slower, more immature parasitoids die, and adults are less fecund in comparison to wasps reared on *T. vaporariorum* on the same host plant (Boisclair *et al.,* 1990; Szabo *et al.,* 1993). In commercial greenhouses, releasing high numbers of *En. formosa* (4-7 wasps per plant per week) failed to control pure populations of *B. tabaci* on poinsettia, even though

the infestation was low at the beginning of the growing period. Consequently, use of this parasitoid for control of *B. tabaci* is not recommended (Hoddle and van Driesche, 1996).

Eretmocerus species perform better than *En. formosa* at temperatures higher than 20ºC (Qiu *et al.*, 2004). *E. eremicus* are able to find hosts more quickly and frequently, with a higher killing capacity of *Bemisia* nymphs after discovering an infested patch than *En. formosa* (Hoddle *et al.*, 1998). The other species, *E. mundus,* was noted for a long time as a controlling factor of *Bemisia* in the Mediterranean vegetable growing system (Avidov, 1956). *E. mundus* is considered the most important whitefly controlling agent in plastic greenhouses in southern Spain as well (Rodriguez-*et al.*, 1994). Laboratory tests indicated that *E. mundus* from Spain parasitized more *B. tabaci* than *Eretmocerus* spp. native to Texas and other exotic parasitoids evaluated (Kirk *et al.*, 2000). *E. mundus* has been successfully applied in the field, and significantly enhanced control of *B. tabaci* (Kirk *et al.*, 2000). Sometimes the two whiteflies occur together. In these cases, biological control of whiteflies might be more successful by mixed releases of two parasitoid species (Gerling *et al.*, 2001).

It is expected that a host may get parasitised more than once, particularly under greenhouses conditions (van Lenteren *et al.*, 1997; Qiu *et al*., 2004). A host can be parasitised more than once by the same species (superparasitism) or by different species (multiparasitism) (Godfray, 1994). As only one parasitoid larva can complete development in a whitefly host (i.e. they are solitary parasitoids), the presence of more immature parasitoid in a host can delay the development of the progeny, increase larval mortality, and result in smaller offspring (van Lenteren, 1981; Vet *et al.*, 1994; Potting *et al.,* 1997). These aspects should be considered to achieve successful biological control.

Overall the knowledge of the employment in biological control of the majority of the parasitoids is still limited except for *En. formosa* (Garling *et al.*, 2001). Hence, based on excellent field experiences with *Eretmocerus* species to control *Bemisia,* I evaluate the efficiency of different species of *Eretmocerus* in more detail. Below, I will summarize some aspects of *Eretmocerus* that had already been studied, prior to my thesis work.

3. Eretmocerus

Eretmocerus species are tiny wasps; the body length has been found to range from 0.72 - 0.77 mm for females, and 0.582 - 0.801 mm for males (Hafez *et al.* 1978). *Eretmocerus* is an ecto-endoparasitoid; females stand beside their host and oviposit between the venter of the host nymph and the leaf surface. Newly deposited eggs are oval and transparent and they turn brown on the next day (Hafez *et al.* 1978). The first instar larva penetrates into the host through a complex procedure, which apparently involves puncturing the host with its mandibles and the host engulfing the young larva (Gerling, 1990). There are three larval stages before pupation. Male and female

immature stages are indistinguishable up to the pupal stage, where the dark segments of antennae are visible in male pupae (Gerling, 1990).

To date two *Eretmocerus* species (*E. eremicus* Rose & Zolnerowich and *E. mundus* Mercet) are used commercially to control whiteflies. *E. eremicus* is native in the United States (Rose and Zolnerowich, 1997). *E. mundus* has been recorded from many parts of the Mediterranean basin (Mound and Halsey 1978), Iran (Anonymous 1998; Ghahhari and Hatami, 2000), and Australia (de Barro *et al.*, 2000).

To be able to choose the best species, population or strain for biocontrol, several biological aspects can be considered. The following section summarizes what was known until now about the biology of *Eretmocerus.* Next I will discuss the criteria that can be used to compare the biocontrol efficiency of these parasitoids.

3.1. Biology

Data on the biology of *E. mundus* and *E. eremicus* can be found in the literature (see table 2), but they are often in disagreement with each other, mainly because they are obtained at different environmental conditions and on different host plants. *Eretmocerus* biology was studied in the laboratory to some extent by several authors (Gameel, 1969; Hafez *et al.*, 1978; Sharaf and Batta 1985; Tawfik *et al.*, 1978; Headrick *et al*., 1999). The effects of temperature on developmental time, fecundity, longevity, and sex ratio of *E. mundus* and *E. eremicus* are summarized in Table 2.

Table 2. Biological characteristics of *E. mundus* and *E. eremicus*.

	T (°C)	D.T. (Days)	Longevity ♀ (Days) ♂	Fecundity (#eggs)	Sex Ratio (%♀)	Host plant	Author
E. mundus	10		6.1 5.0	---	---	Cotton	Tawfik et al.1978
	14	44	11.3 5.7	---	41	Tomato	Sharaf & Batta 1985
	18	---	7.6 ---	14.5		Tomato	Tawfik et al. 1978
	23	---	3.2 1.9	---	89.3	Cotton	Tawfik et al. 1978
	25		9.1 4.5		60	Tomato	Sharaf & Batta 1985
	26.8		3.5 ----	---		Tobacco	Manzaroli et al. 2000
	27.6	----	----	---	35.7	Cotton	Tawfik et al. 1978
E. eremicus	15.5	---	40.5	---	---	---	Gerling 1966
	20	33 35	--	23.5 43	---	Cotton-Cucumber	Powell & Bellow. 1992
	26.7	---	8.6	--	---	---	Gerling 1966
	28	---	5	23.1	---	Cotton	Headrick 1999
	29	16 18	---	20 47	---	Cotton Cucumber	Powell & Bellow 1992

T= Temperature, D.T.= Developmental time

Parasitoid wasps commonly show arrhenotokous reproduction, where fertilized eggs lead to diploid females and unfertilized eggs to haploid males. Thelytokous reproduction may also occur, where females arise from unfertilized eggs, i.e. a form of asexual reproduction. In some cases, both of these reproductive modes have been found to occur in one insect species (Stouthamer and Kazmer, 1994; Arakaki *et al.*, 2000; Schneider *et al.*, 2003). The mode of reproduction of all currently known *E. mundus* populations, which have been reported from different parts of the world, is arrhenotokous, except an Australian population that is thelytokous (de Barro and Hart, 2001). The effectiveness of *E. mundus* as a biological control agent may be enhanced by thelytoky (see below); hence, the Australian *E. mundus* is considered the best candidate to control *B. tabaci* among Australian *Eretmocerus* species (de Barro *et al.*, 2000).

3.2. Behavior

So far, only a few aspects of host searching and host handling of *E. mundus* and *E. eremicus* have been studied (e.g. Foltyn and Gerling, 1985; Headrick *et al.,* 1995; Drost *et al.*, 2000; Hudák *et al.*, 2003; Qiu *et al.*, 2004).

Headrick *et al.*, (1995) showed that the host plants influence searching behavior of *E. eremicus:* females searched for whitefly nymphs faster on cotton leaves than on melon leaves. Moreover, they found that probing a host was repeated less frequently on melon leaves than on cotton.

Walking activity and walking speed of *E. mundus* have been reported to be higher than those of *En. formosa* (Drost *et al.*, 2000).

Foltyn and Gerling (1985) studied the host-handling behaviors in *E. mundus* and found that the third instar *Bemisia* nymphs are preferred for oviposition. In contrast, Headrick *et al.* (1995) found no particular preference for any nymphal instar.

Another aspect of parasitoid behavior is host discrimination between a parasitized and an unparasitized host (van Lenteren, 1981). Drumming movements of *E. mundus* females, by rubbing the hind legs on the host, were reported after oviposition (Foltyn and Gerling 1985). These movements might be associated with marking the host and enable the female to discriminate a parasitized host from an unparasitized one during foraging (Foltyn and Gerling 1985).

4. How to select the best natural enemy for whitefly biocontrol?

Biological control agents should be carefully evaluated and selected to control a certain pest. According to van Lenteren and Manzarli (1999), qualitative criteria, which are primarily used to evaluate natural enemies are the following:

1. Seasonal synchronization with the host; the natural enemy has to be available when the pest occurs. However, sometimes this synchronization may be adjusted through the timing of the application, like applied in greenhouse biological control.

2. Internal synchronization with the host; a natural enemy must not only be able to kill the host but also it must be able to develop a population in order to achieve sustainable and effective control. Therefore, the natural enemy's development must be synchronized with that of the host to allow reproduction.
3. Climatic adaptation; natural enemies should be able to develop, reproduce and disperse in the climatic conditions where they will be used.
4. No negative effect (attack of beneficial organisms).
5. Good culture methods (ability to mass-produce).
6. Host specificity (host range including the pest organism).
7. Great reproductive potential (population growth rate causes substantial mortality).
8. Good density responsiveness.

In addition to the qualitative criteria, several aspects of quantitative criteria, such as a large reproductive potential, host-killing rate, and host-searching capacity, also need to be compared under the same conditions to rank and predict the potential of populations and species (see van Lenteren and Manzarli 1999).

In parasitoids only females are effective to control the pest. Whenever a species or population produces more females, that population or species potentially achieves a higher host killing rate. Therefore, thelytoky (i.e. females produce only female progeny) can boost the effectiveness of a parasitoid as a biological control agent (Stouthamer, 1993). The advantages of thelytoky are: (a) a higher population growth rate and higher oviposition rates, (b) better colonization and establishment at low parasitoid population densities as there is need to find a mate, and (c) more cost effective in mass rearing as production is not 'wasted' on males (Stouthamer, 1993). However, these advantages depend on the fertility of a closely related sexual population (Stouthamer and Luck, 1993).

Under greenhouse conditions, pest densities are usually low, and consequently the rate of population increase (rm) may play a limited role in biological control. Therefore, to evaluate and understand success or failure of biological control, searching- and parasitization behavior of parasitoids at low host densities should be considered (van Roermund and van Lenteren, 1994). In these cases, the time budgets spent on host searching, and the kind of host-selection behavior should be compared under the same conditions, to identify more effective populations or species. Yet, another aspect of host searching behavior, "host discrimination", has an impact on the competition between parasitoids.

Eretmocerus species meet the qualitative criteria, and two of them are commercially used as a biological control agent of whitefly. However, the quantitative criteria should be compared under the same conditions, to evaluate the species and populations, which I deal with in my thesis.

5. Aim and outline of this thesis

The main aim of the work presented in this thesis is to compare the biocontrol efficiency of an arrhenotokous population versus a thelytokous population of *E. mundus*, and partly with an arrhenotokous population of *E. eremicus.* Different aspects that play an important role in the evaluation of the populations/species have been considered, including biology, behavior, and genetic variation.

Research questions were:

1. How is the development of the *E. mundus* parasitoid populations on *B. tabaci*, grown on three host plants? In this study we evaluated the performance of different populations on gerbera, poinsettia, and tomato. The data will provide insight in the pest reduction capacity of the populations.
2. What are the mating challenges in the arrhenotokous populations? We studied the impact of mate finding in arrhenotokous *E. mundus* and *E. eremicus* populations on their efficiency and compare them with a thelytokous population of *E. mundus*.
3. Dose genetic variation support speciation between the arrhenotokous and the thelytokous populations of *E. mundus*? They show different modes of reproduction and different geographical distributions. We compare the divergences of three regions of DNA among *Eretmocerus* species.
4. Does the mode of reproduction (arrhenotoky / thelytoky) have any impact on different behavioral components in *Eretmocerus* species? We studied the host handling behaviors, host discrimination, and competition between *E. mundus* and *E. eremicus*.

Based on the biological and behavioral data that were collected we can estimate the control capacity of the two *E. mundus* populations.

Outline of the thesis

In **chapter 2,** I describe the differences in biology between the two *E. mundus* populations on three different host plants. I investigate if there is any advantage of a thelytokous population over arrhenotokous populations in the use as biological control agent.

In arrhenotokous populations mate finding might pose a challenge, especially in the case of solitary parasitoids where a mate may not be available at the emergence site (van den Assem, 1996). To attract a mate, a female parasitoid may produce volatile, non-volatile sex pheromones, or both. Volatile pheromones enable mate finding by attracting males over long distances to the females and non-volatile pheromones mediate close-range courtship behavior (Quicke 1997). Sometimes males are attracted to sex pheromones of heterospecific females. In that situation the males may, or may not be able to mate successfully with heterospecific females (Post and

Jeanne, 1984; Kimani and Overholt, 1995). If mating does occur, reproductive incompatibility could have negative effects on reproduction of females and will reduce the number of progeny in the next generation (Stouthamer *et al.*, 1996). In **chapter 3,** I deal with these aspects in *Eretmocerus* species and populations. Research questions here are: (1) How are *Eretmocerus* males able to find their mates? (2) Are sex pheromones involved in mate finding? (3) Is there any intra- or inter specific reaction of males to females? (4) Are there any differences between mating behavior? (5) Is there any chance of hybridization?

Wherever several populations of a biological control agent occur they might show differences, which could lead to a variable capacity to control a specific pest (de Bach and Rosen, 1991). In those cases the identification of biological control agents is a fundamental part of a successful biological control program. Such populations can appear morphologically similar, while they might show genetic differences that influence their efficiency in biological control. In these cases, molecular techniques can play an important role in discriminating closely related populations or cryptic species (Landry *et al.,* 1993; Hoy *et al.,* 2000; Caterino *et al.*, 2000). The two *E. mundus* populations that I mainly focus on this thesis show reproductive isolation (chapter 3). We suspect that they might actually be different species, although, they are apparently morphologically indistinguishable (de Barro *et al.*, 2000). The thelytokous mode of reproduction in Australian population of *E. mundus* is assumed to be a direct consequence of infection with *Wolbachia* bacteria. If this thelytoky has lead to reproductive isolation, the *Wolbachia* might have played a role in diversification between the *E. mundus* populations. Therefore, **chapter 4,** describes the genetic divergences of two nuclear genomic regions along with a mitochondrial region in *Eretmocerus* species and populations, to investigate the extent of diversification between *E. mundus* with different reproductive modes, and the possibility to distinguish the *E. mundus* populations with molecular techniques.

To develop a successful biological control program, knowledge of the foraging behavior is also fundamental (Lewis *et al.*, 1990; Godfray, 1994). During foraging, a parasitoid has to be able to find and accept a suitable host in order to achieve reproductive success. After finding a host, parasitoid females should evaluate the host for suitability for reproduction (egg laying) or for maintenance (host feeding) (Burger *et al.*, 2004). Therefore, they may show several kinds of behaviors, such as antennation, probing, and drumming (van Lenteren *et al.,* 1980; Headrick *et al.,* 1995; Higuchi and Suzuki, 1996). In **chapter 5,** the host-handling behavior is presented of different nymphal stages by *Eretmocerus* females for oviposition or host feeding. Comparison between two populations of *E. mundus* (Spanish and Australian) along with *E. eremicus* is discussed within the framework of biological control of whitefly. These observations are used to understand the host-discrimination behavior and to study the occurrence of super- or multi- parasitism.

Sometimes the selected host has already been parasitised. Thus, larvae of parasitoids not only have to defeat the host defense (e.g. encapsulation), but they have

to also compete with other (unrelated) larvae for food sources (super- or multi-parasitism). In general, superparasitism could delay the development of the progeny, increases larval mortality, and results in smaller offspring (Vet *et al.*, 1994; Potting *et al.,* 1997). Therefore, an important element of host selection is to distinguish between parasitised and unparasitised hosts: "host discrimination". In **chapter 6,** I evaluate intra- and interspecific host discrimination, the occurrence of super- and multi-parasitism, and competition in two populations of *E. mundus* and *E. eremicus.*

Finally, in **chapter 7**, I first review the most important results from the studies described in the previous chapters. I suggest directions for future research and also stress the importance of the results to use of *Eretmocerus* populations or species for future applications in biological control.

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Biology of an arrhenotokous and a thelytokous population of the whitefly parasitoid *E. mundus* on three host plants: how do host plants and *Wolbachia* infections influence the parasitoid?

Abstract

A major component of the evaluation of biological control agents is the study and comparison of their biology, including the reproductive mode. In hymenopteran parasitoids, reproductive modes include thelytoky and arrhenotoky. Since a thelytokous population produces only females, it is assumed that they may be better suited for biological control than an arrhenotokous population. Here we compare the effects of these two modes of reproduction on life-history parameters in the whitefly parasitoid *Eretmocerus mundus*. We also determined biological parameters of two populations of *E. mundus* on three host plants (tomato, poinsettia and gerbera) under laboratory conditions, to be able to evaluate the effect of the host plant on the parasitoid. In all situations the developmental times of nymphal stages were not significantly different. Females lived longer on tomato than on poinsettia and gerbera. The number of progeny of both parasitoid populations was highest on tomato, lowest on gerbera and intermediate on poinsettia. Reproduction is high during the first two-days of the female's life for both populations on the different host plants; thereafter it decreases quickly. Arrhenotokous females had a higher fecundity than thelytokous ones. The sex ratio for the arrhenotokous population was 50/50 on the three host plants, while we could find only a few males for the thelytokous population. The intrinsic rate of population increase (r_m) was highest on tomato, intermediate on poinsettia and lowest on gerbera, and similar for the arrhenotokous and the thelytokous populations. The results will be discussed in the framework of selecting the best *Eretmocerus* species/strain for biological control of whitefly.

Introduction

Whiteflies are a serious pest of vegetable, ornamental, and agronomic crops throughout the world. They have caused enormous damage to many crops during the past century (Gerling, 1990; Gerling and Mayer, 1996). Control of whiteflies with chemical pesticides is often problematic because of the wide occurrence of resistance (e.g. Palumbo *et al.*, 2001). Therefore, during the past decades, much research was directed at finding efficient natural enemies of whiteflies (for overviews, see Gerling 1990; Gerling & Mayer, 1995; Gerling *et al.*, 2001). Among different categories of natural enemies to control whiteflies (e.g. predators, parasitoids and microbial agents), parasitoids are the most successful (van Lenteren, 1990; Gerling *et al.*, 2001). So far, several solitary parasitoids of the genera *Encarsia, Amitus*, and *Eretmocerus* have

been reported as potentially efficient biological control agents of the whitefly under greenhouse conditions (van Lenteren, 1990; Drost *et al.*, 1999; Gerling, 2001). Control of *Trialeurodes vaporariorum* with *En. formasa* is very successful. However, *Bemisia tabaci,* another whitefly, is a poor host for *En. formosa* (Boisclair *et al.,* 1990; Szabo *et al.,* 1993) resulting in inferior reproduction of the parasitoid and insufficient control . *Eretmocerus* species are performing better in controlling *B. tabaci* (Hoddle *et al.,* 1998; Qiu *et al.,* 2004). For instance, *E. mundus,* has been noted for a long time as a controlling factor of *Bemisia* in the Mediterranean vegetable growing system (Avidov, 1956; Rodriguez *et al.,* 1994).

E. mundus shows two different modes of reproduction: arrhenotoky and thelytoky. Arrhenotokous females need to mate to lay fertilized eggs that give rise to female progeny; unfertilized eggs develop into haploid males. In contrast, thelytokous females do not need to mate to produce females, and unfertilized eggs give rise to female progeny. As only females are effective in biological control, the thelytokous reproduction can potentially boost the effectiveness of a parasitoid as a biological control agent (Stouthamer 1993). The advantages could be (1) a higher rate of population increase than the arrhenotokous parasitoid, (2) cheaper mass production, as all offspring are females, and (3) more effective biocontrol at low host densities, because the parasitoids do not need to find a mate to produce female progeny (Stouthamer 1993). Nevertheless, advantages and disadvantages of the two modes of reproduction are also depending on other factors such as the number of female offspring produced per thelytokous female versus an arrhenotokous female (Stouthamer, 1993; Stouthamer & Luck, 1993, Silva *et al.*, 2000).

Thelytoky in hymenopteran parasitoids is often associated with the presence of endosymbiotic bacteria of the genus *Wolbachia* (a-proteobacteria) (Stouthamer, 1997). Infection with *Wolbachia* has, in some cases, a severe negative effect on the fecundity of the insect host compared to the arrhenotokous conspecific (Stouthamer & Luck, 1993; van Meer, 1999; Silva *et al.*, 2000). However, the effects might be less extreme for those populations where *Wolbachia* is found throughout the population ("fixed populations") than for those where *Wolbachia* is found only in a part of the population ("mixed populations"; van Meer, 1999). *Wolbachia* is fixed in the thelytokous population of *E. mundus* and all progeny is female (de Barro and Hart, 2001). Hence, the infection with *Wolbachia* is expected not to have a severe negative effect on the fecundity of the infected *E. mundus* population.

To compare the fecundity of arrhenotokous and thelytokous populations of *E. mundus*, *Wolbachia* can be removed from an infected population applying treatment with antibiotics, which leads to an arrhenotokous population. In this case, the progeny of a cured population of *E. mundus* has been reported to be less numerous than the progeny of infected ones (de Barro and Hart, 2001). However, curing with antibiotics not only can remove *Wolbachia,* but also may affect other symbiotic organisms and the biology of the parasitoid (Stouthamer and Mak, 2002). Therefore, comparison of

the biology of a thelytokous with a genuine arrhenotokous population might show different results than comparison with a cured population.

Arrhenotokous populations of *E. mundus* have been reported from many parts of the Mediterranean basin (Mound & Halsey, 1978). Some data on the biology of arrhenotokous populations and the thelytokous population of *E. mundus* are available in the literature (see chapter 1; Greling *et al.,* 2001).

Another factor affecting development and reproduction of the parasitoid is the type of host plant species on which the parasitoid is searching for whiteflies (e.g. van Lenteren *et al.*, 1995; Heinz & Parella, 1994). For *Eretmocerus*, it was found that the number of ovipositions was influenced by the host plant (Headrick *et al.*, 1995; de Barro *et al.,* 2000). Results mentioned in the literature are often in disagreement with each other mainly because they are obtained from different host plants and at different climatological conditions (Gameel, 1969; Hafez *et al.*, 1978; Tawfik *et al.*, 1978; Sharaf & Batta 1985; Manzaroli *et al.,* 1997; de Barro *et al.,* 2000). Here, we report about a study of the biology of two *E. mundus* populations (arrhenotokous and thelytokous) on three different host plants (two ornamentals and a vegetable crop). We investigated (1) the influence of reproductive mode and host plants on biological parameters of the two populations of parasitoids, and (2) the effects of differences in biology on the capability to control *B. tabaci*.

Methods and Material

Maintenance of insects

We used two populations of *E. mundus,* a commercial arrhenotokous population from Spain (ErCal®, Koppert Biological Systems, The Netherlands), and a thelytokous population from Australia, which is a non-commercial laboratory strain (de Barro *et al.*, 2000).

A culture of *B. tabaci* was maintained on poinsettia, tomato, and gerbera plants in a greenhouse (25°C±5°C and 75±10% RH). Each plant was introduced in a cage with approximately a hundred *B. tabaci* adults. The whiteflies were removed from the plants on the next day and the plants were kept in clean cages for 10-12 days, i.e. until *B. tabaci* had reached the 2nd and 3rd nymphal stages. These infested plants were used for experimental work and for rearing the parasitoids. The two populations of *E. mundus* were maintained on the infested plants in two separate climate rooms under 26±1°C, 45±5% HR, and 16L/8D light conditions.

Experimental set up

Parasitoid pupae were collected from the leaves and put separately in a glass vial. The emerging adults were used for experiments on the next day. To record the number of eggs, ten newly emerged females of the two populations from the culture of poinsettia plants (<24 h old) were dissected under a stereomicroscope.

We chose infested leaf parts (4*5 cm) with approximately 25 to 35 *B. tabaci* nymphs for the experiments. Each leaf part was fixed on a moist piece of cotton wool (to prevent desiccation) in a Petri dish (5cm Ø). A few drops of water were added to the cotton to keep them moist during experiments. A female of the thelytokous population or a couple of the arrhenotokous population was introduced in the Petri dishes. The lids of the Petri dishes were covered with netting to keep the parasitoid inside and to allow ventilation. Petri dishes were left upside down in the climate rooms. On the next day the males were removed and each parasitoid female was daily provided with a fresh whitefly-infested leaf part. The leaf parts were kept in the climate rooms and were checked daily until emergence of adult parasitoids. During the transfer of wasps it occasionally happened that the wasp jumped away or got stuck to the cotton. Therefore, the numbers of replications were different for populations and host plants. Longevity of the females, developmental time of the nymphal stages, mortality of the immatures, the total number of progeny, and the sex ratio of progeny were recorded. With these data, we calculated the innate rate of population increase, r_m, of the two *E. mundus* populations on the three host plants.

Data analysis

Mean differences in longevity and developmental time of the nymphal stages were compared between populations on different host plants using ANOVA with SPSS software. The progenies were compared (1) amongst females of the same age, and (2) for a five-day period for females that were still found alive, using ANOVA.

Results

Longevity, developmental time and mortality

The longevity was longer for the Spanish arrhenotokous population than for the Australian thelytokous one, and longer on tomato than on poinsettia and gerbera (Figure 1), but the differences were only significant between the longevity of the Spanish population on tomato and the other host plants ($F_{66:5}$=15, P < 0.01).

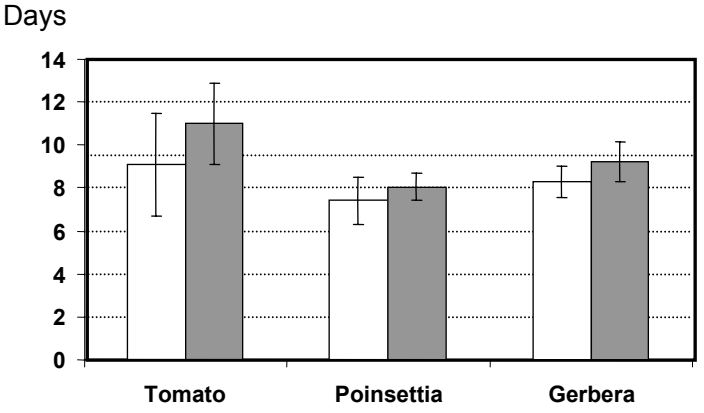

Figure 1. Mean longevity (with S.E.) of *Eretmocerus mundus* populations on different host plants,
= Thelytokous
= Arrhenotokous

The developmental time of nymphal stages ranged between 14 - 19 days on different host plants for both populations (Table 1); significant differences between the populations were not found.

The mortality during the immature stages was higher on gerbera and poinsettia than on tomato (Table 1).

Table 1. Fecundity, mortality, developmental time and population development parameters of two *Eretmocerus mundus* populations on three host plants.

	Host plants	Egg (SD)	Mortality %	sex ratio	R_0	Developmental Time (SD)	%50 Eggs	Generation Time (GT)	LnR_0	r_m
Thelytokous	Tomato	54.6 (7.07)	6.6	1	51	16.1 (1.2)	1	17.1	3.93	0.23
	Poinsettia	28.6 (5.63)	9.4	1	26	15.6 (1.1)	1	16.6	3.26	0.20
	Gerbera	19.0 (506)	10*	1	17	15.4 (1.1)	1	16.4	2.84	0.17
Arrhenotokous	Tomato	117.5 (17.90)	6.9	0.5	55	15.2 (0.9)	2	17.2	4.00	0.23
	Poinsettia	49.4 (6.38)	8.8	0.5	23	15.6 (0.9)	1	16.6	3.11	0.19
	Gerbera	26.8 (7.04)	10*	0.5	12	15.3 (1.0)	1	16.3	2.49	0.15

R_0 = net reproductive rate, r_m = intrinsic rates of increase, * = roughly estimated

Reproduction and sex ratio

Females of both populations were able to parasitize hosts upon emergence, so this species of *Eretmocerus* does not have a pre-oviposition period. Upon emergence, arrhenotokous females had an average of 26.36 (2.9 SD) eggs in their ovarioles, and thelytokous females had an average of 28.75 (3.6 SD) eggs. The post-oviposition period was longer for the thelytokous population than for the arrhenotokous population (Figure 2). The fecundity (and progeny) of both populations was largest on tomato plants and smallest on gerbera plants (Figure 3). A high number of progeny was recorded during the first two-days of the female life time for both populations on all three host plants (figure 2).

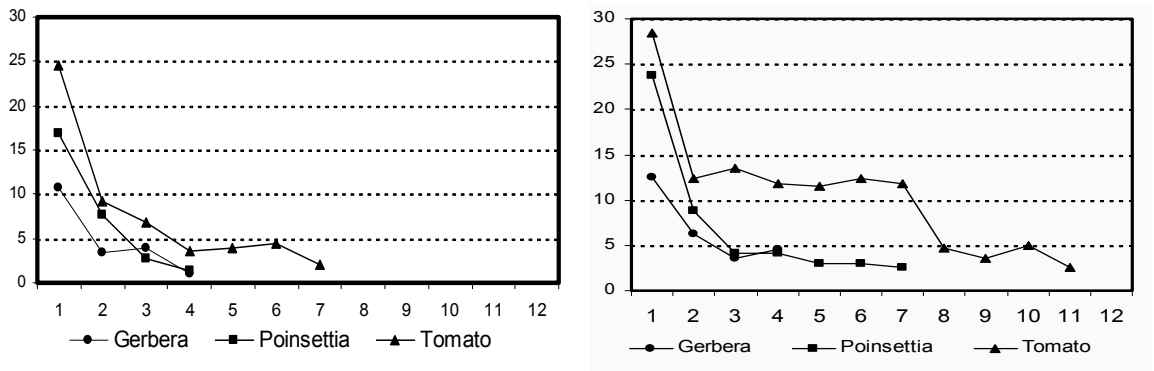

Figure 2. The average number of progeny during adult life of the two *Eretmocerus mundus* populations on three different host plants.

The progeny production decreased sharply after the first day for the thelytokous population. The same was found for the arrhenotokous population, but here daily reproduction was higher and the reproduction period was longer than for the thelytokous populations (Figure 2). Consequently, the total progenies of the arrhenotokous population were significantly larger (approximately twice as large) than those of the thelytokous one except for gerbera ($F_{24:1}$=10.7; P<0.003 for tomato, $F_{45:1}$=23.7; P<0.00 for poinsettia, and $F_{17:1}$=2.7; P<0.124 for gerbera).

The sex ratio of the arrhenotokous population was 50/50, while the thelytokous population produced only females, except for three males.

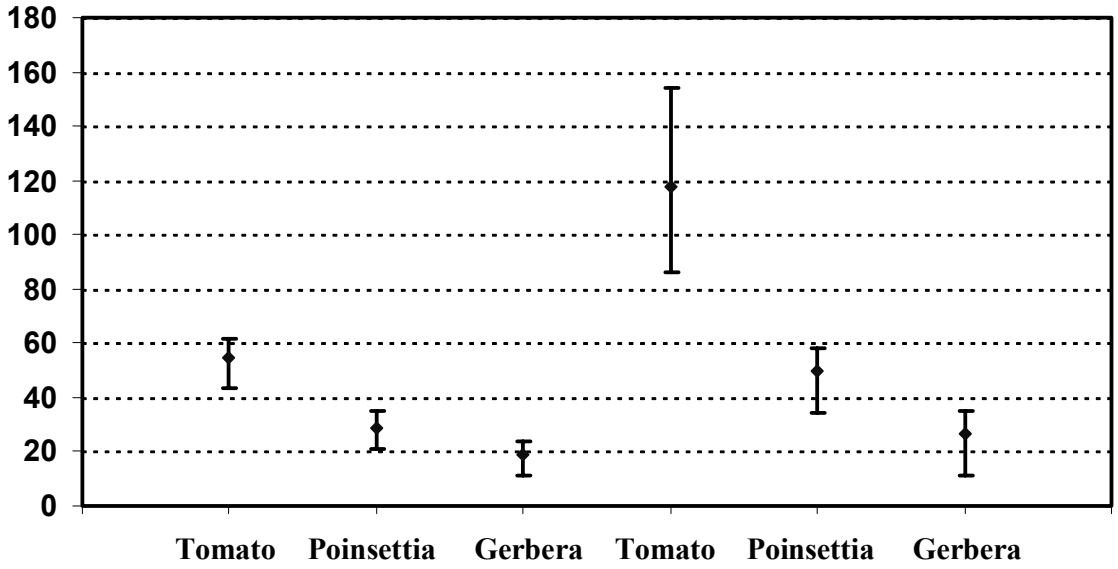

Figure 3. The lowest, highest, and average number of progeny for the two *Eretmocerus mundus* populations on different host plants.

The population growth parameters were similar for both populations on the same host plants, but varied between host plants (Table 2). The intrinsic rate of increase in both populations (r_m) was largest on tomato and lowest on gerbera, with intermediate values for poinsettia (table 1).

Discussion

Life-history parameters

The *developmental time* of *E. mundus* larvae was found to be 15 to18 days on cotton (Gerling & Fried, 2000), 17.1 days on poinsettia (Qiu et al., 2004), 15.4 on sweet potato (Jones & Greenberg, 1998), and 16.0 days on tomato (Sharaf & Batta, 1985). We found a similar range (between 14-19 days) in the developmental time for both populations on different host plants.

The *longevity* of *E. mundus* was reported to be between 10-16 days on cotton (Gerling & Fried 2000), 12.4 days on poinsettia (Qiu et al., 2004), and 9.6 days on tomato (Sharaf & Batta, 1985). We found similar data (range between 7-11 days) for different host plants for both populations (figure 1).

Headrick *et al*. (1999) found a preovipositon period for *E. eremicus*. However, we could not record such period, and females of both *E. mundus* populations began oviposition shortly after emergence, and laid a large proportion of eggs during the first two days of their adult lives.

Contrary to the comparable variation in developmental time and longevity of our data and those of others, fecundity data show large differences. These differences may be caused by other experimental setups, influences of host plants, or differences in *Bemisia*-, and parasitoid strains. Gerling & Fried (2000) measured 81 to 247 eggs per female on cotton, while de Barro et al. (2000) reported 97.8 eggs on cotton, 107.8 on tomato, and 138.3 on rockmelon for the thelytokous population. We found a large variation in fecundity between the two populations, as well as on different host plants (table 1, 2). The cause of differences in fecundity between the two populations will be discussed below. We expect that the host-plant effect on fecundity is the result of differences in host-plant surface that influences the oviposition behaviour of *Eretmocerus*.

The influence of leaf textures of host plants on the foraging behavior of parasitoids has been reported for example for *En. formosa* (e.g. Sütterlin and van Lenteren, 1999). *Eretmocerus* species are ecto-endoparasitoids, i.e. females stand beside their host and oviposit between the venter of the host nymph and the leaf surface (Gerling, 1990). A host plant with smooth leaves, where the margin of the nymph fits level with the leaf surface, results in difficulties during oviposition for the females (Headrick *et al.*, 1996). It is, therefore, no surprise that both for *E. eremicus* and *E. mundus* an effect of the leaf surface on ovipositon was observed (Headrick *et al.*, 1996; de Barro *et al.,* 2000). The fecundity of both *E. mundus* populations used in our experiments was lowest on gerbera, cultivar Maya, with a smooth plant surface and only a few long hairs. Poinsettia and tomato have many short hairs, resulting in easier oviposition by *E. mundus*. Fecundity on poinsettia is lower than on tomato. We suggest that differences in plant chemistry might have had a direct or indirect negative effect on the parasitoid. It is known that poinsettia has a very negatively influence on other natural enemies, such as pathogens (Meekes et al., 2000) and predators (Legaspi *et al.,* 1996).

Table 2. The average numbers of progeny (S.E.) for the two *Eretmocerus mundus* populations on three host plants

	Arrhenotokous population						Thelytokous population					
	Tomato		Poinsettia		Gerbera		Tomato		Poinsettia		Gerbera	
	Average (SE)	n	Average (SE)	n	Average (SE)	n	Average (SE)	n	Average (SE)	n	Average (SE)	n
1	27.64 (1.49)	14	23.75 (1.68)	16	12.44 (1.74)	9	23.32 (1.18)	22	16.94 (0.85)	25	10.67 (1.8)	7
2	12.07 (1.29)	14	8.88 (1.4)	16	6.29 (3.34)	8	8.14 (0.9)	22	7.61 (0.76)	25	3.33 (0.89)	4
3	12.57 (1.29)	14	4.14 (1.01)	7	3.6 (1.4)	6	6.33 (1.18)	12	2.71 (0.37)	8	4 (1.33)	3
4	11 (1.43)	12	4.17 (0.27)	7	4.5 (1.63)	3	3.5 (0.99)	6	1.33 (0.23)	4	1	1
5	11.55 (2.16)	11	3 (1.66)	5			4 (2.18)	3				
6	12.36 (2.03)	11	3 (2.57)	3			4.5 (0.54)	2				
7	11.86 (2.7)	7	2.5 (1.61)	2			2 (1.09)	2				
8	4.63 (1.74)	4										
9	3.5 (0.61)	2										
10	5 (0.4)	2										
11	2.5 (0.2)	2										

Impact of reproduction modes and Wolbachia *infection on population development of the parasitoid*

Negative effects of a *Wolbachia* infection on the fecundity of parasitoids like *Trichogramma* have earlier been reported (e.g. Stouthamer & Luck, 1993; Silva *et al.,* 2000). It is assumed that the severity of the effects of *Wolbachia* infections should be less in a population where the Wolbachia infection is complete ("fixed") than in populations that are partly infected ("non-fixed") (Silva *et al.,* 2000; van Meer, 1999). De Barro (2001) found that the fecundity of an infected *E. mundus* was similar to that of a cured one, although the juvenile mortality was greater in cured females. Our results showed a larger number of progeny for the arrhenotokous *E. mundus,* than for the thelytokous population on all three host plants. Therefore, a negative influence of a *Wolbachia* infection on fecundity is suggested in the thelytokous population. The daily numbers of progeny in both populations were not significantly different during the first days, but the numbers of progeny were significantly larger for arrhenotokous females during the remaining life span than for thelytokous ones.

Eretmocerus *and biological control*

The intrinsic rate of increase (r_m) is a parameter which is often used to compare the efficiency of biological control agents. This rate of increase is a composite value of most of the life-history parameters, including developmental time, juvenile mortality, sex ratio and life-time reproduction. The r_m was highest on tomato, intermediate on poinsettia and lowest on gerbera, and larger in the arrhenotokous population than in the thelytokous one. Consequently, we have to conclude that the type of host plant and mode of reproduction both influence population development of both populations.

The total number of progeny as well as the r_m was larger in the arrhenotokous population than in the thelytokous one. Therefore, the arrhenotokous population might perform better at a high density of whitefly than the thelytokous one. However, at low whitefly densities, which are often found under greenhouse conditions when biological control works well, a high fecundity is no longer important and parasitoid population survival capabilities at low host density becomes crucial. Since thelytokous females do not need to find mates for reproduction, they might have larger survival possibilities at low densities than the arrhenotokous population. I will come back to this issue in the summarizing discussion of the thesis.

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Inter- and intra-specific effects of volatile and non-volatile sex pheromones on males, mating behavior and hybridization in *Eretmocerus mundus* and *E. eremicus* (Hymenoptera: Aphelinidae)

Abstract

Eretmocerus species (Hym. Aphelinidae) are solitary parasitoids of *Bemisia tabaci* (Gennadius). Mate finding and mating behavior of two species, *E. mundus* and *E. eremicus*, were studied under laboratory conditions. We used three populations of *Eretmocerus*: typical arrhenotokous populations of *E. eremicus* (from USA) and *E. mundus* (from Spain), and an atypical thelytokous population of *E. mundus* (from Australia). We studied the intra- and inter-specific responses of males to volatile and non-volatile components of the female sex pheromones, mating behavior, and hybridization between populations and species. In both arrhenotokous populations, males reacted to volatile pheromones by walking towards conspecific virgin females. Males also reacted to non-volatile pheromones by spending more time on and around patches on leaves of poinsettia plants that had been exposed to virgin females. Males of *E. eremicus* showed the same reaction to the non-volatile sex pheromone of *E. mundus* females, but *E. mundus* males did not show any reaction to the non-volatile sex pheromone of *E. eremicus*. There was no response of males of both species to thelytokous females of *E. mundus*. In both species three phases were distinguished in the mating behavior: pre-mating, mating and post-mating. The duration of the phases differed between the three populations. Successful copulation between the two *Eretmocerus* species did not occur. In contrast, we recorded some successful copulations between Australian males and Spanish females of *E. mundus,* but they did not produce any hybrid females.

Introduction

Hymenopteran parasitoids reproduce either thelytokously or arrhenotokously. Thelytokous (or asexual) parasitoids are able to produce female offspring without mating. Therefore, they no longer need to attract males. In contrast, in arrhenotokous (or sexual) parasitoids unmated females can only produce males, thus females must mate to be able to produce female offspring. Mating at emergence sites is likely to occur in gregarious parasitoids because the sexes emerge in proximity to each other (Pompanon *et al.*, 1997).

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Under such circumstances, mate finding is often achieved through tactile and visual stimuli (van den Assem and Jachmann, 1982; Tripathi and Singh, 1990; Yoshida and Hidaka, 1979). However, searching for mates poses a challenge for solitary parasitoids, especially when a mate may not be available at the emergence site (van den Assem, 1996).

To attract a mate, a female parasitoid may produce volatile, non-volatile sex pheromones, or both. Volatile pheromones enable mate finding by attracting males over long distances to the females and non-volatile pheromones mediate close-range courtship behavior (Quicke, 1997). If a male parasitoid perceives volatile pheromones, it may increase its antennal movement, vibrate wings, and follow the female in an accelerated manner (Delury *et al.*, 1999). If a male perceives non-volatile pheromones, it more frequently visits, or stays longer on, substrates with these pheromones. There is evidence that virgin females leave such pheromone marks on the substrate on which they walk (Fauvergue *et al.*, 1998; Shu and Jones, 1993).

Evidence for the presence of volatile and/or non-volatile sex pheromones has been found in species belonging to several hymenopteran families, such as Aphelinidae, Chalcididae, Cynipidae, Pteromalidae, Scelionidae, Braconidae, Ichneumonidae (review in Eller *et al.*, 1984), Eulophidae (Finidori *et al.*, 1996) and Trichogrammatidae (Pompanon *et al.*, 1997). Most sexual pheromones used by parasitoids are volatile (Lewis *et al.*, 1971; Eller *et al.*, 1984; Mohamed and Coppel, 1987). However, among gregarious parasitoid species, where mating at the emergence site is widespread, the emission of long-range sexual pheromones is expected to be uncommon (Godfray, 1994).

There are some examples of insect males reacting interspecifically to sex pheromones. In that situation the males may, or may not be able to mate successfully with heterospecific females (Post and Jeanne, 1984; Kimani and Overholt, 1995). If interspecific mating does not occur, the reproductive ability of the female will not be influenced except for time lost by the mating attempts of the male. However, it will have a negative effect on the mate searching efficiency of males, through the waste of energy and time. In contrast, if mating does occur, reproductive incompatibility could have negative effects on reproduction of females and will reduce the number of progeny in the next generation (Stouthamer *et al*., 1996). These aspects of mating behavior should be considered in biological control when one species is released in the native area of another. This situation may arise in whitefly biological control where related species of parasitoids are released such as *Eretmocerus eremicus* Rose & Zolnerowich and *E. mundus* Mercet (van Lenteren, 2000). *E. eremicus* is a native parasitoid in the United States (Rose and Zolnerowich, 1997). It has been reported as an effective biocontrol agent of *Bemisia tabaci* (Gennadius) on Poinsettia (Hoddle and van Driesche, 1999). *E. mundus* has been recorded from many parts of the Mediterranean basin (Mound and Halsey, 1978). It has been considered the most important whitefly-controlling agent in the plastic greenhouses in southern Spain (Rodriguez *et al.*, 1994). These two species are generally arrhenotokous, but one

population of *E. mundus,* which has been found in Australia, is thelytokous (de Barro *et al.*, 2000). Because this population is thelytokous, it is considered the best candidate for biological control of *B. tabaci* in the dry tropical region of Queensland (de Barro *et al.*, 2000).

To date, several aspects of host finding in *E. eremicus* and *E. mundus* have been studied (e.g. Foltyn and Gerling, 1985; Headrick, 1996). Male behavior and characteristic courtship behavior of *E. eremicus*, when encountering a virgin female, has been described in detail (Hunter *et al.*, 1996). However, the mating behavior of *E. mundus* is not yet studied. Also, it is unknown if females of both species (*E. eremicus* and *E. mundus*) produce volatile or non-volatile pheromones to attract males.

In this paper we provide evidence of the existence of pheromones in *E. eremicus* and *E. mundus.* Further, the inter- and intra-specific responses of males to volatile and non-volatile components of the females' sex pheromones are described. Finally, we compare the mating behavior of these two species and explore the possibility of hybridization between them.

Materials and methods

Maintenance of insects

We used three populations of *Eretmocerus:* the arrhenotokous populations of *E. eremicus* and *E. mundus* that are commercially available (ErCal® respectively BemiPar®, Koppert Biological Systems, The Netherlands), and a thelytokous population of *E. mundus* from Australia, which is a non-commercial laboratory strain (de Barro *et al.*, 2000). Thelytoky in *Eretmocerus* is associated with the presence of an endosymbiotic bacterium of the genus *Wolbachia* (de Barro and Hart, 2001). Therefore, to obtain males of the latter population, newly emerged females were fed a solution of honey with 0.05% rifampicin (antibiotic) (see de Barro *et al.*, 2000). The antibiotic kills the *Wolbachia* resulting in the production of males in the next generation.

All three populations were maintained on *B. tabaci* as host on Poinsettia plants (*Euphorbia pulcherrima* Willd. ex Klotzsch) in a climate room at 25±1°C, 45±5% HR, and 16L: 8D photoperiod.

One day before an experiment, the parasitoid pupae were collected and each pupa was put in a vial separately and the emerging wasps were kept isolated until they were used for experiments. All experiments were carried out in a climate room at 25±1°C, 45±5% HR, and artificial light. The data were analyzed with the Kruskal-Wallis test for differences in behavioral responses between different experimental groups of *Eretmocerus*.

Experimental set-up

Experiment 1: Occurrence of sex pheromones

Volatile pheromones. To test if females produce volatile pheromones, five virgin females (between one and ten hours old), were put in a chamber (1 cm^3) that was connected via fine-mesh netting to a glass tube (11 cm long and 0.6cm Ø)(figure 1). After 10 minutes, while air was flowing through the chamber into the tube (1 L/minute), a conspecific virgin male was introduced at the other end of the tube (figure 1). The time that a male was walking through the tube was recorded. Each experiment was finished when a male reached the end of the tube (the female's position), or when 240 seconds had elapsed. Males were exposed to one-day-old mated and virgin females, as well as to an empty chamber (n=10 for each). The reactions of males to inter-population and heterospecific females (mated and virgin) were also recorded for all combinations (n = 10 for each).

Non-volatile pheromones. To study the presence of non-volatile pheromones, five females (between one and ten hours old) of each population were put in separate glass vials (7cm long and 1cm Ø).

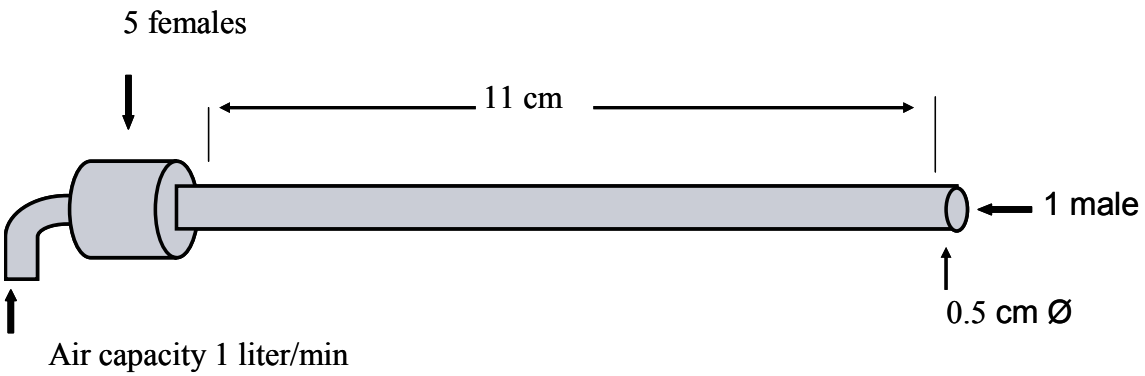

Figure 1. Wind-tube to test volatile pheromone production by *Eretmocerus* females.

We covered the open part of the vial with a Poinsettia leaf on which the female could walk for 30 minutes. After exposure to the female, the leaf was removed and put on a piece of cotton wool in a Petri-dish bottom under a camera, which was connected to a computer. A virgin male, either from the same, or different species or population as the female, was released on the leaf and the behavior of the male was recorded via the program Etho-vision® (Noldus, Information Technology) up to 240 seconds. Each leaf patch was used only once for each male, either from the same, or different species or population as the female. Observations were made with males exposed to one-day-old virgin- or mated- female's patch leaves as well as to clean patch leaves (n=10 for each series).

Experiment 2: Mating behavior, female mating capacity and hybridization

Mating behavior, female mating capacity. To record the mating behavior, a virgin couple of each population between one and ten hours old was introduced on a leaf disk (2 by 3 cm). We used The Observer Program 4.0® (Noldus, Information Technology) to record the duration of different phases of mating under a stereomicroscope. We also recorded the ability of females to mate more than once with the same male or with a conspecific male (n=10 for each series).

Hybridization. To test for any hybridization among the different populations and species, we introduced a virgin inter-population or heterospecific male to a virgin female, and recorded if any successful mating occurred. When a successful mating occurred, the female was collected and offered a leaf disk with hosts on it on a wet piece of cotton wool in a Petri-dish. The leaf disks were renewed daily and the old leaf disks were kept in a climate room to check for any hybridized females in the next generation. Some drops of water were added daily to each piece of cotton wool to avoid desiccation of the leaf disks.

Results

Experiment 1. Occurrence of sex pheromones
Reaction of males to virgin or mated females
There were significant differences between the walking activity of males in the wind-tube towards the chamber containing conspecific arrhenotokous virgin females or controls (mated females or an empty chamber) (Kruskal-Wallis χ^2 (1)=125.8, $p<0.001$). The males went more or less straight-ahead, towards the chamber containing virgin females and reached it in, on average, 34.2± 2.5 SD seconds. The results were the same for newly emerged or one-day-old virgin females (Kruskal-Wallis χ^2 (3)=2.3, p=0.5). (figure 2). In the controls, an empty chamber or a chamber with mated females, the males walked in random directions. In some cases the males reached the chamber, but it took more than 70 seconds to arrive at the chamber. However, the males' reactions did not show any significant difference between empty chambers or a chamber with mated females (Kruskal-Wallis χ^2 (3)=0.6, p=0.9).

In intra-species tests, the Australian males of *E. mundus* showed the same reaction as *E. mundus* males to the females of *E. mundus* from Spain (Kruskal-Wallis χ^2 (1)=3.3, p=0.07). However, the Spanish males did not react to the Australian females. These males either stood still, or walked randomly in the tube. In inter-species tests, the males of *E. eremicus* showed the same reaction as the Spanish *E. mundus* males to the newly emerged or one-day-old virgin females of the Spanish *E. mundus.* Therefore, walking-times towards females were not significantly different; Kruskal-Wallis χ^2 (3)=2.1, p=0.6. In contrast, the *E. mundus* males from Australia or Spain did not show any reaction to the virgin females of *E. eremicus*. In all cases,

mated females of *E. eremicus* or Spanish *E. mundus* were unattractive for males (Kruskal-Wallis χ^2 (5)=1.4, p=0.9).

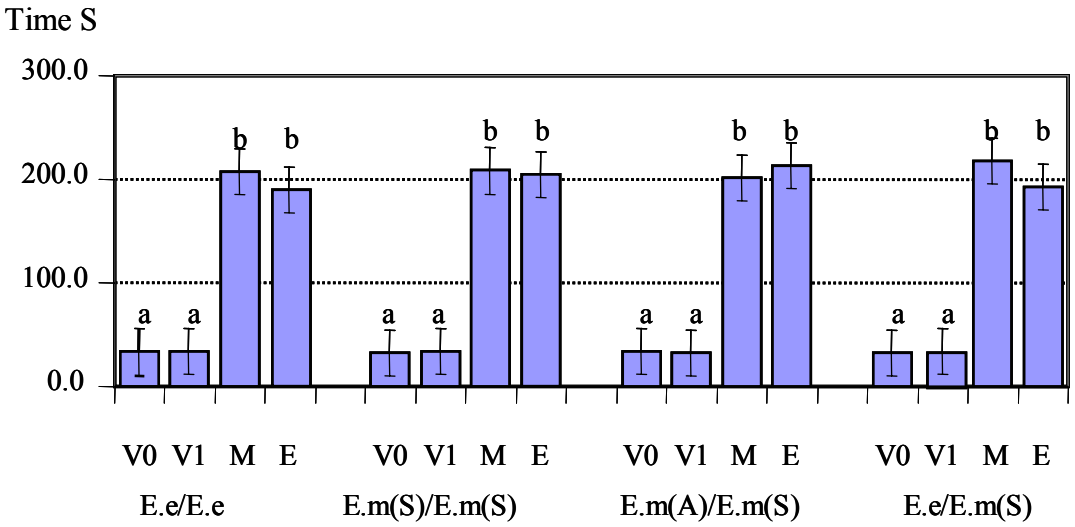

Figure 2. Time to reach end of wind-tube or time spent in wind-tube by *Eretmocerus* males when exposed to volatiles produced by *Eretmocerus* females.
a and b = Significant difference

V0 = Newly emerged female
V1 = One-day-old virgin females
M = One-day-old mated females
E = Empty chamber

♂/♀ = ♂ to ♀ of:
E. e = *E. eremicus*
E. m (S) = *E. mundus* from Spain
E. m (A) = *E. mundus* from Australia

Reaction of males to non-volatile pheromones on leaves

There was a significant difference between the time allocation of male on different patches (arrhenotokous virgin females with mated female patch or clean leaf); (Kruskal-Wallis χ^2 (7)=51.4, p<0.000). In arrhenotokous populations, males spent the whole recording period on or just around the patches where conspecific virgin females had walked earlier (figure 3a).

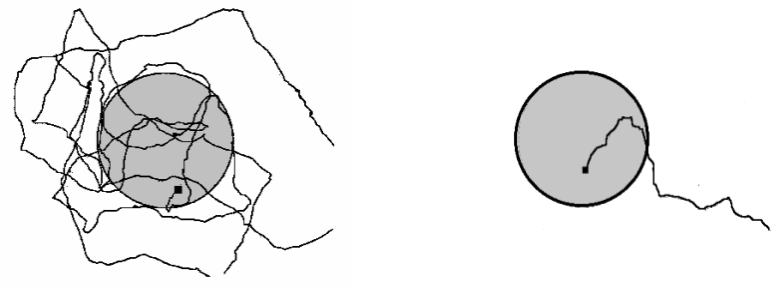

Figure 3. Example of reaction of males to non-volatile sex pheromone of a conspecific female in *E. eremicus* or *E. mundus*.
a. Track of male on patch where a virgin female was present earlier.
b. Track of male on patch where a mated female was present earlier.

However, there was not any significant difference between the walking distance of males on the patches that were visited earlier by conspecific-mated females and clean parts of the leaves (Kruskal-Wallis χ^2 (1)=1.2, p=0.3) (Figure 4). In the latter situation, the males either left the patch or they stood still for a moment and then jumped (figure 3b).

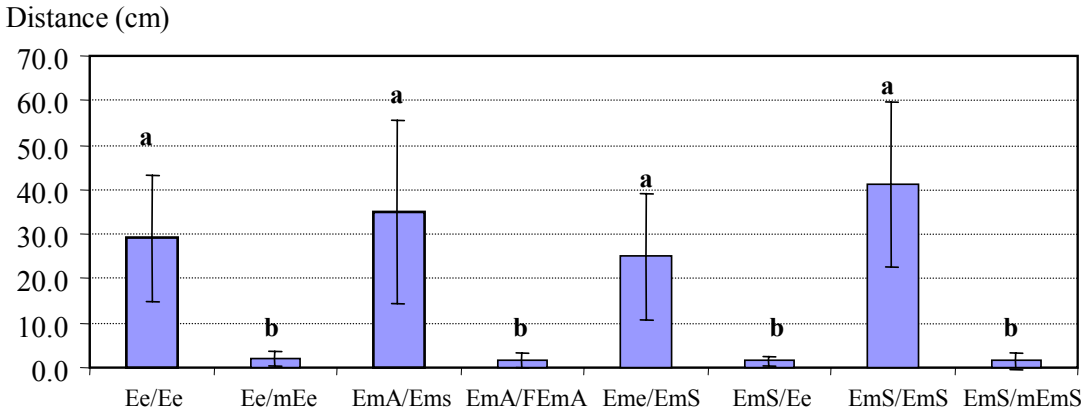

Figure 4. The distance (Mean± SD) that males walked on a conspecific virgin patch **a** and **b**= Significant difference

In intra-species tests, there was not any significant difference between the total distance that the Australian males walked on and around the patches of the Spanish virgin females and the distance that Spanish *E. mundus* males walked (Kruskal-Wallis χ^2 (1) =0.15, p=0.7)(Figure 4). However, Australian or Spanish males did not show any reaction on the patches that were visited by thelytokous females from Australia. In this case they walked away, stood still or jumped.

In inter-species tests, the males of *E. eremicus* searched on and around the patches of virgin females of *E. mundus* from Spain like conspecific Spanish males (Kruskal-Wallis χ^2 (1)=0.57, p=0.4). In contrast, the *E. mundus* males of both populations did not show such behavior to the patches visited by virgin females of *E. eremicus* (Figure 4).

Experiment 2. Mating behavior and hybridization

Mating behavior

After encounter, virgin females responded to males by standing still and allowing mating. Males usually mounted the females from the side, then moved forward, put the fore-legs on the female head near the eyes, grasped her with his mid-legs and put the hind-legs on the female wings. After this the *E. eremicus* males started antennation for several seconds (94.2 ± 25.2 SD) and then rubbed their mid-legs against the anterior edge of the female thorax three times. In contrast, *E. mundus* males antennated only a few times followed by a few seconds of standing still on the back of the female (14.7 ± 1.6 SD). In both species, females put their antennae down after antennation and leg rubbing. The males then bent backwards to mate by putting their fore- and mid-legs on the female's wings, and the hind-legs around her

abdomen. The duration of mating was shorter in *E. mundus* than *E. eremicus* (table I, Figure 5).

After mating, the males of both species mounted the females again. In *E. eremicus* the males started antennating the females for more than 100 seconds (109.0 ± 12.0 SD), whereas the males of *E. mundus* drummed the head of the females with their fore-legs for the same amount of time (113.0 ± 15.0 SD) (table I, Figure 5).

Table I. Comparison of aspects of mating behavior in *Eretmocerus mundus* and *E. eremicus*; (time in seconds ± SD)

Mating behavior	*E. eremicus*	*E. mundus*
Encounter	+	+
Mounted	+	+
Pre-mating antennation	94.2 ± 25.2 SD	15 ± 1.6 SD
Moving of mid-legs	Two or three times	Not
Mating time	5.3 ± 0.9 SD	3.5 ± 1.1 SD
Post-mating antennation	109.2 ± 12 SD	3.7 ± 1.3 SD
Post-mating movement of fore-legs	3.2 ± 0.8 SD	112.7 ± 14.5 SD
Volatile pheromone	+	+
Non-volatile pheromone	+	+
Mating capacity of female	Twice with two males	Twice with two males
Inter-specific response to non-volatile pheromone	+	-

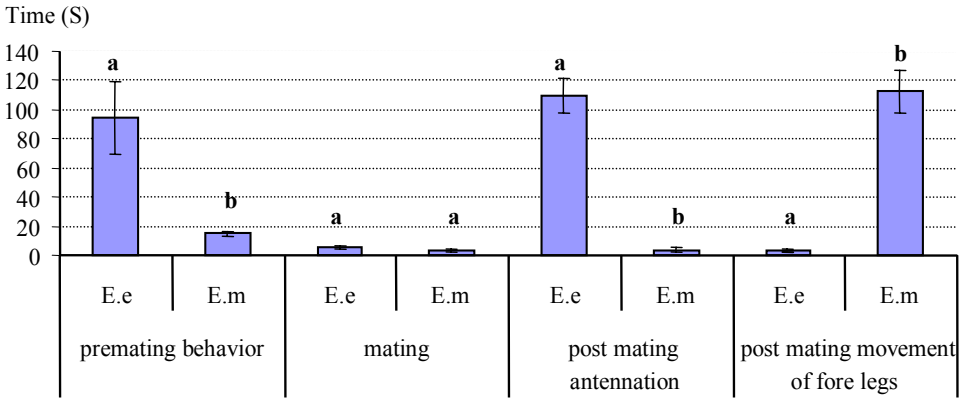

Figure 5. The duration of different phases in the mating behavior of *E. eremicus* (= E.e) and *E. mundus* (= E.m, Spanish population). a and b= Significant difference

In all combinations of intra- or inter-species tests, only the Australian *E. mundus* males and *E. eremicus* males reacted to Spanish *E. mundus* females, and could mount these females easily, and began pre-mating behavior. However, the females either moved forwards or did not lift their abdomen for mating and tried to push away those males with their right hind-leg. In these cases the males moved forwards again in mounted position and the female began to clean her genitalia with her hind-legs. The

pre-mating behavior lasted very long, 5 to 15 minutes, in this situation. Finally the males moved away, but they often came back and mounted the females again. We recorded seven successful matings (copulation) between Australian *E. mundus* males and Spanish females out of 17 attempts (result see below).

In both species, a male was never observed to mate twice with one conspecific female. When a male found a female that had already mated with him, he would leave her. In contrast, a female could mate with two different conspecific males within 10 minutes, but when a third male was introduced she did not accept him anymore (n=15 for each species).

Hybridization

As there was not any attraction between Spanish *E. mundus* males and thelytokous Australian *E. mundus* females, successful matings were not recorded. In contrast, we recorded seven successful matings (out of 17 tests) between *E. mundus* males from Australia and Spanish females of *E. mundus*. However, no hybrid females were produced (table II). In inter species tests, *E. mundus* males were not attracted by virgin *E. eremicus* females, whereas the males of *E. eremicus* were attracted by the virgin females of *E. mundus.* However, no successful matings occurred (table II).

Table II. Reaction to pheromone and possibility of hybridization in *Eretmocerus mundus* and *E. eremicus*.

Female	Male	Reaction to female	Mating	Hybridization
E. mundus (S)	*E. mundus (S)*	Yes	Yes	Conspecific female
E. mundus (A	*E. mundus (A)*	No	-	-
E. eremicus	*E. eremicus*	Yes	Yes	Conspecific female
E. mundus (A)	*E. mundus (S)*	No	No	-
E. mundus (A)	*E. eremicus*	No	No	-
E. mundus (S)	*E. mundus (A)*	Yes	Yes	No female progeny
E. mundus (S)	*E. eremicus*	Yes	No	-
E. eremicus	*E. mundus (A)*	No	No	-
E. eremicus	*E. mundus (S)*	No	No	-

A= Australian S= Spanish

Discussion

Sex pheromones

In arrhenotokous populations, mate-searching efficiency affects the success of parasitoids in establishing a population (Hopper and Roush, 1993) and has important consequences for sex allocation strategies under field conditions. In arrhenotokous populations of *Eretmocerus*, we show in this paper that males appear to respond both to volatile and non-volatile pheromones of conspecific virgin females. Volatile sex pheromones attract males over long distances, and non-volatile sex pheromones enable males to track females at close range. The responses of males to one-day-old virgin females indicate that they release sex pheromones before mating. However, it

seems the mating process influences release of pheromones, where the mated females no longer attract conspecific males.

In inter-population experiments, Australian males of *E. mundus* that are produced by *Wolbachia*-cured females do not show any reaction towards conspecific thelytokous females from Australia. In contrast, they show the same reaction as Spanish *E. mundus* males to the virgin females from Spain. A possible interpretation is that on the one hand Australian males are still capable to react to sex pheromones. On the other hand, as the production of sex pheromones may be costly, this trait may have gone lost in females of the *Wolbachia*-infected Australian population. Laboratory experiments have also shown that in certain circumstances males of *Trichogramma cordubensis* (which were produced by curing a thelytokous population from *Wolbachia* infection) do not attempt to mate with conspecific thelytokous females, whereas they sometimes attempt to mate with heterospecific females (*T. turkestanica* and *T. evanescens)*. It has been suggested that *T. cordubensis* males retain their ability to react to sex pheromones, but that the thelytokous females either do not produce sex pheromones or that they release them in amounts that are too low to excite conspecific males (Silva and Stouthamer, 1997).

E. eremicus males show interspecific reactions to the sex pheromones of *E. mundus*, and are able to mount females of this species. However, they could not achieve mating, possibly because of differences in mating behaviors between the species (table I). The responses of *E. eremicus* males to *E. mundus* females could pose a challenge for male mate finding in situations where both species are present. Therefore, from a biological control point of view, using *E. eremicus* in an area where *E. mundus* is native could negatively affect mate finding in *E. eremicus.*

E. eremicus males do not show any reaction to Australian females of *E. mundus,* and, unexpectedly, Australian *E. mundus* males also show no reaction to *E. eremicus* females. Thus, it may be possible to use both species together without a risk of reducing efficiency through hybridization between them, although other aspects of their biology and behavior should be considered before advising combined releases of the two species.

Courtship behavior

Speciation through reproductive isolation may be facilitated by differences in mating behavior. In general, three different phases of mating behavior can be distinguished in Aphelinid parasitoids: “pre-mating”, “mating” and “post-mating” (Viggiani and Battaglia 1983**).** The discrimination of interpopulation mates before any successful mating takes place (i.e. pre-mating behavior) is expected to have the largest influence on isolation of populations.

The two populations of *E. mundus* from Spain and Australia are geographically and reproductively different. Our Spanish population is arrhenotokous and the Australian one is thelytokous. Since the thelytoky in *E. mundus* is caused by *Wolbachia*, high temperatures may change the mode of reproduction from thelytoky

to arrhenotoky in the field as it is known that high temperatures kill *Wolbachia* like antibiotics do (Rigaud and Juchault, 1998). This situation could lead to successful mating, if one population of *E. mundus* is released in the native area of another. Moreover, because they do not produce any hybrid females, their reproductive success may decrease in the next generation. The lack of hybrid female offspring can have several causes: (1) no insemination takes place, (2) insemination takes place but males develop from fertilized eggs, (3) fertilized eggs die and the all-male offspring produced stem from unfertilized eggs (Stouthamer *et al.,* 1996). To shed more light on these aspects, more study is needed.

The geographically isolated *E. eremicus* and *E. mundus* also show several differences in mating behavior. These differences can be detected in all three phases of the mating behavior: pre-mating, mating and post-mating. The differences are clearest in the pre-mating behavior. *E. eremicus* shows a relatively long period of antennation followed by three times rubbing of the mid-legs against the anterior edge of the female thorax. *E. mundus* males, on the other hand, antennate only during one short bout followed by a few seconds of standing still on the back of the female. It seems that these differences lead to pre-mating isolation between *E. mundus* and *E. eremicus,* through rejection of *E. eremicus* males by *E. mundus* females.

Biological control perspective

In arrhenotokous parasitoids, unmated females are able to produce male progeny. Mate finding is of crucial importance in arrhenotokous parasitoids for long-term natural enemy presence and pest control. Therefore, as the thelytokous *Eretmocerus* population described in this study does not need to mate to produce female offspring, it may have an advantage over an arrhenotokous population, particularly at low pest densities (Aeschlimann, 1990, Stouthamer, 1993, 2003). On the other hand, as mentioned above, releasing of one of the two populations of *E. mundus* in a native area of the other could result in unsuccessful mating between them. In this situation they can not produce hybrid females. Therefore, the progeny will become more male biased, reducing the efficiency of *E. mundus* to control the pest. To design a biological control program, a lot of issues (e.g, biology, foraging behavior, and environmental conditions) should be considered to release one of these populations/species as biological control agent. Therefore, we conclude that mate finding and mating behavior deserves more attention in the study of effectiveness of biological control agents.

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Divergence between sexual and asexual *Eretmocerus mundus* wasps: does the cytoplasmic bacterium *Wolbachia* play a role in speciation?

Abstract

The parasitoid *Eretmocerus mundus* displays both arrhenotokous (sexual) and thelytokous (asexual) reproduction. The latter reproductive mode is induced by the cytoplasmic bacterium *Wolbachia*. We studied the divergences of two nuclear genomic regions (ITS1 and ITS2) and a mitochondrial region (COII) in several sexual populations of *E. mundus* from Europe, an asexual *E. mundus* population from Australia, and a sexual population of *E. eremicus*. Their phylogenetic relationship was analysed using additional data of other populations and species retrieved from Genbank. Analyses of the sequence divergences and constructed trees showed differences among populations and species, while the ITS2 regions showed clearer differences than the ITS1 or COII regions. Trees that were constructed using different clustering methods, and based on sequence differences of the three regions, were congruent. In all cases, sexual European and asexual Australian populations of *E. mundus* formed two different groups, showing genetic diversity exceeding that between recognised species such as *E. eremicus* and *E. warrae*. Since these *E. mundus* populations are also reproductively isolated, we argue that they should be considered different species, and we suggest that *Wolbachia* may have played a role in this speciation through pre-mating effects.

Introduction

Insect may have different modes of reproduction. Hymenopteran parasitoids, for example, commonly show arrhenotokous reproduction, where fertilized eggs lead to diploid females and unfertilized eggs to haploid males. Thelytokous reproduction may also occur, where females arise from unfertilized eggs. In some cases, both of these reproductive modes have been found to occur in one insect species (Stouthamer and Kazmer, 1994; Arakaki et al. 2000; Stouthamer et al. 2001; Schneider et al. 2003). In a number of instances, it has been shown that the cytoplasmic bacterium *Wolbachia* causes the thelytokous reproductive mode (for reviews see Stouthamer 1997; Huigens and Stouthamer, 2003). Such intra-specific differentiation of the reproductive modes leads to the interesting possibility of reproductive isolation between both modes, which may be directly influenced by a *Wolbachia* infection.

This chapter has been submitted to "Molecular Ecology".

In the present study, we examine the amount of sequence-divergence between arrhenotokous and thelytokous populations and species of *Eretmocerus* spp. (Hym. Aphelinidae) to answer the question to what extent a *Wolbachia*-infected thelytokous population of one species of *Eretmocerus* is divergent from arrhenotokous conspecifics, and other species.

The genus *Eretmocerus,* a whitefly parasitoid, contains 53 described species throughout the world (UCD, NM: Universal Chalcidoidea Database, Natural Museum, UK). For instance, arrhenotokous (from now on referred to as sexual) populations of *E. mundus* have been recorded from many parts of the Mediterranean basin (Mound and Halsey, 1978) and occur in other parts of the world as well (UCD, NM). Notably, a thelytokous (henceforth referred to as asexual) population of *E. mundus* has been found in Australia (de Barro *et al.*, 2000). This thelytoky is induced by the cytoplasmic bacterium *Wolbachia* (de Barro and Hart, 2001).

The asexual and sexual populations of *E. mundus* do not show any distinctive differences in morphological characters (de Barro, 2000). However, they do show reproductive isolation even after curing asexual wasps from their *Wolbachia* infection, where they cannot successfully reproduce with sexual wasps (Ardeh *et al*., 2004). Therefore, we embarked on a study of genetic divergences between asexual and sexual populations of *E. mundus*, to investigate if these support the species-status suggested by their reproductive isolation.

Several molecular techniques are used to discriminate between closely related populations or cryptic species (Landry *et al.,* 1993; Hoy *et al.,* 2000; Caterino *et al*., 2000). The most direct molecular techniques involve the comparison of gene sequences to detect the divergence among insect populations and species (Loxdale and Lushai, 1998; Caterino *et al*., 2000; Hoy, 2003). The most critical step is to choose an appropriate region among these sites for a particular systematic question (Hwang and Kim, 1999).

Mitochondrial protein-coding regions, such as cytochrome oxidase I (COI) and II (COII), have been extensively used for phylogenetic and phylogeographic inference in many insect groups (Hoy*,* 2003; Lin and Danforth, 2004). Mitochondrial genes have several advantages (e.g. easier to amplify than nuclear genes and absence of interspersing non-coding regions) (Lin and Danforth, 2004). However, they may show a higher level of homoplasy (Caterino *et al*., 2000, Frati *et al*., 1997; Mooers and Holmes, 2000). Therefore, a combination of mitochondrial and nuclear genes is frequently used to resolve systematic relationships (Lin and Danforth, 2004).

The ribosomal genes are most commonly used in insect systematics, along with mitochondrial genes (Avise 2000; Caterino *et al*., 2000). Ribosomal DNA (rDNA) is a gene complex of coding and non-coding parts found in the nuclear DNA. Functional parts of ribosomal sequences are highly conserved (Gerbi, 1986). Therefore, these parts are used to design primers to amplify the noncoding parts. Other, interspersing parts, such as the 'internal transcribed spacer' (ITS1 and ITS2), show higher levels of polymorphism and have proved useful for comparing closely related insect species,

subspecies, or populations (Caterino *et al*. 2000; Hoy, 2003; Porter and Collins 1991; Stouthamer *et al*., 1999).

Sequence comparison of the ITS1 regions of different species of *Eretmocerus* showed differences between one sexual (Spanish) population and an asexual one (Australian) of *E. mundus.* The variation has been suggested to be intraspecific rather than interspecific (de Barro *et al.,* 2000). However, since these *Eretmocerus* populations are reproductively isolated (Ardeh et al., 2004), we suspect that they should actually be considered different biological species, despite their morphological similarity.

In this paper, we describe sequence divergences of the ITS1, ITS2 and COII regions between several sexual populations of *E. mundus* from Europe, one asexual population from Australia, one sexual population of *E. eremicus,* along with data of other sexual populations and species retrieved from Genbank. The first objective of this study was to examine the utility of the ITS1, ITS2, and COII diversity for distinction of *Eretmocerus* species. The second, and main, objective of our research was to investigate whether the level of intra- and intergenomic variation within the ITS1, ITS2, and COII region was sufficiently great to detect phylogenetic separation between the sexual and asexual populations of *E. mundus*.

Material and Methods

Specimens

Five sexual populations of *E. mundus* from Europe, one asexual population from Australia, along with one sexual population of *E. eremicus* were used in this study (Table 1).

Table 1. The sources and origins of the specimens that have been used for comparison of the ITS1, ITS2, and COII region.

Species	Country of origin	Source
E. mundus	Spain	Koppert company
	Spain	Biobest company
	Italy	BioPlant company
	Italy	Personal collection
	Turkey	Personal collection
	Australia	Personal collection
E. eremicus	USA	Koppert company
E. hayati	Pakistan	*Genbank data base*
E. mundus	Australia	*Genbank data base*
E. queenslandensis	Australia	*Genbank data base*
E. sp.	Ethiopia	*Genbank data base*
E. warrae	Australia	*Genbank data base*

PCR amplification of ITS1, ITS2, and COII, cloning and sequencing

PCR reactions were performed in 50 µl volumes including: 5 µl DNA template, 5 µl PCR-buffer, 1 µl dNTP's (each in a 10 mM concentration), 1 µl each of forward and reverse primer (10 ng), 0.14 µl of *Taq* DNA polymerase, and 36.86 µl of sterile distilled water. The sequences of the primers and the PCR cycling program are shown in Table 2. Each program started with a cycle at 95ºC for 3 minutes and finished with a cycle at 72ºC for 5 minutes.

Table 2. The primer names, sequences, and the PCR-reaction program to amplify ITS1-, ITS2-, and COII regions.

Name	Sequence of primers	Den	Ann	Ext	nC
ITS1 Forward	TCCGTAGGTGAACCTGCGG	94°C	58°C	72°C	35
ITS1 Reverse	GCTGCGTTCTTCATCGATGC	1	1	1.5	
ITS2 Forward	TGTCAACTGCAGGACACATG	94°C	60°C	72°C	35
ITS2 Reverse	ATGCTTAAATTTAGGGGGTA	1	1	1.5	
COII Forward	ATTGGACATCAATGATATTGA	94°C	52°C	72°C	33
COII Reverse	CCACAAATTTCTGAACATTGACCA	1	1	1.5	

Den=Denaturation, Ann=Annealing, Ext= Extension, nC= Number of cycles; Below the temperatures, the duration of each phase of the cycle is indicated in minutes.

Alignment and Phylogenetic analyses

After PCR, electrophoresis of products in 1.5 % agarose gels was done (10 µl). If the PCR product showed a clear band, the remainder of the PCR products were purified with QIAquick PCR purification kits (Qiagen®).

The purified DNA of each sample was ligated into a Pgem-T vector (Promega) and transformed into *Escherichia coli* using heat shock. To check the insertion of the desired fragment, a PCR reaction with the same primers as before was conducted for each sample. DNA was purified from *E. coli* colonies using the QIAprep Miniprep kit (Qiagen®) and sequenced.

The sequences were aligned using the Clustal W option of MegAlign version 4.00 (DNASTAR, Madison, Wisconsin, USA) and were examined further by comparing them with other sequences of *Eretmocerus* species in the GenBank database. Phylogenetic analyses were conducted using the Mega3 program (Kumar *et al.,* 2004). To determine the mean value of genetic divergence within or among the populations and species, the Kimura 2-parameter model was used. Phylogenetic trees were constructed with the Maximum parsimony (MP) and Neighbour Joining (NJ) methods. Node supports were assessed by the bootstrap technique (10000 replicates).

Results

PCR amplification and sequencing results

The ITS1 primers amplified about 540bp of the ribosomal DNA (rDNA) of *E. mundus* populations, while the fragment was longer in *E. eremicus* (approximately 680bp). The ITS2 primers amplified 440bp of the rDNA and COII primers amplified 267bp of mitochondrial DNA across all populations and species. The average of the base

frequencies of the sequences revealed a higher percentage of A+T in the COII sequences (79.2) than in the ITS1 (45.8) or ITS2 (41.8) sequences. The sequences were deposited in the GenBank database (accession numbers for: (1) the ITS1 regions: AY878186- AY878191, (2) the ITS2 regions: AY877317-AY877325, and (3) the COII regions: AY878175-AY878185).

Alignment and divergence of sequences

We had to ignore a part of the termini from each sequence to be able to compare them with the other sequences retrieved from GenBank. Subsequently, alignments and comparisons showed 708 matching characters for ITS1, 457 for ITS2, and 271 for COII. Of those characters, 19% for ITS1, 14% for ITS2, and 26% for COII were informative.

The fragments of the ITS1 region of *E. eremicus* and *E. warrae* were very similar but longer than those of the other species. Therefore, alignment showed several gaps and consequently ambiguous parts along the sequences of other species. In contrast, the fragments of the ITS2 region of *E. eremicus* and *E. warrae* were shorter than the fragment of the other species. Consequently, alignment showed two gaps (13 and 32 base pairs long, respectively) in the first part (5′ side) of the sequences of *E. eremicus* and *E. warrae*, whereas some small gaps (2-8 base pairs long), and consequently ambiguous parts, appeared at the terminal parts (3′ side) of the fragments. The alignment of the COII region did not result in any gap along the fragments.

The sequence divergences within populations were less than 0.017 in ITS1, 0.014 in ITS2, and 0.081 for COII (Table 3), whereas the sequence divergences across populations or species were as large as 0.291 for ITS1, 0.127 for ITS2, and 0.267 for COII (Table 3).

Table 3. The sequence divergences within (bold, diagnoses) and between *Eretmocerus* populations and species

ITS1							
	Au	**Eu**	**Ha.**	**Qe**	**Sp.**	**Ew**	**Ee**
Au.	**0.014**						
Eu.	0.022	**0.011**					
Ha.	0.021	0.033	**0.017**				
Qe.	0.203	0.215	0.211	**0.004**			
Sp.	0.017	0.009	0.028	0.209	**0.000**		
Ew.	0.282	0.293	0.291	0.240	0.286	**0.002**	
Ee.	0.270	0.281	0.280	0.232	0.274	0.016	**0.000**

ITS2					
Au	**Eu**	**Qe**	**Ew**	**Ee**	
0.009					
0.037	**0.014**				
?	**?**				
0.011	0.043	**0.013**			
?	**?**	**?**			
0.123	0.123	0.127	**0.001**		
0.122	0.126	0.126	0.011	**0.000**	

COII					
Au	**Eu**	**Qe**	**Sp.**	**Ew**	**Ee**
0.035					
0.098	**0.081**				
?	**?**				
0.247	0.294	**0.001**			
0.058	0.083	0.233	**0.005**		
0.221	0.267	0.236	0.202	**0.004**	
0.210	0.251	0.224	0.207	0.014	**0.000**

E.e= *E. eremicus*, E.ha= *E. hayati*, E.ma=Australian *E. mundus*, E.me= European *E. mundus,* E.que= E. *queenslandesis,* E.wa= *E. warrae*, "?" = data were not available.

In all cases, the sequence divergences within Australian populations or within European populations of *E. mundus* were lower than between populations. Interestingly, the divergences between Australian and European populations of *E. mundus* were larger than the sequence divergence between the two species *E. eremicus* and *E. warrae* (one originating from the USA, the other from Australia) or between Australian *E. mundus* and *E. queenslandensis* (Table 3).

Phylogenetic analyses and trees

Phylogenetic analyses of the ITS1-, ITS2-, and COII regions with the MP and NJ methods showed several groups in the constructed unrooted trees (analysis excluding gaps). In all trees, the European and Australian populations of *E. mundus* clustered into two separate groups. For instance, in the constructed trees of the ITS1 region the Australian *E. mundus* and *E. hayati* appeared in one cluster, whereas the European populations of *E. mundus* and one species from Ethiopia formed another cluster (Figure 1). The constructed trees for ITS2 regions showed this separation as well, where the Australian *E. mundus* and *E. queenslandensis* formed part of one cluster and the European populations of *E. mundus* another (Figure 2). The results were similar for the COII trees, where the Australian population appeared in one group and the European *E. mundus* and the species from Ethiopia in another (Figure 3).

Re-analysis of the data including gaps did not change clustering into the groups described above; i.e. the in or exclusion of indels did not affect the results. Clearly, there was a large degree of congruence between the phylogenetic trees, with both methods (MP and NJ) and independent of the genomic region.

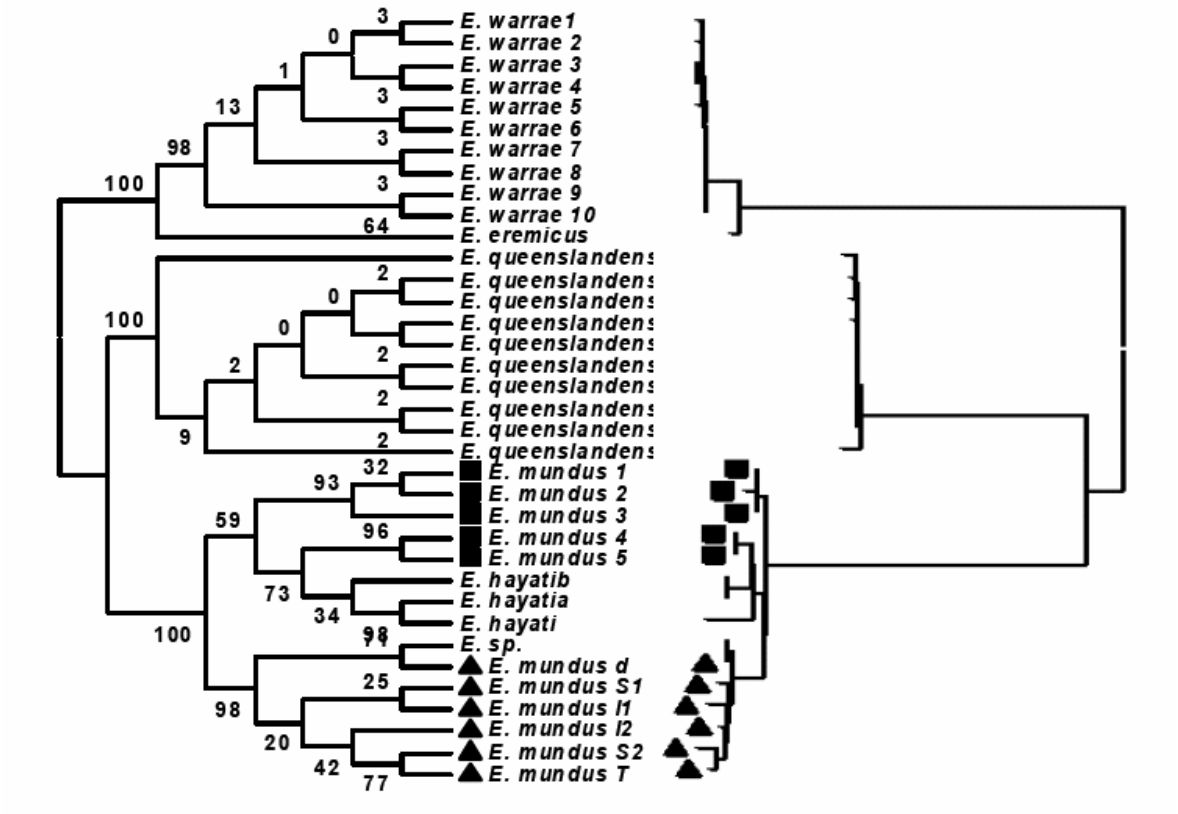

Figure 1. Constructed trees based on MP (left) and NJ (right) methods of the ITS1 regions of *Eretmocerus* spp. (Number shows Bootstrap values, ▲=European populations, ■ =Australian populations, D= de Barro, I= Italy, S= Spain, T= Turkey

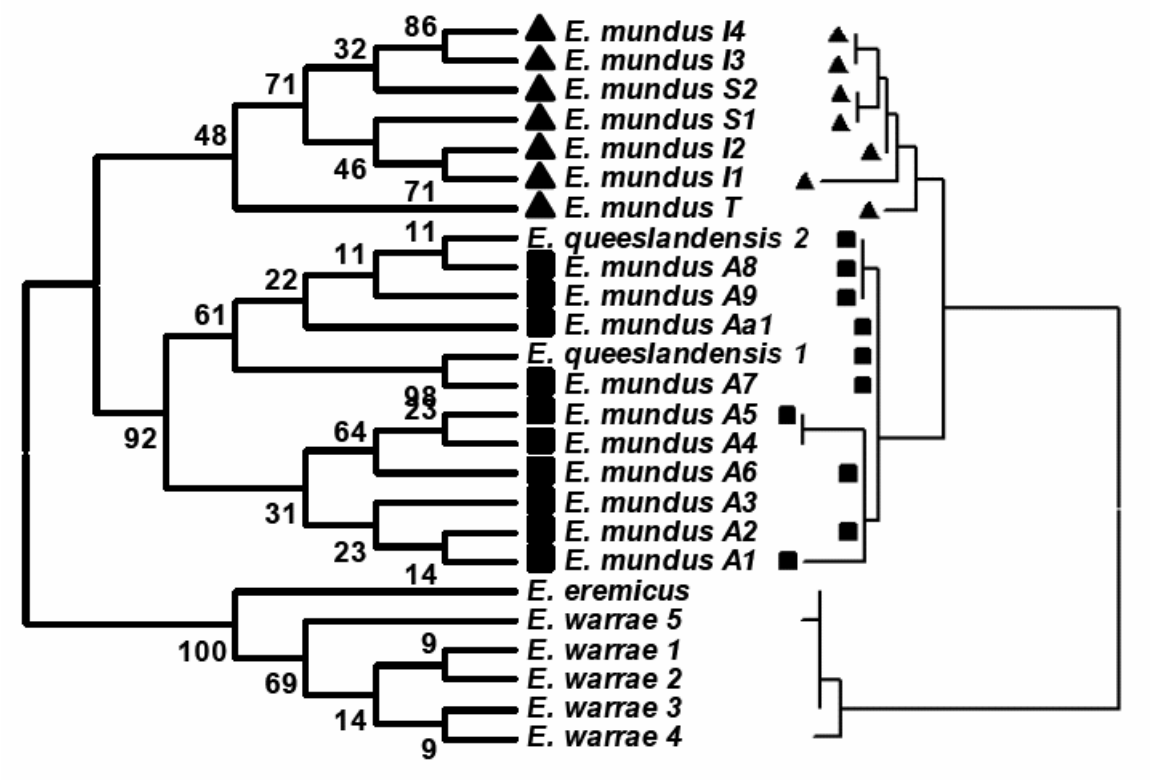

Figure 2. Constructed trees based on MP (left) and NJ (right) methods of the ITS2 regions of *Eretmocerus* spp. (Number shows Bootstrap values,▲=European populations,■=Australian populations, I=Italy, S= Spain, T= Turkey.

Discussion

In addition to the reproductive isolation between sexual *E. mundus* wasps from Europe and the asexual population from Australia, these populations have also diverged at the molecular level, as shown by analysis of the ITS1-, ITS2- and COII regions. The use of internal transcribed spacer (ITS) regions predominates in phylogenetic studies of Hymenoptera (Caterino *et al.,* 2000; Hoy, 2003; van Veen *et al.,* 2003). These regions are noncoding sites, so mutations are more tolerated than in coding regions (Haymer, 1994). Therefore, variation can be recorded both within and between populations (Avise, 1994). COII regions, on the other hand, are more conserved coding parts of the mitochondrial DNA and mutations might be lethal (Caterino *et al.,* 2000). Therefore, the sequence divergences are expected to be less pronounced in these regions than in the ITS1 and ITS2 regions. Yet, as these regions have been used in the study of Australian populations and species of *Eretmocerus* (de

Barro *et al.,* 2000), we included the same primers in our present study to be able to compare the *Eretmocerus* populations and species.

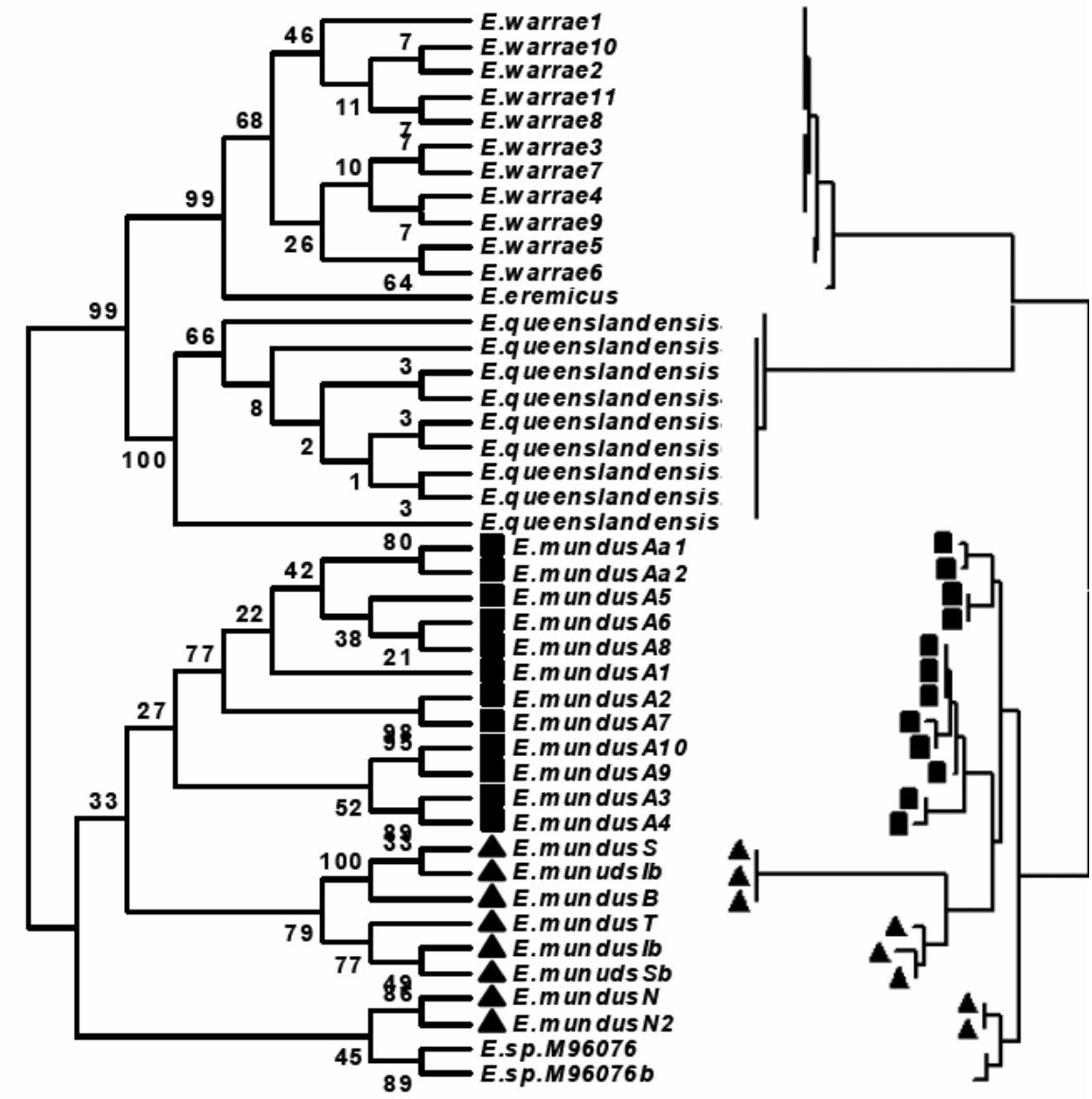

Figure 3. Constructed trees according to MP (left) and NJ (right) methods for the COII regions of *Eretmocerus* spp. (Number shows Bootstrap values, ▲= European populations, ■= Australian populations, A= Australia, I= Italy, S= Spain, T= Turkey

There were some sequence divergences within the European and the Australian populations of *E. mundus* in the ITS1, ITS2, and COII regions. These divergences were larger in the European populations than the Australian ones, which may be influenced by (1) differences in mode of reproduction (sexual vs. asexual) and/or (2) a wider geographical distribution of the European, vs. the Australian, samples. However, the sequence divergences were much lower than those between the two populations of *E. mundus*. Moreover, sequence divergences between *E. mundus*

populations were much larger than the divergence between *E. eremicus* (originally from the USA) and *E. warrae* (originally from Australia) that are considered different species. These divergences had impact on the constructed trees based on both clustering methods (MP and NJ) where the two *E. mundus* populations formed different clusters whereas *E. eremicus* and *E. warrae* formed one cluster. These divergences and separation between the European and the Australian populations of *E. mundus* support our suggestion of speciation within *E. mundus*.

The asexuality in the Australian population of *Eretmocerus* is caused by parthenogenesis-inducing (PI) *Wolbachia* from group B (de Barro and Hart 2001). PI *Wolbachia* infections are common in hymenopteran parasitoids (reviewed in Stouthamer, 1997; Huigens and Stouthamer, 2003) and have been suggested to drive speciation as they may facilitate reproductive isolation (Werren, 1998; Bordenstein, 2003). Reproductive isolation can namely occur due to a loss of sexual functions in the asexual population. In most species completely infected with PI *Wolbachia*, the female sexual traits have degraded (Huigens and Stouthamer, 2003; Bordenstein 2003). Only in infected *E. mundus* and *Muscidifurax uniraptor* wasps also male sexual traits have become non-functional (Ardeh *et al.,* 2004, Gottlieb and Zchori-Fein, 2002). This male-female discrepancy may be explained by male sexual traits that accumulate neutrally, as there are no males in the population, and costly female sexual traits that are actively selected against in infected females. Mutations leading to sexual degradation may arise after all the females in the population have become infected with PI *Wolbachia*, i.e. after the population is fixed for the infection (Pijls et al. 1996; Bordenstein, 2003). Degraded female sexual traits that arise during the spread of the PI *Wolbachia*-infection may, however, also have a selective advantage and eventually help the infection reaching fixation (Huigens and Stouthamer, 2003).

During the initial spread of a PI *Wolbachia*-infection, gene flow should still occur between the uninfected and infected population because infected females still mate. This has been shown in *Trichogramma* wasps where infected and uninfected individuals coexist (Stouthamer and Kazmer, 1994). Therefore, additional barriers besides PI *Wolbachia* are necessary to complete a speciation event. These may be geographical barriers. Geographical isolation alone might, however, also influence speciation (Wu, 2001). The sexual *E. mundus* populations from Europe and the asexual population from Australia are clearly geographically distant from each other. Currently, we can not distinguish whether the reproductive isolation and divergence between them is caused by PI *Wolbachia* alone, geographical barriers alone, or by a combination of both. The genus *Eretmocerus* does, however, provide us with an excellent opportunity to study the role of PI *Wolbachia* in speciation in the future. First, we need more detailed sampling. This might not only reveal more allopatric sexual and asexual wasps, but also sympatric sexual and asexual wasps. When, in contrast to the allopatric sexual and asexual *E. mundus* populations, allopatric sexual *E. mundus* populations do hybridize, this would support a role of PI *Wolbachia* in speciation. Secondly, once more sexual and asexual *Eretmocerus* wasps have been

discovered, a more extensive phylogenetic analysis of the genus *Eretmocerus* should be carried out. Speciation promoted by *Wolbachia* is expected to occur at higher rates than genetically based reproductive isolation as *Wolbachia* may spread faster through populations than nuclear genes causing reproductive isolation (Bordenstein, 2003). A more frequent speciation in infected asexual *Eretmocerus* wasps than in congeneric sexual wasps should demonstrate a role of PI *Wolbachia* in speciation.

The definition of speciation is based on the biological species concept or reproductive isolation (Wu, 2001). Consequently, our present results, combined with the fact that Australian and European *E. mundus* can not hybridize (Ardeh *et al.,* 2004), supports the interpretation that they should be considered different species. We believe that in those cases group level diagnostics based on the sequence divergences of DNA, particularly of the rDNA regions, could be relatively easily developed for identification of species of tiny parasitoid wasps such as *Eretmocerus.*

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Selection of *Bemisia* nymphal stages for oviposition or feeding, and host-handling times of arrhenotokous and thelytokous *Eretmocerus mundus* and arrhenotokous *E. eremicus.*

Abstract

Host-handling behavior is an important aspect of parasitoid foraging behavior. When a parasitoid encounters a potential host, the handling behavior starts with the evaluation of the host and continues if the host has been judged acceptable. Host-handling is usually terminated after egg laying or host feeding and host marking. Host-handling behavior of an arrhenotokous population of two *Eretmocerus* species, *E. mundus* Mercet and *E. eremicus* Rose & Zolnerowich, along with a thelytokous population of *E. mundus* were compared under laboratory conditions. Several elements of host-handling behavior, including encountering, ascending, turning on host, descending, preening, egg laying, and host feeding were recorded. There were no correlations among the durations of these phases across parasitoid populations/species or host nymphal instars. Duration of different phases of host-handling behavior showed only slight and sometimes significant differences between different *Eretmocerus* populations/species. The actual laying of the egg had the longest duration of all host-handling behaviors, and was longer on third nymphal instars than on younger ones. Females of the three populations/species accepted the first three nymphal stages either for egg laying or for host feeding. Females spent a lot of time to make wounds in the host when preparing for host feeding, and eventually killed the host. The implications of these findings for the use of the different *Eretmocerus* populations/species in biological control are discussed.

Introduction

Whiteflies are key pests world wide (van Lenteren & Noldus, 1990; Gerling & Mayer, 1995). They cause direct feeding damage, vector a number of devastating plant viruses, reduce the quality of the harvested product as a result of the excretion of honeydew, and can be the source of various other problems (Drost *et al.*, 1998). Control of whiteflies with chemical pesticides is often problematic because of the wide occurrence of resistance (e.g. Palumbo *et al.*, 2001).

During the past decades, much research was directed at finding efficient natural enemies of whiteflies (for overviews, see Gerling, 1990; Gerling & Mayer, 1995; Gerling *et al.*, 2001).

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To date, several species of parasitoids are used with great success to control whitefly in large commercial greenhouses (van Lenteren, 2000). The most efficient species belong to the aphelinid genera *Encarsia, Eretmocerus* and the Platygasterid genus *Amitus* (van Lenteren *et al.*, 1997; Drost *et al.*, 1999, 2000; Manzano *et al.*, 2000, 2002; de Vis *et al.*, 2003; Qiu *et al.*, 2004).

Gerling *et al.* (2001) list 34 species of *Encarsia*, 12 species of *Eretmocerus*, two species of *Amitus*, and one species each of *Signiphora* and *Methycus* as parasitoids of *Bemisia tabaci* (Gennadius) (Hom; Aleyrodidae), which is the most serious whitefly pest of vegetable, ornamental, and agronomic crops throughout the world (Gerling, 1990; Gerling and Mayer, 1995). Gerling *et al.* (2001) conclude that: "with the exception of *En. formosa* Gahan (Hym.; Aphelinidae)… and despite the frequent use of *Encarsia* species, data on their biological and taxonomic characteristics remain deficient even for commonly used species." One of these genera, *Eretmocerus,* contains two currently important commercial species: *E. eremicus* Rose & Zolnerowich (Hym; Aphelinidae) and *E. mundus* Mercet (Hym; Aphelinidae). *E. eremicus* is a native to the United States (Rose and Zolnerowich, 1997) and is an effective biological control agent of *B. tabaci* on poinsettia (Hoddle and Driesche, 1999). *E. mundus* has been recorded from many parts of the Mediterranean basin (Mound and Halsey, 1978) and is considered the most important whitefly control agent in the plastic greenhouses in southern Spain (Rodriguez *et al.*, 1994). The two *Eretmocerus* species that are now commercially used are arrhenotokous (bisexual). Interestingly, a population of *E. mundus* has been found in Australia, which is thelytokous (asexual) (de Barro *et al.*, 2000). As only females are effective in biological control, thelytokous reproduction can boost the effectiveness of a parasitoid in the form of lower production costs, easier establishment and quicker population growth (Stouthamer, 1993). Therefore, thelytokous *E. mundus* are considered better candidates for biological control of *B. tabaci* than arrhenotokous forms, particularly in the dry tropical regions where establishment is difficult (de Barro *et al.,* 2000).

To develop a successful biological control program, knowledge of the foraging behavior is fundamental (Lewis *et al.*, 1990; Godfray, 1994). During foraging, a parasitoid has to be able to find and accept a suitable host in order to achieve reproductive success. When a parasitoid encounters a potential host, the handling behavior starts with evaluation of the host (van Lenteren *et al.*, 1976). Host evaluation may include several steps such as antennation, probing, and drumming (van Lenteren *et al.,* 1980; Headrick *et al.,* 1996; Higuchi and Suzuki, 1996). In order to select a host, parasitoid females may use chemical cues or physical features of the host such as size, shape and texture (van Driesche and Bellows, 1996). Host selection is influenced by both external and internal factors, e.g. the developmental stages of the host (Vinson, 1998) and egg load of the parasitoid (Casas *et al.*, 2000). In addition to using hosts for oviposition, females of synovigenic species, where eggs develop during the adult life of the parasitoid, often use hosts for feeding to obtain essential nutrients. Host feeding is the consumption of host fluids exuding from a wound,

which is usually made by the female ovipositor (Jervis and Kidd, 1986). Host feeding is rare in pro-ovigenic parasitoids, where eggs are fully developed at the moment the female hatches (Jervis *et al.,* 2001). For example, host feeding usually occurs in *En. formosa* (synovigenic; van Lenteren *et al.,* 1987) but is rare in *A. fuscipennis* MacGown & Nebeker (Hym; Platygasteridae) (pro-ovigenic; de Vis, *et al.,* 2003).

As host feeding may result in killing of the hosts, a parasitoid female may select lower quality hosts for feeding and higher quality hosts for egg laying. Consequently, the female must make a decision whether to use a host for egg laying or for host feeding (Godfray, 1994). Host feeding is often more time consuming than egg laying (Heimple and Collier, 1996), and due to the difficulty of puncturing old nymphal instars, host feeding may occur more frequently on younger nymphs than on older ones (Kidd and Jervis, 1991).

The time budgets spent on foraging, and the kind of host-selection and feeding behavior should be considered to determine parasitoid effectiveness and to select the best species for biological control (e.g. Drost *et al*., 200; Hudak *et al.*, 2003). So far, some aspects of host-searching and host-handling of *E. eremicus* and/or *E. mundus* have been studied (e.g. Foltyn and Gerling, 1985; Headrick *et al.,* 1996; Drost *et al.*, 2000; Hudák *et al.*, 2003; Qiu *et al.*, 2004). However, much information about these behaviors is still incomplete. Therefore, we embarked upon a study of *Eretmocerus* to compare host-handling behavior between two arrhenotokous species (Spanish *E. mundus* and North American *E. eremicus*) and between an arrhenotokous (Spanish *E. mundus*) and a thelytokous population (Australian *E. mundus*). The results are discussed within the framework of biological control of whitefly.

Material and methods

Maintenance of insects

A culture of *B. tabaci* was maintained on poinsettia plants (*Euphorbia pulcherrima* Willd. ex Klotzsch, Euphorbiaceae) in a greenhouse (25°C, 75% RH, and 16L/8D light). Poinsettia plants were daily infested with 20-30 whiteflies and put in a cage. Whitefly infested plants were transferred to another cage after two days while the whiteflies were removed. Leaves of these plants were checked after 10 to 12 days and leaves with the right whitefly stages were removed and used in experiments.

Three populations of *Eretmocerus* were used: (1) an arrhenotokous population of *E. eremicus* (origin North America), (2) an arrhenotokous population of *E. mundus* (origin Spain) that are both commercially available (product name ErCal®, Koppert Biological Systems, The Netherlands), and (3) a thelytokous population of *E. mundus* (origin Australia), which is a non-commercial laboratory population (de Barro *et al.*, 2000). A culture of each parasitoid population was maintained on *B. tabaci* and poinsettia plants in a climate room at 25±1°C, 45±5% HR, and a 16L/8D photo period.

All experiments were done in a climate room at 25±1°C, 45±5% HR.

Host-handling behavior

Infested leaf parts (4x5 cm) with a mixture of different *B. tabaci* nymphal instars (N1, N2, and N3) were offered to the parasitoids. A preliminary experiment had shown that *Eretmocerus* did not accept N4 for oviposition, so N4 nymphs were not offered in the current experiments (Ardeh, unpublished results). Each leaf part was put in a Petri dish (11cm Ø) on a moist piece of cotton wool to prevent desiccation.

Parasitoid pupae were collected and put separately in a glass vial until they had emerged. Females were always used on the first day of emergence. To obtain mated females of arrhenotokous populations, males and females were released on an uninfested leaf part before the experiment until mating had taken place. Next, either a mated arrhenotokous or an asexual thelytokous female was released on an infested leaf part, and their foraging behaviors were recorded using a stereo microscope and The Observer Program 4.0® (Noldus, Information Technology) for a period of maximum one hour or until the female left the leaf part. The following behavioral elements were recorded (Figure 1): walking, standing still, preening (parasitoid cleans her body), encountering (the first contact with the host by the antennae of the parasitoid), probing (parasitoid drums the host with the antennae), ascending (parasitoid climbs on the host), descending and laying egg (moves down from the host and inserts the ovipositor under the host), and feeding of host or honeydew. Each experiment consisted of enough replications to include at least 20 ovipositions under each nymphal instar of *Bemisia* with *E. mundus* by each of the three parasitoid populations.

Data analysis

The data were analyzed with a general linear model (GLM procedure in SAS).

Results

Female behavior on the leaf parts (standing still, walking, preening or feeding on honey) showed substantial variation in frequency and duration (Figure 2). However, females always showed three basic sequences of behaviors upon encountering a host: egg laying, host feeding, and host rejection (Figure 1).

Acceptance of hosts for egg laying:

Females of all populations showed the same sequence of behaviors to select a host for egg laying

1. Probing the host with the antennae.
2. Ascending the host and inspecting its periphery with antennae.
3. Descending, inserting the ovipositor under the host, and laying an egg.
4. Withdrawing the ovipositor and drumming the dorsal part of the host with the hind legs.
5. Preening (antennae and fore legs) and walking away.

No correlations were found between durations of corresponding phases of host-handling behavior across parasitoid populations/species or host nymphal instars (all tests gave insignificant Pearson Correlation Coefficients, data not shown).

The probing phase had the shortest duration compared with other host-handling behaviors, and lasted on average about 4 seconds (Figure 2a). There were no significant differences in the duration of this behavior either between different parasitoid populations/species or between different host nymphal instars, except for the second nymphal instar in *E. mundus* where the duration of probing was significantly shorter.

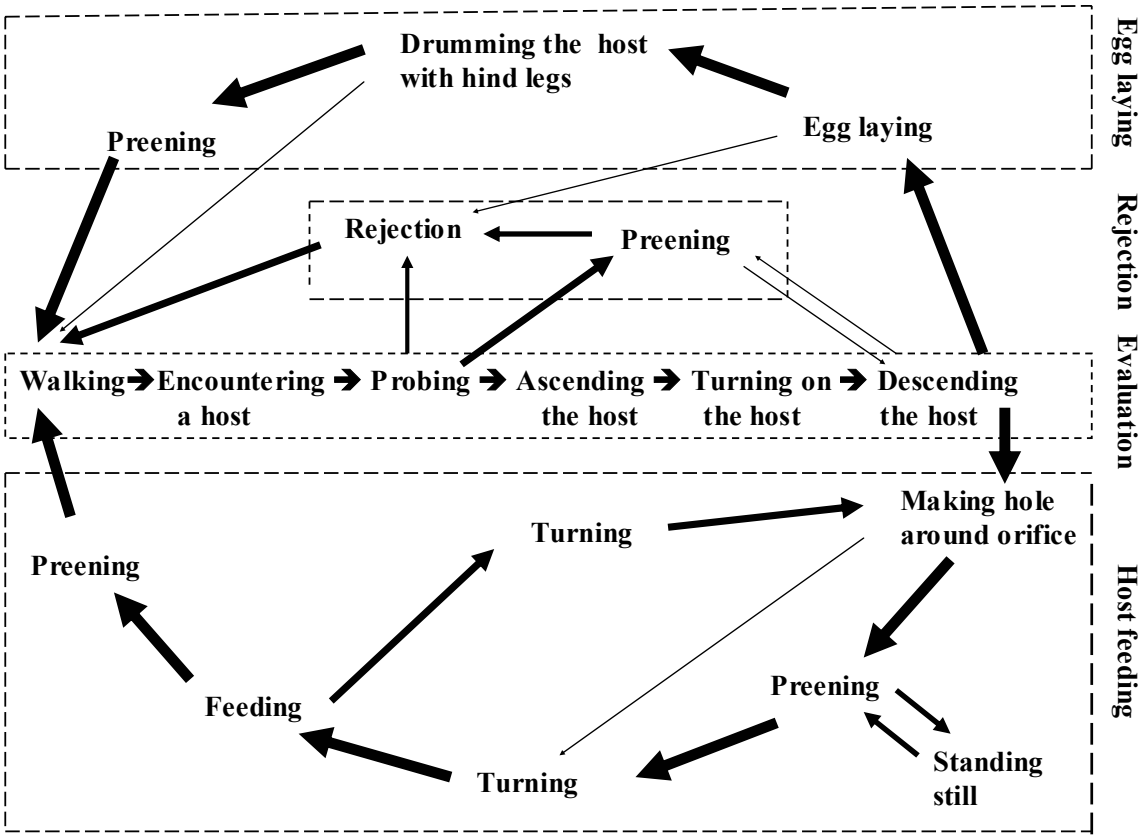

Figure 1. Generalization of the sequence of three basic behaviors (evaluation, egg laying, rejection and host feeding) of *E. mundus* (arrhenotokous and thelytokous) and *E. eremicus* (arrhenotokous) after encountering *B. tabaci* nymphs. The thickness of the arrows reflects the general frequency of transitions between the behaviors.

The third phase during which oviposition takes place, had the longest duration of all host-handling behaviors (on average between 50 and 220 seconds; Figure 2c) and was significantly longer on third nymphal instars than younger ones (Table 1). The duration of oviposition was in most cases not significantly different between parasitoid populations/species, except for oviposition under the 3rd NS (nymphal stage) by *E. eremicus* (longest) and under 1st NS by Spanish *E. mundus* (shortest).

Table 1. Duration of different phases of host-handling behavior among populations of *Eretmocerus* and three nymphal instars (N1, N2, N3) of *Bemisia.*

	Differences among different nymphal instars						Differences among different species				
	E. mundus A		*E. mundus* S		*E. eremicus*		Egg laying N1		Egg laying N2		Egg laying N3
	$F_{(60:2)}$	Pr>F	$F_{(102:2)}$	Pr>F	$F_{(74:2)}$	Pr>F	$F_{(77:2)}$	Pr>F	$F_{(92:2)}$	Pr>F	$F_{(62:2)}$ Pr>F
Enc	1.59	0.21	2.32	0.10	1.72	0.19	1.48	0.23	**8.80**	**0.00**	1.91 0.16
Turn	0.13	0.88	3.98	0.02	0.99	0.38	**12.56**	**0.00**	**21.35**	**0.00**	**11.46 0.00**
Egg	**9.95**	**0.00**	**16.98**	**0.00**	**18.64**	**0.00**	**12.67**	**0.00**	**17.83**	**0.00**	**4.87 0.01**
Drum	0.36	0.26	**8.39**	**0.00**	1.27	0.29	**7.53**	**0.00**	3.89	0.02	**8.31 0.00**
Preen	0.51	0.60	0.82	0.44	1.00	0.37	1.08	0.34	0.55	0.58	1.15 0.32
TH	**7.24**	**0.00**	**17.48**	**0.00**	**10.03**	**0.00**	**14.13**	**0.00**	**14.04**	**0.00**	**8.47 0.00**

The bold numbers indicate significant differences. Enc=encounter, Turn= turn on host by female parasitoid, Egg= egg laying, Drum=drumming the host with the hind legs, Preen= preening the antennae, TH= total duration of host-handling for egg laying, A= Australian S= Spanish.

During the fourth phase, and immediately after having laid an egg, parasitoids started drumming the host with the hind legs and this phase lasted between 10 and 35 seconds (Figure 2d). Duration of this phase was significantly longer in *E. eremicus* than in both *E. mundus* populations (Table 1).

In the last phase of host-handling, females preened the antennae and the head during an average of 5 to 14 seconds (Figure 2e) and then walked away from the host. For this phase, no significant differences in handling times were found (Table 1).

The total host-handling time for laying an egg lasts between 80 and 275 seconds, and variation is largely explained by that of the time needed for oviposition (Figure 2f).

Acceptance of hosts for feeding

Females showed the same probing and ascending behavior for host feeding as for egg laying, but instead of laying an egg, they tried to make a wound with their ovipositor in the orifice region of the *Bemisia* nymphs. Females normally tried two, three or even four times to make a wound, and each time they showed the following sequence of behaviors: preening, turning, probing the wound, and then either feeding from host or turn again for a new attempt to make a wound. Females of the *Eretmocerus* populations/species accepted the three youngest nymphal stages for host feeding. During feeding females fed on the haemolymph of the host, which eventually resulted in killing the host.

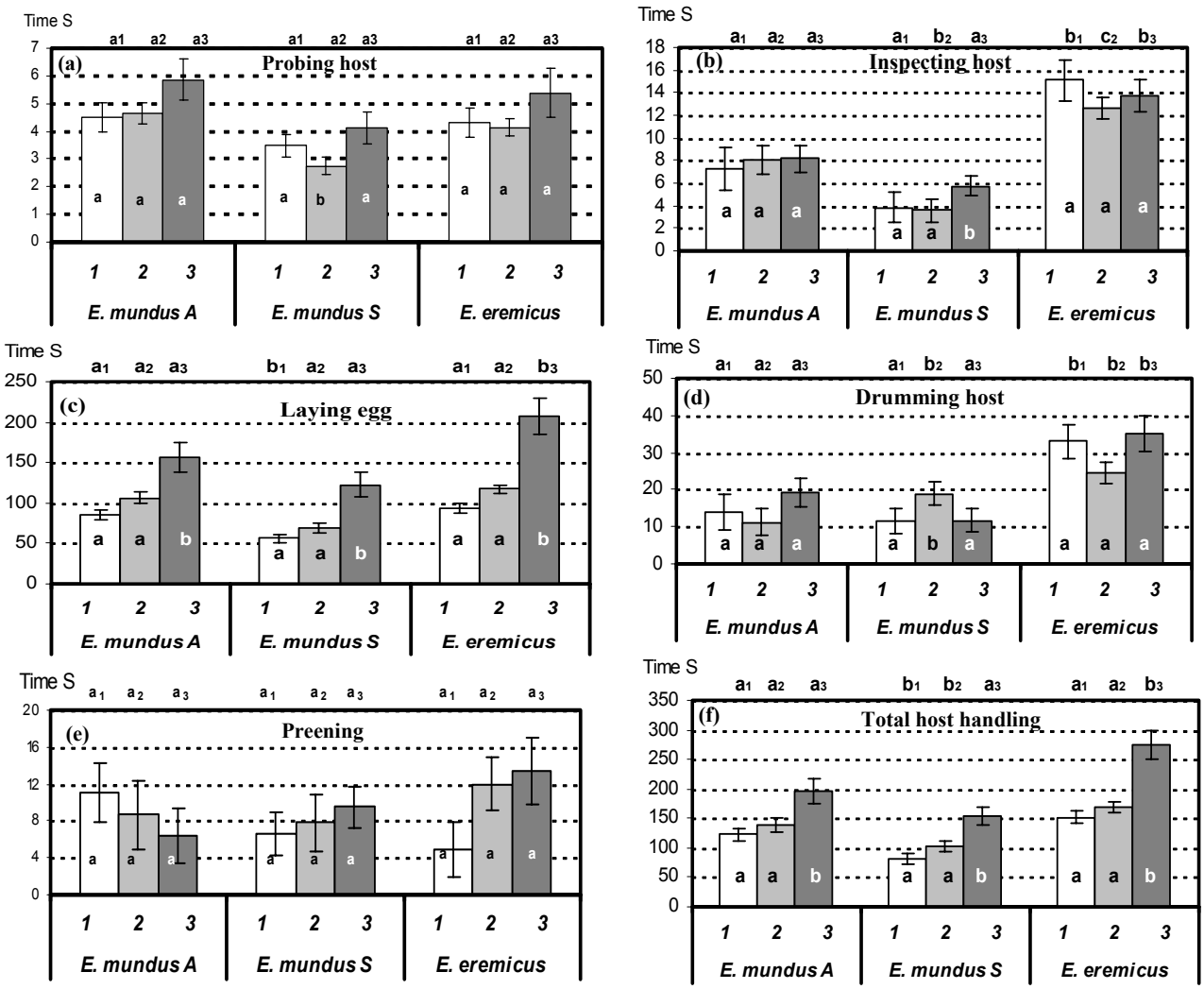

Figure 2. Mean duration (± S.E.) of different behaviors of *E. mundus* and *E. eremicus* females after encountering different stages of *Bemisia* nymphs (N1, N2, and N3, indicated by 1, 2, and 3 respectively on horizontal axis). Statistical comparisons were made of means between populations/species for each nymphal instar separately (significant differences are indicated by different letters above the bars), and between nymphal instars for each species/population separately (significant differences are indicated by different letters in the bars). A=Australia, S=Spain

In both *E. mundus* strains, the frequencies of attempts to make a wound were lower and the total duration of making a wound was shorter in nymphal instars 1 and 2 than in nymphal instar 3 (Figure 3a). For *E. eremicus* there was no difference in number of attempts to make a wound and host-feeding duration (Figure 3a). The duration of host feeding tended to increase with host stage and was shorter for *E. mundus* populations than for *E. eremicus* (Figure 3b). However, due to the infrequent occurrence of host feeding, the number of observations was too low to allow meaningful statistical analysis of its duration.

Rejection of hosts

Most rejections took place at the end of each observation, and *E. eremicus* rejected more hosts in the course of time than *E. mundus* populations (Figure 4). Females did not show any bias among encountered host stages and rejections occurred for all different nymphal instars. When a female did not accept a host, it rejected it either at first touch with the antennae or after drumming the nymph with the antennae, a behavioral component which lasted from less than one second to two seconds.

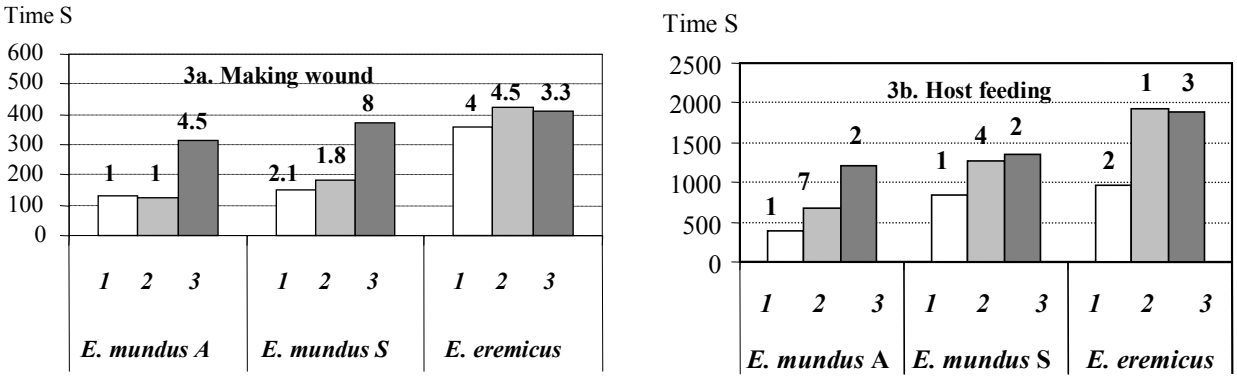

Figure 3. Duration of making a wound and host feeding in *E. mundus* and *E. eremicus* on different nymphal instars of *B. tabaci* (N1, N2, and N3, indicated by 1, 2, and 3 respectively, on horizontal axis). A= Australia, S=Spain.

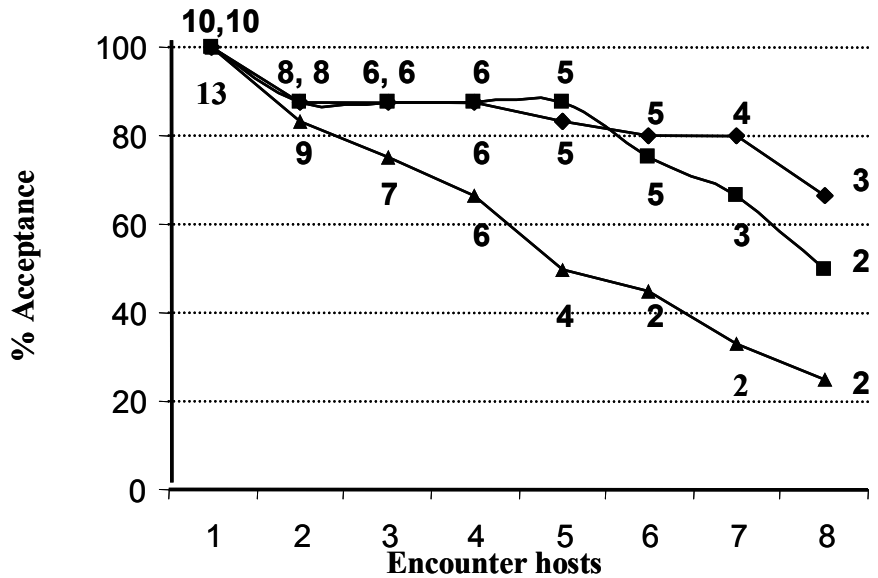

Figure 4) Mean percentage acceptance of hosts (*B. tabaci* nymphs) during foraging by *E. mundus* (two strains) and *E. eremicus*. The number of females still foraging is given above each data point; *E. mundus* Spain (=●), *E. mundus* Australia (=■), *E. eremicus* (=▲).

Discussion

Parasitoids of many species show typical host-handling behavior that can be described by particular phases and sequences (Vinson, 1998). Earlier, *Eretmocerus* host-handling behavior has been divided in three phases (Headrick *et al.,* 1996; Foltyn and Gerling, 1985). Based on our new observations we propose to divide this behavior into five phases: (1) probing the host with the antennae, (2) ascending and turning on the host, (3) descending, inserting the ovipositor and egg laying, (4) drumming the host with the hind legs, and (5) preening antennae and fore legs.

Actual oviposition (phase 3) had the longest duration amongst host-handling behaviors. The duration was significantly longer in third instar nymphs than in younger ones, which might be due to the difficulty of inserting the ovipositor under the host. Foltyn and Gerling, 1985 stated that females put their wings in a vertical position when they lay an egg. However, we could record this behavior only a few times. Our interpretation is that in these cases the females use more force to insert the ovipositor under the host.

After oviposition, many species of parasitoids mark the parasitized host to avoid parasitizing it again (van Lenteren, 1981; Godfray, 1994; Vinson, 1998; Nufio and Papaj, 2001). *Eretmocerus* females started drumming the host with the hind legs after oviposition, and with this drumming we suppose that they apply a chemical mark.

Foltyn and Gerling (1988) reported that *E. mundus* prefers third instar nymphs for oviposition. In contrast, Headrick *et al.* (1996) found that *E. eremicus* did not show a particular preference for any nymphal instar. We found that all three nymphal instars were accepted for egg laying in the sequence as encountered, and a preference for certain host nymphal instars was found neither for *E. mundus* populations, nor for *E. eremicus*.

The three youngest nymphal instars were also accepted for host feeding by *Eretmocerus* females after making a wound in the orifice region of the host. Some authors consider surface feeding on hosts as host feeding (Headrick *et al.,* 1996). However, in this study we only recorded host feeding sensu stricto, which only took place after making a wound. Jervis and Kidd (1986) distinguished four different types of host feeding: (1) Concurrent feeding, where parasitoids use the same host for feeding and oviposition; (2) Non-concurrent feeding, where different hosts are used either for egg laying or host feeding; (3) Destructive host feeding, where hosts die because of feeding; and (4) Non-destructive host feeding, where hosts survive after feeding. As feeding by *E. mundus* and *E. erimicus* in our tests always resulted in killing of the hosts, host feeding in *Eretmocerus* can be described as non-concurrent and destructive. The finding that *Eretmocerus* females use a host either for oviposition or for feeding has been reported for other aphelinids as well (van Lenteren *et al.,* 1980; Gerling, 1990; Headrick *et al.,* 1996).

The *Eretmocerus* ovipositor is not as hard and sharp as that of *Encarsia,* which lays eggs inside the host (Gerling *et al.,* 1998). Therefore, the *Eretmocerus* females select

a soft part of the body in the orifice region to make a wound for host feeding. However, Jervis *et al.* (2001) stated, "it needs to be established whether the females consume mainly the host's haemolymph or mainly the honeydew contained in the host's hind gut". Our observations showed that *Eretmocerus* females spent a lot of time to make a wound in the host and consumed nearly all haemolymph of the host, resulting in an empty exoskeleton of the host. Thus, it is clear that *Eretmocerus* females feed on the haemolymph rather than on honeydew.

As *Eretmocerus* females did not show preference for one of the youngest three host instar stages and these three stages were rejected equally, it seems that rejection of a host is more influenced by internal parasitoid factors (e.g. egg load) rather than a specific host stage. Females rejected parasitized hosts after the first touch of the host with their antennae. Therefore, we suppose that *Eretmocerus* uses chemical cues for recognition of parasitized hosts.

Selection of Eretmocerus *species/populations for biological control*

Some variation occurred in the duration of the different phases of host-handling behavior between populations/species of *Eretmocerus,* but the differences were small and often insignificant. Further, all females equally well accepted all nymphal instars either for egg laying or for host feeding. The different mode of reproduction (thelytoky or arrhenotoky) of the *E. mundus* populations did not influence the duration of host-handling behavior for oviposition and host feeding. Hence, the small differences in host-handling behavior are unlikely to affect the biological control efficiency of populations/species of *Eretmocerus.* The longer host-handling times and the higher host-rejection rate of *E. eremicus* (Figure 4) might make this species slightly less efficient. Taking all current data into consideration, the thelytokous population of *E. mundus* from Australia may be the best candidate for control of *Bemisia*, if the host-location capability and fecundity of the thelytokous population is similar to that of arrhenotokous populations. These characteristics form the topic of our next study.

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Intra- and interspecific host discrimination in arrhenotokous and thelytokous *Eretmocerus* spp.

Abstract

Bemisia tabaci (Gennadius) is a serious pest of vegetable, ornamental, and agronomic crops throughout the world. To control *B. tabaci, Eretmocerus eremicus* Rose & Zolnerowich and *E. mundus* Mercet are considered the most effective parasitoids in dry tropical regions. In parasitoids, choosing the 'right' hosts has direct consequences for their reproductive success and efficiency as biocontrol agent. Therefore, being able to discriminate a parasitized host from an unparasitized one would be important to prevent wasting time, eggs, and to reduce the mortality risk for their offspring. We evaluated intra- and interspecific host discrimination and the chance of super-parasitism or multi-parasitism in two populations of *E. mundus* (sexual and asexual) and *E. eremicus*. Different combinations and sequences of female introduction were carried out for the various populations and species. Experienced females avoided super-parasitism. However, naïve females did lay eggs under hosts that were previously parasitized by conspecific females. *E. eremicus* females avoided to multi-parasitize hosts parasitized by *E. mundus*. However, *E. mundus* females did multi-parasitize the hosts that had been parasitized earlier by *E. eremicus*. In the case of super-parasitism, the outcome showed that neither of the *E. mundus* populations was stronger, whereas in the case of multi-parasitism *E. mundus* appeared stronger than *E. eremicus*. Since those populations and species are morphologically similar a molecular method had to be developed to identify the outcome of super- or multi-parasitism, which is presented in the appendix.

Introduction

Currently, *Bemisia tabaci* (Gennadius) is a serious pest of vegetable, ornamental, and agronomic crops throughout the world. It has caused enormous damage to many crops during the past three decades (Gerling, 1990; Gerling and Mayer, 1996). So far, several biological control strategies have been evaluated for management of *B. tabaci,* e.g. the use of hymenopteran parasitoids, either native or exotic (for a review see Goolsby et al., 1998). Currently, two species of *Eretmocerus* are commercially available: *E. eremicus* Rose & Zolnerowich and *E. mundus* Mercet. *E. eremicus* is indigenous to the United States (Rose and Zolnerowich, 1997). It seems to be effective for control of *B. tabaci* on poinsettia (Hoddle and Driesche, 1999). *E. mundus* is recorded from many parts of the Mediterranean basin (Mound and Halsey, 1978). It is considered the most important controlling agent for *B. tabaci* in the plastic greenhouses in southern Spain (Rodriguez et al., 1994).

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These two *Eretmocerus* species now used are arrhenotokous, but another population of *E. mundus,* which has been found in Australia, is thelytokous (de Barro et al., 2000). Because a thelytokous population only produces female offspring, it is considered the best candidate for biological control of *B. tabaci* (de Barro et al., 2000).

In the evaluation of parasitoids for biocontrol, one aims to select the most effective species. One aspect that may have an important effect on the parasitoid's efficiency is its foraging behavior (Godfray, 1994). During foraging behavior a female parasitoid must make a number of decisions that are relevant to its reproductive success, namely: how long to stay in a patch to search for hosts, and whether to accept a host for oviposition. Part of this last decision is based on whether the host is healthy or already parasitized (see review in Hoffmeister and Roitberg, 1997). Emerging parasitoid larvae should be able to defeat the host defenses (e.g. encapsulation), which are induced by oviposition (Tuda and Bonsall, 1999). If more than one oviposition occurs by females of the same parasitoid species (a phenomenon called super-parasitism), the larvae face competition with other (related or unrelated conspecific) parasitoid larvae. A host can be parasitized more than once by females of the same species (super-parasitism) or by females of a different species (a phenomenon called multi-parasitism) of parasitoid (van Dijken and Waage, 1987). Superparasitism and multi-parasitism can delay the development of the progeny, increases larval mortality, and results in smaller offspring, particularly in solitary parasitoids (e.g. Vet et al., 1994; Potting et al., 1997). Therefore, an important element of host selection is the capability to distinguish between parasitized and unparasitized hosts, so-called "host discrimination".

Host discrimination confers an advantage to parasitoid females by reducing the wasting of time and eggs, and by minimizing the mortality risk for the offspring (van Lenteren, 1976, 1981). Host discrimination is perhaps particularly important in solitary parasitoids because only one larva is expected to complete its development (e.g. van Alphen and Visser, 1990; van Lenteren, 1981; Hofsvang, 1990). Therefore, to avoid competition among its own progeny, intra-specific host discrimination is frequently found in solitary parasitoids but inter-specific host discrimination is rare (van Lenteren, 1981; van Baaren et al., 1994; Royer et al., 1999; Agboka et al., 2002).

Several mechanisms for host discrimination have been described in parasitoids to detect a parasitized host (external, internal or a combination; see e.g. reviews by van Lenteren, 1976, 1981; Potting et al., 1997; Gauthier and Monge, 1999). In many cases "marking pheromones", have been implicated in mediating host discrimination (review in Nufio and Papaj, 2001). Host discrimination can also be mediated by chemical and/or physical changes in hosts induced by the presence of eggs or larvae (review in Nufio and Papaj, 2001). For instance, a hatching larva of an earlier oviposition may change the physiology of the host, enabling discrimination by con-specific parasitoids (Bai, 1991). However, in most parasitoids, the expression of host

discrimination is influenced by internal factors of the adult parasitoid as well, e.g. egg load (Islam and Copland, 2000), different oviposition time intervals (Ueno, 1999; Outreman et al., 2001), and experience of the females (van Lenteren, 1975, 1981; van Alphen and Visser, 1990).

To date, elements of host searching and oviposition behavior have been studied for *E. eremicus* and *E. mundus* (Foltyn and Gerling, 1985; Gerling et al., 1990; Headrick et al., 1995; Greenberg et al., 2002). However, super-parasitism, multi-parasitism, and host discrimination of whitefly parasitoids has been studied only to a limited degree and interspecific discrimination has not been studied at all. Therefore, we embarked upon a study describing host discrimination and competition among *Eretmocerus* species and populations. In this research we evaluate intra- and interspecific host discrimination of the two populations of *E. mundus* (sexual and asexual) and of a sexual population of *E. eremicus*. To obtain better insight in host discrimination among these populations and species, we distinguish different types of discrimination: "self" (where the host has been parasitized by the same female), "intra population" (parasitized by a conspecific female from the same population), "inter population" (parasitized by a conspecific female from another population), and "interspecific" (parasitized by a female from the other species).

Material and methods

Maintenance of the insects

We used three populations of *Eretmocerus: E. eremicus* that is commercially available (ErCal®, Koppert Biological Systems, The Netherlands), and non-commercial populations of *E. mundus* from Spain (sexual) and Australia (asexual). All three populations were maintained on *B. tabaci* and poinsettia (*Euphorbia pulcherima* Willd. ex Klotzsch) plants. A culture of *B. tabaci* was maintained on poinsettia plants in a greenhouse (25°C and 75% RH).

Host discrimination

For the experimental work, leaf parts (3*4 cm) were cut from poinsettia plants infested with *B. tabaci* nymphs. Plant parts were fixed on moist pieces of cotton wool in a Petri dish to prevent desiccation. Subsequently a map of the nymphal distribution was drawn for each leaf part and a one-day-old naïve female parasitoid was introduced. Oviposition events were marked on the map using a stereo microscope; we called this phase the 'initial foraging period'. When the "first females" had achieved some ovipositions, the female was removed and the "second female" was introduced (called the 'test period'), either after 30 minutes or the next day, to study the ability of host discrimination. In the case of self-discrimination, the rejection of parasitized hosts of the same female was recorded during the initial foraging period and during the test period on the next day. The second females were also one day old, either naïve or with oviposition experience. To obtain experienced females we

allowed them to lay one or two eggs under hosts (Vet et al., 1995). After introducing the second females to the leaf, the acceptance or rejection of parasitized hosts was recorded again using a stereo microscope. To see if the presence of a parasitoid egg under the host has any influence on host discrimination, we transferred some parasitized hosts of which the parasitoid eggs had been gently removed, and some unparasitized hosts to a clean leaf part. One hour later, experienced females from each population were introduced onto the leaf parts and host discrimination behavior was recorded.

After each experiment the parasitized hosts were gently removed and checked to make sure how many eggs had been laid underneath them. Different combinations and sequences of female introduction were carried out for each population and species (Table 1). Ten replications were used for all combinations and tests.

Table 1. Different combinations and sequences of introduction of females for the host discrimination experiments (A= asexual, S= sexual).

	First female	Second female
Self discrimination	*E. mundus* (A)	Same female
	E. mundus (S)	Same female
	E. eremicus	Same female
Intra population	*E. mundus* (A)	*E. mundus* (A)
	E. mundus (S)	*E. mundus* (S)
	E. eremicus	*E. eremicus*
Inter population	*E. mundus* (A)	*E. mundus* (S)
	E. mundus (S)	*E. mundus* (A) ◍
Inter species	*E. mundus* (A)	*E. eremicus* ◍
	E. mundus (S)	*E. eremicus*
	E. eremicus	*E. mundus* (A) ◍
	E. eremicus	*E. mundus* (S) ◍

◍ Used to study the outcome of competition in super- and multi-parasitism

Super-parasitism and competition

Naïve females of the two *E. mundus* populations were introduced to study super-parasitism. However, as *E. eremicus* is able to prevent multi-parasitism only *E. mundus* females (either naive or experienced) were introduced to obtain multi-parasitism hosts (see host discrimination results). Therefore, four combinations of super- or multi-parasitism were made among populations and species (Table1). The second females were introduced three hours after the initial foraging period, and when

they showed oviposition behavior under a parasitized host (see Ardeh et al., 2004), the host was marked on the map of the leaf part with hosts. After the behavioral observations, the leaf parts were kept in a climate room to check for emerging parasitoids. Two weeks later the super- or multi-parasitized hosts were collected and each parasitized host was put separately in a glass vial. The emerging parasitoids were preserved for identification with molecular markers at - 20˚C (see appendix).

Analysis of the data

The data were compared with the Fisher exact test using SPSS software.

Results

The two populations of *E. mundus* as well as the *E. eremicus* population showed a high level of self-discrimination. All parasitoid females rejected the hosts parasitized by themselves either during the initial foraging period or during the test period one day later, with the exception of two cases of super-parasitism (out of 32) by *E. eremicus* during the initial foraging period.

In intra- and inter-population experiments, naïve females of all species/ populations accepted parasitized hosts before they had encountered unparasitized hosts, but they rejected all parasitized hosts after an oviposition under an unparasitized host. In contrast, experienced females of all three populations rejected all parasitized hosts (Figure 1).

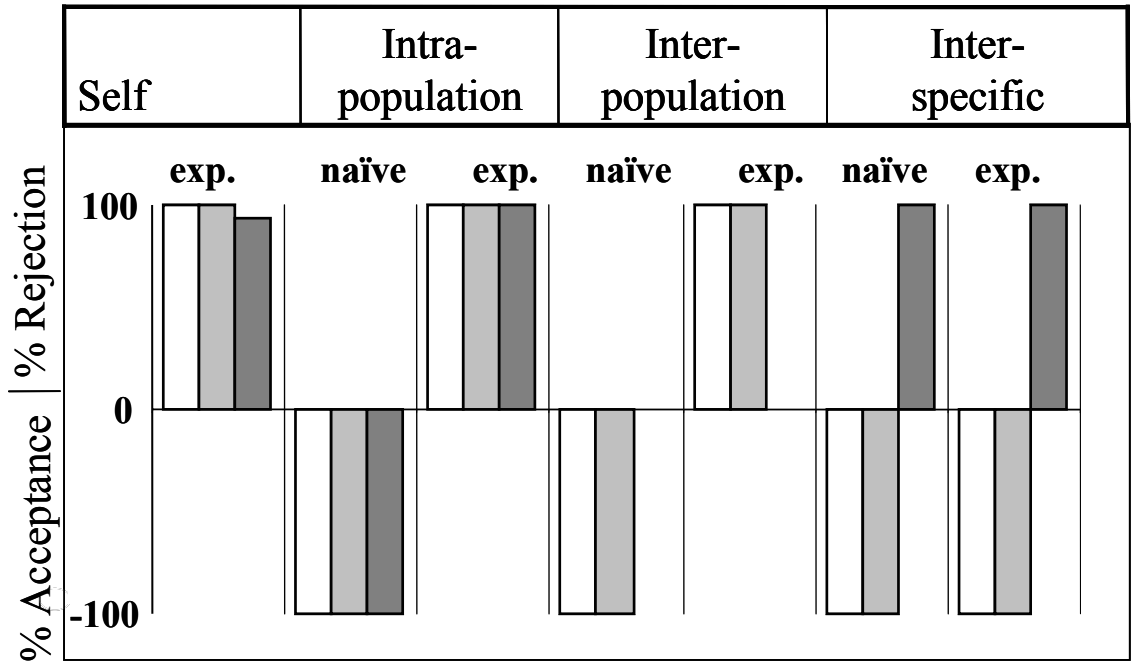

Figure 1. The ability of host discrimination in *Eretmocerus* populations and species. Shown is the percentage of rejections or acceptances of parasitized hosts before the first encounter with a healthy host (each % based on 10 parasitoid females). exp.= with experience, □ = *E. mundus* A, ▢ = *E. mundus S*, ■ = *E. eremicus.*

Experienced females also accepted all unparasitized hosts that had been transferred to another place on the leaf part. In contrast, they rejected all hosts parasitized either by themselves or by conspecific females, even when the parasitoid eggs had been removed from the hosts.

In all cases significant differences were observed for host discrimination between the experienced females and naïve ones (Fisher exact test, $P < 0.001$). The host discrimination results obtained after 30 minutes and after one day were never significantly different for the same combinations tested.

In interspecific host discrimination experiments, all *E. eremicus* females (naïve or experienced) rejected all hosts parasitized by *E. mundus* (both sexual and asexual). In contrast, all females of *E. mundus* (naïve or experienced, both sexual and asexual) did multi-parasitize hosts earlier parasitized by *E. eremicus.* The results were similar after 30 minuets and one day, so these data have been combined (Figure 1). The results were the same for the naïve and experienced females, but there were significant differences between species (*E. mundus* and *E. eremicus*) (Fisher exact test, $P < 0.001$).

Super- and multi-parasitism

In all cases (111 adults) only one parasitoid emerged from a super- or multi-parasitized host. However, in some cases the presence of a second parasitoid larva was clearly visible next to the parasitoid pupa inside the host. The emerging parasitoids from the super-parasitized hosts were a mix of the two populations (Table 2). The results did not show any significant difference in emergence between populations (Fisher exact test, $p > 0.1$). When sexual *E. mundus* females were introduced as the second females, the percentage of emerging males was higher (28.6 %) than when asexual *E. mundus* females were introduced as the second females (Table 2), but not significantly so (Fisher exact test, $p > 0.1$).

Table 2. The outcome of the super- and multi- parasitism experiments.

	E. m S→ E. m A		E m A→ E. m S		E. e → E. m A		E. e → E. m S	
	N	**%**	N	**%**	N	**%**	N	**%**
Total	58	**-**	49	**-**	29	**-**	26	**-**
Mortality	19	***32.8***	14	***28.6***	10	***34.5***	8	***30.8***
Adults	39	***67.2***	35	***71.4***	19	***65.5***	18	***69.2***
Males	10	***17.2***	14	***28.6***	**-**	**-**	10	***38.5***
Females	29	***50.0***	21	***42.9***	19	**-**	8	***30.8***
E. m A	19	***32.8***	10	***20.4***	19	***65.5***	**-**	**-**
E. m S (♂+♀)	20	***34.5***	25	***51.0***	**-**	**-**	18	***69.2***

E. m S= *E. mundus* sexual, E. m A= *E. mundus* asexual, E. e= *E. eremicus.* "→" = Shows the sequence of introducing the females. Total= Total of super or multi-parasitism events. Adults, males, females = the number of adults, males or females that emerged from super - or multi-parasitized hosts, respectively.

Therefore, overall, neither sexual nor asexual populations were stronger than the other one. A Fisher exact test did not show any significant difference in mortality between the sexual and asexual populations (28.6 % compared to 32.6 %, $p > 0.1$).

In contrast, in the multi-parasitism experiments, only *E. mundus* (either sexual or asexual) emerged (Table 2). The ratio of emerging males and females did not show any significant difference in sexual *E. mundus* (Fisher exact test, $p > 0.1$). The mortality of the parasitized hosts varied between 30.8-34.5% (Table 2) and there were no significant differences between sexual and asexual populations (Fisher exact test, $p > 0.1$).

Discussion

Evaluation and eventual use of new biological agents demands consideration of many factors, including interactions among them and their host. Capability of host discrimination is one of these aspects. Several factors influence host discrimination and super- or multi-parasitism. For instance, in the genus *Aphidius* the females of *A. rhopalosiphi* De Stefani-Peres that have low egg loads mostly avoid oviposition in a parasitized host (Islam and Copland, 2000). Females of *A. ervi* Haliday oviposit into recently parasitized hosts, but they will reject the ones that have been parasitized 24 h earlier (Bai, 1991). In some species of parasitoids host discrimination appears to be acquired by learning (e.g. van Lenteren and Bakker 1975; van Lenteren, 1981). Naïve *Eretmocerus* females do not show discrimination between unparasitized hosts and parasitized hosts, whereas experienced females are able to discriminate. So in *E. mundus* it seems that the experience of females is important to prevent super-parasitism, although other factors, such as egg-load, may also play a role. In the field this would result in situations where very little super-parasitism occurs if host densities are high. If host densities are low, super-parasitism will take place until the parasitoid encounters an unparasitized host. Encountering and rejection of parasitized hosts after an oviposition in an unparasitized host might then lead to an increased tendency to leave the patch (e.g. van Lenteren, 1991).

Super- or multi-parasitism results in competition among parasitoid larvae. In gregarious parasitoid species, like *Trichogramma,* a delay in hatching of several hours is sufficient to increase the risk of larval death if it is a member of a second clutch, as they are unable to obtain sufficient food (Klomp and Teerink, 1978; Strand, 1986). As we mostly observed hosts with one parasitoid pupa, and only sometimes hosts with two immature parasitoids, we conclude that both elimination by biting and starvation occurs. However, this tentative conclusion should be substantiated through the study of larvae behavior.

Interspecific host discrimination has been reported less frequently than intraspecific discrimination (Godfray, 1994), and frequently involves closely related species (Vet *et al.*, 1984; Pijls *et al.*, 1995). Thus, it has been suggested that females may use cues shared by both species in recognizing parasitized hosts (Giorgini et al.,

2002). Earlier observations have already shown that *E. eremicus* is weaker than *En. sophia* in the case of multi-parasitism (Collier and Hunter, 2001). Our results show that *E. mundus* females do not discriminate and lay eggs under hosts parasitised by *E. eremicus*, whereas *E. eremicus* females do discriminate and avoid ovipositing under a host parasitised by *E. mundus*. *E. mundus* might not need to discriminate as it is stronger than *E. eremicus* in larval competition, and *E. eremicus* partly prevents being eliminated by being able to interspecifically discriminate. Whenever *E. mundus* and *E. eremicus* share the same niche, *E. mundus* might defeat and replace *E. eremicus* after a few generations because *E. eremicus* might parasitize hosts first, with later multi-parasitisms and *E. eremcicus* eliminations by *E mundus*. This finding may influence the sequence of importation and release of *Eretmocerus* species in biological control.

Another decision to be made is the release of arrhenotokous or thelytokous parasitoid populations. The advantages of asexual reproduction (thelytoky) in parasitoids for biological control programs may include lower costs of mass rearing, faster population growth after release, and easier establishment of the population (van Meer and Stouthamer, 1999). Nevertheless, advantages and disadvantages of the two modes of reproduction also depend on other factors such as the number of female offspring produced per thelytokous versus arrhenotokous females (Stouthamer, 1993; Stouthamer & Luck, 1993, Silva *et al.*, 2000).

Like many other parasitoids the thelytoky in *E. mundus* is caused by *Wolbachia* (de Barro et al., 2000). The bacteria of this genus are obligatory intracellular parasites of arthropods and have been detected in about 70 species of hymenopteran parasitoids (Stouthamer, 2003). *Wolbachia* is transmitted cytoplasmically (maternally) to the next generation (Huigens and Stouthamer, 2003). However, horizontal transmission has been reported in some species of *Trichogramma,* upon super- and multi- parasitism (Huigens et al*.,* 2004). In gregarious *Trichogramma* super- and multi- parasitism may regularly occur between species. However, our present results show that in solitary species like *Eretmocerus*, the occurrence of super- or multi-parasitism seems to be rare in crops with high densities of whitefly, because the parasitoids discriminate. To experimentally achieve transmission of *Wolbachia* between *E. mundus* populations in order to try to change the mode of reproduction from sexual to asexual, we propose to use only naïve females for obtaining super-parasitized hosts.

In general, the fitness of a thelytokous population is expected to be lower than that of the arrhenotokous population when *Wolbachia* is not fixed in the thelytokous population (Huigens et al*.,* 2004). This might explain why, in *Trichogramma* wasps, the survival of thelytokous larvae was much lower than the uninfected larvae when they share the same host (Huigens et al*.,* 2004). In *E. mundus* the *Wolbachia* infection is fixed and, as the super-parasitism results show, the survival of the two populations is not different. However, it is difficult to predict what the mortality of thelytokous

larvae will be of a newly *Wolbachia* infested *E. mundus* strain. This will be the topic of further research.

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Appendix

The molecular identification of arrhenotokous and thelytokous *Eretmocerus mundus* and arrhenotokous *E. eremicus.*

Two different markers were used for identification of *Eretmocerus* species and populations: ITS1 and ITS2. The specimens were collected and preserved at -20C. To extract DNA, each specimen was ground and mixed with 50µl of 5% Chelex®-100 and 2µl of proteinase K (20 mg/ml). The samples were incubated overnight at 56ºC followed by 10 min at 95ºC and two minutes centrifuging at 14000rpm. PCR reactions were performed in 25 µl volumes including: 2.5 µl DNA templates, 2.5 µl PCR-buffer, 0.5 µl dNTP's (each in a 10 mM concentration), 0.5 µl of each of forward and reverse primer (10 ng), 0.07 µl of *Taq* DNA polymerase, and 18.43 µl of sterile distilled water. The primer sequences and the PCR cycles are shown in Table I.

We used the ITS1 marker to distinguish *E. eremicus* from *E. mundus*. This marker resulted in fragments of different size for the two species after running PCR products in 1.5 % agarose gel along with standard ladder (BIOTC) for forty minutes. The ITS1 fragment of *E. eremicus* appeared at about 660 bp and the fragment of *E. mundus* at about 540 bp (figure A1).

To identify different populations of *E. mundus,* we used the ITS2 marker. We incubated 10 µl of the PCR products with 0.5 µl of "Nru I" enzyme, 1.5 µl of the 1X NE buffer and 3 µl of distilled water of 37°C for one hour. The enzyme cut the amplified DNA of the thelytokous population (to 330 and 120 bp) but not of the arrhenotokous population (450bp). The DNA templates were run in 1.5 % agarose gel along with standard ladder (BIOTC) for forty minutes. The population identification was done based on the restriction pattern (Figure A2).

Table I. Primer names and sequences, and the PCR-reaction programs.

Name	Sequence of primer	Den-	Ann-	Ext-	Cycle
ITS1 Forward	TCCGTAGGTGAACCTGCGG	94°C 1min	55°C 1min	72°C 1.5min	35
ITS1 Reverse	GCTGCGTTCTTCATCGATGC				
ITS2 Forward	TGTCAACTGCAGGACACATG	94°C 1min	60°C 1min	72°C 1.5min	35
ITS2 Reverse	ATGCTTAAATTTAGGGGGTA				

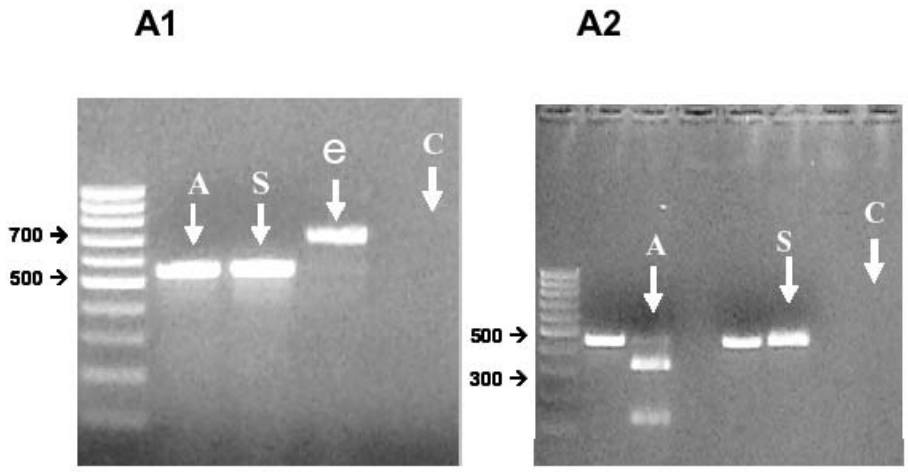

Figure A. differences in band patterns of: (A1) *Eretmocerus* species produced by ITS1 markers and (A2) *E. mundus* populations produced by ITS2 marker after applying "Nru I" enzyme. A=asexual, S=sexual, e = *E. eremicus,* C= control

Summarizing discussion

Many parasitoid species have been successfully used during the past 120 years as biological control agents (e.g. van Lenteren, 2003). Some of these species are cosmopolites, having been recorded in most parts of the world. An accurate evaluation of these parasitoids is of fundamental importance for a successful biological control program. The genus *Eretmocerus* contains 53 described species throughout the world (UCD, NM; Universal Chalcidoidea Database, Natural Museum, London, UK). Two arrhenotokous (i.e. sexually reproducing) species (*E. eremicus* Rose & Zolnerowich and *E. mundus* Mercet) are currently used commercially to control a worldwide key pest: whitefly. Since an asexual population may achieve better pest control than a sexual one, we compared the advantages and disadvantages of the two commercially available parasitoid strains relative to an asexual strain. Comparisons were made of biological, genetical, and behavioral aspects. This chapter summarizes and synthesizes the most important results of the previous chapters. But first I will answer the research questions as formulated in the general introduction:

- Is there any difference between the biology of the asexual and sexual populations of *E. mundus*? *Yes there is: see chapter 2*.
- Is there any mating challenge in sexual populations? *Yes there is, see chapter 3.*
- Does genetic variation support the hypothesis of speciation between the sexual and the asexual populations of *E. mundus*? *Yes it does, see chapter 4.*
- Does the mode of reproduction (sexual / asexual) have an impact on behavioral components in *Eretmocerus* species? *No, it has no impact on host-handling behavior, host discrimination, and competition in the larval stage, see chapters 5 and 6.*

Summary of the previous chapters and main results of my studies

In **chapter 1** I presented an overview of the importance of the damage, biology and management of whiteflies. I explained (1) why we have to use biological control agents, particularly parasitoids, to control whiteflies, (2) why I chose the genus *Eretmocerus* for potential candidates to control *Bemisia tabaci,* and (3) what the aims and the outline of my thesis are.

A major component of the evaluation of biological control agents is the study and comparison of their biology, including the reproductive mode. Therefore, in **chapter 2** I compared the impact of the mode of reproduction (sexual and asexual) in two populations of *E. mundus*. No significant differences were found in the developmental time and mortality of nymphal stages. The number of progeny of both populations was highest on tomato, intermediate on poinsettia and lowest on gerbera. A large number of progeny was recorded during the first two days of the female's adult life for both populations on different host plants. We found that the sex ratio of the sexual

population was 50/50, while only few males were recorded for the asexual population. The intrinsic rate of population increase (r_m) was highest on tomato, intermediate on poinsettia and lowest on gerbera, and similar for the arrhenotokous and the thelytokous populations.

In **chapter 3** I described mate finding and mating behavior of three populations of *Eretmocerus*: sexual populations of *E. eremicus* and *E. mundus*, and an asexual population of *E. mundus*. I found that in both sexual populations males reacted to volatile and non-volatile pheromones of conspecific virgin females. *E. eremicus* males reacted interspecifically to the sex pheromones of *E. mundus* virgin females, but *E. mundus* males did not react to virgin *E. eremicus* females. However, asexual females were not attractive for males of either *E. eremicus* or *E. mundus*. Three phases of mating behavior - pre-mating, mating and post-mating - were distinguished for the sexual populations. We could not record a successful copulation between the sexual and asexual *E. mundus* populations that led to a hybrid female. Based on these results we concluded that speciation might have occurred in *E. mundus*.

In **chapter 4** I studied the divergences of two nuclear genomic regions (ITS1 and ITS2) and a mitochondrial region (COII). Constructed trees showed differences among populations and species, where the ITS2 regions showed clearer differences than the ITS1 or COII regions. The constructed trees, using the sequences of these regions, were congruent with different clustering methods. In all cases, sexual European and asexual Australian populations of *E. mundus* formed two different groups, showing genetic diversity exceeding that between recognized species such as *E. eremicus* and *E. warrae*. Since these *E. mundus* populations are also reproductively isolated (chapter 3), I argue that they should be considered as two different species.

In the next two chapters I investigated the possible impact of reproductive modes on the *E. mundus* female behavior.

In **chapter 5** I compared the host-handling behaviors of the three populations of *Eretmocerus* under laboratory conditions. No correlations were found among the durations of different phases across parasitoid populations/species or host nymphal instars. For some components, however, significant differences were found. The actual oviposition had the longest duration of all host-handling behaviors, and was longer on third nymphal instars than on younger ones. Females of the three populations/species accepted the first three nymphal stages either for oviposition or for host feeding. I found that host feeding takes a lot of time, especially the process of making wounds in the host. It eventually leads to the death of the host.

In **chapter 6** I described how *Eretmocerus* species and populations can discriminate a parasitized from an unparasitized host, which has direct consequences for their reproductive success and efficiency as biocontrol agents. I noticed that experienced females avoided to oviposit under hosts that had previously been parasitized by conspecific females, but naïve females did not. I also found that *E. eremicus* females avoided hosts parasitized by *E. mundus*, thus they prevented multi-parasitism. In contrast, *E. mundus* females do parasitize hosts that had been

parasitized earlier by *E. eremicus.* In multi- or super-parasitized hosts only one parasitoid can develop, and supernumerous parasitoid larvae are eliminated. In the cases of super-parasitism, the outcome in the form of emerged parasitoids shows that neither of the *E. mundus* populations (arrhenotokous or thelytokous) is stronger, whereas in the case of multi-parasitism *E. mundus* appears to be stronger than *E. eremicus*. I developed a molecular method to be able to identify the populations and species in the host-discrimination experiments (appendix to chapter 6).

Comparison of the efficiency of *E. mundus* and other whitefly parasitoids

Three parasitoid genera are currently nominated as the most efficient biological control agents of whitefly: *Amitus, Encarsia,* and *Eretmocerus* (Gerling *et al.,* 2001). The performance of *En. formosa* in the control of *T. vaporariorum* has been studied extensively by many authors (for reviews see chapter 1; van Lenteren and Manzaroli, 1999; Gerling *et al.,* 2001). The biology and behavior of *Amitus* species has also been studied, but to a limited extent (Drost *et al*., 1999; 2000: Manzano, 2000; de Vis 2001, Qui *et al*., 2004). Headrick *et al*. (1999) reported about aspects of the biology of *E. eremicus*, and elements of the biology of *Eretmocerus* and *Encarsia* species are reviewed by Qiu *et al.* (2004). Based on these studies, *Encarsia* and *Amitus* are thought to achieve better control of *T. vaporariorum,* while *Eretmocerus* is more efficient in controlling *B. tabaci* (e.g. van Lenteren and Manzaroli 1999; Gerling *et al.,* 2001; de Vis 2001). In addition, *En. formosa* is capable to control whitefly at relatively low temperatures, whereas *Amitus* and *Eretmocerus* species perform better at temperatures higher than 20ºC (de Vis 2001; Qiu *et al.*, 2004).

To evaluate a natural enemy for control of a certain pest, several criteria may be considered (e.g. van Lenteren and Manzaroli 1999). For the criteria listed by van Lenteren and Manzaroli (1999) I summarize in table 1 the data for species of whitefly parasitoids from the three genera mentioned above.

Based on a comparison of the evaluation criteria listed in table 1, we may conclude that both *E. mundus* strains are promising candidates for biological control of whitefly, as their r_m values are as high as those of *En. formosa*. *Eretmocerus eremicus* scores lower for the r_m value. The developmental time of *E. mundus* and *E. eremicus* is similar at different temperatures on different host plants (for a review see Qiu *et al.*, 2004). Headrick *et al*. (1999) reported a longer generation time for *E. eremicus* (24.2 days on sweet potato and 26.1 days on cotton) than what we found for *E. mundus* in our study on all three host plants (tomato, poinsettia, and gerbera). Moreover, they reported lower numbers for fecundity of *E. eremicus* than I found for *E. mundus.*

We also found that the two *E. mundus* populations have advantages over *E. eremicus* in competition after multi-parasitism. Both *E. mundus* populations appear stronger than *E. eremicus* in the case of multiparasitism.

Table 1. Comparison of evaluation criteria to estimate biocontrol efficiency of four parasitoid species of whiteflies. (The data mentioned in the table have been taken from van Lenteren and Manzaroli 1999 for *Encarsia*; Manzano, 2000 for *Amitus*; de Vis 2001 for *Amitus*; Gerling *et al.,* 2001 for *E. eremicus*; Qiu *et al.*, 2004 for *E. eremicus*; the data for *E. mundus* are my own).

Criteria \ Species		*Eretmocerus mundus*		*Eretmocerus eremicus*	*Amitus fuscipennis*	*Encarsia formosa*
		Sexual	Asexual			
Seasonal synchronization with the host		NA	NA	NA	NA	NA
Internal synchronization with the host		+	+	+	+	+
Temperature range		H	H	H	H	L/H
Humidity range		L	L	L	L	L/H
Mass production		+	+	+	?	++
Host specificity: *Bemisia= B, Trialeurodes= T*		*B*	*B*	*T/(B?)*	*T*	*T/B*
Great reproductive potential expressed as r_m	lowest	0.19	0.17	0.06	0.10	0.10
	highest	0.29	0.23	0.11	0.14	0.28
No negative effects on non-target hosts		No negative effects known, except intraguild predation (see chapter 6), but is not considered negative				
Good searching efficiency		?	?	?	?	++

H = high, L = low, NA = not applicable

This might be the reason that *E. eremicus* females avoid to oviposit under the hosts parasitized by *E. mundus,* whereas *E. mundus* females do oviposit under the hosts earlier parasitized by *E. eremicus* (chapter 6). The advantage of arrhenotokous *E. mundus* is also apparent in its mating behavior because the males are not attracted by *E. eremicus* virgin females, whereas *E. eremicus* males waste time and energy by being attracted by the virgin females of *E. mundus* (chapter 3). Therefore*, E. eremicus* populations may over time be replaced with *E. mundus* if both species are released in the same area.

In conclusion, based on the data for development, behavior, and competition, it is expected that *E. mundus* will be a better natural enemy for control of *B. tabaci* than *E. eremicus.*

Impact of reproductive modes on the biocontrol efficiency of *E. mundus*

In theory the use of asexual natural enemies in biocontrol should have advantages over the use of sexual species (Stouthamer, 1993). This may be the reason why a large number of asexual parasitoid species is used in biological control (Stouthamer, 1997). The frequency of occurrence of asexual reproduction amongst solitary parasitoid species and parasitoid species with extremely small individuals might be high, because for these groups the encounter between sexes might be very difficult (Stouthamer, 1997).

Asexuality (=thelytoky) in parasitoids is caused by *Wolbachia* infection (Rosset *et al*., 1992, Stouthamer and Luck, 1993, van Meer and Stouthamer, 1995). *Wolbachia* infection may have a negative influence on the egg production and other characteristics of the infected females (e.g. Girin and Bouletreau, 1995). Therefore, the fecundity of asexual females might be lower than that of sexual conspecifics. This

effect is assumed to be more apparent when *Wolbachia* infection occurs in a part of a population, i.e. a "mixed population", rather than in a "fixed population" where all the females are infected (van Meer *et al*., 2000). For instance, the relative offspring production of an asexual female was lower than that of cured sexual females in *Trichogramma* species under laboratory conditions (Stouthamer *et al*., 1990; Silva 2000). In the thelytokous *E. mundus* populations that I studied *Wolbachia* has gone in to fixation, and I found that also in these populations the total number of offspring was lower than in the sexual population (chapter 2).

In biological control, however, only females are effective; therefore the number of female progeny is more important than the total progeny. Moreover, in field conditions the host densities are often much lower than the densities which potentially could be parasitised by parasitoids (e.g. Burger *et al*., 2004 a, b). In such cases a good host-searching efficiency is more important than a high fecundity (e.g. van Roermund and van Lenteren, 1994). Therefore, asexual reproduction may have particular advantages for biological control at low host densities as long as the host-searching efficiency is similar to or better than that of sexual populations (van Meer and Stouthamer, 1999).

The *Wolbachia* infection seems to have no negative influence on the host-handling and host-discrimination behavior, and also not on parasitoid larval competition inside the host of *E. mundus*, because results found in sexual and asexual populations were the same for all these aspects.

In conclusion, although the sexual population of *E. mundus* produces more offspring, the asexual population may be a useful natural enemy of whitefly as well, and particularly at low host densities.

What should be done next?

Systematics and speciation in Eretmocerus

Eretmocerus species have been reported from many parts of the world. As the species of this genus are tiny wasps, we expect that a genetic survey of the differences between ITS1, ITS2, and COII regions will enable scientists to resolve the systematic relationships amongst various populations, and to determine the presence of synonymous species, e.g. *E. hayati*, and *E. mundus,* or *E. eremicus* and *E. warrae.* Moreover, the differences in sequences of these fragments can be used to develop molecular markers to discriminate the species easier and more reliably than with morphological characteristics. I have developed markers that have proven to help in the successful identification of the two *E. mundus* populations and the two species, *E. mundus* and *E. eremicus* (chapter 4). These modern methods to recognize species, strains and sexual and asexual populations are very important for correct identification of source material to be used in mass production and release of natural enemies (e.g. van Lenteren et al., 2003)

Several effects of *Wolbachia* infection on the host have been reported

(Stouthamer, 1993). Since the *Wolbachia* infestation may induce reproductive isolation between populations, a possible speciation effect has been suggested (Bordenstein, 2003). The worldwide distribution of the genus *Eretmocerus* provides an opportunity to study the role of PI *Wolbachia* in speciation in the future. If allopatric sexual *E. mundus* populations do hybridize, but sympatric sexual and asexual population do not, this would support a role of PI *Wolbachia* in speciation (see chapter 4).

Foraging behavior of Eretmocerus

Patch marking has been reported in some species of parasitoids next to host marking after oviposition, and marked patched are later only shortly visited (e.g. Hoffmeister and Roitberg, 1997). While I was studying the host-handling behavior of *Eretmocerus*, I noticed that the females barely visit the same part of a leaflet twice. I observed similar behavior during host-discrimination experiments where the experienced *Eretmocerus* females showed a kind of fleeing from the part that had already been exposed to another female. Hence, I suggest that females apply a cue on the visited parts (1) to save time during foraging and (2) to avoid super- (and maybe also multi-)parasitism. Therefore, a study of patch marking is recommended in *Eretmocerus* species to obtain insight in the foraging and dispersal behavior of *Eretmocerus* females.

I did not find any significant differences between host-handing behaviors of the two *E. mundus* populations. Also, they were equally strong during larval competition after super-parasitism. Hence, based on these aspects, they do not have any advantage in efficiency relative to each other. However, due to time constraints, I have not been able to evaluate the searching efficiency of the two strains under greenhouse conditions (the last evaluation criterion mentioned in table 1). Thus, it is advised that this should be studied with priority in future *Eretmocerus* research.

Transmission of Wolbachia *between* Eretmocerus *populations and species*

In an infected population *Wolbachia* is transmitted cytoplasmically by the mother to the next generation of daughters (Huigens and Stouthamer, 2003). To study the effect of *Wolbachia,* an asexual population may be changed to a sexual form with an antibiotic treatment. However, also the opposite situation might be created by infecting a sexual population with *Wolbachia*. Super- and multi- parasitism have shown to result in a number of cases in horizontal transmission of *Wolbachia* from one parasitoid to another (e.g. Huigens *et al.,* 2004). This transfer has actually been obtained in our laboratory for *Trichogramma* species (Huigens *et al*., 2000). Since *E. mundus* is a solitary parasitoid, larval competition takes place in the host after super- and multi-parasitism. Therefore, we expect that transmission of *Wolbachia* between *E. mundus* populations could occur. The experimental conditions for transfer experiments can be deduced from the experiments and results described in chapter 6. Such a transfer would help us in evaluating effects of *Wolbachia* on the host, and

might result in new, more effective asexual *Eretmocerus* strains or species that are also cheaper to mass produce.

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Summary

Whiteflies (Homoptera; Aleyrodidae) are amongst the key pests of vegetable, ornamental, and agronomic crops throughout the world. Because of failing and expensive chemical control, much research has been directed at developing biological control by searching for efficient natural enemies of whiteflies. Among different categories of natural enemies, parasitoids have been efficient control agents and cost effective. The aim of the work described in this thesis was to find an efficient parasitoid to control *Bemisia tabaci*.

As a first step I collected data that were available in the literature to give an overview of the importance of the damage, the biology, and the management of whiteflies. I explained (1) why we have to use biological control agents, particularly parasitoids, to control whiteflies, and (2) why we chose the genus *Eretmocerus* as a potential candidate to control *B. tabaci.*

I concluded from available literature data that the two populations of *E. mundus* might be good candidates for control of *B. tabaci*: the sexual population from the Mediterranean, which is commercially available, and an asexual population, which has been found in Australia. In theory, whenever a parasitoid produces more females, it potentially can achieve better pest control if other aspects of its biology are similar to that of the population that produces fewer females. Therefore, asexuality (i.e. females that produce only female progeny) might boost the effectiveness of a parasitoid as a biological control agent. Therefore, I evaluated the biology of an arrhenotokous (sexual) population versus a thelytokous (asexual) population.

The two populations have already shown compliance with essential qualitative criteria as a biocontrol of *B. tabaci* (chapter 1). I conducted experiments to further compare their characteristics and studied several new elements of their biology. In chapter 2, I compared the impact of the mode of reproduction (sexual and asexual) in two populations of *E. mundus*. I could not find any significant differences in the developmental time and immature mortality between the two populations. However, the number of progeny showed differences. Both populations had the largest progeny on tomato and lowest on gerbera plants, and intermediate offspring production on poinsettia. A large number of progeny was recorded during the first two days of the female's life for both populations on different host plants. The arrhenotokous population produced more progeny than the thelytokous one, but the intrinsic rate of population increase, r_m, did not differ a lot between the two populations.

Sexual females need to find mates to produce female progeny. In chapter 3, I described some challenges in mate finding in sexual populations. To get better insight into mate finding and mating behavior I included a sexual population of *E. eremicus* in the experiments, along with two *E. mundus* populations. I found that in both sexual populations males reacted to volatile and non-volatile pheromones of conspecific virgin females. *E. eremicus* males reacted interspecifically to the sex pheromones of

E. mundus virgin females, but *E. mundus* males did not react to virgin *E. eremicus* females. However, asexual females were not attractive for any male. Three phases of mating behavior, pre-mating, mating and post-mating were distinguished for the sexual populations. I could not record any successful copulation that led to a hybrid female between the sexual and asexual *E. mundus* populations. Based on these results I suspect that speciation might have occurred in *E. mundus*.

In parasitoid wasps the asexual reproductive mode is induced by the cytoplasmic bacterium *Wolbachia.* It has been reported that the infection could lead to speciation. To investigate the speciation hypothesis, I studied the divergences of two nuclear genomic regions (ITS1 and ITS2) and a mitochondrial region (COII) in several sexual populations of *E. mundus* from Europe, an asexual *E. mundus* population from Australia, and a sexual population of *E. eremicus.* Their phylogenetic relationship was analysed using additional data of other populations and species retrieved from Genbank. Analyses of the sequence divergences and constructed trees showed differences among populations and species, where the ITS2 regions showed clearer differences than the ITS1 or COII regions. Trees that were constructed using different clustering methods, and based on sequence differences of the three regions, were congruent. In all cases, sexual European and asexual Australian populations of *E. mundus* formed two different groups, showing genetic diversity exceeding that between recognized species such as *E. eremicus* and *E. warrae*. Therefore, I suggest that *Wolbachia* may have played a role in this speciation through pre-mating effects, and argue that the two *E. mundus* populations should be considered different species.

To investigate any influences of *Wolbachia* infection and genetic variation on the fitness of *E. mundus,* three fundamental aspects of foraging behavior -host handling behavior, host discrimination - and competition between the two populations- were studied.

In chapter 5, I described and compared different components of the host-handling behaviors of *E. mundus* with different reproductive modes along with *E. eremicus* under laboratory conditions. There was no correlation among the durations of different phases across parasitoid populations/species or host nymphal instars. But for some components of the behavior significant differences were found. Overall, the actual oviposition had the longest duration of all host-handling behaviors, and was longer on third nymphal instars than on younger ones. Females of the three populations/species accepted the first three nymphal stages either for oviposition or for host feeding. I recorded a relatively long time for host feeding, especially for making wounds in the host. Host feeding eventually leads to the death of the host.

In the next chapter (6) I described how *Eretmocerus* species and populations can discriminate a parasitized- from an unparasitized host, which has direct consequences for their reproductive success and efficiency as biocontrol agents. I noticed that experienced females avoided to oviposit under hosts that had previously been parasitized by conspecific females, but naïve females did not. I also found that *E. eremicus* females avoided hosts parasitised by *E. mundus*, so they prevented multi-

parasitism. In contrast, *E. mundus* females do parasitize the hosts that had been parasitized earlier by *E. eremicus*, so *E. mundus* does multi-parasitize. In the case of super-parasitism, the outcome shows that neither of the *E. mundus* populations is stronger, whereas in the case of multi-parasitism *E. mundus* appears to be stronger than *E. eremicus*. Since morphological identification of these populations and species are difficult, I used the sequence divergences to develop a molecular method to identify these populations and species (appendix to chapter 6).

In the last chapter, I summarized and synthesized the most important results and I answered the research questions as formulated in the general introduction:

- Is there any difference between the biology of the asexual and sexual populations of *E. mundus*? Y*es there is*.
- Is there any mating challenge in sexual populations? *Yes there is.*
- Does genetic variation support the hypothesis of speciation between the sexual and the asexual populations of *E. mundus*? *Yes it does.*
- Does the mode of reproduction (sexual / asexual) have an impact on behavioral components in *Eretmocerus* species? *No, it has no impact on host-handling behavior, host discrimination, and competition in the larval stage.*

Finally, I have presented the following ideas for future studies on *Eretmocerus*:

- Systematics and speciation in order to be able to select the correct species and populations for biological control.
- Foraging behavior and patch marking in order to be able to determine the host-searching efficiency of the different populations under field conditions.
- Transmission of *Wolbachia* between populations and species to find out if asexual populations can be created that might be cheaper and better biocontrol agents.

Samenvatting

Witte vliegen (Homoptera: Aleyrodidae) vormen wereldwijd ernstige plagen in vele gewassen in het veld en in kassen. Doordat chemische bestrijding vaak faalt en duur is, loopt er veel onderzoek naar efficiënte natuurlijke vijanden van witte vlieg. Vergeleken met andere categorieën natuurlijke vijanden zijn parasitoïden efficiënt en kosteneffectief. Het doel van het in dit proefschrift beschreven onderzoek was het vinden van een goede parasitoïd om de tabakswittevlieg *Bemisia tabaci* mee te bestrijden.

Om te beginnen heb ik een literatuurstudie verricht om inzicht te krijgen van de mate van schade, de biologie en de bestrijding van wittevlieg. Uit de literatuurstudie volgde (1) dat natuurlijke vijanden, en in het bijzonder parasitoïden, geschikt lijken om witte vlieg te bestrijden, en (2) dat parasitoïden van het genus *Eretmocerus* goede kandidaten lijken voor de bestrijding van *B. tabaci*. Uit de literatuur concludeer ik ook dat er twee sterk verschillende populaties van *E. mundus* zijn: een seksuele populatie uit het Middellandse Zeegebied die commercieel verkrijgbaar is, en een aseksuele populatie die gevonden is in Australië. In theorie kan een parasitoïd die meer vrouwelijke nakomeningen voortbrengt een potentieel betere bestrijder zijn indien andere aspecten, zoals bijvoorbeel het zoekvermogen en andere biologische kenmerken van de parasitoïd, hetzelfde zijn als van de populatie die minder vrouwtjes voorbrengt. Aseksuele reproductie waarbij vrouwtjes uitsluitend vrouwelijke nakomelingen krijgen zou de effectiviteit van een parasitoïd als biologische bestrijder flink kunnen verhogen. Daarom heb ik de biologie van een arrhenotoke (seksuele) populatie en een thelytoke (asexuele) populatie met elkaar vergeleken.

Er is reeds aangetoond dat de twee populaties voldoen aan essentiële kwalitatieve eisen voor biologische bestrijding van *B. tabaci* (hoofdstuk 1). Ik heb experimenten gedaan om hun eigenschappen verder te vergelijken en heb een aantal nieuwe aspecten van hun biologie onderzocht. In hoofdstuk 2 beschrijf ik het effect van de voortplantingswijze (seksueel of aseksueel) van de twee populaties van *Eretmoceres mundus*. Ik heb geen significante verschillen gevonden tussen de twee populaties voor ontwikkelingstijd en mortaliteit van de onvolwassen stadia. Het aantal nakomelingen verschilde echter wel. Beide populaties hadden het grootste aantal nakomelingen op tomaat, minder op poinsettia en het laagste aantal op gerbera. De hoogste reproductie werd waargenomen tijdens de eerste twee dagen van het leven van het vrouwtje, en dat geldt voor beide populaties op de verschillende waardplanten. De arrhenotoke (sexuele) populatie kreeg meer nakomelingen dan de thelytoke, maar de intrinsieke snelheid van de populatiegroei, r_m, verschilde niet veel voor de twee populaties.

Seksuele vrouwtjes moeten een mannelijke partners vinden om vrouwelijk nageslacht te kunnen produceren. In hoofdstuk 3 beschrijf ik de moeilijkheden bij het vinden van een partner in seksuele populaties. Voor een beter inzicht in het vinden van een partner en het paringsgedrag heb ik ook een seksuele populatie van *E. eremicus* gebruikt, samen met de twee *E. mundus* populaties. In beide seksuele populaties reageerden mannetjes op vluchtige en niet-vluchtige feromonen van ongepaarde vrouwtjes van dezelfde soort. *E. eremicus* mannetjes reageerden ook op de seksferomonen van ongepaarde *E. mundus* vrouwtjes, maar *E. mundus* mannetjes reageerden niet op ongepaarde *E. eremicus* vrouwtjes. Aseksuele vrouwtjes waren

voor geen van de mannetjes aantrekkelijk. Drie fases van paringsgedrag, pre-paring, paring en post-paring worden onderscheiden voor de seksuele *E. mundus* populaties. Ik heb geen geslaagde copulatie kunnen waarnemen die heeft geleid tot een hybride vrouwtje van de seksuele en aseksuele *E. mundus* populaties. Op basis van deze resultaten verwacht ik dat speciatie kan zijn opgetreden *E. mundus*.

Bij parasitaire wespen wordt de aseksuele reproductie geïnduceerd door de cytoplasmatische bacterie *Wolbachia*. In de literatuur is gesuggereerd dat deze infectie kan resulteren in het ontstaan van nieuwe soorten. Om de soortsvormingshypothese te onderzoeken, heb ik nucleair genomische stukken DNA (ITS1 en ITS2) en een mitochondriaal stuk (COII) onderzocht op verschillen tussen seksuele *E. mundus* populaties van Europa, een aseksuele populatie uit Australië, en een seksuele populatie van *E. eremicus* (hoofdstuk 4). De fylogenetische overeenkomsten zijn geanalyseerd met behulp van extra informatie over andere populaties en soortsinformatie verkregen van de 'genenbank'. Analyse van de sequentieverschillen en de geconstrueerde fylogenetische boom duidden op verschillen tussen de populaties en soorten. Hierbij vertoonde de ITS2 regio's duidelijker verschillen dan de ITS1 of COII regio's . Bomen die geconstrueerd waren met behulp van verschillende clustermethodes en allen gebaseerd waren op de drie verschillende genetische regio's waren overeenstemmend. In alle gevallen vormden de Europese seksuele en Australische aseksuele populaties van *E. mundus* twee verschillende groepen, waarbij de genetische diversiteit tussen die groepen groter was dan tussen twee bekende soorten zoals *E. eremicus* en *E. warrae*. Daarom veronderstel ik dat *Wolbachia* een rol kan hebben gespeeld in soortsvorming door middel van paringsisolatie, en dat de twee *E. mundus* populaties als twee verschillende soorten moeten worden beschouwd.

Om de effecten van een *Wolbachia*-infectie en van genetische variatie te kunnen bepalen op de fitness van *E. mundus* zijn drie fundamentele aspecten van het foerageergedrag onderzocht en vergeleken: gastheerbehandeling, gastheerdiscriminatie en concurrentie tussen de twee populaties. In hoofdstuk 5 heb ik verschillende componenten van gastheerbehandling door *E. mundus* met verschillende voortplantingsmechanismen en ook die van *E. eremicus* onder laboratoriumomstandigheden beschreven en vergeleken. Er was geen correlatie tussen de duur van de verschillende fasen tussen de populaties/soorten, en tussen de ontwikkelingsstadia van de gastheer. Maar voor sommige gedragscomponenten werden significante verschillen gevonden. De eileghandeling duurde het langst van alle gedragshandelingen en was langer bij het derde nimfale stadium vergeleken met jongere nimfale stadia. Vrouwtjes van de drie populaties/soorten accepteerden de eerste drie nimfale stadia voor eileg of voor gastheervoeding. Er weerd een relatief lange tijd voor voeding met gastheerhaemolymfe waargenomen, vooral voor het maken van een gat in de gastheer. Dit voedingsgedrag resulteert uiteindelijk in de dood van de gastheer.

In hoofdstuk 6 heb ik beschreven hoe soorten en populaties van *Eretmocerus* reeds geparasiteerde van niet geparasiteerde gastheren kunnen onderscheiden, wat directe invloed heeft op het reproductiesucces en de efficiëntie als biologische bestrijders. Ervaren vrouwtjes vermijden eieren te leggen in reeds door soorgenoten geparasiteerde gastheren, maar onervaren vrouwtjes deden dit niet. *E. eremicus*

vrouwtjes leggen ook geen eieren in gastheren die al geparasiteerd waren door *E. mundus*, en vermijden zo multiparasitisme. Daar tegenover staat dat *E. mundus* vrouwtjes wel gastheren parasiteren als deze al geparasiteerd waren door *E. eremicus*, en dat heeft dan multiparasitisme tot gevolg. Bij superparasitisme bleek dat bij concurrentie tussend de larven in de gastheer geen van beide *E. mundus* populaties altijd sterker was, maar in de situatie van multiparasitisme lijkt *E. mundus* sterker te zijn dan *E. eremicus*. Aangezien het heel moeilijk is om de populaties en soorten morfologisch te onderscheiden, heb ik op basis van genoom sequentieverschillen een moleculaire methode ontwikkeld om de populaties en soorten te herkennen (appendix bij hoofdstuk 6).

In het laatste hoofdstuk heb ik de belangrijkste resultaten samengevat en de onderzoeksvragen beantwoord zoals geformuleerd in de algemene inleiding:

- Is er verschil tussen de biologie van zich aseksueel en seksueel voortplantende populaties van *E. mundus*? *Ja, dat is er.*
- Zijn er paringsuitdagingen bij seksueel voortplantende populaties? *Ja, die zijn er.*
- Ondersteunen de gegevens over genetische variatie tussen de populaties de hypothese dat soortsvorming is opgetreden tussen de seksuele en aseksuele populaties van *E. mundus*? *Ja, ze doen dat.*
- Heeft het voortplantingsmechanisme (seksueel/aseksueel) invloed op bepaalde gedragselementen van *Eretmocerus* soorten? Nee, het heeft geen invloed op de gastheerbehandeling, gastheerdiscriminatie en concurrentie in het larvale stadium.

Tot slot heb ik de volgende aanbevelingen gedaan voor verder onderzoek aan *Eretmocerus*:

- Systematiek en soortsvorming om de juiste soorten en populaties te kunnen selecteren voor biologische bestrijding .
- Foerageergedrag en plekmarkering om de gastheerzoekefficiëntie van de verschillende populaties onder veldomstandigheden te kunnen bepalen.
- Overdracht van *Wolbachia* tussen populaties en soorten om te kunnen bepalen of aseksuele populaties gemaakt kunnen worden voor goedkopere en betere biologische bestrijding.

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Mjardeh
January 2005, Wageningen

Curriculum vitae

Mohammad Javad Ardeh was born on 12.12.1966 in Tehran. He graduated from the Ostad Motahari high school in Robatkarim city in 1986. In the same year he was accepted as a BSc student in the field of Plant Protection at Tehran University. Afterwards he was accepted as a MSc student at the Entomology Laboratory of the same university in 1991. He obtained his MSc in 1995. He had worked in the Systematic Section of the Plant Pests and Diseases Institute for two years (1995-1996) before he became a scientific member of the Ornamental Research Section in Mahallat (300 km South West of Tehran).

He was working on biological control of thrips, until he was awarded a scholarship to continue his study abroad. He accepted and started his Ph.D. at the Entomology Lab of Wageningen University in 2001.

Publications

Ardeh, M.J., Loomans, A.J.M., & van Lenteren, J.C. (2003) Putative sex pheromone and mating behaviour in the whitefly parasitoid *Eretmocerus eremicus* Rose & Zolnerowich. Proc. Exper. Appl. Entomol. (NEV) 14: 75-80.

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Ardeh, M.J., de Jong, P.W., van Lenteren, J.C., 2005. Biology of an arrhenotokous and a thelytokous population of the whitefly parasitoid *E. mundus* on three host plants: how do host plants and *Wolbachia* infections influence the parasitoid?

در آخرین بخش نتایج حاصله در بخشهای قبلی خلاصه و جمع بندی شده و به سئولات مطرح شده در ابتدای این تحقیق پاسخ مناسب ارائه گردیده است:

- **آیا دو روش تولید مثل (جنسی و بکرزائی) بر روی چرخه زندگی گونه پارازیتوید *E. mundus* تاثیر دارد؟ بله موثر است.**
- **آیا جفت یابی در جمعیت های دوجنسی مشکل ساز است؟ بله همینطور است.**
- **آیا اختلافات ژنتیکی فرضیه جدایی جمعیت های جنسی و بکرزائی را تائید میکند؟ بله تائید میکند.**
- **آیا دو روش تولید مثلی (جنسی و بکرزائی) بر روی رفتارهای دو جمعیت تاثیر میگذارد؟ خیر؛ بر روی رفتارهای بررسی میزبانی، شنا خت میزبانی، و رقابت درون میزبانی موثر نیست.**

در پایان ایده هایی که ممکن است بعنوان موضوع مطالعات آینده قرار گیرد ارائه شده:

- **طبقه بندی و شناسائی گونه های جنس *Eretmocerus* در جهت انتخاب گونه و جمعیت مناسب برای کنترل بیولوژیک.**
- **مطالعه سایر جنبه های میزبان یابی و علائم گذاری بوسیله مواد غیره فرار بر ناحیه مورد جستجو برای درک بهتر رفتارهای میزبان یابی جمعیت های مختلف در سطح مزرعه.**
- **سعی در انتقال باکتری *Wolbachia* بین گونه ها و جمعیت ها جهت افزایش احتمالی کارائی و کاهش هزینه کنترل بیولوژیکی.**

با تشکر ازنظرات آقایان دکتر محبی و زره داران درمورد این خلاصه

دو جنسی و تک- جنسی، نتاجی از آنها حاصل نگردید. بنابراین جدایی جمعیتها و ایجاد گونه جدید از گونه ***E. mundus*** مورد نظر قرار گرفت.

بکرزایی در پارازیتوییدهای راسته **Hymenoptera** اغلب توسط نوعی باکتری از جنس ***Wolbachia*** ایجاد میگردد. تاثیر این باکتری بر جدایی جمعیتها و ایجاد گونه جدید قبلا" گزارش گردیده است. برای مطالعه فرضیه " تفرق ژنتیکی" دوقطعه از اطلاعات ژنتیکی هسته ای (**ITS1 and ITS2**) و یک قطعه از اطلاعات ژنتیکی سیتوپلاسمی (**COII** ازقسمت **Mitochondrial**) در بخش چهارم مورد مقایسه قرار گرفت. برای این منظور ارتباطات شجره ای چندین جمعیت از اروپا، یک جمعیت ازاسترا لیا، و یک گونه از آمریکا در کنار سایراطلاعات موجود در بانک ژن - گزارش شده از استرالیا- مورد تجزیه و تحلیل واقع شد. نتایج بدست آمده، از ارتباطات شجره ای و تفرق اطلاعات ژنتیکی مورد مطالعه، وجود اختلافات را در بین جمعیتها و گونه ها نشان داد؛ این اختلافات در قطعه **ITS2** مشهودتر از قطعه های **1 ITS** و **COII** می نمود.

درختان شجره ای حاصله از بکارگیری روشهای متعدد، که با استفاده از اطلاعات قطعات ژنتیکی فوق صورت گرفت، کاملا" مشابه بودند. در همه موارد جمعیت های اروپایی و جمعیت های استرالیایی در دو گروه جداگانه قرار گرفته، اختلاف بین آنها بیشتر از اختلاف بین دو گونه شناخته شده ***E. eremicus*** و ***E. warrae*** بود. بنابراین جدایی جمعیت ها در گونه ***E. mundus*** و ایفای نقش باکتری ***Wolbachia*** دراین جدایی در مرحله قبل از جفت گیری مورد تایید و تاکید قرار گرفت.

برای مطالعه اثر اختلافات ژنتیکی فوق و نقش باکتری ***Wolbachia*** در کارایی گونه ***E. mundus***؛ سه جنبه از رفتارهای میزبان یابی - بررسی میزبانی، شنا خت میزبانی، و رقابت درون میزبانی- در دو جمعیت فوق مورد مطالعه قرار گرفت.

در بخش پنجم جنبه های مختلف از رفتارهای بررسی میزبانی گونه ***E. mundus***، با دو روش تولید مثلی، در کنار گونه ***E. eremicus*** تحت شرایط آزمایشگاهی مورد مقایسه قرار گرفته است. ارتباطی از لحاظ طول زمانی بین قسمتهای مختلف رفتارهای فوق در بین جمعیت گونه ها و نیز بین سنین مختلف لاروی مشاهده نگردید. ولی اختلاف معنی داری بین قسمتهای مختلف رفتارها وجود داشت. در مجموع مرحله تخمگذاری طولانی ترین زمان را داشت؛ واین زمان برای سنین بالاترلاروی طولانی تر بود. هر سه پارازیتویید تمام سنین لاروی را برای تخمگذاری و یا تغذیه میزبانی (**host feeding**) مورد استفاده قرار دادند. تغذیه میزبانی زمان نسبتا" طولانی را شامل شد و در نهایت باعث تلف شدن میزبان گردید.

در بخش ششم توانائی پارازیتوییدهای مورد مطالعه درتشخیص میزبانهایی که قبلا" مورد تخمگذاری قرار گرفته اند مورد مقایسه قرار گرفت، زیرا این پدیده اثر مستقیمی بر روی توانائی یک عامل بیولوژیک در کنترل آفت مورد نظر دارد. نتایج نشان داد که پارازیتویید یکه قبلا" تخمگذاری کرده است توانائی تشخیص میزبان سالم از میزبان تخمگذاری شده را دارا می باشد، ولی پارازیتوییدی که قبلا" تخمگذاری نکرده این توانائی را ندارد.

گونه ***E. eremicus*** از تخمگذاری بر میزبان تخمگذاری شده توسط گونه ***E. mundus*** خوداری کرد؛ در حالیکه گونه ***E. mundus*** بر میزبان تخمگذاری شده توسط گونه ***E. eremicus*** تخمگذاری نمود (**multi-parasitize**). در صورت تخمگذاری یک میزبان توسط هر دو جمعیت ***E. mundus*** (**super-parasitism**)، هیچکدام قویتر نبوده، احتمال خروج هر دو پارازیتویید وجود دارد. در مقابل اگر یک میزبان توسط هر دو گونه مورد تخمگذاری (**multi-parasitism**) واقع شود، پارازیتویید گونه ***E. mundus*** قویتر خواهد بود.

از آنجایی که شناسایی ظاهری این گونه ها و جمعیت ها آسان نیست - با استفاده از اطلاعات ژنتیکی- روش مولکولی شناسایی برای این منظورتهیه و مورد استفاده قرار گرفت که بصوت ضمیمه در بخش ششم موجود است.

خلا صه

حشرات مکنده خانواده Aleyrodidae از جمله آفا ت کلیدی بسیاری از محصولات زراعی، باغی، و گیاهان زینتی در سراسر دنیا میباشند. به دلیل عدم کارایی وهزینه نسبتا" بالای استفاده از سموم؛ تحقیقات گسترده- ای برای استفاده از دشمنان طبیعی موثر جهت کنترل آنها صورت می گیرد. تا کنون از میان سه گروه اصلی دشمنان طبیعی (پرداتورها، پارازیتوییدها و عوا مل بیماریزا) پارازیتوییدها به عنوان عوامل موثر شناخته شده اند. هد ف اصلی تحقیق دراین پایان نامه بررسی کارایی پارازیتوییدها در کنترل یکی از گونه های آفات ذکر شده (*Bemisia tabaci*) بوده است.

دراولین بخش، اطلا عات مربوط به بیولوژی، خسارت، و کنترل این آفات از منابع علمی موجود جمع آوری گردیده است. سپس لزوم استفاده از عوامل بیولوژیک، بخصوص پارازیتوییدها، برای کنترل این آفات شرح داده شده، و سرانجام دلایل انتخاب جنس "*Eretmocerus*" به عنوان پارازیتویید مناسب کنترل کننده آفت (*B. tabaci*) مورد اشاره واقع شده است.

بر اساس اطلاعات موجود، دو جمعیت از گونه *E. mundus* برای تحقیقات بیشتر انتخاب گردید: (1) جمعیت دوجنسی (دارای نر و ماده) که هم اکنون بصورت تجاری برای کنترل آفت مذکور موجود است؛ (2) جمعیت تک جنسی (نوعی بکرزایی که فقط ماده تولید میکند) که دراسترالیا یافت می شود.

فرض براین است که، جمعیتی که تعداد بیشتری حشره ماده تولید کند، کارآیی بیشتری برای کنترل جمعیت آفت دارا میباشد. بنابراین جمعیت تک جنسی، که فقط حشره ماده تولید میکند، میتواند قابلیت بیشتری نسبت به جمعیتی که هر دوجنس را تولید میکند دارا باشد. برای بررسی این فرضیه، بیولوژیی دو جمعیت مورد بررسی قرارگرفت.

هر دو جمعیت خصوصیات کیفی لازم - بعنوان عامل بیولوژیک کنترل کننده آفت مورد نظر- را دارا بودند (بخش اول). بنابراین در این تحقیق، سایر خصوصیات دو جمعیت مطالعه و مقایسه گردید.

در بخش دوم تاثیر نحوی تولید مثل (دوجنسی و بکرزایی) بر چرخه زندگی دو جمعیت *E. mundus* مطالعه شد. طول دوره و مرگ ومیر درمرحله لاروی تفاوت معنی داری نداشت. ولی تعداد نتاج بین دو جمعیت اختلاف معنی داری نشان داد.

هر دو جمعیت بیشترین تعداد نتاج را بر روی گیاه گوجه فرنگی، متوسط نتاج را بر روی گیاه بنت الغنسول (Poinsettia)، و کمترین تعداد نتاج را بر روی گیاه ژربرا (Gerbera) تولید کردند. در همه موارد، دو جمعیت بیشترین تعداد نتاج را در دو روز اول دوره زندگی تولید کردند. در حالی که تعداد نتاج جمعیت دو جنسی بیشتراز تعداد نتاج جمعیت تک جنسی بود، نرخ رشد جمعیت (r_m) بین آنها تفاوت زیادی را نشان نداد.

حشرات ماده جمعیت دوجنسی برای تولید مثل نیاز به یافتن جنس مخالف دارند. برخی از دشواریهای احتمالی برای یافتن جنس مخالف درجمعیت دوجنسی پارازیتویید فوق در بخش سوم مورد مطالعه قرار گرفته است. برای مقایسه بهتر رفتارهای مورد بررسی، گونه دیگری (*E. eremicus*) بعنوان شاهد در کنار جمعیتهای فوق مطالعه شد.

نتایج این تحقیق، وجود مواد شیمیایی فرار (volatile pheromones) و غیرفرار(non-volatile pheromones)را که توسط حشره ماده منتشر شده و رفتارهای جنس نر را بر می انگیزد، اثبات کرد. گر چه جنس نر گونه *E. eremicus* بطرف حشره جنس ماده گونه *E. mundus* گرا یش میافت، ولی جفت گیری بین آنها مشاهده نگردید. از طرف دیگر، با وجود جفت گیری بین دو جمعیت

کنترل حشرات مکنده خانواده Aleyrodidae به وسیله پارازیتویید جنس *Eretmocerus* با دو روش تولید مثلی

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