Comparison between two species of *Eretmocerus* (Hymenoptera: Aphelinidae): Reproductive performance is one explanation for more effective control in the field

Juan A. Villanueva-Jimenez [a,b], Nancy A. Schellhorn [b], Paul J. De Barro [b,*]

[a] Colegio de Postgraduados, Campus Veracruz. Km 88.5 Carretera Xalapa-Veracruz, Veracruz, 91690, México
[b] CSIRO Ecosystem Sciences, GPO Box 2583, Brisbane, QLD 4001, Australia

HIGHLIGHTS

▶ *Eretmocerus hayati* lived longer and produced three times as many progeny as *E. mundus*.
▶ *E. hayati*'s fecundity is at least twice that reported for other *Eretmocerus*.
▶ Sperm limitation can reduce the proportion of female *E. hayati* progeny.

GRAPHICAL ABSTRACT
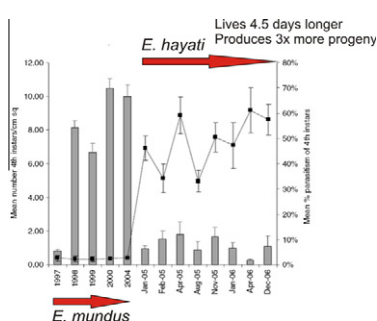
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ABSTRACT

After the invasion of Australia by the *Bemisia tabaci* species Middle East-Asia Minor 1 (MEAM1, commonly known as the B biotype), the native parasitoid *Eretmocerus mundus* (Australian parthenogenetic form) was found to be an ineffective control agent. *Eretmocerus hayati* was therefore introduced and has substantially improved the level of control. A laboratory study was under taken to determine whether superior life history traits were one explanation for the better performance of *E. hayati*. We compared adult longevity, daily fecundity and proportion of female progeny of both mated and unmated females. We also compared the traits across females that were either treated with or without the antibiotic rifampicin, an antibiotic that had already been shown to deplete *Wolbachia* and enable *E. mundus* to produce males. We found that *E. hayati* adults survived longer and produced more progeny than *E. mundus*. Unmated *E. hayati* females produced only males. Rifampcin had no effect on any of the traits for *E. hayati*. In contrast, without rifampicin *E. mundus* females produced mostly female progeny whereas treated females produced mostly males. Recent studies suggest that *E. hayati* co-evolved with MEAM1, whereas the *E. mundus* in Australia co-evolved with the entirely distinct Asia members of the complex. This suggests that the underlying evolutionary relationships within the *B. tabaci* complex may be an important consideration when selecting agents for biological control.

1. Introduction

Bemisia tabaci is a species complex composed of at least 34 morphologically identical species (Dinsdale et al., 2010; Xu et al., 2010; Hu et al., 2011; Alemandri et al., 2012). The reinterpretation of *B. tabaci* as a cryptic species complex indicates that prior to 1993, Australia was home to two distinct species, referred to using the terminology of Dinsdale et al. (2010) as *Australia* (hereafter referred to as AUS) and *Australia–Indonesia*, both members of a cluster of related Asian species (Dinsdale et al., 2010). These were parasitised by two species of *Eretmocerus* (De Barro et al., 2000) and 11 species of *Encarsia* (Schmidt et al., 2001). In 1994, the invasive *Middle East-Asia Minor 1* species (also known as the B biotype or *B. argentifolii* and hereon referred to as MEAM1) was detected

* Corresponding author.
E-mail addresses: javj@colpos.mx (J.A. Villanueva-Jimenez), nancy.schellhorn@csiro.au (N.A. Schellhorn), paul.debarro@csiro.au (P.J. De Barro).

for the first time in Australia. MEAM1 is a member of the African/Mediterranean/Asia Minor cluster (Dinsdale et al., 2010). Over the next 12 months it was detected across the northern half of coastal eastern Australia and inland into southern Queensland and northern New South Wales as well as isolated areas in the Northern Territory and Western Australia (De Barro and Coombs, 2009). The region of New South Wales and Queensland occupied by the invader was also home to AUS (Liu et al., 2007). Surveys had shown that across all hosts, the 4th instar density of AUS averaged 3.4 /cm^2 and ranged up to 15 /cm^2, in contrast densities of MEAM1 averaged 4.9 /cm^2 and ranged up to 40 /cm^2 (De Barro and Coombs, 2009). De Barro et al., (2000) showed that *Eretmocerus mundus* Mercet (Hymenoptera: Aphelinidae; thelytokous Australian form), which reproduced parthenogenetically probably due to the presence of the secondary endosymbiont, *Wolbachia* infection (De Barro and Hart, 2001), was the most promising indigenous parasitoid. However, mean parasitism by *E. mundus* on AUS averaged 11.2% whereas parasitism on MEAM1 averaged 3.4% (De Barro and Coombs, 2009). Furthermore, attempts to control MEAM1 using banker plants to establish *E. mundus* in crops failed to achieve establishment (De Barro unpublished data). Based on an analysis of parasitoid releases against MEAM1 in the USA (Goolsby et al., 2005), it was decided to introduce *E. hayati* Zolnerowich & Rose and the first releases occurred in late 2004 (De Barro and Coombs, 2009). The *E. hayati* released into Australia was imported from field collections in Texas; this population was derived from individuals that had originally come from Pakistan (Hoelmer and Goolsby, 2002). Since then parasitism on MEAM1 has increased to 29.3% (*E. hayati* accounted for 23.6% with 5.7% from the remaining parasitoids combined) of 4th instars and the number of unparasitised whitefly colonies declined from 75% to 24% (De Barro and Coombs, 2009). The consequences have been startling. In the case of one release site in Bundaberg, Queensland, *E. hayati* was released in late 2004. Prior to that, MEAM1 began to outbreak in 1998 and for the next three years averaged between 6 and 11 4th instars per cm^2 on affected crops, during the same period parasitoid numbers, overwhelmingly *E. mundus,* averaged <5% parasitism. After the release, whitefly numbers crashed to <2 4th instars per cm^2 while parasitism increased to >30% (De Barro and Coombs, 2009).

One explanation for the apparent success of *E. hayati* was that it was better adapted to MEAM1 than *E. mundus*. However, available evidence at the time indicated that *E. hayati* had co-evolved with other members of the Asian cluster species that were more closely related to AUS than MEAM1. More recent data has now shown that part of the home range of MEAM1 includes Pakistan (Ahmed et al., 2011; De Barro and Ahmed, 2011), which raises the prospect that *E. hayati* has had the opportunity to co-evolve with MEAM1. A simple question emerged; what was the cause of a much better performance of *E. hayati* compared to *E. mundus*? One possible explanation was that *E. hayati* was better adapted to MEAM1 than *E. mundus* and so possessed greater fitness. To explore this, the biology and fecundity of both species on MEAM1 was studied. We compared adult longevity, daily fecundity and proportion of female progeny of both mated and unmated females. In addition, given the presence of *Wolbachia* in *E. mundus* we took the opportunity to investigate whether this secondary endosymbiont influenced the fitness of *E. mundus*.

2. Material and methods

2.1. Culturing of parasitoids

Cultures of *E. mundus* and *E. hayati* were maintained in separated buildings, in 30 × 30 × 60 cm L–D–H cages with a fine mesh on top, at 24 ± 2 °C, 40–60% R. H. Both species were maintained on MEAM1 feeding on *Hibiscus rosa-sinensis* L. var. Mrs. George Davis. *Hibiscus* plants were maintained, fertilized and pruned appropriately, so that 6–10 fully expanded new leaves across three to four branches were available for whitefly oviposition. Each plant was infested with whiteflies that were left to oviposit until an egg density suitable to achieve a nymphal density of 20–30 /cm^2 was reached after which the adults were removed. Once first and second instar whiteflies were present, parasitoids were added. Leaves were harvested once parasitoids had pupated and placed into emergence containers. Emerged adults were then used to start the next culture.

2.2. Culturing of single leaf Hibiscus cuttings

All experiments were undertaken using single leaf rooted cuttings grown in agar. The first three fully expanded leaves of a *Hibiscus* branch were harvested. Hibiscus cuttings (one leaf plus 8–10 cm of stem) were rinsed and both sides of the leaf were rubbed with a soaked soft cloth to remove any eggs or nymphs. Stems were soaked in a solution of Rootex-L® for 2–3 min following the manufacturer's instructions before being inserted into a plastic tube containing 2% agar. Cuttings were then placed inside a plastic bag for four weeks during which time roots developed. A fertilizer solution (Aquasol® Hortico) was added to the plastic tube in the third week. Once rooted, the single leaf plants were removed from the plastic bag to harden. The rooted cuttings were then placed in a cage along with whitefly adults for 4–8 h until the desired number of eggs (100–250 per leaf) was reached; this gave an egg density that would produce a nymph density of 3–8 nymphs /cm^2. Infested leaves were then kept under natural light in a glasshouse at 24 ± 3 °C for 12 days until second instars were available. Infested cuttings were provided with fertilizer solution as needed.

2.3. Daily parasitism

Individual parasitoid pupae expected to emerge within the next 24 h were harvested from the culture and placed into gelatin capsules the afternoon before the experiment commenced; adults emerge in the morning. Newly emerged adults were then sexed; antenna of live *E. hayati* males have a darker pedicel and a more testaceous club than females (Zolnerowich and Rose, 1998). Male antennae also have funicles bent inwards where live insects accommodate their antennae in front of their face. The required treatment combination (see below) of unmated female (*E. mundus* or *E. hayati*) or unmated female plus male (*E. hayati* only) all without rifampicin, were placed separately inside 100 ml plastic vials containing a small water soaked wick and 2 cm^2 of *Hibiscus* leaf streaked with a honey solution (0.18 ml glycerin, 0.5 ml distilled water, and 1 ml honey). For the rifampicin treated adult, female and male *E. hayati* or female *E. mundus* were placed into the same sized vials as before along with a 2 cm^2 of *Hibiscus* leaf streaked with the same honey solution as before, but that now also contained 50 mg of rifampicin (Sigma®, St. Louis Missouri) prepared according to the method of Sheetz et al. (1997). All vials were then left for 24 h at 24 °C, 40–60% R.H., 14:10 L:D conditions. The required combinations of adults were then added to a clear PVC jar (17.8 × 15.2 × 17.8 cm L × D × H, an 11 cm snap mouth, and a fine mesh across one side) containing an infested cutting and left for 24 h. The adults were then removed using an aspirator and placed into a new cage containing a new cutting. This process was repeated until the female died. Once found dead, males were not replaced and the date of male death was noted. If a female could not be found the replicate was treated as censored data. Censored data means that over time the percentage is calculated using a denominator which is decreased in number by every individual that is

lost. So, if $n = 20$ at day 1 and an individual is lost at day 2 and one dies at day 4 then survival at day 1 is 20/20, at day 3 19/19 and day 4 18/19. However, when calculating overall averages for longevity, fecundity and sex ratio, replicates where the female was lost were not included. Females of both species, treated with and without rifampicin, were analyzed for the presence of *Wolbachia* using the specific *Wolbachia fts*Z specific primers *fts*Zf1 and *fts*Zr1; methodology in De Barro and Hart, (2001). Both species were also tested for the presence of other secondary endosymbionts known to induce parthenogenisis namely *Arsenophonus* and *Cardinium*; both were negative (De Barro PJ, unpublished data).

2.4. Treatments

The treatments were as follows: 1. *E. mundus* female without rifampicin, 2. *E. mundus* female treated with rifampicin, 3. *E. hayati* female without rifampicin and unmated, 4. *E. hayati* female treated with rifampicin and unmated, 5. *E. hayati* female without rifampicin and mated, and 6. *E. hayati* female treated with rifampicin and mated. There were nine replicates of each treatment.

2.5. Statistical analysis

The LIFETEST procedure in SAS v. 9.2, including censored data, was used to compute non-parametric estimates of the survivor function by the Kaplan–Meier method. A Mann–Whitney rank sum test was performed to compare longevity between: a) *E. mundus* females treated with and without rifampicin, b) *E. hayati* unmated females treated with and without rifampicin, c) *E. hayati* mated females treated with and without rifampicin. Once no significant effect of rifampicin on longevity (see results) was shown, results were pooled and retested as follows: d) *E. hayati* mated and unmated females, pooled in both cases for antibiotic treatment e) *E. mundus* females pooled for antibiotic treatment vs. *E. hayati* pooled for both antibiotic treatment and mated status.

To compare fecundity, a Mann–Whitney rank sum test was performed on a) *E. mundus* females treated with and without rifampicin, b) *E. hayati* unmated females treated with and without rifampicin, c) *E. hayati* mated females treated with and without rifampicin, and d) *E. mundus* pooled vs. *E. hayati* pooled. The data on fecundity is based on the numbers of individuals where a parasitoid completes its development, not the number of eggs laid or survival of the immature in the host. As such, it is a measure of realized fecundity i.e. the number of progeny that complete development.

After arcsin transformation, sex ratio (understood as 0 = all males, 1 = all females) was compared by an unbalanced one-way analysis of variance for each variable with GLM procedure in SAS for: a) *E. mundus* females treated with and without rifampicin, b) *E. hayati* unmated females treated with and without rifampicin, and c) *E. hayati* mated females treated with and without rifampicin.

3. Results

3.1. Rifampicin treatment

All the parental *E. mundus* females fed on either the honey solution alone or the antibiotic laced solution and produced the expected 1004 bp product which when sequenced and subsequent Blast comparison, matched sequences belonging to *Wolbachia* (U28175–28211). PCR screening confirmed the presence of *Wolbachia* in the untreated individuals, but not the treated individuals. *Wolbachia* was not detected in *E. hayati*.

3.2. Female survival

The Kaplan–Meier survival curves analyzed by a Wilcoxon test found no significant differences ($X^2 = 0.01$; $df = 1$; $P = 0.91$,) in female survival between *E. mundus* females treated with and without rifampicin. Likewise, no significant difference ($X^2 = 0.50$; $df = 1$; $P = 0.48$,) was observed between unmated *E. hayati* females, treated with and without rifampicin, or between mated *E. hayati* females, treated with and without rifampicin ($X^2 = 0.45$; $df = 1$; $P = 0.51$).

Having no significant differences in survival, the data was pooled. Using the Wilcoxon test, a comparison of survival between the *Eretmocerus* spp. showed *E. hayati* lived significantly longer than *E. mundus* ($X^2 = 5.65$; $df = 1$; $P = 0.02$, Table 1, Fig. 1). When comparing pooled replicates for mated and unmated *E. hayati* females, there was no significant difference ($X^2 = 2.00$; $df = 1$; $P = 0.16$). The comparison of survival of females treated with and without rifampicin of both species pooled revealed no significant difference with the Wilcoxon test ($X^2 = 0.56$; $df = 1$; $P = 0.45$).

3.3. Fecundity

The fecundity of *E. mundus*, treated with and without rifampicin, was not significantly different ($P = 0.15$, Table 1). Similarly, unmated ($P = 0.94$) and mated ($P = 0.75$) *E. hayati*, treated with and without rifampicin, showed no significant difference in fecundity. In each case treated with and without rifampicin data were pooled. Analysis of the pooled data showed that the fecundity of *E. mundus* was significantly less ($P < 0.001$) than that of either mated or unmated *E. hayati* (Table 1).

3.4. Sex ratio

Mean offspring sex ratio of *E. mundus* females treated with rifampicin was significantly different from that of females treated without rifampicin ($F_1 = 56.83$, $P = 0.002$, Table 1). In females treated without rifampicin, approximately 95% of the progeny produced were female. In females treated with rifampicin the percentage of females produced decreased to approximately 43% (Table 1). In females treated with rifampicin, most female offspring were produced over the first 10 days and male production consistently exceeded female production after seven days (Fig. 2).

Statistical analysis was unnecessary in the case of unmated *E. hayati* females, treated with and without rifampicin, as only males were produced (Table 1). When a male and a female of *E. hayati* were mated, females treated without rifampicin and those treated with rifampicin produced the same proportions of male and female progeny ($F_1 = 0.04$, $P = 0.85$, Table 1). There was no difference in the numbers of female and male progeny produced over time and so the data was pooled (Fig. 3). As in *E. mundus* treated with rifampicin, the total sex ratio of mated *E. hayati* was slightly male biased. As time progressed the numbers of female progeny declined and the numbers of male progeny increased, with an overall shift in bias from female to male (Fig 3). Most female progeny were produced by day 10; after day seven the number of male progeny produced consistently exceeded the number of female progeny (Fig. 3). This was consistent for all females and so there was no association between adult mother longevity and relative proportions of male and female progeny.

4. Discussion

As expected, treating *E. mundus* with antibiotic resulted in male and female progeny. *Eretmocerus mundus* from Australia produces mostly females, due most likely to the effect *Wolbachia* has on

Table 1
Mean and median longevity and fecundity, and mean sex ratio of parthenogenetic *Eretmocerus mundus* treated with and without rifampicin, and unmated and mated *Eretmocerus hayati* treated with and without rifampicin. When no differences were obtained, pair was pooled to compare mated or unmated *E. hayati*, and finally to compare both species traits.

Females	Mean (±S.E.) and median longevity (days)	Mean (±S.E.) and median fecundity (adults per female)	Sex ratio (± S.E.) (0.000 = all males, 1.000 = all females)
Eretmocerus mundus			
Parthenogenetic			
Rifampicin	14.25 ± 2.75, 13.0 a	101.60 ± 25.34, 81.0 a	0.417 ± 0.079 a
No rifampicin	15.43 ± 1.57, 15.0 a	55.43 ± 18.51, 42.0 a	0.949 ± 0.028 b
Eretmocerus hayati			
Unmated			
Rifampicin	15.00 ± 4.36, 16.0 a	212.60 ± 46.78, 215.0 a	0.000[*]
No rifampicin	20.40 ± 4.09, 25.0 a	192.38 ± 32.49, 195.0.a	0.000
Mated			
Rifampicin	17.86 ± 1.99, 18.0 a	232.70 ± 34.99, 219.5 a	0.419 ± 0.076 a
No rifampicin	19.33 ± 1.81, 21.5 a	251.36 ± 34.33, 271.5 a	0.437 ± 0.043 a
After pooling			
Eretmocerus hayati			
Unmated	18.38 ± 3.00, 20.5 a	200.15 ± 25.83, 215.0 a	0.000 ± 0.000 a
Mated	18.79 ± 1.34, 20.0 a	243.58 ± 24.49, 229.5 a	0.429 ± 0.039 b
Final pooling			
Eretmocerus mundus	14.00 ± 1.18, 13.0 a	74.67 ± 15.92, 56.0 a	–
Eretmocerus hayati	18.67 ± 1.26, 20.0 b	228.32 ± 18.40, 221.0 b	–

Values for pairs in the same column with the same letter were not significantly different (*p* > 0.05). A Mann–Whitney rank sum test was performed for adult longevity and fecundity. A one-way analysis of variance was performed for arcsin transformation of sex ratio.
[*] No statistical analysis was needed.
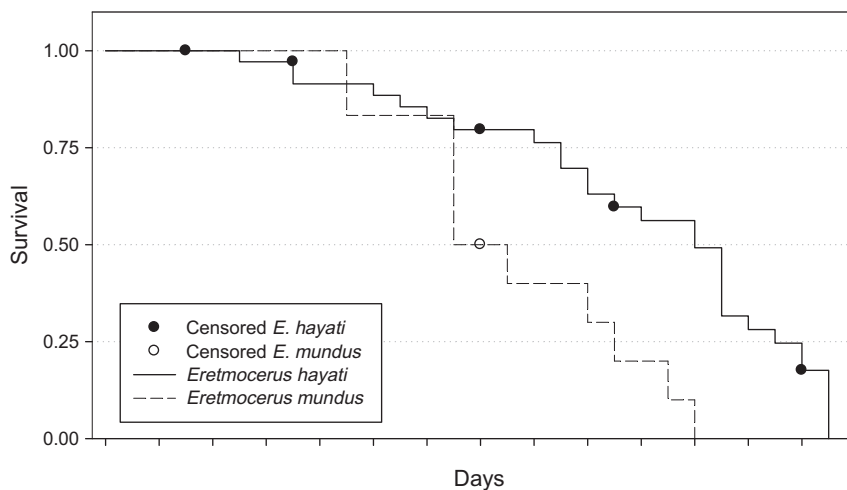
Fig. 1. Survival probabilities of female *Eretmocerus hayati* (solid line) and *E. mundus* (dashed line) over time (days). For *E. hayati* there was no significant difference in the data for mated or unmated individuals that were either treated or untreated with rifampicin; this data was therefore pooled. Likewise, the data for *E. mundus* is pooled in regards to antibiotic treatment status as this did not alter longevity. Censored refers to the inclusion of data where the female was lost and the denominator reduced to account for the loss.

reproduction (De Barro and Hart, 2001). Once treated with rifampicin it is thought that the *Wolbachia* infection is greatly reduced and this results in a marked increase in the production of male progeny. While the fecundity of *E. mundus* appears to be greater when the female was treated with rifampicin, there was no significant difference (Mann–Whitney rank sum test *p* = 0.149) (Table 1). Even when Australian *E. mundus* is treated with antibiotic and so able to produce large numbers of males, it still produced substantially fewer progeny than *E. hayati*. De Barro and Hart, (2001) showed that the testes of the male progeny of antibiotic treated *E. mundus* females contained sperm and that the females (both F1 females and rifampicin treated females) mated with these males had sperm in their spermatheca indicating that copulation did occur. Furthermore, they showed that it was not possible to establish a viable sexually reproducing lineage as mated females produced only

males. De Barro and Hart, (2001) proposed that the effectiveness of Australian *E. mundus* as a biological control agent might be improved by its parthenogenetic reproduction; this does not appear to provide any benefit in terms of efficacy as a potential biological control agent, as its performance both in the laboratory and the field (De Barro and Coombs, 2009) was substantially below that observed for *E. hayati*.

Treatment with the antibiotic had no discernable effect on the reproduction of *E. hayati* and it is only capable of producing female offspring after mating. Given that males were observed to live no longer than five days, it suggests that the observed decline in the number of female progeny produced over time had nothing to do with *Wolbachia* or any other endosymbiont. Rather, the shift in overall bias from female to male was possibly due to a lack of sperm. If this is the case then insemination from a single male
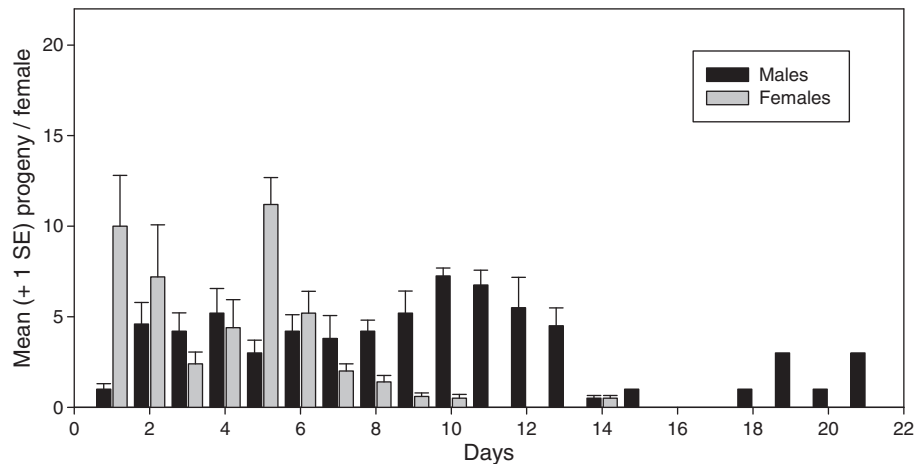
Fig. 2. Mean number (±1 SE) of males (black bars) and females (grey bars) produced by *Eretmocerus mundus*, treated with rifampicin. Not all females survived the whole period of 22 days.
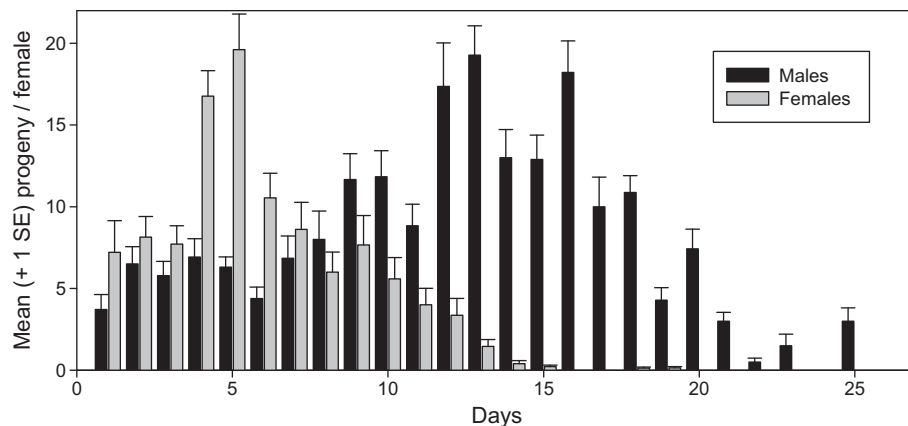
Fig. 3. Mean number (±1 SE) of males (black bars) and females (grey bars) produced by *Eretmocerus hayati* mated females, pooling both treated with and without rifampicin. This data was pooled as there was no significant difference between the two treatments. Not all females survived the whole period of 27 days.

may provide insufficient sperm for the entire female lifespan, and as a consequence more female eggs were left unfertilized in older laying females. However, the same argument cannot be used to explain the decline in female production over time and the corresponding shift to male production observed for *E. mundus* as these females were never inseminated. One explanation could be that the antibiotic's suppressive effect on *Wolbachia* increases with time, possibly because orally administered antibiotics must be absorbed through the gut and then transported to the reproductive tissue (Sakamoto et al., 2008).

Eretmocerus hayati lived on average 4.5 days longer and produced three times as many progeny as *E. mundus* and so may help explain why *E. hayati* performs better in the field in Australia, than *E. mundus*. The average fecundity observed for the Australian form of *E. mundus* falls within the range (27–109) observed by Ardeh, (2004). In terms of the comparative performance of *E. hayati* against other species of *Eretmocerus*, the average fecundity of 228 progeny per female places this species well beyond the averages for sexual *E. mundus* (17–131 progeny per female) (Sharaf and Batta, 1985; Ardeh, 2004; Urbaneja et al., 2007), *E. eremicus* (20–47 progeny per female) (Powell and Bellows, 1992), *E.* nr *furuhashii* (6–34 progeny per female) (Qiu et al., 2005) and *E.* nr *rui* (93 progeny per female) (McAuslane and Nguyen, 1996). There are two likely explanations for the differences observed between the two species. The first is that *E. mundus* simply lays fewer eggs and as such *E. hayati* is simply

a superior parasitoid. An alternative explanation is a difference in co-evolutionary history. This study did not consider whether the different parasitoids were more or less accepting of the different whitefly species found in Australia or whether larval survival in the whitefly host was influenced by the species of host; both would give direct evidence for or against co-evolution. However, De Barro and Coombs, (2009) found *E. mundus* to be the most prevalent parasitoid of MEAM1 in Australia, but levels of parasitism were substantially lower than those observed for the indigenous AUS member of the complex which co-occurred with MEAM1 and shared common hosts both spatially and temporally. One explanation for this is a difference in co-evolutionary history. *Eretmocerus mundus* in Australia is genetically distinct from the sexual form found in Europe (P. De Barro unpublished data) and is likely to be indigenous to Australia; this suggests that it co-evolved with AUS. This member of the *B. tabaci* complex belongs to the Asian group which is separate from the group to which MEAM1 belongs (Dinsdale et al., 2010; Hu et al., 2011). In contrast, *E. hayati* may well have co-evolved with MEAM1 as this species has Pakistan as part of its home range (Ahmed et al., 2011; De Barro and Ahmed, 2011). Goolsby et al., (2005) in their post-release evaluation of biological control of MEAM1 in the south western USA concluded that climate matching was a key element in the success of different parasitoid species in different parts of the USA. The data from this study provides support to the concept that co-evolution is also an important

consideration when selecting potential biological control agents. This is further supported by a recent revision of the genus *Lipaley-rodes* (Dubey et al., 2009) which concluded that it was a junior synonym for *Bemisia* and recent molecular phylogenetic analysis places these species within the *B. tabaci* species complex (Dinsdale et al., 2010). This is of interest because De Barro and Coombs, (2009) showed that while *E. hayati* consistently demonstrated parasitisation levels in excess of 90% for MEAM1, levels were ⩽6% for two species of *Lipaleyrodes*; these species did co-evolve with *E. hayati*.

Acknowledgments

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