Parasitoids are choosy: increase in the capacity to discriminate parasitised tephritid pupae by *Coptera haywardi*

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Abstract This study shows the effectiveness of deliberately selecting for *Coptera haywardi* individuals to increase a population's capacity to discriminate against parasitised hosts. In the 'selected colony' (F1–F4), females were selected based on their ability to discriminate parasitised fruit fly pupae, determined by their host searching, foraging and oviposition behaviour. Female parasitoids of successive generations of the selected colony (F1–F4) showed an increasing discriminatory ability, including reduced host searching and foraging time. The last selected generation, i.e. F4 showed an increase in fecundity compared to the standard colony. In F4 individuals from the selected colony, antennae length increased but the hind tibia size did not, compared to individuals from the control colony. Flight ability and survival remained unchanged across all generations. This selection process could be an effective method of recuperating the discriminatory capacity of a *C. haywardi* colony under mass rearing conditions.

Introduction

The colonization of an insect species inevitably goes through a "bottle neck" phase where, through successive generations, important attributes of individuals are reduced, or even lost (Wajnberg 2004; Parreño et al. 2014). The restoration of these desirable attributes becomes a challenge in many mass-reared colonies where the original genetic status and

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colonization route is unknown (Saul and McCombs 1993; Schütze et al. 2015). A viable option for maintaining important attributes is to select individuals with the desired traits in a practical and targeted way. There are several examples that have demonstrated the feasibility of such a technique for mass-reared insect colonies (Baeshen et al. 2014; Gilchrist and Meats 2014; Quintero-Fong et al. 2016; Tejada et al. 2017) and their natural enemies (Beukeboom et al. 2015; Coelho et al. 2016).

The solitary pupal endoparasitoid, *Coptera haywardi* (Oglobin) (Hymenoptera: Diapriidae) is specific to the tephritid genus *Anastrepha* and has a high capacity for discriminating parasitised hosts (Sivinski et al. 1998; Cancino et al. 2012). The close specificity of *C. haywardi* to pupae of the family Tephritidae, has been linked with its endoparasitic nature (Sivinski et al. 1998). The preference for *Anastrepha* species and the ability to discriminate parasitised hosts makes *C. haywardi* a desirable parasitoid for the concurrent augmentative release with the larval parasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) to enhance biological control of pest *Anastrepha* populations in the field.

Thanks to mass rearing, natural enemies such as parasitoids are used in augmentative releases as a sustainable alternative to chemical control of pests (Mackauer 1976; van Lenteren 2012). When augmentative biological control involves releases of multiple species, an important requirement is that the parasitoids have a high capacity for host discrimination ensuring efficient host searching and thereby reducing conspecific and heterospecific competition in the field (Cancino et al. 2012; Evangelou et al. 2013; Qi-Fu and Tong-Xian 2017). These attributes are particularly important in tertiary or pupal parasitoids that attack towards the end of the host's development period, in order that the parasitoid is able to successfully oviposit and pass on traits from parents to offspring (Scholz and Holler 1992; Ruschioni et al. 2015).

During the mass rearing process, the host searching ability of parasitoids is considered to be one of the main attributes that should be preserved (Mackauer et al. 1996; De Moraes et al. 2000). Host searching is an intrinsic character of a species. However, discrimination ability can decrease under artificial conditions of mass rearing (van Lenteren and Bigler 2010). Maintaining this capacity to discriminate between parasitised and non-parasitised hosts in a mass reared

parasitoid colony is often reduced, or lost under mass rearing conditions where the aim is to maximise parasitoid production, while minimising cost. Recovering discrimination ability can be complex due to the interaction of different factors. For example, this decline may be the result of the use of artificial oviposition substrates or unparasitised hosts used in mass rearing to increase production efficiencies (Lewis et al. 2003). Qualities such as host specificity and the ability to discriminate hosts not previously parasitized are important components of parasitoid searching behavior. However, since they are adaptive attributes, they are sensitive to the selective process that occurs under colonization (van Baaren and Boivin 1998; Lebreton et al. 2008).

To recuperate attributes lost in mass rearing, a common technique is to re-introduce wild individuals (Boller and Chambers 1977; Sorensen et al. 2012). However due to the low percentage of *C. haywardi* parasitism in the field (Sivinski et al. 2000; Aguiar-Menezes et al. 2003; Hernández-Ortiz et al. 2006) and the difficulty of identifying parasitised pupa in the field (Ovruski 1995; Ovruski et al. 2000) this is not practical. Selection (Giunti et al. 2015; Bodino et al. 2016) is another option to optimize an attribute in mass reared colonies. Specialized lines can be used to strengthen the diminished attributes (Coelho et al. 2016) or to produce hybrids with selected attributes (Beukeboom et al. 2015).

This study shows that selection for *C. haywardi* individuals capable of discerning parasitised *Anastrepha* pupae, increases a colony's ability to successfully discriminate. Specifically, this study identified an effective means to increase the discrimination ability of a mass-reared *C. haywardi* colony, which will contribute towards an augmentative biological control program for the control of *Anastrepha* spp.

Materials and methods

Parasitoid source

A population of *C. haywardi* has been maintained for more than 150 generations in the Department of Biological Control of the Moscafrut Program located at Metapa, Chiapas, Mexico was utilised in this study. This *C. haywardi* strain was derived from a colony established in the Fruit Flies Project in INECOL, in

Xalapa, Veracruz, México (Aluja et al. 2009). This population has been reared using pupae of *Anastrepha ludens* (Loew) as the host. In addition, *A. ludens* pupae previously parasitized during the larval stage by *D. longicaudata* have been maintained for over 450 generations and are cultured under the same conditions as *C. haywardi*.

Standard *Coptera haywardi* colony

The *C. haywardi* standard colony was kept under standard conditions of 22 ± 2 °C, RH and a of L:D 12:12 photoperiod. The parasitoid colonies were maintained in mesh-covered aluminum cages (30 × 30 × 100 cm). The host pupae were introduced to the cages on a thin layer of vermiculite in Petri dishes (14.5 cm diameter). The exposed pupae were left for 72 h and unparasitised pupae introduced every 24 h.

Selection for host discrimination by *Coptera haywardi*

All assays were conducted under standard conditions as described above, unless otherwise stated below. Assays were carried out inside a plexiglass cage (30 × 30 × 30 cm) covered with black cardboard to obtain a luminous intensity of 20 lux inside and facilitate the searching, foraging and oviposition behaviour of *C. haywardi* (darkness stimulates oviposition by *C. haywardi*) (Cancino et al. 2012). The *A. ludens* pupae exposed to *C. haywardi* were placed in cylindrical plastic containers (7 cm diameter; 4 cm height) with moistened vermiculite (2 g of vemiculite with 2 ml of water) and maintained at 26 °C for development. 29 days after parasitization, a day before adult eclosion, the pupae were separated from the vermiculite. Eclosed *C. haywardi* adults were placed in plexiglas cages and provided food (one ply paper soaked in honey) and a water soaked cotton wick which was replaced daily.

Females *C. haywardi* were selected for their ability to discriminate non-parasitised pupa based on their host searching, foraging and oviposition behaviour and used to produce four consecutive generations or the 'selected colony'. To 'train', or allow the parasitoids to obtain experience in searching for hosts, i.e. learn, adult females aged 5–6 days were exposed to 100 *A. ludens* pupae for 24 h as previously described.

At day 7, 50 randomly selected individual females with experience in host searching were evaluated separately as described below.

Two pupae, one unparasitised *A. ludens* pupa and the other an *A. ludens* pupa previously parasitized in the larval stage by *D. longicaudata*, were placed 10 cm apart on a Petri dish (14.5 cm diameter) and placed in the assay cage. An individual *C. haywardi* parasitoid female with prior learning experience was released inside the cage and observed for 20 min and a choice was recorded when the female foraged on and oviposited in a pupa. If a female did not make a choice within 20 min, it was marked as 'no choice'. Host searching, foraging and oviposition time were recorded. Host searching time was considered the period elapsed since the release of the female until a choice was made, i.e. a parasitised or unparasitised host was selected for oviposition. Foraging time was considered the period from when a female selected a host, until insertion of the ovipositor. Oviposition time was the period in which the female held the ovipositor within the pupa.

Females *C. haywardi* that discriminated against previously parasitized pupae by ovipositing in the unparasitized pupa were subsequently selected and placed in a 30 × 30 × 30 cm plexiglass cage with water and food. Every 24 h, over a period of five days, 100 unparasitized *A. ludens* pupae were exposed to the females *C. haywardi*. Females who discriminated against parasitised hosts were selected for the subsequent generation. This process was repeated over four consecutive generations to produce the F1–F4 selected lines.

Survival, fecundity and flight ability

The *C. haywardi* F4 line that selected unparasitised pupae and the standard reared colony, were evaluated for survival, fecundity, and flight ability, following the standard procedures of an international protocol (FAO/IAEA/USDA 2014) adapted to this parasitoid species. To determine survival under stress, 15 males and 30 females newly emerged (< 12 h) *C. haywardi* of each line were placed in a Plexiglas cage (30 × 30 × 30 cm) without food and water. Daily mortality counts were recorded, until all parasitoids were dead. To assess fecundity, 35 days old, mated females *C. haywardi* from each line were placed in a Plexiglas cage (30 × 30 × 30 cm). Every 24 h for

five consecutive days, 100 unparasitised *A. ludens* pupae were exposed to the parasitoids. After exposure, pupae were maintained under standard conditions and allowed to develop for 30 days. The number of offspring per *C. haywardi* female were recorded daily until emergence ceased. To determine flight ability, 100 pupae from each line were placed in the base of a black PVC tube (10 cm diameter and 10 cm high). The inside wall of the tube was coated with talc to prevent parasitoids from walking out. The number of adults that emerged and left the tube were counted as flying adults. The number of empty pupae, remaining in the tube on the fifth day after emergence was counted as total emerged adults. The percent of fliers among all emerged parasitoids was computed (adapted from FAO/IAEA/USDA 2014). Survival and fecundity experiments were replicated four times, flight ability experiments were replicated five times.

Body size and antenna length

A sample of ten randomly selected mated females *C. haywardi* from each the standard and the F4 colony were assessed for their size by measuring the length of the hind tibia which is a strong indicator of body size (Rosenheim and Rosen 1992), and the length of their antennae which is considered important for host searching (Quicke 2015; Wang et al. 2016), with a micrometer adapted to a stereoscope at 1.6 × (CARL ZEISS 2000 c).

Data analysis

To analyze discrimination, non-discrimination and no choice of individual female *C. haywardi*, a two-by-two contingency table was used. To avoid problems of dependence between generations, a Bonferroni adjustment was applied dividing the $\alpha = 0.05$ value by five. Searching, foraging and oviposition time was analyzed using a one-way analysis of variance (ANOVA), with the data normalized using a Box-Cox transformation. Flight ability, fecundity and size of the hind tibia and antenna met the assumptions of normality and were analysed using a *t* test. Generational survival was analyzed using the non-parametric log-rank test. All statistical analysis were performed using JMP software (version 7.0.1, SAS Institute Inc.).

Results

Selection for host discrimination by *Coptera haywardi*

The ability to discriminate non-parasitised pupae from *D. longicaudata* parasitised pupae by females *C. haywardi* differed between the standard colony (63.83%), F1 (63.87%) and F2 (71.11%) and the F4 (76.59%) selected population (Table 1; Fig. 1). The percent F4 (23.41%) females that chose parasitized pupae was significantly lower in comparison with the standard colony (36.17%). There was no difference in the percent females that did not make a choice between the standard colony (30.88%) and the selected lines (F1–F4) (range from 22.07 to 23.78%) (Table 1). The searching and foraging time of *C. haywardi* females reduced as the selection process advanced. There was no difference in host searching time by the *C. haywardi* females of the standard colony and the first three selected generations but there was a significant difference between the F4 population and the standard colony ($F_{4,112} = 10.96$, $P < 0.0001$; Fig. 2a). Foraging time for female *C. haywardi* was reduced in all generations of the selected colony compared with the standard colony ($F_{4,125} = 7.37$, $P < 0.0001$; Fig. 2b). The oviposition time was similar for all generations of the selected line and the standard colony ($F_{4,135} = 1.66$, $P = 0.16$), ranging between 10 and 20 min.

Survival, fecundity and flight ability

Survival under stress was similar between the standard colony and F4 of the selected line ($\chi_1^2 = 0.03$, $P = 0.85$) (Fig. 3). The fecundity of *C. haywardi* females was higher in the F4 generation compared with the standard colony ($t_6 = 2.62$, $P = 0.03$; Table 2). There was no difference in flight ability between the standard colony and the F4 selected line ($t_8 = 0.73$, $P = 0.48$; Table 2).

Body size and antenna length

The mean length of antenna increased progressively in the selective line for each generation ($t_{18} = 2.14$, $P = 0.04$; Table 2). The hind tibia length averaged

Table 1 *Coptera haywardi* females from the selected (F1–F4) and the standard colony ability to discrimate *Diachasmimorpha longicaudata* parasitised *Anastrepha ludens* pupae between generations

Parasitoid generation	Host discrimination		Host non-discrimination		No choice	
	χ^2	p	χ^2	p	χ^2	p
Col versus F1	0.000	0.983	0.19	0.662	0.252	0.615
Col versus F2	0.115	0.734	0.314	0.575	0.845	0.358
Col versus F3	0.255	0.613	0.129	0.719	0.042	0.838
Col versus F4	7.812	0.005*	6.936	0.008*	0.448	0.503
F1 versus F2	0.132	0.716	1.023	0.311	2.059	0.151
F1 versus F3	0.233	0.625	0.624	0.429	0.083	0.772
F1 versus F4	7.802	0.005*	1.964	0.161	0.593	0.441
F2 versus F3	0.772	0.395	0.033	0.855	1.23	0.267
F2 versus F4	9.949	0.001*	0.261	0.609	4.391	0.036
F3 versus F4	2.068	0.150	0.434	0.510	1.139	0.285

Contingency table with a df = 1, and using a Bonferroni adjustment of $\alpha/5 = 0.01$ as significance level

'*'Indicates there was a statistical difference between generations at $p < 0.01$. Col = standard colony
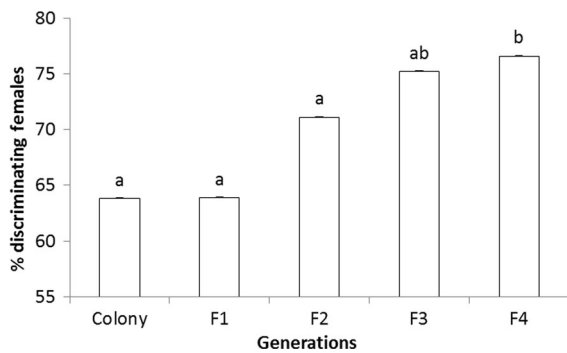

Fig. 1 Percentage of *C. haywardi* females (+ SE) that were able to discriminate from *D. longicaudata* parasitised hosts (i.e. *A. ludens* pupae), in a colony strain and four successive generations after undergoing a selection process to increase the capacity for discrimination. Columns with the same letter do not differ significantly using a contingency analyses with a Bonferroni correction ($\alpha/5 = 0.01$)

1.01 ± 0.02 mm, which was very consistent across both lines and generations ($t_{18} = 0.22$, $P = 0.82$).
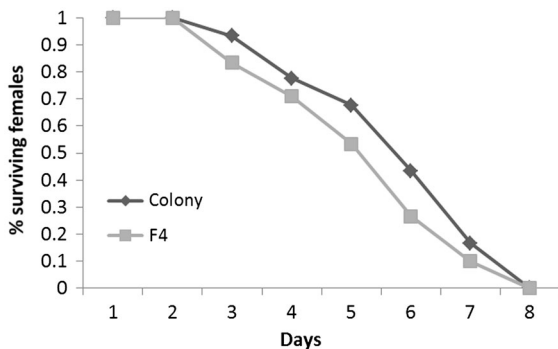
Discussion

The selection of females based on their host searching, foraging and oviposition behaviour was an efficient process to recuperate the ability for host discrimination in mass reared *C. haywardi* populations. By the fourth generation of the selected line, we were able to
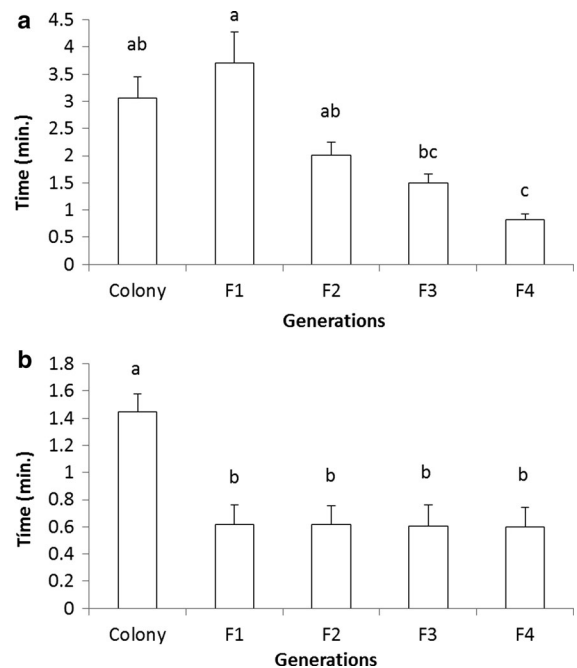
Fig. 2 Mean time (+ SE) spent searching (**a**) and foraging (**b**) for a host pupae (i.e. *A. ludens*) by *C. haywardi* females from a colony strain and four successive generations (F1–F4) developed through a selection process, where only females with the capacity to discriminate unparasitised pupae from those previously parasitized by *D. longicaudata* were selected. Means between columns with the same letter do not differ significantly ($P > 0.05$) using a Tukey test

show that key morphological and performance traits, important for host discrimination, were positively impacted.

In this study, several indicators were used to describe a complex behavior for the host searching of parasitoids which is influenced by several factors, including morphology and genetics (Vinson 1998; Henry et al. 2010). The components of searching behaviour have a high sensitivity to selection (Rolff and Kraaijeveld 2001; Rehman and Powel 2010). The changes in the parasitoid host searching behaviour can be contextualized under two scenarios. The first is that this is an intrinsic activity and that negative changes would imply a decrease in the population and therefore have a direct impact on the adaptation of the species (Boller 1972; Krivan and Sirat 1997; Hassell 2000). Alternatively, searching capability could vary in effectiveness between individuals or populations, but be maintained at a level ensuring the survival of a population (Bautista and Harris 1997; Rolff and Kraaijeveld 2001).

Competition in nature is one of the main challenges facing C. haywardi. Its high specificity requires females to search and find unparasitized pupae in an environment where there is a high likelihood that their hosts have already been attacked by a complex guild of larval parasitoids (López et al. 1999; Ovruski et al. 2000). This means that C. haywardi requires a high capacity for discrimination to maintain population levels. However, this capacity may be affected as a result of a strong trend to homozygous in laboratory strains, which are maintained in an environment where discrimination ability decreases, due to repeated exposure to non-parasitised hosts. Such a purge of the capacity of discrimination can be explained as a response of the genetic variation of this species (Forsman 2015). By selecting for individual C. haywardi females who are able to discriminate, we were able to restore the discrimination capability by the fourth laboratory generation. For C. haywardi, the capacity for discrimination is its most important attribute as a biological control agent of fruit flies. The reduction in the time spent searching for suitable hosts and an increase in fecundity of C. haywardi observed in the current study by the fourth selected generation, are common indicators of favourable lines with a greater capacity for discrimination (Messina and Karren 2003; Boivin 2010).

Host discrimination is very common within a species. However discrimination between species is less common, unless the relationship between the two different species is very close (Vet et al. 1984; Ardeh et al. 2005). C. haywardi are related to larval parasitoids of Anastrepha spp., and they compete for the same reproductive resource. C. haywardi must be able to discriminate and avoid parasitised hosts in order to successfully reproduce. For instance, first instar parasitoids of D. longicaudata within the host usually outcompete C. haywardi (López 2009;

Fig. 3 Longevity of *C. haywardi* obtained through a selection process of females with the capacity to discriminate unparasitized pupae, from previously *D. longicaudata* parasitized pupa (F4) and the standard colony of parasitoids

Table 2 Mean (± SE) antenna length, flight and fecundity of the parasitoid *C. haywardi* from a mass-reared colony and the F4 generation of females selected for their ability to discriminate non-parasitised pupae from pupae previously parasitized by *D. longicaudata*

Generation	Antenna length (mm)	Flight ability (%)	Fecundity (offspring per female per day)
Colony	2. 96 ± 0. 02a	54.93 ± 2.58a	0. 46 ± 0. 20a
F4	3. 16 ± 0. 06b	59.18 ± 2.83a	1. 47 ± 0. 32b

Means within a column followed by the same letter do not differ significantly. *t* test (P > 0.05)

Cancino et al. 2012). The larval parasitoid can dominate the host (De Moraes and Mescher 2005; Harvey et al. 2009) and so coexistence requires a high rate of host discrimination by *C. haywardi*. As this parasitoid is typically reared and released into the field, to increase the level of biological control provided by the larval parasitoid *D. longicaudata,* the expectation is that this second parasitoid, with a high capacity for discrimination, will increase its parasitism rates and together obtain an increased level of control of pest fruit fly populations (Hoffmeister 2000; Cancino et al. 2012).

Parasitoids that are able to effectively discriminate hosts often report higher fecundity and faster host searching (Darrouzet et al. 2007; Lebreton et al. 2008), as we observed in the current study. An increase in wasp fecundity is an important attribute to improve fitness through phenotypic plasticity (Goibin 2010). The discrimination process can involve a cost where the female parasitoids must invest energetic resources to optimise their reproductive success by selecting non-parasitised pupae (Yamada 1988).

The results reported here suggest that increased antennae length favors the identification of unparasitised pupae by *C. haywardi*, leading *A. ludens* pupae previously parasitized by *D. longicaudata* to be rejected. The antennae has been considered as a basic functional structure used in searching behaviour (Lewis et al. 1990; Machtinger et al. 2015; Wang et al. 2016) and selection of hosts by pupal parasitoids (Ruschioni et al. 2015; Wang et al. 2016). Although there is limited published literature about the steps and indicators that guide the selection of a host by *C. haywardi,* it is likely that the antennae play an important role. The two published descriptions of the behaviour of searching and selection emphasise the antennae, which is rubbed on the cuticle of the pupa, to determine acceptance or rejection for oviposition (Fischer et al. 2004; McKay and Broce 2004). Similarly, in the current study we observed *C. haywardi* rubbing their antennae over the pupae before making a choice. Other sensors, including those in the abdomen and the ovipositor, are also thought to be important for discrimination of pupae by parasitoids (Goubault et al. 2011). Vinson (1998) concluded that there is strong selective pressure when host selection involves direct contact. The increase in the size of antennae is possibly a result of a combination a high level of genetic variation in *C. haywardi*

and the strong selection pressure imposed at each generation. Where host-parasitoid relationships are involved, population changes can be relatively rapid (Hughes and Sokolowski 1996; Henry et al. 2010). Changes in survival and the ability to fly were not associated with an increase in discrimination. Both parameters have a close relationship with the quality of the host (Visser et al. 2010), but it is not apparent from this study that improved discrimination is at a cost to the parasitoids with regard to these performance attributes.

It is feasible to assume that the ability for *C. haywardi* to discriminate parasitised hosts was lost through homozygosis. Therefore, under mass rearing conditions, where the priority is to select for highly fecund females, the capacity to discriminate was predicted to decrease due to the pressures placed upon the strain. To maximise the ability of mass-reared parasitoids to discriminate parasitised hosts, there are several options that might be considered: (1) maintaining selected lines that have the highest level of discrimination and that can be mixed in the field with releases of other lines and wild parasitoids (Coelho et al. 2016), (2) hybridisation of lines; different trials have shown the high probability of increasing desirable attributes by combining strains (Gilchrist and Meats 2014), and (3) encourage discrimination by offering parasitised pupae to part of the colony each generation, or intermittently, i.e. every several generations. Further research is required to define and refine which option(s) will be the most effective, while minimizing costs of time and labour.

In augmentative biological programs using the pupal parasitoid *C. haywardi* to complement the activity of larval parasitoids, it is imperative to maintain the capacity for a high level of discrimination. The proposal to select individuals to increase host discrimination is a novel strategy for tephritid parasitoids that will have application in mass-rearing scenarios. However continual maintenance of the desired traits will be required to minimise the return to homozygosity. Further research to understand the mechanisms by which *C. haywardi* searches for, and accepts pupae will clarify aspects of the searching process, and inform better colony management. Genetic evaluation of the strains would also reveal a deeper insight into the capacity for host discrimination by *C. haywardi*.

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