

Interspecific interactions between two *Tuta absoluta* (Lepidoptera: Gelechiidae) larval parasitoids with contrasting life histories

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Abstract

Interspecific interactions between two larval parasitoids of *Tuta absoluta* (Meyrick) with partially overlapping host niches were studied: the idiobiont ectoparasitoid *Dineulophus phthorimaeae* De Santis, and the koinobiont endoparasitoid *Pseudapanteles dignus* (Muesebeck). *T. absoluta* is an important pest of tomato crops worldwide, and its management could be improved by understanding the competitive interactions and potential coexistence between these two parasitoids. Firstly, a 15-min fixed time laboratory test evaluated the host-searching ability of adult *D. phthorimaeae* and *P. dignus* wasps on *T. absoluta* larvae. Secondly, *D. phthorimaeae* host discrimination against endoparasitized and non-endoparasitized hosts by *P. dignus*, at different adult female ages, was experimentally examined. *D. phthorimaeae* wasps spent significantly more time in general searching in the presence of its competitor than in its absence, but, parasitism was only effective by *P. dignus*. Older *D. phthorimaeae* wasps discriminated significantly less than young wasps between *T. absoluta* larvae parasitized and unparasitized by *P. dignus*, and an interaction took place by non-concurrent host-feeding. Intra-guild predation of *P. dignus* larvae by *D. phthorimaeae* female feeding behaviour might have a minor effect in this system. Results are discussed in the context of literature supporting diverse evidence of coexistence in other parasitoid-host systems, with implications for *T. absoluta* biological control.

Keywords: *Dineulophus phthorimaeae*, *Pseudapanteles dignus*, South American tomato moth, parasitoid oviposition behaviour, biocontrol

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Introduction

Coexistence between parasitoid species attacking the same local population of a host species is commonly

observed in nature (Mills, 1992). This coexistence can be established when the parasitoid species specialize in exploiting a particular developmental stage of a particular host, or niche (Waage & Greathead, 1986; Mills, 1992). Simple, classic competition models (Lotka, 1925; Volterra, 1926; MacArthur & Levins, 1967; May, 1973) bear heavily on a set of assumptions regarding coexistence. One such assumption states that two species with partial overlapping niches can coexist by some resource partitioning (Price *et al.*, 2011).

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In parasitoids, larval and adult stages can both compete for hosts. Multiparasitism (i.e., when two or more parasitoid species attack the same host), leads in general, to the death of one of the parasitoid species' larva by competition (Zwölfer, 1971). They can compete directly, by fighting or interfering when occurring simultaneously in a host patch, or indirectly by exploiting a host patch at a different time than a heterospecific competitor. Interspecific competition can be highly asymmetric when the competitors pose different life-history traits. Thus, larval idiobiont parasitoids that are capable of killing or paralyzing previously parasitized hosts are generally considered to be competitively superior to koinobionts, because, of their faster developmental times and lethal host feeding behaviour. However, if they are capable of searching and discriminating healthy hosts, interactions will be reduced (Hawkins, 1994; Mills, 2006; Bernstein & Jervis, 2008). In an applied context, understanding competitive interactions and coexistence among potential parasitoid biocontrol agents is worth elucidating to manage a given insect population.

Besides the classical notion of competition interaction, the concept of intra-guild predation has emerged (Polis *et al.*, 1989; Brodeur & Boivin, 2006). In intra-guild predation, two species belonging to the same guild can switch their trophic relationship and have one species acting as the intra-guild predator and the other species as the intra-guild prey. Therefore, intra-guild predation can be considered as a special case of direct competition, where the immediate energy gain of the intra-guild predator is the most important result (Borer, 2002). For interactions involving exclusively parasitoids, intra-guild predation has been studied for species that can turn from primary to facultative secondary (hyper) parasites (Pérez-Lachaud *et al.*, 2004; Cusumano *et al.*, 2011). However, primary idiobiont ectoparasitoids, that practice non-concurrent host feeding, could eventually prey on previously endoparasitized hosts, and thus engage in a direct trophic interaction. Intra-guild interactions are reported as being common among biological control agents, with either negative or innocuous effect on the efficiency of this pest management control technique (Rosenheim *et al.*, 1995; Brodeur & Boivin, 2006).

In this study we examined aspects of the interaction between two larval parasitoids of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) an important tomato pest (Desneux *et al.*, 2010, 2011), the extremely sinovigenic idiobiont ectoparasitoid *Dineu-*lophus phthorimaeae** De Santis (Hymenoptera: Eulophidae) and the sinovigenic koinobiont endoparasitoid *Pseudapanteles dignus* (Muesebeck) (Hymenoptera: Braconidae). Both species integrate a natural enemies' complex in tomato crops in Argentina and are considered potential biological control candidates of *T. absoluta* (Luna *et al.*, 2010; Savino, 2014). The two parasitoid species exploit the host larval stage but use it in different ways (Colomo *et al.*, 2002; Luna *et al.*, 2007, 2010; Savino *et al.*, 2012; Nieves *et al.*, 2015). In this system, the competitive interaction can occur directly when the *D. phthorimaeae* female performs obligatory non-concurrent or concurrent lethal host-feeding over a host previously parasitized by *P. dignus*. Intra-guild predation can take place if *D. phthorimaeae* attacks *P. dignus*-parasitized *T. absoluta* larvae, yielding an energetic advantage, and consequently, increasing its fitness. In indirect competition, *D. phthorimaeae* host-feeding behaviour will take away suitable hosts for *P. dignus*. It is presumed that any form of competition will increase *D. phthorimaeae* parasitism success.

Regarding the economic importance of *T. absoluta*, the exploration of some significant relationships between the two parasitoid species provide biological foundation for their

manipulation for a *T. absoluta* biological control program (Mills, 1992; Hawkins, 2000).

We hypothesized that both larval parasitoid species, that present partial niche overlapping, would show some level of competition over the same individual host, and because of the broader host resource usage by *D. phthorimaeae*, *P. dignus* will be affected. This work explored interspecific interactions by: (1) observing host search ability by adult *D. phthorimaeae* and *P. dignus* female wasps on *T. absoluta* larvae; and (2) assessing *D. phthorimaeae* host discrimination against *P. dignus* parasitized and non-parasitized hosts, at different adult female ages, and its host feeding behaviour as part of its resource use.

Materials and methods

Insect rearing

Host and parasitoid species colonies were established from material collected from commercial organic tomato crops located at the La Plata Horticultural Belt (La Plata City, Buenos Aires, Argentina), a major agricultural region in the country. *T. absoluta* was reared following the protocol of Luna *et al.* (2007). *T. absoluta* larvae were fed with fresh and water-rinsed tomato leaves, extracted from potted tomato plants maintained in an experimental greenhouse at CEPAVE (CONICET-UNLP, Argentina). Adults were kept in 40 × 40 × 40 cm³ voile-meshed cages, provided with a honey solution (*ad libitum*) with potted tomato plants of approximately 20 cm height, (three fully expanded leaves) as substrate for oviposition. Cages were checked every 2 days to replace plants, which were then placed individually in plastic boxes (20 × 20 × 30 cm³) covered at the top with voile to allow for egg hatching and first-instar larvae strolling to build mines. Thereafter, infested leaves were cut and placed in plastic containers (500 ml) and maintained until pupation with fresh tomato foliage as food.

To establish a *D. phthorimaeae* colony, parasitoid specimens were collected along with those from the host collected in the field. All *T. absoluta* mines in damaged leaves were observed using a stereo microscope (Nikon SMZ 645) for searching immobile hosts and ectoparasitoid larvae and pupae. They were maintained in glass vials (5 ml) until parasitoid adult emergence, sexed and paired up to allow mating. Adults were provided with separate drops of water and honey solution, but without hosts until the experiments were conducted.

The endoparasitoid *P. dignus* colony was reared following Nieves *et al.* (2015) from material collected in the field (cocoons in damaged tomato leaves). Once the adult wasps emerged, they were sexed, paired for mating and placed in oviposition units (transparent glass containers, 1 L volume), and provided with leaves infested with *T. absoluta* larvae. After 48 h of exposure, the adults were removed with a manual aspirator and the host larvae were placed in plastic trays to observe *P. dignus* development until cocoon formation. Pupae were stocked individually in 10 cm diameter × 1.5 cm height Petri dishes with damp filter paper until adult emergence, then sexed and paired to complete a cycle (Nieves *et al.*, 2015). Both colonies were maintained in a walk-in controlled-environment room at 25 ± 2°C, 16-h day-length, and 70 ± 10% relative humidity.

Interaction between D. phthorimaeae and P. dignus

Experiment 1 – Host search ability

To analyze the potential exploitative competition between *D. phthorimaeae* and *P. dignus*, we examined the oviposition

behaviour of adult females in a short-time (15 min) assay. The experimental unit consisted of three-leaved potted tomato seedlings infested with three third-instar *T. absoluta* larvae already settled in feeding mines, enclosed in a cylindrical clear plastic box ($\varnothing = 8$ cm; $h = 10$ cm). Prior to starting the experiment, 24-h old male and female adults of each parasitoid species were randomly chosen from previously isolated pupae from the rearing stocks. They were paired up by species and each couple was kept in a plastic vial ($\varnothing = 7$ cm; $h = 4$ cm) to allow mating. To ensure the parasitoids were not naïve, they were supplied with a 50% honey solution and exposed to hosts. One 5-day old *D. phthorimaeae* and one 2-day old *P. dignus* female wasp were simultaneously released in the unit. Activities displayed in the oviposition behaviour were recorded in the same unit. The following behavioural activities were recorded for 15 min (Al-Wahaibi & Walker, 1999, 2000): (1) general searching, as displayed by fast walking; (2) intensive searching, as slow walking or detention with antennal movements; (3) ovipositor probing; (4) grooming; (5) resting, defined as motionless, non-active state of the wasp in the arena; and (6) parasitizing. Oviposition was distinguished from probing by the typical abdominal contraction the female wasp does when an egg is deposited. The behavioural activities considered are mutually exclusive (i.e., the parasitoid can be engaged in only one kind of behaviour at a time). After the parasitoid exposure, each *T. absoluta* larva was removed and placed individually in a Petri dish ($\varnothing = 8$ cm; $h = 2$ cm) for 24 h to observe *D. phthorimaeae* or *P. dignus* egg/s (the latter upon host dissection) under a stereoscope. Host larvae used only by *D. phthorimaeae* for host-feeding were also recorded. The behaviour of females of *D. phthorimaeae* and *P. dignus* alone in the unit was monitored as in the competition treatment, and considered as controls. Fifteen replicates per treatment in this study (*D. phthorimaeae* and *P. dignus* alone- or both species in competition) were conducted. The experiment was held in a walk-in environmental controlled room at $25 \pm 1^\circ\text{C}$, $70 \pm 10\%$ relative humidity (RH) with a 16.00:8.00 hours light : dark (L:D) photoperiod.

Experiment 2 – *D. phthorimaeae* host discrimination and host-feeding behaviour

The ability of *D. phthorimaeae* to distinguish *T. absoluta* larvae previously *P. dignus* parasitized from those unparasitized was tested in similar environmental conditions and using the same experimental unit as in Experiment 1. Six to eight *T. absoluta* third-stage larvae installed in feeding mines in tomato leaves were firstly exposed for 24 h to one *P. dignus* mated female. After that period, the wasp was removed and the same *T. absoluta* larvae were offered to *D. phthorimaeae* mated females for other 48 h. Since the whole experiment lasted 36 h, parasitoid females of both species used in the experiment were naïve, fed *ad libitum* with honey, and mated during the first 24 h after hatching. Three *D. phthorimaeae* female ages were considered: 1, 5 and 7-days old. Adult female ages and host densities were selected on the basis of the *D. phthorimaeae* reproductive biology information (Savino *et al.*, 2012). Fifteen replicates were performed for each age.

Since *P. dignus* females do not leave any visible marks when parasitizing the host, it was not possible to recognize initially the number of parasitized *T. absoluta* larvae offered to *D. phthorimaeae*. Hence, once the test was finished, all host larvae were checked to verify *D. phthorimaeae* attack, and later dissected to record the number of *T. absoluta* larvae previously parasitized by *P. dignus*. Host larvae attacked by

D. phthorimaeae can be determined using a stereoscopic microscope by detecting signs of attack (i.e., paralysed, cream yellow colour, necrosed larva) or the presence of the egg or the larva on the host's tegument (Luna *et al.*, 2010). *P. dignus* immature stages were recognized using the description in Cardona & Oatman (1971). Four possible responses were considered: (1) parasitized by *P. dignus*; (2) parasitized or host-fed by *D. phthorimaeae*; (3) endoparasitized by *P. dignus* and *D. phthorimaeae* host-fed, or multiparasitized (both parasitoid species larvae in the same host); and (4) healthy.

Statistical analysis

For Experiment 1, the proportion of the time elapsed of each behavioural activity for host search ability (mean \pm SEM) for each parasitoid species, alone or in competition, was compared with a paired *t*-test. Since parasitism was only practised by *P. dignus*, the mean proportion of parasitized *T. absoluta* larvae by this parasitoid species was calculated.

For Experiment 2, a balanced two-way analysis of variance (ANOVA) (including both main effects and an interaction term) was used. The two factors were the *D. phthorimaeae* age and the status of *T. absoluta* larvae offered (i.e., parasitized or unparasitized) on the proportion of host larvae attacked by *D. phthorimaeae*. If an interaction was observed, simple effects analysis was conducted, using one-way ANOVA, on individual factors followed by Tukey's HSD test. Response variables did not deviate from normality and homogeneity of variance assumptions. The data collected were analyzed using the STATISTICA software (Statsoft, Inc., 2007). The proportion values were previously transformed to arcsine and then transformed back to proportions for data presentation.

Results

Experiment 1 – Host search ability

Both parasitoid species showed changes in host search ability, modifying behavioural activities and the time spent searching when exposed to one another in the same arena (fig. 1). *D. phthorimaeae* wasps spent significantly more time in general searching ($t = -3.7$; $df = 33$, $P = 0.0008$) and less in intensive searching in the presence of its competitor than in its absence ($t = 3.60$; $df = 33$, $P = 0.002$), and it was never observed parasitizing *T. absoluta* larvae. In the presence of *D. phthorimaeae*, *P. dignus* females dedicated significantly less time to intensive host searching ($t = 2.99$; $df = 28$, $P = 0.005$) and spent more time resting than in the alone condition ($t = -2.80$; $df = 28$, $P = 0.009$). Interestingly, *P. dignus* exhibited an evasive behaviour against its antagonist by hiding behind leaves or other parts of the unit, and physical contacts between the two parasitoid species were observed in only five out of 15 replicates. Time spent by *P. dignus* females in parasitizing alone or in the presence of *D. phthorimaeae* was similar ($t = -0.38$; $df = 28$, $P = 0.70$). Furthermore, *P. dignus* parasitism rate was unaffected by *D. phthorimaeae*, with a mean proportion of 0.44 ± 0.03 of the offered hosts in a fixed 15 min experiment.

Experiment 2 – *D. phthorimaeae* host discrimination and host-feeding behaviour

Analysis indicated that there was an interaction between host status and *D. phthorimaeae* age on *T. absoluta* larvae attacked (table 1). Older wasps discriminated significantly less

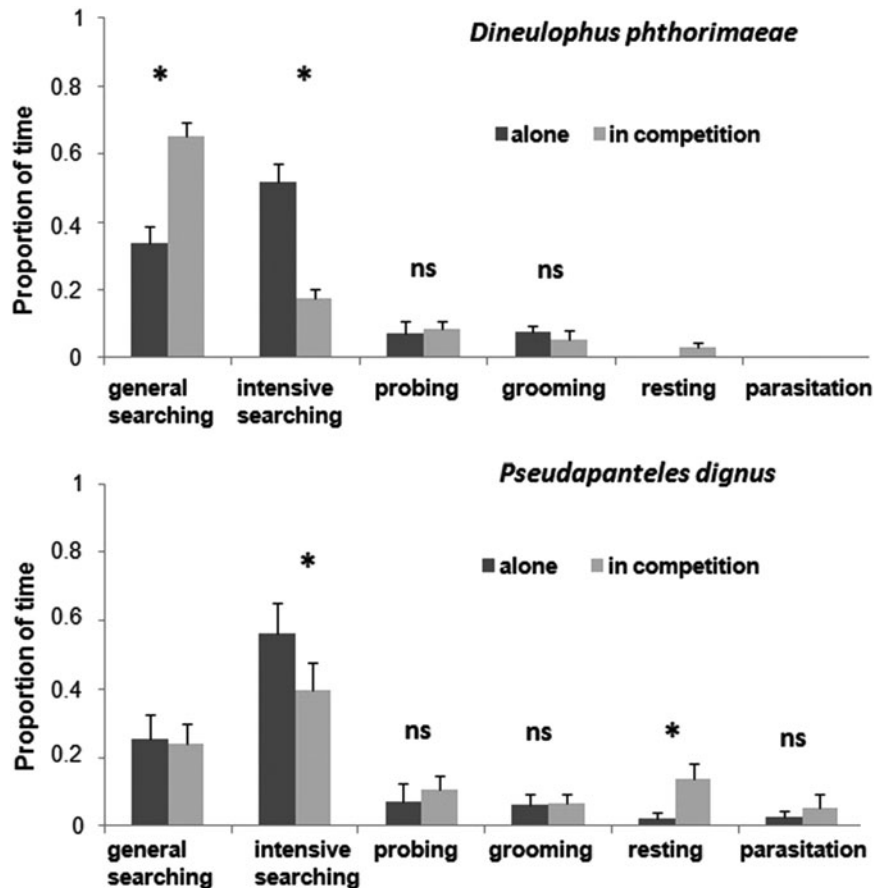


Fig. 1. Proportion of the time (\pm SE) spent in different behavioural activities by female parasitoids of *D. phthorimaeae* and *P. dignus* when alone, or in the presence of its antagonistic female. Asterisks indicate significant differences in behaviour ($P < 0.01$); ns: no significant differences.

Table 1. Results of the two-way ANOVA conducted to jointly assess the age effects of the *D. phthorimaeae* and the status of the *T. absoluta* larvae (parasitized/unparasitized by *P. dignus*) in reference to the ratio of host larvae offered (*significant statistical differences at $P < 0.05$).

Effect	SS	df	MS	F	P
<i>D. phthorimaeae</i> female age	248	2	124	1.3	0.274
<i>T. absoluta</i> larval status	30,050.7	3	10,016.9	105.3	0.000*
<i>D. phthorimaeae</i> age \times <i>T. absoluta</i> larval status	3837	6	640	6.7	0.000*

(fig. 2) between the two host larvae conditions (parasitized or unparasitized by *P. dignus*) whilst *D. phthorimaeae* practised only host-feeding on *T. absoluta* larvae and parasitism was not observed. Furthermore, *D. phthorimaeae* attacks varied significantly with the host status (one-way ANOVA, $F_{3, 176} = 79.1$; $P < 0.0001$), in favour of healthy host larvae. *D. phthorimaeae* adult age was not significant on the proportion of larvae host-fed (one-way ANOVA $F_{2, 177} = 0.44$; $P = 0.644$).

Discussion

Given that only one host individual represents all the resource that a parasitoid needs for its larval development, the competition between species can be intense at this level (Hochberg & Lawton, 1990). Despite this, in nature, the different communities provide sufficient evidence of coexistence among natural enemies by means of a suite of diverse mechanisms, such as niche partitioning, the segregation behaviour through spatial aggregation, or when the hosts' density is intermediate (Godfray, 1994; Mills, 2006).

In this paper we found evidence for the system *D. phthorimaeae* – *P. dignus* – *T. absoluta*, that in the conditions tested, parasitoid female wasps can avoid the same host resource larva. Both species modified their host searching behaviour when confronted with their antagonist. Only *P. dignus* achieved parasitism and the rates were not reduced by the presence of *D. phthorimaeae* females, when exposed simultaneously to *T. absoluta* larvae. Percentage of parasitism by *P. dignus* (~5%) observed in a fixed 15 min experiment, is coincident with some reproductive traits of this species, such as a higher egg load, a moderate sinovigenic strategy and a Type I functional response (Luna *et al.*, 2007; Nieves *et al.*, 2015).

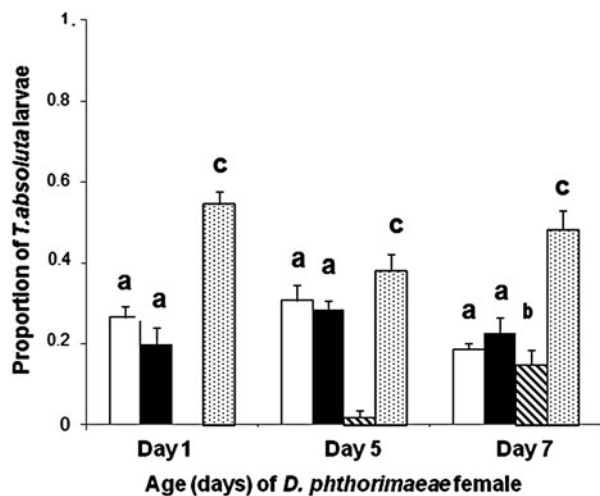


Fig. 2. Mean proportion of *T. absoluta* (+SE) previously exposed to *P. dignus* offered to 1, 5 and 7 day-old adult *D. phthorimaeae* females for 48 h. The figure shows the portion of *T. absoluta* larvae at the end of the experiment that were: parasitized by *P. dignus* only (white bars); the portion of hostfed *T. absoluta* larvae by *D. phthorimaeae* (black bars); the portion of larvae attacked by both species (diagonally striped bars), and the portion of healthy larvae (dotted striped bars). The same letter(s) above the bars for respective histograms for *D. phthorimaeae* age and host larvae status indicate no significant differences (two-way ANOVA; $P < 0.01$).

Conversely, under the same experimental conditions, *D. phthorimaeae* failed to attack *T. absoluta* larvae, and even though it exhibits a similar functional response to *P. dignus*, it is an extremely sinovigenic parasitoid species with a low egg load, which could affect its oviposition behaviour (Savino *et al.*, 2012). We observed that *D. phthorimaeae* did not succeed in parasitizing, which confirmed that this extremely sinovigenic parasitoid, with a potential lifetime fecundity < 10 , behaved as a selective species that allocates eggs only when host physiological and ecological conditions are suitable (Savino *et al.*, 2012; Savino, 2014). Thus, the divergence of the reproductive strategies of *D. phthorimaeae* and *P. dignus* can lead to coexistence by niche partitioning.

Additionally, older *D. phthorimaeae* females discriminated less and attacked, although without ovipositing, unparasitized and parasitized *T. absoluta* larvae by *P. dignus*. Therefore, the competitive interaction took place via the host-feeding behaviour by *D. phthorimaeae*. Host-feeding represents the mechanism by which *D. phthorimaeae* females directly compete with *P. dignus* by eliminating their antagonists' immature individuals through paralysis or venom injection to the host. Collier & Hunter (2001) also observed evidence for direct competition between *Eretmocerus eremicus* (Rose & Zolnerowich) and *Encarsia sophia* (Girault) (Hymenoptera: Aphelinidae), parasitoids of whiteflies, using two mechanisms: the usurpation of hosts in cases of multiparasitism and by lethal host-feeding.

Host resource selection could be then explained by *D. phthorimaeae* reproductive strategy, being that females suffer a significant decrease of egg load with ageing, by resorption (Savino *et al.*, 2012), and they need to perform obligatory host consumption in order to gain energy and mature more

eggs for the next encounter with hosts (Bernstein & Jervis, 2008). Heterospecific host discrimination could be possible by the recognition of parasitized host larvae because of some physical or chemical marks left by *P. dignus*, as well as the detection of movements of the internal larva inside the host (Weisser & Houston, 1993; Godfray, 1994). Further studies would be necessary to answer this point.

The literature supports diverse evidence of coexistence for other parasitoid-host systems that also avoid attacking heterospecific parasitized hosts, including the solitary ectoparasitoids *Cephalonomia stephanoderis* Bretem and *Prorops nasuta* Waterston (Hymenoptera: Bethyridae) (Infante *et al.*, 2001), the pupal endoparasitoid *Coptera haywardi* Loiacono (Hymenoptera: Diapriidae), and the larval-pupal endoparasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) (Cancino *et al.*, 2012). Persad & Hoy (2003) showed that *Lipolexis scutellaris* Mackauer and *Lysiphlebus testaceipes* Cresson (Hymenoptera: Aphididae) promote coexistence by avoiding multiparasitism. Conversely, Leveque *et al.* (1993) found that in the system *Dinarmus basalis* (Rond.) (Hymenoptera: Pteromalidae) – *Eupelmus vuillei* (Craw) (Hymenoptera: Eupelmidae), only the second species increased reproduction in the presence of its antagonist and multiparasitized hosts. Shi *et al.* (2004) demonstrated that when *Cotesia plutellae* and *Diadegma semiclausum* are exposed simultaneously, they attack host larvae already parasitized by the other species, and that this causes a higher joint host mortality. Xu *et al.* (2013) reported for *Encarsia sophia* and *Eretmocerus hayati* that they do not avoid multiparasitism and reproduction of both species is negatively affected. Ulyshen *et al.* (2009) observed that in a similar parasitoid–parasitoid system to that studied in the research presented here, the larval ectoparasitoid *Spathius agrili* parasitized larvae that were previously parasitized by the larval endoparasitoid *Tetrastichus planipennis* completely eliminating the latter in laboratory and field tests.

The prediction that the competition between *D. phthorimaeae* and *P. dignus* could favour the idiobiont ectoparasitoid was partially confirmed. Only older *D. phthorimaeae* wasps killed *T. absoluta* larvae parasitized or unparasitized by *P. dignus*, via host-feeding. Other authors have reported superiority of larval ectoparasitoids in their competition for hosts. Varley (1947) found that ca. 25% of the koinobiont *Eurytoma curta* Walker (Hymenoptera: Eurytomidae) parasitoid larvae were dead by the actions of a complex of idiobiont parasitoid species, with similar biology to *D. phthorimaeae*. Zaviezo & Mills (2001) also showed a predominance of the larval ectoparasitoid *Hyssopus pallidus* (Askew) (Hymenoptera: Eulophidae) over the endoparasitoid *Ascogaster quadridentatus* Wesm. (Hymenoptera: Braconidae) and Ulyshen *et al.* (2009) showed similar results for the idiobiont ectoparasitoid *S. agrili* over the koinobiont endoparasitoid *T. planipennis*.

The intra-guild predation might have only a minor effect in this system since *D. phthorimaeae* females majorly avoid attacking previously parasitized hosts by *P. dignus*, from which it is assumed that the energy gained would be insignificant (Polis *et al.*, 1989; Borer, 2002). Nevertheless, this result indicates that primary larval parasitoid species can eventually be involved in direct trophic interactions by intraguild predation by non-concurrent host feeding.

In conclusion, it is suggested that *D. phthorimaeae* and *P. dignus* are able to coexist and that the intensity of the interaction would depend, among other factors, on the age and the state of nutrition of the *D. phthorimaeae* females. This would be particularly relevant for *T. absoluta* biological control, where

the destructive effect of one of its natural enemies' can be quite high against other species in the complex.

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