For: *Biological Control* (clean version)

**Foraging efficiency and outcomes of interactions of two pupal parasitoids attacking the
invasive spotted wing drosophila**

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Abstract

Two generalist pupal drosophilid parasitoids, *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae), are sympatric and among only a few parasitoid species attacking the invasive *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) in many regions of the world. In this study, we evaluated their foraging efficiency when attacking *D. suzukii* infesting cherry fruit in a laboratory cage experiment; and we examined their potential interspecific interactions, including outcomes of intrinsic competition, host discrimination, and the impact of their interaction on pest control. We show that both parasitoids readily parasitized *D. suzukii* pupae located inside fruit or buried in soil. However, *T. drosophilae* was more efficient than *P. vindemiae* and that parasitism by either parasitoid species was higher in the fruit than in the soil. Generally, the parasitoid species that oviposited first in the host out-competed the later parasitoid in multi-parasitized hosts, we assume, through physiological suppression. Both parasitoids discriminated against hosts parasitized previously by the other species. In an additive-series design experiment with single (*D. suzukii*) or two (*D. suzukii* and *D. melanogaster* Meigen) host species, *T. drosophilae* performed similarly regardless of the presence of a competitor, while *P. vindemiae* achieved a higher parasitism when present alone. The observed parasitism when the two parasitoid species were present together was always lower than the expected one, assuming each species acted independently. This indicates a negative effect by *P. vindemiae* on interspecific competition on host suppression.

Keywords: biological control; *Drosophila suzukii*; interspecific competition; *Pachycrepoideus vindemiae*; *Trichopria drosophilae*

1. Introduction

Interspecific competition among biological control agents could result in the displacement of one species by another species or a decline in the overall biological control effectiveness (Collier et al., 2002; Reitz and Trumble, 2002; Wang et al., 2003, 2008). It is thus important to predict potential interactions, such as competitive outcomes, among natural enemies when designing biological control programs that employ multiple natural enemy species (Mills, 2006). In parasitoids, interspecific competition may occur among species attacking common hosts both extrinsically (among free-living adults) and intrinsically (among immature parasitoids) (Quicke, 1997; Harvey et al., 2013; Cusumano et al., 2016). In extrinsic competition, species may avoid or reduce competition through resource and niche partitioning or differences in life-history and ecological traits (Hawkins, 2000; Bonsall et al., 2002; Amarasekare, 2003; Aluja et al., 2013; Feng et al., 2015; Wang et al., 2015). Intrinsic competition among solitary parasitoids can result in one species eliminating or suppressing the competing species through physical attack, physiological suppression, or both mechanisms (Harvey et al., 2013; Cusumano et al., 2016). In general, the parasitoid that first oviposits into the host prevails in intrinsic competition, largely because the first-instar larvae of many parasitoids often possess sickle-like mandibles that are used to kill the eggs or larvae of other parasitoid species, or the adult parasitoid injects chemicals into the host that create a suitable physiological milieu for the growth and development of her own offspring (Harvey et al., 2013). However, there are some exceptions. For example, some first instar larvae of aphidiine braconids are able to use their mandibles to attack older amandibulate second instars (Chow and Mackauer, 1985) and some adult parasitoids directly kill the eggs or larvae already present in the hosts (Leveque et al., 1992). Also, ectoparasitoids are

likely to out-compete endoparasitoids because the former ones often inject paralyzing venom that not only paralyze the host but also kill endoparasitoids present in the host (Harvey et al., 2013).

Here, we investigated the potential interactions between two solitary parasitoids of fruit flies, *Pachycrepoideus vindemiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae). Both are cosmopolitan species that attack a range of drosophila host species (Carton et al., 1986), including the spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), a pest fly native to East Asia but that has recently expanded its global range to include Europe, North America, and South America (Walsh et al., 2011; Cini et al., 2014; Asplen et al., 2015). *P. vindemiae* appears to be more of a generalist than *T. drosophilae*, as the former species also attacks host species in other families of cyclorrhaphous Diptera as a primary parasitoid (Noyes, 2002) and primary fruit fly parasitoids as a facultative hyperparasitoid (Van Alphen and Thunnissen, 1983; Wang and Messing, 2004a). *P. vindemiae* can even attack and develop from conspecific larvae (Chen et al., 2015). In contrast, the host range of *T. drosophilae* is known to be limited within Drosophilae (Carton et al., 1986). There are biological differences among these pupal parasitoids as well. As an ectoparasitic idiobiont, a female *P. vindemiae* first injects venom to paralyze the host and then places its eggs on the outside of the host body that is enclosed by a protective puparium formed from the hardened exoskeleton of the fly's last larval stage (Wang and Messing, 2004b). In contrast, *T. drosophilae* is an endoparasitic idiobiont, whose female also paralyzes the host after the attack but places her eggs inside the host tissue (Chabert et al., 2012; Wang et al., 2016).

Following the invasion of *D. suzukii*, several recent studies in Europe and North America have evaluated the potential of various indigenous drosophila parasitoids to control *D. suzukii* in the invaded regions (Chabert et al., 2012; Kacsoh and Schlenke, 2012; Gabarra et al., 2014;

Miller et al., 2015; Rossi Stacconi et al., 2015; Wang et al., 2016). Although over 50 hymenopteran parasitoid species have been recorded attacking various drosophilid species, the majority of these parasitoids belong to the *Asobara* (Braconidae), *Leptopilina* and *Ganaspis* (Figitidae) genera and primarily attack host larvae living in fermenting substrates, such as *D. melanogaster* Meigen (Diptera: Drosophilidae), and only a few are pupal parasitoids including *P. vindemiae* and *T. drosophilae* (Carton et al., 1986; Mitsui et al., 2007; Daane et al., 2016). Moreover, most indigenous larval parasitoids appear to be unable to develop from *D. suzukii* because of the strong host immune resistance by *D. suzukii*, whereas both *P. vindemiae* and *T. drosophilae* successfully develop from *D. suzukii* (Chabert et al., 2012; Kacsoh and Schlenke, 2012; Poyet et al., 2013; Rossi Stacconi et al., 2015; Wang et al., 2016). We recently surveyed parasitoid species attacking frugivorous Drosophilidae in California, USA and found that only *P. vindemiae* and *T. drosophilae* emerged from field-collected *D. suzukii* in fruits (Wang et al., unpublished data). Both parasitoids were also found in association with *D. suzukii* in Italy, Spain and the pest's native area in Asia (Gabarra et al., 2014; Rossi Stacconi et al., 2013, 2015; Daane et al., 2016), and *P. vindemiae* attacks *D. suzukii* in Oregon, USA (Miller et al., 2015).

P. vindemiae and *T. drosophilae* are sympatric in California and many other regions of the world and are therefore likely to compete; however, their interspecific interactions and consequences on pest suppression have not been studied. There is also a lack of comparative studies on the relative efficiency between these two parasitoid species when locating and attacking *D. suzukii* on its favorable host plants such as cherry. Unlike other drosophilids that feed typically on decaying fruits, *D. suzukii* females also lay eggs in healthy, ripening fruits using a serrated ovipositor (Walsh et al., 2011; Asplen et al., 2015). Although *D. suzukii* exploits fruit resources in the ripening stages before they become available to other *Drosophila* species, it

can also infest overripe or damaged fruits concurrently with other *Drosophila* species such as *D. melanogaster* (Wang et al., unpublished data). The aims of this study were therefore to determine (i) the parasitoids' relative foraging efficiency in a laboratory trial using cherry fruit infested with *D. suzukii* pupae and (ii) the outcomes of their potential interactions, including mechanisms underlying the elimination of competitors, discrimination ability against hosts previously parasitized by the other parasitoid species and (iii) the impact of interspecific competition on host mortality. Additionally, the immature morphs and developmental stages of both parasitoid species were characterized in order to distinguish the two parasitoids in multiple-parasitized hosts during dissections. In the last experiment, both *D. suzukii* and *D. melanogaster* were used to examine the effect of the presence of an alternative host on interspecific competition.

2. Materials and Methods

2.1. Insects and plant

Studies were conducted under controlled laboratory conditions ($23 \pm 1^{\circ}\text{C}$, 16L: 8D, 40–60% RH) at the University of California's Kearney Agricultural Research and Extension Center (Kearney) in Parlier, CA, USA. Laboratory colonies of the two host species (*D. suzukii* and *D. melanogaster*) and two parasitoid species (*P. vindemiae* and *T. drosophilae*) were initiated from field collections during 2013 in Parlier, and maintained under the same laboratory conditions. *D. suzukii* were collected from infested cherries in the trees and *D. melanogaster* were collected from rotten cherries and peaches on the ground. The parasitoids were reared from parasitized *D. suzukii* and *D. melanogaster* placed in sentinel traps baited with host species pupae, as well as parasitized host pupae from field-collected fruits. To maintain vigor, field-collected insects of each species were introduced into the colonies periodically.

Adult flies were held in Bug Dorm cages (BioQuip Products Inc., Rancho Dominguez, CA), while adult parasitoids were held in smaller screened cages (30 × 30 × 30 cm) (Mega View Science Co. Ltd., Taichung, Taiwan); all insects were supplied with a 10% honey-water solution as food. Fly larvae were reared on a standard cornmeal-based artificial diet in Petri dishes (1.5 cm high, 14.0 cm diameter), as described by Dalton et al. (2011). The Petri dishes were exposed to adult flies for 24 h, and after the flies had developed into 2–3 day old pupae, the dishes were exposed to the adult parasitoids for 2–3 days. The parasitoid-exposed dishes were then transferred to new cages and held for the emergence of adult flies (unparasitized flies emerged in 2–3 days) and parasitoids (emerged in ~20 days).

All bioassays used 12–16 day old female flies and 4–6 day old female parasitoids that had been kept with males since emergence (i.e., assumed to be mated with a high load of mature eggs based on preliminary observations). Cherry ('Bing') branches bearing ripe fruit were cut from an unsprayed orchard and then used within 24 h for all experiments.

2.2. Foraging efficiency

To assess the relative efficiency of *P. vindemiae* and *T. drosophilae* in locating and parasitizing *D. suzukii*, the experiment was carried out in the smaller screened cages containing a 20 cm cherry branch. Each branch was carefully examined under a binocular microscope and any naturally infested and/or excessively ripe fruit was removed. Ten intact fruit and 5 leaves were retained on each branch, and the base was inserted into a plastic container (10 cm high, 5 cm diameter) filled with sterilized soil and water (to keep the leaves and fruit as fresh as possible). Inside the cage, the water filled container was placed on a Petri dish (1.5 cm high, 14.0 cm diameter) filled with sterilized soil and water and positioned so that any fly larvae that emerged

would drop and pupate into this dish. Ten adult *D. suzukii* females were first released into each cage and then removed 2 days later. After another four days, when the flies had developed into pupae, 10 female *P. vindemiae* or *T. drosophilae* were released into each cage for 3 days.

Experiments with each parasitoid species had 10 replicates (i.e., 10 cages).

Once each experiment was initiated, leaves and fruit could be kept fresh for about one week, which was enough time to complete the experiment. After being exposed to the parasitoids, all host pupae were collected from the fruit and soil, and placed on wet tissue paper inside Petri dishes (1 cm high, 5 cm diameter) until the emergence of adult flies or parasitoids, with each replicate kept separately for the two different pupation habitats (fruit or soil). After adult emergence ceased, all remaining (presumably dead) host pupae were reconstituted in water for 1 day and then dissected under a microscope to determine the presence or absence of recognizable fly or parasitoid cadavers. Percentage parasitism was estimated based on numbers of emerged adults and dissected pupae containing a dead parasitoid or fly (see Wang et al., 2016).

2.3. Interspecific competition and host discrimination

To determine how each parasitoid species might eliminate competitors and whether or not they could discriminate against hosts previously parasitized by the other parasitoid species, three experiments were conducted, all in Petri dishes (1 cm high, 5 cm diameter) using 2–3 day old *D. suzukii* pupae. Prior to these experiments, dissections were made of *D. suzukii* pupae that had been exposed to the two parasitoid species at different intervals after exposure in order to develop our expertise in distinguishing the different immature morphs and developmental stages between these two parasitoid species. A subsample of 10 newly laid eggs of each parasitoid species were also measured with an ocular micrometer for their maximum length and width.

First, we determined the mechanisms by which each parasitoid species might eliminate their competitors. Five host pupae were exposed first to one female *P. vindemiae* or one female *T. drosophilae* for 24 h and then to a female of the other parasitoid species for another 24 h. Following the second exposure, all hosts were dissected within 24–48 h under a microscope to determine the developmental stages and survival of all parasitoids inside the hosts. Parasitoid condition could be determined because for both parasitoid species a live egg substantially enlarges before hatching and the embryo matures into a larva within 48 h in most cases, while still confined within the enlarged chorion. In contrast, dead eggs show signs of decomposition of the embryo and gradually become shrunken and uniformly opaque white. Also, a live larva moves actively while a dead larva shows no movement. Using these criteria, 150 exposed hosts were dissected for each exposure sequence (300 total).

Second, we determined the ability of either parasitoid species to discriminate against hosts already parasitized by the other parasitoid species. Two host pupae were exposed to either one female *P. vindemiae* or one female *T. drosophilae* for 6 h (which resulted in approximately 100% parasitism and also gave the oviposition experience for the naïve female parasitoid), and then the same female was presented with two unparasitized hosts and two hosts previously exposed to the female of the other parasitoid species for 6 h. All exposed hosts were dissected within 24 h to determine the presence or absence of both parasitoid eggs and larvae. The experiment had 20 replicates.

Third, we confirmed the host discrimination ability or outcomes of interspecific competition through rearing all exposed hosts. Ten host pupae were exposed to either one female *P. vindemiae* or one female *T. drosophilae* for 24 h, and then five (half) of the previously exposed hosts together with five unexposed hosts were presented to one female of the other parasitoid

species for 24 h. All exposed hosts were reared until the emergence of adult parasitoids or flies.
The experiment had 25 replicates for each combination.

2.4. Effect of interspecific interaction on host suppression

Additive-series design experiments were conducted to assess the effect of interspecific competition between the two parasitoid species on host mortality (parasitism). In other words, the single species treatments with *P. vindemiae* only or *T. drosophilae* only, used half the number of total adult parasitoids as the multiple species treatment (both *P. vindemiae* and *T. drosophilae*). This design is suitable for examining interspecific interactions among natural enemies since it holds intraspecific interactions constant across different levels of diversity (Schmitz, 2007; Hogg et al., 2013).

In the first experiment, twenty 2-day old *D. suzukii* pupae were exposed to (1) one female *P. vindemiae*, (2) one female *T. drosophilae*, or (3) one female of both parasitoid species together in Petri dishes (1 cm high, 5 cm diameter) for 36 h. Then, all exposed hosts were reared until the emergence of flies and/or parasitoids. Parasitism was estimated as described above in the foraging efficiency experiment. The experiment had 21 replicates for each treatment.

The second experiment was designed to determine the effect of alternative host species. We used the smaller screened cages containing a cherry branch and fruit infested simultaneously by both *D. suzukii* and *D. melanogaster*. Cherry branches bearing 10 intact fruit and placed above a large Petri dish with soil were prepared as described previously. Because *D. melanogaster* is unlikely to infest intact fruit, additionally 10 intact fruit were damaged with a 1 cm long surface cut to facilitate *D. melanogaster* oviposition and placed on another soil Petri dish along with branch fruit in each cage. Five *D. suzukii* females and five *D. melanogaster* females were

released into each cage for a 48 h exposure period, and then the flies were removed. Five days later the treatments were established with either (1) 5 female *P. vindemiae*, (2) 5 female *T. drosophilae*, or (3) 5 female *P. vindemiae* and 5 female *T. drosophilae*. The parasitoids had a 3 day exposure periods, after which all fly pupae were collected and separately reared according to host species and pupation habitats (fruit vs. soil). Pupae of *D. suzukii* were easily distinguishable from *D. melanogaster* by the presence of a pair of distinct respiratory tubes on the anterior end. A control treatment without release of parasitoids was also set-up to collect extra data on host abundance and distribution. Each treatment had 15 replicates. Parasitism was also estimated as described previously.

2.5. Data analysis

Mean percentages of parasitism, numbers of hosts that pupated in the two different habitats (fruit and soil), and numbers of different types of hosts (previously parasitized vs. unparasitized) were analyzed among treatments using one-way ANOVA. All data were first inspected for normality and error variance for homoscedasticity and all percentage data were logit transformed as needed before ANOVA.

For the additive-series design experiments, the observed levels of host mortality (parasitism) in the two species release treatment (*P. vindemiae* + *T. drosophilae*) were compared with the expected levels of host mortality, calculated using data from the single species release treatments only. If interspecific interactions among parasitoid species have no effect on the host population (i.e., parasitoids act independently), the levels of host mortality should follow a multiplicative risk model ($H_{\text{exp}} = H_p + H_t - H_p \times H_t$) (Schmitz, 2007). Here, H_{exp} is the expected host mortality when parasitoids were present together, H_p is the observed host mortality by *P. vindemiae* alone,

and H_i is the observed host mortality by *T. drosophilae* alone. The observed and expected levels of host mortality were compared across replicates, using a t-test. All analyses were performed using JMP Pro ver11 (SAS 2011, Cary, NC).

3. Results

3.1. Foraging efficiency

More *D. suzukii* larvae pupated in the fruit than the soil ($F_{1,38} = 7.9$, $P = 0.008$) (Fig.1A). Both *P. vindemiae* and *T. drosophilae* were able to locate and attack *D. suzukii* pupae in fruit and soil, but *T. drosophilae* was more efficient than *P. vindemiae* ($F_{1,18} = 6.7$, $P = 0.018$) (Fig.1B). Parasitism was higher in the fruit than the soil for both *T. drosophilae* ($F_{1,18} = 9.4$, $P = 0.007$) and *P. vindemiae* ($F_{1,18} = 5.4$, $P = 0.032$) (Fig.1C).

3.2. Interspecific competition and host discrimination

The egg of *P. vindemiae* is waxy white (not translucent) and elongate-ovate, more or less cylindrical hymenopteriform, and its larva has six instars. Each instar is similar in appearance, all are conical in shape with the prothoracic segment slightly the widest and the remaining segments narrowing posteriorly. In contrast, the *T. drosophilae* egg is translucent, prolate spheroid with a slight dorsal arch. Newly laid *P. vindemiae* eggs (mean $\pm$ SE, 0.329 ± 0.082 mm) were longer than that of *T. drosophilae* (0.227 ± 0.081 mm) ($F_{1,18} = 154.1$, $P < 0.001$), but the maximum width of both parasitoid species were similar (0.082 ± 0.002 mm for *P. vindemiae* and 0.083 ± 0.002 mm for *T. drosophilae*, $F_{1,18} = 0.097$, $P = 0.758$). The *T. drosophilae* larva has three instars. The first instar is segmented with a pair of large and sclerotized mandibles, and the end of the abdomen has a two-lobed appendage with several teeth on each lobe. Both second and

third instars are similar in appearance: grub-like and lacking the abdominal appendage and large mandibles. It was thus easy to distinguish the morphs between these two parasitoids during the dissections. Also, *T. drosophilae* completed juvenile development 1–2 days earlier than *P. vindemiae*, under the current laboratory conditions, which also aided identification.

Dissections of all *D. suzukii* pupae that were first exposed to *T. drosophilae* and then to *P. vindemiae* found a total of 44 hosts that were parasitized by both parasitoid species (multiple parasitism). Of these, the *P. vindemiae* larva was dead in 22 cases, the *T. drosophilae* larva was dead in 2 cases, and both parasitoid species larvae were dead in 2 cases. In the remaining 18 cases both parasitoid species larvae were still alive at the time of dissection. No dead parasitoid eggs were found. When the hosts were first exposed to *P. vindemiae* and then to *T. drosophilae*, 26 hosts were parasitized by both parasitoids. The *T. drosophilae* larva was dead in 10 cases, both the *P. vindemiae* egg and larva were dead in 1 case, and both parasitoid species larvae were dead in 2 cases. In the remaining 14 cases, both parasitoid larvae were still alive at the time of our dissections.

In all other dissected hosts, they were either unparasitized or parasitized by one parasitoid only. A total of 58 hosts were superparasitized by *T. drosophilae* and in most cases (50) only one first instar survived while all other supernumerary larvae (1–6 larvae per host) were killed. Lacerations on the cuticle of *T. drosophilae* larvae were observed during dissections in superparasitized hosts. In two cases, both first instars were killed and in 6 cases more than one first instar still survived by the time of dissection. A total of 32 hosts were superparasitized by *P. vindemiae* and the supernumerary individuals were dead as eggs (8 cases) or as first instar larvae (14 cases). In 12 cases more than one larva still survived at the time of dissection.

Both *P. vindemiae* ($F_{1,38} = 52.6$, $P < 0.001$) and *T. drosophilae* ($F_{1,38} = 15.6$, $P < 0.001$) accepted fewer hosts previously parasitized by the other parasitoid species, as compared with unparasitized hosts (Fig. 2). More parasitoid offspring emerged from hosts that were not previously exposed than from hosts that were previously exposed to the other parasitoid species for both *P. vindemiae* (2.60 ± 0.41 vs. 1.48 ± 0.30 ; $F_{1,48} = 3.3$, $P = 0.038$) and *T. drosophilae* (2.72 ± 0.28 vs. 1.92 ± 0.31 ; $F_{1,48} = 26.2$, $P < 0.001$). Similarly, fewer offspring were produced when a parasitized host was subsequently exposed to the other parasitoid species for *P. vindemiae* (1.04 ± 0.11 vs. 1.84 ± 0.39 ; $F_{1,48} = 5.1$, $P = 0.015$) and *T. drosophilae* (1.04 ± 0.19 vs. 2.88 ± 0.30 ; $F_{1,48} = 3.4$, $P = 0.032$).

3.3. Effect of interspecific interaction on host suppression

In the first experiment using *D. suzukii* as the only host species, more hosts were parasitized by *T. drosophilae* than by *P. vindemiae* in both single ($F_{1,40} = 4.9$, $P = 0.032$) and two species release ($F_{1,40} = 36.6$, $P < 0.001$) (Fig. 3). The observed parasitism ($64.5 \pm 3.2\%$) was lower than the expected parasitism ($97.1 \pm 0.8\%$), assuming that the two species acted independently ($F_{1,40} = 93.2$, $P < 0.001$).

In the second experiment with *D. suzukii* and *D. melanogaster* simultaneously infesting cherry fruit, *D. suzukii* produced more pupae than *D. melanogaster* ($F_{1,118} = 45.7$, $P < 0.001$) (Fig. 4A). About half ($51.5 \pm 3.5\%$) of the *D. suzukii* pupae were produced from the branch fruit, while only $7.7 \pm 2.4\%$ of the *D. melanogaster* pupae were produced from the branch fruit. Both the percentage of intact branch fruit attacked ($F_{1,118} = 113.8$, $P < 0.001$) and the percentage of host larvae pupating in the fruit ($F_{1,118} = 24.4$, $P < 0.001$) were higher by *D. suzukii* than by *D. melanogaster* (Fig. 4B).

Total parasitism of each or both host species by *T. drosophilae* was always higher than by *P. vindemiae* when the parasitoids were released alone or simultaneously (Table 1). Parasitism by *T. drosophilae* was similar regardless of whether it was released alone or in combination, while parasitism by *P. vindemiae* was higher when released alone and was reduced when the two species were released in combination (Table 1). Both parasitoids did not show a preference over the two different host habitats, except that *T. drosophilae* parasitized a higher percentage of *D. suzukii* pupae in the soil than in the fruit when released alone (Table 1). The observed parasitism when both parasitoid species were released together was lower than the expected parasitism in all host combinations (Fig. 5).

4. Discussion

A key factor in determining the outcome among competing parasitoid species is their intrinsic competition traits (Harvey et al., 2013; Cusumano et al., 2016). We showed that the outcome of intrinsic competition between *P. vindemiae* and *T. drosophilae* offspring was largely determined by which species' egg was first placed in the host, with the parasitoid species that oviposited first generally outcompeting the later. Morphologically, *T. drosophilae* first instars appear to be better equipped for intrinsic competition, possessing well-developed caudal appendages and strong mandibles. Caudal appendages may facilitate its movement through the host's internal tissues and hemolymph to encounter competing larvae, while its strong mandibles may be used to physically kill competing larvae. As an example, the relatively fast movement by first instar *T. columbiana* (Ashmead) is believed to be aided by oxygen obtained through diffusion, whereas second and third instars move slowly and obtain oxygen by attaching to the host's tracheal system (Coon et al., 2014). Adult female *T. drosophilae* did not appear to directly

kill competing species eggs before they laid their own eggs, and the supernumerary individuals were later eliminated by *T. drosophilae*'s early larval stages. Thus, both physical killing and physiological suppression are likely employed to eliminate conspecific competitors by *T. drosophilae*. By contrast, *P. vindemiae* larvae possess small mandibles, lack appendage and remain largely immobile on the host surface in all instars (Tormos et al., 2009). Instead, adult female *P. vindemiae* may kill competitors' eggs in host tissue when they attack hosts parasitized by conspecifics (Goubault et al., 2004a, b). *P. vindemiae* larvae may physically kill conspecific competitors, but their relatively slower movement inside the host may result in a longer period before the competitor is detected.

It is unlikely that the endoparasitic *T. drosophilae* larvae would be able to detect and physically kill the ectoparasitic *P. vindemiae* larvae, or vice versa. For that reason, physiological suppression may play the major role in eliminating the other parasitoid species when multiparasitism occurs with these species. The exact mechanisms of physiological suppression are unclear. It may involve a subtle form of resource competition or asphyxiation that has been monopolized by the first established parasitoid (Harvey et al., 2013), or other forms of physiological suppression such as venom (Vinson and Hegazi, 1998). A previous study reported that, regardless of oviposition order, *P. vindemiae* always prevails in competition against the pupal fruit fly parasitoid *Dirhinus giffardii* Silvestri (Hymenoptera: Chalcididae); apparently the former injects a venom that causes the death of *D. giffardii* larvae in multiparasitized hosts (Wang and Messing, 2004b). But in the current case, neither *P. vindemiae* or *T. drosophilae* seem to be absolutely superior in terms of the physiological factors; in fact, the ectoparasitic *P. vindemiae* did not seem to have an advantage over the endoparasitic *T. drosophilae*.

Interspecific discrimination was mainly observed among phylogenetically close competitors (Vet et al., 1984), or parasitoid species that co-evolved sympatrically for many generations (Gauthier et al., 1999). Previous studies did show that *P. vindemiae* is able to discriminate against hosts parasitized by conspecific females (Goubault et al., 2003; 2004a, b). The current study showed that both *P. vindemiae* and *T. drosophilae* were able to discriminate against hosts previously parasitized by other species. Because of the early-acting superiority, rejection of parasitized hosts could be the best selection strategy by either species as the advantage is self-evident. This could avoid or reduce competition in free-searching environments in natural habitats.

Results obtained also demonstrate that both *P. vindemiae* and *T. drosophilae* adults are able to locate and attack *D. suzukii* in fruit or soil, but highlight a higher foraging efficiency by *T. drosophilae* than by *P. vindemiae*. Several main factors have likely affected their relative differences in foraging efficiency. First is their reproductive strategy: female *T. drosophilae* have a high load of mature eggs (young females contained up to 71.8 eggs, Wang et al., 2016), whereas female *P. vindemiae* produce and hold fewer mature eggs (< 30 eggs) (Wang and Messing, 2004b) but *P. vindemiae* offspring are female-biased (Nadel and Luck, 1992). As a result, the intrinsic rate of increase of *T. drosophilae* (0.124) was similar to that of *P. vindemiae* (0.139) on *D. suzukii* under similar conditions (Rossi Stacconi et al., 2015; Wang et al., 2016). Thus, egg-limitation likely occurs in *P. vindemiae* when the parasitoid is provided with ample hosts (Rossi Stacconi et al., 2015). However, even under the low host density conditions in the later Petri dish experiment, more hosts were attacked by *T. drosophilae* than *P. vindemiae*, suggesting other factors, such as host handling time, might have also affected their relative foraging efficiency. Indeed, *P. vindemiae* examines hosts both externally and internally using her

ovipositor (Goubault et al., 2004a), whereas *T. drosophilae* recognizes hosts through detection of a host contact kairomone (Romani et al., 2002). There may be involving in a high cost in host handling by *P. vindemiae*.

Our results suggest that interspecific competition between these two parasitoids may reduce the overall impact on the host population. Under the simple conditions presented in our Petri dish experiment with *D. suzukii* only, parasitism by each parasitoid was reduced when they were present in combination, indicating strong interspecific competition. However, under the manipulated laboratory cage conditions, *T. drosophilae* performs similarly regardless of whether it was released alone or in combination. On the contrary, *P. vindemiae* achieves a higher parasitism when released alone, while it suffers from *T. drosophilae* competition when the two species are released in combination. In the latter, *T. drosophilae* seems to have an advantage over *P. vindemiae*. We also observed that *T. drosophilae* develop faster than *P. vindemiae*. Such differences in terms of the duration of embryological development may not be shown when hosts were exposed to both parasitoids subsequently, but it could play a major role in determining winners and losers when both parasitoids were released simultaneously, as in this additive-series design experiment.

In nature, sympatric species may be able to avoid or reduce competition through exploring different resources or habitats (i.e., niche separation) (Amarasekare, 2003). Although *T. drosophilae* and *P. vindemiae* did not show a preference to *D. suzukii* vs. *D. melanogaster* hosts or fruit vs. soil habitats, the presence of an alternative host did appear to relax interspecific competition between these parasitoid species. Both parasitoid species may attack a diverse array of *Drosophila* species that breed on different habitats and co-exist through exploiting different hosts in natural habitats. Thus, long-scale field investigations on population abundance taking

into account host-parasitoid dynamics are required to confirm our results. It is also important to study the effects of factors such as host fruit species, host species and habitat characteristics on the coexistence of the two parasitoid species. More importantly, all of these factors need to be investigated to determine how the competitive interactions between the two species could influence their efficiency in suppressing *D. suzukii*.

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 555

Table 1. Mean ($\pm$ SE) parasitism of *D. suzukii* (DS) and *D. melanogaster* (DM) by *P. vindemiae* (PV) and *T. drosophilae* (TD) when the parasitoid species were present singly or simultaneously in an additive design experiment in laboratory cage

Treatment	Parasitoid species	Total parasitism of each or both host species (%) ¹			DS parasitism in different habitats (%) ²		DM parasitism in different habitats (%) ³	
		DS	DM	DS + DM	Fruit	Soil	Fruit	Soil
Single species	PV	16.7 $\pm$ 2.8 b	27.6 $\pm$ 8.4 b	17.0 $\pm$ 2.6 b	16.9 $\pm$ 3.7 A	15.0 $\pm$ 3.4 A	24.3 $\pm$ 11.7 A	31.5 $\pm$ 9.3 A
Single species	TD	49.6 $\pm$ 3.4 a	62.7 $\pm$ 7.0 a	53.1 $\pm$ 3.5 a	33.1 $\pm$ 6.4 A	66.2 $\pm$ 5.7 B	69.6 $\pm$ 8.6 A	46.4 $\pm$ 10.0 A
Both species	PV	4.3 $\pm$ 1.5 c	10.1 $\pm$ 6.2 c	6.1 $\pm$ 2.6 c	3.6 $\pm$ 1.6 A	4.7 $\pm$ 2.2 A	3.6 $\pm$ 3.6 A	4.1 $\pm$ 4.1 A
	TD	33.9 $\pm$ 3.8 a	44.3 $\pm$ 6.9 a	35.5 $\pm$ 4.3 a	29.8 $\pm$ 4.1 A	39.0 $\pm$ 6.6 A	36.1 $\pm$ 7.5 A	53.4 $\pm$ 10.1 A
	PV + TD	38.2 $\pm$ 3.1 a	54.4 $\pm$ 6.9 a	41.6 $\pm$ 3.3 a	33.4 $\pm$ 3.7 A	43.7 $\pm$ 6.2 A	39.8 $\pm$ 9.3 A	57.4 $\pm$ 9.2 A

¹ Different letters (lower case) within columns indicate significant difference (ANOVA, $P < 0.05$).

² Different letters (upper case) within row indicate significant difference (ANOVA, $P < 0.05$).

³ Different letters (upper case) within row indicate significant difference (ANOVA, $P < 0.05$).

Figure captions

Fig. 1. Foraging efficiency by *P. vindemiae* and *T. drosophilae* on *D. suzukii* pupae held in cages containing cherry fruit and soil. (A) Number of host larvae pupated in the fruit and soil, (B) total percentage parasitism of host pupae by each parasitoid species, and (C) percentage parasitism of host pupae in the fruit and soil by each parasitoid species. Bars refer to mean $\pm$ SE and different letters above the bars indicate significant difference (ANOVA, $P < 0.05$).

Fig. 2. Discrimination by adult *P. vindemiae* and *T. drosophilae* between unparasitized *D. suzukii* pupae and parasitized *D. suzukii* pupae that had been recently parasitized by the other parasitoid species. Bars refer to mean $\pm$ SE of numbers of the two different types of hosts (2 pupae per host type) parasitized by the parasitoids based on the dissection of exposed hosts and different letters above the bars indicate significant difference (ANOVA, $P < 0.05$).

Fig. 3. Numbers of *P. vindemiae* and *T. drosophilae* adults that emerged from *D. suzukii* pupae when the parasitoid species were present singly or simultaneously in an additive design experiment in Petri dish. Bars refer to mean $\pm$ SE and different letters above the bars indicate significant difference (ANOVA, $P < 0.05$).

Fig. 4. Abundance and distribution of pupae produced by *D. suzukii* and *D. melanogaster* when presented hosts in fruit or soil habitat. (A) Total number of pupae produced by each host species; and (B) percentage of pupae produced from the intact branch fruit and percentage of larvae

pupated in fruit by each host species. Values are mean $\pm$ SE and different letters above the bars indicate significant difference between these two different host species (ANOVA, $P < 0.05$).

Fig. 5. The observed and expected parasitism of *D. suzukii* and *D. melanogaster* by *P. vindemiae* and *T. drosophilae* when the parasitoid species were present singly or simultaneously in an additive design experiment in laboratory cage containing infested cherry fruit and soil by both host species. Values are mean $\pm$ SE and different letters above the bars indicate significant difference between the observed and expected parasitism (ANOVA, $P < 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5