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Effect of fruit and host fly species on the associative learning by *Fopius arisanus*

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Abstract

Parasitoids, released in augmentative biological control programmes, which display a rapid host-location capacity, have a higher likelihood of successfully controlling target pest species. By learning to associate sensory cues to a suitable oviposition site, might parasitoids used as biological control agents, locate hosts more rapidly, and perhaps increase the efficacy of e.g. Tephritidae fruit fly management. We studied associative learning of *Fopius arisanus* (Hymenoptera: Braconidae) and tested its range of learning in natural and conditional hosts and host fruits, i.e. *Bactrocera dorsalis*, *Zeugodacus cucurbitae*, *Ceratitis capitata* and *Ceratitis cosyra* (Diptera: Tephritidae) and on fruits (papaya, tomato, banana). Naïve female *F. arisanus* were compared with experienced wasps, which had been offered infested and non-infested fruit, and been allowed to oviposit. Preferences for olfactory cues from infested fruits were thereafter assessed in a two-choice olfactometer. Naïve and trained parasitoids preference differed in general and non-responders to infested fruits were higher among naïve parasitoids. The trained wasps preferred the fruit infested in the training more than the control fruit, for all combination, except when *C. cosyra* infested the fruits, hence avoidance behavioural response was observed towards the odour of the infested fruit. *Fopius arisanus* was capable of behaviourally respond to the learned information, e.g. associative odour learning was achieved, yet limited depending on interaction level, fruit fly and fruit combination. To create *F. arisanus* preference of an associated odour, it might hence be needed to ensure oviposition in perceived suitable host and host fruit, for the parasitoid learning to become favourable in a biological control setup.

Keywords: Braconidae, Tephritidae, preference, behaviour, conditioning

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Introduction

During the release of parasitoids in a biological control programme, it is expected that females rapidly find and oviposit in the target host, in the target plant or fruit. How fast the biological control agent can locate its host and oviposit is one factor that can affect the insect efficiency in pest management (Kroder & Messing, 2010). The longer time the parasitoids take to locate their host, the higher is the risk that they will be attacked by predators and be affected by other biotic and abiotic factors. Energy investment will also increase with a longer location time. Insect host location is an innate behaviour but also a behaviour that can change by learning (Raine & Chittka, 2008; Wei *et al.*, 2013). Learning is defined as a

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modification of behaviour based on past experience. If the learned behaviour is a result of previous experience associating a stimulus with a reward or a punishment, it is defined as associative learning. Parasitoids can learn to associate host-related odours (Ngumbi *et al.*, 2012; Canale *et al.*, 2014) and visual cues (Segura *et al.*, 2007; Lucchetta *et al.*, 2008; Desouhant *et al.*, 2010), with their hosts. Individuals that can learn which environmental stimuli are associated with mates and food may have a fitness advantage over those that cannot and therefore should be favoured by natural selection (Dukas & Duan, 2000).

Associative learning is previously documented for various Braconidae parasitoids (Müller *et al.*, 2006; Ngumbi *et al.*, 2012) and for Braconidae wasps that develop in Tephritidae fruit fly species (Lewis & Takasu, 1990; Seino & Kainoh, 2008; Giunti *et al.*, 2016). Parasitoids preference for odour cues that orient them to host patches may change with the associative learning (Giunti *et al.*, 2015). Inexperienced wasps respond innately to stimuli that are derived from their hosts or that indicate appropriate hosts (Turlings *et al.*, 1993). A positive oviposition experience by *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae) on a preferred host causes additionally an increased attraction, due to the positive association generated of plant volatiles and the reward, given as host presence (Harris *et al.*, 2012). Experience of non-infested host fruit can subsequently reduce parasitoid acceptance during later encounter with the same host substrate, even if the latter is infested (Thiel & Hoffmeister, 2009). Experience of parasitation that gives a positive association with accompanying cues might enhance the host location capacity subsequently, thus laboratory studies have demonstrated that learning of host-associated sensorial cues reduce the time of decision and enhance the host location efficiency (Papaj & Vet, 1990; Canale *et al.*, 2014; Giunti *et al.*, 2015). Researchers have proposed that from an applied perspective, odours could be used to train mass-reared parasitoids prior to release, to potentially improve their efficacy in the field, i.e. by allowing the parasitoids to associate a good oviposition site with a sensorial cue that are emitted from the target fruit fly and host fruit (García-Medel *et al.*, 2007; Benelli & Canale, 2012). *A priori* host exposition stimulates host discrimination and enhances fruit fly parasitism (Gonçalves *et al.*, 2017). However, a learned preference in laboratory does not always translate into a change in parasitization preferences in semi-field, as observed for the parasitoid *Cotesia glomerata L.* (Hymenoptera: Braconidae) (De Rijk *et al.*, 2018).

Fopius arisanus Sonan (Hymenoptera: Braconidae) is an egg-pupal koïnobiont endoparasitoid able to attack and survive in at least 20 Tephritidae species from the genera *Bactrocera*, *Ceratitis*, *Anastrepha* (Quimio & Walter, 2001; Rousse *et al.*, 2006), and possibly also *Euphranta* and *Philophylla* (Chinajariyawong *et al.*, 2000). Successful introductions of *F. arisanus* into Hawaii and French Polynesia demonstrate that this parasitoid is an efficient biological control agent, causing a high level of parasitized egg, which result in a reduction of populations of, e.g. *Bactrocera dorsalis* Hendel and *Ceratitis capitata* Wiedemann (Quimio & Walter, 2001; Vargas *et al.*, 2007, 2010). Releases of this Asian parasitoid have additionally been conducted in Guatemala (Rendon *et al.*, 2006), Kenya (Ekesi *et al.*, 2016), Benin (Gnanvossou *et al.*, 2016), Senegal (Ndiaye *et al.*, 2015) and Australia (Carmichael *et al.*, 2005) with different results. Augmentative release of Braconidae parasitoid species from the subfamily Opiinae occurs frequently and is hence a relatively established management method of Tephritidae pest species (Sivinski *et al.*, 1996; Montoya *et al.*, 2000; Vargas *et al.*, 2004; Aluja *et al.*, 2009)

The host location behaviour by *F. arisanus* is guided by olfactory cues from the host fruit (Altuzar *et al.*, 2004) and from Tephritidae eggs (Rousse *et al.*, 2007; Quilici & Rousse, 2012). Semiochemicals emitted from fruit fly-infested fruits are additionally stimulating the searching behaviour of parasitoids that parasitize the larval stage of the flies (Ero & Clarke, 2012; Sivinski & Aluja, 2012). Specific compounds emitted as fruits are infested and fruit fly parasitoids are able to differentiate odour from infested and non-infested fruits (Carrasco *et al.*, 2005). The combination of odours from both fruit and fruit fly, as in infested fruits, is preferred by *F. arisanus* over non-infested fruits (Liquido, 1991; Altuzar *et al.*, 2004; Rousse *et al.*, 2007).

By using different fruits infested with fruit fly species eggs, we investigated to which extend the wasp *F. arisanus* developed a preference for an odour after training and if association learning ability is related to the different combinations. By comparing the innate behavioural odour response with the response of parasitoids that has previous experience of the same odour, we examined *F. arisanus* associative learning capacity. We investigated the ability of mated female *F. arisanus* to associate cues of the fruit fly species *Zeugodacus cucurbitae* Coquillett, *B. dorsalis*, *Ceratitis cosyra* Walker and *C. capitata* (Diptera: Tephritidae), with the fruits papaya *Carica papaya* L. (Caricaceae), tomato *Solanum lycopersicum* L. (Solanaceae) and banana *Musa sp*, Diekmann, (Musaceae). We conducted parasitoid preferences assays in a Y-tube olfactometer to examine the effects of association learning experience and to investigate if the capacity to learn depended on the fruit fly species and/or the fruit combination.

Materials and methods

Parasitoid F. arisanus

An initial population of *F. arisanus* started in 2008 at the International Institute of Tropical Agriculture station in Benin (IITA-Benin), with 1000 individuals (70% females) provided by the International Centre of Insect Physiology and Ecology (icipe), Kenya. *Fopius arisanus* were released in Benin and specimens recovered from the field (300 individuals, 86% females), in 2010, gave rise to a new colony, from which we obtained female *F. arisanus* used in the bioassays in this study. The climate chamber was kept at 25 ± 2 °C and RH 75 ± 5% with a photoperiod of 10 L:14 D. Parasitoids were reared with *B. dorsalis* as a host and papaya as the main larvae food substrate. Fruit fly infestation of papaya sections were done in transparent Plexiglas cages (20 × 20 × 20 cm) during 4 h with 50 couples of mature *B. dorsalis*. The infested papaya sections were thereafter introduced to cohorts of 50 couples of 7–15 days old *F. arisanus*, allowing parasitization during 48 h. After incubation of the papaya for 10 days, pupae were placed in nylon mesh-covered (100 μm gauge) containers, which permitted the emerging parasitoids to leave but hindered the fruit fly species leaving. The adult wasps were thereafter kept in cages (20 × 20 × 20 cm) that were placed near windows to provide natural sunlight every day (10 am to 16 pm) as male *F. arisanus* require bright light to initiate mating (Hagen, 1953; Ramadan *et al.*, 1992; Sime *et al.*, 2008). Pure honey and water were provided *ad libitum*. Mature, mated 7–11 days old female parasitoids were used for the experiments.

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Tephritidae species

Tephritidae species *B. dorsalis*, *Z. cucurbitae*, *C. capitata* and *C. cosyra* were reared under laboratory conditions with 25 ± 2 °C, 75 ± 5% RH and 12 L:12 D photoperiod. *Bactrocera dorsalis*, *C. capitata* and *C. cosyra* larvae were reared on papaya and *Z. cucurbitae* were provided with zucchini *Cucurbita pepo L.* (Cucurbitaceae). Whole fruits, grown at the IITA station, were introduced into the rearing cages during 48 h and then incubated for 10–15 days. Pupae were collected and transferred to cages (40 × 40 × 50 cm), where emerged male and female adults were kept together. Flies were provided with water and a mixture of a dry diet of red sugar and enzymatic-hydrolysed yeast (CAS: 100684-36-4, Affymetrix, Santa Clara, CA, USA) at a ratio of 3:1, respectively, *ad libitum*.

Fruit material

Papaya *C. papaya* L. (Caricaceae), var. Solo, and tomato *S. lycopersicum* L. (Solanaceae), var. hybrid Thorgal F1 Mill, were cultivated within IITA-Benin station. Banana *Musa sp*, Diekmann (Musaceae), were bought from the local market. Fruits with a similar size were used, hence the bigger fruits were chopped into comparable sizes, approximately 100 g fruit^{-1}. Ripe fruits were used, evaluated by ocular observations of colour, where <¾of the papaya were yellow, <¾ of tomato red and the whole banana yellow.

Bioassays

Each bioassay consisted of three parts; training of parasitoids, two-choice test and post-olfactometer observation. Nineteen bioassays were conducted; each one testing *F. arisanus* response to one combination of two fruits infested with different fruit fly species (table 1). By using two training methods, with different levels of interaction (low interaction (LI) and high interaction (HI), see below) with the host fruit fly species, we assessed whether the level of interaction had an effect on the learning ability (table 1). Comparisons between naïve and trained insects allowed us to assess the effect of the learning experience.

Fruits were infested naturally by the fruit fly species, both for the training session and for the choice assays. One fruit was introduced per cage (15 × 15 × 15 cm) together with 30–50 fruit fly females of the respective fruit fly. Infestations of fruits by *Z. cucurbitae* and *B. dorsalis* were completed during 1 h and *C. capitata* and *C. cosyra* were allowed to oviposit during 2 h. Observations were thereafter made with a stereomicroscope (WILD M3R, Heerbrugg, Switzerland, 40× magnifications) to confirm the presence of at least 30 fruit fly eggs per fruit. All experiments were conducted in a room with uniform lighting to avoid phototaxis. Environmental conditions were 25 ± 2 °C and 75 ± 5% RH.

Training procedure

One-half of the parasitoid cohort was trained one time before the bioassays, while the second half was kept naïve. The training consisted of exposing the wasps simultaneously to one infested fruit and to one non-infested fruit. During the training method named low-interaction level (LI), parasitoids were introduced into a small cage (15 × 15 × 15 cm) during h. A total of approximately 60 parasitoids, in groups of 15, were trained per bioassay. The parasitoids were able to touch the fruits, palp, probe and oviposit in the fruit fly eggs. For the first 15 m in the cage, the parasitoids were observed and their behavioural activities were recorded. In the training method named high-interaction level (HI), parasitoids were introduced into a small cage (15 × 15 × 15 cm). A total of approximately 50 parasitoids were trained per bioassay, in groups of five. Each parasitoid that was observed ovipositing was removed from the cage. The parasitoids were considered trained only if it had adopted oviposition behaviour, i.e. when the parasitoid drilled its ovipositor in the cluster of eggs, had its antennae raised, and stayed motionless for at least 25 s. The training was performed between 10.00 and 12.00 am. The interval between the training and the testing phase was 1–4 h. Hence, all the parasitoids in the high-interaction level oviposited in the fruit fly eggs, while parasitoids in the low-interaction level were in contact with the fruits for 1 h but did not for sure oviposit.

Table 1. Fruit and Tephritidae fruit fly species used in bioassay during the training and in the two-choice olfactometer test.

Bioassay	Fruit in training		Fruit fly species	Training method[1]
	Infested	Non-infested		
1	Tomato	Papaya	*Z. cucurbitae*	LI
2	Tomato	Papaya	*B. dorsalis*	LI
3	Tomato	Papaya	*C. cosyra*	LI
4	Tomato	Papaya	*C. capitata*	LI
5	Banana	Papaya	*Z. cucurbitae*	LI
6	Banana	Papaya	*B. dorsalis*	LI
7	Banana	Papaya	*C. cosyra*	LI
8	Banana	Papaya	*C. capitata*	LI
9	Tomato	Banana	*Z. cucurbitae*	LI
10	Tomato	Banana	*B. dorsalis*	LI
11	Tomato	Banana	*C. cosyra*	LI
12	Tomato	Banana	*C. capitata*	LI
13	Tomato	Papaya	*Z. cucurbitae*	HI
14	Tomato	Papaya	*B. dorsalis*	HI
15	Tomato	Papaya	*C. cosyra*	HI
16	Tomato	Papaya	*C. capitata*	HI
17	Banana	Papaya	*Z. cucurbitae*	HI
18	Banana	Papaya	*B. dorsalis*	HI
19	Tomato	Banana	*Z. cucurbitae*	HI

[1] Low-interaction level (LI), high-interaction (oviposition) level (HI).

Two-choice assays

The naïve and the trained parasitoids were compared in two-choice assays to measure the effect of learning. The treatments in each bioassay (1–19) consisted of two fruits infested with the respective fruit fly species (table 1). We investigated the olfactory response of the parasitoids towards volatiles of infested fruits in an olfactometer. The system consisted of a compressor (KNF Neuberger, D-79112, N-type 035, 230 V, 1.7 A, Bj 10/1997, Pmax 4.0, IP 44 Kw 0.23, 50 Hz), which generated the air stream through the olfactometer. The air was first pushed through an activated charcoal filter and thereafter through a bottle of water to clean and humidify the air. The air then was divided and passed through two glass bottles containing two different odour sources. In each bottle, one infested fruit was placed. Odours from the bottles were then led into each glass arm in the Y-tube olfactometer (3 cm diameter). The airflow was 4L min^{-1} per arm.

Trained and naïve female *F. arisanus* were individually, alternately and gently transferred into the opening of the olfactometer. The wasps were observed during 5 min or were

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discarded as non-choice insect if they did not make a choice within 5 min. Time of activation, time of choice and the odour source chosen were recorded. The olfactometer arms were swapped and cleaned after every ten females tested (five trained and five naïve females). The olfactometer bioassays were performed between 13.00 and 16.00 pm. For each bioassay, 80–90 female *F. arisanus* were tested individually; 40–45 trained and 40–45 naïve.

Post-olfactometer observation

Directly finishing the two-choice assay, parasitoid (the once that had made a choice) were taken for an additional behavioural test. The same two fruits used in the olfactometer test were infested (4 h *a priori*) with the corresponding fruit fly species (same species used in the previous olfactometer assay) and placed in cubic cages (15 × 15 × 15 cm). Batches of five parasitoids, either trained or naïve females, were introduced in each cage. The parasitoids behavioural activities such as contact with the fruit, probing and ovipositing were observed for 15 min per cage. Parasitoids were collectively observed and number of time each behavioural activity was performed was recorded. Thereafter, the fruits were left with the parasitoids for 24 h and then placed into incubation as described by Ayelo *et al.* (2017).

Data analysis

For each two-choice assay, a likelihood χ^2 was done to compare the frequency of choice of a given odour cue by trained and naïve parasitoid females. If the number of responding wasps were <5 for one treatment, Yates correction was used. The activation time and the time spent for each chosen cue were tested with a generalized linear model (glm) with a γ distribution, with inverse link function. The observed behavioural activities after olfactometer were tested both with a glm with Poisson distribution and with a likelihood χ^2-test. The parasitoid emergence from each fruit was tested using a glm with Poisson distribution, with log link function. The probability among trained wasps of making a positive association with the odour of the infested host and fruit presented during the training was estimated using a three-step algorithm. A binary success probability test of random samples of the real observations was made primarily for all treatments. Thereafter the first step was replicated with new observations created by bootstrap ($B = 9999$), and a new random vector was considered, indicating if $P < 0.05$. Bootstrap created new observations based on the real observed values and proportions test with continuity correction then done with the cloned data. All tests were done with R v.3.2.2 (R Development Core Team, 2009).

Results

Choice of fruit by naïve and trained parasitoids

Naïve and trained parasitoids made overall a different choice of fruit, between fruit infested in the training and the non-infested control ($\chi^2 = 7.5187$, df = 1, $P = 0.0061$), even if the numerical difference was small (55.9 and 44.1 for the trained and 50.1 vs. 50.0 for naïve). The trained wasps chose the fruit infested in the training more than the control fruit ($\chi^2 = 7.6640$, df = 1, $P = 0.0056$), while there was no difference between naïve choices of fruit ($\chi^2 = 0.0010$, df = 1, $P = 0.9750$). The difference between naïve and trained parasitoids was detected in six olfactometer assays, while no difference was found in 13 cases (fig. 1). In the occasions where the choice for fruit differed between naïve and trained parasitoids, the trained preferred the fruit that had been infested during the training for all combination, except when *C. cosyra* infested the fruits (fig. 1). In both bioassays with LI, and HI-trained parasitoids, we observed cases where the choice of fruit differed between naïve and trained parasitoids. However, overall comparisons showed that the choice of fruit differed between naïve and HI-trained parasitoids ($\chi^2 = 12.285$, df = 1, $P = 0.0005$), while there was no difference in choice between naïve *F. arisanus* and the LI-trained parasitoids ($\chi^2 = 0.595$, df = 1, $P = 0.4403$). Naïve parasitoids did not have a preference for banana, papaya or tomato ($\chi^2 = 1.795$, df = 2, $P = 0.4076$) and no difference between fruit choice was observed in any of the two-choice assays (table 2).

The number of parasitoids that did not make a choice was higher for naïve than for trained *F. arisanus* (glm, 17.33 ± 0.98, 14.53 ± 0.92 naïve and trained, respectively, bioassay^{-1}, $z = 2.072$, $P = 0.0383$). When fruits were infested with *Z. cucurbitae*, the number of parasitoids that did not make a choice in the olfactometer was lower than when infested with the other fruit fly species (fig. 2).

Activation time and time spent by F. arisanus *in each olfactometer arm*

The activation time (time taken to respond to odour compound in the olfactometer) was lower for trained than naïve parasitoids, i.e. the trained *F. arisanus* were faster to get activated and move in the two bioassays when fruits were infested with *Z. cucurbitae* (table 2). Other bioassays did not result in a difference in activation time between the parasitoid groups and there was no overall difference between activation time for naïve and trained *F. arisanus* (glm 76.47 ± 3.08, 71.56 ± 2.77 s (±SE) for naïve and trained parasitoids respectively, $t = 1.187$, $P = 0.235$).

The time spent in each olfactometer arm did only differ between naïve and trained parasitoids in very few bioassays, while in most assays there were no differences in time spent between the two treatments. Trained wasps spent more time in the presence of tomato odour than banana when the fruits were infested by *C. cosyra* (glm, 275 ± 80, 145 ± 108 s (± SE), respectively, $P = 0.05$). *Fopius arisanus* spent shorter time with tomato than papaya when infested by *C. capitata* (glm, 169 ± 91 and 243 ± 84 s (± SE), respectively, $P = 0.026$) and shorter time with tomato than papaya when infested with *B. dorsalis* (glm, 91 ± 97, 197 ± 95 s (± SE) for naïve and trained parasitoids, respectively, $P = 0.022$).

Post-olfactometer observations

Fopius arisanus post-olfactometer behavioural activity oviposition did not differ between naïve and trained parasitoids, while probing and contact was higher for trained parasitoids than for naïve wasps (table 3).

Probability of positive association

We calculated the probability to learn, i.e. to respond positively to odours of host-infested fruits, with which previous experience was made. This probability of success was dependant on the tritrophic levels interaction as it depended strongly on the infesting fruit fly species and also slightly on the three

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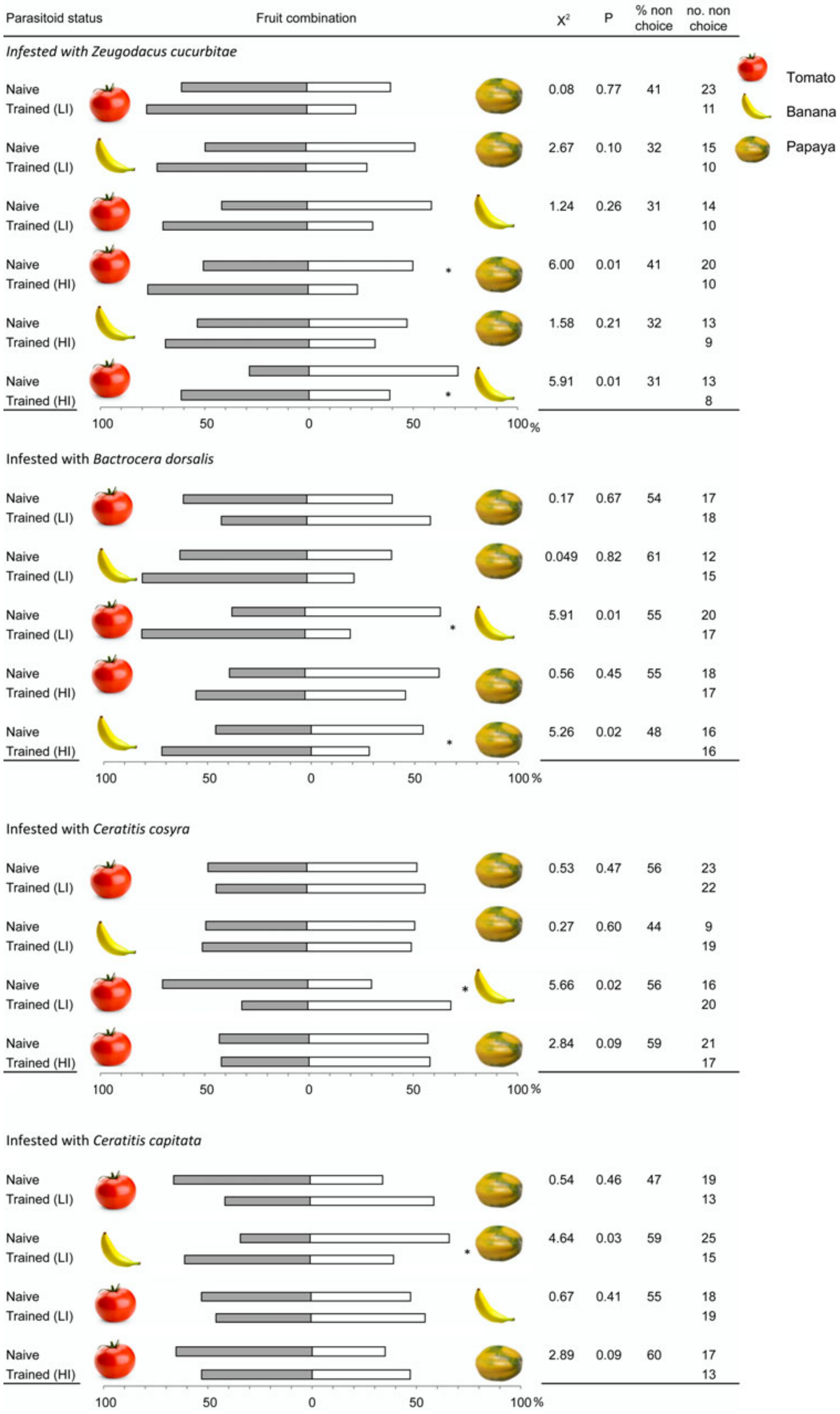

Fig. 1. Choice of infested fruits in two-choice olfactometer, comparison of naïve and trained *Fopus arisanus*. HI = high-interaction and LI = low-interaction training, grey bars = fruit infested during training, white bars = fruits not infested during training, *choice between naïve and trained differed (χ^2 test).

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Table 2. Olfactometer results. (A) Naïve parasitoids preference for fruits and (B) activation time in olfactometer assays by naïve and trained *Fopius arisanus* females.

	A. Naïve parasitoids preference for fruits		B. Activation time (sec ± SE)		
Bio-assay	χ^2	*P*	Naïve	Trained	*P*
1	0.477	0.490	116.85 ± 25.93	107.53 ± 22.22	0.786
2	0.727	0.394	103.90 ± 76.66	79.84 ± 70.61	0.249
3	0.053	0.819	53.64 ± 49.63	74.42 ± 62.11	0.209
4	2.130	0.144	46.14 ± 47.70	37.20 ± 37.98	0.488
5	2.695	0.101	64.17 ± 11.51	54.51 ± 8.90	0.503
6	0.800	0.371	80.28 ± 68.71	52.55 ± 57.71	0.182
7	0.032	0.858	48.77 ± 48.83	60.50 ± 50.69	0.374
8	1.667	0.197	56.77 ± 58.58	56.18 ± 50.58	0.904
9	0.702	0.402	88.24 ± 9.08	66.40 ± 6.41	0.047*
10	1.800	0.180	52.42 ± 57.32	84.48 ± 58.16	0.093
11	3.522	0.061	83.77 ± 71.10	81.97 ± 70.33	0.928
12	0.048	0.827	51.46 ± 50.80	45.23 ± 43.39	0.571
13	0.000	1.000	77.77 ± 14.41	78.71 ± 11.73	0.960
14	0.834	0.361	91.83 ± 15.24	123.19 ± 21.40	0.233
15	0.022	0.883	116.05 ± 21.00	81.96 ± 14.50	0.185
16	1.371	0.242	57.00 ± 12.84	61.28 ± 12.44	0.813
17	2.751	0.090	87.73 ± 11.81	54.63 ± 6.81	0.015*
18	0.467	0.944	96.59 ± 12.38	93.10 ± 11.51	0.837
19	0.801	0.391	87.52 ± 14.26	82.74 ± 12.69	0.803

* activation time for naïve and trained differed.

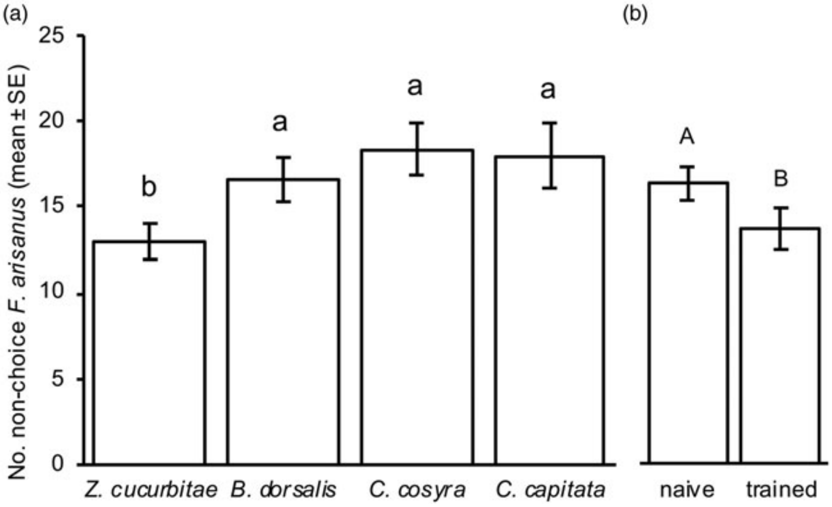

Fig. 2. Number of non-choice *Fopus arisanus* per bioassay in relation to (a) infesting fruit fly and (b) parasitoid status; naïve or trained (glm).

Table 3. Activity (contact, probing and ovipositing) by naïve and trained *Fopus arisanus* during post-olfactometer test (glm, mean ± SE *F. arisanus*/cage).

Behaviour	Naïve	Trained	*Z*	*P*
Contact	1.55 ± 0.11	1.75 ± 0.12	1.205	0.228
Probing	0.92 ± 0.09	1.20 ± 0.10	2.127	0.033
Oviposition	0.58 ± 0.07	0.70 ± 0.08	1.211	0.226

fruit combinations tested (fig. 3). When fruits were infested with *Ceratitis*, the chance of learning (making a positive association) was lower than 50% and parasitoids had highest chance to make a positive association of fruit and fruit fly odours in the presence of *Z. cucurbitae*, followed by *B. dorsalis*, *C. capitata* and least with *C. cosyra* (fig. 3).

Parasitoid emergence

The parasitoid *F. arisanus* emerged only from fruits infested with *B. dorsalis* while no emergence of the parasitoid was recorded from *Z. cucurbitae*, *C. capitata* and *C. cosyra*-infested fruits. With the exception of emergence of *C. cosyra* from tomato, all flies emerged from all fruits. The emergence of *F. arisanus* in relation to total emergence of fruit flies and parasitoids was on average 23%, which did not differ between fruits (glm, $z = 0.1291$, $P = 0.1962$). It is however imperative to recognize that we observed *F. arisanus* emerging from tomato in a very

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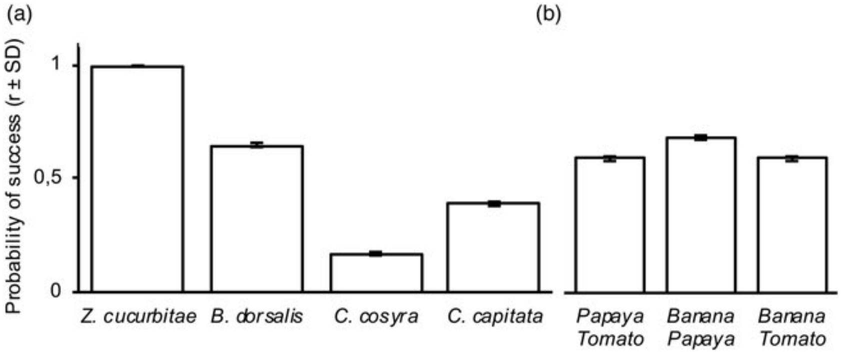

Fig. 3. Probability of success by *Fopus arisanus* to make a positive association in relation to (a) fruit fly and (b) fruit combination (bootstrap, $B = 9999$).

low number of cases. The number of *F. arisanus* emerging was positively correlated to the number of fruit flies (*B. dorsalis*) emerging from the same fruits ($R = 0.69$).

Discussion

A difference between naïve and experienced *F. arisanus* in their response to odours was considered a result of the association of odours generated while in contact with the fruit fly eggs in the fruit. Based on that supposition, we observed a high number of parasitoids that were not affected by the associative learning activity they were confronted with, as few assays showed odour-response differences. A modest associative learning was observed for the egg parasitoid *F. arisanus* in general. The trained wasps were in a limited number of assays performing a positive chemotaxis towards odours of infested fruit in which they had previous experience. In yet fewer assays, trained female *F. arisanus* displayed a negative chemotaxis towards the odour related to their previous experience. The learning ability was nevertheless closely related to Tephritidae species.

The few positive odour associations created by *F. arisanus* were observed in assays with experience of *Z. cucurbitae*-infested tomato, *B. dorsalis*-infested tomato, *B. dorsalis*-infested banana and after experience of *C. capitata*-infested banana. Previous studies showed that *F. arisanus* have an ability to associate host with fruit types after experience (Dukas & Duan, 2000), nonetheless we observed a limit to which *F. arisanus* can learn to prefer host flies and fruits, possibly linked to the combination of fruit fly species and fruit. Positive associations can be generated with a brief exposure to sugar reward in combination with an odour, as observed for *Psyttalia concolor* Szépligeti (Hymenoptera: Braconidae), which afterward preferred odours associated with the reward (Canale *et al.*, 2014). In our study, presence of fruit fly eggs was given as reward and we found that the capacity by *F. arisanus* to react positively to odours associated with a host could be partly related to the parasitoid performance in the different fruit fly species eggs (Segura *et al.*, 2016). *Bactrocera dorsalis* is a more optimal host for *F. arisanus* than *Ceratitis* spp. yet *F. arisanus* develop in *C. cosyra* and *C. capitata* (Mohamed *et al.*, 2010; Ayelo *et al.*, 2017). *Fopius arisanus* rarely emerge from *Z. cucurbitae* (Harris & Bautista, 1996; Bautista *et al.*, 2004; Rousse *et al.*, 2006). Positive associations after experience with *B. dorsalis* were hence expected and marginal learning was observed after experience with *B. dorsalis* in which *F. arisanus* develop well. The calculated probability to learn was accordingly highest with *B. dorsalis*. Positive learning probability was <50% in *Ceratitis* species, which are comparably less preferred hosts than *B. dorsalis* (Mohamed *et al.*, 2010; Ayelo *et al.*, 2017), and produce less offspring (Harris & Bautista, 1996; Harris *et al.*, 2007).

We observed some cases where the associations made with the fruit fly eggs were negative, hence avoidance behavioural response was observed towards the odour of the infested fruit after training. Negative association was obtained when *C. cosyra* eggs were infesting tomato and it was observed as a preference for banana by the experienced *F. arisanus*, as opposed to attraction towards tomato by naïve wasps. This implicated that a negative association was formed with experience of the *C. cosyra* eggs in tomato and the wasp acted upon this with repulsion. Fruits infested by *C. cosyra* generated a negative association and emergence of *F. arisanus* was null. As the combination of fruit and host is important in parasitoid choice of oviposition site (Harris & Bautista, 1996), the sub-optimal fruit for *C. cosyra* was probably part of the reason why the unrewarding stimulus caused avoidance. The development of the fruit flies, in which parasitoid develop, is affected by the quality of the substrate, in which the host is found. Hence, the capability of *F. arisanus* to survive differs between fruits, in relation to fruit fly species. *Zeugodacus cucurbitae* is known to survive in *Musa* spp., tomato and papaya (Mcquate *et al.*, 2017), *B. dorsalis* survive in banana, tomato and papaya (Liquido *et al.*, 2015), *C. capitata* in seed banana *Musa balbisiana* (Colla) (Musaceae), tomato and papaya (Liquido *et al.*, 1990), while *C. cosyra* is not known to survive in banana, yet emerge occasionally from tomato (Kambura, 2016) and develop in papaya (Steck, 2015). Thus, among the species of Tephritidae fruit flies used in our study, some combinations are sub-optimal hosts for *F. arisanus* yet positive association creation and learning capacity was not straightforwardly linked to performance in host and host fruit.

Learned avoidance behaviour in response to an odour source, i.e. negative associative learning, is documented for other Braconidae wasps (Takasu & Lewis, 1996), and perhaps it is more pronounced in specialist Braconidae wasps than in generalist (Steidle & Van Loon, 2003). Low-quality reward such as oviposition in a non-host species might cease the response to previously attractive odours (Takasu & Lewis, 2003). Danger in form of an electric shock can cause the parasitoid *P. concolor*, to respond by avoidance to an innately attractive Tephritidae-host-induced odour (Benelli *et al.*, 2014). Unsuccessful host-foraging experiences and oviposition in sub-optimal host species do however not always cause

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aversive odour association (Costa *et al.*, 2010; Harris *et al.*, 2012), hence oviposition experience by *Diachasmimorpha kraussii* (Fullaway) (Hymenoptera: Braconidae) in non-host (*Drosophila melanogaster*, Diptera: Drosophilidae) infested host fruit do not increase the ability to discriminate between host (*Bactrocera tryoni* (Froggatt), Diptera: Tephritidae) and non-host-related odours (Masry *et al.*, 2018). While this disability to reduce attraction after unrewarded and/or non-host oviposition might be related to a lack of experience to the non-host during evolution (Costa *et al.*, 2010), perhaps it is also related to the genetic relatedness between host and non-host species. *Bactrocera dorsalis* and *Z. cucurbitae* belonged until recently to the same genus (De Meyer *et al.*, 2015), yet does *F. arisanus* parasitism in the latter not allow survival (Nishida & Haramoto, 1953; Vargas *et al.*, 2012).

The method of giving mass-reared biological control agents pre-release experience of a suitable oviposition site, in association with a host sensorial cue, is proposed to enhance parasitoid location to find the target fruit and fruit fly species. This is to ensure an efficient and rapid host location during the release phase and to ideally improve their efficacy in the field (García-Medel *et al.*, 2007; Benelli & Canale, 2012). The method is proposed based on the insects' capacity to increase the ability to discriminate between host and non-host odours after experience (Giunti *et al.*, 2015; Masry *et al.*, 2018) and to increase the number of parasitized host eggs, resulting in a higher number of parasitoid offspring (Dukas & Duan, 2000). Associative learning could also result in a reduction of host location searching time (Dukas, 2008). Correspondingly, can learnt odours related to danger cause fruit fly parasitoids to spend more time with the control than in the presence of ethyl-octanoate and decanal associated with the threat (Benelli *et al.*, 2014). However, we found only in two occasions did the trained *F. arisanus* responded earlier to the fruit odours than naïve wasps. This is more in line with previous research by Ngumbi *et al.* (2012) and Canale *et al.* (2014), where time reduction of host finding was not, or only rarely, obtained by experienced wasps, compared with naïve even if learning of infestation-induced volatile compounds was attained. It is also possible to change an innate behaviour, and increase host parasitism for a novel host (Li & Lui, 2003; Wei *et al.*, 2013). Perhaps could pre-release experience also reduce attraction to non-target hosts, before the release of the parasitoid in the field to, e.g. reduce attraction to species that acts as sinks, where the parasitoid parasitizes but where no viable progeny develops.

Previous experience with infested fruits increased responsiveness for *F. arisanus*, since trained were more active to respond (less non-responders) to the fruits odours than the naïve. Oviposition experience (high interaction) furthermore changed the fruit preference, since overall fruit choice differed slightly between experienced *F. arisanus* and naïve parasitoids, while experience in the low interaction method did not result in an overall difference in fruit choice compared with the naïve parasitoids. In the high-interaction level, all females oviposited in the fruit fly eggs, while in the low-interaction level, the females were in contact with the fruits for 1 h but did not with certainly oviposit. For *P. concolor*, only 20 s of interaction with odour and a sugar reward can be sufficient for a positive association (Canale *et al.*, 2014), while repeated exposure of odours and sugar reward might be needed to learn to respond to odours associated with their hosts (Ngumbi *et al.*, 2012), and yet subsequent training might increase effective location of the target host (Minoli *et al.*, 2012). To create *F. arisanus* preference of an associated odour, it might hence be needed to ensure oviposition in perceived suitable host and host fruit, for the parasitoid learning to become favourable in a biological control setup. Our study has enlightened restrictions in odour learning and showed that the learning capacity of *F. arisanus* might be limited to certain fruit fly species and fruit combinations and interaction level, as the results show that among different combinations of egg and fruit species, there are different behavioural effects of the associations created. We are yet to understand to what extent *F. arisanus* is able to recognize the species of fruit flies and what are the criteria for the parasitoid to accept or reject fruit fly eggs. Further studies about the extent to which laboratory results can be translated into the field are needed, as the learning and change in preference obtained in laboratory assays might not always translate in preference change in the field (De Rijk *et al.*, 2018). The development of mass-rearing methods to enhance searching behaviour in biological control agents is of interest, e.g. to elucidate the cues used to associate and orient towards the target host, to increase efficiently during the critical first time after release.

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