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▶ **To cite this version:**

Hossein Kishani Farahani, Ahmad Ashouri, Seyed Hossein Goldansaz, Martin S. Shapiro, Jean-Sébastien Pierre, et al.. Decrease of memory retention in a parasitic wasp: an effect of host manipulation by Wolbachia?. Insect Science, 2017, 24 (4), pp.569-583. 10.1111/1744-7917.12348 . hal-01305485

HAL Id: hal-01305485

https://univ-rennes.hal.science/hal-01305485

Submitted on 21 Jun 2016

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ORIGINAL ARTICLE

Decrease of memory retention in a parasitic wasp: an effect of host manipulation by *Wolbachia*?

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Abstract

Several factors, such as cold exposure, aging, the number of experiences and viral infection, have been shown to affect learning ability in different organisms. *Wolbachia* has been found worldwide as an arthropod parasite/mutualist symbiont in a wide range of species, including insects. Differing effects have been identified on physiology and behavior by *Wolbachia*. However, the effect of *Wolbachia* infection on the learning ability of their host had never previously been studied. The current study carried out to compare learning ability and memory duration in two strains of the parasitoid *Trichogramma brassicae:* one uninfected

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and one infected by *Wolbachia*. Both strains were able to associate the novel odors with the reward of an oviposition into a host egg. However, the percentage of females that responded to the experimental design and displayed an ability to learn in these conditions was higher in the uninfected strain. Memory duration was longer in uninfected wasps (23.8 h and 21.4 h after conditioning with peppermint and lemon, respectively) than in infected wasps (18.9 h and 16.2 h after conditioning with peppermint and lemon, respectively). Memory retention increased in response to the number of conditioning sessions in both strains, but memory retention was always shorter in the infected wasps than in the uninfected ones. *Wolbachia* infection may select for reduced memory retention because shorter memory induces infected wasps to disperse in new environments and avoid competition with uninfected wasps by forgetting cues related to previously visited environments, thus increasing transmission of *Wolbachia* in new environments.

Key words associative learning; memory duration; spacing learning; *Trichgramma brassicae*; *Wolbachia* infection

Introduction

Learning is widespread throughout the animal kingdom and plays a central role in adapting to local spatial and temporal environmental conditions (Ardiel & Rankin, 2010; Pravosudov & Smulders, 2010; Mery, 2013). Learning occurs throughout an animal's lifetime, impacting all aspects of life, for example, from searching for a mate, locating and choosing resources, and identifying predators or conspecifics (van Baaren *et al.*, 2005, Dukas, 2003, 2008a; Giunti *et al.*, 2015). The acquisition of information plays a critical role in predicting future conditions and therefore needs to be stored and retrieved in the memory.

Although it is generally assumed that learning is beneficial, direct measurements of the benefits of learning and memory in terms of fitness reveal that learning and memory may also

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be costly (Dukas & Bernays, 2000; Dukas, 2008b; Raine & Chittka, 2008). Tradeoffs can therefore be expected between the importance of memory for fitness gains and the energy consumed for the production, consolidation, or retention of memory. In various species, the retention of memory ranges from a few seconds to several years (Rankin *et al.*, 1990; Portavella *et al.*, 2004; Giurfa, 2007; Giurfa & Sandoz, 2012; Bruck, 2013). This duration is determined by numerous factors such as the number of training sessions (Toppino & Bloom, 2002), diet (Suzuki *et al.*, 1998; Petursdottir *et al.*, 2008), aging (Gallagher & Rapp, 1997; Page & Peng, 2001; Schaie *et al.*, 2004; Weiler *et al.*, 2008; Bartolo *et al.*, 2010), stress (Nishio *et al.*, 2001; Shors, 2004), reward intensity and value (Adcock *et al.*, 2006; Hoedjes *et al.*, 2010; Kruidhof *et al.*, 2012), or cold exposure (van Baaren *et al.*, 2006). External biotic factors such as viruses, bacteria, and fungi can also affect the learning ability of animals. The influence of parasitic infections on cognitive functions has undergone considerable study in vertebrates (Kavaliers & Colwell, 1995; Kavaliers *et al.*, 1995; Braithwaite *et al.*, 1998; Cox & Holland, 2001), but has received less attention in the invertebrates (Gegear *et al.*, 2006; Iqbal & Mueller, 2007; Kralj *et al.*, 2007). However, in invertebrates, parasitic infections can have detrimental effects on cognition. For example, the use of floral information was impaired in bumblebees [*Bombus impatiens* Cresson (Hymenoptera: Apidae)] infected with the gut protozoan parasite *Crithidia bombi* Lipa and Triggiani (Kinetoplastida: Trypanosomatidae) (Gegear *et al.*, 2006). In addition, adult honey bees [*Apis mellifera* L. (Hymenoptera: Apidae)] infected with *Varroa destructor* Anderson and Trueman (Parasitifomres: Varroidae) displayed a reduced learning capacity (Kralj *et al.*, 2007) and lower rates of return to their colony, possibly due to a loss of learning and cognition (Kralj & Fuchs, 2006).

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The number of learning experiences during an animal's lifetime may affect memory duration (Roitberg, 1993; Pearce, 2008). A range of animals have been studied (*Apis mellifera*, *Drosophila*, the marine mollusks *Aplysia* and *Hermissenda*, rats and rabbits) with regard to the effects of repeated experience on memory formation (Carew *et al.*, 1972; Fanselow & Tighe, 1988; Tully *et al.*, 1994; Spieler & Balota, 1996; Kogan *et al.*, 1997; Hermitte *et al.*, 1999; Muzzio *et al.*, 1999; Beck *et al.*, 2000; Wu *et al.*, 2001). Honeybees (*Apis mellifera*) have been successfully used to describe the dynamics of memory formation after single or multiple-trial appetitive learning both in color learning by free-flying bees and in olfactory proboscis extension conditioning (Menzel & Müller, 1996; Menzel, 1999; Giurfa, 2007; Giurfa & Sandoz, 2012), indicating that a consolidation process occurs within a few minutes after learning. In all cases, multiple learning trials facilitate memory consolidation into long lasting memories extending over one or several days.

Wolbachia is an α-proteobacteria living as an obligatory endosymbiont (Taylor & Hoerauf, 1999) and has been found in numerous arthropod species, including spiders, terrestrial isopods, such as filarial nematodes, mites, and insects (Goodacre *et al.*, 2006; Werren *et al.*, 2008; Cordaux *et al.*, 2012). In arthropods, *Wolbachia* has evolved a large number of strategies to manipulate host reproduction including parthenogenesis induction, feminization, and male killing to enhance its transmission (Werren *et al.*, 2008). Up to 10% of species of parasitoids belonging to the *Trichogramma* genus are known to be infected with *Wolbachia* in which it induced thelytokous forms (Poorjavad *et al.* 2012). Species belonging to the *Trichogramma* genus are endoparasitoids, and most species are associated with eggs of Lepidoptera, although some can also attack dipteran, coleopteran, and symphytan eggs (Hoffmann *et al.*, 1995; Pinto, 1998b; Mansfield & Mills, 2002). *Trichogramma brassicae* (Westwood) is a biological control agent that is widely used against various pest species. In

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nature, one uninfected and one infected strain of *T. brassicae* coexist with the infected strain only producing female offspring (Farrokhi *et al.*, 2010; Poorjavad *et al.*, 2012).

Most of the studies have focused on the effects of *Wolbachia* infection on arthropod traits such as fecundity, longevity, adult size, parasitism rates, and the rate of emergence of *Wolbachia*-infected wasps (Hohmann *et al.*, 2001; Grenier & De Clercq, 2003; Miura & Tagami, 2004). *Wolbachia* affects several life history traits in some species, either in a way that increases its transmission (Silva *et al.*, 2000; Hohmann *et al.*, 2001; Zchori-Fein *et al.*, 2001; Panteleev *et al.*, 2007) or negatively due to side-effects of *Wolbachia* infection (Fialho & Stevens, 2000; Weeks *et al.*, 2007). In the strains studied in this paper, we have shown that *Wolbachia* infection can impair the decision-making process during patch exploitation, potentially increasing transmission, but at the expense of a decrease of fitness of the infected individuals (Kishani Farahani *et al.*, 2015). Here we investigated the influence of *Wolbachia* infection on three experience-dependent behaviors: (i) the ability to learn; (ii) the ability to retain memory and (iii) the effect of additional experience on memory retention. The main methodology is a Pavlovian conditioning assay in which female parasitic wasps associate the reward (a host) with an odor, using a flight chamber to observe the response of *T. brassicae* after conditioning.

Material and methods

Parasitoids

In this paper, we compare two strains of *T. brassicae*: one that is *Wolbachia* infected and one that is not (uninfected). Both strains came from cultures maintained by the Biological Control Research Department (BCRD) of the Iranian Research Institute of Plant Protection (IRIPP). The original source of these parasitoids were collected from north Iran (Baboulsar

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Region, South of Caspian Sea) in the same fields in 2012. The current study was conducted on two naturally infected and uninfected wasp populations. It has been shown that these strains have the same genetic background and thus all observed difference will be due to *Wolbachia* prevalence (Kishani Farahani *et al.* 2015). In many comparative studies investigating the effects of *Wolbachia* between stains, uninfected strains are produced via antibiotic treatment to an infected strain. However, there is evidence to suggest that such antibiotic treatment may have negative effects on *Wolbachia* treated arthropods (Timmermans and Ellers 2009; Dedeine *et al.* 2001) impacting both physiology and behavior. As such, the possibility that observed differences are due to a negative effect of antibiotic treatment could not be ruled out.

Genetic similarity was performed based on the size of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer 2 (ITS-2) region amplified through PCR. The sequences were aligned and then subjected to a maximum parsimony analysis (with MEGA5 software), with heuristic search and TBR branch-swapping algorithm, in order to phylogenetically assess the identification and common origin of our strains (Kishani Farahani *et al.*, 2015).

In our infected strain, *Wolbachia* prevalence was determined in female wasps which produced only female offspring (Huigens & Stouthamer, 2003). *Wolbachia* presence was verified by a PCR method based on the *Wolbachia* surface protein (wsp) (81F/691R primers; Braig *et al.*, 1998). PCR-reactions were performed as described for *Trichogramma* species identification with the following two modifications: (1) the primers used to amplify the *wsp* region were 5′-TGGTCCAATAAGTGATGAAGAAAC-3′ (forward) and 5′ AAAAATTAAACGCTACTCCA-3′ (reverse), and (2) the cycling program was: 3 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by 5 min at 72 °C after the last cycle (Braig *et al.*, 1998). The *Wolbachia* infected line was monitored

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throughout the experimental duration by PCR tests to ensure that all different behaviors were due to *Wolbachia* presence. All female wasps which produce only female progeny were proved to be infected by *Wolbachia*.

Parasitoids were reared at 25 ± 1ºC, 50% ± 5% RH and 16 : 8 L : D on eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). *E. kuehniella* eggs were used in the following experiments for both infected and uninfected wasps. Eggs were obtained from a culture maintained at the Insectary and Quarantine Facility, University of Tehran. The culture was reared at 25 ± 1ºC on wheat flour and yeast (5%). About 20 mated female moths were kept in glass containers (500 ml) to provide eggs and eggs were collected daily to ensure that living eggs used in the experiments were no more than 24 h old. Prior to use in experiments, wasps were reared for 15 generations on *E. kuehniella*.

Flight chamber

The flight chamber used in the experiments was similar to that previously described (Yong *et al.*, 2007) with some modifications of the structure (Fig. 1). Air was driven through the main tunnel chamber (200 × 50 × 50 cm, l × w × h) by a fan located at the upwind end, and extracted outside by a fume hood at the downwind end. A smaller chamber (50 × 20 × 20 cm, l × w × h), centered within the main chamber and open at both the upwind and downwind end, served as the experimental arena. The walls of the main chamber and experimental arena were made of transparent acrylic material. All flight responses were tested at 25°C, 50% RH, and a light intensity of 2000 lux.

In order to isolate parasitoid females for our experiments, small squares from egg cards (eggs were glued on cards (1×5 cm) with 10% honey solution) were placed into emergence

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canisters and kept in incubators at 25 ± 1ºC, 16 L : 8 D and 50% ± 5% RH. Emergence canisters consisted of closed cardboard cylinders (500 mL, 63×161 mm) with a glass vial (50 mL, 26×93 mm). Once approximately 20 parasitoids had emerged into a glass vial, they were removed and provided with undiluted honey as a food source. The vials were subsequently closed with a ventilated plastic cap to serve as a holding container until the females were 24 h old.

Preliminary test: Test of an innate preference for the odor of peppermint or lemon

To study the innate preference of the wasps towards one odor (peppermint and lemon odors were tested) against a filter paper without odor, 50 naïve wasps of each strain were exposed to either the odor of peppermint or the odor of lemon in the wind tunnel and their responses were recorded. To do this, single naïve female wasps were introduced into the flight chamber described above using a 5 mL shell vial. The tested odor was presented on strips of filter paper (1× 2 cm) on which 1 μL of one or other solution (peppermint or lemon) had been placed on one filter paper, and no odor on the second filter paper. Each filter paper was attached to a glass pipette placed vertically on a stand and spaced 10 cm apart. Twenty-five of the 50 wasps underwent this procedure using the peppermint odor (at least 97% pure) and the other 25 underwent the procedure using the lemon odor (at least 97% pure). The responses of the wasps to the odors were observed in the flight tunnel during a flight time of 15 min. Any individual that landed or hovered on an odor site for more than 2 minutes was recorded as a responder wasp. Females that did not complete a flight or did not fly after 5 min were scored as displaying no response. Preliminary tests revealed that if females alighted for more than 2 minutes on an odor, they remained on the odor until the end of the 15 minute test

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period. If females failed to fly during the first 5 minutes, the female would choose neither odor during the experimental duration.

Conditioning

Sixty-five one-day old naïve females were exposed to host eggs for 15 minutes to gain oviposition experience and to avoid the variability in sequence and duration of behavioral events associated with learning from the first host encountered (Mills & Kuhlmann, 2004). As some females died, were lost, or failed to oviposit during the procedure, sixty wasps in vials (2×10 cm) with 100 host eggs (one-day old eggs glued onto cardboard) were transferred into conditioning tanks (25×25×25 cm). The conditioning odor was pumped into the tanks during conditioning at an air speed of 1 m/s. Thirty wasps underwent this conditioning procedure using peppermint odor, and the other thirty underwent it using lemon odor. This procedure was carried out using both uninfected and infected females, and lasted a duration of 2 hours.

Experiments

Experiment 1: test of odor preference after conditioning Fifteen minutes after conditioning, females of both strains were placed individually in the flight chamber as described above. The responses of 50 female wasps (randomly selected from amongst the survivors of the 60 originally conditioned), 25 of which had been conditioned on peppermint and 25 conditioned on lemon, were tested for both strains. Females that demonstrated a preference for the conditioned odor (i.e. any individual that landed or hovered on the conditioned odor site for more than 2 minutes), were scored as having learned to associate this odor (that was perceived as neutral in the preliminary test) with oviposition. The numbers

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of neutral responses displayed by the wasps were compared to identify the effects of *Wolbachia* on changes in the behavioral response in both strains and the ability of the strains to learn.

Experiment 2: memory duration after one and multiple experiences Memory (retention) was defined as being present when wasps displayed a post-conditioning significant preference for the conditioned odor (peppermint/lemon) in the case of wasps conditioned on peppermint or lemon respectively, compared to the unconditioned odor, at the time tested after the conditioning. To determine the duration of memory, the responses of conditioned wasps of both strains were observed 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, and 30h after conditioning. Their responses for the odors of peppermint and lemon were recorded in the flight tunnel under the conditions previously described. For each time interval, 50 wasps of each strain (i.e. 25 conditioned for each odor) were observed in the wind tunnel as described above, amounting to a total of 1200 wasps tested.

The wasps were conditioned 1 to 5 times consecutively to show how the number of additional experiences affects memory duration of females. To do this, we divided the total conditioning time (120 min) into 1 to 5 periods, i.e. 120, 60, 40, 30, 24 min for one to five learning sessions, respectively. During each training session, when the wasps were in contact with the odor, they were offered 100 host eggs, as described above, and their responses were tested in the flight tunnel 15 min after the last conditioning. The time interval between successive training sessions was 6 h between the first and second session, and 4 h between each of the other training sessions, so that the wasps were no more than 24 h old after all 5 training sessions. During the intervals between the training sessions, the conditioned wasps were kept at 25 ± 1ºC, 50% ± 5% RH and 16 : 8 L : D and fed with a 10% honey solution. For each test, the responses of 50 wasps of each strain (25 conditioned with peppermint, and

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25 with lemon) were recorded. The wasps' responses were observed every 2 hours, so that a total of 11,400 wasps were tested (5700 per strain).

Statistical analysis

The innate responses of both strains were compared by Chi-Square tests using SAS software (SAS Institute, 2003). To compare the responses of the two strains before and after conditioning we used the Generalized Linear model implemented in the procedure GENMOD of SAS software (ver. 9.1), with the binomial family error and logit link. After this global test, the least square estimates of the proportions in each level were compared by the Chi-square approximation (an option offered by GENMOD).

The estimation of forgetting relies on a series of observations recorded at different times t_1; t_2; …t_n after conditioning. At each time, a set of n_t subjects was subjected to a choice test with three possible responses: *a*; *b*; and *c*, which correspond respectively to a preference for the odor side, a preference for the opposite side (no odor), and to a null choice, i.e. no answer. The time courses of these three responses are illustrated. The forgetting of conditioning results in a switch from a high level to a lower level of positive responses, a simultaneous switch from a low level to a high level of null responses, and a switch from a very low to a moderate level of negative responses. A constraint links the three responses as $n_a + n_b + n_c = n_t$ or $n_c = n_t - n_a - n_b$. The course of these three responses over time can be described by two logistic functions written here as probabilities,

p_a, p_b, p_c, constrained by $p_a + p_b + p_c = 1$:

$$p_a = k_a - \frac{k_a - a_a}{1 + e^{(-b_a(t-t0))}} + a_a \quad (1)$$

$$p_c = \frac{k_c - a_c}{1 + e^{(-b_c(t-t0))}} + a_c \quad (2)$$

$$p_b = 1 - p_a - p_c \quad (3)$$

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k_a, respectively k_c, and a_a, respectively a_c define the sill and baselines of the logistic models (1) and (2): the baselines are a_a and a_c, and the seals are $k_a + a_a$ in model(1), $k_c + a_c$ in model (2). $k_a + a_a$ estimates the initial state in model (1), and a_c the final state. It is the inverse in model (2), where a_c is the initial state and k_c+a_c the final state.

A supplementary restriction lies in the fact that, as t0 represents the mean time to oblivion, i.e. the inflection time point of the logistics functions; it has to be the same in all three equations. The data consist of a vector of three counts: $V_t = (n_{at}, n_{bt}, n_{ct})$ the respective number of subjects responding a; b or c at time t. An R script was written to do this. The experimental design was a balanced factorial design with two factors: firstly, the type of strain, which had two levels (uninfected and infected), and, secondly, the number of conditioning experiments, which had five levels. This design resulted in ten crossed levels. The model defined by equations 1 to 3 was fitted individually on each set of ten data. The maximization of the likelihood cannot be fully automatic, and requires an initial guess of the seven parameters ka; aa; ba; kc; ac; bc; t0. This was done by a visual evaluation of each graphic representation of the crossed levels. The maximization of the likelihood provides a starting point for each of the seven parameters to the algorithm. This was done by considering each graphic representation of the crossed levels (Bouvier & Huet 1994). As an example, in figure 4 (Uninfected wasp (lemon)), we begin by t_0 which represents the common inflection point of the three curves, initially set to 21. The initial level of response (seal) which represents $k_a + a_a$ was then set to 20, and a_a, representing the final level of response, was set to 9. As a result, k_a was set initially to 20 - 9 = 11. The exponential coefficients (b_a equation (1) and b_c equation (2)), which are more difficult to evaluate graphically, where set to an arbitrarily low value such as 0.01, which ensures a nice convergence of the algorithm. The

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function returns an error message if the algorithm does not reach the convergence, and in this case, other initial values were tried. Non-linear regression is also susceptible to a well known pitfall: the convergence toward a local maximum. In this case, the fitted curves poorly fit some parts of the data, and other initial values must be chosen to obtain the best fit. All these procedures where used when needed, and the correct fit was obtained in all cases. Only the final values for t_0 are given. In the example, the initial guess was 20, and the final convergence was obtained at 21. The algorithm provides the standard error for the final parameters, which enables us to assign them a confidence interval (Table 2). After maximization, we obtained seven estimates of each parameter, along with an estimate of their standard error through the hessian matrix, which is provided on demand by the *optim* function of R (R core team 2013). All correlations between pairs of coefficients were lower than 0.9; a threshold commonly accepted as critical for the correct inversion of the hessian matrix [Bouvier (1996), by Bouvier & Huet, (1994)]. As our main focus was t0, we will give only the results for this parameter, which was accurately estimated with standard errors ranging from 0.3 to 1.8 hours.

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Results

Preliminary test: Test for an innate preference for peppermint or lemon odor

Naïve females of both the uninfected (χ^2 = 0.13, *P* = 0.93, df = 2, *N* = 50) and the infected (χ^2 = 0.69, *P* = 0.70, df = 2, *N* = 50) strains displayed no significant preference for the odor of lemon or this of peppermint against the filter paper with no odor or for an absence of a response (χ^2 = 0.95, *P* = 0.62, df = 4, *N* = 100) (Fig. 2).

Experiment 1: test of odor preference after conditioning

For uninfected wasps, the rate of an absence of response decreased significantly after conditioning for both odors, whereas it did not for the infected wasps (GENMOD of SAS, lemon odor: χ^2 = 6.36, *P* = 0.0117, df = 1, *N* = 25; peppermint odor: χ^2 = 5.67, *P* = 0.0173, df =1, *N* = 25 for uninfected wasps and lemon odor: χ^2 = 0.37, *P* = 0.5451, df = 1, *N* = 25; peppermint odor: χ^2 = 0.1, *P* = 0.7578, df = 1, *N* = 25 for infected wasps)(Table 1, Fig. 3a). For both strains, the rate of positive responses for the odor in the presence of which they had been conditioned increased significantly after conditioning (Proc GENMOD of SAS: lemon odor: χ^2 = 10.59, *P* = 0.0011, df = 1, *N* = 25; peppermint odor: χ^2 = 9.08, *P* = 0.0026, df=1, N=25 for uninfected wasps and lemon odor: χ^2 = 6.18, *P* = 0.0129, df = 1, *N* = 25; peppermint odor: χ^2=7.55, *P*=0.006, *df*=1, *N*=25 for infected wasps (Table 1, Fig. 3b). Infection by *Wolbachia* then has no effect on this parameter. The rate of opposite responses were 1.0% ± 0.5% and 4% ± 1% for uninfected wasps conditioned with peppermint and lemon respectively, while this rate was 8% ± 1% and 12% ± 2% for infected wasps conditioned with peppermint and lemon.

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Experiment 2: memory duration after one and multiple experiences

For a particular strain, the time to oblivion increases with the number of experiences undergone by the animals, and for a defined number of conditioning experiences, the uninfected strain retained the conditioning for longer than the infected strain (Table 2, Fig. 5). A three-factor variance analysis was done to verify these conclusions. Differences between the types were highly significant ($F_{1,\ 393}$ = 376.2, P < 0.001), as were the number of experiences ($F_{4,393}$ = 305.3, P < 0.001), odor type ($F_{1,\ 393}$ = 1.004, P < 0.001), and their interaction ($F_{1,390}$= 0.024, P=< 0.001). The interaction clearly arises from two different levels (1 and 2) of the number of experiences, where the difference between uninfected and infected strains was much lower than at other levels (Fig. 5). Figure 5 also shows that in both strains, memory duration increased in response to the number of learning experiences undergone.

After one training session, the memory duration of the uninfected wasps was 23.88 and 21.47h, on lemon and peppermint respectively. For infected wasps on the same odors, these values were 18.94 and 16.27h respectively. After five training sessions, the memory duration was 57.86 and 58.29h for uninfected wasps on lemon and peppermint respectively, while it was 40.27 and 41.23h for infected wasps on the same odors.

Discussion

Our results showed that the infected wasps presented a different behavior and particularly a reduced memory duration. After conditioning, the number of no responses decreased significantly for the uninfected wasps, whereas infected wasps showed the same number of no responses before and after conditioning: almost 30% of females of the infected strain

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never displayed orientation to conditioned odor. However, the memory duration of the infected wasps who were able to learn (i.e. who displayed an orientation towards the conditioned odor), was significantly shortened, regardless of the number of training sessions undergone. As our two strains had the same genetic background (Kishani Farahani *et al.*, 2015), their difference in memory retention should be attributed first to the *Wolbachia* infection of the infected strain. This reduced memory duration could result from the manipulation of host behavior by *Wolbachia*, intended to increase *Wolbachia* transmission. Another possibility is that this reduced memory retention is more adaptive for infected wasps for their own fitness or may be due to some other unknown activity of host manipulation by *Wolbachia*.

The infected females, which were able to learn or to use their learning ability, always displayed shorter memory duration, irrespective of how many training sessions they had undergone. It has been predicted that information will only be used if it is useful for predicting the future (Stephens, 1989), and it has been suggested that factors such as the number of particular events, the reliability of the information and the rate of environmental change could all influence the cost–benefit balance of information use (Roitberg *et al.*, 1993; Stephens, 1993; Dukas, 1998). The impact of the utility of the information on the learning ability was elegantly demonstrated by Theil *et al.* (2013) who studied two strains of *Venturia canescens*, one thelytokous and one arrhenotokous, but unlike the strains used in our study, their strains live in different habitats and the thelytokous form is not infected by *Wolbachia* (Foray *et al.*, 2013). The thelytokous wasp *V. canescens* lives in habitats with relatively stable host–substrate associations and may encounter high levels of hosts. They showed a preference for a new odor after only a single experience. However, the response faded within 24 h, even when the learning experiences had been spaced out, and the response did not

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develop into a long-term memory. The arrhenotokous wasp *V. canescens* lives in habitats where hosts are scarce and are likely to be found on a variety of substrates. Unlike the thelytokous wasps, arrhenotokous wasps learned a new odor after a single experience, and the memory lasted more than 24 h. The authors hypothesized that in a habitat in which hosts are encountered relatively frequently, memory that simply covers the interval until the next host encounter might be sufficient, particularly if the cost of constructing this memory can be reduced by not using protein synthesis-dependent memory. This is the first example demonstrating a difference in learning ability between two strains of a parasitoid species with different sexuality, but in this case the difference in learning ability seems to be adaptive and linked to the fact that the two strains use different habitats.

In our case, it is impossible to separate the effects of infection from the effects of reproductive mode, but both our strains inhabit the same microhabitat, and it is unlikely that the decrease in memory retention could be adaptive for the infected strain, subjected to the same ecological constraints of that of the uninfected strain. If it is not adaptive for the infected wasps to have a decreased memory capacity, two hypotheses remain: firstly, the differences in memory retention could be attributable to host manipulation by *Wolbachia* to increase their transmission or secondly, such differences could be attributable to a negative by-product of the infection. Concerning the hypothesis of host manipulation, according to Gautestad and Mysterud's (2013) theoretical model of memory, a wider dispersal range (i.e. using a larger space) is to be expected for wasps with a shorter memory horizon (duration). This phenomenon could increase the likelihood for a transition to full dispersal, making returns to previously-visited environments unlikely. Simulation studies suggest that memory can eventually shape the spatial distribution of the population (Bernstein *et al.*, 1988, 1991). If this applies to our case, the shorter memory duration may induce a greater dispersal rate of

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infected wasps. This behavior would be useful to the *Wolbachia* as it enables the parasite to contaminate new hosts. Indeed, *Wolbachia* can be transmitted via the superparasitism behavior of their hosts by horizontal transmission (i.e. when a female oviposits into a host that has already been parasitized, the contaminated larvae can transmit the *Wolbachia* to the other larvae present in the host, (Huigens *et al.*, 2000, 2004)). A previous study (Kishani Farahani *et al.*, 2015) has shown that infected females display higher superparasitism rates. As infected wasps produce only female offspring, it would lead to lower genetic diversity among this population in comparison to uninfected populations (Stelzer 2011; Simon *et al.* 2003). On the other hand, Huigens *et al.* (2004) showed that competition ability of *Wolbachia* infected wasps was lower than this of sexual wasps. Thus lower memory duration may help infected wasps to disperse in new environments and avoid competition with uninfected wasps by forgetting cues related to previously visited environments. This phenomenon may lead to a larger potential for a transition towards full dispersal, with a small chance of returning to previously visited environments. Simulation studies suggest that learning can eventually shape the spatial distribution of the population (Bernstein *et al.*, 1988, 1991). Lower memory duration allows wasps to disperse in new environments and to mix with other populations, thus leading to greater genetic variations among populations.

We found that memory duration in individuals of both strains increased as a function of the number of experiences. In classical conditioning experiments, conditioning trials that are spaced out over time produce better conditioning than those that are clustered together in time (Terrace *et al.*, 1975; Rescorla & Durlach, 1987; Barnet *et al.*, 1995; Beck *et al.* 2000). Interest in trial spacing has been renewed in recent years, because of claims that the perception of time is central to conditioning. A number of mechanisms can contribute to the trial spacing effects in classical conditioning (Barela, 1999). According to Gallistel and

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Gibbon (2000), the acquisition of conditioned responses depends on the animal deciding that the rate of reinforcement under the conditioned stimulus (CS) condition is higher than that in the background (Gibbon & Balsam, 1981). By repeating learning events, the animals will be confronted by the stimuli several times, and this leads the animals to overestimate the extent of conditioned stimuli. A higher rate of encountering CS can reveal the importance of this stimulus for the animal, and thus it will be considered to be a high value stimulus in the animal's life. According to our results, both strains responded positively to repeat conditioning and increased their memory duration. However, in all additional experience tests, memory duration was shorter for the infected wasps than for the uninfected wasps. This may have been due to host manipulation by *Wolbachia*. Lower memory retention can be an adaptive strategy for time limited animals such as parasitoids. In other short-living species such as *Anaphes victus* (Hymenoptera: Mymaridae), for which longevity is around 2 days, it was shown that an associative learning ability allows the females to gain around 2 minutes at each oviposition and is thus adaptive (Van Baaren & Boivin, 1998). It has been shown that *T. brassicae* survive 3–4 days when provided with food and oviposition (Lundgren & Heimpel, 2003). As memory formation is costly (Burger *et al.*, 2008; Gleiss *et al.*, 2013), we can hypothesize that *Wolbachia* infection prevents the formation of longer-term memory to save energy as an adaptive strategy increasing *Wolbachia* transmission.

Several studies have shown that *Wolbachia* infection can change body organ function and structure, leading to changes in arthropod life style, in their use of hosts, and in their spread throughout the natural environment. Two recent reports have shown that *Wolbachia* infection of *Drosophila melanogaster* increases resistance to four types of RNA viruses, some of which are common in both field and laboratory cultures (Brun & Plus, 1978; Ryder *et al.*, 2004; Zambon *et al.*, 2006). Similar protection from natural enemies has sometimes been

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observed in other arthropods (Gil-Turnes *et al.*, 1989; Gil-Turnes & Fenical, 1992; Haine *et al.*, 2005). This suggests that adaptation to specific conditions may depend on direct selection of symbionts expressing ecologically-important traits and simultaneously on indirect selection of the host. The effects of *Wolbachia* infection on memory duration can have a major impact on host ecology by widening the host's niche, thus allowing the host to exploit particular resources. Endosymbiotic *Wolbachia* bacteria are known to affect the fecundity and dispersal of infected strains (Stouthamer & Luck, 1993; Silva, 1999). Some studies have shown that *Wolbachia* can infect the Central Nervous System of adult and juvenile hosts (Albertson *et al.*, 2013; Strunov *et al.*. 2013). *Wolbachia* may affect parts of the nervous system, such as the mushroom bodies which make hosts forage in more patches, thus spreading the *Wolbachia* infection and increasing the probability that genes will be dispersed in the natural environment. However, further research should be done to investigate the effects of *Wolbachia* infection on the central nervous system or on other organs involved in lifetime information acquisition. Moreover, these behavioral differences could either be due to negative side effects on mobility behavior, or on perception capacity for example, or due to a negative effect on learning ability, although our experiments did not allow us to differentiate. As nothing is known about the possible other heritable symbionts associated with these lines (e.g., Rickettsia, Spiroplasma, Cardinium, etc), we cannot be completely sure that they have no role. However, more research is required to investigate the molecular and neural bases of learning and memory formation, for example, tracking the neurotransmission processes in uninfected and infected *T. brassicae* brain structures.

Acknowledgments

We would like to thank Akbar Golshani, Pouria Abrun and Valérie Briand, for the technical support provided and also appreciate the assistance of Dr. Lucy Alford and Monica Gosh for

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help with the language and writing. This study was financially supported by University of Tehran, but the sponsor had no involvement in the study design, the collection, analysis, or interpretation of data, the writing of the report or in the decision to submit the paper for publication.

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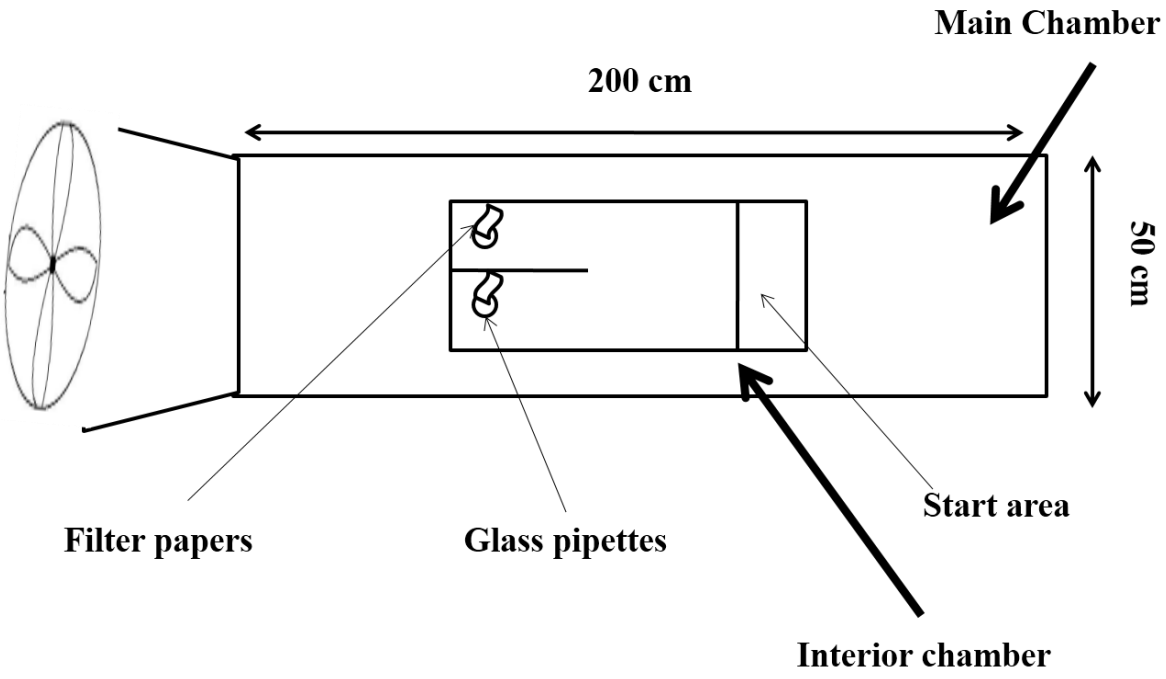
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Accepted March 13, 2016

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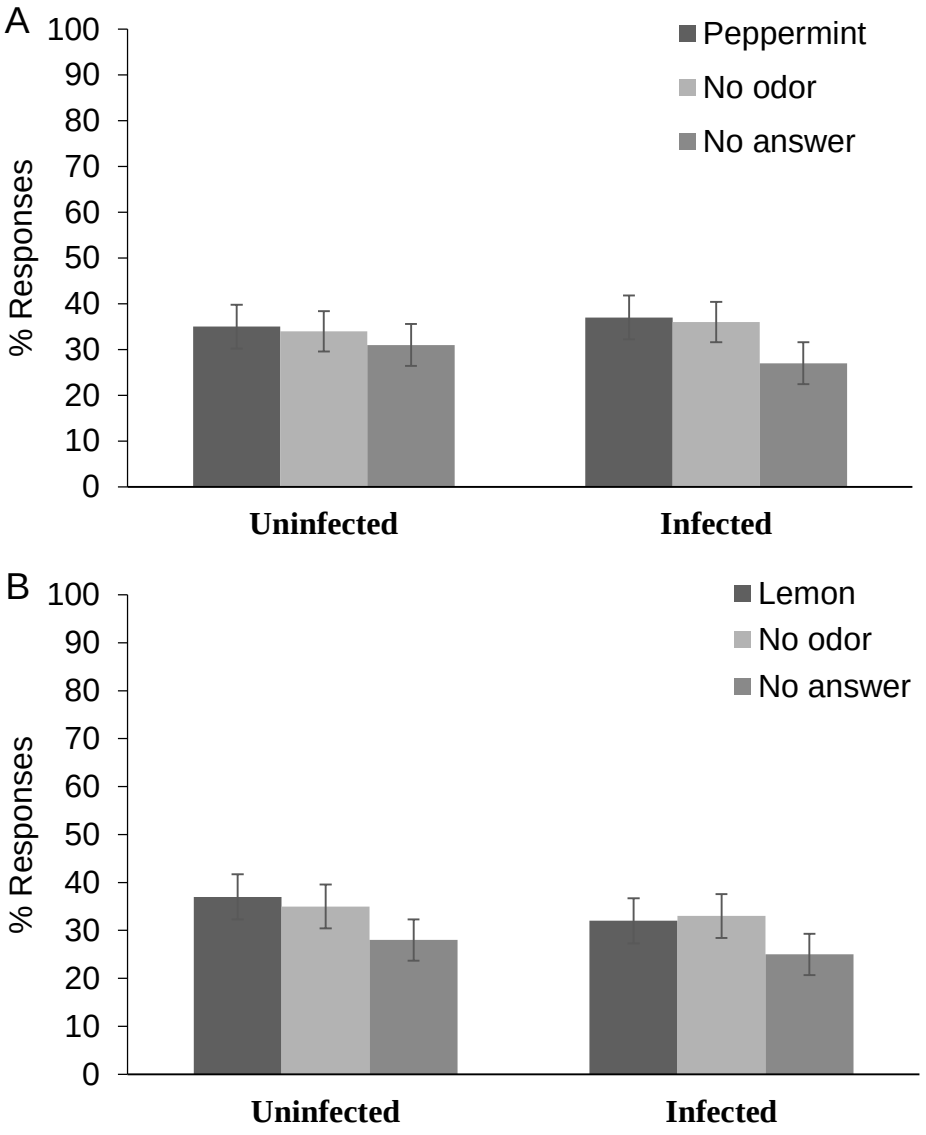
Figure legends

Figure 1. Diagram of the wind tunnel. Air was driven through the main tunnel chamber at a speed of 2.5 m/s, peppermint and lemon odor (at least 97% pure). Arrows show the dimension of each part of wind tunnel. To avoid positional effects, after each 5 experiments the positions of the peppermint and lemon were displaced.



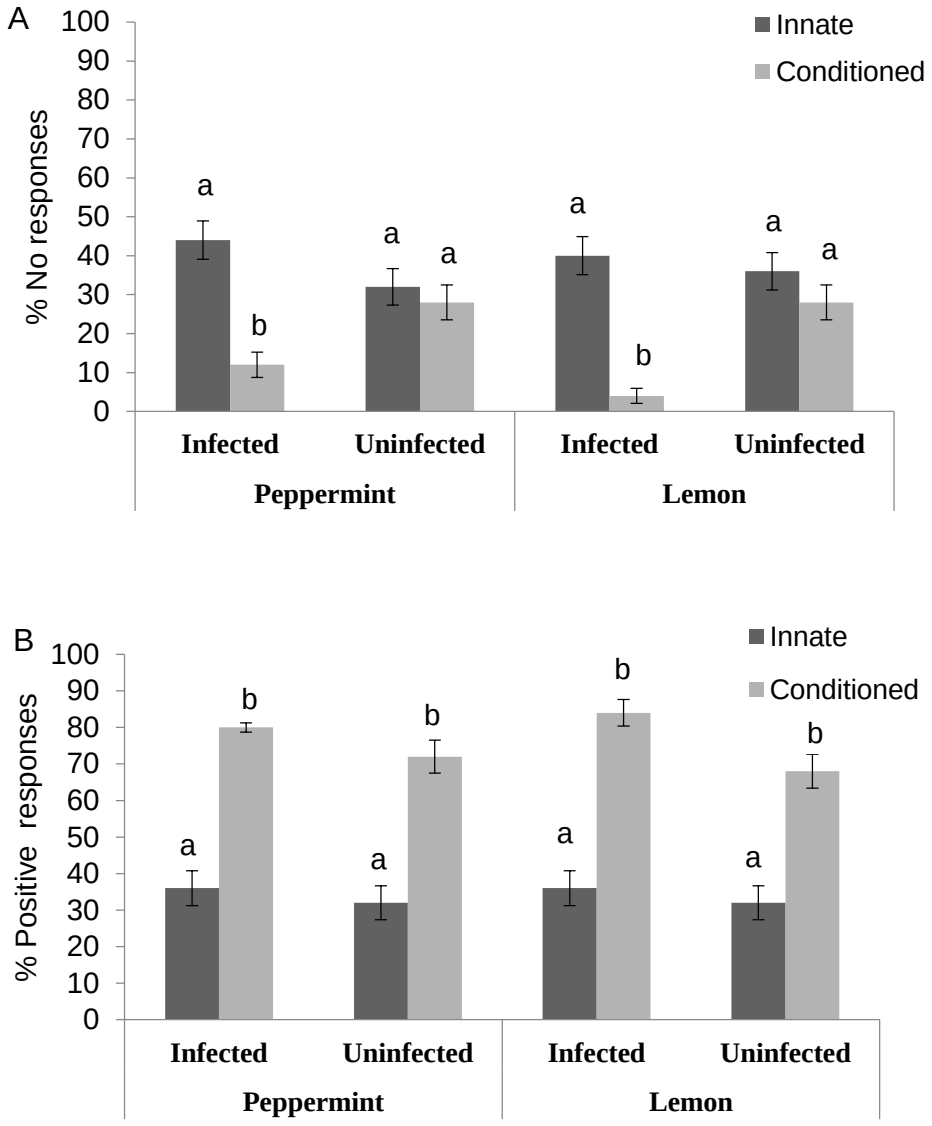
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Figure 2. Percentage of recorded responses by naïve uninfected and infected wasps toward peppermint (A) or lemon (B) odor. The error-bars represent the confidence interval on percentage.



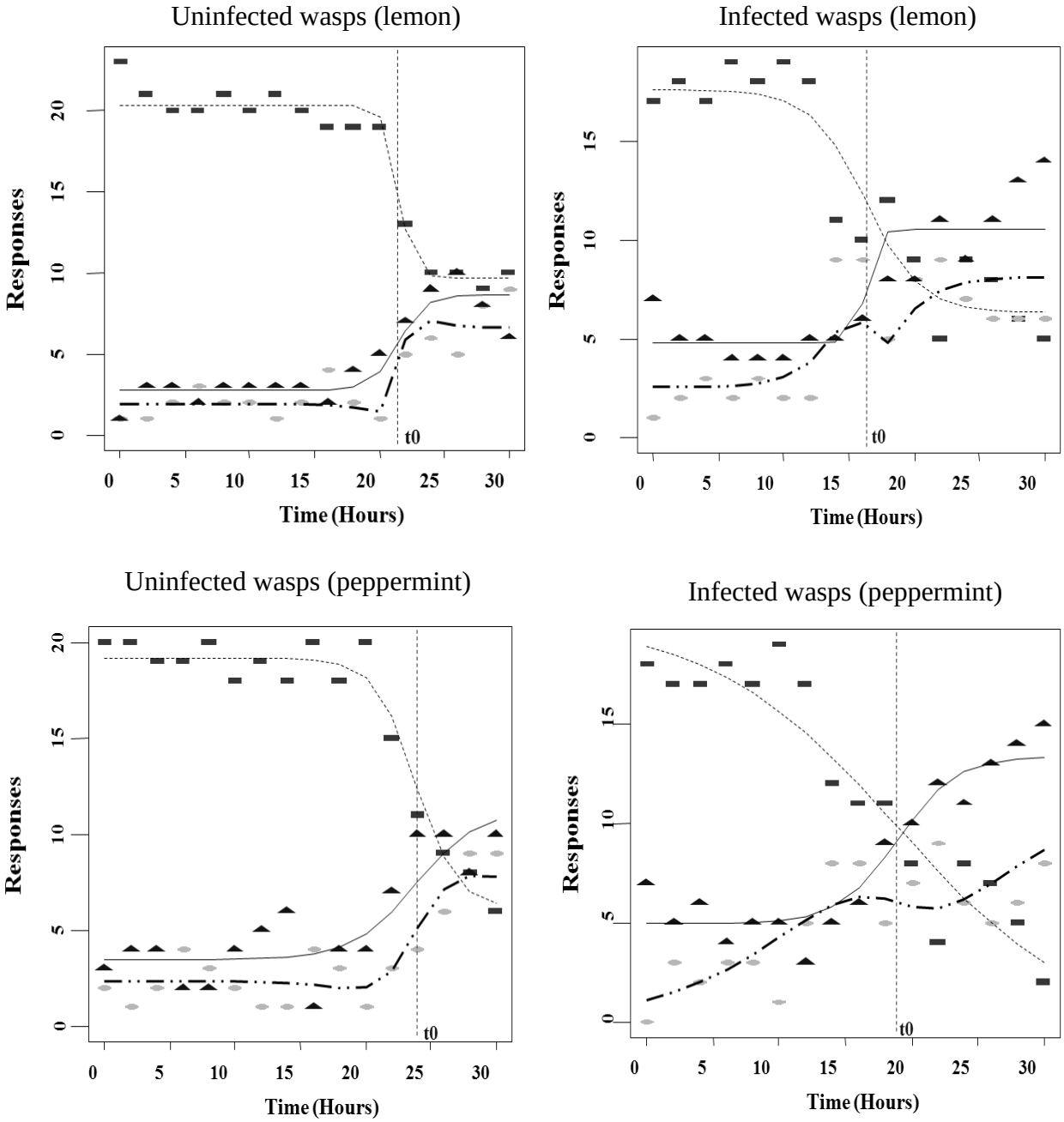
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Figure 3. Percentage of recorded responses by wasps: (A) no response (B) positive response by uninfected and infected wasps after and before conditioning by peppermint and lemon odor, respectively. The responses of 25 wasps (uninfected, females only, and infected) to peppermint/lemon odor was compared. Different letters indicate statistically significant differences.



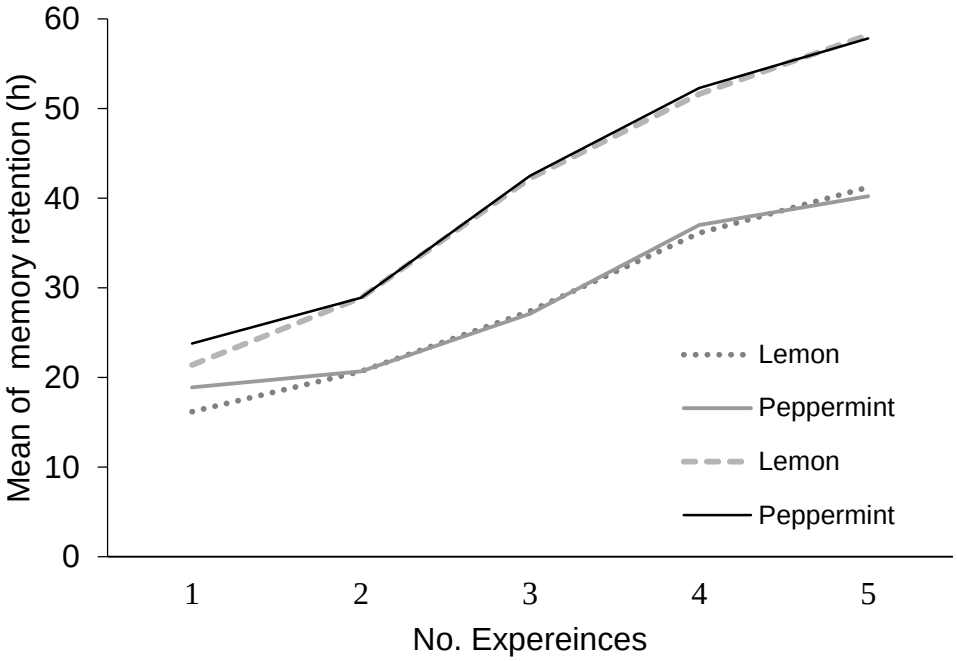
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Figure 4. Time course of the three possible responses to odor of a set of a conditioned parasitoid after one previous experience. The dotted line indicates positive responses, the gray line negative responses, and the dot-dashed line the neutral responses over time.



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Figure 5. Differences in the forgetting mid-time depending on the number of conditioning experiences (abscissa) and the type of strain.



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Table 1 Effects of the strain, conditioning, and interaction of these two factors on the wasps' responses. Significantly different results are shown in bold.

		Strain		Conditioning		Interaction between strain and conditioning	
		Chi-Square	Pr > ChiSq	Chi-Square	Pr > ChiSq	Chi-Square	Pr > ChiSq
Peppermint	Neutral	0.32	0.5745	4.35	**0.037**	2.76	0.0965
	Positive	0.48	0.4883	18.54	**<0.0001**	0.09	0.769
Lemon	Neutral	3.33	0.0679	8.47	**0.0036**	4.65	**0.0311**
	Positive	1.43	0.2322	18.91	**<0.0001**	0.64	0.4251

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Table 2 Values (t0), standard errors (SE), 95% lower (lower) and upper (upper) confidence interval bound of the estimates of the mid-time oblivion of each type (strain) and each number of experiments (number exp.).

	Strain	Number of experiences	t0	SE	Lower	Upper
Peppermint	Uninfected	1	23.8	1.4	21.0	26.7
	Infected	1	18.9	2.4	14.0	23.8
Lemon	Uninfected	1	21.4	0.7	20.0	22.8
	Infected	1	16.2	0.5	15.1	17.3
Peppermint	Uninfected	2	29.8	1.0	27.7	31.8
	Infected	2	20.8	0.7	19.4	22.2
Lemon	Uninfected	2	28.9	0.7	27.4	30.4
	Infected	2	20.7	1.1	18.4	23.0
Peppermint	Uninfected	3	42.5	2.9	36.8	48.2
	Infected	3	27.1	0.6	25.9	28.4
Lemon	Uninfected	3	42.2	0.7	40.8	43.6
	Infected	3	27.4	0.5	26.4	28.4
Peppermint	Uninfected	4	52.3	0.9	50.4	54.3
	Infected	4	37.0	0.6	35.8	38.2
Lemon	Uninfected	4	51.6	0.8	49.9	53.2
	Infected	4	36.1	0.4	35.2	36.9
Peppermint	Uninfected	5	57.8	0.9	56.0	59.7
	Infected	5	40.2	1.2	37.8	42.7
Lemon	Uninfected	5	58.2	2.0	54.3	62.2
	Infected	5	41.2	0.6	40.0	42.4