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Hossein Kishani Farahani[1*], Ahmad Ashouri[1], Pouria Abroon [2], Jean-Sebastien Pierre[3], Joan van Baaren[3]

1. *Department of Plant Protection, Faculty of Agriculture and Natural Resources, University of Tehran, Karaj, Iran*
2. *Department of Plant Protection, college of Agriculture, University of Bu Ali, Hamadan, Iran*
3. *University of Rennes 1, UMR-CNRS 6553 EcoBio, Avenue du Général Leclerc, Campus de Beaulieu, 35042 Rennes Cedex, France.*

* Corresponding Author: Hossein Kishani Farahani

Address: *Department of Plant Protection, Faculty of Agriculture and Natural Resources, University of Tehran, PO Box 4111, Karaj, Iran.*

Email: Kishani@ut.ac.ir

Phone: +989122054324

Fax: +982612240593

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Abstract:

Upon encountering a host, a female parasitoid wasp has to decide whether to learn positive or negative cues related to a host. The optimal female decision will depend on the fitness costs and benefits of learned stimuli. Reward quality is positively related to the rate of behavioral acquisition in processes such as associative learning. *Wolbachia*, an endosymbiotic bacterium, often plays an impressive role in the manipulation of its arthropod host's biology. Here we studied the responses of two natural *Wolbachia* infected/uninfected *Trichogramma brassicae* populations to theoretically high- and low- reward values during a conditioning process and the consequences of their responses in terms of memory duration. According to our results, uninfected wasps showed an attraction response to high value rewards, but showed aversive learning in response to low value rewards. Memory span of uninfected wasps after conditioning by low-value rewards was significantly shorter compared to high-value rewards. As our results revealed, responses to high quality hosts will bring more benefits (bigger size, increased fecundity and enhanced survival) compared to low-quality hosts for uninfected wasps. Infected wasps were attracted to conditioned stimuli with the same memory duration after conditioning by both types of hosts. This was linked to the fact that parasitoids emerging from both types of hosts present the same life-history traits. Therefore, these hosts represent the same quality reward for infected wasps. According to obtained results it can be concluded that *Wolbachia* manipulates the learning ability of its host resulting in the wasp responding to all reward values similarly.

Keywords:

Associative learning, aversive learning, *Trichogramma brassicae*, *Wolbachia*, fitness benefits

Introduction:

The fitness benefits of decision-making processes play important roles in uncertain environments, such as foraging for food or learning (Katz et al., 2015; Addicott et al., 2017). Through decision making, animals maximize rewards whilst minimizing costs by learning (Korn and Bach, 2015; Budaev et al., 2019). Tradeoffs exist between decision costs and benefits in many ecologically relevant tasks such as; spatial exploration strategies, choosing between plant species by pollinators; and prey and predator choice (Chittka et al., 2009; Chittka and Raine, 2006). This trade-off characterizes the balance between reaping the benefits of spending energy to learn new cues or acting innately.

Several factors such as stress (Nishio et al., 2001; Shors, 2004), aging (De Bartolo et al., 2010; Weiler et al., 2008), repetition (Toppino and Bloom, 2002), and reward value (Adcock et al., 2006; Kruidhof et al., 2012; Hoedjes et al., 2011) may affect the learning ability and memory retention of animals. Reward value can affect different behaviors such as flower odor preference in honeybees (Russell et al., 2016), win-shift tendency in birds (shift away from/avoid locations that have recently yielded food) (Sulikowski and Burke, 2007), as well as changes in the duration of memory for, e.g., odors in fruit flies (Burke and Waddell, 2011). Kruidhof et al. (2012) hypothesized that consolidation of different memory forms may be induced by exposing to the different value of same type of rewards.. The importance of reward value in behavior modification of an organism is variable in relation to association ability between learned information and the reward value (Savage and Ramos, 2009, Kaiser et al., 2017). To study reward value effects on memory duration in an ecologically relevant context, parasitic wasps are ideal model organisms as learning to associate odor cues with host presence may lead to optimize their host searching efficiency (Hoedjes et al., 2011).

Wolbachia (Rickettsiales: Anaplasmataceae) (α- proteobacteria) is the most widespread endosymbiotic bacterium and infects arthropod species, including a high proportion of insects (Kageyama et al., 2017; Kajtoch et al., 2019). Several researches have been focused on effects of *Wolbachia* on bionomics (Hohmann et al., 2001; Grenier and De Clercq, 2003; Miura and Tagami, 2004) and behavior (Almeida et al. 2010, Ramirez-Romero et al. 2012, Furihata et al., 2015) of parasitic wasps, and resulted in contradictory conclusions. Wasps of the genus Trichogramma (Hymenoptera: Trichogrammatidae) like many other parasitic wasps harbor this symbiotic bacterium (Hurst et al., 1999; Werren, 1997). The parasitoid wasp *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) has been considered as the most used biological control agent against various Lepidoptera pest species in agricultural systems to attack pest egg stages (Hoffmann et al. 1995; Pinto 1998; Mansfield and Mills 2002; Babendreier et al. 2003; Kuske et al. 2003). It has been shown that two mode of reproduction in Trichogramma wasps frequently happens as it also occurs in *T. brassicae* individuals. Arrhenotokous parthenogenesis, in which unfertilized eggs develop into haploid male offspring which are viable and fertile (Hohmann et al. 2001; Pannebakker et al. 2004; Ma and Schwander 2017). Thelytoky parthenogenesis, which female offsprings are produced from unfertilized eggs (Hohmann et al. 2001; Poorjavad et al. 2012; Ma and Schwander 2017). In these particular cases, Thelytoky parthenogenesis, researches have shown that infection to endosymbiotic bacterium *Wolbachia* will lead to thelytoky and consequent offspring sex ratio will be female biased (Farrokhi 2010, Kishani Farahani et al. 2015, Poorjavad et al. 2012, 2018).

Kishani Farahani et al. (2017) showed differences in learning ability and memory retention between *Wolbachia* infected and uninfected *T. brassicae* and stated that it may be due to negative side effects of *Wolbachia* infection. However it has been neglected that learning impairment

between infected and uninfected strains may be due to obtaining different benefits from learning tasks. The main question of this paper is: "Do *Wolbachia* infected wasps make decisions, learn and memorize new stimuli, without considering the future fitness benefits of their decisions?" We hypothesize that *Wolbachia* infected wasps will face the same fitness benefits from learning low- and high-quality rewards due to exhibiting the same resultant life history traits irrespective of reward quality. We expect that uninfected wasps will show differences in behavior, learning ability and memory retention, according to host quality. In contrast, we predict that *Wolbachia* infected wasps will display the same learning ability and the same memory duration irrespective of reward quality.

Material and methods:

Parasitoids

In this paper, we compared two strains of *T. brassicae*: one infected with *Wolbachia* (*Wolbachia* wBaT.bra registered as FJ441291 in Genbank) and one not (uninfected). Both wasp strains were provided by the Biological Control Research Department (BCRD) of the Iranian Research Institute of Plant Protection (IRIPP). The original strains of these parasitoids were collected from same corn fields located in Baboulsar Region (southern coastline of Caspian Sea, 2016) .It has been proved by previous studies, carried out based on ITS2 and COI sequencing tests, that the both infected and uninfected lineages carry the same genetic background (Kishani Farahani et al., 2015; Poorjavad et al., 2018). The wasps' genetical background characteristic allowed us to avoid the potential confounding effects, physiological and behavioral, of antibiotics treatments against infected linage (Timmermans and Ellers, 2009; Dedeine et al., 2001; Hoffman et al., 1990).

To determine *Wolbachia* prevalence in female wasps which produced only female offspring (Huigens and Stouthamer, 2003), a PCR method based on the *Wolbachia* surface protein (wsp)

was implemented (81F/691R primers; Braig et al., 1998). *Wolbachia* presence was verified by a 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). Throughout the entire experimental duration the *Wolbachia* presence in infected line was screened by PCR tests to ensure that all different behaviors are due to *Wolbachia* presence (kishani Farahani et al. 2015).

For all experiments described below, we used the flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae) to rear parasitoids. Lepidoptera eggs were obtained from a culture maintained at the Insectary and Quarantine Facility, University of Tehran. The host eggs were maintained at 25±1ºC on host larvae fed by wheat flour and yeast (5%). glass containers (500 ml) were used to keep newly emerged and mated moths to provide moth eggs. Eggs were collected daily. Also egg cards (2×5 cm), loaded with 200 eggs of *E. kuehniella* were used to rear Parasitoids on. The egg cards were placed into emergence canisters, 500-ml cardboard cylinders (63×161 mm) with a 50-ml glass vial (26×93 mm), and held in incubators at 25±1ºC, 16L: 8 D and 50±5% RH more than five generations. All lab-reared wasps were fed daily on a droplet of 10% honey solution, spread and rubbed on the internal surface of each tube to avoid damaging the wings, and consequently the behaviors, of the wasps.

In order to isolate parasitoid females for our experiments, we placed small squares of egg cards into emergence canisters and held them in incubators at 25±1ºC, 16L: 8 D and 50±5% RH. Emergence canisters were inserted through the lid. Females are more strongly phototactic than

males, facilitating the collection of newly emerged mated females in the glass vials (Morrison et al., 1978; Kishani Farahani et al., 2015). Once approximately 20 parasitoids had emerged into a glass vial, they were removed, provisioned with undiluted honey as a food source, and closed with a ventilated plastic cap to serve as a holding container until females were 24 h old.

Experimental design:

We tested the effects of *Wolbachia* presence and reward value using young *E. kuehniella* eggs, less than 12 hours old, as hosts of high value and old eggs, 30 days old, as hosts of low value, on adult wasps learning ability and memory retention. The factorial design comprised of four treatments: T1: uninfected wasps conditioned with young eggs, T2: uninfected wasps conditioned with old eggs, T3 infected wasps conditioned with young eggs and T4: infected wasps conditioned with old eggs.

Life history traits:

To study trade-offs in adult decision making of learning a new odor, we recorded life history traits of wasps reared on high- and low-quality rewards for both uninfected and infected wasps.

Morphometric measures: To correlate body size with fitness parameters, the length of the left hind tibia and wing surface of each individual was measured using a binocular microscope (0.5×6.3, Olympus SZ-CTV) connected to a video camera (JVC KY-F). Tibia length is a commonly used as an indicator of body size in parasitoid wasps and correlates strongly to others measures, e.g. dry mass (Godfray, 1994). From photographed images, wing area and tibia length of 40 wasps per strain (from each host quality, totally 160 wasps) were determined using Image J software. Since body mass is thought to correlate with size, for each strain reared on high- and low-quality hosts, a minimum of 40 females (per strain reared on high- or low-quality hosts, i.e. a

total of 160 females) were selected randomly and frozen in liquid nitrogen on emergence, and weighed on a microbalance to an accuracy of ±0.1 µg (Mettler Toledo XP2U) (Ismail et al., 2012).

Longevity: Following wasp emergence, adult longevity without food (but with access to water) was measured to estimate longevity with only available capital resources, amount of energy reserves within the body after development, (n= 40 females per strain reared on high- or low-quality hosts, i.e. a total of 160 females). Individual adults were placed in small tubes (1.5 cm in diameter and 10 cm long) and were monitored until death (Kishani Farahani et al., 2016).

Innate preference:

Innate preference of 50 wasps towards one odor (peppermint and lemon (essential oil, at least 98% purity, by Adonis Gol Darou Group, Iran) were tested as described by Kishani Farahani et al. (2017) in the wind tunnel as previously described by Yong et al. (2007) and their responses were recorded. We performed the cognitive tests in a flight tunnel (200 x 50 x 50 cm l ×w ×h) made of transparent Plexiglas. A smaller chamber (50×20×20 cm, l×w× h), centered within the main chamber and open at both the upwind and downwind ends, served as the experimental arena. The experimental room was illuminated with 2000 lux lights provided by LED lights (Pars Shahab Lamp Co., Iran). Air was driven through the flight tunnel by a fan located at the upwind end, and extracted outside by a fume hood at the downwind end. The end opposite to the start zone of the tunnel was divided by a glass separator wall in two decision chambers. Each decision chamber contained an odorant stimulus presented on a filter paper attached to a glass pipette placed vertically on a stand. Wasps were exposed to odor versus clean air. To do this, single naïve female wasps were introduced into the flight chamber. Twenty-five of the 50 wasps underwent this procedure using the peppermint odor and the other 25 underwent the procedure using the lemon

odor. 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 over the start area in the flight chamber were scored as displaying no response (Kishani Farahani et al., 2017).

Conditioning

The ability of wasps to learn was determined using a Pavlovian conditioning procedure whereby an odor stimulus was associated with the reward of ovipositing (van Baaren et al., 2005; Bleeker et al., 2006). Both strains of wasps were naïve without any former oviposition experiences to avoid the variability in sequence and the retention of behavioral events associated with learning from the first host encountered (Mills and Kuhlmann, 2004). Sixty five one-day old naïve females were exposed individually to host eggs for 15 minutes to gain oviposition experience. This allowed wasps to spend conditioning time to oviposit on hosts and associate oviposition with the odor in the conditioning tank. Since some wasps died, were lost or did not oviposit during the manipulation, approximately sixty wasps per treatment were kept for the experiment. Half of the tested individuals (n=30) were conditioned using peppermint odor and the remaining half with lemon odor. For conditioning, one adult wasp was introduced to a vial (2×10 cm) containing 100 host eggs, high quality or low quality hosts, glued on a cardboard and was transferred into the conditioning tank (25×25×25 cm). During experiments, the conditioning odor (either peppermint or lemon) was pumped into the tanks at a speed of 1 m/s speed. The conditioning process lasted a total of 2 hours and was repeated for both uninfected and infected females (60 females of each of the treatments T1 to T4). The conditioning time of 2 hours was set based on the average time of patch leaving for 100 adult wasps exposed to 100 eggs.

Test of odor preference after conditioning

Fifteen minutes after conditioning, wasps were placed individually in the flight chamber. The response of 50 female wasps (randomly selected from the surviving wasps of the 60 conditioned), 25 conditioned on peppermint and 25 conditioned on lemon, were tested for the four treatment groups (totaling 4 x 50 females). The responses of the wasps to the conditioned odors were observed in the flight tunnel during a flight time of 15 min. If females displayed a preference towards the conditioned odor (i.e. the individual landed or hovered on the conditioned odor site for more than 2 minutes), it was assumed that associative learning between the odor and the reward of oviposition had occurred. The number of wasps which landed on an unconditioned odor and the number of non-responding wasps were recorded to determine behavioral response variation by both strains. Females that did not complete a flight or did not fly after 5 min were scored as displaying no response. All flight responses were tested at 25°C, 50% RH, and a light intensity of 2000 lux (Kishani Farahani et al., 2017).

Test of memory duration

Memory (retention) is defined as present when wasps show a significant preference for the conditioned odor (peppermint/lemon) when conditioned on peppermint or lemon respectively, as compared to the unconditioned odor, after learning. To determine the duration of memory, experienced wasps of both strains were kept 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, and 30h after training until used in a bioassay in the same conditions as previously described. For each time interval, 50 wasps of both strains were observed in the wind tunnel as described, representing a total of 1600 tested wasps (Kishani Farahani et al., 2017).

Statistical analysis:

Before running statistical analyses, all data were tested with Kolmogorov–Smirnov test (by procedure UNIVARIATE, SAS software (ver. 9.1)) for normality.

Life history traits:

Numerical data (weight, longevity, wing surface, and tibia length) were analyzed by Generalized Linear models based on a Poisson distribution and log-link function. In all cases, strain and host quality were considered factors. When a significant effect of treatment was found, a Bonferroni's *post hoc* multiple comparison test was performed, and the two-by-two comparisons evaluated at the Bonferroni-corrected significance level of $P = 0.05/k$, where k is the number of comparisons. The statistical analyses were performed using SAS software (SAS Institute Inc., 2003).

Learning:

The innate responses of both strains were compared by Chi-Square using SAS software (SAS Institute, 2003). To compare the responses of the two strains before and after conditioning, we used a 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).

Memory retention:

To estimate memory duration of uninfected and *Wolbachia* infected wasps, we developed a dynamic statistical model (Kishani Farahani et al., 2017). Briefly, 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 peppermint side, a preference for the lemon side, and to a no choice. Forgetting conditioning results in a switch from a high level to a lower

level of correct responses, a simultaneous switch from a low level to a high level of no choices, and a switch from a very low to a moderate level of incorrect choices. 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 $p_a + p_b + p_c = 1$:

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

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

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

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 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. To reveal significant differences in memory retention of both uninfected and infected wasps

conditioned with high- and low-quality rewards, we carried out a three-factor analysis of variance to verify the conclusions. The experimental design was a balanced factorial design with two factors: the type of strain, with two levels, uninfected and infected, and the reward value, young or old hosts (Kishani Farahani et al. 2017).

Results:

Life history traits

Host quality and strain effects and interaction of these parameters on life history traits of uninfected and infected wasps are shown in Table 1. Weight ($\chi 2=61.83$, $P<0.0001$), longevity ($\chi 2=27.2$, $P<0.0001$), Tibia length ($\chi 2=7.75$, $P=0.005$), and wing area ($\chi 2=33.61$, $P<0.0001$) were significantly higher for uninfected wasps reared on high quality hosts than for uninfected wasps reared on low-quality hosts (Figure 1).

For infected wasps, we observed no significant difference in weight ($\chi^2=1.29$, $P=0.259$), longevity ($\chi^2=0.49$, $P=0.489$), tibia length ($\chi^2=0.08$, $P=0.779$), and wing surface ($\chi 2=8.2$, $P=0.004$), between females reared on high- and low-quality hosts (Figure 1).

Test of odor preference after conditioning

The effects of reward quality and conditioning, and the interaction between these factors, on the responses of uninfected and infected wasp are shown in Table 2.

Uninfected wasps with High- and Low-quality reward

Associative learning towards conditioned stimuli was observed by uninfected wasps conditioned with high quality reward, with the wasps displaying attraction responses towards the conditioned stimuli. Uninfected wasps associated the conditioned odor with oviposition (Lemon odor: $\chi^2=6.30$,

P=0.012, N=25; peppermint odor: χ^2=21.67, P<.0001, N=25) (Figure 2a). In addition, the neutral response by uninfected wasps was significantly lower after conditioning (Lemon odor: χ^2=14.62, P=0.001, N=25; peppermint odor: χ^2=30.39, P<.0001, N=25) (Figure 2b).

Conditioned uninfected wasps with low-quality rewards displayed aversive learning by showing significantly less positive responses towards the conditioning odor (Lemon odor: χ^2=8.47, P=0.003, N=25; peppermint odor: χ^2=4.86, P=0.0275, N=25) (Figure 2a). Neutral responses of uninfected wasps (Lemon odor: χ^2=4.51, P=0.033, N=25; peppermint odor: χ^2=32.97, P<.0001, N=25) were significantly lower after conditioning (Figure 2b).

Infected wasps with High- and Low-quality reward

Infected females significantly preferred the odor in the presence of a high-quality reward to which they were conditioned (i.e. olfactory learning) to the unconditioned odor (Figure 3a) (Lemon odor: χ^2=7.59, P=0.005 N=25; peppermint odor: χ^2=13.9, P=0.0002 N=25). Infected wasps showed no significant differences in neutral responses before and after conditioning (Lemon odor: χ^2=1.58, P=0.20, N=25; peppermint odor: χ^2=2.36, P=0.124, N=25) (Figure 3b).

Positive responses of infected wasps (Lemon odor: χ^2=11.43, P=0.0007, N=25; peppermint odor: χ^2=8.7, P=0.003, N=25) showed a significant difference after conditioning (Figure 3a). Furthermore, no significant differences were observed in neutral responses of infected wasps conditioned with a low-quality reward (Lemon odor: χ^2=3.3, P=0.067, N=25; peppermint odor: χ^2=2.76, P=0.096, N=25) (Figure 3b).

Memory retention

Differences between the strains were highly significant (F= 7.36, p= 0.02), as were the reward value (F = 12.03, p= 0.008), while odor type (F =1.43, p =0.26) and interaction (strain× reward

value) (F=0.54, p=0.71) were not significantly different. For the uninfected strain, the memory retention was longer for wasps conditioned with a high-quality reward than with a low-quality reward (P= 0.02 for lemon odor; P= 0.03 for peppermint odor) (Figure 4). For the infected strain, the memory duration did not vary significantly with reward value (P= 0.49 for lemon odor; P= 0. 8 for peppermint odor) (Figure 4). Memory retention of infected wasps was lower than uninfected wasps when conditioned with high quality hosts (P= 0.03 for lemon odor; P= 0.01 for peppermint odor). However, memory retention did not significantly differ between infected and uninfected wasps when conditioned with low-quality hosts (P= 0.73 for lemon odor; P= 0.35 for peppermint odor).

Discussion:

In accordance with our hypotheses, uninfected wasps changed their behavior in response to reward value. Conditioned uninfected wasps with low-value rewards showed aversive learning and avoided the conditioned odor. On the other hand, conditioned uninfected wasps with high quality hosts were attracted to conditioned stimuli and showed olfactory learning. Uninfected wasps, conditioned with high-quality rewards, showed significantly longer memory retention than those conditioned with low-quality hosts. *Wolbachia* infected wasps showed associative learning in response to high- and low-value rewards and were attracted to conditioned stimuli. Memory retention of infected wasps did not significantly differ after conditioning with high- and low-quality rewards and their memory duration was lower than uninfected wasps conditioned with high quality rewards. According to the obtained life history traits, different host qualities result in different fitness benefits for uninfected wasps by producing bigger and thus likely more fecund individuals. However, infected wasps obtained the same fitness benefits from both host qualities which displayed the same value as the reward for infected wasps.

The host represents the sole physiological and nutritional environment during immature development for insect parasitoids (Jervis and Kidd, 1986). Consequently, host quality is important for overall parasitoid growth and development and may affect the immature developmental time, longevity, mortality rate, and fecundity (Harvey and Strand, 2002; Sampaio et al., 2008). For example, Kant et al. (2012) showed that *Diaeretiella rapae* females emerging from high-quality hosts produced 62 % more offspring than females emerging from low-quality hosts. Body size has an impressive role in ecology and evolution theories (Angilletta et al., 2004), since it affects all aspects of physiology, life history, and fitness of animals (Thorne et al., 2006; Chown and Gaston, 2010; Wilder et al., 2016). Larger body size is linked with greater fitness in several animal species (Kingsolver and Huey, 2008; Amarillo-Suárez et al., 2011; Goldbogen, 2018; Chown and Gaston, 2010). According to Lacoume et al. (2007) small males of the parasitoid wasp, *Dinarmus basalis* (Hymenoptera: Pteromalidae), possessed a reduced size and sperm stock when compared to larger males. As a result, the smaller males fertilized fewer females in competitive situations. According to our results, uninfected wasps reared on low-quality hosts showed a smaller size compared to those reared on high-quality hosts (reward). As a consequence of parasitizing low-quality reward hosts, smaller offspring would be produced. Therefore, this could be a reason for aversive learning towards low-quality hosts observed by uninfected wasps.

In the current study, low-quality rewards led to aversive behavior in uninfected wasps. The importance of reward value in the modification of behavior varies depending on the ability to associate the learned information with the reward value. This ability, in turn, has consequences for the fitness of the organism. It allows the organism to focus exclusively on learned cues which profit the organism with more efficient feedbacks. Different kinds of aversive stimuli have been studied such as electric shock and inedible food (Mery and Kawecki, 2005; Zhao et al., 2004;

Salloum et al., 2011; Kandori and Yamaki, 2012; Unoki et al., 2005). In the case of aversive learning, the contributions made on insects have been thus far limited to a few species such as the fruit fly, *Drosophila melanogaster* (Diptera: Drosophilidae), in which flies learn to associate an odor and an electric shock (Malik and Hodge, 2014; Le Bourg, 2012). Zhang et al. (2005) showed that *Caenorhabditis elegans* (Rhabditida: Rhabditidae), modifies its olfactory preferences after exposure to pathogenic bacteria, avoiding odors from the pathogen and increasing its attraction to odors from familiar nonpathogenic bacteria. This behavior may lead to increased dispersion of uninfected wasps to avoid low-quality hosts while infected wasps may spend more time in patches containing low-quality hosts.

It has been shown by Kruidhof et al. (2012) that different values of reward, even of the same reward type, induce consolidation of a different memory form in a parasitoid wasp. The importance of a reward may vary in relation to its benefit to an animal's fitness and the reliability of the association between the learned cues and the linked reward (Kruidhof et al., 2012). Soldati et al. (2017) showed that red-footed tortoises (*Chelonoidis carbonaria* (Turtle: Testudinidae)) are able to recall the learned information about the stimuli as indicators of relative reward values, which demonstrates a memory for the relative quantity and quality of food. Since longer memory is more energetically costly than shorter-lasting memory, longer memory consolidation by low reward value may not be optimal (Mery and Kawecki, 2002). However, consolidation of longer memory may not be beneficial if making irrelevant associations is a high-risk action (Smid et al., 2007). The observed similar memory retention and host size of infected wasps reared on high- and low-quality hosts suggests that there are similar fitness benefits to be gained from both high- and low-quality hosts.

As our results showed, we observed two different memory forms, aversive and attraction, and different durations by uninfected wasps when conditioned with different reward qualities. Based on our results, we observed aversive memory that was significantly shorter than attraction learning of uninfected wasps. Avoiding and remembering associatively learned predictors of unpleasant stimuli is crucial for survival (Krypotos et al., 2015). Memories can, however, become counter-adaptive when they are overly generalized to harmless cues and contexts (König et al., 2017). Associatively learning the predictors of noxious events is useful for survival as it enables pre-emptive avoidance. Depending on the nature of the unpleasant experience, its memory can last for different durations (Davis and Squire, 1984; Dudai, 2012; Kandel et al., 2014). Amano and Maruyama (2011) showed that the nematode *Caenorhabditis elegans* displayed shorter memory retention when conditioned by negative reinforcements compared to nematodes conditioned with pleasant stimuli. Memory formation is a costly process which consumes much of the daily energy budget (Burns et al., 2011; Placais et al., 2017; Liefting et al., 2019). Energy efficiency is indeed put forward as a major factor of the selective pressure driving the evolution of nervous system function and efficiency (Placais et al., 2017). As we have shown, uninfected wasps will display a smaller size on low-quality hosts which may lead to less energy resources. However, it seems that shorter aversive memory retention may play an evolutionary role for uninfected wasps as these animals are time-limited foragers and therefore may be able to learn new stimuli related to higher reward presences.

Our results showed that *Wolbachia*-infected wasps associated the conditioned odor with both types of reward, high and low quality, which means they behave similarly towards hosts that represent different qualities for uninfected wasps. This behavior can aid *Wolbachia* transmission towards uninfected individuals. Indeed, in addition to vertical transmission of *Wolbachia*, which ensures

the durability of intracellular symbioses, horizontal transmission must also occur (Tolley et al. 2019). Horizontal transfer was observed when infected and uninfected parasitoid larvae shared the same host (Huigens et al. 2004). According to Huigens et al. (2004), horizontal transfer can occur in nature only when a transferor and a recipient host are in close contact. Huigens et al. (2000) stated for the first time that high rates of natural horizontal transfer occur between conspecifics in *T. kaykai*, when *Wolbachia*-infected and uninfected *T. kaykai* larvae shared the same host. According to Kishani Farahani et al. (2015), *Wolbachia*-infected *T. brassicae* lack host parasitism status, evaluation ability, and superparasitize hosts more frequently than uninfected *Wolbachia*-strains. It seems that the higher superparasitism rate and the behavior of parasitizing all host qualities may allow *Wolbachia* to be transmitted between Trichogramma strains horizontally. As both strains share the same niches, associative learning may allow *Wolbachia*- infected wasps to find all type of host qualities, oviposit on them and hereby increase the chance of infection of new individuals, which will lead to an increase in infection rate within the species.

In conclusion, learning represents trade-offs between the cost of learning and the associated benefits. Subsequently, if infected wasps gain similar benefits from ovipositing in high- and low-quality hosts, there would be no additional benefit of associated learning and enhanced memory retention, and thus a fixed memory retention would be favored. It seems that uninfected wasps make decisions based on the future fitness benefits; the wasps will learn a cue if it's beneficial to future behavior and fitness. In contrast, decision making by infected wasps is detrimentally affected by *Wolbachia*. The behavior of infected wasps such as decision making may be manipulated by *Wolbachia* to enhance dispersal to new environments and through new hosts. However, other possibilities such as potential negative side effects of *Wolbachia* infection on mobility behavior, perception capacity, or learning ability should be considered.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Hossein Kishani Farahani, Ahmad Ashouri; Methodology: Hossein Kishani Farahani, Joan van Baaren; Validation: Hossein Kishani Farahani, Ahmad Ashouri; Formal analysis: Hossein Kishani Farahani, Jean-Sébastien Pierre; Investigation: Hossein Kishani Farahani, Pouria Abroon, Joan van Baaren; Resources: University of Tehran, Iran, University of Rennes 1; Writing - original draft: Hossein Kishani Farahani, Joan van Baaren ;Writing - review & editing: Hossein Kishani Farahani, Joan van Baaren; Supervision: Ahmad Ashouri, Joan van Baaren; Project administration: University of Rennes 1; Funding acquisition: University of Tehran

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Data availability

Should the manuscript be accepted, the data supporting the results will be archived in an appropriate public repository (Dryad) and the data DOI will be included at the end of the article.

Animal welfare ethics: During all experiments all wasps were reared on flour moth larvae in the laboratory conditions at 25˚C with a 16:8 L: D photoperiod and 50± 5% R.H. Adult parasitoids were fed on undiluted

honey. All wasps were maintained and tested under the same conditions, and throughout all experiments, one day old wasps were fed on a 10% honey solution. All animals were obtained from a culture maintained at the Insectary and Quarantine Facility, University of Tehran. After finishing the experiments all wasps were kept at the same condition and were fed with undiluted honey, and during this period they were exposed to hosts to have routine life stages including feeding and oviposition.

All methods were carried out in accordance with Iranian and European regulations. Experimental protocols were approved by the University of Tehran. The study was carried out in compliance with the ARRIVE guidelines.

The current study was not included any **potentially harmful manipulations and Invasive samples.**

Supplementary information

There is no supplementary data.

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Table 1. Effects of strain, host quality and the interaction of these two factors on longevity, tibia length, wing surface and weight of wasps. Significant results are shown in bold.

	Host quality		Strain		Interaction	
	Chi-Square	Pr > ChiSq	Chi-Square	Pr > ChiSq	Chi-Square	Pr > ChiSq
Longevity	17.07	**<0.0001**	3.62	0.057	9.73	**0.0018**
Tibia length	4.97	**0.0258**	1.66	0.1981	3.4	0.065
Wing surface	3.28	0.07	66.9	**<0.0001**	36.44	**<0.0001**
Weight	38.65	**<0.0001**	53.07	**<0.0001**	20.78	**<0.0001**

Table 2. Effects of host quality, conditioning and the interaction of these two factors on responses of uninfected and infected wasps. Significant results are shown in bold.

			Reward quality		Conditioning		Interaction	
			χ^2	P value	χ^2	P value	χ^2	P value
uninfected	Peppermint	No response	4.5	**0.034**	6.06	**0.013**	8.44	**0.003**
		Positive	21.67	**<.0001**	6.3	**0.012**	12.12	**0.005**
	Lemon	No response	13.7	**0.0002**	7.63	**0.0057**	12.42	**0.0004**
		Positive	3.95	**0.045**	12.58	**0.0004**	12.12	**0.0005**
infected	Peppermint	No response	0.19	0.662	0.44	0.507	0.18	0.67
		Positive	0.18	0.67	15.37	**<.0001**	1.03	0.309
	Lemon	No response	0.89	0.345	0.11	0.739	0.11	0.739
		Positive	1.03	0.309	30.39	**<.0001**	1.03	0.309

Figure legends:

Figure 1. Weight (A), longevity (B), Tibia length (C) and wing area (D) of uninfected and infected wasps reared on high quality- (HQR) and low quality- (LQR) rewards. Each box plot represents the spread of the sample and variability is indicated by the distance between the whiskers. The box plot shows the median (bold line), the interquartile range (length of box), and extreme, maximum and minimum, data points. Generalized Linear models based on a Poisson distribution and log-link function were implemented. Different letters indicate significant differences based on Bonferroni-corrected significance level of $P = 0.05/k$, where k is the number of comparisons. N=40 wasps per strain (from each host quality, totally 160 wasps).

Figure 2. A) Positively responding individuals of uninfected wasps conditioned with peppermint/ lemon odor, and B) Non-responding individuals of uninfected wasps. Responses were observed in a flight chamber in a choice situation between conditioned stimuli (CS) or unconditioned stimuli (US). Female wasps were exposed to high quality- (HQR) and low quality- (LQR) quality rewards. Each box plot represents the spread of the sample and variability is indicated by the distance between the whiskers. The box plot shows the median (bold line), the interquartile range (length of box), and extreme, maximum and minimum, data points. Generalized Linear Models (GLM) were implemented with the binomial family error and logit link. Different letters indicate significant differences based on Bonferroni-corrected significance level of $P = 0.05/k$, where k is the number of comparisons. N= 50 wasps from each host quality (totally 100 wasps).

Figure 3. A) Positively responding individuals of *Wolbachia* infected wasps conditioned with peppermint/ lemon odor, and B) Non-responding individuals of *Wolbachia* infected wasps. Responses were observed in a flight chamber in a choice situation between conditioned stimuli (CS) or unconditioned stimuli (US). Female wasps were exposed to high quality- (HQR) and low quality- (LQR) quality rewards. Each box plot represents the spread of the sample and variability is indicated by the distance between the whiskers. The box plot shows the median (bold line), the interquartile range (length of box), and extreme, maximum and minimum, data points. Generalized Linear Models (GLM) were implemented with the binomial family error and logit link (N.S.) stands for Non-significant differences. Different letters indicate significant differences based on Bonferroni-corrected significance level of $P = 0.05/k$, where k is the number of comparisons. N= 50 wasps from each host quality (totally 100 wasps).

Figure 4. Memory retention (t0) of uninfected and infected wasps after conditioning with high quality - (HQR) and low quality- (LQR) rewards. Vertical notched bars indicate the 95% confidence interval of the estimates. Memory retentions were compared using one way analysis of variance (ANOVA). Different letters indicate significant differences based on Bonferroni-corrected significance level of $P = 0.05/k$, where k is the number of comparisons. 50 wasps per strain (from each host quality, totally 2400 wasps).

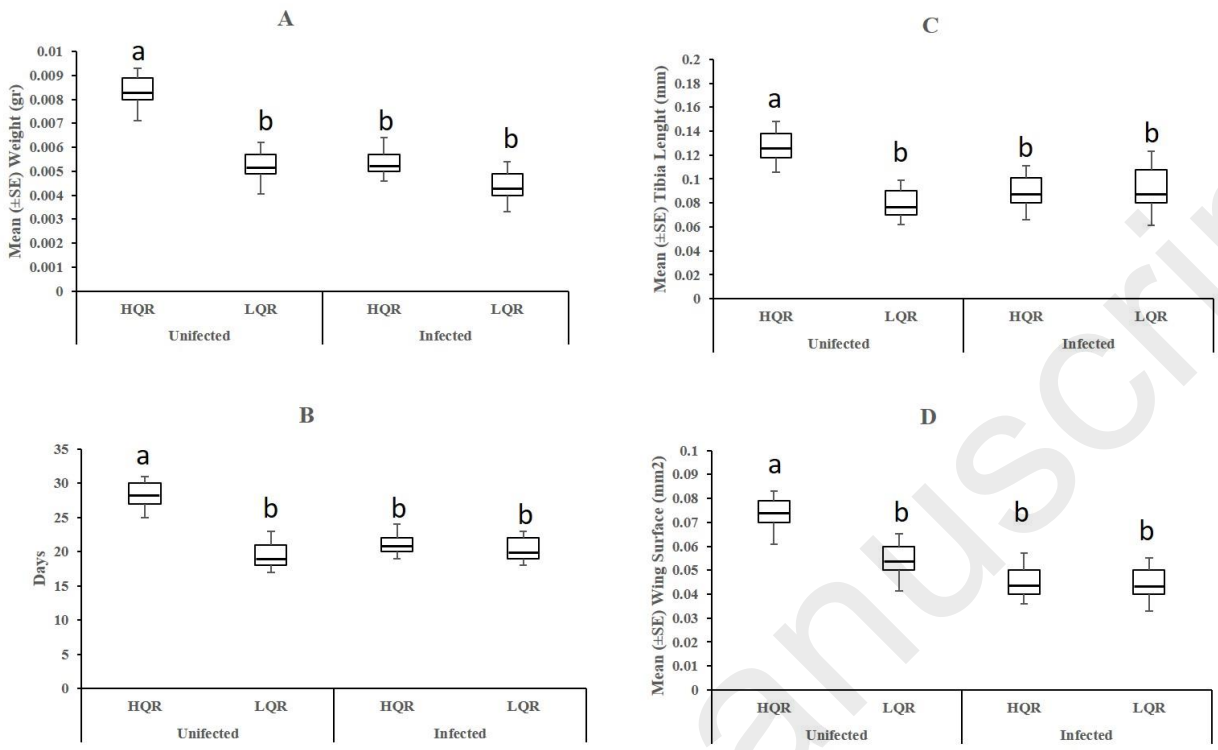

Figure 1

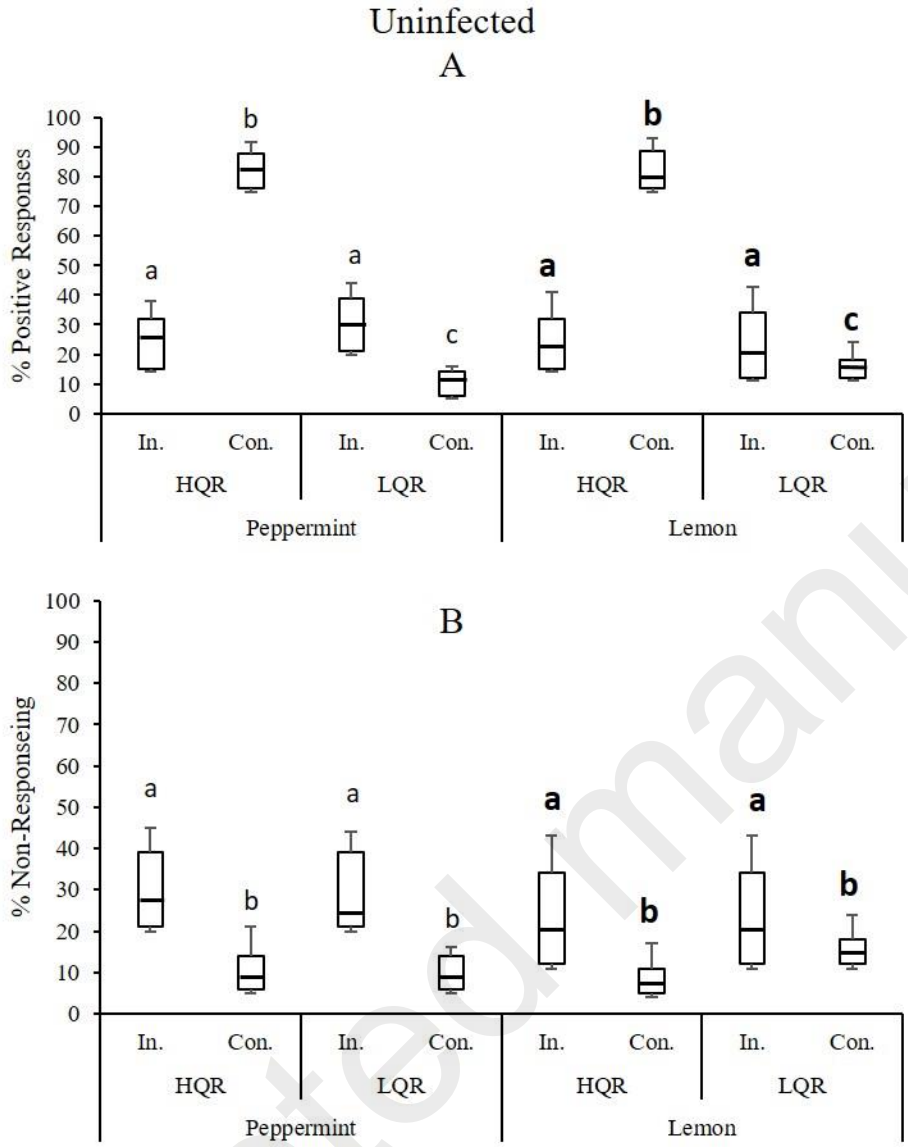

Figure 2

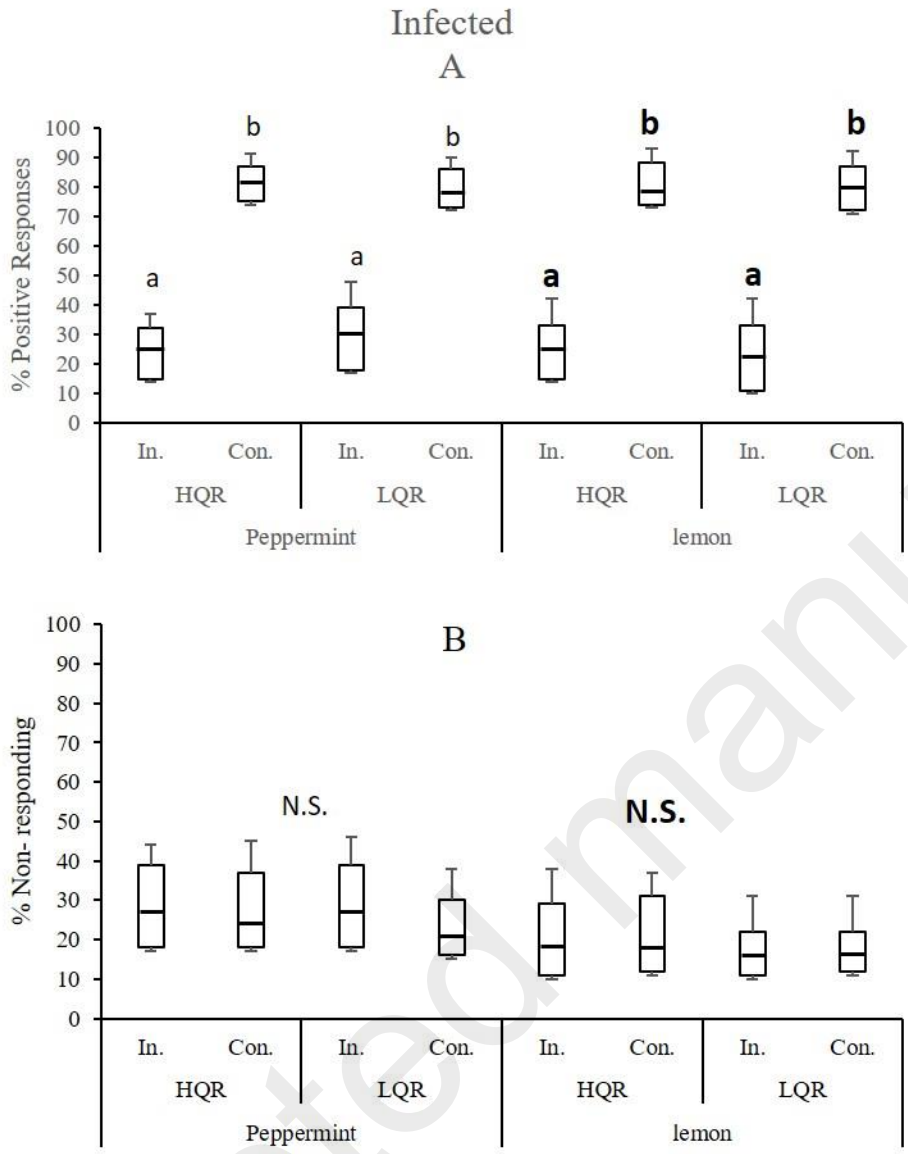

Figure 3

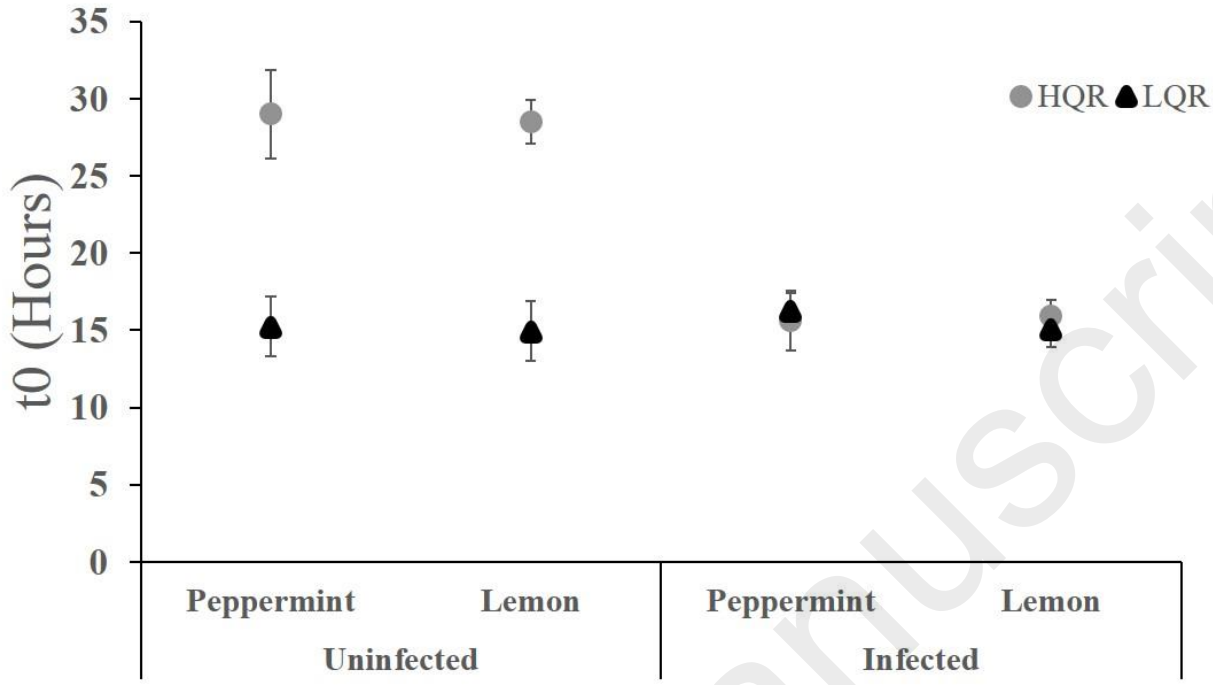

Figure 4