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Associative learning and memory duration of *Trichogramma brassicae*

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ABSTRACT

Learning ability and memory duration are two inseparable factors which can increase the efficiency of a living organism during its lifetime. *Trichgramma brassice* Bezdenko (Hym.: Trichogrammatidae) is a biological control agent widely used against different pest species. This research was conducted to study the olfactory associative learning ability and memory duration of *T. brassicae* under laboratory conditions. According to our results, *T. brassicae* showed olfactory learning ability in response to conditioned odors, and this learned olfactory stimuli lasted for 20 hours. In a second experiment, the effects of frequent experiences on the memory duration of females were studied. A direct relationship between frequent experiences and memory duration was observed. When exposed to a conditioned odor, wasps' memory duration increased in response to the number of experiences. Memory was observed at 28 h after one extra conditioning. The duration of the associative memory lasted 42 hours when 2 extra experiences were given, 50 hours after 3 experiences, and 58 hours after 4 extra conditioning experiences. Our results showed that *T. brassicae* can associate new odors to host existence, and they will show increased memory duration after multiple experiences.

Keywords: associative learning, memory duration, olfactory, Trichogramma brassicae.

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Introduction

Two of the most important behavioral aspects of a living organism are its learning ability and its memory duration. Learning is defined as changes in behavior that occur due to past experiences (1). Several studies have documented the learning observed in many invertebrate species, such as snails (2), the nematode *Caenorhabditis elegans* (3, 4), isopods (5), and several insect species. Among the insects, dipterans (5, 6), lepidopterans (7), and hymenopterans (8, 9) provide the best and oldest known examples of learning. However, learning has been studied in different genera from parastici wasp families, such as Agrochrysis, Asobara, Brachymeria, Bracon, Compolitis, Cotesia, Diaeretiella (10-23). Learning may lead to a number of changes in innate behavioral aspects of individuals, such as increased accuracy in stimuli selection related to a required source, modified preferences, and totally decreased mutability of a parasitoid's response to a cue (24, 19).

Different stimuli types including visual, olfactory, and tactile, even those that are ecologically relevant, can be learned by parasitoids as new stimuli (25, 26, 27). Novel learned stimuli enable parasitoids to focus on the most reliable cues (19, 23, 24) which can increase a female's efficiency in nature in finding the host habitat faster and saving energy by avoiding random searches for a host habitat.

The Trichogramma genus is a generalist egg parasitoid that primarily parasitizes Lepidopteran species (28, 29). *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) is a polyphagous parasitoid that parasitizes a wide range of lepidopterous hosts (30). It is being used globally as a biological control agent against its hosts. An estimated 32 million hectares were treated with *T. brassiace* and other parasitoids of the family Trichogramma in 1993 (31), whereas recent statistics show that 15 million hectares are currently being treated with Trichogramma (32, 33). In Iran, this parasitoid is the dominant Trichogramma species and has been reared and released for the biological control of some local key pests (34). Despite several studies on different biological features, the learning ability and memory duration of this species has not been studied well.

Identifying the potential of natural enemies to be control agents is the first step toward developing a biological control program. Because of the importance of this species in biological control programs and in order to learn how to increase female parasitoid efficiency in nature, this investigation was conducted to study the olfactory learning abilities and memory duration of *T. brassicae*.

Material and Methods

Host eggs

Eggs of *Ephestia kuehniella* Zeller (Lep.: Pyralidae) were used as hosts for all observations. 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 plastic containers (500 ml) to produce eggs. Eggs were collected daily to ensure that those used in the experiments were not more than 24 h old.

Parasitoids

The stock colony of wasps came from cultures maintained by the Biological Control Research Department (BCRD) of the Iranian

Research Institute of Plant Protection (IRIPP). The wasps were originally collected from northern Iran (Baboulsar region, south of the Caspian Sea) in 2012. The colonies were reared on *E. kuehniella* for more than 25 generations. 200 fresh host eggs (maximum 2 h old) were glued onto lengths of cardboard (1×5 cm) with a 10% honey solution. The cards were exposed to a 24-h-mated female in a glass vial (50 ml, 26×93 mm). 24 h later, they were removed and maintained in separate glass vials (50 ml, 26×93 mm) in incubators at 25±1ºC, 16L: 8 D and 50±5% RH. The glass vials were checked daily until wasps emerged.

Experiment Design

Conditioning

Sixty-five mated females (naïve and one day old before any host encounter experience) were exposed to host eggs (only 25 host eggs were glued onto cards). The wasps were maintained in vials for 30 minutes to gain oviposition experience. Females were given initial experience to avoid variability in sequence and duration of behavioral events associated with learning from the first hosts encountered. Insects were maintained in vials (2×10 cm) with 300 host eggs (one-day-old eggs, glued onto cardboard with a 10% honey solution). Vials were transferred to conditioning tanks (25×25×25 cm) for 4 hours. During this time, a peppermint odor (at least 97% pure) for half of the wasps and a lemon odor for the rest were pumped into the tanks at 1 m/s.

Flight chamber

The flight chamber used in the experiments was similar to the one previously described by Yong et al. with some modifications to the structure (35). Air was pumped through the main tunnel chamber (200×500×50 cm, l×w×h) by a fan at the upwind end and exhausted out by a fume hood at the downwind end. Wind speed in the wind tunnel was 0.5 m/s. Layers of netting between the fan and main chamber created laminar airflow. A smaller chamber (50×20×20 cm, l×w×h) was centered within the main chamber and opened at the upwind and downwind ends to serve as the experimental arena. The walls of the main chamber and experimental arena were clear acrylic. All flight responses were tested at 25°C, 50% RH, and a light intensity of 2000 lux. The schematic of the wind tunnel setup is shown in Figure 1.

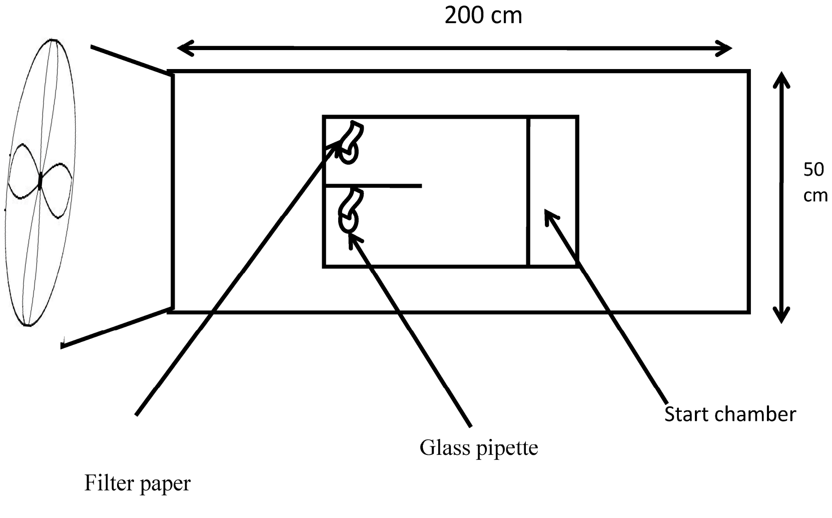

Figure 1. Schematic view of wind tunnel used for flight assays

Testing

Fifty wasps were selected from among the conditioned wasps to test in the flight chamber. Fifteen minutes after conditioning, the female wasps were introduced into the flight chamber using a 5 ml shell vial. One ul of each type of odor (peppermint and lemon) was placed on strips of filter paper (1 x 2 cm). These strips were attached to a glass pipette, placed vertically on a stand, and spaced 10 cm apart. The responses of wasps in the flight tunnel to the conditioned odors were observed over the course of 5 minutes. The number of landings on the target (peppermint) or alternate source (lemon odor) was recorded. Females that did not complete a flight or that did not take flight after 5 min were scored as no response.

Memory (retention) was defined as being present when wasps showed a significant preference for the previously associated odor. In order to determine the duration of memory, 50 experienced wasps were kept 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 24 h, 26 h, and 30 h after training until a bioassay was conducted for each time. All wasps (650 individuals) were kept at 25±1ºC, 50%±5 RH and 16:8 L: D and fed with 10% honey solution. Responses of wasps in the flight chamber from each period were observed as explained.

The wasps were conditioned consecutively to show how frequent experiences affect memory duration in females. Wasps were re-conditioned 4 hours after the first conditioning with 100 host eggs, and this conditioning period lasted 2 hours. Wasp responses were tested 15 minutes after the second conditioning. Four hours after the second conditioning, wasps were re-conditioned for 2 hours. This procedure was repeated to study the effects of 3 and 4 extra conditioning events. Between each trial, conditioned wasps were kept at 25±1ºC, 50%±5 RH and 16:8 L: D and fed with 10% honey solution. For each test, sixty-five one-day-old naive females of both strains were conditioned, and the responses of 50 wasps in the flight chamber were recorded as described. All flight bioassays were conducted under laboratory conditions at 25±1ºC, 50%±5 RH.

Statistical analysis

Differences in the total number of wasps that completed a flight to one odor within each two-choice experiment were analyzed by χ^2 statistics using SAS software, Proc Freq., (36). To test memory duration, responses of wasps in each period were recorded and analyzed by Chi-Square test. Whenever the difference was found to be significant, we concluded that a preference for the more frequently visited odor existed.

Results

As Figure 2 shows, no significant differences were observed in the wasps' innate preference for the peppermint or lemon odor ($\chi2$= 0.17, P= 0.914, N=50).

A significant difference was observed between the responses to the two odors ($\chi2$= 13.82, P= 0.001, N=50), with a greater preference for the previously-associated odor (Figure 2). This learned stimuli lasted 20 h ($\chi2$= 6.714, P= 0.0348, N=50). Results showed that memory was present 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 h after a conditioning event with host eggs in the parasitoid *T. brassicae.* Wasp responses during the period in which lasted are shown in Figure 3. However, at 24 h, 26 h, and 30 h after such a training event, this parasitoid did not associate the peppermint odor with the reward of parasitizing a host egg; i.e., the memory was no longer present. At this time a significant difference was observed between responses, but the proportion of neutral responses was not significantly different.

The wasps were conditioned consecutively to show how frequent experiences affect memory duration in females. Memory was observed at 28 h after one extra conditioning when wasps were conditioned 6 hours after the first conditioning procedure (χ^2=6.768, P=0.0339). The memory lasted 42 hours when 2 extra experiences were performed (χ^2=8.091, P=0.0175). The memory lasted for 50 h after 3 and 58 h after 4 extra conditioning experiences (χ_3^2=7.084, P_3 =0.029; χ_4^2=8.605, P_4 =0.0135). Response trends are shown in Figure 4.

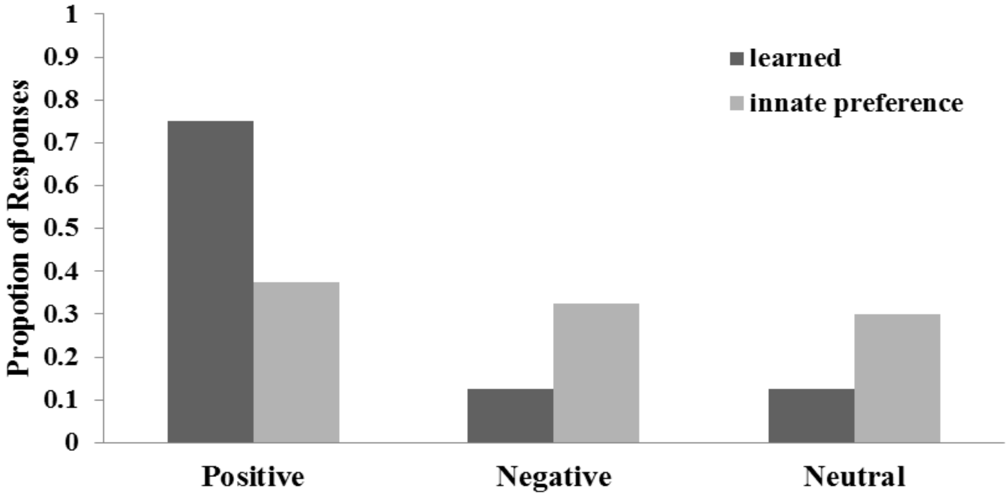

Figure 2. Proportion of innate and learned responses to tested odor by *T. brassicae* females

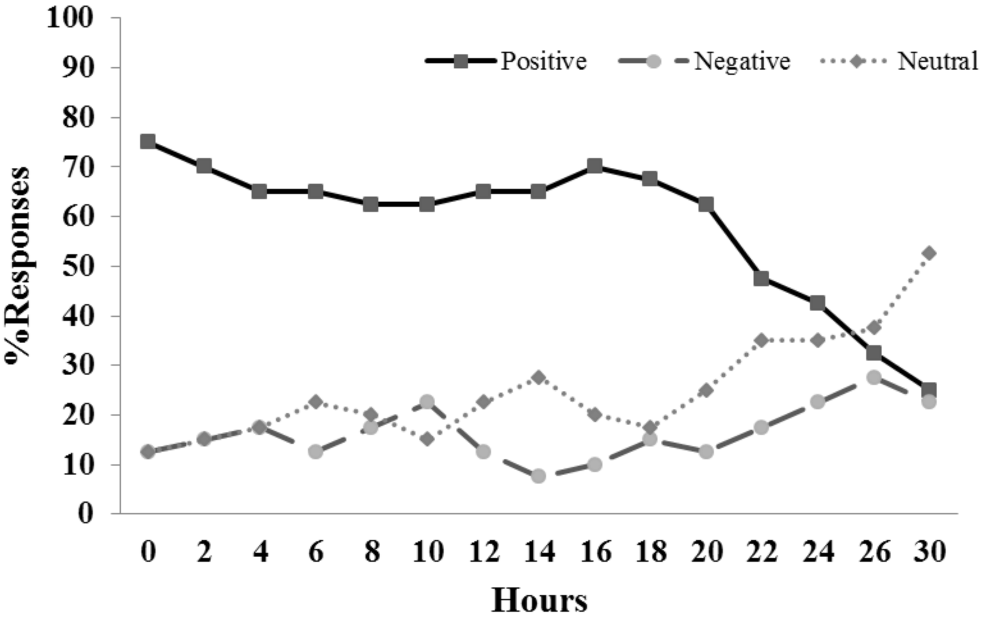

Figure 3. Response trend by *T. brassicae* females during period in which memory lasted.

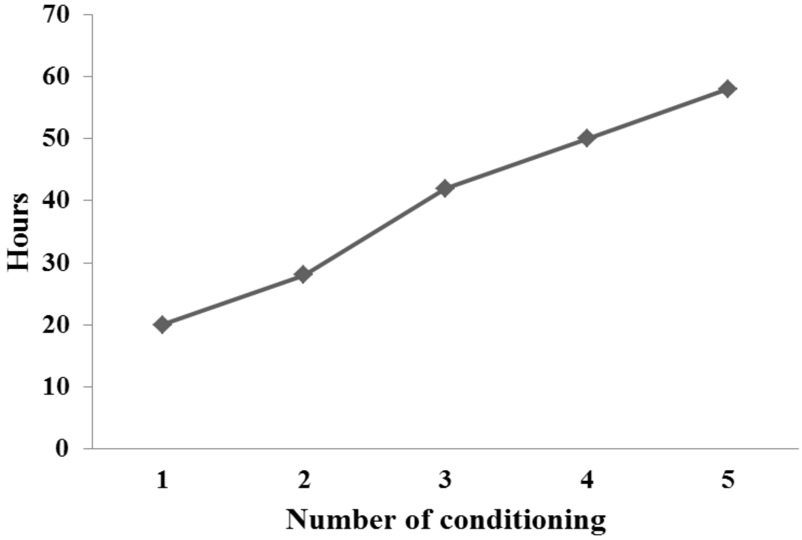

Figure 4. Wasps' memory duration in response to number of experiences

Discussion

According to our results, *T. brassicae* showed associative learning ability in response to conditioned odors. This learned olfactory stimuli lasted for 20 h. Females exhibited a direct relationship between frequent experiences and memory duration. Their memory duration increased in response to the number of experiences when they were exposed to conditioning odors.

Trichogramma wasps are tiny egg parasitoids of lepidopteran eggs which use host cues to find their hosts, and most of these wasps encounter various hosts (from different species) in various qualities in different environments. Therefore, during their lifespan, they parasitize different hosts. Thus, by learning reliable stimuli which guide them to find hosts of higher quality, they will increase their efficiency. *T. brassicae* is a generalist parasitoid which encounters a broad range of stimuli that are related to host existence. Generalist parasitoids usually must deal with wider stimuli types during habitat selection than do specialists, and thus, learning has been proposed as a more highly profitable strategy to find the most suitable habitat. It has been suggested that learning is especially more important for generalist species (37, 7). Several pieces of empirical evidence confirm this idea (38, 12). This mechanism would enable foraging parasitoids both to cope with the variability of either direct cues from the hosts or indirect cues produced by the host's food plant (20, 38, 21) and to adjust their response to changes in the abundance and quality of potential hosts (39, 7, 21).

We found that number of trials (experiences) is the dominant factor in memory duration of *T. brassiace*. In classical conditioning, conditioning trials that are repeated during an animal's lifetime produce better conditioning than trials that are repeated once (40, 41). There are a number of mechanisms that can contribute to the effect of number of experiences in classical conditioning (42). The acquisition of conditioned response depends on the animal deciding that the rate of reinforcement in the conditioned stimulus (CS) is higher than that in the background (43). To implement the idea, the animal is assumed to calculate the ratio of the estimated rate of reinforcement in the CS over the rate of reinforcement in the absence of the CS. Through repetition of learning events, the animal will be faced with the stimuli several times, which will introduce to them the importance of the conditioned stimuli. Higher rates of encounter with CS can show the importance of this stimulus to the animal; thus, it will be considered a high value stimuli in the animal's life. According to our results, *T. brassicae* showed a positive response to repeated conditioning and increased memory duration. When females were exposed to frequent experiences, they showed increased memory duration, lasting for 58 h. This amount of time can help females search for hosts more reliably and save valuable energy and time.

Memory duration and learning are two entirely interdependent components. Memory acts as a safe in which learned information is stored. It will be affected by type and number of conditioning events and experiences. Memory type is classified into several forms based on the time that learned information can be recalled in the animal kingdom. After one single training, short-term memory (STM, also called anaesthesia-sensitive memory (ASM)) will be obtained. STM lasts only a few hours, is unstable, and will quickly be disrupted by other factors such as an application of anaesthesia shortly after learning (44). More durable memory forms of stabilized memory which are resistant to anaesthesia are generally organized after several training events in separated times (spaced training). After several consecutive experiences, LTM (long term memory) must generally be formed, but some parasitoid wasps form LTM after only one learning event (45, 46). *Cotesia glomerata* showed LTM after one single parasitism on its preferred host, *Pieris brassicae,* whereas *C. rubecula,* a closely related parasitoid, showed LTM only after several spaced learning events with its solitary host, *P. rapae* (46). Other species of Trichogramma genus, like *T. evanescens*, despite their extremely small brain, showed LTM which was present 24 h after one single encounter with a hitch-hiking and oviposition experience (46, 47). According to our results, *T.brassicae* showed durable memory after just one training event, and it lasted for 20 hours after conditioning.

As our results show, *T. brassicae* showed long term memory and a positive relation to frequent experiences. This associative learning ability may enable females to show more efficiency in the field in host-seeking processes. As this species is a generalist parasitoid, learning will play an important role in its efficiency in mass release biological control programs. However, more studies must be conducted on its learning ability in the wild to show how this parasitoid will change its innate host-seeking behaviors and its total parasitism efficiency.

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