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Automated high-throughput individual tracking system for insect behavior: Applications on memory retention in parasitic wasps

Jessica A.C. de Bruijn^{a,*}, Louise E.M. Vet^b, Maarten A. Jongasma^c, Hans M. Smid^a

^a Laboratory of Entomology, Plant Sciences Group, Wageningen University, Wageningen, the Netherlands

^b Department of Terrestrial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands

^c Plant Research International, Wageningen University, Wageningen, the Netherlands

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ABSTRACT

Background: Insects are important models to study learning and memory formation in both an ecological and neuroscience context due to their small size, behavioral flexibility and ecological diversity. Measuring memory retention is often done through simple time-consuming set-ups, producing only a single parameter for conditioned behavior. We wished to obtain higher sample sizes with fewer individuals to measure olfactory memory retention more efficiently.

New method: The high-throughput individual T-maze uses commercially available tracking software, Ethovision XT[®], in combination with a Perspex stack of plates as small as 18 × 18 cm, which accommodates 36 olfactory T-mazes, where each individual wasp could choose between two artificial odors. Various behavioral parameters, relevant to memory retention, were acquired in this set-up; first choice, residence time, giving up time and zone entries. From these parameters a performance index was calculated as a measure of memory retention. Groups of 36 wasps were simultaneously tested within minutes, resulting in efficient acquisition of sufficiently high sample sizes.

Results: This system was tested with two very different parasitic wasp species, the larval parasitoid *Cotesia glomerata* and the pupal parasitoid *Nasonia vitripennis*, and has proven to be highly suitable for testing memory retention in both these species.

Comparison with existing methods: Unlike other bioassays, this system allows for both high-throughput and recording of detailed individual behavior.

Conclusions: The high-throughput individual T-maze provides us with a standardized high-throughput, labor-efficient and cost-effective method to test various kinds of behavior, offering excellent opportunities for comparative studies of various aspects of insect behavior.

1. Introduction

Learning and memory formation are universal traits in the Animal Kingdom (Dubnau, 2003), which makes it possible to study them in a wide range of animal species with varying levels of brain complexity, including insects, such as fruit flies (*Drosophila melanogaster*), bees (*Apis mellifera*) and parasitic wasps (Chen and Tonegawa, 1997; Galizia et al., 2011; Margulies et al., 2005; Smid et al., 2007). For ecological and neuroscience studies insects are ideal models due to their small size, behavioral flexibility and enormous ecological diversity.

Memory retention is an important parameter in studies of learning and memory formation, and it is generally assessed by measuring conditioned behavior. Many different bioassays have been used to study memory retention in insects such as the proboscis extension reflex

(Bitterman et al., 1983), the two-choice wind tunnel (Geervliet et al., 1998b), the Y-tube olfactometer (Wäckers 1994), the static two-chamber olfactometer (Huigens et al., 2009), the four-quadrant olfactometer (Vet et al., 1983), the locomotion compensator (servosphere) (Vet and Papaj, 1992) and the T-maze olfactometer (Hoedjes et al., 2012; Jiang et al., 2016). These bioassays measure memory retention through conditioned behavior in different ways and each has its own strengths and weaknesses. The two-choice wind tunnel, the four-quadrant olfactometer and servosphere bioassays allow for detailed recording of biologically relevant behavioral responses of individual insects, but are time consuming. Wind tunnels also require expensive equipment and ample space. The T-maze olfactometer is used with groups of insects, which is more time efficient, but data points are formed per group and therefore many conditioned animals are required

* Corresponding author at: Laboratory of Entomology, Plant Sciences Group, Wageningen University, PO Box 8031, 6700 EH Wageningen, the Netherlands.
E-mail address: jessica.debruijn@wur.nl (J.A.C. de Bruijn).

per experiment to obtain sufficient sample sizes. Furthermore, information on different parameters of individual behavior are not recorded (Lin et al., 2015) and social behavior may affect the observed behavioral response (Kohn et al., 2013).

A bioassay consisting of a video setup with automated tracking software and a well-designed test system can solve several of the above described drawbacks. Automated tracking software allows for detailed recording of many behavioral parameters and has already been used in several studies, but generally only with recordings of a single individual or with group release where individual identities are lost (Beshel and Zhong, 2013; Faucher et al., 2006; Lin et al., 2015; Reza et al., 2013; Smith and Raine, 2014; Spitzen et al., 2013). Recently, further technological advancements in studies on insect behavior have been realized with video tracking software, where the behavior of individual insects in multiple arenas are simultaneously recorded, allowing for both detailed individual behavioral recording and high-throughput (Kloth et al., 2015; Thoen et al., 2016).

In this study a novel bio-assay was designed for memory retention testing in parasitic wasps. This setup consists of a block with 36 individual olfactory T-maze arena's in combination with a video setup and tracking software, and allows for simultaneous automated behavioral tracking of 36 individual wasps. We used complex, commercially available odor extracts and compared the sensitivity of the wasps for these odors using the electro-antennogram technique. To test this novel bioassay, we used two unrelated and ecologically different parasitic wasp species, *Cotesia glomerata* and *Nasonia vitripennis*. *Cotesia glomerata* (Braconidae: Microgastrinae) is a parasitic wasp that lays her eggs in first instar caterpillars of Pieridae butterflies. It forms long term memory (LTM) for specific host-plant odors when they are rewarded with an oviposition in a caterpillar of the large cabbage white butterfly, *Pieris brassicae*, on that plant (Smid et al., 2007). The jewel wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae) lays her eggs in pupae of several fly species. It forms LTM for natural odor extracts after a single oviposition experience in a pupa of the bluebottle blowfly, *Calliphora vomitoria* (Hoedjes and Smid, 2014). To optimize the bioassay for use with these species, sensitivity, preference and memory retention experiments were conducted. The combined results suggest this system can be used for a broad range of parasitic wasp species and may be further extended to include many more insect species and research fields.

2. Materials and methods

2.1. Insect cultures

Cotesia glomerata (Hymenoptera: Braconidae) females were obtained from a colony which is re-established each year from individuals collected from cabbage fields around Wageningen, The Netherlands. Wasps were reared on *Pieris brassicae* L. (Lepidoptera: Pieridae) caterpillars, which in turn were reared on cabbage plants (*Brassica oleracea*) as described in Geervliet et al. (1998a). Parasitoid cocoons from this rearing were placed in cages (40 × 30 × 30 cm) in a climate chamber (20–22 °C, 50–70% relative humidity, photoperiod L16:D8) where wasps were supplied with honey and water. From these cages, two-day-old female wasps were collected and placed in a separate cage with water and honey until experiments started. Female wasps of 3–5 days old were used in all experiments.

Nasonia vitripennis wasps (strain AsymCx) were reared as described in Hoedjes et al. (2012), in polystyrene rearing vials (28.5 × 95 mm) with foam stoppers (Genesee Scientific, San Diego, CA, USA). Wasps were kept in a climate chamber at 25 °C, 50–70 % relative humidity, with a photoperiod of L16:D8. Pupae of the fly species *Calliphora vomitoria* (Kreikamp, Hoevelaken, the Netherlands) were used as host, as described in Hoedjes et al. (2012). Female wasps were fed honey and water and used at 2–4 days after emergence.

2.2. Odors used for conditioning and memory retention testing

Four different commercially available, complex odor blends for this study: 2x Royal Brand bourbon Vanilla extract, Natural Chocolate extract, Pure Coffee extract, and Natural Almond extract (Nielsen-Massay Vanillas Intl., Leeuwarden, the Netherlands). The choice for these odors was based on earlier studies on *Nasonia* learning and memory (e.g. Hoedjes et al., (2012, 2014, 2015); Liefing et al. (2018); van der Woude et al. (2018)). These blends were chosen, since they were not expected to evoke high innate responses to the wasps, as they are not present at host or food sites, but, since they are composed of many different odorants, are also unlikely to remain undetected. Odor detection was previously confirmed for *N. vitripennis* using electroantennogram (EAG) analysis (Hoedjes et al., 2012), showing that at the antennal level, these odors showed doses-dependent responses. For *C. glomerata*, such EAG experiments were performed in this study. For behavioral bioassays, concentrations of these odors could be fine-tuned to obtain a 50%-50% choice from unexperienced wasps in a T-maze and clear-cut conditioned responses to each side of the T-maze (Hoedjes et al., 2012). The additional advantage of using odor blends, which are unrelated to the biology of the wasps, is that such odors provide the best opportunity to get unbiased results in memory studies, where different species are compared.

2.3. Electroantennogram analysis for *C. glomerata*

An electroantennogram (EAG) analysis was conducted to assess the sensitivity to several complex natural odor blends at the level of the olfactory sensilla on the antenna, because potential differences could affect the detection of memory retention in subsequent experiments.

The EAG setup was adapted from Hoedjes et al. (2012), and based on a commercially available set-up from Syntech, Hilversum, The Netherlands. We performed EAG analysis with commercially available odor blends. The odor extracts were dissolved in a 50 ml 4% agarose (A9539-500 g, Sigma) solution in deionized water, at odor concentrations of 1%, 4%, 16% and 64%. Odor blends were heated to 80 °C in a water bath and were then added to the agarose solution at the same temperature, and mixed with a magnetic stirrer. The control agarose solution was made without odor extract. Solutions were poured on a flat plastic sheet (OHP Transparency film, Nobo ACCO Brands Cooperation, England). The agarose was allowed to spread out on the sheet, to level out and dry for 30 min. Strips of 40 × 5 × 2 mm agarose were cut from the center of the dried agarose solutions and a strip was placed against the inner wall of a Pasteur pipette, where it would not block the airflow. Pasteur pipettes were subsequently sealed with parafilm until the start of the EAG analysis. Just before the start of the experiment the Pasteur pipettes were flushed with 250 ml of clean air to standardize odor release.

Unconditioned *C. glomerata* females were anaesthetized by putting them briefly on ice, after which they were decapitated and the last segment of one of the antennae was cut off. The base of the head was connected to the ground electrode of the EAG setup and the cut antenna to the recording electrode. We used 4% almond as a standard odor and corrected with the unscented control agarose to calculate relative EAG responses as described in Hoedjes et al. (2012).

2.4. Conditioning procedures

2.4.1. *Cotesia glomerata*

Female wasps were given an associative learning experience using a classical conditioning procedure, adapted from Bleeker et al. (2006). In the original procedure, wasps learned to associate plant odors as the conditioned stimulus (CS) with suitable hosts as the unconditioned stimulus (US), after a single oviposition experience with a caterpillar on a plant leaf. This type of conditioning is considered a form of classical (Pavlovian) conditioning, where the host-searching phase is excluded.

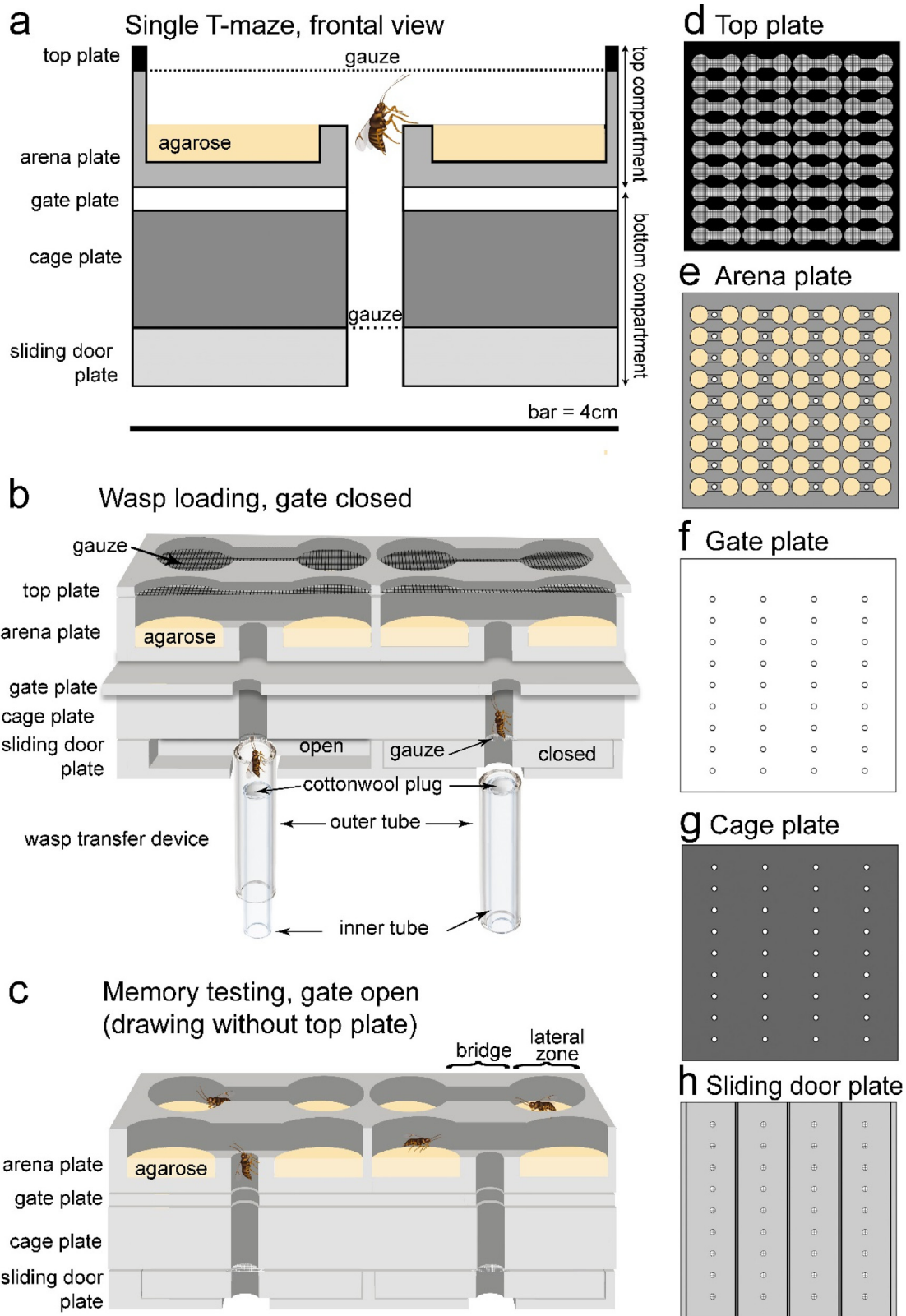


Fig. 1. Drawings of the T-maze set up. All elements are labeled in the figures. One T-maze consisted of multiple layers of Perspex plates. The bottom compartment consisted of the bottom sliding door plate and the cage plate. Above the bottom compartment was the gate plate. The top compartment consisted of the arena plate with the agarose zones and the top plate (a) Cross section of one single T-maze, frontal view, out of the complete block of 36. This drawing has been made to scale (scale bar = 4 cm). The odorized agarose is colored yellow, the other elements have different shades of grey to distinguish them, but all plates are of the same transparent Perspex material. (b) Drawing in perspective of a set of 4 arena's and connected cages, in wasp loading position, with the gate closed. The left row has the bottom sliding door opened, and a wasp can be loaded from the bottom using the wasp transfer device. The right row has the bottom sliding door closed, and the wasp is in the cage compartment. (c) After loading of all wasps, the gate is opened allowing the wasps to enter the arenas from their cages and start exploring the two fields of odorized agarose. (d–h) The 5 different plates of Perspex that together form the block with 36 T-mazes, from top to bottom. The grey shades correspond to those used in Fig. 1a (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Smid et al. (2007) later found that his single trial conditioning method resulted in the formation of robust, protein synthesis-dependent long-term memory (LTM). In the current study, odorized agarose was used as CS, instead of plant leaves, on which caterpillars and so-called frass (feces and silk produced by the feeding caterpillars) were placed. The agarose odorized with vanilla or coffee extract was made as described above at a 4% odor concentration. A globular sphere of odorized agarose was made by dipping the cone of a micro pestle (SIAL501ZZ0, Sigma-Aldrich) 5 times in the odorized agarose solution at intervals of 30 s, resulting in a globular shaped substrate (15 mm diameter) on a stick. The agarose was allowed to cool at room temperature for 30 min after which 150–200 first instar caterpillars and frass were placed on top of the agarose.

Two hours before conditioning, wasps were retrieved from the climate cabinet and placed in the laboratory to acclimatize. For conditioning, 15 wasps were individually transferred to polystyrene rearing vials and sequentially offered the odorized agarose stick with the caterpillars to allow each wasp a single oviposition in a host. Upon offering the stick with odorized agarose, caterpillars and frass, wasps initiated oviposition immediately and a single oviposition was generally completed within 30 s. After oviposition wasps were captured by letting them walk into a clean vial. The wasps were then transferred to a small cage (Dimensions 17 × 17 × 17 cm, Bugdorm type 41515, Megaview Science, Taiwan) with water and honey and kept in a climate chamber until testing 24 h later. Wasps were reciprocally conditioned with two different odors for memory retention experiments: 15 wasps were given an oviposition experience on vanilla scented agarose and 15 wasps on coffee scented agarose.

2.4.2. *Nasonia vitripennis*

Conditioning trials for *N. vitripennis* were done as described in Hoedjes et al. (2012). This conditioning procedure is known to induce protein synthesis-dependent LTM in this strain (Hoedjes and Smid, 2014). Coffee and vanilla extracts were used to train and test wasps. Wasps were given an associative learning experience with a reciprocal, differential classical conditioning procedure, where half of a group of wasps was first given an associative learning experience with vanilla odor and a host (CS+), after which it was exposed to coffee odor without a host (CS-). The other reciprocal half of the group was conditioned with the same odors, but in opposite order, so coffee odor as CS+ and vanilla odor as CS-. The associative learning experience was conducted by placing wasps individually in a well of a 12-well microtiter plate (Greiner Bio-One, Alphen aan den Rijn, the Netherlands), each well containing two *Calliphora vomitoria* pupae and a piece of filter paper (0.75 cm²) with 1 µl pure vanilla or coffee extract. During a 1 h period the wasp would drill and host feed while experiencing the odor to form the association. A group of 12 wasps was given this experience individually. Actual oviposition does not take place with this conditioning protocol, but previous experiments have shown that deposition of eggs is not required to form LTM (Hoedjes and Smid, 2014). Wasps that did not start drilling within the first 30 min of conditioning were excluded from experiments. After the CS+ experience, wasps were transferred to a polystyrene vial for 15 min. They were then exposed to the CS- for 15 min. Here, the odor was offered in the form of a glass capillary (ID 1.3 mm, cut to 30 mm; Fisher Emergo, Landsmeer, the Netherlands) filled with pure odor extract and covered at one end with pure petroleum jelly (Vaseline original, Unilever Nederland B.V., Rotterdam). This entire procedure was defined as one single conditioning trial and lasted 90 min. This difference in duration of a conditioning trial with *C. glomerata* reflects the difference in duration of the oviposition behavior between the two species. After conditioning, wasps were transferred to a clean polystyrene vial with honey and water and placed back in the climate chamber until testing the following day.

2.5. Memory retention bioassays

2.5.1. High-throughput individual T-maze design

The high-throughput individual T-maze design is based on the video tracking setup described in Thoen et al. (2016) and Thoen (2016) for thrips, which is here redesigned for use with parasitic wasps. The system consisted of a stack of five Perspex plates with dimensions of 180 × 180 mm and thickness of 2, 5 or 10 mm (PyraSied BV., Leeuwarden, The Netherlands). In these plates different openings were made, using a computer guided laser cutting machine (BRM 6090 laser machine, BRM Lasers, Winterswijk, the Netherlands). Together, they formed 36 T-maze arenas for individual testing of 36 wasps simultaneously (Fig. 1).

The different layers of transparent Perspex plates were divided into two compartments (Fig. 1). The bottom compartment served as 36 cages to load and hold 36 wasps (Fig. 1b) until their release at the start of the experiment, whereas the top compartment consisted of the actual T-maze arenas situated directly above each of the 36 cages. The bottom compartment could be closed or opened towards the top compartment by a gate plate, to allow for simultaneous release of wasps from the cages into the T-maze arenas (Fig. 1b, c).

The bottom compartment with the cages and the gate was formed by four layers, from top to bottom: one gate plate (195 × 180 × 2 mm) with 36 circular holes of 5 mm diameter (Fig. 1f). By sliding this plate back- or forwards, the holes in this plate could be aligned (Fig. 1c) or closed (Fig. 1b), thereby opening or closing the connection between the cages and arenas. The second plate (180 × 180 × 10 mm) formed the actual cages, with 36 5 mm cylindrical openings where wasps were trapped until testing commenced (Fig. 1g).

Below this second layer was a third layer, the bottom sliding door plate, which consisted of four slides (180 × 41 × 5 mm) which could move on a Perspex plate of 180 × 180 × 2 mm (Fig. 1h). To allow free movement of these slides, the bottom plate had two 180 × 5 × 5 mm Perspex pieces glued on the left and right sides and 180 × 2 × 5 mm spacers glued between individual slides. In the center of the slide opening of the bottom plate, four longitudinal slits of 160 × 10 mm were made to allow access to the slides from the bottom. Each slide had nine holes, positioned directly underneath the cage cells, and were covered on the top with gauze (Monodur, PA 250; Nedfilter b.v., Almere, the Netherlands) for bottom ventilation of the cells. The slides allow for opening (Fig. 1b, left) and closing (Fig. 1b, right) of each consecutive cell by sliding them backwards or forward while loading wasps from below directly into the cage cells thereby using the natural, negative geotaxis of the wasps.

Above the bottom compartment (cage and gate) is the top compartment, which consisted of the arena plate and the top plate. The arena plate (180 × 180 × 10 mm) consisted of 36 two-choice arenas (Fig. 1e). Each arena was made of two circular lateral zones of 15 mm across and 8 mm deep, connected by a bridge (10 × 8 × 5 mm) (Fig. 1a, c). The bridge is 3 mm higher than the lateral zones so that each lateral zone could be filled with a 3 mm (odorized) agarose layer. After application of the agarose layer, the bridge and lateral zones are at equal level (Fig. 1a). In the middle of the bridge, at equal distance to each of the lateral zones, a 5 × 5 mm circular opening was made in line with the cages to allow wasps to enter the arena, when the gate is aligned with that opening (Fig. 1c). The system was closed with a top plate (180 × 180 × 2 mm) where the area above each arena was cut out and covered with gauze for ventilation (Fig. 1d). The stack with all plates was aligned and kept together in a holder with an opening of 180 × 180 × 24 mm to prevent movement of plates and ensure exact alignment of the 5 mm openings of the cage, gate and central opening of the arenas through which wasps could walk.

For *N. vitripennis* a prior model of the high-throughput individual T-maze was used, where the top plate (Fig. 1d) had no opening for ventilation, where wasps were loaded from the top into the bottom compartment (the cage, Fig. 1b) instead of from the bottom and only 32

instead of 36 could be loaded in the system. Furthermore, the central circular opening in the bridge of the arena was 6 mm instead of 5 mm (Fig. 1d). The design of the arenas was exactly the same.

2.5.2. Use of the high-throughput individual T-maze

Before experiments, odorized agarose solutions were prepared and 0.5 ml was pipetted into the lateral zones of each arena after which it was left to dry at room temperature for 30 min. Odorized agarose was prepared with either vanilla, chocolate or coffee extract at different concentrations (0.5, 1, 2 and 4%) or control agarose, where no odor was dissolved in the agarose. Combinations of two odor pairs in different concentrations were used according to results obtained with un-experienced and experienced wasps as described in Sections 3.2 and 3.3. The lateral zones of each arena were always filled with two different odor solutions to present a two-choice situation. The location of a specific odor was alternated in every other arena. Once the agarose had dried, 36 wasps were taken from their cage using a transfer device (Fig. 1b). This transfer device consisted of an outer glass tube (outer diameter 8 mm, inner diameter 6 mm, length 6 cm) in which an inner tube capped with cotton wool was placed (outer diameter 5 mm, inner diameter 4 mm length 6.5 cm). With this device wasps could gently be pushed forward out of the transfer device and loaded into the bottom compartment. Hereafter the high-throughput individual T-maze was placed underneath the camera setup.

Upon opening of the gate of the system, to allow the simultaneous release of the wasps into the two-choice arenas, behavior was recorded for 10 min. Per recording 36 *C. glomerata* wasps, 12 wasps per treatment, were tested. For *N. vitripennis* groups of 29–32 vanilla or coffee conditioned wasps were tested.

All experiments were repeated on at least three different days, and treatment groups were loaded in a single plate in a randomized block design for *C. glomerata*. After testing, agarose was removed and plates were cleaned with soap (Bosmanite AL-42, Rogier Bosman Chemie B.V., Dinteloord, the Netherlands) and warm water.

2.5.3. Camera setup

The complete high-throughput individual T-maze was placed on a backlight (FL tubes, 5000 K) on 15 mm spacers, in a camera setup (Fig. 2), which consisted of a digital camera (GigE, Basler acA2040-25-gc) with a varifocal lens (Kowa LM35HC 1" 35 mm F1.4 manual iris c-mount). The entire setup was shielded from daylight during recording by a black curtain with a white inner liner facing the setup. Behavior in the high-throughput individual T-maze was recorded using Debut Video Capture Software (v 1.88, © NCH Software) at 2046 × 2046 pixel resolution, a frame rate of 12.76 fps and. mp4 file format.

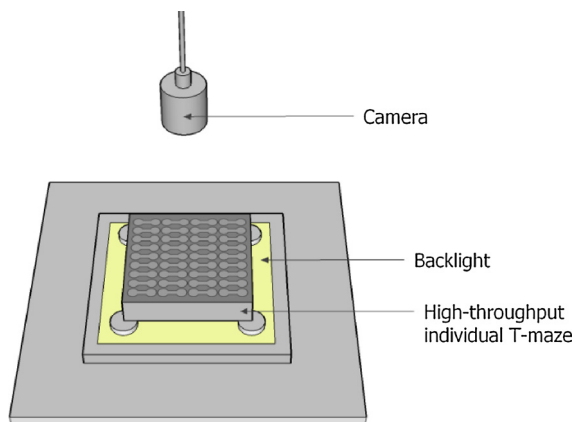


Fig. 2. The camera setup with the high-throughput individual T-maze in place. The high-throughput individual T-maze is placed on top of a backlight. The camera was positioned directly above the center of the bioassay for an optimal view of all arenas.

2.5.4. Video analysis

Video recordings were analyzed with EthoVision[®] XT version 11.5 (Noldus Information Technology B.V., Wageningen, The Netherlands). Each arena was defined in EthoVision as consisting of 3 zones, two lateral zones in which the two odor sources were present, and a neutral zone, which consisted of the bridge and entry hole. Walking behavior of the individual wasps was tracked using Ethovision's differencing method at a detection sensitivity value of 13. Wasps were not tracked when in the bridge zone or when their velocity dropped below 0.21 cm/s, and tracking started again above 0.25 cm/s. Tracking started once a wasp entered one of the lateral zones and paused when the wasp either stopped moving, or when it was present in the neutral zone. Behavior was recorded until the total time spent moving in the lateral zones accumulated to 30 s. From the Ethovision[®] XT data output the following behavioral parameters were used; latency until first zone entry, latency until first zone exit (zone alteration), residence time and frequency of zone entry. Latency until first zone entry consisted of the time from wasp release, till its first entry in the lateral zone. Latency until first zone exit, defined as zone alteration in Ethovision[®] XT, consisted of the time from wasp release till the first time it exited a lateral zone. Residence time was defined as the total time a wasp spent moving in a lateral zone. Frequency of zone entry consisted of the number of times a wasp entered a lateral zone in the total recorded time. With this data we created the behavioral parameters first choice and giving-up time. First choice was determined by selecting the zone with the lowest latency until first zone entry. Giving-up time was determined by subtracting latency until first zone entry from latency until first zone exit (zone alteration). Residence time and zone entries (frequency of zone entry data) were used directly from Ethovision[®] XT. Wasps that did enter a lateral zone, but did not have 30 s of movement in the lateral zones in the 10 min recording, were only included in the analysis of first choice data. Their data for the other parameters was discarded.

2.5.5. T-maze for group testing

In order to compare the results obtained from high-throughput individual T-maze for memory retention in *N. vitripennis* with the previously used T-maze for groups (Hoedjes et al., 2012), we compared the two methods, following the same protocol and set-up as used by Hoedjes et al. (2012). Briefly, the T-maze consisted of three Plexiglas tubes, a central tube with a small opening in which the wasps were introduced and two lateral tubes through which an airflow of 100 ml/min was blown towards the central tube, where it could leave the system through ventilation slits covered by gauze. Odor was provided by placing two capillaries filled with either pure vanilla or coffee odor extract, in the airflow lateral to each arm of the T-maze. Groups of 9–12 wasps were released in the central tube and after 10 min the final choice was recorded by counting the number of wasps in each lateral tube. Wasps that did not make a choice, by remaining in the central tube, were regarded as non-responding. A total of 12 groups was tested for memory retention, 6 groups with vanilla as CS+ and 6 with coffee as CS+. Note that the final choice behavioral parameter, which was obtained from this bio-assay, cannot directly be compared with the first choice parameter measured in the high-throughput individual T-maze, since we only used the choice after 10 min. Furthermore, the size dimensions of the T-maze for group testing are much larger, and as a consequence, wasps are expected to switch between the two odors at a much lower frequency than in the high-throughput individual T-maze. Thus, final choice in the T-maze for group testing, as recorded after 10 min, may not necessarily be the first choice, but rather results from both choice behavior, residence time and patch leaving tendency in the two lateral tubes of the T-maze.

2.6. Data analysis

For *C. glomerata* the relative EAG responses were analyzed by a two-way ANOVA using SPSS, version 23 (IBM, Armonk, NY, USA), to test for

differences in EAG response between the four odors and for concentration effects. Normality and equal variance assumptions were checked with normality and residual plots, after which pairwise comparisons were made using a Tukey's LSD.

First choice results of the odor preference experiment were statistically analyzed using a binomial test. For memory retention testing in the high-throughput individual T-maze, Performance index (PI) scores of all four behavioral parameters were based on two wasps, one CS1 + (conditioned with odor 1, vanilla) wasp and one CS2 + (conditioned with odor 2, coffee) wasp. Their scores were combined to form one PI, as described below. The two corresponding wasps that contribute to one PI score were tested in a two-choice arena at the same position in plates analyzed directly after each other. No PI score was formed if one of the two wasps did not respond during recording. In case of the binomial first choice results, the PI was calculated for each wasp pair as 100 if both the CS1 + wasp would first enter the conditioned agarose zone with odor 1 and the CS2 + wasp first entered the zone with odor 2. If one of the two wasps entered the alternative zone first, then the PI would be 0, if both would enter the alternative zone first, the PI was -100. No PI score was formed if one of the two wasps did not respond during recording. For giving up time and total residence time, PI scores were calculated per wasp pair by subtracting the percentage of active searching time that the CS2+ wasp spent on the CS1 zone from the percentage of time the CS1 + wasp spent on the CS1 zone (PI = % time CS1 + wasp on CS1 - % time CS2+ wasp on CS1). The same was done for zone entry data, but here instead of the percentage of time, the percentage of visits to either zone was used (PI = % visits CS1 + wasp to CS1 - % visits CS2+ wasp to CS1). All datasets from these calculations consisted of PI values ranging between -100 to 100, where a value of -100 represented a negative effect of conditioning, 0 represented no effect of conditioning and 100 a maximum effect of conditioning. More details on how these PI scores were calculated can be found in the supplementary information. Since not all datasets were normally distributed, all average PI scores were statistically analyzed with a one-sample Wilcoxon's signed rank test, to test if their values were significantly higher than 0, which would indicate memory retention (Hoedjes et al., 2012).

For the T-maze for group testing, we used performance index (PI) scores for memory retention experiments as described in Hoedjes et al. (2012), with data of reciprocally tested groups. One group was given a conditioning trial in combination with odor 1 as CS (CS1 + wasps), the other with odor 2 as CS (CS2 + wasps). After testing, the percentage of CS2+ wasps that had chosen odor 1 was subtracted from the percentage of CS1 + wasps that had chosen odor 1. These PI scores were also statistically analyzed with a one-sample Wilcoxon's signed rank test. In all cases, we used an alpha value of 0.05 as cut-off for significance.

3. Results

3.1. Electroantennogram recordings of *C. glomerata*

EAG analysis (Fig. 3) showed a significant effect of odor, odor concentration and the interaction between odor and odor concentration (odor: $F_{3,256} = 110.612, P < 0.001$; concentration: $F_{3,256} = 87.678, P = 0.000$; odor \times concentration: $F_{9,256} = 24.273, P = 0.000$). Pairwise comparisons show that wasps were more sensitive to almond than to any of the other odors (Tukey's LSD, $P < 0.001$, Table 1). Sensitivity to vanilla, chocolate and coffee was not different. Due to the high sensitivity to the almond extract, this odor was not selected for further experiments.

3.2. Odor preference of *C. glomerata*

The selection of the odor pair for conditioning of *C. glomerata* was based on odor preference of unconditioned wasps (Fig. 4). First, we tested the preference for each type of 1% odorized agarose vs.

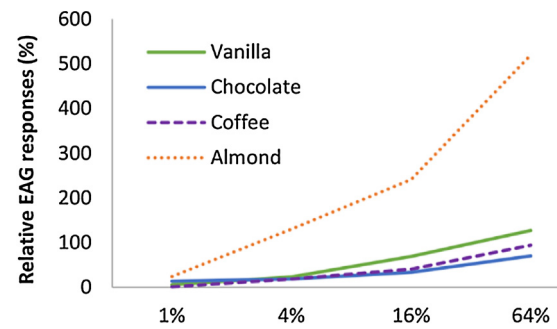


Fig. 3. Relative EAG responses of *C. glomerata* with various concentrations of vanilla, chocolate, coffee and almond odors. Results were calculated by using 4% almond odor as a standard and by correcting with control odor results. There was a significant effect of both odor and concentration and their interaction, with sensitivity to almond being significantly different from vanilla, chocolate and coffee.

Table 1

Pairwise comparison results of the different odors of the EAG of *C. glomerata*. Since a significant effect of odor was found in the EAG experiment with *C. glomerata*, the various odors were compared to find out which odors differed from each other.

Odor 1	Odor 2	P-value
Almond	Chocolate	< 0.001
	Coffee	< 0.001
	Vanilla	< 0.001
Chocolate	Coffee	0.720
	Vanilla	0.078
Coffee	Vanilla	0.160

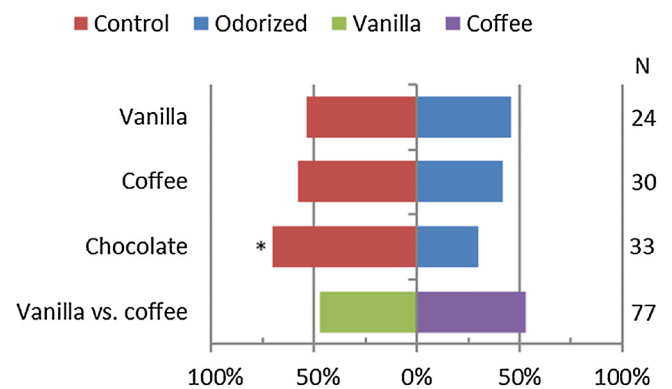


Fig. 4. First choice results of unconditioned *C. glomerata* wasps with differently odorized agarose. Wasps were either tested with 1% odorized vs. control agarose, where vanilla, coffee and chocolate were the different odor options, or with 1% vanilla vs. 1% coffee agarose.

unscented control agarose in the high-throughput individual T-maze. Three groups of 12 wasps were tested for each type of odorized agarose. First choice data showed that unconditioned wasps have an aversion to 1% chocolate ($F_1 = 30\%, p = 0.043$), whereas there was no preference for vanilla ($F_1 = 46\%, p = 0.839$) or coffee ($F_1 = 42\%, p = 0.487$) over the control agarose. Therefore, the chocolate extract was excluded from further testing. Combining the two remaining odors, and testing three groups of 36 unconditioned wasps with 1% vanilla vs. 1% coffee, showed no preference for either odor (Fig. 4, F_1 vanilla = 47%, $p = 0.649$).

3.3. Memory retention in *C. glomerata*

Vanilla and coffee extracts were selected for conditioning

Table 2

Memory retention results of *C. glomerata* for the different behavioral parameters in the high-throughput individual T-maze, given different test odor concentrations. A One-Sample Wilcoxon's Signed Rank Test was used to test memory retention. Vanilla and coffee conditioned odor preference results were analyzed using a Chi² test for first choice and a Wilcoxon's Signed Ranks Test for other behavioral parameters. The number (n) of PI scores used in the analysis is represented in the table.

Test with 1% vanilla and 1% coffee									
Parameter	Vanilla conditioned			Coffee conditioned			PI (n = 19)		
	% in zone			% in zone			PI value	P-value	Std. error
	Vanilla	Coffee	P-value	Vanilla	Coffee	P-value			
First choice	74	26	0.039	47	53	0.819	26	0.025	10.38
Residence time	74	26	0.011	48	52	0.825	26	0.010	8.57
Giving up time	81	19	0.004	53	47	0.872	21	0.013	6.96
Zone entries	69	31	0.007	49	51	0.916	25	0.010	8.01

Test with 1% vanilla and 0.5% coffee									
Parameter	Vanilla conditioned			Coffee conditioned			PI (n = 26)		
	% in zone			% in zone			PI value	P-value	Std. error
	Vanilla	Coffee	P-value	Vanilla	Coffee	P-value			
First choice	65	35	0.117	27	73	0.019	38	0.012	13.67
Residence time	47	53	0.694	32	68	0.012	15	0.045	9.05
Giving up time	45	55	0.694	36	64	0.258	8	0.301	9.47
Zone entries	49	51	0.931	37	63	0.006	17	0.034	7.99

Test with 2% vanilla and 1% coffee									
Parameter	Vanilla conditioned			Coffee conditioned			PI (n = 17)		
	% in zone			% in zone			PI value	P-value	Std. error
	Vanilla	Coffee	P-value	Vanilla	Coffee	P-value			
First choice	71	29	0.090	29	71	0.090	41	0.035	17.28
Residence time	72	28	0.023	38	62	0.154	34	0.010	10.99
Giving up time	80	20	0.007	23	77	0.121	37	0.008	11.71
Zone entries	60	40	0.155	51	49	0.832	29	0.023	10.23

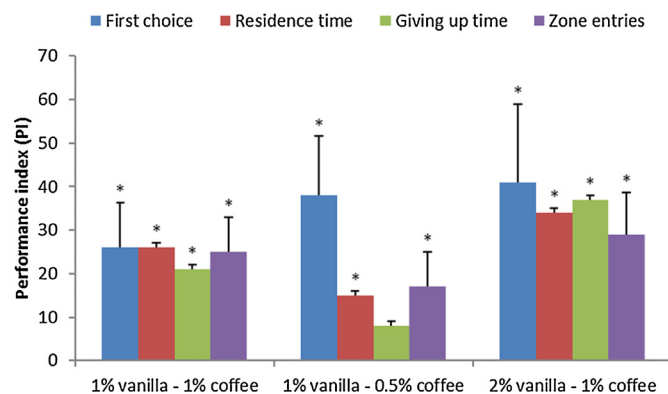


Fig. 5. PI scores of the behavioral parameters of *C. glomerata* in the high-throughput individual T-maze with different test odor concentrations. *C. glomerata* was tested in the high-throughput individual T-maze with test odor concentrations of 1% vanilla and 1% coffee (N = 19), 1% vanilla and 0.5% coffee (N = 26) and 2% vanilla and 1% coffee (N first choice = 17, other parameters N = 16). Significant PI scores (P < 0.05) are indicated with an asterisk (*).

(concentration 4%) and memory retention testing (concentration 1%) in accordance with above results. PI values of all tested parameters showed significant memory retention (Table 2, Fig. 5). Response levels of vanilla conditioned wasps were 68% (n = 23) and for coffee conditioned wasps 75% (n = 27). Underlying odor preference scores show a clear preference for vanilla with vanilla conditioned wasps, but no preference for coffee with coffee conditioned wasps (Table 2).

Since no preference was found for coffee with coffee conditioned wasps, different odor concentrations were tested to optimize the system; 1% vanilla vs. 0.5% coffee and 2% vanilla vs. 1% coffee. Testing with 1% vanilla and 0.5% coffee improved preference results of coffee conditioned wasps, but at the expense of vanilla conditioned wasps (Table 2). Response levels of vanilla conditioned wasps were 78% (n = 28) and for coffee conditioned wasps 86% (n = 31). PI values of all except first choice dropped and the giving up time parameter was no longer significant (Table 2, Fig. 5).

Testing with 2% vanilla and 1% coffee resulted in low P-values for both vanilla and coffee conditioned wasps, though not all significant (Table 2, response vanilla 67% with n = 24, coffee 72% with n = 26). PI scores, however, were high and significant for all behavioral parameters (Table 2, Fig. 5).

3.4. Memory retention in *N. vitripennis*

Testing *N. vitripennis* in the high-throughput individual T-maze resulted in highly significant PI scores and significant results for almost all odor preference parameters (Table 3, Fig. 6). Testing in the T-maze for group testing resulted in a significant PI score for final choice (Table 3, Fig. 6). Response levels in the high-throughput individual T-maze ranged from 75% to 84%, response levels of the T-maze for group testing ranged from 80 to 81%.

4. Discussion

Behavioral assays for insects have undergone a clear technological evolution in the past two decades. Time consuming methods using

Table 3

Memory retention results of *Nasonia vitripennis* for the different behavioral parameters in the high-throughput individual T-maze and the T-maze for group testing. A One-Sample Wilcoxon's Signed Rank Test was used to test memory retention. Vanilla and coffee conditioned odor preference results were analyzed using a Chi² test for first choice and final choice. For other behavioral parameters a Wilcoxon's Signed Ranks Test was used.

High throughput individual T-maze										
Test with 1% vanilla and 1% coffee agarose										
Parameter	Vanilla conditioned			Coffee conditioned			PI			
	% in zone		P-value	% in zone		P-value	PI value	P-value	Std. error	N
First choice	63	37	0.069	8	92	< 0.001	55	< 0.001	8.07	51
Residence time	71	29	< 0.001	15	85	< 0.001	56	< 0.001	5.25	48
Giving up time	75	25	< 0.001	21	79	< 0.001	52	< 0.001	6.27	48
Zone entries	58	42	0.005	22	78	< 0.001	45	< 0.001	5.29	48

T-maze for group testing										
Test with vanilla and coffee extract capillaries										
Parameter	Vanilla conditioned (n = 55)			Coffee conditioned (n = 56)			PI			
	% in zone		P-value	% in zone		P-value	PI value	P-value	Std. error	N
Final choice	69	31	0.005	25	75	< 0.001	43	0.028	8.63	6

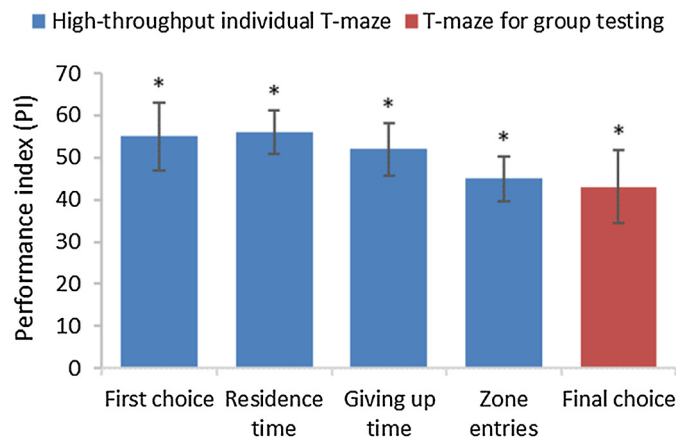


Fig. 6. PI scores of the behavioral parameters of *N. vitripennis* in the high-throughput individual T-maze and the T-maze for group testing. *N. vitripennis* was tested with 1% vanilla and 1% coffee agarose in the high-throughput individual T-maze (N first choice = 51, other parameters N = 48). With the T-maze for group testing only final choice could be assessed (Final choice N = 6). Significant PI scores ($P < 0.05$) are indicated with an asterisk (*).

observations of individual insects have been redesigned with the latest advances in video tracking technology (Beshel and Zhong, 2013; Faucher et al., 2006; Jiang et al., 2016; Lin et al., 2015; Reza et al., 2013; Smith and Burden, 2014). Whereas various of these studies still test single insects (Faucher et al., 2006; Reza et al., 2013; Smith and Burden, 2014), our high-throughput individual T-maze makes it possible to load 36 wasps in individual cages from which they can simultaneously be released into their own two-choice arena. The camera setup was combined with commercially available video software and multiple arena tracking software (Noldus et al., 2001), which allows for tracking of many individual wasps. Though simultaneous tracking of multiple insects in one arena has been reported previously (Beshel and Zhong, 2013; Jiang et al., 2016; Lin et al., 2015), individual identities of insects are often lost when walking tracks cross one another and social interactions may influence the results. The multiple arena tracking

module of EthoVision makes it possible to assign many arenas in which individual wasps can be tracked. This allows for both high-throughput and recording of detailed individual behaviors, without social interactions and the need for massive amounts of insects. We showed that our system was able to detect multiple behavioral parameters suitable for measuring memory retention levels, thereby providing robust datasets in an efficient manner.

The conditioning and test protocols we used were designed to make them easy to standardize and reproduce with commercially available, natural odor blends. Our results emphasize that odor selection for conditioning and testing should be done carefully with both EAG and preference tests. Even though three natural odor extracts (vanilla, coffee and chocolate) showed an equal sensitivity in the EAG experiment, and are known to be used for conditioning parasitoid wasps (Gutiérrez-Ibáñez et al., 2007; Hoedjes et al., 2012; Lewis and Takasu, 1990; Lewis and Tumlinson, 1988; Zhou et al., 2015), our odor preference results of unconditioned wasps showed a clear aversion for the agarose odorized with chocolate vs. control agarose, whereas this was not the case for vanilla and coffee. An equal preference level of these odors to unconditioned wasps makes it easier to detect effects of conditioning. A final round of fine-tuning was performed by testing different concentration of odorized agarose in the individual T-maze.

In order to find the best memory retention results it is important to assess if it is possible to induce a preference with each of the two odors used in the bioassay. Our results show that PI scores could be substantially increased when both odors showed significant conditioning effects.

The reciprocal design of treatments eliminates any remaining odor bias and allows for the creation of performance index (PI) scores. These PI's are commonly used in studies on learning and memory formation as a parameter to measure conditioned behavior, but usually these PI's are based on groups of insects (Hoedjes et al., 2012; Jiang et al., 2016; Kohn et al., 2013). With the development of a high-throughput individual T-maze, we were able to calculate PI scores based on two individual wasps, which increased sample sizes and therefore statistical power compared to PI's based on groups of insects. Robust PI scores based on each of 4 behavioral parameters for both *C. glomerata* and *N. vitripennis* were obtained in this study, demonstrating the suitability of

this set-up for testing of memory retention. Using *N. vitripennis*, we compared the high-throughput individual T-maze with the T-maze for group testing, which showed comparable PI scores, but substantially better p-values, using a similar number of insects. This suggests that the required number of insects for the individual T-maze per experiment may be lower than for the T-maze for group testing

The high-throughput individual T-maze is a strong tool to advance knowledge of learning and memory dynamics in ecologically diverse groups such as parasitoid wasps. Results of *C. glomerata* and *N. vitripennis* show the system is likely to be suitable for a broader range of parasitic wasp species and possibly also for other model insect species like *D. melanogaster*. Furthermore, due to the use of commercially available, natural odor blends, which are unrelated to odors wasps are exposed to in nature, it is possible to design comparative experiments with different species. Many types of preferences can be measured in this bioassay, such as food, color and odor preferences, but also other types of behavior such as mate choice and courtship behavior, in line with what was done by Reza et al. (2013). The system allows for the selection of the most relevant and statistically strong behavioral parameters, allowing users to make species-specific selections to record various kinds of behaviors. Adaptations to the bioassay, to meet specific requirements of species, can be implemented easily, because of the flexibility of the laser-cutting methodology for manufacturing of the arenas and the low cost of the Perspex plates.

The selected behavioral parameters of the high-throughput individual T-maze; first choice, residence time, giving up time and zone entries, are all highly relevant for foraging success (Wajnberg, 2006). Although the conditions in the set-up described here are artificial, the fact that significant PIs were obtained from these 4 different behavioral parameters show that learning affects different aspects of foraging behavior that contribute to foraging success for hosts, and thereby to realized fitness of the wasps. Our *Cotesia* model system provides excellent opportunities to validate how the results from our current high-throughput bio-assay translate into natural or agricultural situations, since *C. glomerata* is a well-known model species for behavioral studies in field, semi-field and wind tunnel situations (Benson et al., 2003; Bleeker et al., 2006; De Rijk et al., 2018; Geervliet et al., 1998a, b; Kruidhof et al., 2012; Lucas-Barbosa et al., 2014; Smid et al., 2007). In addition, the set-up could be useful for efficient screening of relevant behavioral parameters of candidate species for biological control.

In conclusion, the high-throughput individual T-maze combines the benefits of high-throughput and individual testing. It provides us with a standardized high-throughput, labor-efficient and cost-effective method to test various kinds of behavior and offers excellent opportunities for comparative studies of various aspects of insect behavior.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: <https://doi.org/10.1016/j.jneumeth.2018.09.012>.

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