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The use of parasitic wasps as bio-indicators for explosives: which species to use and how to train them.

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Table of Contents

Abstract

The detection and removal of explosives is a major issue in law enforcement today. A possible option for the detection of explosives is the use of parasitic wasps as indicators. By training them to respond to the odour of explosives and specifically DNT, the wasps could possibly be used to indicate the presence of this odour. The goal of this project is to determine which wasp species is the most suitable and which method is most useful in training the wasps. To test this, four wasps were chosen to be used. However, eventually only two of these were tested extensively. To answer the question of which method is most suitable, multiple methods were tried, mostly with *Cotesia glomerata*.

Testing with *Cotesia glomerata* was found to be extremely problematic. Observation of the behaviour of these wasps seems to indicate an aversion to the odour, but specific aversion testing showed no significant result. Aversion testing with *Diadegma semiclausum* shows no indication of an aversion either, but how the wasps responded to conditioning could not be sufficiently tested to give a viable result. Other wasps could not be sufficiently tested at all.

Because *C. glomerata* was the only wasp that could be sufficiently tested with different methods, the question of which wasp is most suitable can not be answered. It is clear however that *Cotesia glomerata* seems to be unsuitable for use as an indicator for explosives, due to the extreme difficulties with conditioning. Which method is most useful can also not be answered sufficiently, as *C. glomerata* is the only wasp tested extensively with more then one method.

1. Introduction

1.1. Goal

The detection and removal of explosives is a major issue in law enforcement today. Finding explosives using a metal detector is becoming increasingly difficult, because more and more explosives are made without any metal parts. An alternative to using a metal detector is using animals to detect the explosives. The animals used would have to be able to detect the specific odours coming from explosives. An alternative to using dogs is the use of insects. Honey bees are able to learn a wide range of odours and can be trained to find a wide range of chemicals. Research by the Montana University has shown that honeybees can be trained to detect odours from landmines and other explosives. These honeybees were able to reliably find explosives at levels similar to those occurring in landmine fields (Bromenshenk et.al. 2003). Research on *Microplitis croceipes* has shown that these wasps can also be trained to distinguish odours associated with explosives, such as 3,4-dinitrotoluene (Olson et.al, 2003). Other species of parasitic wasps, although not trained on odours associated with explosives, have shown to be able to learn and be trained on a large variety of odours (Kaiser & De Jong, 1993; Kaiser et.al, 2003; Kerguelen & Cardé, 1998).

The goal of this research project is to select the parasitic wasp best suitable for use as a bio-indicator for explosives. Also, this project will look at the best method for training these wasps and how to use them as bio-indicator for explosives.

This leads to the following research questions:

1. Which wasp is most suitable?
    a. Which wasp shows the highest strength and stability of the CR?
    b. Which wasp has the highest sustainability as a detector?
2. Which method of conditioning gives the best result?
    a. What type of reward gives the best result?
    b. How should the conditioning trials be set up?
3. Which method of maintaining the wasps gives the best results?

<u>1a</u>. To use the wasps as a bio-indicator it's necessary for the wasps to show a strong and reliable response to the conditioned stimulus. The reliability of the response must be very high for the wasp to be used effectively in the field. Ideally the wasp needs to give a response every time it is presented with the conditioned stimulus. This is not realistic of course and therefore the wasp with the frequency closest to this ideal is considered the wasp with the most stable conditioned response. To test this, the different species of wasps will be tested under similar conditions and their responses will be compared.

For the wasps to be useful in the field, the wasps also need to be able to ignore environmental stimuli and only give a response when the conditioned stimulus is encountered. When confronted with a mixture of odours, the wasp needs to give a response only when the conditioned odour is present. Therefore the wasps will be tested with different stimuli/ odours, separate from the conditioned stimulus as well as in combination with the conditioned stimulus, to see if other stimuli affect the conditioned response.

<u>1b</u>. The life expectancy of a wasp as well as the amount of extinction greatly influence how long a wasp can be used effectively as a bio-indicator. Obviously a wasp with a longer life expectancy can be used longer; however, because of extinction it's not always possible to use a wasp effectively for its entire life without reinforcing the conditioning. The different species of wasps will be compared with regard to life expectancy and extinction.

2a. The type of reward that is used can have an influence on the way the wasp responds. The two types of reward considered here are a food reward and an oviposition experience as reward. The expected CR associated with these two reward types is quite different. A food reward results in feeding specific behavior, involving the proboscis, whereas an oviposition experience is expected to result in an ovipositor probing response (Wäckers, 2001; Olson et al., 2003). However, a general increase in activity could be the resulting response in either case. The most important response for use in the field is the flight response. The wasps are expected to fly towards a source of the conditioned odour after conditioning. Both types of reward are expected to result in a positive flight response after conditioning, although some slight differences may occur. The choice for either reward type is dependent on these expected responses as well as practical aspects associated to the two reward types. These practical aspects include things like the availability of the reward and how to use the reward in the setup (i.e. placement).

2b. There are a lot of factors associated with the conditioning trials. These factors range from practical aspects, such as the actual setup, to factors that can't be controlled, such as the differences between groups of wasps and individual wasps. To be able to use the wasps as a bio-indicator it's important to find out how these factors influence the outcome of the conditionings trials. To successfully condition the wasps to the odour of explosives, the factors that influence the conditioning in a negative way should be eliminated or at least minimized as much as possible.

3. Maintaining the wasps is important for the use of the wasps as a bio-indicator. The longer a wasp can be used as a bio-indicator the more useful it is from a practical point of view, as less time is needed to train new wasps. To this effect it's important to know how the wasps can be maintained in the best way, as to be useful for the longest period of time. The most important factors in this are the average lifespan of the species as well as how they are reared and kept.

1.2. Background

Animal Learning

A lot of different definitions exist for the term animal learning. One of these definitions is that animal learning consists of experience resulting in a change in the reaction to a certain situation. With this definition animal learning strongly depends on the combination of stimuli and their consequences. Often in an animal's environment one event will reliably predict another or the action of an animal may result in consistent consequences. An animal that knows about these relationships will be able to anticipate future events, giving the animal a greater chance to survive (Pearce, 1997).

In an unchanging world it would suffice for knowledge of these relationships to be given down through the generations and no learning would be required. However, the world is always changing. Learning is therefore necessary for animals to survive in changing environments. This knowledge of relationships between stimuli and consequences can be achieved by associative learning. Associative learning can be said to have taken place when there is a change in an animal's behaviour as a results of one event being paired with another (Pearce, 1997).

This definition of associative learning forms the basis for conditioning. Conditioning consists of pairing a stimulus to an event in experiments, thereby inducing associative learning in the test animals. There are two main methods of conditioning that have been used in different forms to study associative learning.

Conditioning methods

Pavlovian/ Classical conditioning:

Pavlovian conditioning consists of presenting the animal with a conditioned stimulus (CS) followed by an unconditioned stimulus (US). The CS is a stimulus that has no particular ecological importance to the animal (e.g. a tone). The US consists of something ecologically important to the animal (e.g. food). By pairing the CS with an ecologically important event (the US) associative learning of the CS should occur, according to the definition of associative learning.

The unconditioned stimulus triggers certain behaviour in the animal. For example, when a bee is touched on the antennae with sugar water (the US) it will extend its proboscis. When the US is paired with the CS and associative learning occurs, presenting the animal with the CS will result in this same behaviour. When this occurs the animal performs the conditioned response (CR). With Pavlovian conditioning associative learning is thought to have occurred when presenting the CS is followed by the CR.

Most of the time the CS serves as a signal for the occurrence of the US, this is called excitatory conditioning. However, it’s also possible for the CS to indicate omission of the US; in this case the process is called inhibitory conditioning. This research uses excitatory conditioning.

If an animal displays what is thought to be a CR, it may not always be due to associative learning. The response that is the CR may also occur when CS and US are not paired (Pearce, 1997). To make sure that the CS is genuine control groups need to be used in the research on associative learning. With these control groups it’s possible to see if a CR occurs even if the CS and US are not paired.

It’s also important to consider the type of CR one can expect to occur. The presentation of a CS is often thought to substitute the US and therefore give a similar CR as the ‘normal’ response to an US. This may not always be the case. The CR can take on two forms: one of these is the consummatory response where the CR mimics at least a component of the response to the US, the other form is the preparatory response, where the CR is not intimately tied to the response elicited by the US and may for example consist of a general increase in activity. The CS itself may also have an influence on the CR causing it to differ from the CR that one would expect. All in all a lot of things about the CR are still unclear and this can cause some difficulties when trying to determine whether a CR has occurred or not. If this is not taken into account with an experiment the CR may be missed entirely and wrong conclusions can result from this.

Instrumental conditioning:

Instrumental conditioning also uses the pairing of events to induce associative learning. However, instead of using a conditioned response to predict the US, instrumental conditioning depends on the animal responding in a certain way to a stimulus, after which the event is delivered. The response of the animal constitutes one event; the outcome of that response constitutes the second event (Pearce, 1997). Because the animal learns about a response, the animal forms a response-US (R-US) association, instead of a CS-US relation as with Pavlovian conditioning.

Memory

Conditioning may result in the growth of a connection between internal representations of CS and US. This in turn may lead to the retention of the association between CS and US over a longer period of time. How long this memory of the CS-US association lasts differs between animals as well as within a separate individual. A distinction is made between two types of memory: short-term retention and long-term retention. Depending on the type of information (or association) the information is stored for a shorter or longer time. Most animals can retain large amounts of information and remember for a remarkably long time, but time does result in loss of some aspects of information.

This gradual loss of information over time, when conditioning is concerned, is also called extinction. If conditioning is used to study animal intelligence and cognition, this extinction may not be that big of a problem (as long as the extinction is not to extreme). However, if conditioning is used for more practical applications where long-term retention is desirable (such as with this research project) extinction may cause serious problems. Therefore, extinction plays a big role in the choices made when using conditioning for these purposes.

Additional learning trials can help to counteract some of the problems with extinction. This consists of repeating the conditioning trials (or a part of the conditioning trials) at a later time, with the goal of re-establishing the associations made with the first conditioning. The additional learning trials usually don't have to be equally extensive as the original conditioning trials, as the association is usual still there in some way and only needs to be re-established.

Reward

Food is a much used and effective US to use in conditioning Hymenoptera (Menzel et.al., 2001; Tertuliano et.al., 2003). When training parasitic wasps, food is not the only available reward type. Parasitic wasps not only use chemical cues to find food, but they use chemical cues to find hosts as well (Lewis & Takasu, 1990). These hosts and the subsequent oviposition experience can be used very effectively as an US (Kaiser et.al., 2003).

'Hunger' State

When conditioning is done with food, hunger state can have an influence on the response to conditioning. Research with M. croceipes has shown that the hunger state prior to conditioning as well as during the test has an effect on the response when testing. Wasps that were relatively hungry maintained high seeking behaviour when repeatedly exposed to the CS in absence of a food reward (Tertuliano et. al., 2003). A high seeking behaviour is highly desirable when using the wasps as a bio-indicator. It's desirable for the wasps to maintain a high response as long as possible. This would suggest that training the wasps in a hungry state is beneficial to the use of those same wasps as a bio-indicator.

The concept of 'hunger' as an elevated motivational state does not necessarily have to apply to food only. A wasp that has not been able to oviposite for a while can be considered 'hungry'. Not for food but for the opportunity to reproduce, this is also called a high egg load. This assumption would suggest that wasps that have a high need to reproduce will give a higher response to the odour then wasps that have had ample opportunity to reproduce.

1.3. Practical aspects

The ultimate goal of this project is to teach wasps to recognize and respond to the odour of explosives. The wasps need to associate the odour of explosives with some kind of important event. Pavlovian conditioning was used to accomplish this association. The odour of the explosives constitutes the CS.

The primary form of the US used was oviposition experience. The choice for this US over for example food was based mostly on the ‘hunger’ concept mentioned above. When working with naïve wasps, the initial hunger state of the wasps increases with each day after hatching. Hunger for food has to be created by a regime of absence of food before the conditioning trials, without killing the wasps. Such a regime is not needed to create ‘hunger’ for oviposition. Therefore, oviposition is easier to use as far as the ‘hunger’ concept is concerned. This practical reason is not the only reason why oviposition was considered the best choice. When the wasps are used as free flying wasps they will encounter food sources in the field and associative learning of these odours in the field could interfere with the conditioning on the explosives.

The choice of species to be used was mostly a choice of availability. The Entomology Department of the Wageningen University already has a rearing program for *Diadegma semiclausum, D. fenestrales, Cotesia glomerata and C. rubecula.* The NIOO institute has a rearing program for *Microplitis croceipes.* Because these species were available the choice fell to a selection of these species. To keep the number of species to be tested to a minimum *D. fenestrales* and *C. rubecula* were hardly used within this project. More experience was available on the research of *D. semiclausum* and *C. glomerata*, so these were primarily used. Together with *M. croceipes*, 3 species of 3 different genera were available for this research based on availability.

A drawback of all these species is that they are quite small. This could present some problems with handling them. A bigger species would be easier to handle. For this reason a rearing program was started with two bigger species: *Pimpla turionellae* and *Pimpla hypochondriaca*. The rearing program of *P. turionellae* was a little more successful and therefore this species was used to some extend in this research. Together with the other 3 species this made 4 different species that were available for this research.

For any animal to be effectively used in the field a method must be found to reliably measure the response of the animal(s). The research done by the Montana University (Bromenshenk et.al. 2003) made use of a specialized LIDAR system. This system however is dependent on a large quantity of free moving ‘detectors’, in this case flying bees. Using a free flying detection system with bees has several advantages such as low costs, limited time needed for training and greater safety for the handlers as they don’t need to handle the bees constantly (Bromenshenk et.al. 2003). All of these advantages are also true for a free flying detection system with parasitic wasps. If, and how this method can be used on a non-social species like parasitic wasp species is not clear.

2. Methods

2.1. Odour

The odour used for this research is 2,4-dinitrotolueen (abbreviation: (2,4-)DNT). This odour was chosen for this research because it is a major component of most explosives and constitutes a large portion of the mix of odours emanating from explosives like landmines. Also, DNT is one of the few components of explosives that are available without needing specific clearance from the government.

2.2. Rearing

All of the wasps used were reared in a cage containing water and honey as food. These cages were kept at 20°. The hosts that were u sed differed for each wasp. *Cotesia glomerata* as well as *Diadegma semiclausum* were reared on the large cabbage white (*Pieris brassicae*), *Cotesia rubecula* was reared on the small cabbage white (*Pieris rapae)* and *Pimpla turionellae* was reared on the Greater wax moth (*Galleria mellonella)*. All of the wasps used were between 5 and 20 days old.

2.3. Conditioning arena

In this setup the actual conditioning was done in a so-called 'arena'. This 'arena' consists of a Petri dish with two holes in the side to facilitate the airflow and a hole in the bottom where the wasp can be let in (Figure 1). The 'arena' was closed off with the lid of the Petri dish. The reward was placed around the hole in the bottom, so the wasp would come into contact with the reward when it crawled into the 'arena'. This setup was large enough for the wasp to move, but small enough to optimize the contact of the wasp with the reward and the odour.

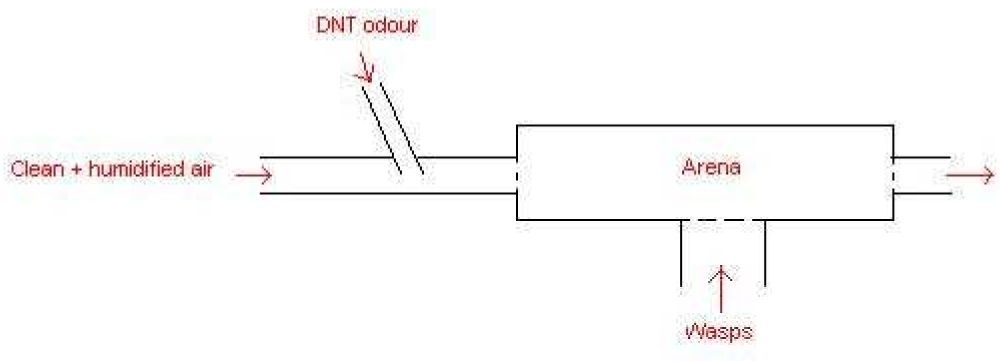

Figure 1. Schematic representation of the conditioning arena.

To effectively train the wasps to the odour, mechanical stimuli as a result of the airflow were kept to a minimum. The airflow was set at 100 ml/min with a flow-meter; this is high enough to carry the air through the arena, but low enough not to disturb the wasp too much. The air was humidified to prevent static. This way the air pumped in has a negligible mechanical effect on the wasp. T he conditioning arena had a volume of roughly 67 cm² (diameter 5.4 cm; height 1.25 cm).

Before the air was pumped into the 'arena', it went through a modified tube where the odour was administered. The modification consists of a hole, where a cartridge (see below) containing the odour can be put. By blowing air through the cartridge, the odour is blown into the tube, where the continuous airflow carries it further into the 'arena'. The airflow through the cartridge has a lower airspeed then the continuous airflow. The airflow trough the cartridge is regulated by a machine (Syntech Stimulus Controller CS-55) which controls the length and strength of the burst of air. This way the administration of the odour could be regulated without interrupting the continuous airflow.

The cartridge with the odour was made by inserting a small strip of filter paper into a glass pipette. On this piece of paper 100µl of a 1% DNT solution was pipetted. The DNT solution was made in dichloromethane. Another cartridge was made with cinnamon. This was done by placing a small piece of cinnamon into the pipette instead of a filter paper with DNT. The cartridge was then placed in the hole in the tube where the continuous airflow flowed trough.

Because DNT is a slightly toxic substance, it had to be pumped out of the arena en disposed off. Between the 'arena' and the pump a flow-meter was placed to regulate the speed of the airflow and to keep it at 100 ml/min. The DNT was pumped out into the air suction device of an acid chamber. The DNT was thereby disposed off without affecting the general airflow.

To optimize the conditioning it's important that the start of the odour administration precedes the first contact off the wasp with the reward, the reward consisting of caterpillars. The reward was placed in a way that the wasps came into contact with it as soon as they entered the 'arena'. The odour was administered just before the wasps came into contact with the frass. The placement of the reward prevented the wasps from walking around aimlessly and made it easier to time the administration of the stimulus.

With this conditioning arena only *Cotesia glomerata* was used. The wasps were 5-10 days old. With each wasp 3 trials were done, with intervals of about 5 minutes. The reward used for *C. glomerata* was the large cabbage white (*Pieris brassicae*); the caterpillars were 1-2 days old.

Observation

During the conditioning the wasps were observed. These observations were to show what kind of behaviour the wasp displays when coming into contact with the reward and the odour. The behaviour shown during conditioning was later expected to occur with the tests as this shows if the wasp has learned to associate the odour with the reward.

About an hour after the last conditioning trials the wasps were tested for a response to the odour. All the conditions within the 'arena' were kept the same as during the conditioning, with the exception of the reward. No reward was placed in the 'arena'. The odour was administered just as the wasps climb into the arena. The wasps were then observed to see if they displayed any of the behaviour that is associated with the reward, as observed during the conditioning trials. A control was done where wasps were let into the arena with the reward, but without pumping the odour into the arena (clean air was pumped through instead).

The wasps were also released in the wind tunnel to test their attraction to the odour.

2.4. Wind tunnel

To test the attraction of the wasps to the odour, they were placed into a wind tunnel (Geervliet et.al., 1994). Humidified air is blown through the wind tunnel. In the middle of the wind tunnel the wasp is released. Upwind from the release spot two round pieces of green carton with a diameter of approximately 5 cm are placed, one with a filter paper on it with the odour, the other with a blank piece of filter paper on it. A positive response is when the wasp lands on the piece of carton with the odour, a negative response is when the wasp lands on the piece of carton without the odour and a 'No response' is when the wasp lands on neither (and usually lands somewhere else in the wind tunnel). The number of positive responses is an indication of the attraction to the odour.

2.5. Conditioning with *Nasturtium*

Another method of conditioning that was used was to condition the wasps on plants with feeding damage from caterpillars. The caterpillars were left on the leaf and were used as the reward for the conditioning. *Nasturtium* was used instead of cabbage, because cabbage has a strong odour itself that might be stronger than the DNT odour applied. *Nasturtium* is less fragrant itself and caterpillars of the cabbage white can feed on *Nasturtium* as well. The conditioning consisted of applying odour on the leaf and putting the wasps on the leaf (i.e. on the frass) and using the caterpillars as reward.

Two methods of applying the odour were used. One method that was used was applying the odour as small drops around the caterpillars by means of a pipette. With the other method the solution with the odor was sprayed onto the leaf with an air-brush paint device (figure 2), which was held a couple of centimeters from the surface of the leaf. With this second method the caterpillars were covered with an Erlenmeyer or other glass receptacle with a diameter (at the covered surface) of 2.5 cm. This was done to prevent spraying the solution on the caterpillars, as the dichloromethane in the solution is toxic for the caterpillars. With both methods the odour was applied directly around the caterpillars to optimize the conditioning, but without damaging the caterpillars.

The solution used for the conditioning with *Nasturtium* was basically the same as the solution used in the conditioning arena, but here different concentrations were used. Solutions were used containing 0.01, 0.1 and 1% DNT. Ten wasps were trained for each percentage and tested in the wind tunnel with that same percentage. Also, ten wasps were trained on 0.01% DNT and tested on 0.1% DNT.

Observation

During the conditioning observations were made on the behaviour of the wasps. The first observations made were the observations when putting the wasp on the leaf. Special attention was given to the eagerness or reluctance of the wasp to get on the leaf. Observations were also made on the behaviour of the wasp on the leaf after contact with the odour and reward (search behaviour and oviposition). These observations were made to give an idea if this method of conditioning had any effect on the behaviour of the wasps.

Afterwards the wasps were again tested in the wind tunnel, but instead of using pieces of carton, leaves of the *Nasturtium* plant were placed in the wind tunnel, one sprayed with DNT, the other without DNT.

Figure 2. An air brush device made by manufacturer Revell, as was used to spray DNT on the leaves.

2.6. Conditioning with cabbage/separate pupae

Similar to conditioning on *Nasturtium*, conditioning was done with cabbage leafs. The solution with the DNT was sprayed on the leaf in the same way as was done with the *Nasturtium* conditioning. The wasps were placed directly on the leaf, which contained caterpillars and frass. This method was mostly the same as with *Nasturtium*, making it possible to compare the effect of a different plant on the conditioning.

Two different wasp species were conditioned in this way. The two wasps used were *Cotesia glomerata* and *Cotesia rubecula*. For *C. glomerata*, the large cabbage white (*Pieris brassicae*) was used, while for *C. rubecula*, the small cabbage white was used (*P. rapae*). The conditioning was done with a solution of 0.1% DNT and a solution of 1% DNT. A comparison could be made between *C. glomerata* and *C. rubecula* by using this method with both species.

The wasps were tested afterwards in the wind tunnel. Instead of using pieces of carton, cabbage leafs were used.

Separate Pupae

For *Pimpla turionellae* a different method was used. The method used for the smaller *Cotesia* wasps was not an option for *P. turionellae* as this wasp uses a different host stage to lay their eggs in. Pupae of the Greater wax moth (*Galleria mellonella*) were used as host for *P. turionellae*. A couple of pupae were sprayed with a 1% DNT solution and placed in a small cage containing 5 *P. turionellae*. The pupae were left in the cage for the wasp to oviposite. After about an hour, the pupae were removed from the cage. When the wasps oviposited in the pupae they were in direct contact with the odour, which should lead to conditioning.

During the conditioning the behaviour of the wasps was observed to see what kind of behaviour these wasps show when ovipositing. Afterwards the wasps were tested in the wind tunnel by placing one piece of filterpaper with DNT and one piece without DNT in the wind tunnel and observing the direction of flight of the wasps. Ten wasps were conditioned and tested.

2.7. Four-branch olfactometer

To test for a possible aversion of the wasps against the DNT odour, a 4-branch olfactometer was used (figure 3). With the 4-branch olfactometer air is pumped in at four sides and flows out through a hole in the middle. The air pumped in forms 4 separate areas inside the olfactometer if it's pumped in at the right speed. This speed was found to be 150-160 ml/min. The air was cleaned with activated coal and humidified. On one side of the olfactometer, the odour is pumped in with the airflow. Effectively the inside of the olfactometer contains 3 pockets of clean air and 1 pocket of air containing the odour. The total volume of the 4 areas together was 86.6 cm^3.

The odour was pumped in by letting the cleaned and humidified air flow through a bottle containing a piece of filter paper with the odour on it. Tests were done with 1% DNT and 5% DNT solutions. These solutions were pipetted onto the filter paper (0.1 ml). The air flowing through the bottle would mix with the odour before flowing into the olfactometer.

An unconditioned wasp was then released into the olfactometer through the hole in the bottom. Observations were then made on how long the wasp would stay in each of the 4 areas. The total observation time was 6 minutes. Of these 6 minutes the first was not included into the calculations, as this was considered time for the wasp to get used to the airflow. Also, this time was needed for the pockets of air to form correctly, as the airflow was disturbed every time a wasp was extracted again from the olfactometer. The average amount of time that the wasps would spend in each clean air area could be compared to the average time they spent in the area with the odour. If the average time spent in the area with the odour is lower then the average time spent in each of the other areas, this would be an indication that the wasps experience an aversion against the odour.

A control group was also tested. Everything was kept the same, only the bottle which contained the odour with the test group was now left empty. Ten wasps were used for the test and control group.

Species

The species used in this experiment were *Cotesia glomerata, Diadegma semiclausum* and *Pimpla turionellae.*

Olfactometer as a conditioning method

A test was also done to see if the 4-branch olfactometer could be used for conditioning the wasps. A cabbage leaf with caterpillars was placed in the area with the odour and the wasps were then released into the olfactometer. After they found the reward and made an oviposition they were taken out of the olfactometer. All other factors were kept the same as in the aversion test, with the exception of the presence of the reward in the area with the odour.

After this conditioning the wasps were tested for preference to the area with the odour in a similar way as they were tested to the aversion.

Statistical analysis

On the results of the Olfactometer tests a Friedman test and a Wilcoxon-Wilcox test (Köhler et.al. 1996) were done to see if any area differed from another and if this was true, which area differed from which other area.

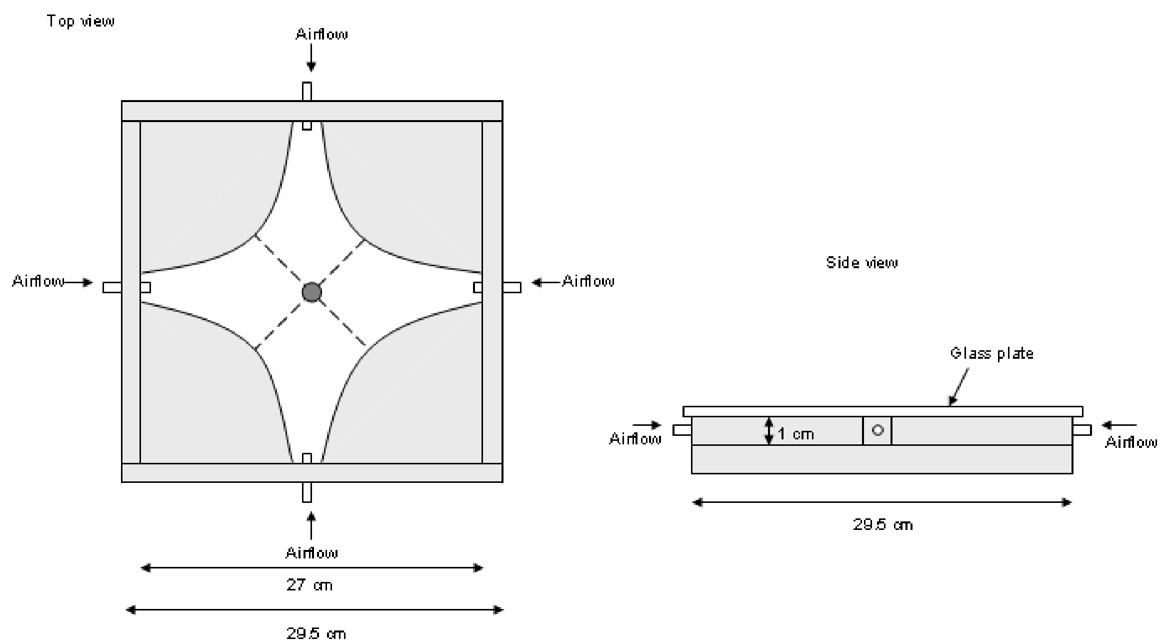

Figure 3. Schematic representation of a 4-branch olfactometer.

2.8. Y-tube

Conditioning

Before testing with the Y-tube, the wasps were conditioned in a similar way to the *Nasturtium* tests. Instead of a leaf, a piece of filter paper was used. On this filter paper the solution was pipetted and then caterpillars and frass were placed on the paper. The rest of the conditioning was done in the same way as with the *Nasturtium* tests. Three conditioning trials were done with each wasp.

A control group was also tested. This group was not conditioned before being released into the Y-tube. This group would show if the wasps had any innate preference for the odour or not and if the wasps are suitable for this method.

Setup

The Y-tube consist of, as the name implies, a glass Y shaped tube (figure 4). The diameter of the glass tubes is 3.6 cm. Through two of the branches air is blown and this air flows out of the Y-tube by means of the remaining branch. Through both branches air is blown that has been cleaned with active coal and has been humidified. The air is blown through the Y-tube at 4 l/min. At one of the branches the air is led through a bottle containing a filter paper with the odour, similar to the way the odour is administered with the 4-branch olfactometer tests. The clean air and odour come together in the remaining branch and are pumped out there. If the wasps are attracted to the odour, they should walk towards the branch where the odour is blown in.

To test if the wasps have a preference for the odour, a finish line is drawn on the y-tube at the end of both branches. The wasp is observed for 5 minutes, in which time it must have crossed one of the finish lines, otherwise it's considered a No Response (NR). If it crosses one of the finish lines, it has to stay in that branch for 15 seconds. If this occurs it is considered a positive or negative response. Positive if this happened in the arm containing the odour, negative if it was the other branch with the clean air. A positive response after conditioning is considered an indication that the conditioning worked.

The wasps were also tested in the wind tunnel in addition to the Y-tube. These tests were attempted with two species of wasp: *Cotesia glomerata* and *Diadegma semiclausum*.

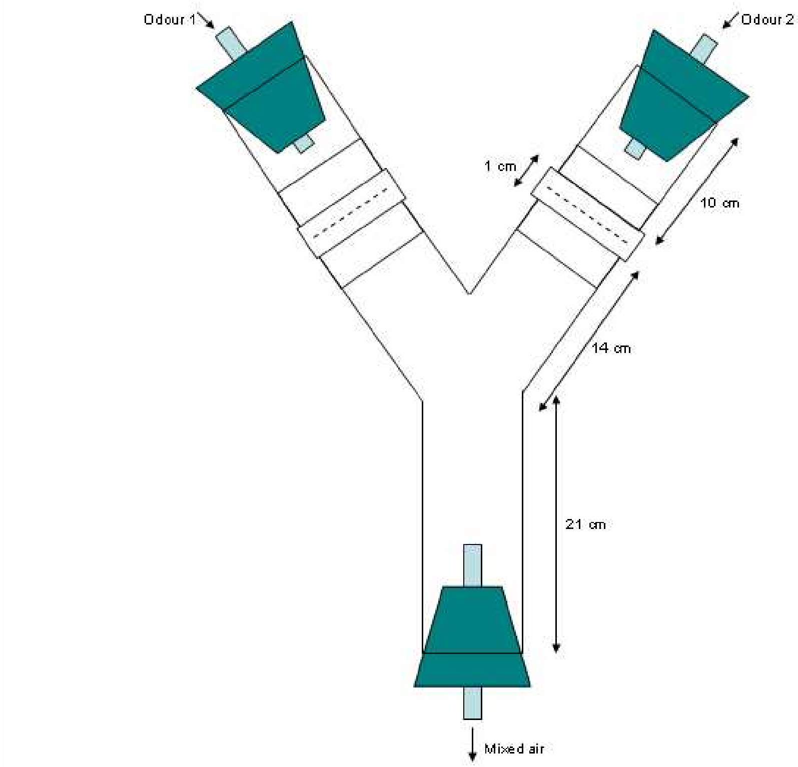

Figure 4. Schematic representation of a Y-tube.

3. Results

3.1. Conditioning arena

Observations

One of the first problems to arise with the conditioning arena was the reluctance of the wasps to get into the arena. Normally, *Cotesia glomerata*, when put into a glass tube, tries to climb to the top of the tube. This means the wasp climbs out of the tube if the opening is at the top. However, in this case the wasps stayed at the bottom of the tube. This could be due to some of the air flowing through the hole in the bottom of the arena and into the tube. To solve this problem, plastic tubes were used, which were prepared so that both ends were open. This way the wasps could be 'forced' out of the top of the tube and into the arena by blowing air into the bottom end of the tube. In the end it was possible to get the wasps into the arena, but with some difficulty.

A second problem with this setup was the timing of the stimulus (the DNT odour). To best condition the wasps the odour should be administered a couple of seconds before the wasp comes into contact with the reward. To accomplish this, the odour was administered just before the wasp would crawl out of the tube, but this proved hard to time. This resulted in the gaps between odour and reward would be different for different wasps and some errors occurred where the odour was even administered too late. A real solution for this problem could not be found, the only thing that could be done was time as precisely as possible.

When the wasps came into contact with the frass their behaviour changed noticeably. They showed clear searching behaviour, where they would follow the frass to the caterpillars, feeling with their antennae and probing with their ovipositors. We looked for these oviposition-specific behaviours in the tests.

Wasps that were conditioned were then tested in the same arena. This time, as the wasps crawled into the arena, they would walk around aimlessly and soon end up on the top of the arena. Some of the wasps did feel around with their antennae, but this was seen as more exploratory behaviour then searching for caterpillars. The wasps didn't show any probing with their ovipositor. None of the wasps showed the behaviour that they showed in the conditioning trials, nor did they show any other indication of recognizing the odour.

Wind tunnel experiments

When the wasps were tested in the wind tunnel, they showed no directed response to the odour either. Most of the wasps did not fly in the direction of the odour and landed in different places inside the cage, indicating a No Response. The few wasps that did fly in the direction of the odour did not show a preference for either the piece of cardboard with the odour or the clean piece of cardboard. None of the wasps showed a positive response, or a negative response for that matter. The results of these tests indicate that the wasps did not associate the odour of DNT with the reward and the chance to reproduce.

Conclusions

These tests raised more questions then they gave answers. The reason the wasps didn't learn to associate the odour with a reward could be a number of things. It could be that the wasps are not able to learn this odour. It could be that the method was wrong. Or maybe it was the testing method that was inadequate. Previous studies with *Cotesia glomerata* had shown that this wasp was capable to learn a wide variety of odours. Also, the wind tunnel was used before for tests with this wasp. This left the method as the primary candidate for causing the problems with conditioning these wasps to DNT. To see if the method was the problem, other methods were devised and tested.

3.2. *Nasturtium*

Observations

The biggest problem with this method was the reluctance of the wasps to get onto the leaf. The first trials were done with 1% DNT solution and this appeared to cause a high aversion in the wasps. The wasps would not even get close to the leaf and if they were put on the leaf they would fly away immediately. A lower concentration of 0.1% DNT solution showed some decrease in the aversion of the wasps. A concentration of 0.01% appeared not to have any aversion effect on the wasps. It proved impossible to train on 1% DNT solution. Training with the other two concentrations was possible, although sometimes with some difficulty. Applying the odour solution to the leaves showed some problems initially, as the solution is made with dichloromethane, which caused some corrosive effects on the leaves.

Wind tunnel experiments

The wasps were again tested in the wind tunnel. In the 0.1%/0.1% (conditioning/wind tunnel) and 0.01%/0.01% test all of the wasps gave a No Response. In the 0.01%/0.1% test, 3 wasps gave a negative response, and 7 gave a No Response. Again, the tests seem to show that the wasps do not associate the odour with a reward.

Conclusions

Again, this method seemed to raise more questions than give answers. Next to the same questions of the conditioning arena (was it the method, the testing or the wasps that gave the problems), another question was raised: is the DNT odour aversive for the wasps? The reluctance of the wasps to go onto the leaf certainly pointed in this direction. To rule out some of the aspects of the method, another method was used to see if that gave better results.

3.3. Cabbage /pupae

Observations

Cotesia glomerata showed the same reaction with cabbage leaves as the wasps did with the *Nasturtium* leaf. The wasps were reluctant to walk on the leaf and would fly away as soon as they came into contact with the sprayed leaf. Using a cabbage leaf instead of a *Nasturtium* leaf did not solve the problem with this method as far as *C. glomerata* is concerned.

C. rubecula appeared to show less of an aversive reaction then *C. glomerata*. They showed the same response to high concentrations (1%), they didn’t walk on the leaf and flew away as soon as they came into contact with the odour. However, these wasps didn’t seem to have as much trouble with the lower concentrations (0.1%). With this concentration they showed the desired behaviour of searching and ovipositioning. Eventually 10 *C. rubecula* were conditioned successfully using the cabbage leaf and a 0.1% solution.

Conditioning with *Pimpla turionellae* was far less problematic then with the *Cotesia* wasps. They did not show any aversive reaction to the odour and made several ovipositions in the prayed pupae. Ten wasps were conditioned on 1% without any problems.

Wind tunnel

When testing *C. rubecula* in the wind tunnel, they showed the same behaviour as *C. glomerata* in earlier tests. The wasps did not fly in the direction of the odour and all of the tested wasps showed a No Response. This test seemed to indicate that *C. rubecula* did not associate the odour with a reward.

When *Pimpla turionellae* was tested in the wind tunnel, another problem occurred. The tested wasps also didn't fly towards the odour, but they showed different flight behaviour then the *Cotesia* species. Where the *Cotesia* species just flew in different directions then towards the odour, all of the *Pimpla* wasps flew against the ceiling of the wind tunnel and hovered there. Apparently they were attracted to the light or some other factor influenced the wasps to show this behaviour. It seemed that *P. turionellae* is not suitable for testing in the wind tunnel.

Conclusions

The tests (and especially the conditioning attempts) with both *Cotesia* species seemed to indicate that these species have an aversion against the DNT odour. To see if this aversion is the reason for the absence of learning in the previous tests, a test was devised to test the wasps for aversion.

3.4. Four-branch olfactometer

Observations

Diadegma semiclausum and *Cotesia glomerata* show very different behaviour in the 4-branch olfactometer. While *D. semiclausum* runs around through the areas and almost never sits still in one area for long, *C. glomerata* does the exact opposite and crawls into the olfactometer and just sits still for most of the time. Sometimes *C. glomerata* changes position once or twice, but then the wasp continues sitting still again. This behaviour is not optimal for use in the 4-branch olfactometer, because the setup depends on the wasp choosing between the areas. With *C. glomerata* it's not clear if the wasp sits still in an area because it likes it there, or because it just happens to be the first spot it finds. With *D. semiclausum* it's clear that the area it spends the most time in is the area it prefers, as here the wasps run through all the areas. Therefore it seems *D. semiclausum* is better suitable for testing with this setup.

One of the main problems with this setup is that all factors have to be controlled very precisely. The lighting has to be the same in each area (the light has to be centred above the setup) otherwise the wasps could prefer a side because it's lighter there. Also, the airflow has to be the same in all the areas because this could influence the wasps as well. The airflow also has to be of a particular speed, this is important to get 4 specific areas in the olfactometer. If the speed of the air is too high or too low, the 4 pockets of air are not formed properly. During the testing with this olfactometer all of these problems came up at least once. But after some failed experiments, the ideal setup was eventually established as much as possible.

Graphs and tables

The test with *C. glomerata* showed no aversion of the wasps to the odour. The wasps on average did not spend less time in the area with the odour (figure 5). They even spend more time on average in that area then the control group (figure 6). The wasps spent significantly more time in the area with the odour then in the area directly opposite of the odour (Friedman and Wilcoxon-Wilcox tests $P<0.05$, $n=8$; Köhler et.al. 1996), but not significantly more then in the other two areas (Friedman and Wilcoxon-Wilcox tests $P>0.05$, $n=8$; Köhler et.al. 1996). This result was completely different from the expected result, but was mostly due to the immobility of this wasp species inside the olfactometer. With the testing group more wasps landed first in the north quarter then in the control group, resulting in these graphs. None of the areas in the control group differed significantly (Friedman test $P>0.05$, $n=8$; Köhler et.al. 1996). These tests with *C. glomerata* are hardly conclusive, because of the relative immobility of the wasps influencing the results more then the odour.

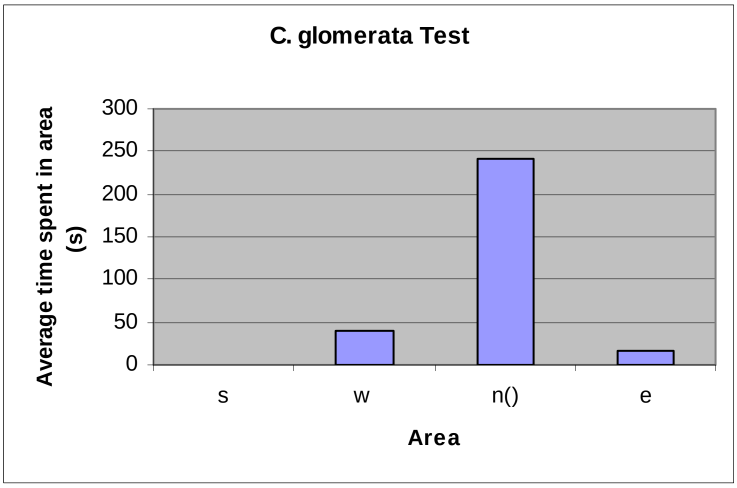

Figure 5. Average time spent in each area by the test group of *C. glomerata*. The () sign indicates the quarter where the odour was administered.

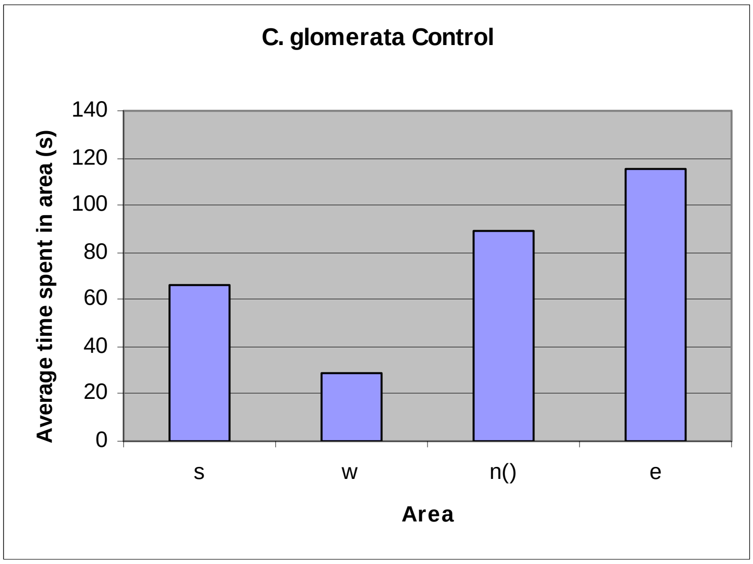

Figure 6. Average time spent in each area by the control group of *C. glomerata*. The () sign indicates the area where the air was pumped through an empty bottle.

The tests with *D. semiclausum* showed no aversion response either. The test group spent a little less time in the area with the odour (figure 7), but the control group also spent less time in that area then in the other areas, therefore the result may have some other factors causing it instead of aversion to the odour (Figure 8). The test group spent significantly less time in the test area then in the area opposite of the test area (Friedman and Wilcoxon-Wilcox tests $P<0.05$, $n=8$; Köhler et.al. 1996), but not significantly less time then in the other two areas (Friedman and Wilcoxon-Wilcox tests $P>0.05$, $n=8$; Köhler et.al. 1996). The control showed no significant preference (Friedman test $P>0.05$, $n=8$; Köhler et.al. 1996). The tests with *D. semiclausum* are a bit more reliable, as this species did not stay stationary in the olfactometer and was therefore exposed to all the areas.

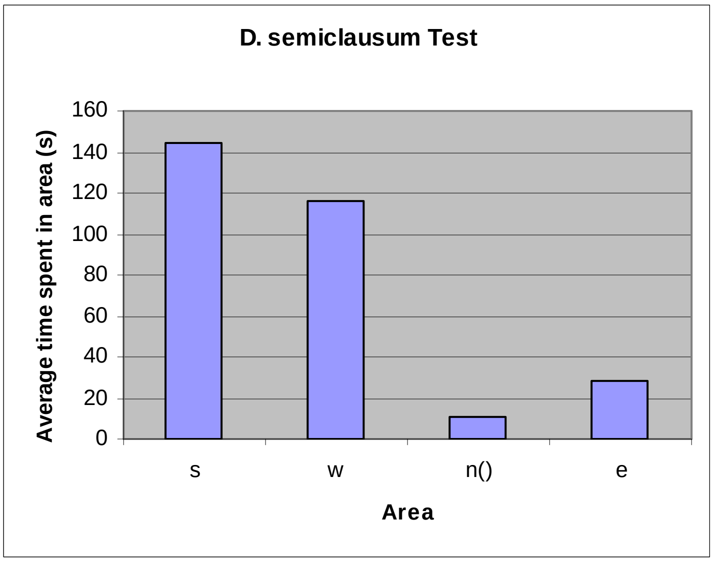

Figure 7. Average time spent in each area by the test group of *D. semiclausum*. The () sign indicates the area where the odour was administered.

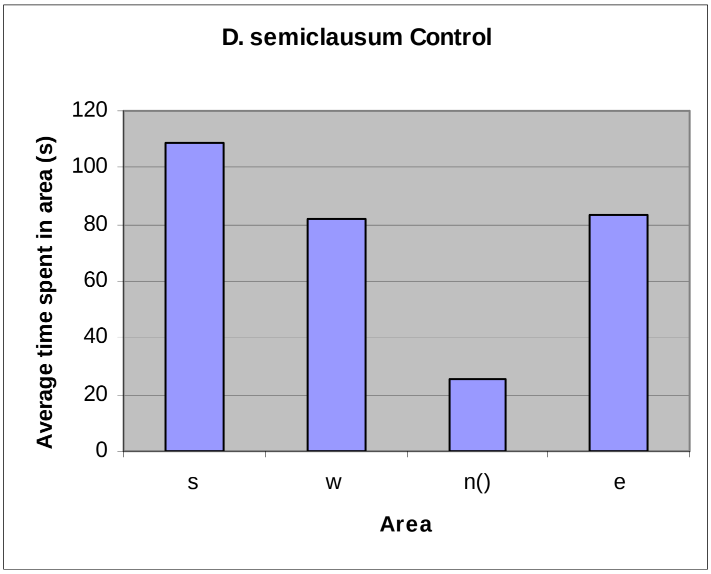

Figure 8. Average time spent in each area by the control group of *D. semiclausum*. The () sign indicates the area where the air was pumped through an empty bottle.

The tests seem to indicate that neither species has any aversion against the odour of DNT. Although the test results of *Cotesia glomerata* were mostly due to the movement (or lack thereof) of the wasps, it was still an indication that no aversion occurred. This was concluded, because if the wasps would have had an aversion to the DNT odour, they would probably have moved out of the area despite there normal stationary behaviour. *D. semiclausum* showed the behaviour needed for this test to work and showed hardly any reluctance to go into the area with the odour. Aversion was considered for now not to be the reason the wasps showed so much difficulty with this odour, so the reason had to be something else.

The tests with *Pimpla turionellae* showed no aversion towards the odour. However, there were only 5 specimens of *P. turionellae* available. Also, the space inside the olfactometer seemed to be a bit restricting for these much larger wasps. The limited availability of these wasps and the sub-optimal conditions inside the olfactometer make the result less reliable and make it virtually impossible to draw conclusions from these tests. For a reliable comparison with the other species, an olfactometer that is better suited to the size of *P. turionellae* needs to be used, and more wasps need to be tested.

The test with the olfactometer as a method for conditioning proved useless for *C. glomerata*. The *C. glomerata* used showed the same stationary nature as with the other tests with the olfactometer. The conditioning was already a problem, because the wasps were not all able to find the cabbage leaf. The area with the cabbage leaf had to be the area where the wasp landed first for the conditioning to have any affect, and even then it would result to basically the same setup as the conditioning arena used. Again, the stationary nature of *C. glomerata* proved the limiting factor for use of this setup.

This same test with *D. semiclausum* did not have the same problems. Like the aversion tests, the wasps moved around enough and all of them found the reward. When testing these conditioned wasps, however, they showed no significant preference for the area with the odour (figure 9) (Friedman and Wilcoxon-Wilcox tests P>0.05, n=8; Köhler et.al. 1996).The wasps did not seem to associate the odour with a reward.

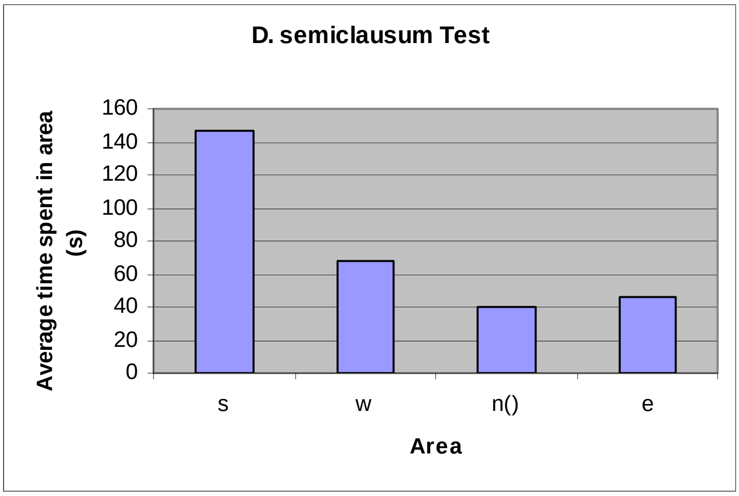

Figure 9. Average time spent in each area for the conditioned test group of *D. semiclausum*. The () sign indicates the area where the odour was administered.

3.5. Y-tube

Cotesia glomerata

Testing with the control group showed that the Y-tube experiment has the same problems with the stationary nature of *C. glomerata* as the olfactometer experiments. All of the wasps would stay in one place near the entrance of the Y-tube and would not walk towards either of the branches. One wasp did go into one of the branches, but this was due to a mistake with inserting the wasp into the tube, which was done to forcefully. This wasp was not counted in the results. Therefore, the result was a No Response for all of the wasps tested.

With conditioning the wasps showed some of the same problems as with the *Nasturtium* tests. They showed an extreme reluctance to go onto the paper with the solution and flew away immediately when coming into contact with the paper. The difference to the *Nasturtium* tests however was that this behaviour was less evident in the lower concentrations. Only with concentrations higher then 1% DNT did this problem become too difficult to overcome. Some wasps (8) were eventually successfully conditioned with a 1% solution and tested with the Y-tube and wind tunnel.

Testing with the Y-tube resulted in the now familiar problems with the stationary nature of the wasps. None of the wasps tested moved to either of the two branches. All of the wasps therefore gave a No Response. This test therefore gave no conclusive answer to the question if the conditioning worked.

The test with the wind tunnel with these same wasps gave no conclusive results either. All of the wasps gave a No Response when put into the wind tunnel. None of them flew in the direction of the filter papers with the odour. Again these tests raised more questions then they answered.

Diadegma semiclausum

The control group of *D. semiclausum* did not give the same problems with the Y-tube. Like the tests with the olfcatometer, the wasps moved around a lot and frequently walked into one of the two branches. Only one of the 10 tested wasps gave a positive response, but this was not because of any kind of problem with the wasps or the setup. This was more probably because the other wasps did not have a preference for either of the two branches and would therefore not stay in one of these branches for the required 15 seconds. It was assumed that if a preference was present the wasps would stay for the required 15 seconds. This test seemed to indicate that the Y-tube is potentially a good testing method for *D. semiclausum*.

The conditioning with *D. semiclausum* caused a lot more problems, however. *Diadegma semiclausum* showed the same problems as the *Cotesia* wasps did with this method and the *Nasturtium* method. However, *D. semiclausum* displayed this reluctance at much lower concentrations then *Cotesia*. Concentrations as low as 0.1% gave the same response as a 1% or 5% concentration did with *Cotesia*. In the end it was impossible to condition the wasps with a high enough concentration for the test to be effective.

Conclusions

The tests with the Y-tube did not answer the question about the ability of the wasps to learn the DNT odour. It did show a couple of other things, though. It clearly showed that this conditioning method is not suitable at all for *D. semiclausum*. It also showed that the Y-tube method is not a suitable method of testing for *C. glomerata*, due to its tendency to stay stationary. More methods of testing as well as conditioning methods have to be tested to answer the question about learning the DNT odour.

4. Discussion

Bees that were trained to find explosives were successfully trained on 2,4-DNT and this seemed to be a logical choice to use in this project as well. But research done on *Microplitis croceipes* with 2,4-DNT and 3,4-DNT showed the remarkable result that 3,4-DNT could be learned by the wasps, but that conditioning on 2,4-DNT was not successful (Olson et.al., 2003). *M. croceipes* is perhaps a better reference for the choice of chemicals then bees. The choice to use 2,4-DNT in this project may therefore have been a slight mistake. However, the choice of chemicals to use was limited due to restrictions on the use of chemicals associated with explosives.

Most conditioning with honeybees is done by placing the bees in a harness and using food as an US (Gerber & Smith, 1998; Menzel et.al. 2001). This method was rejected for this project, as most parasitic wasps used in this project are two small to use such a harness effectively. This method may have been used to effectively condition bees on DNT by the Montana University (Bromenshenk et.al. 2003), but simply replicating this successful method with parasitic wasps was not possible.

The conditioning arena setup was inspired by setups described in earlier research (Pérez-Maluf & Kaiser, 1998; Tertuliano et.al., 2003), with a few alterations. This previously described setup was used with food as a reward, but within this project oviposition was used instead. This made it necessary to alter the arena to accommodate the oviposition reward and also to ensure that the wasps could be removed easily. The second alteration was needed because of the light toxicity of DNT. To minimize the danger of this toxicity, the air had to be actively pumped out of the conditioning arena. These two major alterations were made out of necessity. However, the main function of the conditioning arena was to blow the odour over the wasps while they came into contact with the reward and it was thought that this main function was not lost despite the alterations. However, seeing the conditioning arena method was not successful in conditioning the wasps, where the methods on which it was based were successful (al be it with other odours and wasps) raise the question if the alterations did indeed alter the function of the arena too much.

At first a lot of effort was put into standardizing as much as possible all the factors that influence conditioning. The conditioning arena was built specifically for this purpose. By standardizing all these factors, it would be possible to study independently how all these factors influence the conditioning. Controlling these factors proved more difficult then anticipated. The conditioning arena showed that a lot of factors were not properly standardized. Especially the timing of the odour administration proved very difficult to standardize. This was largely due to difficulties with getting the wasps into the area where conditioning was to take place.

However, even though the conditioning trials were not as standardized as expected, they were thought to be sufficient nonetheless. Therefore, it was expected that the wasps would at least show some form of CR or otherwise indicate a degree of associative learning. The absence of this raised serious questions, not only about the conditioning method, but also about the ability of the wasps to learn the odour.

Because the conditioning arena was only used with *Cotesia glomerata* these problems could very well originate from the wasps and not from the method used. At the time of the conditioning arena only *C. glomerata* was available to use in the research, therefore the conditioning arena could not be tested with other wasps. The choice to change methods was made out of necessity, because of the unavailability of other wasp species to test with the conditioning arena. By the time other wasps became available, the focus of the research had shifted from the question of which method is most suitable, towards a more primary question: can the wasps be conditioned on DNT? The choice was made not to rebuild the conditioning arena when these other wasps became available, as this method was thought to be more suitable to test a particular conditioning method after it was established that the wasps could indeed be conditioned on DNT. As this question was not answered to satisfaction and because of time issues, the conditioning arena was eventually not used with other wasps.

The Nasturtium tests were based on a method that had some success in the past. However, that method was used mostly with natural plant odours (using the leaf of the corresponding plant with the conditioning). With this test this was not possible as DNT is not a natural plant odour. This meant finding a way to place the odour on the plant leaf. Spraying or pipetting the DNT solution on the plant gave some problems itself, as the solution was made with dichloromethane, which has some corrosive effect on leaves and also on the caterpillars for that matter. The method was still usable, but the effect of corrosion on the odour coming from the leaf could not be measured and it could be that the corrosion of the leaves was a part of the cause of the reluctance of the wasps to get on the leaf.

The later tests with the olfactometer and with conditioning on filter paper seemed to indicate that the wasps show this reluctance to the DNT odour whether a leaf is used or not. However, it seems that the reluctance was stronger at lower concentrations when a Nasturtium or cabbage leaf was used. The exact cause of this is unclear; perhaps it has something to do with a chemical reaction between DNT and the plant substances.

The wasps seemed to show this reluctance only when confronted with a surface sprayed or pipetted with DNT, not with airborne DNT odour. One possibility is that some other factor then olfactory senses influence the presence or absence of the distaste of the odour. However, this would not explain the fact that the wasps would show some reluctance to get near the sprayed surface even when they had not yet touched the surface. And the wasps also showed reluctance to get in the conditioning arena setup from the tube, which could be caused by the airborne odour (it’s not certain if this was the cause). The olfactometer tests and the tests with the Y-tube indicate that the wasps do not show any form of distaste for the odour when airborne and this information could be used in the administration of the odour in future conditioning trials and testing.

The data obtained for *C. glomerata* in the olfactometer and Y-tube tests need to be considered with certain scepticism. The wasps of this species showed a strong tendency to stay in one place no matter what the circumstances in that spot. This behaviour was already known for this species, but the olfactometer and Y-tube tests were nonetheless the only viable options for this research. The stationary nature of the wasps has most probably altered the outcome of these tests and no definite conclusions can therefore be drawn from these tests. The tests do give at least some indications towards the reaction of *C. glomerata* to DNT odour and conditioning, but the result is not as usable as the results with *D. semiclausum*, which moves around more and is more suitable for these tests.

In previous research with *Microplitis croceipes* where DNT was used (Olson et.al., 2003) the wasps were conditioned using food and not oviposition. Within this project the choice was made to use oviposition. However, other research with *M. croceipes* has shown that the wasps can be conditioned with oviposition experience as well (Meiners et.al., 2003). Therefore, this use of a different reward was expected not to have a big impact on the effectiveness of the conditioning of the wasps. Using oviposition as a reward required no alterations to the rearing program of the wasps and was therefore found to be easier to use in the case of this project.

4.1. Answer to research questions

Most suitable wasp

The question of which wasp is the most suitable is the main question for this research, but a satisfactory answer was not found for this question. Two of the four species to be tested were not sufficiently available to be tested properly. Also, the third species (*Diadegma semiclausum*) only became available later on in the research. Some comparison can be made between *Cotesia glomerata* and *D. semiclausum*, but only with regards to the last two test setups used, the olfactometer and the Y-tube. Neither species showed any aversion reaction to the odour in the olfactometer, therefore not indicating any difference between the species on this level. The Y-tube tests showed that *D. Semiclausum* seems to have a higher reluctance to contact DNT on a filter paper, but if this is a result of the method or a characteristic of the wasp is not clear. Therefore, the question which of these two wasps is most suitable can not be answered in a satisfactory or significant way.

With the results of this project it may not be possible to compare species and make any conclusions on which is the best one to be used, but the extensive testing with *C. glomerata* gives us at least some information about the suitability of this species. The occasional *C. glomerata* that was successfully conditioned still didn't show any response to the odour. This, combined with the great difficulty in training the wasps and the contradictory results of the olfactometer, leads to the conclusions that *C. glomerata* is definitely not suitable for use with DNT. This much can be said, even though the reasons for this unsuitability are still relatively unclear. Further research with *C. glomerata* may indicate the reasons for these problems, but until these reasons can be researched and be used to solve the problems, it would be unwise to use *C. glomerata* as bio-indicator for explosives.

Conditioning method

Cotesia glomerata is the only species of the four to be extensively tested with multiple different methods of conditioning. All of the tried methods had their drawbacks and problems, but they all had in common that the wasps that were successfully conditioned showed no response to the DNT odour when tested in the wind tunnel or otherwise. This could be the fault of the methods used, indicating that none of these methods is suitable. But, as no other wasp species were conditioned with the same number of methods, one can not rule out that it was because of some problem with this species that caused the disappointing results. Therefore, the question of which method is most successful also cannot be answered in a satisfactory way, although testing indicates that the tested methods are at least not suitable for *C. glomerata*.

Maintaining the wasps

None of the wasps were successfully conditioned to associate DNT odour with a reward. Therefore the question about maintaining the wasps as a bio-indicator could not be addressed.

4.2. Difficulties with the research

The main problem encountered during this project was the unavailability of some of the wasp species. *Cotesia glomerata* was the only wasp of the 4 chosen in advance which was available for the entire length of the project. *Diadegma semiclausum* did become available later on, but even then only two species could be extensively tested. *Microplitis croceipes,* the one wasp with which previous similar research has been done was not available, while this species especially would have been a very useful species for reference purposes and comparison with literature. Because of this unavailability of the wasps, the main research question could not be answered fully, only the usefulness of *C. glomerata* could be tested extensively enough to reach some conclusions.

The other main problem in the project was the reluctance of most of the wasps to even come close to the odour. The reason for this can be a lot of things (toxicity of the odour or aversion to the odour among other things), but this problem did cause the conditioning to be a lot more difficult then initially anticipated. Also, the wasp species that was mainly used, seemed to show the most reluctance to the odour, compared to the (small number of) wasps tested of other species. The combination of this reluctance of *C. glomerata* and the unavailability of the other wasps limited the options within the project.

4.3. Recommendations & future projects

Cotesia glomerata seems to be of limited use for the purpose of bio-indicator, but other species might be better suitable for this purpose. Therefore the first course of actions to further this research would be to actually try other species. Some research has been done with *Microplitis croceipes* on this subject (Olson et. al., 2003) and including this species in the project would give a frame of reference for use with other wasp species. Also, the small number of *Pimpla turionellae* in this research project didn't seem to show the same reluctance as *C. glomerata*, indicating that this wasp species would at least be easier to condition. The testing of other wasps would give a better perspective on the viability for using parasitic wasps as bio-indicators for explosives.

Another angle for future research is the type of conditioning method used. Within this project only a small amount of methods was used and most of these with only one species. The second research question for this project; which method is most suitable, is a very important for actually implementing the wasps in the field. The better the method the more practical the use of wasps as bio-indicator would be. To establish this, a much wider range of methods needs to be tested to give a better view of how to implement them.

An aspect of the method used is the reward (or US) used to condition the wasps. In this project only oviposition was used. An angle for future research could be to use food in comparison with oviposition.

These other methods could also be tried on *C. glomerata*; this could give some indication on the reasons for the problems in this project, and may even indicate a solution to the problems with *C. glomerata*. The reasons for the reluctance of *C. glomerata* to come near DNT could be a nice angle for future research, even if it would not contribute directly to the main goal of using wasps as bio-indicators.

5. Literature

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