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TITLE:

A Wind Tunnel for Odor Mediated Insect Behavioural Assays

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SUMMARY:

Here, we describe the construction and use of a wind tunnel for odor mediated behavioural assays with insects. The wind tunnel design facilitates the release of odor sources by several methods, with and without visual stimuli. Wind tunnel experiments are important methods to identify behaviorally active volatile chemicals.

ABSTRACT:

Olfaction is the most important sensory mechanism by which many insects interact with their environment and a wind tunnel is an excellent tool to study insect chemical ecology. Insects can locate point sources in a three-dimensional environment through the sensory interaction and sophisticated behavior. The quantification of this behavior is a key element in the development of new tools for pest control and decision support. A wind tunnel with a suitable flight section with laminar air flow, visual ques for in-flight feedback and a variety of options for the application of odors can be used to measure complex behaviour which subsequently may allow the identification of attractive or repellent odors, insect flight characteristics, visual-odor interactions and interactions between attractants and odors lingering as background odors in the environment. A wind tunnel holds the advantage of studying the odor mediated behavioural repertoire of an insect in a laboratory setting. Behavioural measures in a controlled setting provide the link between the insect physiology and field application. A wind tunnel must be a flexible tool and should easily support the changes to setup and hardware to fit different research questions. The major disadvantage to the wind tunnel setup described here, is the clean odor background which necessitates special attention when developing a synthetic volatile blend for field application.

INTRODUCTION:

The wind tunnel is an important tool in insect chemical ecology studies that allow laboratory testing of insect flight responses to semiochemicals. By releasing odors into a controlled wind stream, the insects' behavioural response to these stimuli can be directly monitored by studying their upwind flight towards the source. Olfaction is the most important sensory mechanism by which many insects interact with their biotic environment[1]. Insects use odor cues to find suitable partners for mating. Similarly, they use odor bouquets from host resources to find food for themselves, or the offspring. Plants release floral odors in combination with nectar and pollen rewards to secure insect pollination efficiency. All these volatile cues diffuse passively into the environment and insects need to identify and interpret their individual relevance. As volatiles are released into the environment, the molecules travel with the wind as filaments, retaining the initial concentration for long distances downwind, before eventually being broken up and diluted by turbulence and diffusion[2]. Insects can detect minute changes in the volatile signal and direct their movement upwind, towards the source. Insects display a flight behavior with fast upwind surges when in contact with an attractive odor, and casting sideways upon the loss to relocate the odor plume[3,4]. The co-localized arrangement of olfactory neurons in the sensilla of the insect antennae can facilitate behavioral responses to the onset and loss of plume contact with remarkable high resolution[5] and enable the insects to distinguish between similar odor molecules originating from different sources[6]. Visual feedback while in flight, termed optomotor anemotaxis, is fundamental to identify wind direction, objects and relative displacement[2,7]. By the use of sensory interaction and sophisticated behavior, insects can locate point sources in a three-dimensional environment.

The identification of insect attractants and repellents can have several important applied aspects. Sex pheromones (intraspecific signals) of many pest insects can be synthesized and released into the air to disrupt the mating behavior[8]. Both pheromones and kairomones (interspecific signals) can be used for mass trapping, attract and kill in monitoring traps to give direct information of pest status. Insect repellents, such as for mosquitoes[9], can also be studied in wind tunnel bioassays. These methods play an important part of integrated pest management and decision support systems for farmers.

Wind tunnel bioassays, where the odor mediated behavior repertoire of a species can be monitored, is a powerful method to identify potential new tools for pest control to replace or reduce the impact of pesticide use.

The theoretical reasoning behind the wind tunnel design is thoroughly described[10]. Here, we describe the wind tunnel construction, odor application and flight behaviour that has been used in several experiments to determine the wind tunnel bioassay protocol. The wind tunnel (**Figure 1**) at Nibio (Ås, Norway) is constructed from scratch resistant transparent polycarbonate. The flight arena is 67 cm high, 88 cm wide and 200 cm long. In front of the flight arena, there is an additional polycarbonate section, 30 cm long. This part of the wind tunnel serves as a utility section for the application of odors. If the volatiles get into contact with the polycarbonate housing in the flight arena, they may later on be re-released and contaminate between sessions. On each end of the utility section, there is therefore a perforated metal grid. Both grids restrict the airflow and create a slight overpressure on the

upwind side. This results in increased laminar flow on the downwind side. The upwind grid is made from a perforated metal plate with 8 mm holes evenly dispersed across the cross section of the tunnel to provide 54% open area. The downwind grid has holes of 3 mm and a 51% open area. This reduces the turbulence and ensures that the odor plume travel centrally down the length of the flight arena. The odor plume will have the shape of a narrow cone and can be visualized by the use of smoke. On the floor of the flight arena, plastic or paper circles of varying sizes (from 5 to 15 cm in diameter) are laid out to give insects visual feedback during the flight. There is a 25 by 50 cm access door on the upwind end of the flight arena and in the utility section. Between the downwind end of the flight arena and the exhaust filter section, there is a 60 cm open area for insect handling. This access area is covered on the sides with a 0.8 mm meshed fabric to prevent the insects escaping into the room.

Air is drawn into the first filter housing by a fan. The air passes through a dust filter before it is purified by 24 high capacity active charcoal filters and released in the tunnel. The air exiting the tunnel is passed through a similar filter housing before being released back into the room. It might be beneficial to exhaust the air to the outside of the building through a fume hood. The fans on both filter housings are run with equal flow. Both fans have a continuous dimmer switch and are calibrated to different wind speeds using a flowmeter. The air speed is dependent on the species tested. 30 cm s^{-1} is often a good starting point. For small insects, the ideal air speed may be reduced, and for strong flyers, the airspeed can be higher to increase the relative flight distance.

The wind tunnel room facilitates the control of temperature, humidity and light intensity. LED strips are placed behind a 3 mm opaque poly(methyl methacrylate) pane to create a diffuse light source above and behind the flight arena. Both light sources can be controlled independently.

Odor application can be achieved by several means. Generally, odors are released into the airflow in the center of the upwind end of the flight arena. Depending on the research questions at hand, the release point can be exposed or covered. A glass cylinder (10 cm diameter, 12.5 cm long) with a metal mesh (2 × 2 mm mesh size) on the downwind side can visually block the odor source and at the same time serve as a landing platform for insects. In many experiments, a horizontal glass platform can be used for presenting odor sources, or visual signals close to the release point. There is also the opportunity to release two odors at the same time, side by side, to facilitate choice assays. The release points are then placed 20 cm apart and the odor plumes overlap from halfway down the tunnel. The choice can then be identified by which plume the insect is following upwind.

The wind tunnel design facilitates numerous volatile release methods. For example, a specific odor can be released in front of a background odor such as emitted by a crop plant[11,12]. Also, different visual stimuli can be tested[13,14]. The experimental setup must be adapted to each species and research question.

Natural odor sources, such as plant parts and synthetic odors from dispensers can be introduced directly into the flight arena. To isolate odor mediated behaviors from visual, the odor source can be covered, or the volatiles carried into the flight arena via a charcoal filtered laboratory air supply from the outside. The odor source is then confined to a glass jar and the

air is pushed through the jar into the wind tunnel via Teflon tubes and glass pipes. The airspeed at the release point should match the wind speed in the arena.

To release odors at specific blend ratios, a sprayer can be used. The sprayer is an ultrasonic nozzle with a conical tip and an inserted microbore to facilitate a liquid flow at 10 µL min^{-1}. The nozzle is connected to a broadband ultrasonic generator and operates at 120 kHz. A syringe pump is pushing the odor sample into the sprayer nozzle. Fluorinated ethylene propylene (FEP) tubing with 0.12 mm inner diameter is connecting the 1 mL gastight syringe and the nozzle. Tubing adaptors that swell in ethanol and shrink in air, facilitate tight fitting with no internal volume. The aerosol droplet size generated from the vibration of the nozzle is frequency dependent and depends on the specific solvent used. The small droplets evaporate and are brought down the wind tunnel as volatiles. Other sprayer designs also exist and a cheaper version utilizing a piezo driven glass capillary provides a similar solution[15].

Synthetic mixes or headspace collections can be used with the sprayer. The samples are diluted with pure ethanol to the desired concentrations. With volatile collections, the sample can be diluted to correspond to the collection time. This means that a volatile collection sampled over 3 h should be diluted to 1800 µL, which at a release rate from the sprayer at 10 µL min^{-1} corresponds to 3 h.

The identification of the flight behavior can be done directly by manual observation or by post hoc video analysis. The oriented flight should be distinguished from random flight. Odor mediated behavior can be recognized by the following characteristics: zig-zag flight across the odor plume, straight upwind flight when inside the plume, and looping back if the contact with the plume is lost. Upon the loss of an attractive plume, the insects can also start to zig-zag with increasing arches to reconnect to the lost plume[3,4]. This behaviour is fundamental in a field setting where the insects following an attractive odor need to cope with turbulence and shifting wind directions. The flight pattern is not uniform and varies across the insect orders. As an example, strong flyers such as blowflies have a faster upwind orientation with wider casting pattern than moths, and the wind speed should be increased to facilitate a longer relative flight path.

The flight of an insect can also be filmed. With a single camera, simple flight characteristics can be described by plotting the x y coordinates[16]. By using two cameras with synchronized frame capture, the 3D flight can be reconstructed using an external software[17]. The flight track can then be analyzed to give information about the flight speed and distance, the flight angles with respect to wind direction and the details about the flight characteristics in relation to the odor plume. There are both custom and commercial equipment and software available which enable automatic frame by frame tracking. The calibration frames should be used to reference real world space, and rectilinear wide-angle lenses should be used to minimize lens distortion. Care should be taken to reduce visual background noise, such as edges and corners in the wind tunnel arena, and to maximize insect to background discrimination. By using an infrared light source, the reflection (*e.g.*, from nocturnal mosquitoes) can be filmed with monochrome CCD cameras[17].

PROTOCOL:

1. Preparing Glass Tubes

1.1. Prepare the glass tubes (*e.g.*, 2.8 cm diameter, 13 cm long) and close one end with a plastic snap cap.

1.2. Separate 10 insects into the capped glass tubes and cover the remaining end with gauze using a rubber band. Allow the insects to acclimatize to the temperature, light conditions and humidity of the wind tunnel room for at least 2 h.

Note: The number of insects inside each tube depends on the species and research question.

2. Preparing Odor Sources

2.1. Sprayer protocol.

2.1.1. Fill the 1 mL gastight syringe with a diluted headspace collection or a synthetic odor mix.

Note: The content of the diluted headspace or synthetic odor mix depends on insect species and research question. In general, the concentrations used should correspond to the natural release rates of the authentic odor source.

2.1.2. Connect the syringe tip to the FEP tubing using tubing adaptors and insert the syringe into the syringe pump.

2.1.3. Start the syringe pump.

2.1.4. Start the broadband ultrasonic generator.

Note: The release of the aerosol from the sprayer can be confirmed by pointing a torch light from below the release point.

2.1.5. Run the sprayer with 96% ethanol for a minimum of 10 min between the treatments to clean the inside of the tubes and nozzle. Use a separate syringe, dedicated to pure ethanol, for all cleaning.

2.1.6. Clean the syringes and the tip of the nozzle with 96% ethanol after use.

2.2. Authentic odor source protocol.

2.2.1. Insert the authentic odor sources into the wind tunnel or into a 2 L headspace collection jar.

2.2.2. Connect the headspace collection jar to the laboratory air flow and release into the wind tunnel.

Note: Collect plant material as close to the start of the experiments as possible and prevent wilting by inserting the cut end into a small vial with water. The amount and type of plant material, or other authentic odor source, depends on insect species and research question.

2.3. Position the glass tube with the insect(s) onto a holder 180 cm downwind from the odor/visual source and 30 cm from the ground. The capped end should point upwind.

3. Starting the protocol

3.1. Use two cameras to capture two different views. Mount the cameras above the flight arena and angled to capture two different views.

3.2. Open the cap.

3.3. Start the timer.

Note: Different species require different time frames to respond, *e.g.*, the apple fruit moth (*Argyresthia conjugella*) will respond within 4 to 5 min[11], but the grapewine moth (*Lobesia botrana*) require up to 20 min to respond[18].

3.4. Observe the flight pattern and pay special attention to the flight characteristics and upwind orientation. Score the flight performance according to predefined behavioral categories, *e.g.*, take off, oriented flight over a short distance (minimum 20 cm), oriented flight over longer distance (< 5 cm from the source) and landing.

Note: Filming and 3D tracking using two cameras can be used to give more detailed information on the flight characteristics. In general, the cameras are mounted above the flight arena and angled to capture two different views.

3.5. Collect the insects landing on the walls of the wind tunnel, outside the odor plume, and replace back onto the holder.

Note: The insects can be given one chance to respond to odors with upwind flight and not be replaced.

3.6. Change to clean glass hardware between the treatments.

Note: Frequent control treatments should be run to identify possible contamination sources.

3.7. Euthanize the insects with CO_2 or by freezing after the experiments.

Note: Depending on the research question, females can be scored for egg development or visually inspected to verify the condition of wings or antennae.

4. Cleaning

4.1. Wash all metal and glass hardware with ethanol and water and leave until dry.

4.2. Heat all metal and glass hardware to 300 °C for 6 h to remove the contaminants.

REPRESENTATIVE RESULTS:

Blowflies responds strongly to odors from dead animals which represents an ephemeral larval growth substrate[19,20]. Using dead mice as a natural odor source, we investigated the details of the flight behavior of 15 day old, mated female *C. vicina,* with or without, a visual stimuli next to the odor release point[13]. To eliminate the natural visual cue, we used the glass jar system described above. With a wind speed of 30 cm s^{-1} blowflies displayed, more than 80% take-off and oriented flight behavior in the downwind end of the wind tunnel and more than 60% of them advanced with controlled and oriented movement into the upwind part of the tunnel (**Figure 2**). There was no significant effect of the visual cue on oriented flight behavior. However, to efficiently display the full range of behaviors that under natural conditions will provide blowflies with the opportunity to deposit the eggs on a carcass (take-off, oriented flight and landing), an addition of a visual cue is needed to mark the odor source. The use of a visual cue significantly increased landings at the source from 14% to 40%. This simple extraction of a larger wind tunnel experiment[13] illustrates the need for a full account of the sensory modalities used in resource location by insects, and it shows that expanded information regarding behavior may strengthen the results and subsequently allow more knowledge to be extracted from each experiment.

To answer which volatile cues the pea moth is using to find a suitable host plant for oviposition, we studied the upwind orientation of 5 – 7 day old mated females towards pea plants in different phenological stages (leaf, bud, flower, pod) in the wind tunnel[14]. Wind speed was 30 cm s^{-1}, light intensity 1000 lux, temperature 20 – 22 °C and RH 60 – 70%. We used a combination of visual and olfactory cues by placing the living plants directly into upwind end of the wind tunnel arena and comparing the behavioural response with the corresponding headspace extracts. We also tested a synthetic blend of ten antennaly active pea plant volatiles. The behavioural response of the pea moth (**Figure 3**) showed that mated females were much more attracted to pea plants with flowers (58%) and buds (52%) than to pea plants in the leaf (10%) or the pod stage (24%). Similar responses were observed using the corresponding headspace extracts. Mated females were most attracted to the headspace extracts obtained from flowering pea plants (56%), followed by pea plants with buds (42%), and low responses were recorded for the pod (28%) and the leaf stage (10%). Testing a sprayed syntheic blend of ten antennaly active pea volatiles as stimuli, resulted in a landing response of 34%.

The results show the link between host plant phenology and the corresponding plant odor, as well as its impact on the behaviour of mated *C. nigricana* females. Mated females have a clear preference for pea plants during flower development and the associated odor profile is crucial for the host location. Moreover, this experiment is showing that mated *C. nigricana* females can distinguish between different phenological stages of pea plants when sensing only volatile cues. Sensory integration is important for host location and may increase the ability to percieve minor differences, particularly in females[13,21,22]. Nevertheless, in this wind tunnel experiment, the landing responses on headspace extracts from flowering pea plants (56%) without the presence of visual cues were the same as the responses to the real plant

(58%). The similarity between headspace extracts only and real plants imply that the odor is the fundamental host plant cue for mated *C. nigricana* females.

One of the main challenges when developing kairomone lures from wind tunnel behavioural assays, is translating the finished blend into the field environment. The wind tunnel has a clean odor background, whereas the field conditions are permeated by odors from the surrounding vegetation which may alter the chemical information.

Experiments in the wind tunnel was performed with field collected females at the wind speed of 30 cm s^{-1}, light intensity of 5 lux, temperature of 19 – 20 °C and a RH of 55-65%. The experiments with and without background odors aided in the development of a plant volatile based field lure for the apple fruit moth *Argyresthia conjugella*[11]. The results show that the odor dispensers with a complex blend (7 components) and a simple blend (2 components) have similar upwind attraction when presented alone on a clean background (**Figure 4A**). In a choice assay, however, with the field dispensers embedded in an apple volatile background, the apple fruit moth females preferred the complex but not the simple blend (**Figure 4B**).

The results show that the blend complexity is a key player to overcome the plant background influence and that the background interaction needs to be considered when developing kairomones for field use.

FIGURE LEGENDS:

Figure 1. Schematic of wind tunnel located as NIBIO, Ås – Norway. The wind tunnel is placed in a climate controlled room. The airflow is filtered by activated charcoal filters before and after odor application, and is then circulated back into the room.

Figure 2. Average (±SE) percent behavioural response of *Calliphora vicina* to natural odor sources with and without visual stimuli. The odor stimuli was confined to a glass jar and introduced into the wind tunnel by a charcoal filtered air stream. This figure has been modified from [Aak, A. & Knudsen, G. K. (2011) Sex differences in olfaction-mediated visual acuity in blowflies and its consequences for gender-specific trapping. Entomologia Experimentalis et Applicata 139 25-34]. Significant differences are identified by t-tests (significance level: p = 0.05) on a total of 50 flies per experimental treatment, with the averages based on the proportion of responders among 10 flies tested on five separate days.

Figure 3. Percent landing response (±SE) of *Cydia nigricana* to pea plants in different phenological stage, the corresponding headspace collection and a synthetic volatile blend of 10 antennaly active compounds. The headspace and the synthetic blend was released from an ultrasonic sprayer. The plant material was placed directly into the flight arena. This figure has been modified from [Thöming, G., Norli, H. R., Saucke, H. & Knudsen, G. K. (2014) Pea plant volatiles guide host location behaviour in the pea moth. Arthropod-Plant Interactions. 8 (2), 109-122]. Significant differences are identified by ANOVA (significance level: p = 0.05). For all treatments, at least 50 females were tested and the moth had 6 min to respond to the odor.

Figure 4. Percent *Argyresthia conjugella* approaching complex and simplified attractants (<5 cm). (A). Upwind attraction to field dispensers without the background. (B). Upwind

attraction to field dispensers embedded in the plant volatile background. This figure has been modified from [Knudsen, G. K. & Tasin, M. (2015) Spotting the invaders: A monitoring system based on plant volatiles to forecast apple fruit moth attacks in apple orchards. Basic and Applied Ecology 16 (4), 354-364]. Significant differences are identified by ANOVA (significance level: $p = 0.05$). For all treatments, at least 45 females were tested and the moth had 5 min to respond to the odor.

DISCUSSION:

The wind tunnel is a helpful tool for identifying both attractive and repellent odors for many insects[4,9]. With sound knowledge of the ecology, biology and behaviour of the insect studied, its flight characteristics can be easily identified and the environmental conditions, wind speed, visual stimuli and odor application can be tailored to fit. It is recommended when starting out with a new species, to fine tune the wind tunnel parameters using the most attractive source possible. With kairomones, this is usually live host plant material or a natural food source and with pheromones, a caged calling female or male (depending on the insect studied). To identify the repellents, there is also a need for an attractive source on which to measure the antagonism[9]. These initial results will also serve as a baseline for further experimentation.

The wind tunnel dimensions should be considered to give room for free movement and display of innate flight characteristics. With strong flyers and large insects, large flight arenas may be necessary. For smaller insects a portion of the arena may be used. The wind speed may be specific to the insect species studied and should be adjusted to fit the flight capacity. With all odorants, but in particular with pheromones care should be taken to avoid contamination and frequent control treatments should be made to avoid false conclusions.

The activated charcoal filters will supply a blank canvas for the application of the desired odors. But there may be a flip side to clean laboratory conditions, because natural background odors may interact with the developed field dispensers[11,23]. Translating wind tunnel blends of ubiquitous volatiles to the field conditions may then be less straight forward and options to include background odors in the wind tunnel should be considered. With pheromones, which are distinct volatiles, the translation to field conditions are less of a problem. This also applies to volatiles from rare resources, such as cadaver kairomones utilized by Calliphorid flies[24].

The sprayer is an excellent tool for releasing volatiles at known concentrations and blend ratios. The sprayer circumvents the problem of calculating release rates which quickly turns complicated. With sprayed headspace, it is easy to compare the efficiency of a volatile headspace collection by comparing the attraction to living plants and intact plant materials. The content in volatile collections can be identified with a gas chromatograph coupled to a mass spectrometer. The sprayer utilizes a solvent diluted sample. The solvent may interact with insect flight performance and care should be taken to identify the impact. Ethanol is an attractant for some insect species, *e.g.*, woodboring beetles[25]. As the sprayer functions by vibrating at ultrasonic frequencies, care should also be taken to consider the anti-predatory response on certain species capable of detecting bats[26].

Attraction of crawling insects could also be tested in a wind tunnel[27]. A raised platform, parallel to and in the centre of the odor plume could then be fitted inside the arena[28]. However, particular care should be taken to handle contamination issues.

The wind tunnel can also be a powerful tool to validate biotechnological solutions, as a study on genetically modified *Vitis vinifera* plants with altered kairomone emission ratio and corresponding *L. botrana* attraction has shown[22]. Transgenic plants, headspace extracts and synthetic blends were tested for *L. botrana* attraction in the wind tunnel and resulted in reduced attraction compared to control plants of *V. vinifera*[29].

There are, however, some limitations to the use of wind tunnels which should be carefully considered in each particular case. As insects normally are used in the arena only once, species with long generation time is less ideal for wind tunnel experiments. Also red listed species can be controversial to test in laboratory behavioural studies. However, pest insects, for which there is a considerable need for attractive lures, usually have short generation time, and sufficient numbers can be gained from field collection or a rearing protocol.

The wind tunnel certainly has its place in chemical ecology behavioural studies. It can be constructed in various ways, depending on the budget, and functionality can be added to fit with various research questions. The wind tunnel has the benefit of allowing observations and measurements of the flight behaviour of insect responses to odors and other sensory stimuli.

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None

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Figure

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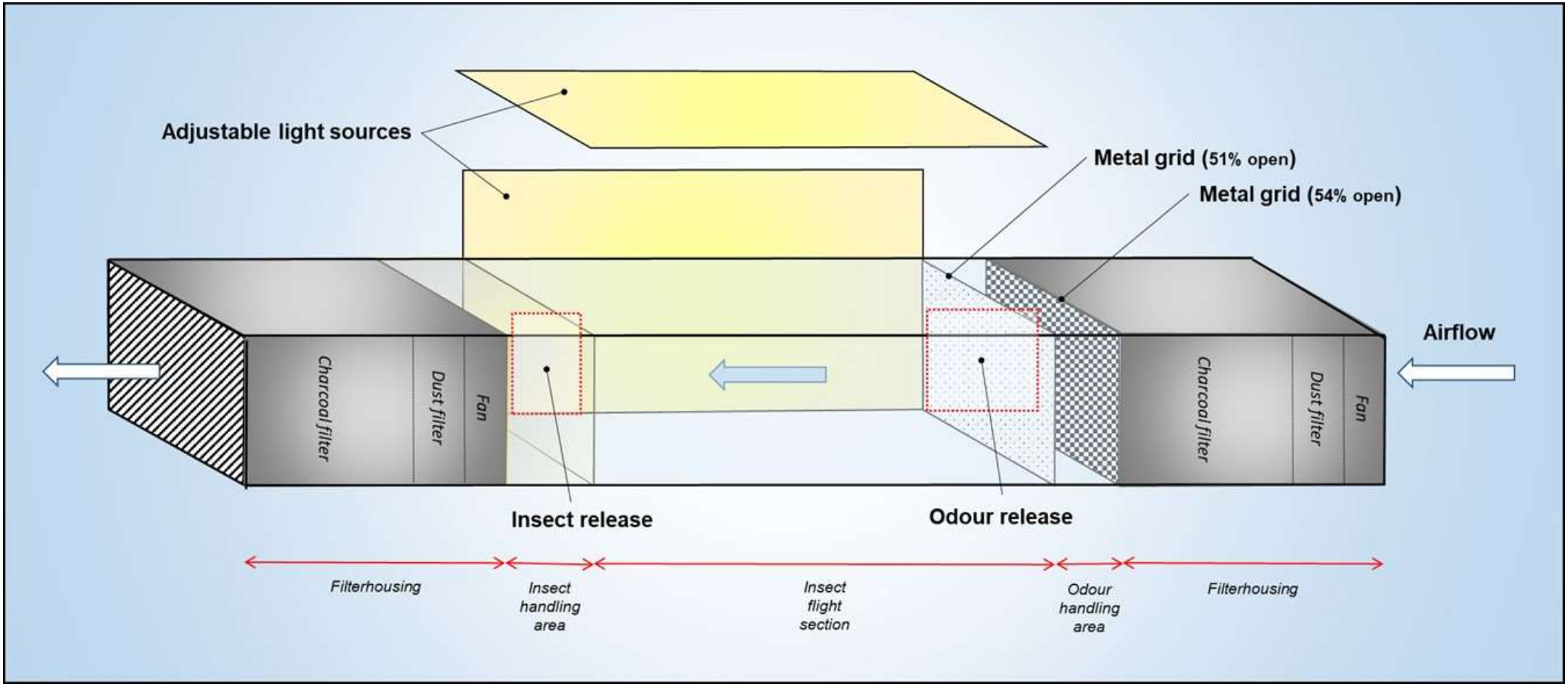

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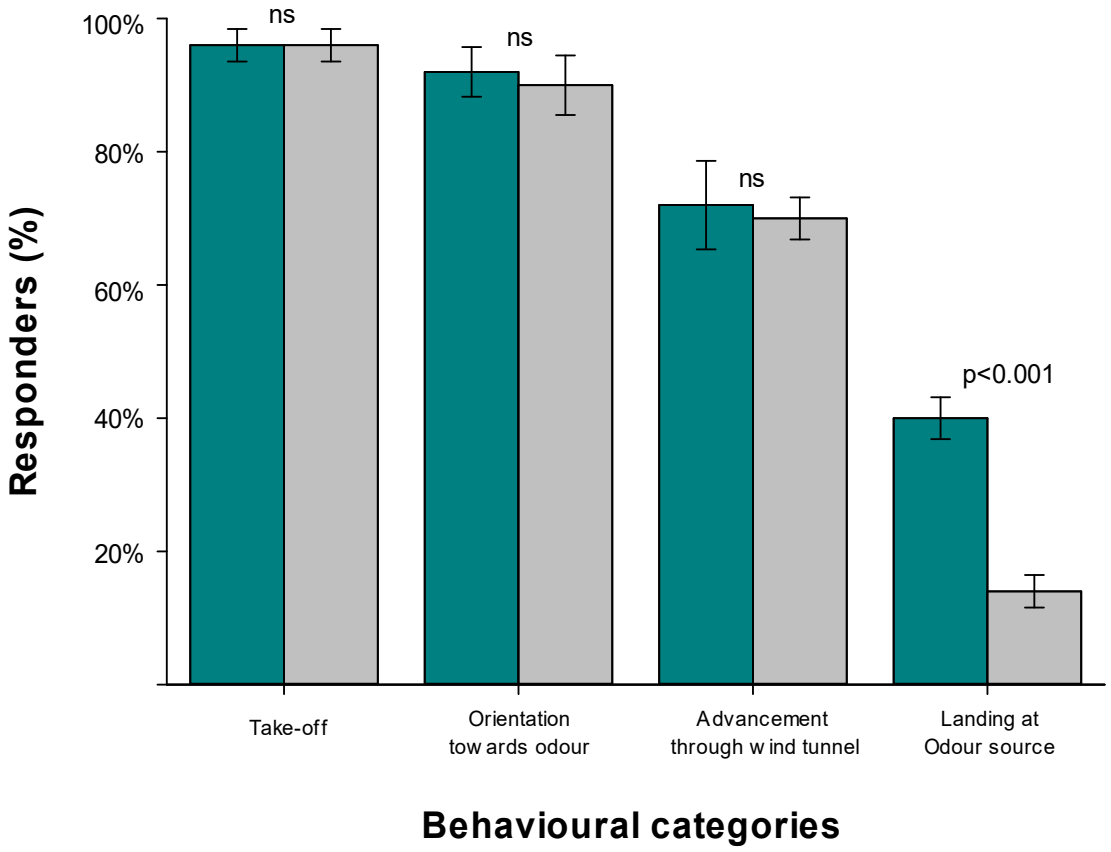

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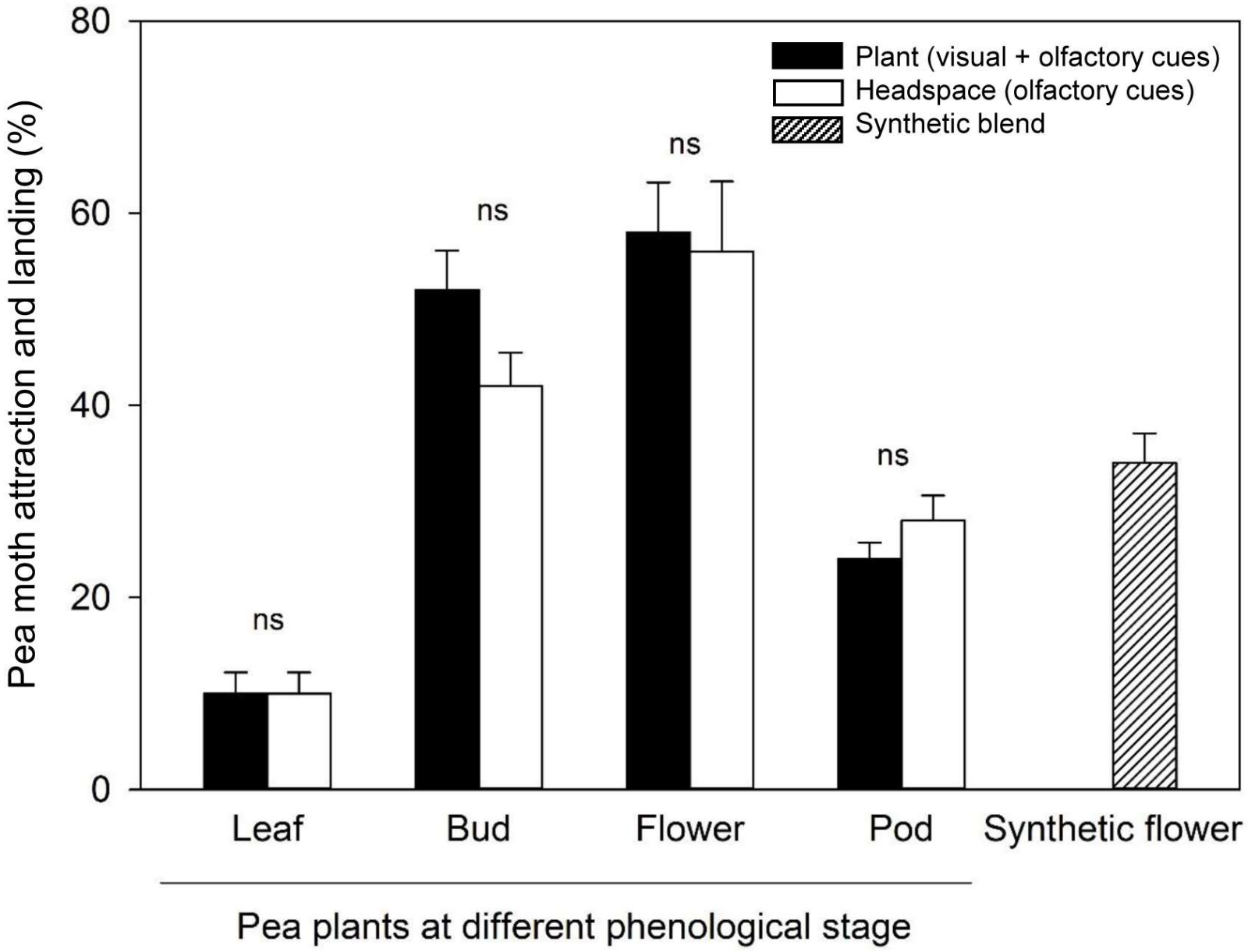

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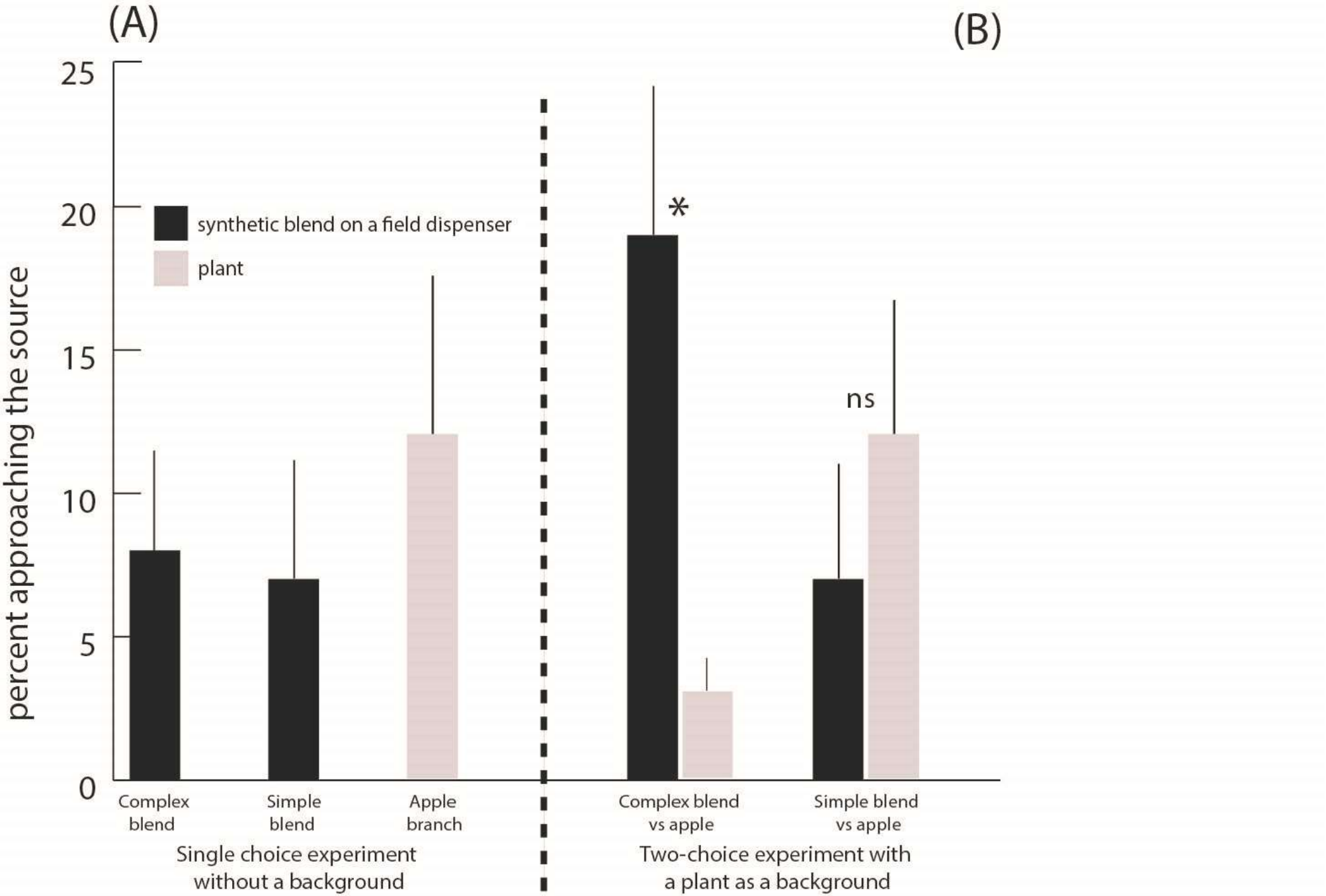

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Flight arena	any	NA	Construct to fit the filter housing
Filter housing x 2	Camfill Farr		Contains the dust and charcoal filters
Fan x 2	Fischbach	Model D640/E35	Silent fan with continous dimmer switch
Perforated grids	any	NA	Two different open areas are needed, e.g. 54 and 51%
Flowmeter	Swema air	Swema air 300	Identifying the wind speed
Ultrasonic sprayer	SonoTek		Sprayer nozzle with conical tip and inserted microbore
Broadband ultrasonic generator	SonoTek		Function generator
Syringe pump	CMA microdialysis	CMA 102	Liquid delivery
FEP tubing	CMA microdialysis		0.12 mm inner diameter
Tubing adaptors	CMA microdialysis		Connectors for zero internal volume
Gastight syringe	any	NA	1000 µL syringe for headspace collections and synthetic b
Gastight syringe	any	NA	1000 µL syringe for cleaning sprayer
Torch	any	NA	Small light source for checking sprayer release
Timer	any	NA	Timer with alarm function
Holder for insect release	any	NA	Metal construction
Lighting	any	NA	LED is preferable due to low heat production
Moisturiser	any	NA	Size depends on volume of wind tunnel room
Temperature control	any	NA	Temperture range depends on species
Glass tubes	any	NA	Tubes (2.8 cm diameter, 13 cm long) for insects
Snap cap	any	NA	Snap cap that fits the glass tube
Gauze	any	NA	Fabric to close the glass tube
Rubber band	any	NA	To hold gauze in place
Glass cylinder	any	NA	Cylinder for odour containment and landing platform (10
Glass jars	any	NA	Glass jars for dynamic headspace collection
Connectors and tubes	any	NA	Tubes and connectors depends on type of glass jars
Air supply	any	NA	From laboratory air or bottles
Charcoal filters	any	NA	For cleaning the outside air sypply
Vial	any	NA	Small vial with water to keep plant material fresh
Oven	any	NA	Heat metal and glassware to 300 degrees to decontamina

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cm diameter, 12.5 cm long)

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Author(s): GEIR K. KNUDSEN, MARIO TASIN, ANDERS AAK and GUNDA THÖMING

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Response to editor

Dear editor,

We have now responded to your comments below. Wind tunnel constructions, and bioassays using wind tunnels, have the benefit of being highly customizable depending on species tested and research question. We feel it is important to convey that this customization is what gives wind tunnel studies its versatility. We have therefore opted to give general descriptions in the protocol. Notes are added to show that several options are available.

1. Step 1.3.1.1: Please provide the detailed preparation procedure or the composition of the diluted headspace collection/a synthetic odour mix.

Reply: A note has been added to show that the odour content of the headspace collection and synthetic mix depends on species and research question.

2. 1.3.2.1: What plant is used? How to prepare the potted plants or freshly cut plant material? How much is used? How to collect the plant materials?

Reply: We have changed 1.3.2 to “Authentic odour source” to recognize the fact that other odour sources than plant material can be tested in the wind tunnel. E.g. for calliphorid flies, mouse cadavers can be used. Point 1.3.2.2 is added, in case a headspace collection jar is used. A note has been added to show that amount and type of odour source depends on species and research question.

3. 1.7: Two cameras are used to monitor the flight. How to set up the camera? Where to set up the camera?

Reply: Added a note on general placement of cameras. The use of cameras is described in the introduction. In general, setup and calibration depends on what type of tracking software is used and manufacturer recommendations should be followed.

4. 1.11: How to wash the hardware? Using what?

Reply: This has been specified.

5. Please sign the new Author Agreement License, which is attached to this email. Please upload it to your Editorial Manager account when you submit your revision.

Reply: This will be sent later. The corresponding author, Gunda Thöming, is away on summer vacation and not available online.

Best regards,

Geir K. Knudsen

Nibio