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MINI REVIEW

Scents and sensibility: Best practice in insect olfactometer bioassays

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Abstract

Olfactometers have been used for more than 100 years and are integral to experimental chemical ecology. Studies utilising olfactometer bioassays form the foundation for understanding the behavioural responses of invertebrates to chemical stimuli under standardised laboratory conditions. Widely used olfactometry apparatuses include two-arm olfactometers for binary responses through to four- and six-arm arenas to evaluate more complex behaviours. Despite its prevalence in chemical ecology studies, there has never been a review of experimental best practice in olfactometry. This review critically evaluates both olfactometry methods and applications as well as experimental design and analysis. We aim to outline a standard of good practice to improve experimental design and reporting for studies involving olfactometry, thereby establishing a reference guide to build a robust experimental workflow for olfactometry bioassays.

KEYWORDS

behaviour, binary response, bioassay, chemical ecology, chemical stimuli, complex behaviours, experimental design, olfactometer, reference guide, standard of good practice, statistical analysis, volatile compounds

INTRODUCTION

The olfactometer is an invaluable tool used by chemical ecologists to study insect behaviour in response to chemical stimuli. Such bioassays are the fundamental first step in characterising the identity and function of biologically active volatile chemical compounds that underpin chemically mediated interactions between organisms. Olfactometry has been extensively used in studies investigating chemically mediated insect behaviour across a range of taxa for over a century (Barrows, 1907; Knolhoff & Heckel, 2014; Barbosa-Cornelio et al., 2019). There are three main types of 'moving air' olfactometer designs used in such behavioural studies: (1) two-arm (Barrows, 1907; McIndoo, 1926; Roberts et al., 2019), (2) four-arm (Hardee et al., 1967; Pettersson, 1970; Vet et al., 1983), and (3) six-arm (Beerwinkle et al., 1996; Turlings et al., 2004). Although less common than their 'multi-way' counterparts, one-way olfactometer designs have also been successfully used to study insect behaviour but these are not a primary consideration for this manuscript (e.g., Piersanti et al., 2014; Bertoldi et al., 2019). In addition to 'moving air' olfactometers, 'still air' olfactometers are also widely used by chemical ecologists (Barrows, 1907; Wood et al., 1966; Weeks et al., 2011). As there are relatively few well-established methods it could be expected that experimental design and statistical analysis of olfactometry data is similarly standardised. However, it can be argued that many published studies have used statistical analyses that do not fully adhere to traditional assumptions required for the type of data collected in olfactometer bioassays.

Appropriate statistical analysis is important and incorrect use may have unintended consequences on the interpretation of olfactometer data, its reproducibility,

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effect size calculations, and any conclusions drawn from such bioassays. As experimental design choices are tightly linked to statistical analysis methods, our intention here is to provide a set of 'best practice' guidelines for researchers carrying out olfactometer bioassays and analysing the data collected from them appropriately to facilitate reproducible research. These guidelines aim to highlight key olfactometer bioassay experimental design challenges and how to implement modern statistical methods into data analysis workflows. We predominantly focus here on insect olfactometry, but the approaches described are more widely applicable to behavioural choice studies of different contexts and taxonomic groups.

COMMONLY USED OLFACTOMETER DESIGNS

A range of olfactometer apparatuses are used to study chemically mediated insect behaviours. Here we describe each moving-air olfactometer design as well as the still air olfactometer and outline how they are typically used by chemical ecologists.

Still air olfactometers

One of the earliest reports of an olfactometer being used in a behavioural bioassay was that describing a simple still air olfactometer (Barrows, 1907). Such olfactometers typically consist of an enclosed arena in which one or more chemical stimuli being investigated are located before adding the study subject(s) (Figure 1A). Behaviour in relation to the stimulus or stimuli is scored in one of several ways: counting study subjects moving toward or touching a stimulus, time spent by study subjects within a pre-determined distance of the stimulus, or time taken for study subjects to respond to the stimulus (Knolhoff & Heckel, 2014). Although many studies have successfully used still air olfactometers for different insect taxa (e.g., Weeks et al., 2011; Hennessy et al., 2022; Pouët et al., 2022), there are experimental limitations to using this apparatus.

Still air olfactometers often present an unrealistic scenario to study subjects as there are few environments with static air (Renou & Anton, 2020). The most significant limitation, however, when using still air olfactometers is that without airflow it is not possible to directly observe anemotactic behavioural responses in study subjects (i.e., those in response to the direction and intensity of air currents) (Kennedy, 1977). Such behaviour is commonly used in combination with chemotaxis to locate odour sources in a process called odour modulated anemotaxis (van Breugel & Dickinson, 2014; Saxena et al., 2017). Using still air olfactometers also limits the ability to accurately control odour dispersion within the olfactometer and maintain an active concentration gradient (Cardé & Willis, 2008). Depending on the experimental hypothesis, this may restrict any conclusions drawn from still air olfactometer bioassays as many insect species use concentration gradients to locate odour sources over 'short' distances (Renou & Anton, 2020). Nevertheless, there are examples where odour source location has been found to be more effective in still air environments compared to those with moving air (Lacey & Cardé, 2012). In all cases still air olfactometers offer a simplified, low-cost method to study insect behaviour toward volatile chemical stimuli.

Two-arm olfactometers (Y-tube, T-tube, and linear track)

Two-arm olfactometers are a widely used design reported in the scientific literature and they are available in several configurations. The idea of a two-choice olfactometer was conceived by Barrows (1907) but the T-tube olfactometer design recognisable today was developed by McIndoo (1926). This design consists of a main tube (stem) that divides into two tubes (arms), creating a T-shape where the arms are opposite one another and the angle between each arm and the stem is 90° (Figure 1B). Y-tube olfactometers are like the T-tube but with each arm meeting the stem on opposite sides at an angle between 130 and 150° as described in Girling et al. (2006) and Kissen et al. (2009). This angle helps to position study organisms so that they are simultaneously exposed to both odours within the two airflows as they meet (Girling et al., 2006). In some bioassays, a wire 'walkway' may be inserted into a two-arm olfactometer to create a linear track olfactometer that controls how the study organism reaches the junction between the stem and the two arms (Sakuma & Fukami, 1985). As the position of a study organism relative to the two airflows as they meet at the junction cannot always be directly controlled, it is important to consider how this may influence their behaviour and therefore any conclusions drawn from a biased experimental set-up.

Air entering two-arm olfactometers is typically charcoal-filtered then humidified by passing it through deionised water before being split into two airflows of equal flow rate (Roberts et al., 2019; Meza et al., 2020). One airflow is pumped over the stimulus and the second airflow is pumped over the control or a second stimulus before being separately pumped into each of the olfactometer arms towards the stem (Roberts et al., 2019; Meza et al., 2020). Study organisms are introduced, either individually (Meza et al., 2020) or in groups (Roberts et al., 2019), into the base of the olfactometer stem where they travel toward the junction between the two arms. Once at the junction between the stem and two arms, study organisms are exposed to the two airflows and may discriminate between them. The preference of a study organism for each stimulus is scored by recording when a point along the arm is reached or when the study organism remains beyond a pre-determined point along the arm for a specific duration (Roberts et al., 2019; Meza et al., 2020).

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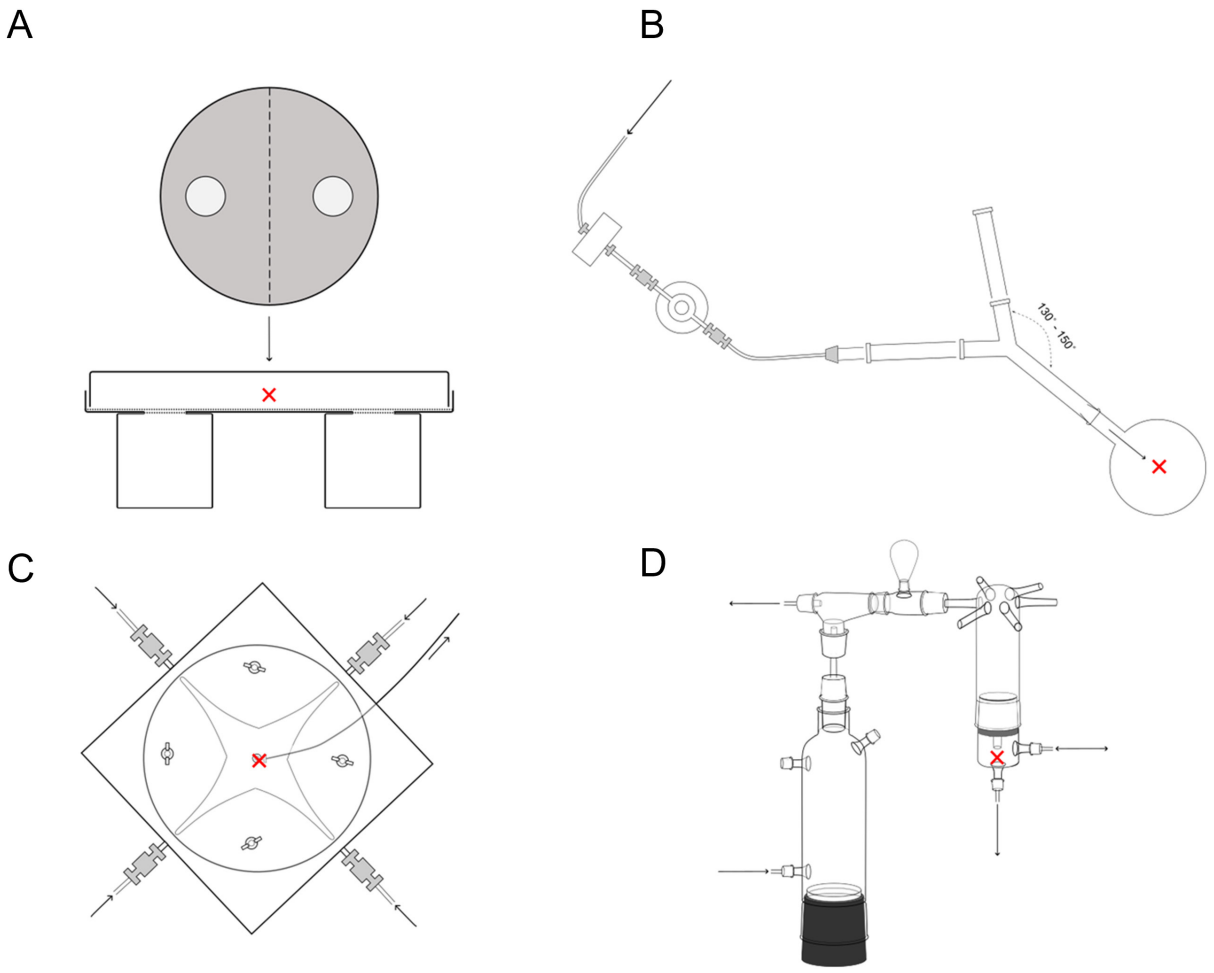

FIGURE 1 Standard designs of (A) still air olfactometer (Weeks et al., 2011), (B) Y-tube (two-arm) olfactometer (Roberts et al., 2019), (C) four-arm olfactometer (Vet et al., 1983), and (D) upright six-arm olfactometer (Turlings et al., 2004), with attached sample chamber. Arrows indicate direction of airflow and schematics exclude pumps, activated charcoal filters, and multiple sample repositories. × = introduction of study organism(s).

Four-arm olfactometers

Four-arm olfactometers are another widely used design. The initial concept and design was conceived by Hardee et al. (1966, 1967) to study the boll weevil, *Anthonomus grandis* Boheman, and subsequently adapted for studying aphid behaviour by (Pettersson, 1970). The Pettersson design was further modified by Vet et al. (1983) to improve the odour field boundaries within the arena and ensure that they are truly discrete. This design is primarily used to study a wide range of small (<5 mm) walking insects but has also been used to study the walking behaviour of flying insects (Figure 1C). These olfactometers have an arena that is connected to four arms and these lead into a central zone. Air is pumped out of the olfactometer through a hole located in the centre of the top or bottom of the central zone, drawing air into the open ends of each of the four arms. In this way the olfactometer consists of four discrete odour fields, one in each arm, plus a central zone where the test subject is exposed to all odour fields before being drawn from the olfactometer by a vacuum pump.

Study organisms are typically introduced individually into the central zone of the arena so they may choose to enter and remain for a period within one of the four odour-fields. It is important to note that once a study organism enters an olfactometer arm it is no longer able to detect the other odour fields present within the olfactometer. The study organism is free to move between each arm of the olfactometer but must pass through the central zone where the odour fields mix to move between each arm. Although four-arm olfactometers can be used to test multiple chemical stimuli simultaneously, it is common to have one treatment arm and three control arms in an olfactometer bioassay (Bruce et al., 2005; Webster et al., 2010; Ali et al., 2021). The logic behind this experimental design is that the observation of choice by a study organism for a single treatment arm is a stronger indication of preference as there is a 25% probability of a study organism choosing the treatment arm by chance compared to experiments featuring two treatment arms as this probability increases to 50%. However, if experimental design incorporates several different treatments (e.g., three arms containing unique

odour fields and one control arm) it is possible to facilitate higher-throughput data collection (Turlings et al., 2004).

Four-arm olfactometer bioassay scoring typically involves recording the cumulative amount of time individuals spend in each arm as well as the number of times each arm is entered. Scoring can either be done manually with proprietary software such as OLFA (Nazzi, 1995) and Noldus Observer (Mizuno et al., 2022) or automatically with Noldus EthoVision (McCormick et al., 2016). There are many open-source alternatives, such as JWatcher (Bertoldi et al., 2019), to the proprietary software packages used in behavioural analysis. This topic is out-of-scope for this review, so please refer to the extensive review of the open-source video tracking software environment by Panadeiro et al. (2021) for further information and guidance on software selection.

Six-arm olfactometers

Six-arm olfactometers are the most recent design, first described by Beerwinkle et al. (1996) to study plant-moth interactions. Turlings et al. (2004) refined the six-arm olfactometer system to be a 'high-throughput' approach that allows for six odours to be simultaneously assessed. This olfactometer design has a central arena with six separate tubes connected to it and typically features a vertical release tube to introduce subjects into the arena (Figure 1D). Like four-arm designs, the chemical stimulus or corresponding control are drawn by negative air pressure through each of the six tubes. Study organisms are released, either individually or in groups, at the bottom of the release tube and any individuals entering a horizontal tube are trapped within a glass bulb fitted to the tube (Turlings et al., 2004). In this way the study organisms selecting each arm can be counted after a pre-determined period.

PRACTICAL CONSIDERATIONS FOR OLFACTOMETER BIOASSAYS

There are no best practice standards published for olfactometer bioassays despite being used in chemical ecology laboratories globally. Variation in bioassay approaches increases the likelihood that studies may be subject to errors that confound data collection and its subsequent analysis. This ultimately impacts the reliability of any conclusions drawn from them, which is elaborated on below in the 'Experimental design and analysis' section after outlining typical olfactometer bioassay considerations.

Olfactometer design

Olfactometer design must be appropriate to the research question and insect species studied. For example, consider whether a flying insect will respond to odours in the same way whilst walking as they do in flight. Although anemotactic responses may be recorded when using a 'moving air' designs (Kennedy, 1977), the odour gradient is flattened along a line toward the source to create a situation like that found at distance in the field. There is typically a steep concentration gradient that permits the detection of chemotaxis or chemoklinokinesis, which are unlikely to occur at distance in the field (Cardé & Willis, 2008). Locomotory responses involved in finding an odour source may be further grouped into those operating at distance and those operating at close-range (Kennedy, 1977). Although both distant and close-range responses include taxis, the mechanisms used are distinct. This is because at close range steep local gradients of odour exist, whrease at distance these gradients become disrupted and shallow. As a result, close-range responses are often chemotactic whereas distant responses may be operational, with directional cues provided by other features such as wind. Olfactometer designs, however, do not discriminate between distant and close-range behavioural responses (Kennedy, 1977). The net effects of several responses (e.g., orthokinesis, klinokinesis, and chemotaxis) are recorded together rather than on an individual basis. This fact may be particularly important for insects where their mode of locomotion is flight at distance and walking at close-range (e.g., aphid parasitoid wasps).

Olfactometers should ideally be constructed from chemically inert materials such as borosilicate glass or polytetrafluoroethylene (PTFE) wherever possible to prevent cross-contamination between replicates through chemical adsorption directly into the olfactometer structure. Although olfactometers should be large enough to not constrain insect movement, they should be small enough to enable the insect to easily explore each odour field within the experimental arena to make informed decisions in the time permitted. Similarly, the air flow inside the olfactometer should be regulated to ensure that it is identical in each arm and appropriate for the size of insect being studied (i.e., smaller insects may require a reduced air flow to limit the effect on their movement) as well as the chemical stimulus being tested (i.e., reduces odour plume turbulence) (Tichy et al., 2020). This can be determined by directly observing insect movement in preliminary bioassays. General information on olfactometer apparatus (size, air flow, sample chambers, etc.) can usually be derived from the wider literature on so called 'model' organisms that have been extensively studied, including *Drosophila* spp. (Diptera) (Faucher et al., 2006; Semmelhack & Wang, 2009; Mazzetto et al., 2016), *Cotesia* spp. (Hymenoptera) (Steinberg et al., 1992; Bogahawatte & van Emden, 1996; Gohole et al., 2003), *Spodoptera* spp. (Lepidoptera) (Carroll et al., 2008; De La Rosa-Cancino et al., 2016; Revadi et al., 2021), and *Schizaphis* spp. (Hemiptera) (Pettersson, 1970; Dawson et al., 1988). However, referring to studies that use closely related insect species may be more valuable to determine key olfactometer bioassay apparatus details if less well characterised

study organisms are being used. It is recommended that pilot olfactometer bioassays are carried out prior to data collection, testing several experimental treatments and olfactometer apparatus configurations building on those identified from the literature to ensure that the apparatus is functional and study organisms behave as expected.

Olfactometer apparatus validation

Movement of air through the olfactometer can be visualised before recording behavioural responses to ensure that odour fields are discrete (Pope, 2004). This is most easily done using a smoke test. Different methods may be used to carry out a smoke test, such as combining the vapours of concentrated hydrochloric acid and ammonia to produce a thick white smoke of ammonium chloride, which can be observed and photographed as it is drawn through the olfactometer (Pope, 2004).

Given the wide range of factors other than odour that may influence insect behaviour within an olfactometer it is important to determine the presence of any directional bias before and during testing behavioural responses to chemical stimuli. This can be done by recording insect movement when controls are placed in each arm of the olfactometer (Turlings et al., 2004) or through observing behaviours such as antennal lateralisation (Sakuma & Fukami, 1985). An appropriate number of replicates, guided by power analysis calculations, should be carried out to draw firm conclusions on any inherent directional bias to determine whether the experimental set-up is fit for purpose and likely to generate reliable data for hypothesis testing (Hoffmeister, 2005). These results can then be used to guide experimental design and limit any sources of directional bias resulting from chemical contaminants or visual interferences within the wider experimental environment. It is good practice to present results for directional bias testing when the olfactometer design has been optimised and, even where there is no directional bias apparent, to alternate the position of the odour source for each pre-defined replicate (e.g., Roberts et al., 2019).

Environmental conditions

The environmental conditions, such as temperature and humidity, under which an olfactometer bioassay was carried out should be reported. Where possible these environmental parameters should be controlled due to the impact that they may have on insect behaviour. As insects are ectothermic organisms, they cannot maintain a constant body temperature through homeostatic processes. The temperature within an olfactometer may therefore interfere with a study organism's metabolism and influence its behaviour (Abram et al., 2017). A secondary effect of uncontrolled environmental temperatures is that study organisms may be responding to temperature gradients within an olfactometer, using thermosensors located on their antennae, rather than the chemical stimuli being tested (Budelli et al., 2019).

Air water content may also influence behaviour inside an olfactometer. This is because many insects can detect changes in humidity through hygroreceptors located on the distal portion of their antennae (Altner & Loftus, 1985; Tichy & Loftus, 1996). Without controlling for the effect of humidity on study organism behaviour, their response to the chemical stimuli being tested may be masked. The influence of humidity on insect behaviour in an olfactometer has been demonstrated by Martinez & Hardie (2009), who showed that an aphid parasitoid preferentially moved toward humid air when given a choice between this and drier air. It is therefore important to consider the chemical stimulus itself and how this might affect airflow humidity (e.g., biological samples, such as plant leaves, vs. a pure compound applied to an otherwise dry piece of filter paper).

Light spectra and intensity should be standardised through use of artificial light sources with similar characteristics to natural light rather than relying on natural sunlight. To facilitate reproducibility, measurements of radiant energy from light sources should include the type of lamp apparatus used as well as the related electrical characteristics (voltage, type of electrical current, etc.). Insect flicker fusion frequencies are also important to consider when choosing an artificial lighting source to illuminate bioassays (Shields, 1989). For example, alternating current using standard ballasts and incandescent lamps should be avoided as they flicker at 120 cycles per s, which is within the 20–300 cycles per s range readily observable by insects (Shields, 1989). Phototaxis, both negative and positive, caused by lighting source flicker has been demonstrated in several insect species from a range of orders (Chu et al., 2006). The impact of light flicker frequencies can be overcome using fluorescent lamps with integral electronic ballasts as these produce an output frequency of 40 kHz, which is outside the known observable range of insects (Shields, 1989). Another consideration is the biology of the study organism and whether light should be excluded from the bioassay arena completely, with nocturnal insects being studied under darkness using infra-red lights or red-light conditions to facilitate natural behavioural responses (Lihoreau & Rivault, 2011).

External stimuli

As olfactometer bioassays aim to assess the behavioural response of study organisms to volatile chemical stimuli it is essential to control, wherever possible, any external stimuli from the bioassay as they may cause directional bias. If these stimuli cannot be controlled, it is difficult to confidently conclude that behaviours observed in an olfactometer are a true response to just the chemical stimuli being assessed. Many insects use visual cues to gain information about their environment, for instance

herbivores (Prokopy & Owens, 1983; Hendry et al., 2018), parasitoids (Cochard et al., 2019), predators (Wang et al., 2015), and pollinators (Rachersberger et al., 2019). To minimise the influence of visual stimuli on study subject behaviour, opaque material screens that surround the entire olfactometer should be used where possible to exclude them. Because a variety of methods can be employed in this aspect of olfactometer experimental design they should be reported in the bioassay description (e.g., Liu et al., 2016; Niassy et al., 2019). Where steps taken to exclude visual stimuli from the olfactometer bioassay are not reported it is often not possible to determine whether the authors have considered this to be standard experimental practice or that it was not a part of the bioassay protocol and could therefore limit any conclusions drawn from the bioassay.

Olfactometer rotation

Another common method of reducing directional bias resulting from external stimuli or environmental factors in four-arm olfactometer bioassays is to periodically rotate the entire olfactometer during the bioassay (Birkett et al., 2004; Bruce et al., 2005; Webster et al., 2010; Ali et al., 2021). This ensures that the treatment arm is moved through 360° during a bioassay, but this process could itself influence the behaviour of the study organism. Insects are known to be highly sensitive to vibrational cues within their environment and rotating an olfactometer during a bioassay is highly likely to cause vibrations that influence study organism behaviour (Polajnar et al., 2015). To prevent this, directional bias should be mitigated using other approaches, such as rotating the position of the treatment arm by 90° between biological replicates rather than during each replicate. Directional bias could also be mitigated by increasing the number of treatment arms in four- or six- arm olfactometers to two, with the treatment arms being identical but directly opposite one another (Turlings et al., 2004).

Stimuli presentation

Chemical stimuli are typically introduced into an olfactometer arena as volatiles originating from either biological material or as purified synthetic chemicals in a stream of purified and humidified air. In both cases the odour source should be placed into a suitable chamber made of an inert material, such as a borosilicate glass chamber or polyethylene terephthalate (PET) bag (Stewart-Jones & Poppy, 2006). Air inlets and outlets should be offset (e.g., top and bottom) so that air is drawn across the odour source and not simply across one section of the odour source. Consideration should also be given as to where air passing through the olfactometer is vented to prevent odours recirculating back into the olfactometer (Du et al., 1998).

Where biological materials are presented, it is important that they are presented in a state that, as much as possible, is representative of conditions found in the field. Mechanical damage to the biological material, such as snapping plant stems or crushing leaves, can affect the odours produced (Dicke et al., 1990). Careful consideration should also be given to the use of appropriate controls when biological samples are used. For example, if a study seeks to characterise the behavioural response of natural enemies to plants infested with potential prey then an appropriate control would be an uninfested, non-prey infested, or artificially damaged plant of the same species rather than clean air (Dicke et al., 1990; Kissen et al., 2009). Where purified chemicals are presented, it is important to consider how the use of different solvents will affect the concentration of the chemical presented and its release rate. For example, use of liquid paraffin (Roberts et al., 2019) is likely to result in the insect being presented with test chemical at a lower concentration for a longer period than if the same test chemical had been presented in hexane (Dawson et al., 1988; Campbell et al., 1993) or diethyl ether (Du et al., 1998).

Odours originating from biological material can also be collected onto an adsorbent and eluted using a standardised volume of solvent whereas synthetic chemical stimuli sources are typically prepared to a predetermined concentration in a solvent. A range of solvents is routinely used by chemical ecologists, but it is important to consider their physio-chemical properties to ensure that chemical stimuli are introduced into the olfactometer arena in a controlled, reproducible way. Chemical stimuli prepared in volatile solvents, such as diethyl ether, have increased release rates. This may result in the study organism being exposed to higher concentrations of chemical stimuli at the start of the bioassay than at the end of the bioassay. Such variation in stimulus delivery over time may increase with bioassay duration or if stimuli are used to test multiple study subjects. Variation in release rates could, therefore, influence insect behaviour due to changes in both stimulus concentration (Roberts et al., 2019) and the ratio (Webster et al., 2010). Preparing chemical stimuli in less volatile solvents, such as paraffin oil, can minimise such effects but care must be taken that release rates are sufficiently high to elicit behavioural responses in the study organism. In recent years there has been work to develop more precise and reliable systems to deliver chemical stimuli to study subjects in olfactory studies (Burton et al., 2019; Tichy et al., 2020; Guo et al., 2022), but these are not widely applied to insect olfactometer bioassays. Other key factors that may influence release rates include environmental conditions, such as temperature (Stipanovic et al., 2004; Zhu et al., 2015) or airflow rate (van der Kraan & Ebbers, 1990) as well as the physio-chemical properties of the chemical stimulus itself (Nielsen et al., 2019). It is, therefore, important to report the environmental conditions in which an olfactometer bioassay is carried

out and to standardise these wherever possible as they impact both insect behaviour and stimulus delivery.

Olfactometer cleaning

Olfactometers should be thoroughly cleaned between replicates to prevent cross-contamination arising from the chemical stimuli being assessed or semiochemicals deposited by a study organism during a bioassay. Initial cleaning should see olfactometers soaked in a diluted fragrance-free laboratory detergent, such as 5% Decon 75, for 15 min to remove frass and other bodily fluids before rinsing with warm water (Salisbury et al., 2012). Additional cleaning steps are largely dependent on the olfactometer material. Glass olfactometers can be rinsed with solvents such as HPLC-grade acetone before baking in a dedicated glassware oven at a minimum of 120 °C for at least 15 min (Bradburne & Mithen, 2000; Roberts et al., 2019). Greater care is required when cleaning plastic olfactometers to prevent damaging their structural integrity, so any additional cleaning should use 'soft' solvents such as ethanol and left to dry within a fume hood rather than in an oven. If glass chambers are used to hold the chemical stimuli sources being tested in an olfactometer experiment, then they must also be solvent cleaned between replicates as described above whereas PET bags should be considered as single use and disposed of between replicates.

Consideration should also be given to other contaminant-prone components within the olfactometer apparatus. Activated charcoal filters used to remove chemical contaminants from the air passing over a chemical stimulus must be periodically regenerated as they have a limited adsorption capacity (Dutta et al., 2019; Roberts et al., 2019). This typically involves heating the charcoal to 220 °C under a stream of inert nitrogen gas for up to 60 min (Dutta et al., 2019). Tubing used to connect each component of the olfactometer apparatus should be constructed from chemically inert materials (e.g., PTFE) as should the connectors themselves (e.g., brass Swagelok fitted with PTFE ferrules) (Roberts et al., 2019).

Physiological state of study organism

The influence of physiological state on insect behaviour is well known (Dawson et al., 1988; Browne, 1993). There are many examples of physiology influencing insect behaviour in olfactometer bioassays, including developmental stage (Reynolds et al., 1986), nutritional state (Defagó et al., 2016), mating history (Saveer et al., 2012), and pathogen infection (Wan et al., 2020). Physiological state should not only be reported but also be a key experimental design consideration for the scenario being simulated in the olfactometer bioassay, ensuring that behaviours observed in response to chemical stimuli are representative of those observed under natural conditions. Careful consideration should also be given to the time of day in which insect behaviour is recorded. Behavioural responses to chemical stimuli can change throughout the day (Weeks et al., 2013), meaning that olfactometer bioassays carried out across multiple days should be temporally standardised (i.e., carried out during the same time period each day) to account for such variability.

Physiological variability, along with any variability introduced due to environmental factors such as temperature, is often accounted for in experimental design and analysis through blocking. A common approach to experimental blocking in olfactometer bioassays is to block by day (Rojas et al., 2006; Logan et al., 2008). Bioassays should be planned so that each day of the bioassay includes a representative sample for the conditions or treatments being tested, but the order in which these are tested each day should be randomised (Casler, 2018). Blocking can also be included in statistical analysis to help identify whether any variation is due to factors other than the chemical stimuli being tested (Casler, 2018).

Releasing study organisms – groups or individuals?

The decision to release insects either individually or in groups when carrying out an olfactometer bioassay depends on several key factors. It is important to consider how study organisms may interact in an enclosed environment and whether this may impact behavioural responses being recorded in the bioassay. For example, Turlings et al. (2004) demonstrated that female parasitic wasps do not influence the behaviour of other females when released in small groups but males in mixed-sex groups preferentially orientated toward females over the chemical stimulus being tested. In experiments where collective decision-making or social behaviours are of interest it may be appropriate to introduce groups of study organisms into an olfactometer, though there are limited examples where this approach has been used (Mburu et al., 2009). Group releases may also be used to increase the throughput of olfactometer bioassays for 'solitary' insects (Turlings et al., 2004; Roberts et al., 2019), theoretically enabling more data to be collected in a shorter time period.

Although there are clearly some advantages to releasing groups of study organisms in olfactometer bioassays, caution must be exercised in analysing such data as the behaviour of individual insects within the same group is not independent and this leads to pseudoreplication (Ramírez et al., 2000). To avoid pseudoreplication in olfactometer bioassays, each group release should be treated as a single experimental unit or replicate, rather than treating each individual insect as a replicate. Although this reduces the effective sample size of an experiment, and therefore the statistical power, it provides a more accurate representation of variability in the data. Statistical methods to account for pseudoreplication are discussed in more detail below.

EXPERIMENTAL DESIGN AND ANALYSIS

Although the principles of experimental design and statistical inference were initially founded in the natural biosciences (Fisher, 1949), the practice of statistics and numeracy training for practitioners is widely recognised as a challenge. There are various probable causes for this that are discussed in the literature (Barraquand et al., 2014; Smaldino & McElreath, 2016), but a few specific and pernicious problems are common and widely recognised, such as pseudoreplication (Hurlbert, 1984), poor statistical power (Cohen, 1992), and poor consideration of the effect size (Nakagawa & Schielzeth, 2010). Although there is broad awareness of these common issues, specifying definitive solutions has proved somewhat challenging. Our goal here is to define key experimental design and analysis issues before highlighting their relevant solutions.

Pseudoreplication

Pseudoreplication is the failure to account for the assumption of independence of observations that can affect both experimental and observational data. This has been highlighted as a frequent problem in different fields, e.g., ecology (Arnqvist, 2020) and psychology (Freeberg & Lucas, 2009). This common assumption of independence relates to the idea of randomisation in experimental design, which in turn relates to veracity of statistical evidence that can be gleaned from a bioassay. Pseudoreplication in olfactometer bioassays has been extensively reviewed by Ramírez et al. (2000). Issues of pseudoreplication in olfactometer bioassays can be mitigated for by ensuring that there is sufficient starting material (e.g., chemical stimuli or study organisms) and using good experimental design to avoid: (1) using the same study individual subject for multiple replicates, (2) using the same odour source for multiple replicates, (3) using the same olfactometer apparatus for multiple replicates without cleaning, or (4) bioassaying groups of study subjects but using individuals from these groups as replicates. It is also possible to account for pseudoreplication using generalised linear mixed models as described below.

Statistical power

Statistical power is typically defined as the probability of correctly detecting a significant experimental effect when the null hypothesis is false (Cohen, 1992). Statistical power is positively associated with sample size, and so the use of arbitrary sample size in experimental designs is rarely, if ever, appropriate. Using arbitrary sample sizes without evidence of effect-size thinking is often the legacy of poor experimental design practices being propagated through the peer-reviewed literature to become an 'accepted' practice. Although statistical power is clearly an important consideration in experimental design, it is a pervasive issue that affects many disciplines beyond insect ethology, e.g., ecology (Jennions, 2003) and psychology (Abraham & Russell, 2008). There is evidence that this practice may stem from insufficient training in numeracy and statistics for young scientists (Horton & Hardin, 2015). One suggestion is to place more emphasis in curricula and peer review on the statistical effect size to guide best practice reform.

The concept of effect size is considered of high importance in good experimental design and scientific practice, being an expectation of the magnitude of difference in a measure relative to observed variation. However, there is little specific training or emphasis on this, even though it has a directly interpretable meaning (e.g., biologically) and can be used to directly compare the strength of results across bodies of work as in meta-analysis. Failure to consider the effect size in experimental design, and to explicitly report it routinely, may contribute to scientific bias by selective publication of studies with marginally significant statistical effects (Head et al., 2015). A general remedy for this is to design curricula in the applied sciences to incorporate 'effect size thinking' into statistics basic training (Horton & Hardin, 2015).

It is possible to estimate the appropriate number of replicates needed for an experiment through power analysis (Ismail et al., 2021). This is a method for determining the number of replicates to detect an effect of a given size with a certain degree of assurance. It considers the variability in the data (e.g., standard deviation), the desired significance level (i.e., 0.05), and the desired power (i.e., 0.8) (Cohen, 1992). Power analysis can be carried out using existing data (e.g., mean time spent in an olfactometer arm and standard deviation) for a study organism that has been reported in the literature or by completing pilot studies to generate data (Cohen, 1992). These data can then be used to calculate an effect size for use in a power analysis.

Statistical analysis

Here we briefly outline remedies for improving statistical analysis of olfactometer bioassays. The basics of 'best practice' include capacity building and a move toward the use of modern regression methods in experimental design for these studies. This may include the use of the generalized linear model to account for non-Gaussian data (e.g., Nakagawa & Schielzeth, 2010), mixed effects models to account for correlated effects in olfactometer experimental design (e.g., Arnqvist, 2020), and justifying the sample size for an expected effect size using power analysis or through other heuristic methods (Festing & Altman, 2002).

Data from olfactometer bioassays are analysed using a narrow range of statistical analyses depending on the olfactometer design used to collect them. This is largely due to the olfactometer design ultimately dictating the type of data that can be collected. Bioassays using still air

olfactometers or two- and six-arm olfactometer designs are used to collect count data, for example of the total number of individuals in each olfactometer arm at the end of a pre-determined observation period. Four-arm olfactometer designs are used to record temporal data related to the duration that an individual spends in each olfactometer arm and the number of times each arm was entered.

Binary count data collected using still air and two-arm olfactometers are commonly analysed using a χ^2 goodness of fit test (Du et al., 2018; Kpongbe et al., 2019; Osei-Owusu et al., 2020) or binomial exact test (Roberts et al., 2019; Meza et al., 2020). Such analyses seek to identify deviations from an expected frequency (χ^2) or 50:50 probability distribution (binomial exact test). There is 50% probability that an individual will travel down an olfactometer arm when presented with an unbiased experimental setup. Although such statistical analyses are valid, they cannot be applied to nested data and this data structure is relatively common in olfactometer bioassays. It is recommended that modern regression methods are applied to binary count data using a generalised linear mixed model fitted to a binomial distribution with a logit link function (binary or multiple logistic regression) (Mas et al., 2020; Rondoni et al., 2022).

Exact binomial tests identify deviations from expected probability distributions whereas generalised linear mixed models facilitate modelling non-independence in data (i.e., pseduoreplication). This is especially pertinent if, for example, a test individual or odour source is used more than once in a bioassay. Random factors in a generalised linear mixed model can be used to account for pseudoreplication and avoid overdispersion (van Neerbos et al., 2023). Applying generalised linear mixed models to binary count data also allows control for confounding variables. As olfactometer bioassays are time-consuming and replicates are often carried out over several days or weeks it is possible that behavioural variation arising from physiological or environmental effects may inadvertently be introduced into the data. By including 'replicate' as covariate in the generalised linear mixed model such variability may be accounted for. Count data collected using four- or six-arm olfactometer designs can also be analysed using modern regression methods (generalised linear mixed model fitted to a Poisson distribution with a log link function) to overcome the previously outlined issues.

Temporal data collected using multi-arm olfactometers requires additional consideration during statistical analysis. A major challenge with four- and six-arm olfactometer experimental designs is that each of the arms cannot be considered independent of one another as the time spent in one arm directly influences the time spent in the remaining arms (i.e., correlated). This is further compounded by the fact that chemical stimulus presentation is often unbalanced as there are often multiple 'control' arms and one 'treatment' arm. Such data has, historically, been statistically analysed by calculating the mean time spent in the 'control' arms and then directly comparing this to the 'treatment' arm using a paired t-test (e.g., Birkett et al., 2000; Bruce et al., 2003). Using this approach does not facilitate identification of individual variability in each 'control' arm to determine whether the experimental setup has directional bias. If data collected from the 'control' arms is assumed to be homogeneous in this way it can lead to significant effect size underestimations (Arnqvist, 2020). With these issues being better recognised there has been a shift in how four-arm olfactometer data are analysed. As this is compositional data (Aitchison & Egozcue, 2005), the duration spent in each olfactometer arm can be converted into a proportion of the total time spent in all four arms then logratio transformed for analysis using a generalised linear mixed model fitted to a Gaussian distribution (Epel, 2013). Including random effects in the model can account for the correlated nature of the data collected from four-arm olfactometers.

INTERPRETATION OF BEHAVIOURAL RESPONSES

Describing insect behaviours has historically proven problematic (Knolhoff & Heckel, 2014). It is important to accurately describe insect behaviours to avoid misinterpretation of data collected using olfactometers. Dethier et al. (1960) proposed five standard terms to describe the effect of chemical stimuli on insect behaviour: 'attractant', 'repellent', 'stimulant', 'arrestant', and 'deterrent' (Table 1). In terms of insect locomotion within an olfactometer, 'attractant' and 'repellent' would only apply if the behavioural outcome is also considered. Kennedy (1977), however, cautioned that 'attractant' and 'repellent' are portmanteau concepts as they often combine the meanings of the other terms. For example, insects may aggregate at a spot due to an orientated movement from distance or the action of an 'arrestant' after a chance arrival by the insect. Although the standard terms proposed by Dethier (1960) have served as a foundation to describe insect behaviour, emerging subfields of entomology in the following decades have led to

TABLE 1 Description of categorisations of semiochemicals in terms of insect behavioural responses (Dethier et al., 1960).

Behaviour	Categorisation	Definition
Stops / slows	Arrestant	Reduce progression of insect through reduction of speed or increase in turning rate
Starts / speeds	Stimulant	Increase of dispersal from an area containing chemical; increase speed or decrease turning rate
Positions towards	Attractant	Purposeful movement toward chemical source
Positions away	Repellent	Purposeful movement away from chemical source

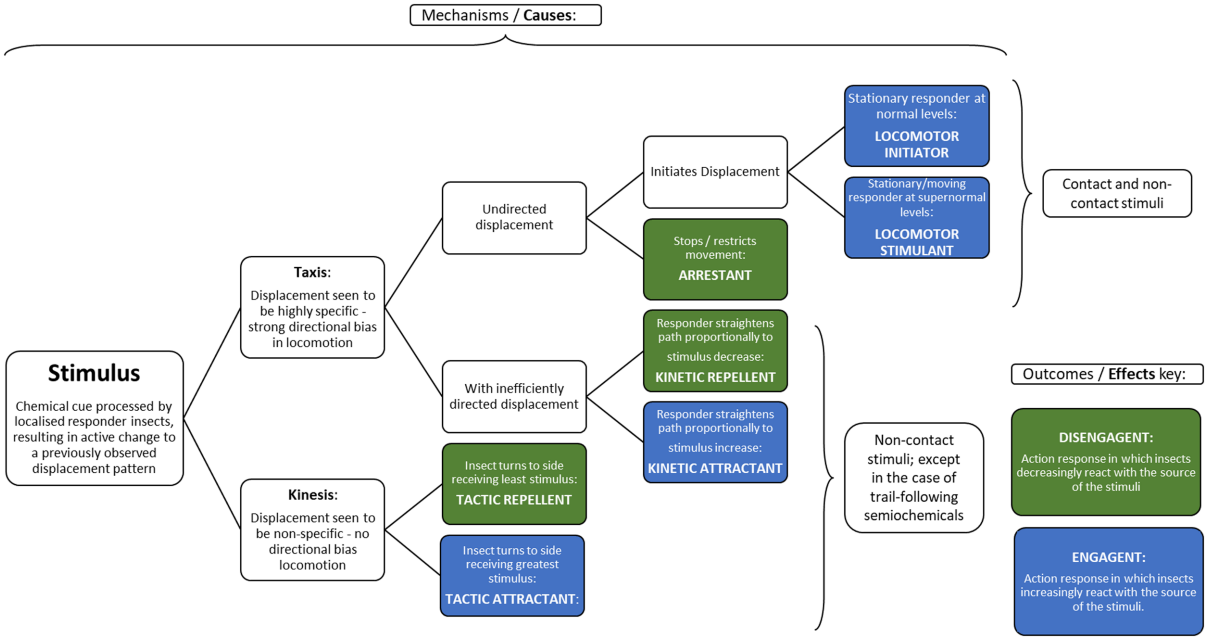

FIGURE 2 Updated terminology to describe chemically-mediated insect behaviours. Adapted from Miller et al. (2009).

a need for an updated framework to describe insect behaviour more generally (Grieco et al., 2007). Such a framework that builds upon the earlier work by Dethier (1960) has been proposed by Miller et al. (2009) (Figure 2). In most cases, however, unless the specific behaviour is observed, it is best practice to describe an insect's behavioural response to a chemical stimulus as simply positive or negative where only the end point is recorded.

CONCLUSION

Olfactometry remains a key tool for chemical ecologists globally and has been pivotal in furthering our understanding of chemically mediated insect behaviours. Our review fulfils a critical role in synthesising olfactometry techniques and their application while promoting best practices for carrying out these bioassays. By outlining appropriate olfactometer use, experimental design, and data analysis we have set a benchmark for reproducible research in insect ethology studies using olfactometers. This will ensure that behavioural observations derived from olfactometer bioassays, and their interpretation, are accurate and informative while streamlining the bioassay process.

AUTHOR CONTRIBUTIONS

Joe M. Roberts: Conceptualization (lead); project administration (lead); writing – original draft (lead); writing – review and editing (equal). **Ben J. Clunie:** Visualization (lead); writing – review and editing (supporting). **Simon R Leather:** Conceptualization (equal). **W. Edwin Harris:** Conceptualization (equal); writing – original draft (equal); writing – review and editing (equal). **Tom W. Pope:** Conceptualization (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no known conflict of interest related to this work.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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