



Using T- and Y-mazes in myrmecology and elsewhere: a practical guide

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Abstract

T- and Y-mazes are powerful tools for studying the behavioural ecology and cognition of animals, especially ants. Such mazes are cheap, easy to deploy, and very flexible, allowing diverse subjects to be studied. These include cue and route learning, pheromone following, and testing for cognitive abilities such as associative and concept learning. However, while simple, the use of bifurcation mazes has many pitfalls, especially for ants which deposit pheromone trails. In this methods primer, I discuss the strengths and limitations of using bifurcation mazes to study ant behaviour, and outline some of the pitfalls to be avoided. I also provide a description of a suggested Y-maze design, and methodological details about using Y-mazes to study associative learning, route learning, and trail pheromone following. Although the description of the uses of bifurcation mazes, and their pitfalls, is not exhaustive, this methods primer hopes to be of use to both beginners and more experienced researchers in designing and deploying bifurcation mazes in their research. While primarily aimed at myrmecologists, much of the content of this methods primer will be broadly applicable to animal behavioural research.

Keywords Y-maze · T-maze · Bifurcation · Methods · Experimental design

Introduction

T- and Y-mazes (henceforth bifurcation mazes) are perhaps the most commonly used maze design in ethological research. In essence, they are extremely simple—a path, called the ‘stem’, leading to a single bifurcation from which two paths, termed the ‘arms’, lead. Bifurcation mazes are commonly deployed for testing a wide range of taxa including vertebrates such as rats and mice (Wenk 2001; Deacon and Rawlins 2006), fish (Warren 1960; Darland and Dowling 2001), and birds (Jones et al. 1999), and invertebrates such as fruitflies (Tully and Quinn 1985), woodlice (Hughes 1967), bees (Giurfa et al. 2001), and, of course, ants (Lubbock 1884). While this short review and methods primer focuses on the use of bifurcation mazes in myrmecology and in the lab, many of the examples of use, the methodological suggestions, and potential pitfalls highlighted, apply to a broad range of study organisms, and also apply to the use of bifurcation mazes in the field.

Strengths and weaknesses

Bifurcation mazes represent a very good trade-off between the lack of control of more open designs, and the invasiveness and constraint of more restrictive designs. Many of the following strengths and weaknesses are presented as compared to either more or less restrictive designs.

Strengths

Simplicity

Data collection from bifurcation mazes can be very straightforward. Their use requires little training, which allows high-quality data to be collected rapidly, even by students with only minimal training. Data collection does not rely on motion-tracking software and the associated well-controlled filming conditions, and so can be deployed in the field. This also allows more extensive manipulations during experiments, which would spoil automated video analysis. Lastly, the simplicity of the system can often result in very rapid data collection, depending on the amount of pretraining needed. However, there are several obvious and not-so-obvious pitfalls that must be avoided, even given the simplicity of the system (see below).

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Flexibility

Bifurcation mazes can be used to study a very broad array of behaviours, both in the lab and in the field. Table 1 provides some examples of research topics studied using simple bifurcation mazes, with accompanying references, so that interested readers can follow-up on how the mazes were used to tackle each research topic.

Low interference

Compared to more restrictive designs, bifurcation mazes can allow behaviour in a semi-natural and biologically relevant context. This is perhaps the most important advantage of such experiments. Individual animals can be allowed to make repeated return visits to a maze, or naïve animals allowed to explore a maze, without any direct interference. This makes them a much less invasive method than other bioassays, such as the maxilla-labium extension response (MaLER, the equivalent of the proboscis extension response assay for bees) (Guerrieri and d’Ettorre 2010) or the mandible opening response (MOR, the equivalent of the sting extension response assay for bees) (Guerrieri and d’Ettorre 2008).

Importantly, bifurcation mazes in which the studied individual enters the maze of their own volition can in many cases ensure that the individual is in the correct motivational state. This is in stark contrast to harness-based techniques or other forced tests, in which the animals cannot choose not to participate. In such tests, there exists a real danger that the tested animal is alarmed, or otherwise not in an appropriate motivational state. Of course, efforts must nonetheless be made to ensure the correct motivational state: food deprivation in appetitive learning experiments is important to ensure ants are foraging and motivated to forage and learn (Oberhauser et al. 2018). Task state and context (for example, whether an ant is scouting, returning to the nest, or following a pheromone trail) may or may not also affect the behaviour of ants on mazes (Jander 1990; Czaczkes et al. 2017). Similarly, alarming test subjects before giving access to the maze should be avoided, to ensure that ants are attempting to forage, not escape.

Cost effectiveness

Bifurcation mazes are cheap, and serviceable mazes can be made rapidly from household materials. This allows ad hoc experiments to be carried out in field conditions using locally sourced materials. The cost effectiveness of the

Table 1 Examples of how simple bifurcation mazes can be used

Research direction	Example references
Studying memory and cognition	
Location learning	Grüter et al. (2011)
Associative learning	Vowles (1964); Dupuy et al. (2006); Josens et al. (2009); Czaczkes et al. (2014); Yilmaz et al. (2017)
Concept learning	Giurfa et al. (2001)
Abstract association learning	Czaczkes et al. (2014)
Unreinforced learning using SAB paradigm	Dember and Fowler (1958); Dingle (1962); Deacon and Rawlins (2006)
Re-learning	Czaczkes et al. (2015a)
Studying signal and cue following and preference	
Trail pheromone following	Hangartner (1969); von Thienen et al. (2014); Czaczkes et al. (2017)
Home-range marking effects	Wüst and Menzel (2017)
Odour cue preference	Schatz and Hossaert-McKey (2010)
Visual cue preference	Yilmaz et al. (2017)
Attraction to substrate-born vibrations	Roces et al. (1993)
Path angle preference	Jackson et al. (2004); Gerbier et al. (2008); Forster et al. (2014)
Cue discrimination	Dupuy et al. (2006)
Surface coarseness	Bernadou and Fourcassié (2008)
Physical communication of direction	Popp et al. (2017)
No entry signal	Robinson et al. (2005)
Information use strategies	
Information conflict (e.g., memory vs pheromones)	Aron et al. (1993); Grüter et al. (2011); Josens et al. (2016)
Information transfer	Provecho and Josens (2009); Saverschek and Roces (2011)

Some references fall into more than one category

methods also lends itself to high-risk projects, or student-run research projects, as the costs of failure are low.

Objectivity

Unlike more continuous behaviours such as mandible gaping or sting or proboscis extension, the scoring of bifurcation maze data is relatively robust to observer bias if clear decision criteria are used (e.g., “antennae crosses a decision line”). Nonetheless, such biasing—conscious or unconscious—is still possible, and so blind data collection using video recording should be used wherever possible (Kardish et al. 2015; Holman et al. 2015). At a minimum, subsets of data should be double-checked using blinded methods to test for bias.

Control

Compared to many field study techniques, using bifurcation mazes allows careful control of interfering variables. This includes variables such as bifurcation symmetry, wind direction, visual cues, and importantly social cues and signals from other ants, all of which can affect behaviour and path choice (Graham et al. 2003; Devigne et al. 2004; Müller and Wehner 2007; Forster et al. 2014; Czaczkes et al. 2015b). Some of these benefits can be exported out of the lab using bifurcation mazes in field (Harrison et al. 1989; Czaczkes and Ratnieks 2012).

Weaknesses

Lack of control

Compared to other laboratory techniques, bifurcation maze experiments can have less control. In bifurcation tests in which individuals can decide to take part or not, the delay between training events, or training and testing events, cannot be precisely controlled. In some situations, differences of even a few seconds can very strongly affect learning and performance (Menzel 1968). Thus, bifurcation tests may be inappropriate for the detailed dissection of memory processes. Individuals may even choose to stop cooperating partway through an experiment, especially if motivation levels are not high (Oberhauser et al. 2018). In addition, in many designs, the test animal is self-selected, by choosing to enter the maze, or being the first to reach the food source. This may not be a bad thing, if the aim is to study the behaviour of foragers, but the non-randomness of the tested ants should be kept in mind.

Binomial data

Behaviour in bifurcation mazes is often taken in terms of arm choice. This is a binomial variable, meaning that each data point can only take one of two forms—left or right. This is often referred to as success or failure in textbook descriptions of binomial data (Crawley 2007). Binomial data is not as statistically powerful as continuous data, as it contains less information per data point. This means that higher sample sizes are needed. Residence duration in each arm—a continuous variable—can also be taken (Dupuy et al. 2006). However, this may not be possible for all experiments, and care must be taken to choose an appropriate cut-off to residence time data collection, as eventually subjects will become frustrated, resulting in some proportion of random behaviour.

Pitfalls in bifurcation maze use

Pheromone contamination

A major issue when using bifurcation mazes to study ants is contamination by trail pheromone and other chemicals, notably cuticular hydrocarbons, which can also affect ant behaviour (Devigne and Detrain 2002; Wüst and Menzel 2017). In other animals, other substrate-deposited cues can also bias results. Some ant species only deposit pheromone after finding a resource, while others deposit pheromone while exploring as well. A simple method for removing pheromone is by covering the maze with disposable paper overlays (Czaczkes et al. 2013). However, if the overlays do not cover the entire maze surface, or the ants walk on the side of the maze, contamination can still occur (Popp et al. 2017). Likewise, simply replacing the maze arms or the overlay may not be sufficient as, due to corner cutting and wall following by ants, pheromones can build up on one side of the stem (see Fig. 1). Depending on the setup required, it may be possible to completely replace all components of the bifurcation maze every ant visit, which is ideal (Saverschek and Roces 2011).

It is best to test ant preference individually, with only one ant on the setup at a time, rather than in batches or via continuous testing. This helps avoid potential olfactory contamination. It also avoids the problem that ants can be attracted to the sight of other ants, or learn to associate other ants with a reward via local enhancement (shown not only in bumble bees (Leadbeater and Chittka 2005; Avarguès-Weber and Chittka 2014), but also present in ants (Graham P, unpublished data).

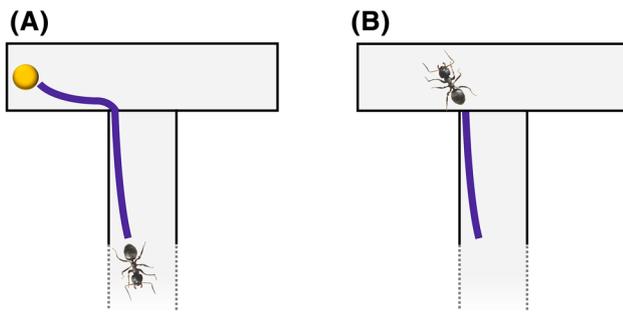


Fig. 1 Corner cutting leads to directional pheromone trails on maze stem. **a** An ant returns from a feeder (yellow circle) on one arm of the T-maze, depositing a pheromone trail (thick line). **b** The pheromone on the T-maze head is removed (for example by replacing a disposable overlay), but the trail on the stem remains. Due to corner cutting by returning ants, the pheromone trail on the stem channels ants to the left. This can also occur if only a small section of the pheromone trail on the stem is replaced. Adapted with permission from Popp et al. (2017). (Colour figure online)

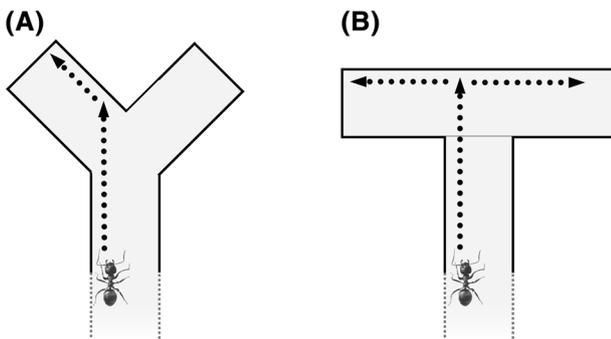


Fig. 2 Channelling results in default choice in Y-mazes. **a** In a standard Y-maze with no constriction at the bifurcation, animals reaching the far end of the stem section find themselves in either one side or the other of the point of the Y bifurcation, and may continue ahead, following that arm by default without making an active choice. **b** In a T-maze, animals reaching the far end of the stem are forced to make an active directional choice, as continuing ahead is not an option

Channelling and default choice in standard Y-mazes

Bifurcation mazes assume the ant is making a choice (one arm or the other). However, in standard Y-maze ants may be channelled into one arm or the other passively without being forced to make an explicit choice (see Fig. 2a). Ants arriving by chance on one side of the central Y-maze point may be channelled down one arm without having sensed the other arm at all. The default choice problem can be exacerbated by wall and side following, or thigmotaxis (Dussutour et al. 2005; Casellas et al. 2008), in which the ants simply walk along one side of the maze stem, and are thus channelled smoothly in one arm. Wall following effects are stronger for shallower turns, as found on Y-mazes (Casellas et al. 2008). Thus, wall-following

effects should be weaker, but may not disappear, in T-mazes. The default choice problem can also be minimised using a T-maze (Fig. 2b), or using a narrowing Y-maze point (see Fig. 3).

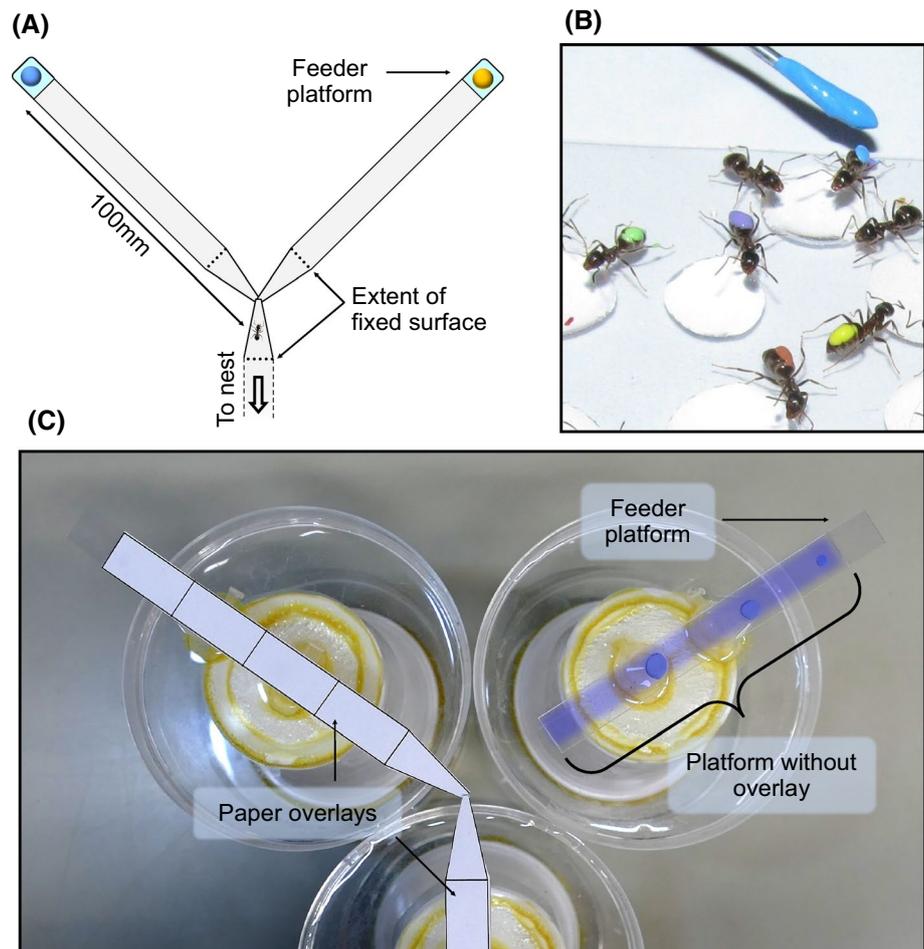
Lack of realism in T-mazes

90° angles do not often occur in the branching networks ants travel on, be they pheromone trails or plants. Natural trail bifurcation angles tend to be around 50°–55° (Jackson et al. 2004). Making such abrupt directional changes may disrupt behaviour such as pheromone trail following, increasing the proportion of U-turns and the number of random choices made (Helanterä et al. 2011). Unpublished data by Grüter and Czaczkes showed no effect of trail bifurcation angle on pheromone following accuracy in symmetrical bifurcations in *Lasius niger*. Similar unpublished results for *L. niger* are reported (Garnier, Pers. Comm.), but otherwise I am aware of no other data on this potential problem. As such, the lack of realism in T-mazes may not, in fact, be a problem. Nonetheless, all else being equal, it may be best to avoid such unnatural bifurcation angles.

Lack of learning motivation

The energetic costs for ants of walking the distances used in a laboratory are negligible (Gissel Nielsen 2001). Thus, without a negative reinforcer on the wrong maze arm there may be little motivation for the ants to learn, bar saving time. Indeed, even when learning does not take place, food retrieval rates on a bifurcation maze can increase over time, by virtue of the ants running faster, and learning to quickly check the other maze arm if their first choice was unrewarded (Bernadou and Fourcassié 2008). A standard negative reinforcer used is a quinine solution (see below). However, it should be kept in mind that this results in a very artificial situation. More naturalistic would be to increase the time costs of travel by increasing the distances used, but this slows data collection considerably. When using negative reinforcement it may not be clear whether the subjects are learning to choose the positively reinforced cues, avoid the negatively reinforced cues, or both (Giurfa et al. 1999), unless this is specifically tested for (Josens et al. 2009). Note that learning can be very rapid over short distances even with no negative reinforcer (Grüter et al. 2011), and that when trained with both positively and negatively reinforced cues, learning is driven overwhelmingly by the positive reinforcement in at least some ants (Josens et al. 2009). Thus, even without increased costs or negative reinforcement, sufficient motivation to learn is often present.

Fig. 3 A suggested basic Y-maze design. **a** A schematic view of the apparatus used in my lab to study *Lasius niger* (pictured to scale). Fixed plastic surfaces extend almost the entire length of the arms and stem, ending at the dotted lines (see **c**). These are covered by disposable paper overlays. At the end of each arm a plastic platform is affixed, on which visually identical drops of sucrose solution (reward), water (no reward), or quinine solution (punishment) can be placed. **b** Many ants can be easily marked individually while feeding at the end of the Y-maze arm by dabbing their abdomen with a dot of acrylic paint. **c** A picture of the suggested basic Y-maze with disposable paper overlays on the stem and left arm. The fixed plastic surfaces are raised on stilts over water moats



Preference for something over nothing, or nothing over something

Many studies use bifurcation mazes to study signal or cue attraction, especially pheromone following. A common procedure is to mark one arm with the putative pheromone or signal, and the other with a solvent which is allowed to evaporate, or with nothing. This is problematic, as many insects, including some ants, are innately attracted to a wide variety of cues (Becker and Mannesmann 1968; Birch et al. 1970). This may sometimes be related to novelty, and indeed even specialists such as leafcutter ants have a preference for novel forage (Cherrett 1972). This may also be related to generalist predators being attracted to the chemical signals of their prey (Zuk and Kolluru 1998). In other words, given a choice, ants may be attracted to something versus nothing. It thus becomes important to also test for generalised attraction to novel cues in the study species. Testing for preference for a compound against an arbitrarily selected control compound may not be a good solution, as other problems might arise: an apparent preference for one compound might be in fact

avoidance of the other. Demonstrating that a compound is a true trail pheromone is a complex process. Ideally, the putative trail pheromone compound would be found on the trail (Choe et al. 2012). However, as trail pheromone concentrations of even well-established trails may be below the range of current detection technology, this may be impossible. As an alternative, the glandular source of the pheromone must be found, and the gland contents tested in a pheromone-following assay (Simon and Hefetz 1991). Eluted gland content fractions should also be tested to pinpoint the precise active compound, and demonstrate that the other fractions are behaviourally neutral. Behavioural observations of the involvement of the glandular source with trail deposition are critical—an attractive compound stemming from the head is unlikely to be a trail pheromone if trail laying involves abdominal dipping. Lastly, bifurcation mazes are useful for testing compound attractiveness, but may not be appropriate for testing for the release of trail following behaviour: open arenas with a trail drawn onto them (for example, using the capillary tube method described below) are preferable (e.g. Chen et al. 1998). Such open arenas allow natural trail-following behaviours

to be observed, such as antennae lowering or tropotaxis-linked zigzagging over the trail (Simon and Hefetz 1991; Jackson et al. 2006).

Ants may also be attracted to nothing over something due to neophilia, and may even actively recruit to new territories unmarked by home-range markings (Hölldobler and Wilson 1977; Fourcassié and Deneubourg 1994), requiring an extra level of caution when interpreting bioassay results.

Physical issues—angle biases, step-ups and downs, and side biases

Ants tend to prefer moving in a straight course away from the nest (Garnier et al. 2009; Helanterä et al. 2011; Forster et al. 2014). Thus, given a choice between an arm almost in line with the stem, and one very much at an angle to the stem, ants prefer the one which is more in line with the stem (Garnier et al. 2009; Forster et al. 2014). It is thus critical to keep the angle of the bifurcation bilaterally symmetrical. This must be especially attended to if, for experimental reasons or to avoid pheromone contamination, each arm and the stem must be individually removable. Similarly, ants may have a preference for climbing upwards (Czaczkes et al. 2018a), and thus uneven slopes in the two arms may cause biases.

Related to this, ants tend to dislike taking a small step down, and may dislike taking a step up. Again, if the arms are not affixed to the stem and each other, care must be taken to avoid discontinuities in the walking surface.

Lastly, it is very common to see side preference biases in bifurcation data. These may arise from various reasons such as uneven lighting in the room, the presence of conspicuous attractive landmarks including the experimenter or cameras (Graham et al. 2003), or other sources. It is often difficult or impossible to find the cause of the bias or eliminate it, and such side biases may be innate, and have been repeatedly reported in the behaviour of ants and other animals (Hunt et al. 2014). It is thus imperative that all trials must be balanced for cue side.

Recommended methods

Basic Y-maze setup suggestion

The following describes a Y-maze design that overcomes many pitfalls commonly associated with other bifurcation maze designs.

The Y-maze is composed of three identical arms, one of which acts as a stem (see Fig. 3a). The arms come together at a central bifurcation, each offset at 120° from the others. This angle is chosen for convenience and to increase the difference in view when facing down each of the two arms—I

am not aware of any evidence for the bifurcation angle of a symmetrical Y-maze affecting arm choice. However, it is important to note that in natural ant trails (and elsewhere in nature) outgoing bifurcations tend to be at an angle of 50°–55°, which can also provide polarity to a trail network (Jackson et al. 2004). Thus, a bifurcation of 55° may result in a more natural behaviour.

Each arm narrows at the bifurcation to a point the width of the ant to be studied (for example, 1–2 mm for *Lasius niger*). Each arm is composed of a disposable paper overlay, which are brought together to allow the tips to overlap. However, the surface on which the overlays are placed does not extend for the entire length of the overlays. Rather, it stops where the overlay narrowing begins (see Fig. 3c). This means that the bifurcation point does not have an underlying surface which can be contaminated by trail pheromone. Additionally, I can anecdotally report that *L. niger* ants are reluctant to walk onto smooth plastic surfaces, while paper substrates are not avoided.

The overlay-holding platforms are raised on stilts over a water moat, to prevent ants escaping the setup. However, mid-sized- or bigger ants tend not to leave the maze surface, especially once trained (Jander 1990). Alternatively, plastic walls coated with Fluon can be affixed to the sides of the overlay holders, to prevent ants from leaving the setup, especially during pretraining (Dupuy et al. 2006).

An alternative to using overlay platforms and paper overlays is to use standardised disposable arms, for example composed of toothpicks (Saverschek and Roces 2011). This has the advantage of making the entire bifurcation setup disposable, but sometimes irregularities in the shapes of the toothpicks result in bending arms or difficulties in creating a fully symmetrical, flat maze. Ants may also tend to walk on the sides or bottom of the toothpick, making observation of behaviours on the maze, such as pheromone depositions, difficult. This can be mitigated using flat toothpicks.

Ants can be introduced onto the setup via a paper bridge or drawbridge mechanism. Alternatively, they can be brought onto the setup by allowing them to crawl onto a toothpick or a piece of paper and then moving them to the start of the maze—the appropriate method will depend on how easily disturbed and how ready to crawl onto objects the ants being studied are. Note that, if training an ant to a food source at the end of the maze arm, the ant should be allowed to make an outward journey and find the food item by itself, ideally making an undisturbed journey from the nest to the food source on the first visit. This will encourage food location learning (Graham and Collett 2006). Bringing the ant directly to the reward should be avoided. Similarly, if the ant has to perform several visits to the maze, it is advisable that it returns to the exit of the maze unaided.

The arm choice of the ant can be taken when a predefined part of the ant (e.g., antenna, head, whole body) crosses

a ‘decision line’ partway along the arm, or when the ant reaches the end of the maze arm or contacts the food droplet on the feeder platform. I recommend taking both decision line data (initial decision) and data on reaching the end of the maze (final decision). A comparison of these data allows quantification of ‘switching’—whether the ant changes her decision partway through her run. For cue-attraction experiments, ants may be allowed to spend extended periods of time on the maze, and the proportion of time spent on the various parts of the maze measured (Dupuy et al. 2006; Bernadou and Heinze 2013). Ideally, data should be collected blind to treatment, or video-recorded, treatment blinded, and checked for bias (Kardish et al. 2015; Holman et al. 2015).

Cue training

Training ants to cues allows a very broad array of research questions to be tackled, such as preference assessment, defining the limits of workers’ cognitive abilities, or exploring information use strategies (see Table 1).

First step—feeder design and marking ants

It is likely that ants will require multiple return visits to a cue-associated feeder to reliably learn an association. For example, *L. niger* workers require on average 2–3 visits to a feeder to ensure that most ants have learned that one side of a maze is rewarded (Grüter et al. 2011; Oberhauser et al. 2018), and 2–4 visits to each of two quality-associated odour cues to link a food quality to an odour cue (Czaczkes et al. 2018b). In some *Camponotus* species, differential odour learning may take up to 16 visits (Dupuy et al. 2006), though side learning is much faster. To achieve multiple return visits, offering a food reward at the end of the maze, and marking individual ants, will be necessary (see Fig. 3b).

A simple and effective feeder design is a small square of plastic, which can be affixed to the end of the overlay holder, or placed directly on the end of a maze arm (see Fig. 3c). Alternatively, if the overlays will not be replaced in between the outwards and return visit, rewards, including liquid food, can be placed directly onto the end of the paper overlay. For nectarivorous ants, using a sucrose solution of known molarity is preferable to using dilute honey, as this allows repeatability and comparability of results. A molarity of 1M works well, balancing the high motivation from high sugar content with the increased feeding time due to increased viscosity (Josens et al. 1998; Detrain et al. 1999; Paul and Roces 2003; Falibene and Josens 2011; Sola and Josens 2016), although some ants will recruit to concentrations as low as 0.01M (0.5% w/w, Roces 1993). Ideally, the sucrose drop and feeder plate should be replaced after every visit, as evaporation can increase the molarity of small drops over time. For predatory ants, piles of *Drosophila* often work well (Macquart et al.

2008), although specialised ants may not accept them. Even piles of brood can be successfully used to train ants (Lubbock 1884). For granivorous ants, offering two or three seeds at the end of a maze arm works well, with the seeds being replenished after each visit. Seeds should be all of the same size and appropriate for study species, so that a single ant can carry the seed easily (Ladevese et al. 2007).

On the unrewarded arm of the bifurcation, a drop of water (a visually identical neutral stimulus) or a drop of quinine solution (negative stimulus) can be used. A quinine solution of 0.06 M as a negative stimulus coupled with a 1M sucrose-positive stimulus enhances visual discrimination in honeybees in a Y-maze (Aavarguès-Weber et al. 2010), and concentrations of 0.08 or 0.01 M have been reported to act as effective negative reinforcers for two *Camponotus* species (Dupuy et al. 2006). However, in at least some cases, ants do not seem to learn the negative association, only the positive association, in differential conditioning trials (Josens et al. 2009).

Most ants can be marked easily while they are drinking from a drop of sucrose solution. A toothpick or paperclip dipped in acrylic paint works well (see Fig. 3b). Although training multiple ants concurrently is not advised (see above), it can be done if different colour combinations are used, and for medium sized ants (3–4 mm in length) at least two colour dots can be placed on the gaster.

Simple paint marking may not be possible for some ants which are either very greasy, covered in hairs, or are extremely anxious. In such situations, coloured or fluorescent powders (or ‘dusts’) can be used to mark the ants (Hagler and Jackson 2001). Alternatively, for species with small colonies, all the workers in a colony can be marked. For automated data collection systems, miniature RFID tags (Robinson et al. 2012) or two-dimensional barcodes (QR codes) (Greenwald et al. 2015) can also be used. As paint and tags can be groomed off, affixing a wire or fibre around the gaster is also possible (Stuart 1986). Such marking techniques require immobilisation of the ants. CO₂ and cold anaesthesia are commonly used. However, immobilising insects for marking using anaesthesia should be used with caution, as anaesthesia is well known for disrupting insect learning and behaviour in a variety of ways (Nicolas and Sillans 1989; Poissonnier et al. 2015). In honeybees, CO₂ narcosis can trigger ovary activation and changes in gene expression, which may have downstream effects on behaviour (Koywiwattrakul et al. 2005). An alternative method for immobilising ants is to use a weak vacuum which sucks the ants head into a pipette tip (Heinze 1993), or to clamp ants between two pieces of sponge.

After feeding at a feeder, ants must be allowed to unload their food to nestmates before testing or the next round of training.

Odour cues and cue training

Odour cues are likely to be the most effective form of cue for training non-location information, such as food quality and composition, risks and aversive stimuli, and abstract concepts. The odour can be in the food, on the maze surface, or in an air stream being blown over each arm. However, these odour sources can be learned differently. After five visits, *L. niger* foragers do not learn to associate odour present on the path alone with food quality (Oberhauser, unpublished data). However, they do learn food odour in the absence of odours on the path in an identical setting, and then respond to odours on a Y-maze arm during choice tests. Food can be infused either with artificial food flavouring or essential oils. If essential oils are used, care must be taken to shake the mixture well before each use. Paper maze overlays can be odour-impregnated by storing them in a sealed container containing an odour source, such as a few drops of essential oil on a glass petridish. Finally, different ant species may show innate preferences for some odours, so careful controls and pilot studies should be used to find a pair of odours that are equally attractive. Even when these are found, experiments should balance different odours and their associations. Similarly, some ant species show strong innate preferences for certain light wavelengths (Yilmaz et al. 2017).

An effective method for training ants to a cue is to first offer the various cue/reward combinations sequentially at the end of the straight path. The path may be covered by overlays impregnated with the reward scent at the end. Once the ant has made multiple visits to the food via the straight path, experiencing all the different cue/reward combinations to be trained, preference can be tested by offering a Y-maze with scent-cued overlays. Ants can also be trained to cue/reward associations using trophallaxis from nestmates (Provecho and Josens 2009; Josens et al. 2016), or in leaf-cutting ants, by allowing them to encounter successful foragers on the path or in the nest carrying a scented food item (Roces 1990, 1994).

Especially when using a new maze design on a species for the first time, care must be taken not only that the odour cues can be perceived by the ants at the bifurcation point but also that the odour of the two arms can be differentiated at that point. In closed flow-through olfactometer bifurcation mazes, too strong an air flow from one arm can push odour cues up the other arm. Air flow patterns can be visualised using smoke created by mixing diethylamine [(C₂H₅)₂NH] and acetic acid (CH₃COOH) in a glass vial (Pham-Delegue et al. 1991). If using passive odour diffusion with odour cues emanating from the end of the maze arms, it is important to ensure that both cues can be detected from the central bifurcation. More generally, as with other bioassays, a positive result implies that the setup is adequate, but a negative

result may be due to a design flaw in the setup or a mismatch between the setup and the study species.

Visual cues and other cues

Visual cues are effective for side learning. Ants can be easily taught to follow only the left arm of a bifurcation maze, or the arm of a maze with a bright light at the end (Yilmaz et al. 2017). For fixed side learning, unmodified lab surroundings are usually sufficient for good learning; no explicit visual cues are required (Grüter et al. 2011). If the ant is to learn to follow a distinct visual cue, light sources have been reported to show good success (Fourcassie and Beugnon 1988; Yilmaz et al. 2017). Some ants, such as *L. niger*, fail to distinguish salient visual cues such as A4 colour sheets with a large high-contrast geometrical shapes on them (Oberhauser, unpublished data). It must be kept in mind that ants might have an innate attraction to large, salient objects, or light of various wavelengths (Graham et al. 2003; Yilmaz et al. 2017).

Various other cues could, in principle, be used to train ants. Bernadou and Fourcassie (2008) report that *L. niger* cannot use substrate coarseness as a cue. Kleineidam et al. (2007) trained *Atta wollenwiederei* to seek food using temperature cues.

Pheromone trails

When studying trail pheromone following, a standardised amount of trail pheromone must be applied to one or both arms of the bifurcation maze. There are three basic sources of trail pheromone that can be used: synthetic trail pheromones (Choe et al. 2012), gland dissections (von Thienen et al. 2014), and naturally laid trails (Grüter et al. 2011; Czaczkes et al. 2017). As substrate characteristics and temperature affect pheromone trail evaporation (Jeanson et al. 2003; van Oudenhove et al. 2011), these should be noted, and ideally controlled.

Synthetic pheromones offer the advantage of exact standardisation, replication, and control. However, they are only available for a handful of species. Moreover, while the main active compound of a pheromone trail may be known, naturally deposited trails may involve a mixture of various chemicals at specific concentrations, and thus synthetic trails may not elicit completely natural behaviour. Moreover, other behaviourally relevant cues, such as home-range markings (Devigne et al. 2004), will be completely absent from the trail. Similarly, a biologically reasonable trail strength needs to be calculated (for an example, see von Thienen et al. 2014) to avoid unrealistically strong trails. Trail pheromones that are attractive and elicit trail-following behaviour may become repellent or release alarm behaviour at very high concentrations (Simon and Hefetz 1991).

As synthetic pheromone trails are usually mixed into a volatile solvent, depositing an even pheromone trail along a maze arm can be challenging. One method is to use a glass capillary to draw up a set amount of solution, and use the capillary as a pen to draw an even line on the maze arm overlay (Edwards and Pinniger 1978; Czaczkes and Beckwith 2018). With practice, an even line can be reproduced repeatedly. A microsyringe can also be used to apply known quantities of pheromone along a trail (Hölldobler et al. 1994). The control arm should be marked with the same solvent but free of the synthetic pheromone (Hölldobler et al. 1994).

Gland extracts are a good alternative to synthetic pheromone trails. Glands can be dissected out under a binocular microscope, and immersed in a solvent such as dichloromethane (Hölldobler et al. 1994; Czaczkes and Beckwith 2018). This will result in a more complex chemical blend than an artificial trail, but will also likely include chemicals from the gland tissue or the damaged ant which are not naturally found on trails. Gland content can also be extracted using a microsyringe or capillary tube directly from the gland (Hillery and Fell 2000). Care must be taken not to puncture other glands when dissecting out the trail pheromone gland. The gland extract can be fractionated to remove non-target compounds (Simon and Hefetz 1991). Again, home-range marking cues will be absent. A good method for within-experiment replicability is to make a stock solution of glands in solvent and store this at -20 or -80 °C. If sufficient quantities are made, only one stock solution can be used throughout the course of an experiment, extracting small amounts at a time as they are needed. Stock solutions can be diluted systematically to systematically test the effect of various trail strengths. As with synthetic pheromones, artificial trails made using gland extracts must be calibrated to find a biologically relevant concentration (von Thienen et al. 2014). Application of gland extract solution is similar to synthetic trails.

The alternative to experimenter-deposited trails are trails naturally deposited by the ants. Ants can be allowed to forage at a food source while confined to a straight path whose surface is covered with a paper overlay. After sufficient pheromone has been deposited, the overlay can be trimmed to the shape of the bifurcation arm (Jackson et al. 2004; Grüter et al. 2011). This method ensures a completely realistic trail, with all accompanying secondary compounds, gland contents, and home-range markings. However, standardisation can be problematic. In many formicines, pheromone deposition can be seen by eye (Beckers et al. 1993), and the number of pheromone depositions can be taken as a measure of trail strength. However, it is likely that the amount of pheromone in each ‘dot’ varies between and within ants. As pheromone begins to evaporate as soon as it is deposited, the time between pheromone deposition and testing will strongly affect trail strength (Jeanson et al. 2003). Pheromone-marked

overlays can be stored in a freezer until they are needed, but the effect this will have on trail strength must be tested for. Ants may also respond differently to the home-range markings of non-nestmates (Wüst and Menzel 2017). To avoid this, and to avoid satiation and prevent tested ants from being in the wrong task state (Czaczkes et al. 2017), trails formed by ‘donor’ colonies can be used. These can stem from a colony fragments of the test colony, or if the species shows no intraspecific trail or home-range marking specificity, from another colony (Jackson et al. 2006).

Concluding remarks

T- and Y-mazes are a very powerful and flexible methodology for studying a wide range of subjects. They are cheap, easy to deploy, and robust. However, while they are easy to deploy, there are many pitfalls that are to be avoided to ensure high-quality data. I hope that this work helps other researchers avoid many of these pitfalls. However, the methods and potential issues outlined here are far from exhaustive or definitive. Methods will have to be adapted to different situations, and this will result in more potential problems to be overcome. By carefully watching the behaviour of the animals on the maze, and aiming for as much biological realism and as little disruption as possible, many unforeseen problems can be identified and overcome. Bifurcation mazes are well worth using, and well worth using right.

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