

Lessons in cognition: A review of maze designs and procedures used to measure spatial learning in fish

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Abstract

The use of different mazes to assess spatial learning has become more common in fish behavior studies in recent decades. This increase in fish cognition research has opened the door to numerous possibilities for exciting and diverse questions, such as identifying ecological drivers of spatial cognition and understanding the role individual variation plays in navigational abilities. There are many different types of mazes, each with its own specific considerations, making it challenging to determine exactly which spatial test is the most relevant and appropriate for a particular experiment. Many spatial mazes, such as the T-maze and Y-maze, have been successfully adapted from rodent studies, particularly with respect to zebrafish, a widely accepted non-mammalian model in biomedical studies. Standardization across studies is increasing with these easily accessible maze designs, validating them for use in fish; however, variations in design (e.g., length of arms and scale) and procedure still exist, and the impact of these variations on results is largely unknown. The efforts to standardize mazes outside zebrafish work are also more limited. Other mazes have been developed specifically for use on fish, with design modifications varying widely, making it difficult to draw comparisons. In this review, we have highlighted the many design and procedural elements that should be considered for the acquisition of reliable behavioral data, with the goal of drawing readers' attention to aspects of experimentation that are often not given the careful consideration that they deserve. We then argue that additional focused research and reporting is needed to produce more reliable methods in spatial learning research across a broader range of subjects.

KEY WORDS

fish cognition, maze design, spatial learning, zebrafish (*Danio rerio*)

1 | INTRODUCTION

Learning plays a central role in behavioral ecology; animals integrate sensory information and use multiple environmental cues simultaneously to adapt their behavior. One such type of learning is spatial

navigation, which is the ability to learn and remember locations from past experiences (Shettleworth, 1993). Like other vertebrates, fish rely on the ability to navigate efficiently around their environment to find and return to new foraging patches, remember the location of safe refuges, and avoid predators. As environments change, it is

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increasingly important to understand how animals learn to navigate their world and balance exploring new areas with remembering past information. The existence of individual behavioral variation within species, and how much this intraspecific behavioral diversity influences community structure and ecosystem function, further intensifies the challenges of understanding how populations move through their environment.

Controlled studies in the laboratory have begun to illuminate the world of spatial learning in fish. Much of the work in fish has been adapted from the extensive field of learning and memory in other lab animals, notably rodents (e.g., Arakawa & Iguchi, 2018), but fish offer the opportunity to ask a variety of unexplored questions as well as deepen our knowledge of spatial learning through comparative research. The ecological diversity of fish has made them ideal organisms to answer several advanced questions related to spatial learning. Unique life histories have proven enticing to researchers to ask nuanced questions about, for example, how the diversity of spatial variation an individual has faced influences learning ability. For instance, amphibious killifish (*Kryptolebias marmoratus*) that have had terrestrial experience exhibit improved spatial learning relative to individuals that remain in the water (Rossi & Wright, 2021). The sensing capacities of fish have also been leveraged to ask questions relating to internal drivers of spatial abilities; weakly electric fish (*Gymnotus* sp.) have been used to study how active sensing behaviors change over the course of spatial learning (Jun et al., 2016). Due to the range of weakly electric fish's electrosensory capabilities and the ability to measure electric organ discharges, the researchers were able to more directly link the animal's exploratory behaviors to the active sensing that occurred at specific locations during learning. Although questions surrounding environmental or internal mechanistic drivers of spatial learning are intriguing, less effort has been focused on more basic, but equally important, questions related to the different testing methods.

A thorough investigation of how maze design and procedures impact participation and success in learning trials is needed to make studies more comparable and research effort more efficient. Many of the mazes and methods used to study spatial learning in fish have been adapted from research in rodents, where standardizing and directly testing the appropriateness and applicability of tests has been emphasized to further understanding of behavioral performance (Arakawa & Iguchi, 2018). Recently, standardizing and considering design-related issues in mazes developed for zebrafish (*Danio rerio*) has also received attention (Benvenutti et al., 2021). Zebrafish are a model organism for studying neurobiological mechanisms of learning and memory; as such, there has perhaps been more of an emphasis on consideration of the standardization and efficiency of the behavioral assays used for this type of research. Behavioral ecologists working on fish more generally have typically not focused on the benefits of standardized tests in spatial learning (although calls for improved reporting and standardization of other aspects of behavioral ecology are on the rise, e.g., Roche et al., 2023). We fear that in the pursuit of answering "flashier" research questions, we have failed to devote appropriate time and research effort to understanding what the most efficient way of measuring learning in fish is and lack a full

understanding of how design can influence our results (although this is not always the case, see, e.g., Jones et al., 2023). In drawing attention to the variation in the dimensions and shapes of mazes used in zebrafish learning research, Benvenutti et al. (2021) have called attention to the needs of zebrafish researchers; however, this message is important for the larger field of spatial learning in fish. We are not trying to replicate Benvenutti et al.'s (2021) work, and have thus, for example, not included a table with dimensions of mazes used in research as they have so excellently done for mazes used in zebrafish. We are instead attempting to amplify this call for behavioral ecologists to consider carefully the methods and procedures used in spatial learning research. This field is actively growing and now is the time to set a strong foundation for generating high-quality data that support our ability to have a broad understanding of how fish navigate their environment.

This review is by no means a comprehensive evaluation of spatial learning in fish (for this consider Ahlbeck Bergendahl et al., 2024). It is also not a general argument for enrichment or more ecologically relevant housing conditions, although these factors can influence cognitive performance in fish (Johnsson & Näslund, 2018; Salvanes et al., 2013), or the reporting of husbandry and enrichment specifically (as has been done by Jones et al., 2021). In fact, in many places we do not make a specific recommendation for design. This is on purpose, as our goal is to draw attention to the lack of focused research on which to base design decisions. We fear that specific recommendation may limit the type of experimentation that we think is much needed to understand how design influences our ability to detect variation in spatial learning abilities. Our goal is to argue for the importance of thoughtful design of mazes used in spatial learning tests to ensure easy-to-interpret results that are more comparable across studies. To achieve this goal, we take a narrative approach as opposed to a more formal meta-analysis as we think that, particularly outside of zebrafish, currently there is no sufficient research to draw general conclusions about any of the design-based issues that we highlight. We think a meta-analysis would rely too heavily on results obtained in zebrafish, and our goal here is specifically to encourage this type of work in a diverse range of species to understand how much variation there is in the effects of different design decisions. Thus, in the following review we have often first summarized what is known from research on zebrafish in the neurobehavioral field for a specific maze type before expanding to consider its use in other species of fish and more ecological questions. We included spatial learning focused research from studies we were already aware of as well as papers they cited or that cite them and conducted extensive searches in Google Scholar using search terms such as "spatial learning fish" and "maze design fish."

We hope this review can serve as the impetus and justification for specific studies related to the issues of maze design and experimental procedures in spatial learning research. We will first synthesize what is known about maze design, with a particular focus on commonly used constructions, and experimental procedures. We will then highlight avenues for future research and suggest ways to incorporate basic design-related questions into existing research programmes.

2 | WHY DESIGN MATTERS

With a tantalizing array of ideas related to spatial learning open for investigation, it is tempting to develop complicated experiments designed to answer nuanced questions; however, there are many

basic questions related to the design of the experimental apparatus and procedures that must be decided on prior to beginning any spatial learning experiment (Figure 1). Unfortunately, the implications of many of these design considerations have not been explicitly tested. Many experiments mimic the methods of previous studies, which also

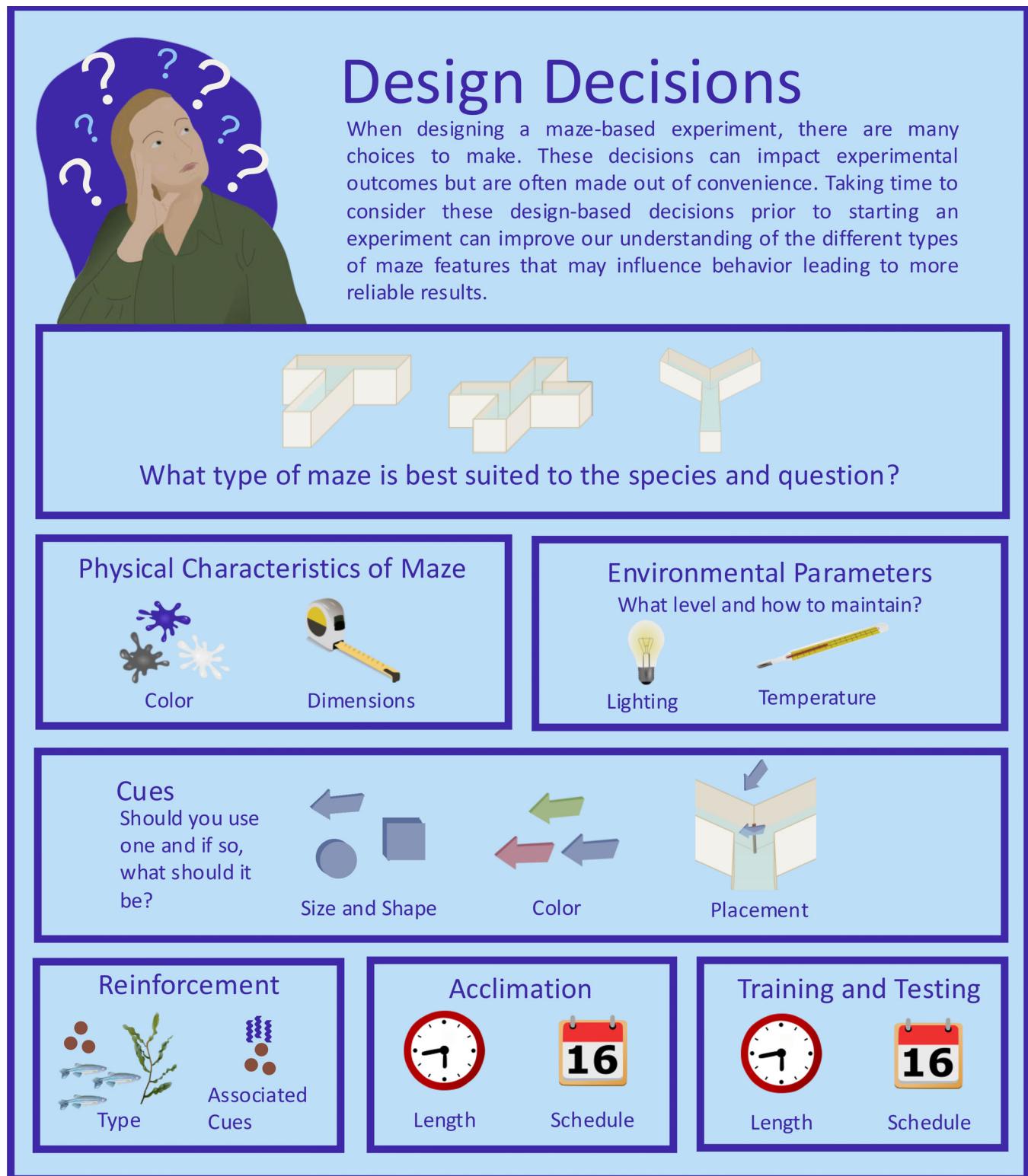


FIGURE 1 Key design factors to consider when planning experiments that utilize a behavioral arena or maze to investigate cognition in fish.

may have originally been decided arbitrarily or with convenience in mind. Although convenience and efficiency are important considerations when designing experimental procedures, design elements that have the potential to improve the quality of results should not be ignored. Importantly, changes in experimental procedures or differences in maze design (e.g., time between trials [Cognato et al., 2012], presence of spatial cues [Hughes & Blight, 1999], maze size and number of choices [Jones et al., 2023]) can influence whether animals participate in the experiment, and the animal's performance.

One major goal when making design decisions is to create a test that accurately measures what we think we are measuring (Agrillo et al., 2017; Toms et al., 2010). Although this may sound simple, there are potential pitfalls that can limit the interpretation of results. Low levels of participation in a test, particularly participation that is biased to favor one phenotype over others, can be a challenging issue with spatial learning experiments. For example, maze-based experiments often begin with the fish in a start box; if the design of the maze does not lead to the fish feeling safe, the fish may fail to leave the start box. This is a challenging result to interpret. Do individuals that do not leave the start box have poor spatial navigation abilities, or are they fearful? Particularly because fish may consistently differ in fearfulness and neophobia (Coleman & Wilson, 1998), this can lead to biased participation, which may also reduce meaningful variation in other performance metrics and limit our ability to answer experimental questions. There are also links between neophobia and exploration and cognition: responding to a stimulus because of its novelty requires both memory and the ability to classify the stimulus as novel (Greggor et al., 2017). Differences in neophobia and exploration may thus alter an individual's likelihood of entering a maze and interacting with the rewarding stimuli but may also be important for understanding spatial learning. Factors like color and size of maze, as well as external stimuli, are all important for creating mazes that increase participation. Given sufficient acclimation time, most individuals will begin to explore new areas, so when considering how to maximize the participation of fish in trials, time to acclimate should be part of that consideration. If design choices prolong acclimation, fewer trials can be accomplished. Spatial learning experiments can often be quite time intensive requiring multiple days of testing and training for every individual. Therefore, design is not just successful if most individuals eventually participate; ideally they begin to interact with the test arena relatively quickly.

Although ideally we want all, or most, individuals to participate in a test, identifying variation in spatial learning ability is often also important. If tests are not appropriately difficult, it is impossible to distinguish between individuals. Too hard and all or most individuals fail, too easy and they all succeed. The goal therefore is typically for all individuals to participate but to exhibit variation in success or time to success. Experimenters must consider the number of choices an individual has to make, the number of options available for every choice, and the size of the maze among other potential factors to make the test appropriately challenging. The outcome of these decisions is not merely cosmetic. For example, the relationship between metabolic

traits and cognitive performance differs depending on the number of options available in a maze (Cortese et al., 2024). In simpler mazes, fish with a higher metabolism are faster and more successful at learning to locate a food reward; however, fish that have a lower metabolism perform better when there are more choices. Ultimately, unless we achieve these design goals it is difficult to compare results across trials and species.

Although there is still limited information to guide design decisions when building mazes for fish, research with zebrafish and other model organisms such as rodents has begun to highlight the importance of design and can be used as a valuable starting framework to guide future research with more diverse species. From an ecological perspective, zebrafish studies may seem an unlikely source of information for investigating the effects of individual variation on behavior and cognition; however, their increased popularity in neurobehavioral studies, which rely on standardized behavioral tests, makes the zebrafish a powerful behavioral candidate (Kalueff et al., 2013; Miklosi & Andrew, 2006). Standardized testing allows for easier comparisons across studies. Finding a balance between standardized testing conditions and the inclusion of ecologically relevant aspects of the focal species' environment is crucial for species-level comparisons and meta-analyses.

3 | MAZE TYPE

3.1 | T- and plus-mazes

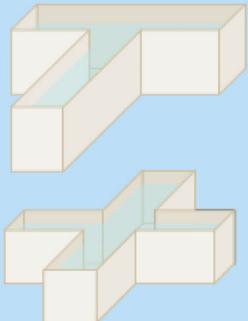
T-mazes (Figure 2) have been used to probe spatial learning abilities of a variety of species of fish. The T-maze consists of a long arm with a start box at one end and two perpendicular arms on the other end (in the shape of a "T"). The T-maze design is based on behavioral protocols in rodents (Deacon & Rawlins, 2006). Spatial abilities of an individual can be tested either by requiring the animal to remember which of the alternative arms it has previously visited or the location of a reward across a set number of trials.

The plus-maze (Figure 2) is a common variant of the T-maze developed in rodent studies in which there is one starting arm and three choice arms. Unlike the T-maze, all arms in the plus-maze are typically the same length, so upon leaving one chamber, the starting arm may appear as an identical option as well. However, the plus-maze can have the added complication that animals may prefer to continue into the arm directly across from the starting arm as it does not require any turning that may need additional analysis considerations to account for (Jones et al., 2023).

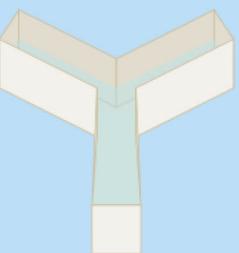
In zebrafish both T- and plus-mazes have been used in a variety of studies to examine the neurobiological underpinnings of spatial learning and memory (Al-Imari & Gerlai, 2008; Benvenutti et al., 2021; Ninkovic & Bally-Cuif, 2006). Compounds that are known to disrupt spatial learning and memory in rodents also have a negative effect on spatial learning in the T- and plus-mazes in zebrafish (e.g., ethanol: [Fernandes et al., 2014; White et al., 2000], MK-801: [Sison &

Types of Mazes

T- / Plus-Maze

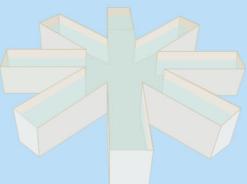


Y-Maze



Alternative Mazes

Additional Options



Radial Arm Maze

More Complicated



Bifurcating Maze

Simpler Construction



Arena Maze

differences in the ratio of the length of the starting arm to the choice arms (e.g., 3:2 [Darland & Dowling, 2001], 2:1 [Babkiewicz et al., 2021], 1:1 [McAroe et al., 2016]), settling time (e.g., no settling period [Wang et al., 2022]; 30 s [Ngoc Hieu et al., 2020]; 5 min [Babkiewicz et al., 2021]), and length and frequency of training trials (e.g. three trials across 3 days [Darland & Dowling, 2001]; 10 trials per day per training block [McAroe et al., 2016]). Despite these differences in methodology, studies generally report an increasing association between unconditioned stimuli (e.g., food, shelter, social groups) and conditioned stimuli (e.g., location, cue), suggesting that these mazes are a robust method for studying spatial learning. Although data that indicate learning has occurred are important for validating the apparatus generally, the wide range of specific methods and apparatus used makes comparisons between studies challenging and can lead to inefficient tests if researchers conduct more trials or build larger mazes than is necessary to address a question.

3.2 | Y-maze

Also adapted from work with rodents (Kraeuter et al., 2019), the Y-maze consists of three equilength arms (Figure 2). Unlike the T-maze in which two arms are typically shorter and perpendicular to the main arm, all three arms in a Y-maze are at a 120° angle from each other. This type of maze can be used for a variety of spatial learning tests but relies on the tendency of animals to explore previously unvisited areas. Some versions of this test incorporate a flow-through aspect in which a controlled flow of water (sometimes containing either a positive or negative cue) enters the maze from the two target arms and leaves through the start zone (Grella et al., 2010).

Cognato et al. (2012) developed a standardized two-trial version of the Y-maze for zebrafish with a glass Y-maze (25 cm long, 8 cm wide, 15 cm high). White paper geometric forms were placed on the outside of each arm and then covered with black plastic self-adhesive film, so the visual cues were visible to fish inside the maze. A starting arm was randomly assigned to each fish; one of the remaining arms was open for both trials, and the other arm was closed during the first trial and open during the second. Cognato et al. (2012) then compared different intervals between trials (1, 3, 6 h) and the effects of compounds that disrupt learning in rodents (MK0801 and scopolamine) on the performance of zebrafish. In the second trial, fish preferred the previously unexplored arm when there were short time intervals between trials and when unexposed to drugs; increased time between trials and exposure to memory-disrupting drugs both decreased the preference for the novel arm (Cognato et al., 2012), suggesting that this version of the Y-maze can be used as a rapid method for measuring spatial memory of previously visited locations. The protocol used by Cognato et al. (2012) has become well established in the zebrafish literature with many studies replicating the exact methods (e.g., Bortolotto et al., 2014; Capatina et al., 2020; Zanandrea et al., 2018) or similar methods (e.g., Sheng et al., 2014). Despite the benefits of a standardized test, however, this protocol has rarely been

FIGURE 2 Schematic diagram of different arena shapes reported in the fish spatial cognition literature. Alternative maze designs are organized by general strategy with an example of a maze that accomplishes that goal.

Gerlai, 2011; van der Staay et al., 2011]), suggesting these mazes are a valid technique for measuring spatial memory in fish. Further, a range of experimental procedures have been used, including

adapted for other species of fish; instead, the use of Y-mazes in other fish species follows a range of protocols and designs. For example, Davis et al. (2014) used a three-dimensional Y-maze in which the arms were cylindrical and the whole arena was suspended and could be rotated in four possible directions to assess horizontal and vertical movements of *Corydoras aeneus*. Instead of using a standard “Y” shape for their Y-maze, where two arms are at 45 degrees, Takahashi et al. (2010) tested the spatial abilities of *Trachurus japonicus* using rectangular glass tanks that were partitioned to create three different zones: one zone at one end and two at the other. Although the authors described the arena as a Y-maze, it could be argued that the maze does not follow the typical “Y” shape and cannot be classified as such. Comparing the results of these studies with results found in other species is thus challenging because it is unclear if differences in performance reflect species differences or design differences.

3.3 | Alternative mazes

Not all spatial learning research questions fit neatly within the standard T- or Y-maze arena type. In some cases, the species' ecology also lends itself better to other designs. For these situations, alternative mazes have been constructed. These mazes can take a variety of forms, with the general idea to create more options, make a maze more challenging, or make construction simpler (Figure 2). Various alternative mazes have been adapted from standard T- or Y-mazes. For example, plus-mazes have been modified to include additional arms, sometimes called radial arm mazes (DePasquale et al., 2020; Faillace et al., 2018; Long & Fu, 2022) or larger compartments (Pittman & Lott, 2014). T-mazes may also be made more complex by combining a series of multiple T-junctions to create complex bifurcating mazes (Lucon-Xiccato & Bisazza, 2017). Although alternative mazes can take different forms, here we summarize general types of alterations that are commonly made.

T-mazes may be simplified to include one large start chamber and two choice compartments without the “arms” separating the start position and location of the reward; the choice compartments are then visually occluded with a small opening a fish must cross to enter the compartment (e.g., Karekla et al., 2017). Plus-mazes have similarly been modified to include a central starting chamber and a choice of four compartments that are accessible from the central area with no “arms” separating the choice from the reward (Brown & Braithwaite, 2004; DePasquale et al., 2016; Roy & Bhat, 2017; Spence et al., 2011). Unlike the original plus-maze test, individuals are typically started in the central compartment of these mazes, but, similarly, only one of the compartments contains access to the social or food reward, whereas all other compartments are “false” choices that act as decoys for the correct choice.

Other mazes consist of one central reward chamber that is accessible by different paths that can be either open or fully/partially blocked to allow different types of access to the reward (e.g., Gómez-Laplaza & Gerlai, 2010). Similar mazes, sometimes called “arena

mazes,” consist of a large open field with a start box on one side and a reward chamber that may be accessible using a “pre-chamber” on either side (Cogliati et al., 2019). The general design of this type of maze is that fish starts on one side of the maze and all choices are presented across an open field along the opposite wall (e.g., Salvanes et al., 2013). Physical as well as visual and olfactory access to the reward chamber can be controlled by inserting and removing solid or mesh doors (Závorka et al., 2020). These mazes can be made more complicated with multiple reward chambers and access points (Jones et al., 2023) or a start box that faces in the opposite direction (Roy & Bhat, 2016). This type of maze has the benefit of controlling for visual or chemical cues associated with the reward by having only one reward that is potentially accessible from different paths. A similar maze used multiple starting chambers and target chambers in a rectangular tank (Fernandes et al., 2016). These mazes are often built to fit inside standard aquaria so they can be added or removed to increase the functionality of existing behavioral setups; this also makes them easy to construct, not requiring specific precision water-tight construction (Fernandes et al., 2016). Other mazes may similarly have a reward on one side and a start chamber on the other, but with different types of barriers blocking access that fish must learn to navigate (Girvan & Braithwaite, 2000; Lucon-Xiccato & Bisazza, 2017).

There can be obvious benefits of using unique alternative mazes. They can often be easily constructed and fit in with existing behavioral apparatus in a lab. They may also be necessary to ask a specific question or for a species' particular ecology. However, if there is no specific reason to use an alternative maze, there can be drawbacks associated with their use. The results may be harder to compare across species and studies. Importantly, they may also require additional pilot testing to confirm their validity and fine-tune the details of design and training protocols.

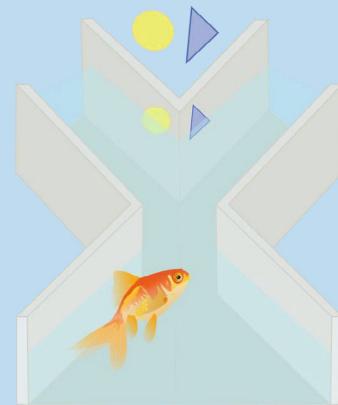
4 | GENERAL DESIGN CONSIDERATIONS

4.1 | Maze structure

After the type of maze is decided to use for an experiment, there are several additional general decisions related to the design of all mazes that must be made. The color of the maze, for example, is important as the background color can influence avoidance and approach behavior (scototaxis). Light/dark behavioral assays in different species of fish rely on a preference for dark backgrounds as a measure of anxiety-like behavior (e.g., Maximino et al., 2007; Maximino et al., 2010a; Maximino et al., 2010b). Indeed, this preference for dark backgrounds is found in mazes as well: in a plus-maze in which two arms were dark and two arms were light, zebrafish spent more time in the dark arms, but this was reversed with exposure to anxiolytic compounds (chloridiazepoxide and ethanol) that tend to reduce anxiety (Sackerman et al., 2010). However, using dark backgrounds may not always be ideal as they can make it challenging if one uses computer software to track the location of the fish in a maze. Human viewers

BOX 1 Using design to understand how fish navigate the world.

There are two different proposed strategies for processing navigational information (Paul et al. 2009). Individuals may encode the location of rewards relative to the position of cues (allocentric strategy) and/or encode their own orientation relative to external cues (egocentric strategy) (Burgess, 2008). Whereas, egocentric navigation uses specific landmarks in the environment that act like beacons (Braithwaite and De Perera, 2006; Johnsen and Rytter, 2021) or a series of learned directional responses (left, right, forward, backward) (Paul et al. 2009; Johnsen and Rytter, 2021), allocentric navigation involves the formation of a 'cognitive map' that is built from possible different routes using detailed spatial relationships between multiple landmarks (Poucet, 1993; O'keefe and Nadel, 1978). Thus, egocentric navigation involves components of simple associative learning (e.g. turn left at the brick house) (Wolbers and Wiener, 2014), whereas, allocentric navigation is more robust and flexible, and considered more cognitively demanding (Negen et al. 2020; Vijayabaskaran and Cheng, 2022). In the 1990s, a series of studies



emerged using goldfish and mazes and procedures that were explicitly designed to distinguish between different navigational strategies and their neural mechanisms (Rodriguez et al. 1994; Salas et al. 1996a,b). By varying the need for goldfish to use extra-maze cues to solve a spatial task, these studies showed that fish were capable of complex forms of spatial learning. This paved the way for exploration into allocentric and egocentric navigation in other fish species, and the importance of geometric information as a cue in solving spatial tasks (Lee et al. 2013; Yashina et al. 2019; Baratti et al. 2020).

may also find it difficult to locate fish against a dark background in videos. Most programmes recommend using white or light backgrounds to increase the contrast between the subject and background. Similarly, many fish prefer to stay close to walls over moving in the open (thigmotaxis) and prefer deep to shallow water (geotaxis) (Maximino et al., 2012), which should be considered when designing mazes. For example, reward locations that are easier to access while remaining close to the walls may be preferred by fish to ones that require them to cross open spaces (Jones et al., 2023). These preferences can also be leveraged in the design: a plus-maze with deep and shallow arms was proposed to be akin to sheltered and open arms in elevated plus-mazes used in rodent research (Walsh-Monteiro et al., 2016). Deeper reward chambers have also been incorporated into different maze designs in zebrafish studies (Darland & Dowling, 2001; DePasquale et al., 2016; Wang et al., 2022).

The size—both overall and the ratio of the lengths of different portions of the maze—may also be important. Maze size should consider the general size of the test species; for example, a maze that is a suitable size for a salmonid may not be suitable for smaller fish species such as stickleback and vice versa because the distances required to swim would not be ecologically relevant. In addition, the life stage of the animals should be considered, because mazes may need to be

resized as the animals grow (Varga et al., 2018). In research with zebrafish, the length of the start arm has varied, and in some cases more than doubles across studies (e.g., from 21 cm [McAroe et al., 2016] to 50 cm [Gaikwad et al., 2011]). Although the length of the choice arms is usually shorter than the start arm in the T-maze (but see Wang et al., 2022) the length of the choice arm also varies widely, from 8 cm (Ngoc Hieu et al., 2020) to 12 inches, approximately 30 cm (Darland & Dowling, 2001). The relative scale of the arms has been suggested as a modification to increase experimental throughput, because a shorter distance to the target arm is thought to reduce acclimation and training time (Ngoc Hieu et al., 2020). Indeed, fish in mazes with longer arms took longer to leave the start chamber and, in many cases, did not leave at all, reducing overall participation (Jones et al., 2023).

Other factors of the maze, including the lighting and temperature, should also be considered, as maintaining these at a constant level can require planning. As discussed earlier, zebrafish are scototoxic and tend to prefer dark areas, so uneven lighting can lead to shadows biasing where zebrafish want to spend their time. Concerningly, few authors report lighting conditions (Benvenutti et al., 2021). Although a variety of water parameters may affect performance and participation, as ectotherms, the behavior and physiology of fish is highly affected by the water temperature; evidence suggests that, within their

thermal tolerance zone, fish learn at a faster rate in warm water compared to cold water (Babkiewicz et al., 2021). Aside from ensuring lighting and water quality parameters are within the normal range for the focal species, these environmental factors should be held constant across trials for validity and repeatability of trials.

4.2 | Cues

Many experiments rely on visual cues to direct fish in the learning process. The presence (or absence) of a cue and the location of the cue (inside vs. outside the maze) in a spatial learning maze are important. For example, intra- versus extra-maze cues were used to distinguish between potential navigational strategies used in the spatial learning process (Box 1). The presence of a visual cue typically increases the rate of spatial learning (Wuxin et al., 2023), and fish may develop different search patterns in the presence or absence of visual cues (Hughes & Blight, 1999). Babkiewicz et al. (2021) used color cue cards located at the end of the correct choice arm to act as an intra-maze landmark. In contrast, McAroe et al. (2016) attempted to control for intra-maze cues, forcing fish to use visual cues outside the maze or depend on directional information. Similarly, Gaikwad et al. (2011) relied on visual cues in the room (e.g., table and door) to act as landmarks.

Aside from color and location, other features of the visual cue may also be important and influence preference for a particular path in a maze. For example, in the development of standardized Y-maze protocols, Cognato et al. (2012) found that zebrafish spent less time in arms marked with a cross compared to squares and triangles but similar amounts of time in every arm when they were marked with a circle, square, and triangle. Whether these preferences are the same for other species though is untested. Evidence shows that color also influences preference. Famously, female guppies (*Poecilia reticulata*) prefer reds, oranges, and yellows (Houde & Endler, 1990). Barramundi (*Lates calcarifer*) prefer blues and greens, but this preference can be changed depending on the rearing environment (Ullmann et al., 2011). Although results differ slightly depending on the exact comparison, zebrafish tend to prefer blue and red over green and yellow (Avdesh et al., 2012; Kim et al., 2017; Park et al., 2016; Peeters et al., 2016; Zhang et al., 2018). Importantly, pairing preferred colors with food rewards resulted in faster learning compared to unpreferred colors or no color cues (Kim et al., 2017), so considering cue color is important when designing maze trials, particularly if speed of learning is important for the research question.

Even when specific visual cues are not used in mazes, rewards often come with their own associated cues that must be considered to ensure that fish are not locating rewards based on unconditioned knowledge (e.g., finding food because of its smell). In a poorly designed test, fish can use the associated cues to locate the reward, making learning unnecessary. To avoid this, rewards are often placed in every possible end location but are accessible only from certain predetermined paths (e.g., Jones et al., 2023), making tracking by olfactory senses more difficult. Water changes between trials can also be

used to reduce olfactory cues associated with rewards and previous test subjects (e.g., McAroe et al., 2016). Various procedural modifications have also been used, such as floating feeder rings (Babkiewicz et al., 2021) or developing unique jelly-like bait (Gaikwad et al., 2011) to contain the food reward and limit unwanted visual cues.

5 | EXPERIMENTAL PROCEDURES

5.1 | Unconditioned stimuli and the motivation to learn

In spatial learning tasks that require an individual to find a specific location, perhaps the most important question is the identity of the unconditioned stimuli to reinforce that location. This decision should not be made lightly, particularly because individuals differ in their motivation for stimuli. Food is commonly used, potentially because it is used in the training of a variety of other species, but there are drawbacks associated with using food. Fish satiate relatively quickly, so they may lose motivation if there are multiple trials in a day (Sison & Gerlai, 2011). Additionally, standardizing hunger levels is challenging. Typically, researchers feed all individuals after a trial to attempt to make all fish similarly motivated for subsequent trials, but intrinsic metabolic differences between individuals mean that individuals likely still differ in their motivation to forage (Biro & Stamps, 2010; Killen et al., 2011). Using special preferred foods during trials may keep trials as rewarding experiences, but new foods can require specific habituation (Babkiewicz et al., 2021; Gaikwad et al., 2011). Further, many fish will not forage when alone, and social dynamics alter the amount fish will eat (Gil & Hein, 2017), which presents a challenge for trials where fish are typically tested in isolation. This issue can be overcome by conducting trials using groups of fish (Spence et al., 2011) or slowly reducing the number of fish as trials progress until eventually a single fish is tested (Gaikwad et al., 2011; Grossman et al., 2011).

Aside from food, access to a social group can also be used as the unconditioned stimuli (e.g., Al-Imari & Gerlai, 2008; Burns & Rodd, 2008; DePasquale et al., 2016). Sometimes access to a social group is also combined with a food reward (Lucon-Xiccato & Bisazza, 2017). For social species that spend the majority of their time schooling this may be an ideal stimulus. Even individuals of social species differ in the amount of time they choose to spend with a social group (Gartland et al., 2022; Laskowski & Bell, 2014) and individual social behavior may be related to other behaviors (Sih et al., 2004) including cognitive abilities (Lucon-Xiccato & Bisazza, 2017). Social status is also related to cognitive abilities in some fish (Wallace et al., 2021), and status within a group could lead to further biases in motivation to engage with a social group. Housing fish individually may decrease variation in motivation to spend time with a social group (Munson et al., 2021) but reduce cognitive abilities, irrespective of motivation or activity (Brandão et al., 2015). Fish can be housed individually during testing for easy identification (e.g., three-spine sticklebacks, Jones et al., 2023); however, highly social species such as zebrafish may be more sensitive to the negative effects of social

isolation making comparisons across species challenging (e.g., McAroe et al., 2016).

Refuges located in a deeper area of the maze, sometimes with enrichment in it (e.g., Darland & Dowling, 2001), have also been used as the unconditioned stimuli. These areas may be used as a shelter; however, like social tendency, boldness can differ between individuals (e.g., Coleman & Wilson, 1998; Dahlbom et al., 2011; Ehlman et al., 2019), potentially leading to biased motivation to participate and locate the stimulus. Although many potential benefits and drawbacks are associated with each of these different kinds of stimuli, a gap still exists in the literature because, to our knowledge, no study has directly tested the influence of different stimuli on participation in trials and rate of learning.

5.2 | Acclimation, training, and probe trials

Other procedural questions that may influence spatial learning relate to acclimation and the timing and frequency of learning. Acclimating individuals to the testing apparatus can be a useful way of reducing neophobia and can promote learning and exploration (Brown, 2001); however, how best to acclimate individuals is not always clear. Acclimation may occur at the start of the trial by keeping fish in an enclosed area prior to release (e.g., Jones et al., 2023). A settling period after the fish is initially placed in the testing arena can be important to minimize stress from handling and transfer (Brydges et al., 2009; Brydges & Braithwaite, 2009). How long this period should be is not well known though, and different lengths of time have been used (e.g., 5 min [Babkiewicz et al., 2021]; 30 s [Ngoc Hieu et al., 2020]). Special acclimation trials may also be included in which fish—either individually or as a group—are allowed to explore the experimental apparatus prior to rewarded trials (Babkiewicz et al., 2021). Acclimating to the maze conditions as a group may minimize social stress; group size can be slowly reduced over trials until fish are tested by themselves (Gaikwad et al., 2011).

The length, number, and frequency of behavioral trials may also impact participation and perceived learning. Even within the same species there has been considerable variation in these factors. Zebrafish perhaps represent the best example of this; as few as 3 trials across 3 days have been reported (Darland & Dowling, 2001; Wang et al., 2022), but as many as 10 trials per day per training block have also been used (McAroe et al., 2016). What marks the end of a trial also differs across experiments. Trials can be ended as a function of time (e.g., Jones et al., 2023) or when the fish has successfully found the reward (e.g., McAroe et al., 2016). Trials that are terminated on completion are more demanding for the experimenters, who must watch fish in real time, but may be beneficial, particularly if returning to the home tank is reinforcing for fish. Watching fish in real time requires humans to be present though; trials that are of a set time have the benefit of being able to be video-recorded. This means more fish may be able to be run simultaneously and experimenters can leave the room, potentially reducing distractions and improving participation rates. Without focused testing though, which method

results in the most efficient learning and highest participation rates is hard to know definitively.

6 | FUTURE RESEARCH DIRECTIONS

Although we feel that there is not yet sufficient evidence to design the perfect maze, we hope to encourage this type of research in the future, with the goal of making spatial learning trials more efficient, comparable and rigorous for all research questions. Many spatial learning studies require multiple trials per individual and multiple individuals to obtain robust results. The time required to conduct this type of research naturally limits our ability to address every spatial learning question immediately. However, careful experiments that deliberately test the impacts of different design features on participation and learning rates are much needed. Not only will they direct the design of future studies, making the results easier to generalize across studies and labs, but they may be able to improve participation rates, making studies more rigorous by reducing bias, and increase learning rates. Improved participation and increased learning rate also have the added benefit of potentially making the experiment run faster, opening up more time to address additional questions and better understand the variation in spatial learning present in fish.

Design-based questions may not be the main focus of any one research group; however, finding ways to address these questions is the responsibility of all researchers interested in spatial learning. One way to begin generating research-backed evidence on the influence of design on learning to better inform maze design is by incorporating additional treatment groups in studies designed to answer more ecologically relevant questions (e.g., Jones et al., 2023; Cortese et al., 2024). Additionally, collaborating with multiple labs to simultaneously study the same design question in multiple species can improve the applicability and robustness of results (Jones et al., 2023). Even just improving the reporting of methods and specific results may facilitate meta-analyses that could lead to more specific guidelines (as has been done for other experimental methods [e.g., Killen et al., 2021]). Including all recorded behavioral measures in supplementary materials (e.g., time to leave the start box and time to reach the reward) would also allow future researchers to compare the impact of design features on useful behavioral metrics. Similarly, gathering as much information about the study animals (e.g., STRANGE framework [Webster & Rutz, 2020]) and housing conditions (e.g., DETAILS framework [Jones et al., 2021]) and procedures prior to and during testing can help inform other studies and reduce potential biases that may impact cognitive performance. Finally, formalizing and publishing preliminary tests that are designed to validate methods of a specific study can reduce the need for future researchers to “reinvent the wheel” when making design decisions. With some forethought and appropriate controls even short trials prior to an existing planned experiment can be used to answer numerous important design questions. Particularly for early career researchers, conducting short trials like this prior to committing to longer experiments may be a beneficial way to run multiple studies in succession and gain experience in

conducting trials prior to starting more complicated studies. The more we, as a field, get into the practice of conducting these preliminary tests in a systematic, scientific manner, the more data we will accumulate in different species that can then be used to draw general conclusions about best practices to inform future research. Any of the aspects of design and procedure that we discussed in this paper would benefit from formal testing to either gather information about best practices or rule out as important in influencing meaningful measures of learning. In our opinion, issues relating to color and size of the arena as well as time to acclimate to the arena and length of trial are particularly important. A study directly testing the efficacy of different rewards across species on participation and learning rates would also be exceptionally valuable for all researchers.

Technology will likely change the way we measure a variety of traits in fish, including spatial learning, and with it will come new design challenges. More knowledge about current methods will only facilitate our ability to integrate new technology smartly into our research programmes. Maze trials are often recorded with the researcher absent, so a key consideration when making experimental design decisions is how to extract meaningful data from the videos in a time-efficient and unbiased manner. Tracking software has already made recording activity of fish, including in mazes, easier (e.g., Walter & Couzin, 2021). However, with it come additional considerations, including the color of the background and the height of the maze to avoid visual occlusion. Technological advances may also change the way we are able to test the spatial learning abilities of fish in the future. For example, an automated learning device has been built for captive baboons (Fagot et al., 2015). In this system, baboons can roam around a naturalistic enclosure where they have free access to computerized systems where they can interact with cognitive experiments on a voluntary basis. Radio frequency identification (RFID) systems are used to make the tests and results individual specific. If a similar RFID system was used to incorporate mazes into housing conditions for fish such that they could access them when they wanted, many of the issues of motivation and neophobia inherent to mazes could be reduced or systematically studied more easily. As we move forward with our understanding of how design influences our ability to detect differences in learning, we need to consider the ways we can adapt our understanding of current tests with future technology.

Ultimately the conclusions we draw about spatial learning are only as robust as the data we can collect. Fundamental to the acquisition of reliable behavioral data is a well-thought-out protocol and experimental design. Future research should be dedicated to deliberately and explicitly investigating how different design decisions impact behavioral outcomes with the goal of improving the robustness of spatial learning experiments to better understand how fish navigate their world.

AUTHOR CONTRIBUTIONS

C.D. conceived the idea for the review. A.M. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the final manuscript.

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