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TITLE:

Using Enclosed Y-Mazes to Assess Chemosensory Behavior in Reptiles

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SUMMARY:
Y-mazes enable researchers to determine the relevance of specific stimuli that drive animal behavior, especially isolated chemical cues from a variety of sources. Careful design and planning can yield robust data (e.g., discrimination, degree of exploration, numerous behaviors). This experimental apparatus can provide powerful insight into behavioral and ecological questions.

ABSTRACT:
Reptiles utilize a variety of environmental cues to inform and drive animal behavior such as chemical scent trails produced by food or conspecifics. Decrypting the scent-trailing behavior of vertebrates, particularly invasive species, enables the discovery of cues that induce exploratory behavior and can aid in the development of valuable basic and applied biological tools. However,

pinpointing behaviors dominantly driven by chemical cues versus other competing environmental cues can be challenging. Y-mazes are common tools used in animal behavior research that allow quantification of vertebrate chemosensory behavior across a range of taxa. By reducing external stimuli, Y-mazes remove confounding factors and present focal animals with a binary choice. In our Y-maze studies, a scenting animal is restricted to one arm of the maze to leave a scent trail and is removed once scent-laying parameters have been met. Then, depending on the trial type, either the focal animal is allowed into the maze, or a competing scent trail is created. The result is a record of the focal animal's choice and behavior while discriminating between the chemical cues presented. Here, two Y-maze apparatuses tailored to different invasive reptile species: Argentine black and white tegu lizards (*Salvator merianae*) and Burmese pythons (*Python bivittatus*) are described, outlining the operation and cleaning of these Y-mazes. Further, the variety of data produced, experimental drawbacks and solutions, and suggested data analysis frameworks have been summarized.

INTRODUCTION:

Y-mazes are common, simple tools in studies of animal behavior that allow for a variety of questions to be addressed. In addition to being widely used in laboratory studies, Y-mazes are also functionally compatible with various field environments to study wild animals in relatively remote settings. Researchers have examined the behaviors of wild vertebrates using Y-mazes in a wide variety of taxa across similarly diverse field applications (e.g., lampreys[1]; cichlid fish[2]; poison frogs[3]; lacertid lizards[4]; garter snakes[5]).

Many researchers are focused on how and to what degree chemical cues drive animal behaviors in reproductive, spatial, and foraging ecology[6]. A variety of chemical stimuli can be tested in Y-mazes and at fine scales, such as two chemical trails that only differ slightly in concentration[7], or detection ability based on the reproductive status of the target species[8]. Chemical trails—the principal stimulus used in Y-maze tests—can be naturally created by conspecifics or specifically placed in the environment by a researcher using a defined chemical source[1,5]. Stimuli can also be tested in unique combinations to determine multimodal influence of cues such as changing contexts of cue presentation (airborne vs. substrate trails[9]; visual plus chemical cues[10]). Although there are many other methods for assessing chemosensory responses in reptiles (see discussion section), Y-mazes allow for searching behavior(s) to be assessed and at multiple temporal and spatial scales, which can lead to greater levels of behavioral inference.

Reptiles have been broadly tested for their reliance on chemical cues in reproductive and foraging ecology, and researchers often employ Y-mazes in these studies[11,12]. The chemical ecology of reptiles continues to be deciphered by studies employing Y-mazes to address a variety of evolutionary and behavioral questions that are valuable to wildlife managers. For example, recent tests with invasive snake and lizard species have revealed that chemical cues alone can influence choice and time allocation within the novel environment of a Y-maze[13-15].

The use of large Y-mazes for moderately sized focal animals (e.g., large-bodied reptiles) is generally restricted to laboratory settings where the focal animals can be housed easily over the long term, experimental factors (e.g., climate, light, external stimuli) can be controlled, and access to infrastructure (e.g., power, running water) is unlimited. Studies on wild animals, however, are often restricted to specific locations for various reasons (e.g., logistics permitting). As a result, challenges arise that must be addressed through creative problem solving and methodological adjustments to maintain consistent and comparable results.

Here, two experimental setups have been described using Y-mazes and remote monitoring tools to assess reproductive chemical ecology of invasive squamate reptiles (i.e., snakes and lizards) in different field scenarios: wild-caught, captive Argentine black and white tegu lizards (*Salvator merianae*) in Gainesville, FL, and wild-caught Burmese pythons (*Python bivittatus*) in Everglades National Park, FL. As implied by its name, the Y-maze apparatus creates an experimental environment in which an animal enters a main passageway (the base of the Y; “base”) which then leads to two divergent passageways (the arms of the Y; “arms”). In these experiments, two types of animals are used for a single trial: scent-laying animals (provide the stimulus scent in a restricted area of the maze) and focal animals (data are collected on this animal as it explores the scent trail).

As an experimental apparatus in chemoecological studies, any Y-maze must be constructed in a way that allows easy removal of the animal within and can be dissembled for thorough cleaning and reset. Also discussed are the constraints inherent to these different testing environments (e.g., diurnal vs. nocturnal animals, infrastructure differences) that prompted methodological adjustments. Although the focus was on tegu lizards and Burmese pythons, these designs can be applied to a wide range of reptile species. In this research on invasive reptiles, Y-mazes benefit the rate and scale of inference because they enable rapid collection of data to inform management goals that shift in-step with the invasion threat posed by a given species. In particular, studying chemoecology of invasive species is critical for the development of effective chemical control tools.

Discrimination is the key observation from empirical tests using Y-mazes where a focal animal chooses between two stimuli and that decision-making process is assessed. A swath of behaviors can also be scored in Y-maze trials during the trial itself (live) or after the trial (video) to expand inferential power. The complexity of the *a priori* objectives of a given study dictate whether live observation or archived recordings best suit the design. Here, Y-maze methods have been described in detail for addressing chemoecological questions to inform future studies by researchers interested in similar questions on reptile behavior, especially in chemical ecology.

PROTOCOL:

All procedures involving the use of live vertebrates were approved by the Institutional Animal Care and Use Committees of the U.S. Department of Agriculture and the U.S. Geological Survey.

NOTE: Because these studies focus on invasive vertebrates, compliance with containment standards must also be met, which impose specific constraints on the design and execution of experiments. Although many of the methods are similar between the two study locations and diurnal vs. nocturnal study timing, distinct methods have been described in each of the following two sections.

1. Y-maze setup and diurnal protocol for the U.S. Department of Agriculture (USDA) Animal Plant Health Inspection Service (APHIS) Wildlife Services National Wildlife Research Center Florida Field Station: on-site testing of wild-caught, captive tegus

NOTE: Plans for all components of the Y-maze and containment structure are provided in **Supplemental File 1**.

1.1. Y-maze dimensions and design

1.1.1. Use a bottom piece (1.22 m x 2.44 m fiber cement siding panels) to anchor the Y-maze. Drill holes in the top layer to allow carriage bolts to pass upward for the attachment of the maze pieces. For specific directions, see **Supplemental File 1**.

1.1.2. Construct the walls of the maze out of white PVC trim board; internal dimensions of the base are 120 cm L (side walls) x 42 cm W x 14 cm H.

NOTE: Passageway width was designed to accommodate 2x focal animal width. Extra width allows flexibility for two, animal-deposited scent trails to be created.

1.1.3. Make sure that the internal dimensions of the arms are 120 cm L (side walls) x 40 cm W x 14 cm H.

1.1.4. Assemble the maze using separate bottom, side, and top components secured together prior to running a trial. Make the top out of clear acrylic to enable visualization of animals within maze. For specific directions, see **Supplemental File 1**.

1.1.4.1. When a single scent trail is created, use an internal partition in the base to restrict space access for the scent-laying tegu. For specific directions, see **Supplemental File 1**.

1.1.4.2. When two scent trails are deposited by different animals in sequence, use a system of partitions to block alternating arms of the maze and exclude each animal from alternating halves of the base. For specific directions, see **Supplemental File 1**.

1.1.5. Use boxes to allow transport and collection of animals used in Y-maze trials. Make sure that all boxes are opaque and fitted with removable lids and acrylic doors that are easily secured.

1.1.5.1. Ensure that the base box (109 cm L x 56 cm W x 46 cm H) is at the opening of the base of the Y-maze. Use it to transfer scenting or focal animals to the maze and for acclimation prior to opening the door and permitting voluntary access of the animals into the maze.

1.1.5.2. Make sure that the arm boxes (83 cm L x 50 cm W x 44 cm H) are at the terminal ends of the arms of the Y-maze to facilitate the capture of either scenting or focal animals.

1.1.5.3. For specific directions on construction and assembly, see **Supplemental File 1**.

1.2. Camera setup for diurnal video acquisition

1.2.1. Camera specifications: Ensure that project cameras can record continuous video under variable light conditions and are suitable for outdoor use under prevailing temperature and humidity conditions.

1.2.1.1. With the camera mounted to the underside of the study enclosure, ensure that the entire Y-maze can be captured in the camera's field of view. Adjust the lens or height of the camera to increase or decrease the field of view. When the field of view is set, ensure that sufficient behavioral detail, such as tongue-flicks, can be captured.

NOTE: If the height of study enclosure is fixed (e.g., 180 cm H) thereby limiting adjustments to the field of view, multiple cameras can be used to obtain complete coverage of the Y-maze interior. Ensure that cameras are set to enable "wide dynamic range" when used in outdoor applications.

1.2.2. Power specifications: Ensure that each camera has an adequate power supply to record continuous video for the duration of the planned trial (e.g., use an Uninterruptible Power Supply (UPS) with a built-in backup battery to ensure continuous power).

NOTE: If there is no AC power supply available, POE (power over ethernet) cameras can be powered via network cables connected to a digital video recorder (DVR) or POE switch where a network video recorder (NVR) is used.

1.2.3. Recording specifications: When choosing a DVR or NVR, make sure that it meets project requirements including sufficient storage capacity and enough POE connectors (DVR) or camera channels (NVR) to accommodate the number of cameras used. Select recording parameters to suit the video quality desired, keeping the size of the data files in mind (e.g., H264 compression rate and an image rate of 10 frames per second [FPS]).

1.2.4. Protocol for obtaining and processing video:

1.2.4.1. Begin recording the video from the moment the animal begins to enter maze to the moment of capture or preset timeframe (1.3.3.4).

1.2.4.2. Using software that can be installed on one or more computers and allows viewing of live or recorded video, export files using the video format of choice.

1.2.4.3. Be sure to export the same time window and duration of video for each camera used to allow simultaneous review of multiple feeds.

1.2.4.4. Be sure to export the data regularly because many systems will overwrite older data with new data if DVR/NVR file storage capacity is limited.

1.3. Protocol for running scent-laying animals

1.3.1. Assessment of bias

1.3.1.1. Prior to running experimental trials, assess a Y-maze for bias by assembling the maze, as described below , but without presenting the scent on the paper. Acclimate the focal animal, and start the trial.

NOTE: Depending on the design of the study (e.g., repeated measures using the same focal animals vs. testing of novel focal animals each time), bias trials will establish that the maze itself, by design, does not bias a focal animal's choice. Many factors contribute to bias such as elevation, sunlight, and visual markers.

1.3.1.2. If reorienting or adjusting other physical aspects of the maze do not remove side bias, randomize the arm designated to receive an experimental scent in a given trial.

NOTE: Over a set number of trials, an unbiased maze results in a choice probability of 0.5 for either arm, and a binomial test is performed (**Figure 2**).

1.3.2. Trial preparation and Y-maze assembly

1.3.2.1. Wear nitrile gloves throughout when handling any surfaces that the animal can explore to avoid scent contamination. Change the gloves between trials and within the setup of a trial if multiple scent trails are being created.

1.3.2.2. Prepare new, clean scenting paper (white butcher paper, minimum 61 cm wide) on a clean surface. Cut to appropriate length such that the paper for each section can overlap at the junction of the Y and extend past the ends of the base and the arms to fit under the boxes.

1.3.2.3. Sweep the bottom of the maze and then cover either with paper directly or with a boundary layer between the paper and bottom (e.g., plastic sheeting) to facilitate clean-up if the animal defecates or musks in the maze.

1.3.2.4. Secure the paper in place by piercing it with carriage bolts in the bottom, working from one end to another to keep the surface smooth. Overlap the papers at the junction such that the base paper is on top.

1.3.2.5. Place the sides of the maze into position over the carriage bolts, but do not secure them to the bottom.

1.3.2.6. Insert and secure the partition(s) needed for the trial type to be tested (see single-scent 1.3.3 vs. double-scent trials 1.3.4).

1.3.2.7. Slide the acrylic top pieces into slots, and secure with flat head nails.

1.3.2.8. Secure the sides to the bottom by tightening the wing nuts to the carriage bolts.

1.3.2.9. Put the clean arm boxes into place, and secure with thumb screws. Secure the box lid using cable ties. Ensure that the doors have been removed.

1.3.3. Single-scent trials

NOTE: The purpose of these trials is to present a single scent trail in the Y-maze that runs from the base through one arm.

1.3.3.1. Before fitting the acrylic top, secure the partition to block the untreated arm. Select the scented arm randomly (e.g., coin toss, random number generator).

1.3.3.2. Place the scent-laying animal into the clean, dry base box. Secure the lid of the base box (e.g., cable ties, bolts) and the door (e.g., thumb screw). Transport the holding box to the study enclosure, and secure it to the end of the base of the Y-maze with thumb screws.

NOTE: Ensure that the door to the base box is in place prior to loading the animal.

1.3.3.3. Acclimate the animal in the box for a set, consistent period (e.g., 60 min). Remove the base box door, and allow the animal to enter the maze freely.

1.3.3.4. Monitor animal activity remotely using video feed (see below). After the animal has traveled from the base box to the arm box, remove the animal from the maze as scenting is complete.

1.3.3.4.1. If the animal is inside any box, insert and secure the removable door, remove the box, and return the animal to its enclosure.

1.3.3.4.2. If the animal is back in the maze, wait near the maze until the animal is seen returning to the box, and then remove the box.

NOTE: Squamates defecate defensively and create alarm cues that contaminate the scent being tested, so avoid startling the animal.

1.3.3.4.3. If the animal does not return to a box, slowly approach the maze and use visual cues (e.g., slow hand waving) to encourage the animal into the box, and then remove the box.

1.3.3.5. Clean and dry the base box (1.5.5).

1.3.3.6. If defecation occurred, collect and absorb as much as possible with a paper towel, but do not wipe to prevent spreading.

1.3.3.7. Partially disassemble the maze to allow removal of the interior partition, and then reassemble. Clean the partition (1.5.5).

1.3.3.8. Proceed to section 1.4 for protocol for running focal animals.

1.3.4. Double-scent trials

NOTE: The purpose of these trials is to present two different scent trails simultaneously in the Y-maze, with both running from the base through their respective, randomly chosen arm.

1.3.4.1. Before fitting the acrylic top, secure the partitions to block the arm not chosen for the first scent and half of the base opposite to the blocked arm.

1.3.4.2. Follow the procedures outlined above for a single scent trial (1.3.3 to 1.3.3.8) with one exception. When the acrylic door is removed (1.3.3.3), insert a half-size door into the opening on the side that is to remain blocked to ensure that the scenting animal can only move in the open section of maze.

1.3.4.3. Partially disassemble the maze, remove the partitions, and clean (1.5.5). Dry with clean towels.

1.3.4.4. Reinstall the partitions, but flip them to block the now-scented area of the maze. Reinstall the acrylic top.

1.3.4.5. Repeat step 1.3.4.2 for the second scent-laying animal.

1.3.4.6. Partially disassemble the maze and remove the partitions. Reassemble the maze.

1.3.4.7. Proceed to section 1.4 for protocol for running focal animals.

1.4. Protocol for running focal animals during diurnal hours

1.4.1.1. Follow steps 1.3.3.2 to 1.3.3.3 with the focal animal planned for that trial.

1.4.1.2. Monitor animal activity remotely using video. If observing over a set window of exploration time, start the timer when the animal has completely emerged from the base box.

1.4.1.3. At the completion of the trial, remove the animal (1.3.3.4).

1.5. Breakdown and clean-up

1.5.1. Detach the remaining boxes from the maze and disassemble all the boxes. Wear fresh nitrile gloves throughout the disassembly and cleaning.

1.5.2. Remove the acrylic top pieces and set them aside in a safe location for cleaning to avoid scratching or cracking. Be sure to avoid scratching pieces when removed (clear field of view for video-monitoring behavior must be maintained). Disassemble the sides of the maze and set them aside for cleaning.

NOTE: Minimize scratches and UV degradation to Y-maze materials by keeping them always shaded.

1.5.3. Remove the paper (and plastic) in a consistent motion by rolling it up to avoid contamination of the bottom and discard it.

1.5.4. Use an odorless, laboratory-grade soap and soft scrub brush or microfiber cloths to clean all the surfaces of the Y-maze pieces and all the boxes. Clean the acrylic top pieces and removable doors with the same soap, but with soft sponge or microfiber cloths to prevent scratching.

NOTE: The known chemical signals in squamate reptiles are lipid-soluble compounds, and washing with a detergent is the standard protocol for cleaning lipid cues and other scents from polymer-based apparatuses in terrestrial vertebrate studies[11,12,21].

NOTE: In field applications, sanitation protocols may be required. If so, spray all the inner surfaces of the maze (floor, walls, partitions, acrylic pieces, boxes) with an appropriate sanitation solution, let it sit for 10 min, and then wipe with a microfiber cloth.

1.5.5. Rinse the cleaned components with water by wiping the surfaces with clean, wet microfiber towels, and avoid allowing soap residue to dry prior to rinsing; do not pour water into the maze.

1.5.6. Allow the pieces to air dry or pat them dry with fresh microfiber cloths.

1.5.7. Once dry, reassemble the maze pieces if running another trial immediately.

2. Y-maze setup and crepuscular timing protocol for the U.S. Geological Survey (USGS) trials in collaboration with National Park Service: relatively remote testing of wild-caught Burmese pythons

NOTE: Plans for all components of the Y-maze and containment structure are provided in **Supplemental File 2**.

2.1. Y-maze components and rationale for changes to USDA design

NOTE: The Y-maze described was significantly altered to expand potential research species and in isolated conditions. The vertical depth was increased to accommodate a variety of species, and different materials and construction methods were used to improve outdoor durability and cleaning. See **Figure 1** for a visualization of the completed maze. For specific directions on construction and assembly, see **Supplemental File 2**.

2.1.1. Cut Y-maze components from white polypropylene, and heat-weld all cut pieces that are to be permanently fixed (e.g., maze bottom and side walls).

2.1.1.1. Anchor the bottom of the Y-maze (244 cm L x 122 cm W) made of plywood sheets fastened together with deck screws, attaching it via an aluminum angle bracket riveted along the bottom of the outer side walls of the maze.

2.1.1.2. Ensure that the base of the Y-maze is 120 cm L x 42 cm W x 23 cm H, and that each outer arm side wall is 120 cm L, inner arm side wall is 108 cm L (for specific directions, see **Supplemental File 2**).

2.1.1.3. Slot the acrylic top into place using an aluminum angle fixed to the side walls with screws every 30 cm (for specific directions, see **Supplemental File 2**).

NOTE: Screws placed at regular intervals at the top edge of the Y-maze side walls also serve as static visual markers when analyzing the video from the trials and provide scale.

2.1.1.4. Ensure that each opening of the Y-maze (base, arms) has an additional baseplate (42 cm W x 30 cm H) on the end of the side wall that attaches to a box so that the baseplate frames a central opening (34 cm W x 16 cm H; for specific directions, see **Supplemental File 2**).

2.1.1.5. Use a partition piece to restrict access of the scenting animal (for specific directions, see **Supplemental File 2**). Secure a blocking plate (46 cm W x 22 cm H) in place using fastener tape. Anchor the partition and the plate using improvised, easily cleaned weights (e.g., plastic jug filled with water; 2.3.6; **Figure 1**).

2.1.1.6. Ensure that the acrylic pieces make up the top of the maze (0.6 cm thick, clear). For specific directions, see **Supplemental File 2**.

2.1.2. Use opaque boxes fitted with a sliding door and lids that are easily secured to allow for transport and collection of animals in Y-maze trials (**Figure 1**).

2.1.2.1. Modify the boxes (21.6 cm L x 27.9 cm W) with drain holes in the bottom, fit the lids with small screws and nuts, and provide a single opening for ingress/egress (the door). For specific directions, see **Supplemental File 2**.

2.1.2.2. Fasten the box to the end of the Y-maze by attaching the box faceplate to the Y-maze faceplate using bolts and wingnuts or locks.

NOTE: When in place, boxes also anchor the acrylic top pieces in place.

2.2. Camera setup for crepuscular video acquisition: See **Figure 1** for a snapshot of the camera field of view.

2.2.1. Camera specifications: Ensure that the project camera can record continuous video under variable light and temperature conditions to accommodate crepuscular and nocturnal study species.

2.2.1.1. With the project camera mounted on the ceiling crossbeams of the enclosure, ensure that the entire Y-maze can be captured within the camera's field of view. Raise or lower the height of the tent to increase or decrease the field of view (project camera mounted at a height of ~3 m). Ensure that the reflection of the infrared light emitted from the camera on the acrylic top does not obscure critical portions of the frames in the overnight footage.

2.2.2. Power specifications: Ensure that each project camera has an adequate power supply to record continuous video for overnight filming (approximately 20 h).

NOTE: If there is no AC power supply available, power can be supplied using deep cycle sealed lead acid 12-volt batteries (e.g., two 12-V 20-Ah gel batteries wired in parallel).

2.2.3. Recording specifications: To minimize the file storage volume, record the lowest quality video that is still adequate to enable counting of tongue-flicks in the Y-maze.

NOTE: High-resolution footage requires large storage volume, and lowering the resolution is a very effective way of ensuring that file sizes are manageable.

2.2.3.1. Limit the framerate (frames per second, FPS) of footage to the minimum needed to detect tongue-flicks (e.g., recording resolution of 800 x 450 with a maximum framerate of 25 FPS results in approx.120 GB of footage per trial).

2.2.4. Protocol for obtaining and processing video

2.2.4.1. Arm the camera at the beginning of each scenting event (2.3.10), and let it record continuously through to the end of the focal event (approximately 20 h).

2.2.4.2. After each trial is complete, power off the camera and retrieve the SD card (2.4.4). Transfer the footage to the desired storage location.

2.2.4.3. As SD cards frequently force recording devices to record footage in 5-min clips, combine these clips using movie processing software for ease of processing.

2.2.4.4. Review the footage using a media file reviewing program that allows variable playback speed and customizable forward-jump intervals.

NOTE: This reduces review time from about 20 h down to a maximum of 1 h if fine-scale resolution is not required during video processing.

2.3. Protocol for running scent-laying animals

NOTE: Steps in this section will take approximately 1.5 days to complete due to longer acclimation times for wild reptiles.

2.3.1. See bias preface in section 1.3.1 to ensure that no bias can be found in the maze.

2.3.2. Wear nitrile gloves throughout when handling any surfaces or study animals to avoid scent contamination.

2.3.3. Place the scenting or focal animal into its box at least 24 h prior to the trial for acclimation.

NOTE: To minimize stress effects, the box is left in a shaded area as close to the maze as possible without being disturbed by cleaning or other activities. Ensure that all animals tested (scenting, focal) are acclimated this way.

2.3.4. Prepare new, clean scenting paper on a clean surface and of sufficient length to overlap at the junction of the Y, and cover the entire bottom surface (2 arm papers = 121.9 cm; 1 base paper = 152.4 cm).

2.3.5. Secure the ends of the papers near the boxes and the Y-junction with masking tape.

2.3.6. Install the partitions to block half of the base arm (left or right side) with a long partition, and block the entrance to the opposite arm with a short partition. When installing the barriers, do not rip the scenting paper. For large scenting animals, affix a heavy object that can easily be removed and cleaned behind the barrier as a brace to prevent barrier failure (2.1.1.5).

NOTE: The scent trail must always start on one side of the base, then cross to the opposite arm so that focal animal's choice is clear.

2.3.7. Slide the acrylic top into place, one section at a time, and ensure that the angles meet completely. Use clear plastic tape to cover any gaps.

2.3.8. Attach both the arm boxes to the maze by connecting the faceplates using wingnuts and/or padlocks, and ensure that the doors are locked open.

2.3.9. Two hours before sunset, attach the base box (containing the scenting animal), and ensure all movements are slow and steady to minimize stress to the animal.

2.3.10. Arm the camera, and open the door to the base box, being sure to latch the door in place using both barrel bolt locks. Remain out of the animal's view, and exit the area.

2.3.11. After 3 h (1 h after sunset), note the location of the animal within the maze as well as the ambient conditions. If the animal is in transit, wait until it enters the box.

2.3.11.1. If the animal is in any box, close and secure the box door, remove the box, and then remove the animal, taking care to prevent defensive scent deposition in the box.

2.3.11.2. If the animal is motionless inside the body of the maze, use visual cues (e.g., long rod or hand waving) to stimulate its movement into a box. If the animal remains, remove the arm box(es) so that the acrylic top can be removed, and the animal can be collected manually and transferred to a bag.

NOTE: Proper personal protective equipment (PPE) is always to be worn when handling large animals (e.g., puncture-resistant gloves, eye protection).

2.3.12. Partially disassemble the maze to allow removal of the interior partitions (avoid disturbing the scent paper) and then reassemble. If defecation occurred, collect and absorb as much as possible with clean microfiber cloths, but do not wash the area.

2.3.13. Proceed to section 2.4 for protocol for running focal animals.

2.4. Protocol for running crepuscular focal animals

NOTE: Steps in this section will take approximately 2 days to complete and must begin around the same time as the start of section 2.3.

2.4.1. Acclimate the scheduled focal animal in the box for at least 24 h prior to being run in maze.

2.4.1.1. During the final hours of focal animal acclimation, run the scent-laying animal prior to moving to the next step (2.3.9).

NOTE: Time the scent-laying step as close as possible to the time of introduction of the focal animal to the maze to reduce scent degradation.

2.4.2. Attach the base box (containing the focal animal) using wing nuts and/or padlocks to the base of the Y-maze. Use slow, steady movements when holding/transporting the box to minimize stress to the focal animal.

2.4.2.1. Ensure that both the arm box doors are latched opened. Begin the focal trial by opening and latching the base box door using barrel bolts. Remain out of the animal's view and exit the area.

NOTE: With wild nocturnal reptile trials, focal animals are given overnight to explore the maze.

2.4.3. Four hours after sunrise, return to the maze and follow section 2.3.11.1 to remove the focal animal.

2.4.4. Collect the camera SD card and recharge the batteries if needed. Discard the used paper from the maze and proceed to cleaning (section 1.5).

Insert Figure 1 here

REPRESENTATIVE RESULTS

A multitude of variables can be recorded and/or scored from Y-maze trials. The design of the study should be primarily driven by the desired outcomes/deliverables. Further, if the study is relying on repeated measures (e.g., repeated use of the same focal animals), proper testing and analysis structures are required. For example, as the USDA trials relied on repeated testing of focal tegus, the planning of experimental trials was fully randomized.

Choice data: The majority of studies using Y-mazes report simple binary choice data and analyze the results with parametric statistics such as a binomial test. The chief limitation here is sample size, which directly affects the power of any statistical analysis. In **Figure 2**, a series of statistical thresholds per study sample size are depicted that demonstrate how many "successes" would need to occur for a given binomial test to yield statistically significant results. These are mathematically derived and therefore generalizable to any Y-maze test. Binomial statistics are easy to generate using online freeware. For calculating probabilities, one-tail distributions are used if an *a priori* rationale is given; otherwise, the two-tail distribution should be used.

Choice of an arm is often determined by the distance the focal animal moves in a given arm. The simplest way to set this threshold is by establishing a landmark within the maze. For most Y-maze studies, the landmark is the entrance of the arm box. Because reptiles conduct all chemosensory assessment with the chemical-sensing organs in the anterior region of the head, the head is the focal point during a trial. For example, because Burmese pythons are often longer than the entire maze itself, choice is best and most efficiently determined by the movement of the head past a landmark. Other options for determining choice are time spent in an arm and complete movement of the focal animal into a box. Failure is determined by a focal animal not making a choice within a specific period.

More fine resolution analyses can be derived from choice data in the Y-maze. For example, researchers can generate a choice penalty score[16] Here, researchers must track the degree to which the focal animal explored the non-target arm of the maze. Non-target can be defined as the arm the researchers determine *a priori* that the focal animal will not choose based on the alternative hypothesis tested. The simplest example of a non-target arm would be the unscented arm when only one arm contains a target scent. More complex examples would be the choice between two scents from the same source, but presented at different concentrations[7]. When the experimental design is multi-level and/or the data go from binary to incremental, as with choice penalty, an appropriate statistical approach should be used such as repeated measures analysis of variance (ANOVA) or other methods used with continuous or proportional datasets.

Behaviors: Throughout the duration of an experiment in which focal animals are observed, a variety of individual behaviors can be quantified. This number of variables can either be determined *a priori* depending on what is known[16] or *post hoc* following preliminary observations on a subset of data[14,15]. The study objectives and their degree of resolution determine what behavioral assessments should be made within the maze, if any (i.e., in many studies, only choice data are quantified[17]). Behaviors can be assessed throughout the maze, in sections, or during specific time periods; for instance, behaviors seen only in the base or at the junction of the arms may be prioritized[8]. Video recordings facilitate behavioral scoring, although the resolution of the video and its length—factors that impose data storage constraints—should be considered before experimentation begins.

Temporal variables: As with behavioral variables, many temporal aspects of animal performance can be quantified during Y-maze trials. For example, researchers can time latency periods (e.g., latency to emerge from the box[8]). Most temporal variables are associated with exploration of the maze such as total trailing time or time spent in each arm. These variables are usually analyzed in a multi-factor analysis such as multi-way ANOVA.

Observer bias: With any studies involving animal behavior, observer bias significantly influences data collection[18]. Therefore, observers should be blind to the treatment being tested. The simplest way to do this is to code the video files numerically and then randomly sort them (e.g., random number generator) prior to assigning them to observers. Controlling for observer bias is difficult-to-impossible when live data collection is the only option. In a field setting, this would require two cooperators: an observer blind to the treatment and a coordinator who sets up the trial. Extensive reviews summarize the effects of experimenter bias on data collection and interpretation in behavioral and ecological studies[18,19].

Insert Figure 2 here

FIGURE AND TABLE LEGENDS:

Figure 1. Layout of the USGS Y-maze. On the left, a schematic shows the components of the Y-maze with a scale bar for perspective. On the right, a snapshot from the video camera demonstrates the field of view for behavioral recordings.

Figure 2. Sample sizes and P-values for binomial tests from Y-maze results. Each given sample size represents a set number of trials where a scent is tested in one arm of the Y (target arm) while the other could be a control (non-target). Top number above each bar is the one-tail P-value for that number of target arm choices, bottom is two-tail. Numbers within the top bar represent the maximum number of non-target choices that are still traditionally statistically significant ($P < 0.05$).

DISCUSSION

While Y-mazes are very powerful tools to investigate chemical ecology in reptiles, their limited design can preclude other avenues of inquiry. However, a diversity of other options is available[11,12,20-22]. For example, tongue-flick assays are simpler to execute and allow simultaneous assessment of behaviors exhibited to an array of chemical stimuli relative to control odors[23-26]. Open-field tests are another option where a focal animal freely explores an enclosure until it encounters a source of chemical cues, and its behavioral reactions are subsequently scored[27,28]. Combinations of these approaches can assess discriminatory capacities of reptiles in varying contexts such as presenting a mix of artificial and natural odors along with refugia[29]. Y-mazes can also be modified to expose animals to airborne chemical cues alone or in combination with substrate-borne cues[16,30], and *post hoc* inference can be used to redesign data collection if archived video data are available[31]. Bioassays should be designed to simplify data collection and minimize conflicting stimuli, especially when a specific source of cues is being assessed (e.g., chemical cues[21]).

Researchers in animal behavior often observe and quantify focal animal responses in novel, artificial environments (e.g., an enclosed maze with a featureless landscape), and care should be taken to assess whether a given animal is exhibiting natural, exploratory behavior versus avoidance, agitation, or similar distressed behavior. Distressed animal behavior in experimental apparatuses is primarily attributed to neophobia: fear of novelty[32]. An example is escape behavior, where the focal animal pushes against the joints or the edges of the apparatus to achieve egress. Another example is shyness, where the focal animal demonstrates reluctance to enter the maze, the degree of which can be quantified by latency of maze entry. Apparatus (re)design can facilitate engagement of the focal animal to avoid these confounding effects of distress. The most common approach is repeated introduction of the focal animal to the apparatus to remove the novelty of the environment before testing begins, and contemporary statistical models (e.g., generalized linear mixed models) allow for test animals to be used in multiple trials. An important aside relevant to ecological considerations in behavioral testing is that reduced neophobia is associated with the success of invasive species[33]. Thus, depending on *a priori* knowledge of the species in question, neophobia may have variable importance as an experimental design consideration.

Acquisition of behavioral data from videos imposes multiple constraints that become major bottlenecks in experimental timelines. For example, the length of a given trial can exponentially increase data extraction time. One workaround is to analyze behavior only until a threshold is met (e.g., total time active). The threshold can be based on the longest video available for a given trial. Alternatively, machine-based observation (e.g., artificial intelligence) can be developed, although this is time- and resource-consuming with considerable effort required for quality control. Another issue is data management: videos must be of sufficient quality to enable behavioral scoring and assessment, resulting in data storage constraints. While cloud storage is now accessible, upload/download rates are often problematic, especially when data acquisition occurs in remote field locations. Additional challenges manifest in the limitations of recording tools that affect the integrity of behavioral observation. Clear viewing of focal animal behavior is always necessary, but visibility is often impeded by uncontrollable factors (e.g., moisture, insects, wind movement). Further, when recordings come from a single perspective (e.g., bird's eye view), behaviors occurring in the vertical plane (e.g., head raises[14]) are difficult to assess. A solution is to provide multiple camera angles per trial. Lastly, the time of day significantly affects behavioral recording. Nighttime behavioral analysis requires a camera with a nighttime mode and minimal light projection to avoid obstructive glare on the Y-maze surface or attraction of insects that can interrupt the camera feed. Considering the above, foreknowledge of the study site or species biology can inform which constraints are likely to occur with what frequency and thus inform desirable sample sizes.

Behavior is tightly coupled with physiology, and the utility of Y-mazes for evaluation of behavioral endocrinology in a variety of species has been demonstrated. However, this paper emphasizes some variation in the execution of these experiments depending on the target species, research question, and resources available. Therefore, the selection of materials and dimensions of each testing setup should be carefully considered for potential subsequent research expansion. Section 2 describes modifications made to materials outlined in section 1, which were incorporated to accommodate future, more complex behavioral trials with tegus. The increased vertical depth of the Everglades mazes will allow new questions about chemical ecology in wild-caught tegus to be answered without unduly protracting project design and setup, further demonstrating the translatability of this experimental apparatus.

When employing the above-described techniques in a relatively remote setting (see section 2), there are several limiting factors that must be considered, and project planning is paramount. Depending on the statistical power needed for the prescribed treatment experiment and biological timing of the target species (e.g., seasonality), the resources and labor required will be affected. Further, if single or repeated use of focal animals are desired, careful attention to reducing potential stressors is necessary. Each of these factors will either extend the project timeline or require increased labor, space, and materials. For example, section 2 presents the use of wild-caught male pythons as focal animals trailing another group of wild-caught and hormonally manipulated males, all of which require approximately 24 h of quiet acclimation time in holding boxes to minimize stress effects. Although these acclimation periods extended trial times to over two days, stress due to captivity and handling affect wild animal behavior and must be minimized to generate clean datasets[34,35].

In summary, Y-mazes are powerful, adaptable tools that can be used to investigate the chemical ecology of diverse wildlife under widely variable conditions, provided there is vigilant *a priori* planning. Careful consideration must be taken to choose appropriate questions and to properly design the experimental setup for given taxa and conditions. Researchers and managers can significantly benefit from using Y-mazes to better understand animal chemosensory biology as these tools enable flexible experimental designs that provide large volumes of fine-scale behavioral data, especially when combined with remote monitoring tools.

ACKNOWLEDGEMENTS

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None

DISCLOSURE

None

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Figure 1

Click here to access/download;Figure;ParkerCurrylowetal-JoVE-Figure-1.jpg

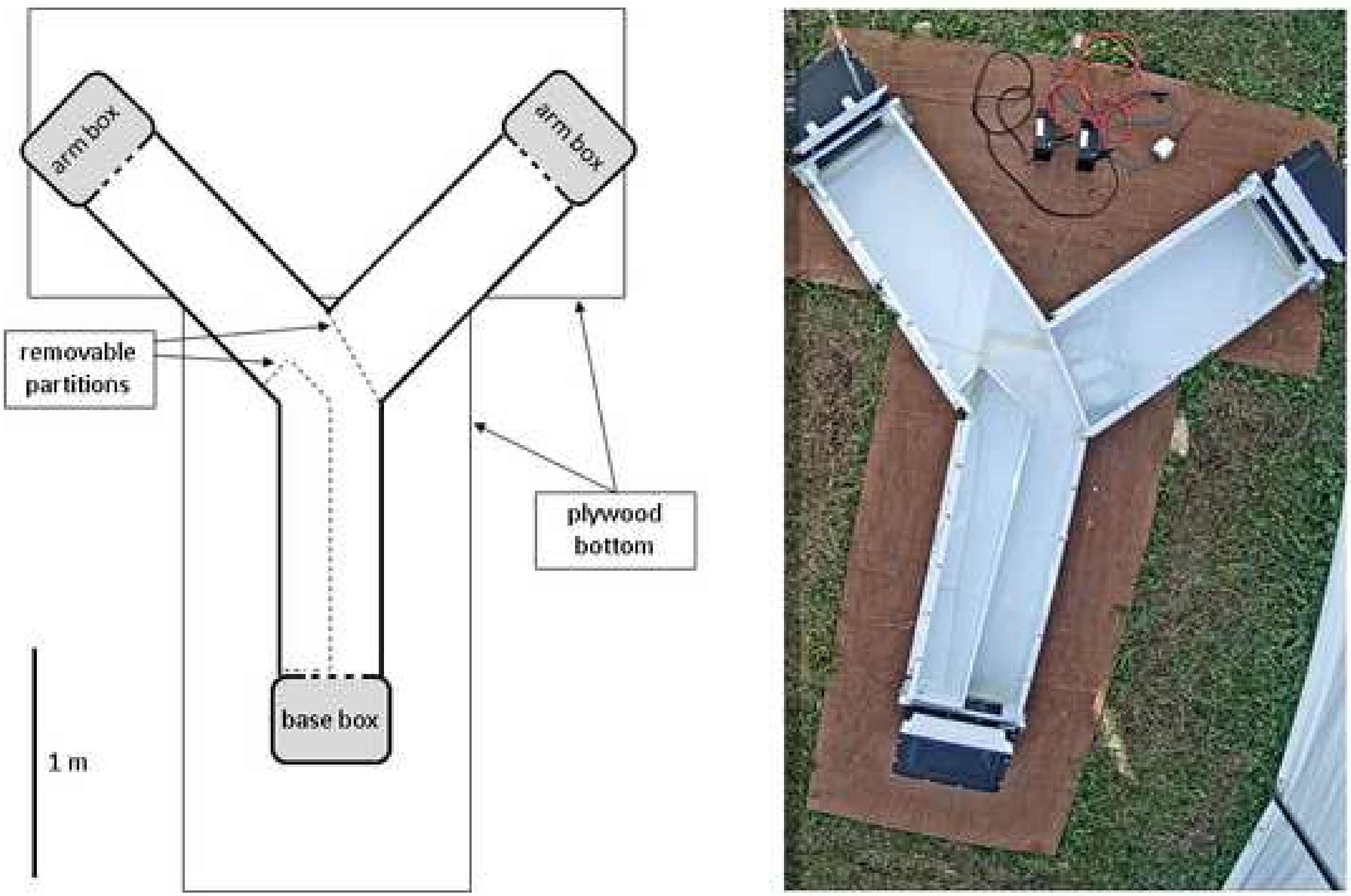
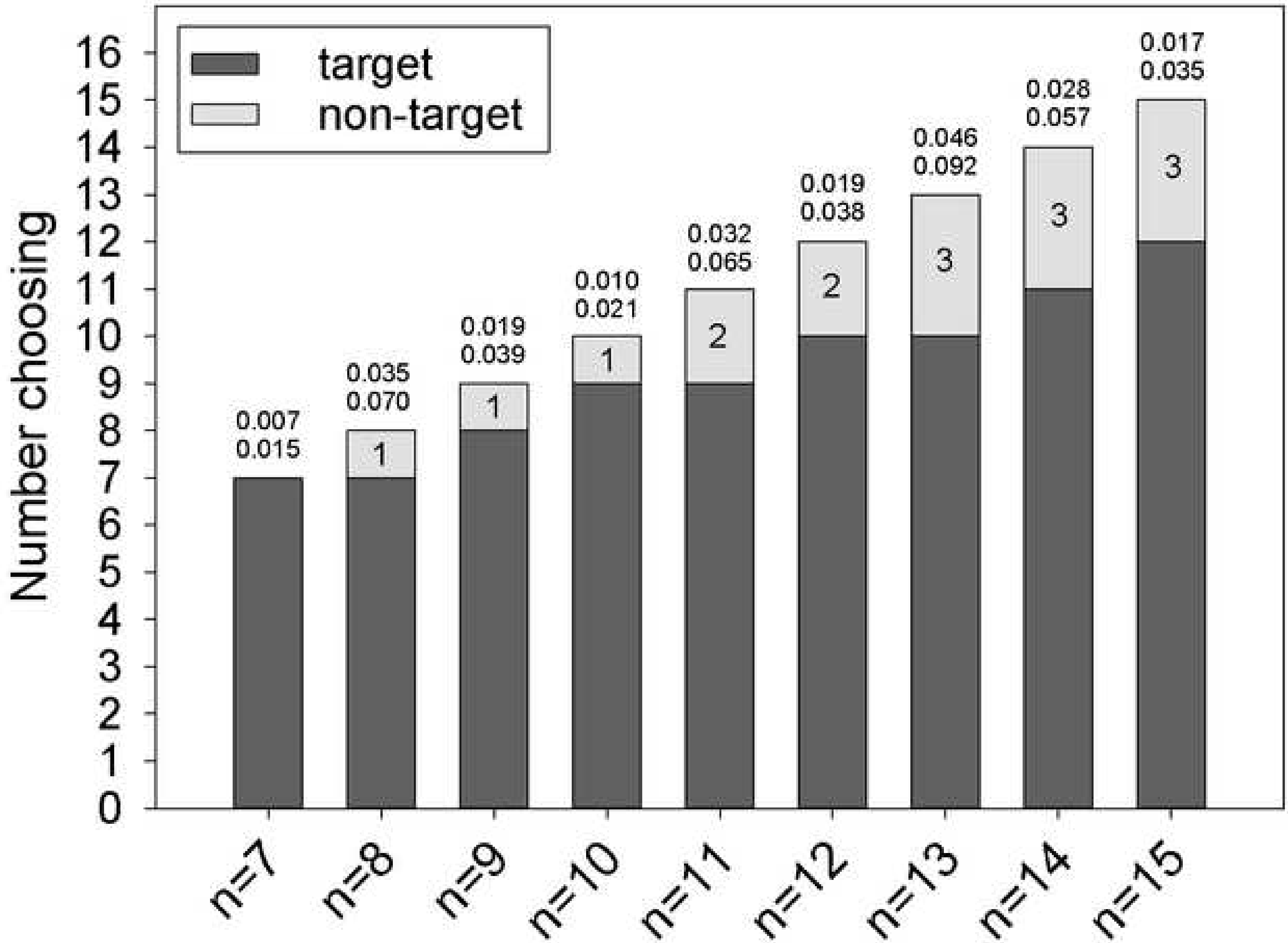


Figure 2


Number choosing
0
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non-target
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0.015
0.035
0.070
1
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0.039
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0.010
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0.065
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3
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n=8
n=9
n=10
n=11
n=12
n=13
n=14
n=15

Name of Material/ Equipment
1" Steel zinc-plated corner brace
121.92cm W x 304.8cm L x 1.27cm H white polypropylene Extended Range High-Heat UHMW Sheet
182.88 cm L x 81.28 cm W x 0.64 cm Thick Clear Acrylic Sheet
2.54 cm W x 2.54 cm H x 243.84 cm L Mill-Finished Aluminum Solid Angle
4.5 kg spool of 5 mm Round Polypropylene Welding Rods
5 mm Plain Aluminum Rivets
Aluminum angle, 1.9 cm
Aluminum angle, 2.5 cm
Aluminum angle, 3.2 cm
Aluminum flat bar 1" x 1/8" thick
Avigilon 2.0 MP camera
Avigilon NVR
Clear acrylic sheet (5.6 mm thick)
Fillet Weld Nozzle 3/16" x 15/32" / 4.5 x 12 mm
Hanging File Folder Box
HardiePanel HZ10
Heat Welding Gun
Kraft Butcher Paper Roll, 24"
Kraft Butcher Paper Roll, 46 cm wide
Micro-90 Concentrated Cleaning Solution
MKV ToolNix - Matroska tools for linux/Unix and Windows
Network Camera
Palight ProjectPVC 1/4"
Palight ProjectPVC 1/8"
Privacy windscreen (green)
Protective Glove, Full-Finger
REScue Disinfectant
Reversable PVC trim, 1/2" x 24"
S4S / Veranda HP TRIM
S4S / Veranda HP TRIM (1" x 8" Nominal)
ScotchBlue 24 in. Pre-taped Painter’s Plastic
Sterilite 114 L tote box
Sterilite 189 L tote box
Super Max Canopy
VLC Media Player
White Pavilion Tent

Company	Catalog Number
Everbilt, The Home Depo	13619
TIVAR	UHMNV SH
Plexiglass	32032550912090
Steelworks	11354
HotAirTools	AS-PP5N10
Arrow	RLA3/16IP
Everbilt, The Home Depo	802527
Everbilt, The Home Depo	800057
Everbilt, The Home Depo	800037
Everbilt, The Home Depo	801927
Avigilon, a Motorola Sol	2.0C-H4SL-BO1-IR
Avigilon, a Motorola Sol	D-NVR3-VAL-6TB-N
United States Plastic Cor	44363
TRIAC	107.139
Sterilite	18689004
James Hardie Building Pr	9000525
TRIAC	141.227
Bryco Goods	24 inch x 175 FT
Bryco Goods	BGKW2100
International Products C	M-9050-12
Moritz Bunkus	v.48.0.0
Axis Communications	M3104-LVE
Palram	159841
Palram	156249
MacGregor	Size to fit
ArmOR Hand	HS1010-RGXL
Virox Animal Health	44176
UFP Industries, Veranda	H120XWS17
UFP Industries, Veranda	H190OWS4
UFP Industries, Veranda	827000005
3M	PTD2093EL-24-S
Sterilite Company	1919, Steel
Sterilite Company	1849, Titanium
ShelterLogic	25773
VideoLAN	v.3.0.11
King Canopy	BJ2PC

Comments/Description

See Supplemental File 1, Step 2.1 "90 degree 2.5 cm steel corner brace"
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.1. "white polpropyl
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.1.6. "Acrylic pieces"
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.1.1. "aluminum ang
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.1. "heat weld")
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.1.1. "rivet")
See Supplemental File 1, Step 1.2 "aluminum angle (1.9 cm x 1.9 cm x 0.16 cm thick)"
See Supplemental File 1, Steps 1.2 and 2.2.2 "aluminum angle (2.5 cm x 2.5 cm x 0.16 cm thick)"
See Supplemental File 1, Step 1.2 "aluminum angle (3.2 cm x 3.2 cm x 0.16 cm thick)"
See Supplemental File 1, Step 3.2.1 "aluminum strap"
See "1.5 Camera set-up and video acquisition" (step 1.5.1 "Avigilon 2.0 MP")
See "1.5 Camera set-up and video acquisition" (step 1.5.3 "NVR")
See Supplemental File 1, Step 1.3 "clear acrylic sheet" and step 3.2.1 "clear acrylic door"
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.1. "heat weld")
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.2.1. "Boxes")
See Supplemental File 1, Step 1.1 "fiber cement siding"
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.1. "heat weld")
See "1.2 Protocol for running scent-laying tegus" (step 1.2.1.2 "butcher paper")
See "2.3. Protocol for running scent-laying pythons" (step 2.3.4. "scenting paper")
See "1.4 Breakdown and clean-up" (step 1.4.4 "laboratory-grade soap")
See "2.2. Camera setup and video acquisition" (step 2.2.4.2. "movie processing software")
See "2.2. Camera setup and video acquisition" (step 2.2.1. "Project camera")
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.2.3. "faceplate")
See "2.1. Y-maze components and rationale for changes to USDA design " (step 2.1.2.1. "door")
See Supplemental File 1, Step 4.2 "green heavy duty shade cloth"
See "2.3. Protocol for running scent-laying pythons" (step 2.3.11.2. NOTE: "puncture-resistant glove")
See "1.5. Breakdown and clean-up." (step 1.5.4. NOTE "sanitation solution")
See Supplemental File 1, Step 2.1 "PVC board partition", and step 3.2.1 "thinner PVC trim boards"
See Supplemental File 1, Steps 1.2, 2.2.2, and 2.2.3 "PVC board"
See Supplemental File 1, Steps 3.2.1 "PVC trim board"
See "1.2 Protocol for running scent-laying tegus" (step 1.2.1.3 "plastic sheeting")
See Supplemental File 1, Step 3.2 "arm box"
See Supplemental File 1, Step 3.2 "Base box"
See Supplemental File 1, Step 4.3 "white canopy"
See "2.2. Camera setup and video acquisition" (step 2.2.4.3. "media file reviewing program")
See Supplimental File 2 "3. Enclosure materials and consideratons" (step 3. "pavilion tent")

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Department of Biology
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October 14, 2020

Dear Dr. Jewhurst,

We are submitting our revised manuscript, "Using enclosed Y-mazes to assess chemosensory behavior in reptiles," for additional review at *Journal of Visualized Experiments*. We made all of the editorial changes suggested by the editor and the majority of the suggested changes provided by the reviewers. There were minor additional edits required by both the U.S. Department of Agriculture and the U.S. Geological Survey that have been added. All changes are indicated using Track Changes, and a clean copy is also provided. We also include the updated Author License Agreement.

The writing of the manuscript and establishment of the methods described were conducted by federally employed scientists from USDA and USGS. The methods are the result of many years of collaboration between the corresponding author and the scientists from these two entities.

We look forward to your feedback on our resubmission.

Sincerely,

M. Rockwell Parker, Ph.D. (corresponding author)
mrockwellparker@gmail.com +1 (541) 740-5070

To the editor and reviewers,

Thank you for the feedback and constructive comments that improved the manuscript.

Below, we address the suggested changes by the editor as well as the reviewers. Our responses are italicized and immediately follow comments that called for changes. All changes were tracked. Further, we made additional, minor changes that were required by internal review at USGS and USDA, and these changes had no effect on the content of the article nor the protocol described. These changes included edits in the supplemental documents. We also added a line in the acknowledgments thanking the reviewers for their feedback.

Editorial comments: Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We updated the manuscript.

2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol.
We updated the text in the protocol section of the manuscript.

3. Please upload a signed copy of the USGS revised author license agreement as well.
We included the updated and approved version of the ALA.

4. Please include an ethics statement that specifies that the proper institutional approval has been obtained for the animal work.
We included an ethics statement at the beginning of the Protocol.

Reviewer comments:
Reviewer #1:
This paper outlines a method to study trailing in large reptiles, a lizard and a snake. While put forth as a way to study invasive species, the design could be applied to more basic research and not just on conspecific trailing. The paper covers the construction and testing details in great detail. The authors also detail testing protocols and controls. There are many details for construction and running tests. Though I have no doubt this method can (and does) work, I wonder how many labs will actually use it.
While we agree that the application of the approach we describe may be viewed as restricted/niche, this is the first comprehensive description, to our knowledge, of how to scale-up a Y-maze for chemosensory tests of terrestrial animal behavior with an enclosed design that works for very large vertebrates. Further, design plans like those we include in the supplemental files provide excellent detail that is simply not possible in other journals, which is why we chose to write this manuscript for JoVE. Here, we could focus exclusively on methodological details that can make our studies seem more possible for other researchers. Lastly, a Y-maze is an

investment in experimental infrastructure. Researchers at multiple types of institutions need only invest in the construction of one then pursue any number of research questions to assess in any focal animal species.

This is another y-maze snake paper involving prey, not conspecific trailing, but this may also be important to determine what prey the species are most attracted to chemically. Kojima Yosuke and Mori Akira, 2015. Active foraging for toxic prey during gestation in a snake with maternal provisioning of sequestered chemical defences. Proc. R. Soc. B.28220142137
We thank the reviewer for this reference and have included it in the manuscript.

Major Concerns:
The paper seems a bit disorganized and repetitive. It should be made clear initially that the maze is for field vs lab use. Issues of lab vs field testing need to be discussed and laid out at the beginning. The details also seem applicable, but of course I do not know directly. However, the writers are important and accomplished researchers on reptile chemosensory behavior and know their animals and behavior systems well.
We added verbiage in the Introduction to address this, especially that our design of the Y-maze is to adapt to field conditions that are unconsidered/irrelevant in lab studies.

Minor Concerns:
Could not find any figure legends.
The legends were embedded within the manuscript as dictated by JoVE requirements. The legends are located where the figures will be displayed in the manuscript (~lines 420 and 488 in the clean copy), just prior to the Representative Results and the Discussion sections.

Reviewer #2:
This contribution is a very useful and detailed methods paper that will be a handy addition to JoVE's video library of methods. It is also very well written. I only have a few comments although a few of them will require some careful thought. I also wondered if the authors could make the paper more general? The title suggests a general paper, but the paper itself is very species-specific. In my opinion, the two methods described should be applicable to a wide range of lizards and snakes. I suggest a way to deal with this below. My two major comments below apply to the cleaning protocol and side-bias.

49-50 "to one half of the maze" might be clearer if you say one arm of the maze, or do you mean the maze itself?
We made this change.

57 When I read the word "reparations" I think of payments for injustices such as slavery or starting a war!
We changed this to “solutions” and edited this sentence for grammar/flow.

The introduction is well written and clear but there is no mention of why a Y-maze is superior to other tests of chemical discrimination such as presenting scent on cotton buds or labelled tiles

or even scented refuges. I can think of reasons why they may be advantageous compared to these methods and I have no doubt the authors can too. A few sentences to this effect may strengthen their case.
We added this as suggested, and we note in the Introduction that this is covered in the Discussion as per JoVE standards (experimental constraints/considerations/alternatives are addressed in the Discussion and not Introduction).

171 VLC is also a good option because it is free and can read a wide range of formats.
We added this as suggested.

179 In my opinion, one of the biggest unspoken issues with Y-mazes and reptiles is when they have a side-bias. I realise you are selling a design here (using a Y-maze) and this is a confounding variable related to the animals' biology. However, you have raised the issue of "bias", so how do you reconcile what might be bias due to some property of the maze vs a 'property' of the animal?
We discuss bias in Section 1.3 of the Protocol and added a line about the fact that randomizing the arm receiving a target scent is a measure put in place to counteract any bias.

188 How often/when would you change to new gloves?
New gloves should be worn with every assembly step to avoid contamination between phases. There is no evidence, to our knowledge, that reptiles can sense human skin lipids, so the use of gloves is a preventative measure. We added verbiage that mentions the frequency of glove change.

In the methods, this is all about tegus. I'm just wondering why this has not been generalised to lizards? I understand that you used this on tegus, but obviously this design would apply to most lizards, or certainly those that tongue-flick while exploring? In the introduction you could mention that this design was used successfully on tegus but can be generalised to a wide range of species and as such, you will refer to "lizards" in the methods. A similar comment applies as above, with respect to whether to refer to pythons or snakes.
We thank the reviewer for this suggestion and have included the verbiage along with changing "tegu" and "python" or "snake" to "animal" throughout the Protocol section.

249-269 to me this is one of the most critical sections because if cleaning is not effective, your next test animal may be picking up residual cues from the previous trial. How do you know that this protocol is effective? This is not a particularly detailed or specific protocol. For example, if you look up papers by Sharon Downes and Rick Shine on Australian velvet geckos, they outline a protocol that they tested. In other words, do x and y and the test animal will not detect any residual cues based on our assays. You could do the same or at the very least provide more justification about what is essential for ensuring an odor-free maze for the next animal.
Downes & Shine 1998 (Animal Behaviour) was a great study (we now included it in the Discussion; it is an excellent example of how to combine open field tests using refugia and traditional chemosensory metrics). However, the thoroughness of their cleaning was due to 1) their use of natural substrates (rocks) compared to our use of non-porous polymers and 2) their

use of pungent, artificial odors as control scent (cologne). The cleaning protocol we describe goes beyond the standards in squamate chemical ecology utilizing artificial environments like the Y-maze (typically only the paper and other disposable surface materials are discarded and the next trial is set up). The references cited (especially the reviews) address the purpose of cleaning, but we added verbiage to refer to those studies in this section of the Protocol.

308-309 Burmese pythons are nocturnal. Do you need to say more about filming at night or under low light conditions?
We thank the reviewer for pointing out the lack of our description of this consideration and have updated the language throughout the manuscript to reflect this.

JoVE –Supplemental File 1 to Parker, Currylow et al, *Using Enclosed Y-Mazes to Assess Chemosensory Behavior in Reptiles*

USDA National Wildlife Research Center Y-maze materials and dimensions – Here, the basic design and construction of the apparatus used in diurnal Protocol section 1 of the manuscript has been outlined.

1. Maze bottom, sides, and top

1.1. The bottom or base of the maze is made of four 1.22 m x 2.44 m panels of fiber cement siding in two layers of two panels each. The panels are placed together along the long edge, such that they form a 2.44 m square. The top layer of panels is oriented perpendicular to the bottom layer for added support. Holes drilled in the top layer allow carriage bolts to be passed up through the top for attachment of the maze (locations determined when the top and sides are assembled on the base). When the carriage bolts are in place, the two layers of panels are secured together using masonry screws.

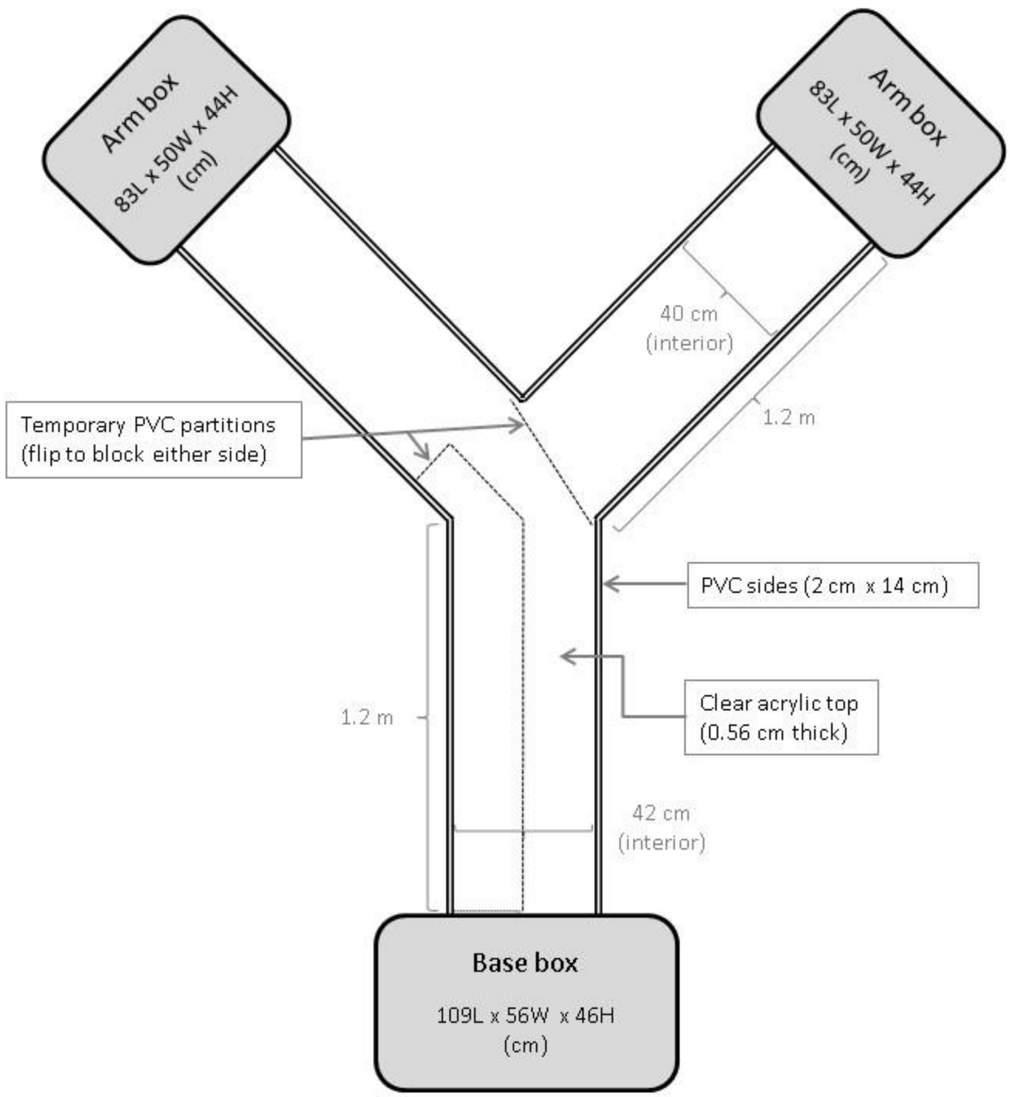
1.2. The sides of the Y-maze are made using cellular white polyvinyl chloride (PVC) trim board (1.9 cm x 13.9 cm). The smooth side faces the interior of the maze environment and the wood-textured side faces out. For stability during maze assembly and to secure the sides to the cement siding base, aluminum angle (2.5 cm x 2.5 cm x 0.16 cm thick) is attached using stainless steel screws to the outside edge of the board along the full length with the angle facing out. Where the angle meets the Y-maze base, two holes are drilled at opposite ends of the board to allow carriage bolts from the base to pass through. Another piece of aluminum angle (1.9 cm x 1.9 cm x 0.16 cm thick) is attached along the full length of the outside top edge of the side with the angle facing in. A 0.7 cm gap is left between the angle and PVC board creating a slot to allow the maze top to be inserted. The arm ends of the main passageway sides are mitered at 45° to allow a flush fit with the arm sides. Where the ends of the sides meet with the holding and capture boxes, a small piece of aluminum angle (3.2 cm x 3.2 cm x 0.16 cm thick) is attached, allowing the boxes to be secured to the maze. Holes drilled in the angle allow the use of thumb screws to attach the boxes.

1.3. The top of the Y-maze is a clear acrylic sheet (0.56 cm x 48 cm), inserted into the slots at the top of the Y-maze sides. Two pieces are used to cover the main passageway from the box end to the V-end and one piece each for the arms. The piece at the end of the main passageway is cut to match the V-shape of the maze and the arm pieces are cut at an angle to meet with straight edges of the main passageway piece. When assembled, all top pieces fit flush together forming a completely enclosed top. Holes drilled through the aluminum angle, acrylic top, and PVC sides approximately every 35 cm allow the top pieces to be secured during trials using simple flat top nails (inserted into the aligned holes).

2. Internal partitions are used within the main passageway of the Y-maze to control space use by scent-laying animals and thereby restrict scent deposition to defined areas of the maze.

2.1. For trials where a single scent trail is deposited by a scent-laying animal, a 46 cm L x 13.7 cm H x 0.8 cm thick PVC board partition is used to completely block the passageway of the control/untreated arm. The ends of the partition are beveled so that the interior face of the partition meets flush to the interior sides of the maze. For stability, a 30 cm long x 3.8 cm x 9 cm H untreated wood board is attached to the rear face of the partition using stainless steel screws. A 90° 2.5 cm steel corner brace attached to the bottom edge of the wood board in the center allows the partition to be secured with a deck screw to the base of the maze during trials. A pre-drilled hole made in the base and marked conspicuously (using black or red wax pencil) makes attachment easier, as the location of the hole will be faintly visible through the scenting layer.

2.2. For trials where two scent trails are deposited by different animals, a system of partitions is used to block alternating arms of the maze and exclude each animal from alternating halves of the main passageway (see **Supplemental Figure 1**).



2.2.1. The partition described in 2.1 is used to block the unused arm of the maze.

2.2.2. A 1 … ength of the main p … uminum

Supplemental Figure 1. Dimensions and layout of Y-maze at USDA NWRC.

angle (2.5 cm x 2.5 cm x 0.16 cm thick) is attached to both sides of the base of the board along the full length. At the distant ends of the partition, holes are drilled into the aluminum angle at the base; one hole at each end on each side (4 total). Using these holes, the partition is secured to the base using deck screws (again holes in the base predrilled and conspicuously marked).

2.2.3. An L-shaped partition is used to enclose the arm end of the main passageway, on the side opposite the blocked arm. This partition is constructed of two pieces of 13.7 cm H bx 1.9 cm thick PVC board, such that the long side is 30 cm L and the short side 11 cm L. The pieces are permanently joined by a butt joint using stainless steel screws. A rebated butt joint cut into the end of the long side allows the partition to overlap the 45° point of the main passageway partition. A small bevel cut into the outside tip of the butt joint makes for a flush 45° union. A small 90° 2.5 cm steel corner brace attached to the inside of the L partition allows it to be secured to the base using a deck screw. Attaching one bracket to the top side and one to the bottom side allows the L-partition to be flipped for use on either side of the main passageway.

3. Base and arm boxes

3.1. A box secured to the base end of the Y-maze holds the scenting or test animal prior to its release into the apparatus. Boxes secured at the terminal ends of the Y-maze arms facilitate capture of the animal at the end of the trial.

3.2. Base and arm boxes are grey plastic storage containers modified to include openings that match the ends of the Y-maze, with removable doors.

3.2.1. Openings cut into a bottom side of each box are reinforced with externally mounted pieces of PVC trim board (1.9 cm x 18.4 cm) with similar openings cut to match. Two layers of thinner PVC trim boards (0.8 cm x 18.4 cm) are attached to the outside of the first trim board and matching openings are cut. The top of the middle board is mostly cut away to create a slot in which a removable 0.56 cm thick clear acrylic door can be inserted. The opening assembly is secured to the box using stainless steel screws with a backing of aluminum strap for reinforcement. All seams are sealed with clear acrylic caulk. To secure the door while the animals are in the boxes, a thumb screw is secured through a hole in the top of the acrylic door into a threaded insert screw. Two threaded insert screws installed on each side of the opening (matching openings in the aluminum angle on the sides of the maze) allow the boxes to be secured to the maze using thumb screws.

3.2.2. Holes drilled through the top lid and top edge of each box, approximately 25 cm apart, allow the top to be secured using small plastic cable ties. With the lid in place, cable ties are passed through the holes and around the box edge and tightened.

3.2.3. Seven holes (2.5 cm diameter) along the top edge of both long sides of each base box provide ventilation. An additional row of six 1.2 cm holes in the upper middle sides of the base box provides extra ventilation.

3.2.4. The dimensions of the base box are 108.6 cm L x 55.9 cm W x 45.7 cm H. The base box has two clear acrylic doors, one full size and one half-size. The half-size door is used to block one half of the opening to the main passageway during two-scented trials.

3.2.5. The dimensions of the arm boxes are 82.9 cm L x 50.2 cm W x 43.8 cm H.

4. Enclosure for maze

4.1. If Y-maze trials are conducted outdoors under ambient conditions, additional shelter is required to provide a safe and comfortable environment free of distraction from outside influences. Additional security (secondary and/or tertiary layers of caging) is also suggested and may be required when working with non-native study animals.

4.2. Place the entire Y-maze apparatus within a secondary cage enclosure (6.1 m L x 3.0 m W x 1.8 m H) covered with wire mesh on the roof and sides. A walk-through door provides access. Secure green, heavy-duty shade cloth to the top and upper sides of the enclosure to provide shade and a visual barrier.

4.3. A 6.1 m L x 3.7 m W white canopy (12 ft. x 20 ft. 2 in. 8-leg canopy) erected over the secondary cage enclosure provides additional shelter from the sun and rain.

USGS Invasive Species Science Remote (Everglades NP) Y-maze materials and dimensions: Here, the modifications used in nocturnal Protocol section 2 of the manuscript have been outlined. The materials were selected for outdoor durability and ease of cleaning.
Each Y-maze's three separate components (a base and two arms) were fastened together onto two sheets of plywood for stability and security during trials.

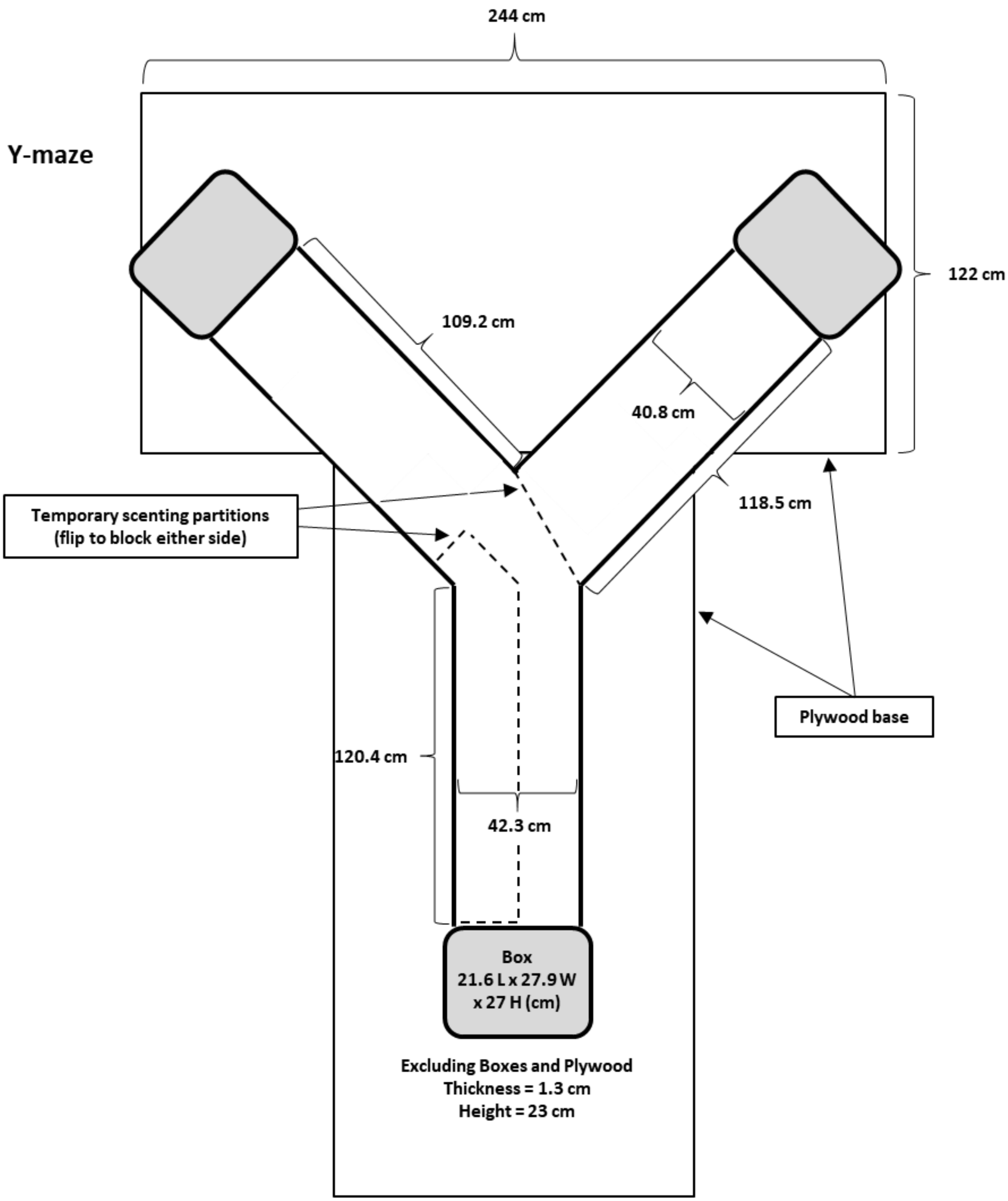


Figure 1. USGS Field Y-maze layout. Schematic of field Y-maze components and dimentions with partitions in place.

1. Construct the Y-maze sides and bottom from two H.O.T. white polypropylene sheets (304.8 cm L x 121.9 cm W x 1.3 cm H). Heat weld the walls onto the bases using a heat gun fitted with a weld nozzle (4.5 x 12 mm) and spool of 5 mm polypropylene welding rod. Heat welds are used to ensure a strong bond with minimal seams (see **Figure 1**).

1.1. Y-maze base: Cut the base floor to measure 120.4 cm L x 42.3 cm W x 23.0 cm H with an inner shorter length of 108 cm L angling to a point at the end (42.8 cm on each side to the midline) where the two angled arms fit together.

1.2. Y-maze arms: Cut each arm to a mirrored angle at the base (118.5 cm along the outside length and 109.2 cm on the inside shorter length) and 40.8 cm wide with walls along the lengths and 23.0 cm high.

1.3. To create a channel for the acrylic top (see section 1.6) to slide onto, use mill-finished aluminum solid angle (243.8 cm L x 2.5 cm W x 2.5 cm H) cut to the length of the base walls (120 cm L) and both arms (120 cm long sides, 110.5 cm short sides).

1.3.1. To affix the aluminum angle to the polypropylene walls, use Arrow 5 mm plain aluminum rivets with a grip length of 12 mm at approximately 30-cm spacing along the top edge of all the walls. Ensure that the aluminum angle is facing inward and at a height of 0.7 cm above the top of the walls to allow space for the acrylic top to slide in.

1.4. Construct temporary partitions for the base and non-scenting arm (see **Figure** 1) out of the same H.O.T. white polypropylene sheets used for the Y-maze bottom and walls.

1.4.1. Cut and heat weld the temporary partition for the base of the Y-maze as pictured in **Figure 2**.

1.4.2. Cut a rectangular piece of the polypropylene to measure 46 cm W and 22 cm H as the temporary partition plate for the non-scenting arm. This should completely block access to one arm.

NOTE: Temporary partitions can be affixed to easily cleanable weights (e.g., 1-gallon jugs of water) using hook and loop tape to keep them in place.

1.5. Affix baseplates to each opening of the Y-maze that will allow the boxes (see section 2) to attach. Baseplates are constructed out of the H.O.T. white polypropylene.

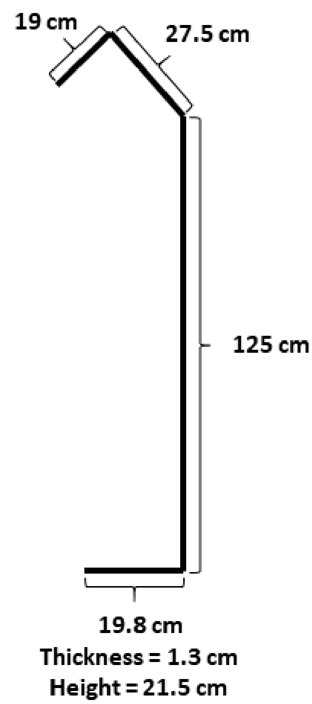


Figure 2. Temporary base partition dimensions. Partitions are made from 0.6-cm thick polypropylene. See section 1.4.1.

1.5.1. Cut baseplates to measure 42 cm W x 30 cm H and with a center “window” opening measuring 34 cm W x 16 cm H at 2 cm from the bottom of the baseplate and 13 cm from either end.

1.5.2. Affix the baseplates flush to the bottom of the Y-maze openings using aluminum angle and rivets on the outside walls.

1.6. Construct the top of the Y-maze from 0.6 cm-thick clear acrylic sheets (182.9 cm L x 81.3 cm W) to fit atop the base and each arm of the maze (see **Figure 3**).

1.6.1. Cut the base acrylic top to measure 182.0 cm L x 46.0 cm W with a v-notch cut at one end measuring 32.3 cm L on either side and meeting in the center to accommodate the two angled arm pieces (see **Figure 3**).

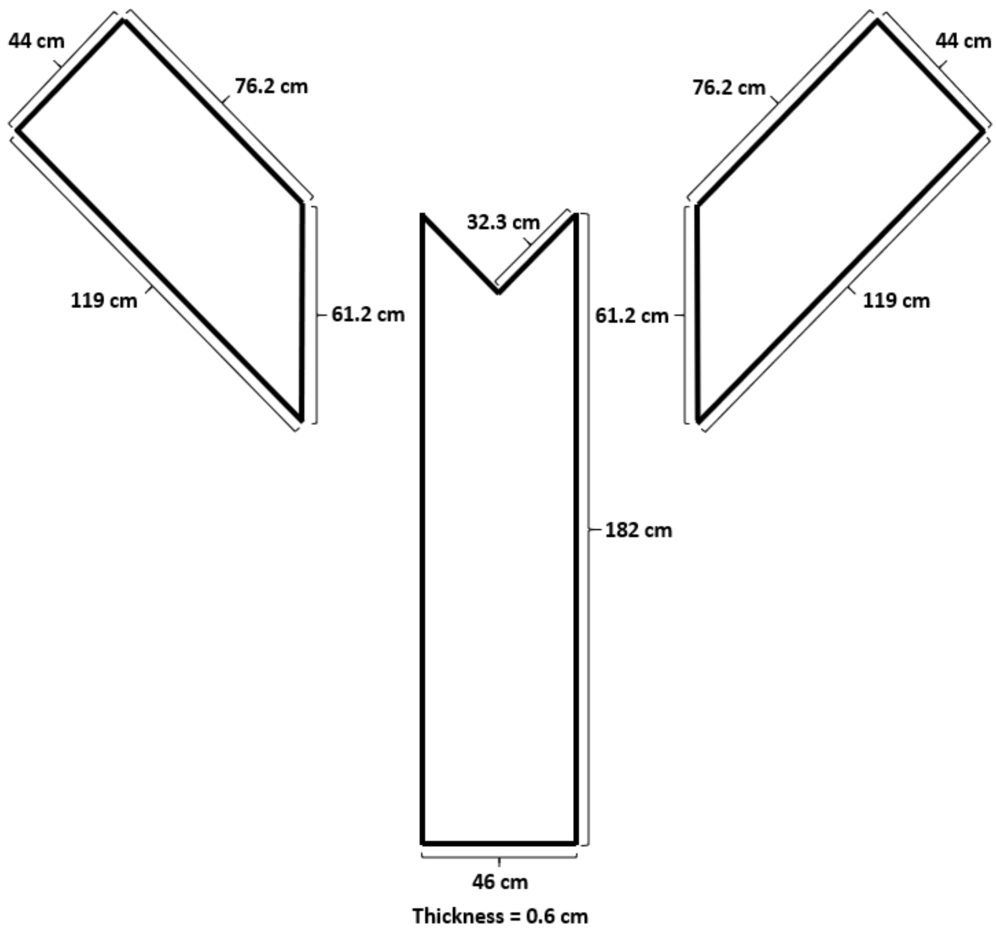


Figure 3. Acrylic top dimensions. Schematic of Y-maze acrylic top pieces and dimensions. See section 1.6.

1.6.2. Cut the arm acrylic to 119.0 cm L x 44.0 cm W on the longer outer length, and 76.2 cm L on the inner shorter length. Cut one angled end across measuring 61.2 cm W to fit along the base (see **Figure 3**).

1.7. To secure the Y-maze in place and prepare for outdoor use, affix it in place to 122 cm x 244 cm plywood sheets.

1.7.1. Orient the plywood sheets side-by-side with the shorter end of one sheet meeting the center of the long end of the other forming a "T" so that they can fully support the base and wide arms of the Y-maze (see **Figure 1**).

1.7.2. Affix the maze to the plywood base using deck screws drilled through an aluminum angle bracket riveted along the bottom of the outside walls of the maze.

2. Box materials and modifications: The three boxes (i.e., hanging file folder boxes, 54.9 cm L x 70.9 cm W) that will be fastened to the three ends of the Y-maze. These boxes also anchor the acrylic top in place (see **Figure 4**).

NOTE: Choose a dark-colored box to reduce light stress to the animals.

2.1. Drill drain holes in the bottom of each box and affix its lid into place using small screws and nuts.

2.2. On the long side of each box, cut a rectangular "window" opening (2.5 cm from each side) for egress/ingress (see **Figure 5**).

2.3. Cut a 37-cm long narrow slot, centered along the inside of the long edge of the lid on the edge closest to the side window, for the sliding door (see Error! Reference source not found.).

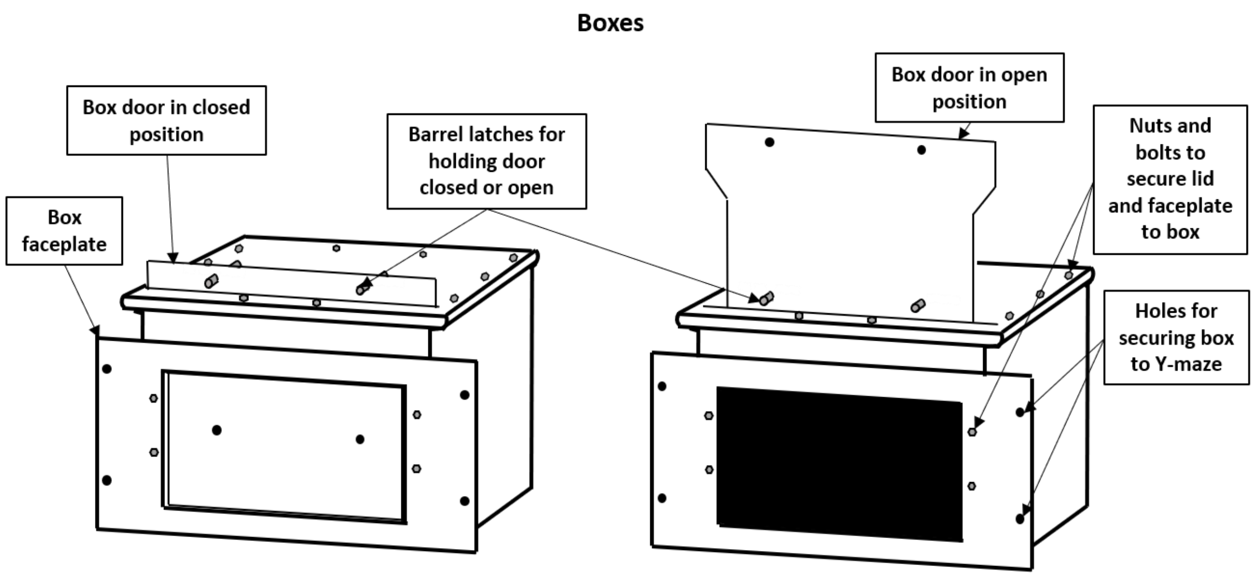


Figure 4. Box components and assembly. See section 2.

2.4. Bolt two barrel latches (centered and spaced at 13 cm apart) onto the lid of the box just behind

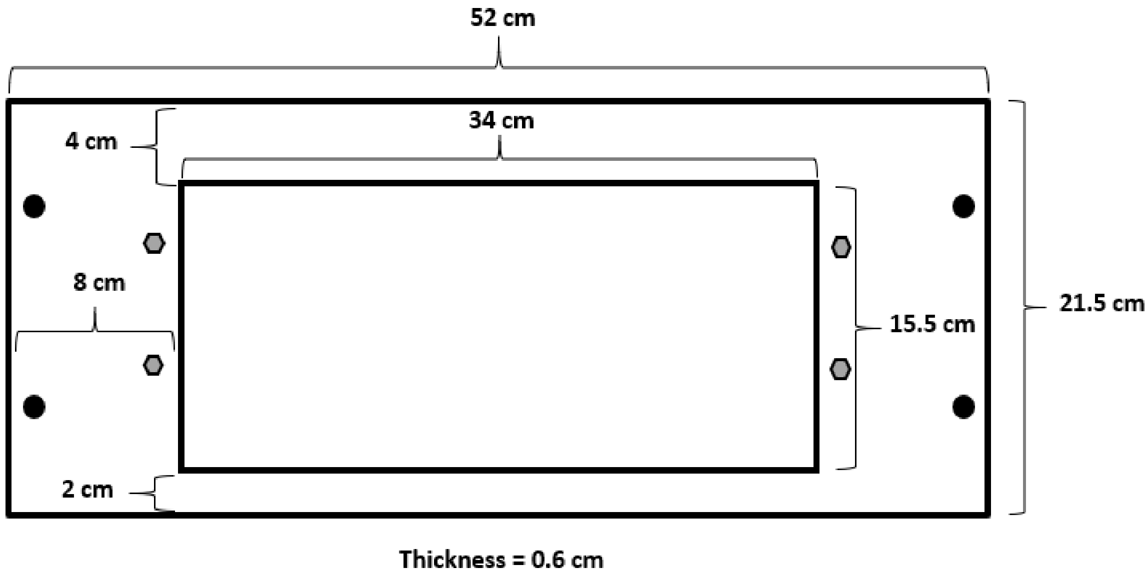


Figure 5. Box faceplate dimensions and modifications. Faceplate used on boxes is made from 0.3 cm plastic board. See section 2.2

the slot (see Error! Reference source not found.). These latches will be used to secure the sliding door closed or open.

2.5. Construct a box door from 0.3 cm plastic board to the dimensions shown in **Figure 7** and a box face plate with an internal opening from the polypropylene sheet to the dimensions shown in.

2.6. Place the box door into the slot and use the latches as references to drill two sets of holes into

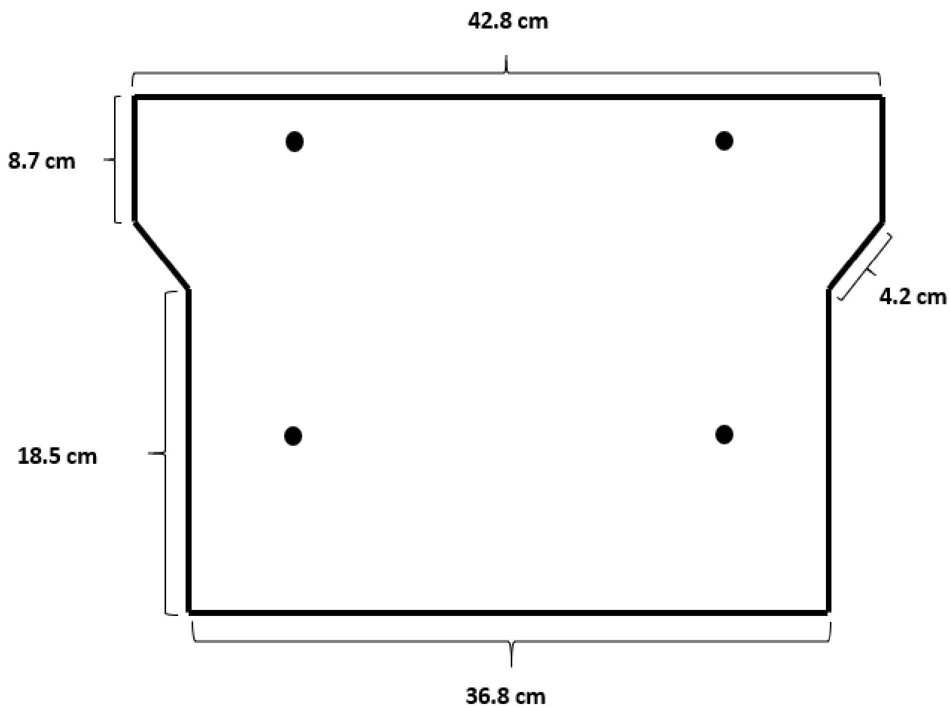


Figure 6. Box door dimensions and modifications. Box doors are made from 0.3 cm plastic board. See section 2.5.

the sliding door; one set lined up with the latches when the sliding door is closed, and another set lined up with the latches when the door is open.

2.7. Affix the box faceplate to the box using small nuts and bolts and fill any gaps with epoxy for good measure.

2.8. Align the box faceplate with the Y-maze arm faceplates to ensure a flush connection, then drill aligned holes through both so that they can be secured together with bolts and wingnuts (for easy removal).

NOTE: The first box's faceplate can be used as a template when drilling the rest of the faceplate holes and ensures that every box is compatible with every Y-maze opening.

2.9. Align each box faceplate to the Y-maze baseplates and affix using bolts and wingnuts or locks. When these boxes are affixed, they also anchor the acrylic top in place.

3. Enclosure materials and considerations: Each Y-maze is enclosed by a 610 cm L x 300 cm W x 280 cm H white pavilion tent to shield it from direct sunlight and prevent overheating the study animals. Center these tents directly overtop the middle of the Y-mazes and their polls are used to affix the monitoring cameras for behavior studies.

NOTE: Extra stabilizing measures are highly recommended if the tents must withstand wind gusts (e.g., fasten with 0.6 cm bolts to 0.9 m U-posts driven into the ground at every tent leg, with each junction in the tent frame also bolted together using 0.6 cm bolts).

3.1. To minimize any potential bias, evaluate the location of each enclosure, both independently and across enclosures, for the following factors:

3.1.1. Elevational gradient: Ensure that each enclosure is on a completely level surface and are at the same elevation.

3.1.2. Wind direction and force: Ensure that the enclosure is oriented so that narrower ends of the tents will face prevailing winds. Ensure any additional enclosures are not too proximal or lined up along prevailing wind directions that would allow any scent from one enclosure to potentially contaminate another.

3.1.3. Sun exposure: Ensure that each enclosure is oriented so that sunlight hits each portion of the internal Y-mazes in a balanced way (i.e., if one Y-maze gets sun along its long side at 10 AM, any others should also get sun at the same time along the opposite long side to equalize exposure to the arms). Enclosures should not be too proximal as to avoid casting shadows on one another.

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Title of Article: Using enclosed Y-mazes to assess chemosensory behavior in reptiles

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