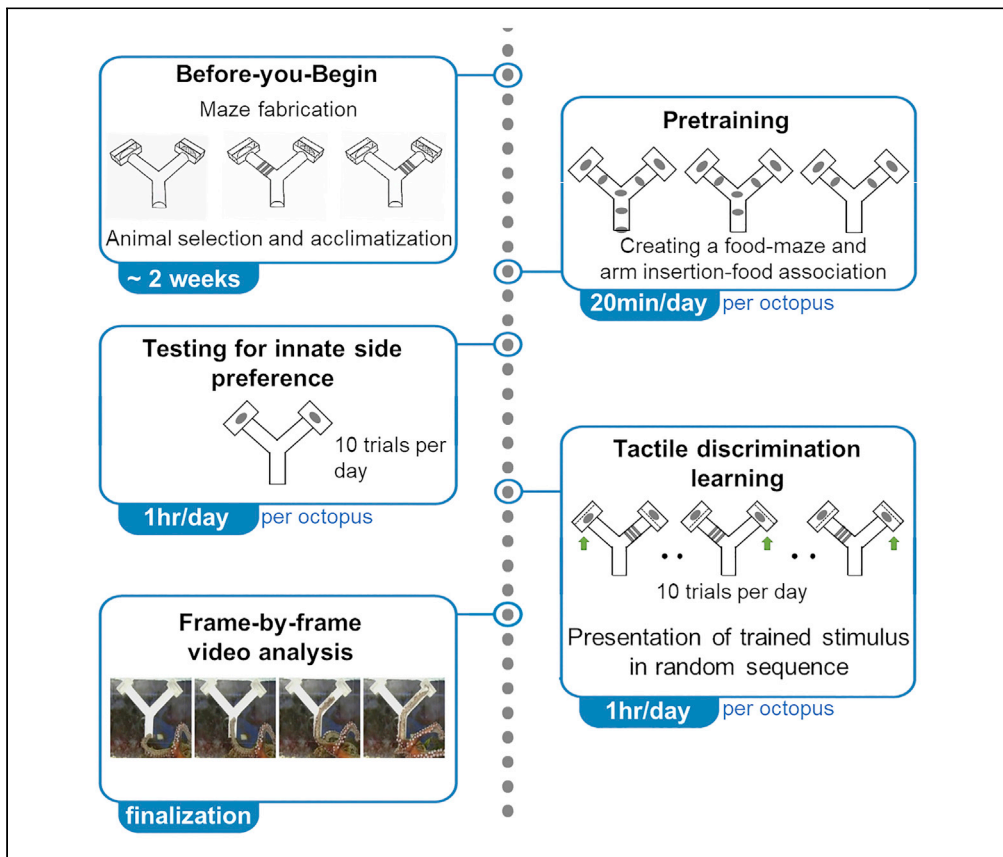# STAR Protocols

Protocol

Protocol for controlled behavioral testing of octopuses using a single-arm tactile discrimination two-choice task

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Letizia Zullo,
Binyamin Hochner,
Michael J. Kuba

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Highlights

Protocol for training octopuses to use single arms in a Y maze

Present stimuli to single arms and assess responses

Test the arm sensory system – central nervous system (CNS) interaction

Tactile input from the arm is used by the CNS to direct arm movement

Due to their unique body, standard behavioral testing protocols are often hard to apply to octopuses. Our protocol enables controlled behavioral testing of the sensory systems in single arms while allowing observation of the arm motion. The protocol allows the researcher to exclude the sense of vision without surgical manipulation and selectively test peripheral sensory input-derived learning and motor behavior. Applying the protocol requires systematic and multistage training of octopuses to associate correct maze interaction with food reward.

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Protocol for controlled behavioral testing of octopuses using a single-arm tactile discrimination two-choice task

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SUMMARY

Due to their unique body, standard behavioral testing protocols are often hard to apply to octopuses. Our protocol enables controlled behavioral testing of the sensory systems in single arms while allowing observation of the arm motion. The protocol allows the researcher to exclude the sense of vision without surgical manipulation and selectively test peripheral sensory input-derived learning and motor behavior. Applying the protocol requires systematic and multistage training of octopuses to associate correct maze interaction with food reward. For complete details on the use and execution of this profile, please refer to Gutnick et al. (2020).

BEFORE YOU BEGIN

Successful experiments with octopuses require prior setup of an appropriate salt water aquarium system which is used for both housing and testing octopuses. The experimental maze must be constructed, animals introduced to the system and reward food must be selected.

Experimental animals

For this experiment we used both male and female adult or sub-adult *Octopus vulgaris*. This species is a diurnal, generalist predator, which is commonly found in the Mediterranean Sea. Currently, there are no laboratory breeding protocols for *Octopus vulgaris*, therefore, our experimental animals were wild-caught live by local fishermen.

> *Alternatives:* Several types of octopuses can be purchased from commercial distributors, these are usually smaller octopus species. *Octopus bimaculoidis* is a commonly available species, but we have found them much more difficult to train in this type of maze.

1. Acquire octopuses from provider, and select octopuses with all arms intact. Since it is not possible to determine the age of a wild-caught octopus, choose animals of approximately the same size.
2. House each octopus individually.

> ⚠ CRITICAL: Check for local regulations regarding permits for keeping and testing cephalopods. Although not all countries require specific keeping and testing approvals for this work, we recommend that all keeping and experiments should adhere to the guidelines of the EU Directive 2010/63/EU for cephalopod welfare (Fiorito et al. 2014, 2015).
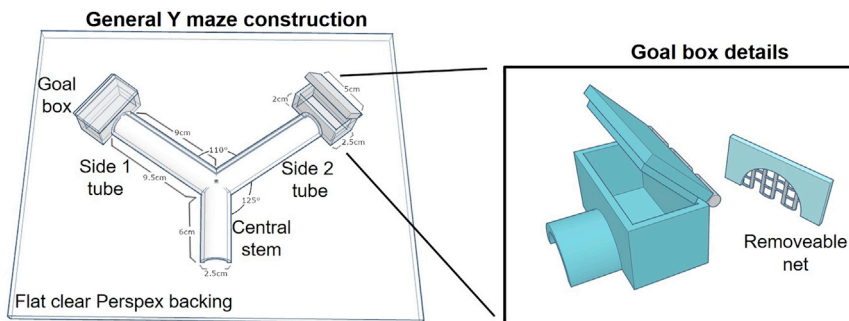
Figure 1. General Y maze construction with goal box and separation net detail

Housing/testing aquarium system

Our aquaria were custom built, 300- to 500-liter, escape proof (Asada et al., 2021), glass tanks. We used 40 × 50 × 40 cm tanks. Each artificial sea water system (Instant Ocean Sea Salt) included a custom-built stainless-steel rack with 8 glass tanks. Each rack had its own filter tank, which included a physical filter (100–200 micron) and protein skimmer (Aqua Medic GmbH) and a biological filter (Bacto Balls, Aqua Medic Gmbh).

> *Alternatives:* We had best results with Instant Ocean Sea Salt, but many additional brands are available. Semi-closed natural sea water and flow-through natural sea water systems will also work.

The tanks were enriched with clay-pot dens, gravel, rocks and green algae (like *Caulerpa prolifera*). It is important that the tanks are of equal size and have the same basic enrichment elements. Our pump (Aqua Medic GmbH) was set to provide a flow rate of one full exchange of the tank volume/ hr. We kept the temperature of the holding rooms at about 19°C. Day and night cycles were simulated by artificial illumination for 12 h (standard aquarium illumination system providing 3500k).

> *Note:* In this protocol, we provide the currently available items, which are equivalent to the equipment used in our system. There are many more options for salt water systems, local salt water aquarium experts can provide advice, equipment and help setting up a new system.

Acclimatization

> ⏱ Timing: 2 weeks

3. Acclimatize the octopuses to the holding/testing system for at least 14 days. During this period, the octopuses should get accustomed to the feeding, cleaning and general maintenance schedule of the facility. Shorter periods might affect the success of the experiment.
   a. Select a feeding schedule for the octopuses:
      We fed octopuses once every other day with either dead shrimp or pieces of fish (10–15 grams).

> *Note:* This is a normal feeding schedule for octopuses. Other labs, depending on availability, use feeding schedules of up to 1–2 small clams per day. There is no evidence that any form of food deprivation increases motivation and learning speed in octopus.

   b. At this stage, exclude from the experiment octopuses that do not eat regularly.

Selecting a reward

> ⏱ Timing: approx. 20 min/day on feeding days per octopus
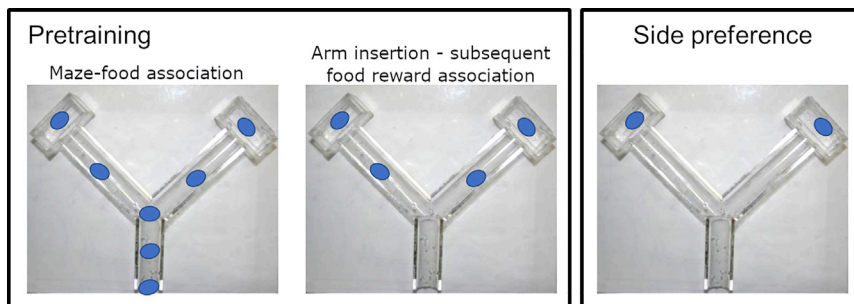
Figure 2. Food reward locations for pretraining maze-food association, pretraining arm insertion – subsequent food reward association, and side preference

During the 2-week acclimatization period a size and type of food reward should be selected. Depending on the species and origin of the octopuses the preferred food might differ (Kuba et al., 2006; Maselli et al., 2020).

4. Test several food types (availability of fish, shrimp, clam etc. depends on location) by cutting them into small portions and feeding them to the octopus one by one.
5. For *Octopus vulgaris* from the Mediterranean Sea, we used pieces of defrosted, uncooked, shrimp. It is important that:
   a. The food should always be available, and can be portioned into equal reward sizes.
   b. The size of the rewards needs to be determined depending on the size of the octopuses. The octopuses should accept at least 10 pieces of reward sized food during a single feeding.

   Note: Switching food items during the experiment might affect the octopuses' behavior. For this reason, high quality frozen food is a good option since larger quantities can be stocked. Freezing fresh food is also a good option, but be sure to test that the octopuses accept the thawed version.

Experimental mazes: basic Y maze
We designed and custom built a Plexiglas two-choice Y shaped maze that attached to the front glass of the home tank.

6. The design of the maze is as follows (Figure 1):
   a. Acrylic glass, with the flat transparent surface toward the observer and the opaque Y shaped maze toward the octopus
   b. The Y shaped maze has a central stem which opens into two side tubes, each ending in a goal box.
      i. The length of the entrance-to-goal box should be approximately 2/3 the length of a fully extended arm.
      ii. In our maze, the length from entrance to the central stem to entrance to the goal box was 17 cm.
   c. The tube diameters allow the octopus to insert only a single arm at a time.
      i. The diameter should not exceed the diameter of the thickest part of the arm (proximal arm area).
      ii. We used 2.5 cm diameter tubes with an interior diameter of 2 cm.
   d. The goal boxes can be opened for inserting the food reward.
   e. A removable net can be inserted into the goal boxes, such that food placed in the goal box behind the net could not be pulled out by the octopus.
      i. Construct the removable net from a plexiglass rectangle that slots tightly into the goal box.
      ii. Cut a hole, or arch into the rectangle, leaving at least a 0.5 cm frame for stability.
      iii. Cover the hole with a section of netting, cut from a standard aquarium hand net, and secure to it with instant glue.
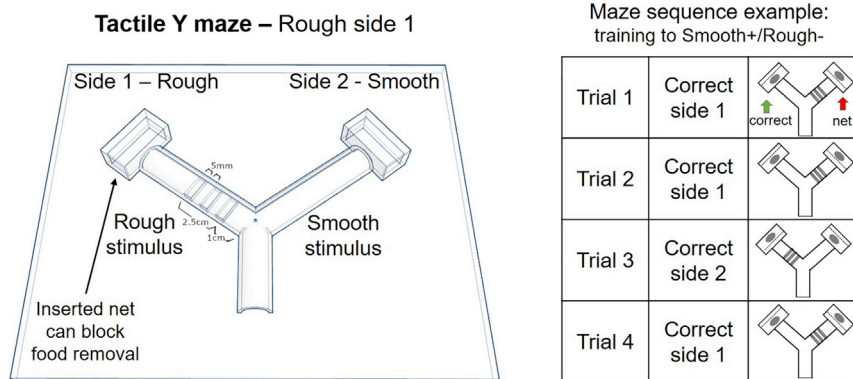
Figure 3. Tactile Y maze construction and example for using 2 mazes to present the tactile stimulus in side 1 and side 2 tube locations following a random sequence

Note: We used water-resistant tape to create a maze that is opaque to the animal. An alternative would be to use opaque plexiglass. Any material should be tested against bright light to ensure the there is no shadow of an arm visible to the animal from the room lights or filming lights.

7. For our experiments we constructed the maze flat surface from 1 cm thick plexiglass. The maze tubes were constructed from 2.5 mm–3 mm thick plexiglass. A thick plexiglass flat surface prevents the octopus from bending the maze and inserting arms between the flat surface and the front glass of the tank. All sizes and thicknesses should be adjusted depending on the size of the octopuses used.
8. The maze should be flushed with clean sea water between every trial.

   Note: This base maze was used both for pretraining and side preference phases (Figure 2).

Experimental mazes: tactile discrimination maze
To test tactile discrimination, we modified the basic Y maze design to include tactile stimuli in the flat surface of the side tubes of the maze (Figure 3).

9. The tactile stimuli are three 2–3 mm deep grooves in the flat surface of one of the side tubes. Each groove was approx. 5 mm with 5 mm between grooves.
   a. The side of the maze with the grooves is called rough.
   b. The side of the maze in which the surface inside the tube remained flat is called smooth.

   Note: Two versions of this maze must be constructed in which the rough stimulus is present on opposite sides, and which are otherwise identical.

   Note: The removable net could also be inserted into either of the goal boxes.

Setup for filming

10. Octopuses should be tested in their home tanks. The maze apparatus is designed to fit on the front glass of the tank. At the start of each trial the maze is attached to the front glass of the tank using clamps, and at the end of each trial it is removed.
11. Cover the back and side walls of the glass tanks with opaque lining.
12. Place the video camera centrally in front of the tank, with one experimenter on each side of the camera (for details see step-by-step method details- Side preference). The camera view should cover the entire front glass of the tank (Figure 4).

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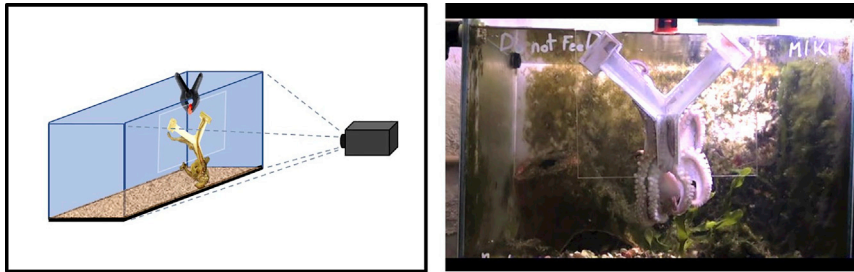
Figure 4. Camera and tank setup for experiment

> *Note:* During the experiment, any electronic devices, including the camera must be protected by plastic, as octopuses will often and accurately spit water at them.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Octopus vulgaris	Local fishermen	N/A
Software and algorithms		
The Observer XT	Noldus	https://www.noldus.com/observer-xt
SPSS Statistics	IBM	https://www.ibm.com/products/spss-statistics
Microsoft Excel	Microsoft	N/A
Other		
Plexiglass maze	custom made	N/A
Clear acrylic sheet 10 mm thickness	N/A	N/A
Acrylic Pipe Rigid 25 mm outer diameter	N/A	N/A
Spring clamps 150 mm/6"	KENDO tools	40103
Aquarium, Full glass	custom made	N/A
Aquarium rack, Stainless steel	custom made	N/A
Ocean Queen 160 protein skimmer	Aqua Medic	412.320
Aqualine T5 Reef Blue 39W 85 cm	Aqua Medic	88700
Bacto Balls 5l Eimer	Aqua medic	410.81
AC Runner 3.2	Aqua medic	100.432
Instant Ocean Sea Salt	Instant Ocean	SS15-10
Marina Nylon Fish Net - 20 cm	Marina	UPC: 015561112772

STEP-BY-STEP METHOD DETAILS

Starting from pretraining and until the completion of the experiment, octopuses should only be fed during training.

Our pretraining and training schedule was the same as the feeding schedule, with pretraining/ training sessions every other day.

> *Note:* While any form of food deprivation is not recommended, feeding only during training enhances motivation to participate in the trials.

Pretraining: maze-food association

⊙ Timing: 20 min/day per octopus

In this phase the octopuses learn to generally associate the maze with food, and then associate inserting arms into the maze with food.

> *Note:* For this phase, use the basic Y maze.

For this stage the entire maze is baited with food: both goal boxes, both side tubes and throughout the central stem, and especially in the maze opening which is visible to the octopus (Figure 2).

1. Experimenter 1 uses an aquarium hand net to ensure that the octopus remains in the den or the back of the tank, preventing the animal from interacting with the maze before it is properly positioned and secured to the front glass (see example in Methods Video S1).

2. Experimenter 2 attaches the opaque plexiglass maze to the front glass of the tank, ensuring that water fills the entire maze and goal boxes.

   ⚠ CRITICAL: In order to secure the food in the vertical maze the pieces of food must be cut large enough to wedge into the maze tubes and not fall out. Care must be taken that when placing the baited maze into the water the water reaches throughout the whole maze.

3. When the maze is secure, remove the net.
   a. If the octopus does not approach the maze, lure it to approach with movement near the front glass (see example in Methods Video S1). This usually results in the octopus reaching an arm or swimming to the front and then accidentally encountering the food at the entrance to the maze.
   b. If the octopus tries to insert more than one arm into the maze at the same time, or if it tries to open the goal boxes directly (see goal box detail in Figure 1), gently push it back with the hand net.
4. Allow the octopus to insert its arms multiple times and remove multiple pieces of food, until the food is removed from one of the goal boxes.
5. When the food is removed from one of the goal boxes remove the maze from the tank.

   > *Note:* The maze is removed from the tank to prevent an arm reaching a goal box without getting a food reward.

   ⚠ CRITICAL: The removal of the maze should be immediate, after food is removed from a goal box. At this stage the octopus is trained to associate the maze with food, and inserting an arm into the maze as the interaction that is required to get food. Reaching an empty goal box from which food cannot be retrieved is information we want to add only in the experiment phase, in which the octopus must learn that not all interactions will result in a food reward.

6. Repeat this procedure until the octopus has consumed a total of 10 pieces of shrimp.

   > *Note:* This stage is completed once the octopus purposefully approached and inserted arms into the maze for 2–3 sessions.

Pretraining: arm insertion - subsequent food reward association

⏱ Timing: 20 min/day per octopus

In this stage the octopuses learn to associate inserting an arm through the central stem and into the side tubes, with a subsequent food reward. Food is placed only in the side tubes and goal boxes, and not at the central stem or intersection. No food is visible at the entrance to the maze (Figure 2).

7. Repeat steps 1–6

Note: This stage is completed once the octopuses consistently recovered the food from the maze and entered the arm all the way into a goal box for at least 2 sessions.

Side preference

⏱ Timing: 2–4 days; 1 h/day per octopus

Note: For this phase, use the basic Y maze.

As in pretraining, side preference training sessions are conducted according to the feeding schedule. We conducted trials once every other day.

In this phase of the training, each session consists of 10 trials.

8. Place available food (no net insert) in both goal boxes (Figure 2).
9. Start the filming camera.
10. Experimenter 1 uses an aquarium hand net to ensure that the octopus remains in the den or the back of the tank. (See example in Methods Video S1).
11. Experimenter 2 attaches the opaque plexiglass maze to the front glass of the tank, ensuring that water fills the entire maze and goal boxes.
12. When experimenter 1 removes the aquarium hand net, experimenter 2 starts a timer.
13. If within 1 min from the beginning of the trial the octopus does not make any attempt to approach the maze, lure it with movement outside the tank.
14. The trial ends when the octopus arm passes into either of the goal boxes. At this point the octopus can retrieve the food reward.
    a. Once the arm has grasped the reward the maze should be removed. It is important that the arm only reach one goal box.
    b. Experimenter 2 marks the trial duration, the side choice, whether the animal was lured and whether it took the food.

⚠ CRITICAL: After the food in the goal box is grasped, the removal of the maze should be immediate. The animal should not be allowed to make a second choice.

15. If within 3 min the octopus does not make a choice, end the trial and remove the maze.
16. Switch the locations of the experimenters.
17. Wait until the food reward is fully eaten before starting the next trial.
18. Complete steps 10–16 until reaching a total of 10 trials.
    a. If the octopus does not approach the maze in three consecutive trials end the session.

A minimum of 20 trials should be conducted to assess a potential side preference.

At the end of the trial, the time, the choice made, whether the animal was lured and whether it took the food, were recorded.

Note: The experiment is best performed with two experimenters in the room, their position relative to the camera switches between trials. In order to perform the experiment in a balanced manner with only one experimenter, the position of the experimenter relative to the camera should be randomized. In this case, preferably 20 trials per experimenter position should be conducted (a total of at least 40 trials).

Tactile discrimination experiment

⏱ Timing: varies; 1 h/day per octopus

Note: For this phase, use the tactile discrimination maze.

As in pretraining and side preference, tactile discrimination sessions are conducted according to the feeding schedule. We conducted trials once every other day.

As in side preference, each session consists of 10 trials.

19. Assign each of the octopuses a correct and incorrect stimulus-
    a. Half of the octopuses should be assigned **rough** as the correct stimulus (rough+/smooth-).
       For them a correct trial is inserting an arm to the goal box of the rough side tube.
       Use the tactile discrimination maze with the net in front of the food on the smooth side goal box, and behind the food on the rough side goal box.
    b. The other half should be assigned **smooth** as the correct stimulus (smooth+/rough-). For them a correct trial is inserting an arm to the goal box of the smooth side tube.
       Use the tactile discrimination maze with the net behind the food on the smooth side goal box, and in front of the food on the rough side goal box.

20. Create a random presentation sequence for the position of the correct goal box. In order to randomize the position of the correct goal box, use the two tactile discrimination mazes, and present them following a restricted randomness sequence (Fellows, 1967).

The octopuses are prevented from using chemical information to find the correct side, by placing food in both goal boxes, but blocking the food with the net on the incorrect side.

Note: Using a restricted randomness sequence in a 2-choice task (Fellows, 1967), ensures that an animal cannot reach a significantly correct level of performance using any other rule, such as win-switch or a win-stay strategies, but must follow the discrimination cue in order to succeed.

21. Following the stimulus presentation list, and according to the trained octopuses' correct stimulus, out of view of the animal:
    a. Place food in both goal boxes.
    b. Place a net in front of the food in the incorrect goal box.
    c. Place a net behind the food in the correct goal box
22. Start the filming camera.
23. Experimenter 1 uses an aquarium hand net to ensure that the octopus remains in the den or the back of the tank. (See example in Methods Video S1).
24. Experimenter 2 attaches the opaque plexiglass maze to the front glass of the tank, ensuring that water fills the entire maze and goal boxes.
25. When experimenter 1 removes the aquarium hand net, experimenter 2 starts a timer.
26. If within 1 min from the beginning of the trial the octopus does not make any attempt to approach the maze lure it with movement outside the tank.

27. The trial ends when the octopus arm passes into either of the goal boxes.
    a. If the arm entered the correct goal box, allow the octopus to grasp the food reward and then remove the maze.
    b. If the arm enters the incorrect goal box, immediately remove the maze.

    ⚠ CRITICAL: When an incorrect choice is made, removal of the maze should be immediate. If the octopus manages to make a second choice, it cannot learn the association between the correct stimulus and the location of the correct goal box.

    c. Experimenter 2 marks the trial duration, the choice, whether it was correct or incorrect, whether the animal was lured and whether it took the food.

28. If within 3 min the octopus does not make a choice, end the trial and remove the maze.
29. Switch the locations of the experimenters.
30. Wait until the food reward is fully eaten before starting the next trial.
31. Repeat steps 21–30, at 10 trials per session, until reaching the learning criterion.
    a. If the octopus does not approach the maze in three consecutive trials end the session.

    Note: In order to prevent unintentional food deprivation, if the octopuses make more than seven mistakes, it is necessary to feed the animal additional food outside of the training trials. In this case it is important to wait at least an hour between training and feeding so that the octopus does not associate the feeding with the experiment.

End of experiment

The experiment ends when the learning criterion is reached. We chose a criterion of 8 of 10 correct choices in a single training session. This is a statistically significant difference between correct and incorrect trials (Post experiment video analysis is used to confirm the learning result – see video analysis below).

    Note: For all the stages it is important to let the octopus finish consuming the food before beginning a new trial. Octopuses can hold food in the mouth area and consume it for long periods of time while still continuing to interact. However, this might influence motivation and which arm they choose to use to interact with the maze.

Video analysis

    ⏱ **Timing: At least twice the amount of recorded hours**

32. Import all videos to Observer XT, for manual frame-by-frame analysis (Figure 5).

    Note: Video analysis can be done by any behavioral observation video analysis software.

Analyze videos without consulting the notes and times written during the experiment.

The tactile discrimination task requires interaction with a stimulus once the arm is in a side tube, and only then a decision regarding whether the side is correct is made.

33. Several measures can be collected to evaluate the presence of stimulus interactions, decision making after stimulus interaction and information transfer between arms.
For this purpose, we recorded time points for:
    a. First touch of the maze – This sometimes helps determining motivation to participate in the experiment.
    b. Arm insertion into the central stem – The start of movement into the maze
    c. Arm at bifurcation point- The time at which one of the side tubes is first entered.
    d. Arm entered side 1; Arm entered side 2 – Measure for first stimulus encountered and potential change between sides.
    e. Choice time – Used for determining the duration of the arm insertion to choice.
    Each trial was also marked with:
    f. Movement strategy of the arm inside the maze (Gutnick et al. 2011, 2020)– In our experiment two strategies were seen – search or straight. Search movements allowed for potentially more interaction time with the stimulus area required for making a choice. Comparison of

Post experiment video analysis options – Octopus trained to Smooth-/Rough+

Arm insertion | Arm enters side 1 | Arm redirected to side 2 | Correct choice

Arm in maze – r1 | interaction with smooth stimulus

Measured duration of movement (by frame or sec) - from arm insertion to goal box choice
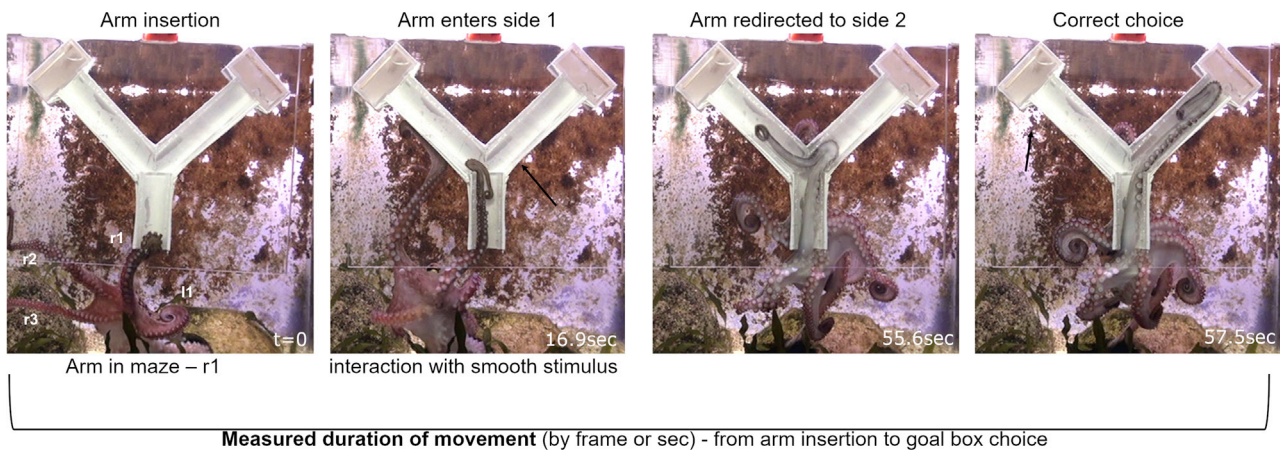
Figure 5. Example of post experiment video analysis of experimental trials - arm insertion, stimulus interaction and movement duration

arm insertion to choice duration confirmed that these movements were different in time, with search movements being significantly longer.

g. The identity of the individual arm making the choice (r1, r2, r3, r4, l1, l2, l3, l4) – This is used to determine if information is transferred between arms or learned by each arm independently. It can show whether the learning is at arm level or at the level of the CNS and then communicated to the arms.

h. The number of entries to each maze side– This can indicate restarting or redirecting the arm to find the correct stimulus side.

i. Position of the animal relative to the maze (as marked by eye level and position of the mantle: side 1, side 2, below, above) – In some cases this might influence the identity of the arm inside the maze. Usually if the animal sits covering the maze, it can only use hind arms for the task.

34. Additional measures of learning;

a. Learning should be confirmed by comparison with performance in the beginning of the experiment, as well as comparison with choices in side preference trials.
The restricted randomness sequence (Fellows, 1967) ensures that criterion cannot be reached by following non learning rules, such as repeating a sequence, repeated choice of a single side or following a win-switch or lose-switch strategy.

b. The additional details regarding the movement of the arm in the maze help confirm learning. In our experiment animals shifted from using the straight and search arm movement strategies equally in the beginning of the experiment to primarily using the search movements. These details showed that one aspect of the learning process was shifting to the more successful movement strategy.

35. Test observer reliability by comparing choices recorded from video analysis with choices recorded during the experiment. Additionally, an independent evaluator, not involved in filming, should fully analyze at least 20% of the trials and those results compared to analyzed data.

EXPECTED OUTCOMES

Depending on the discrimination task, the process of training and experiment requires up to 30 sessions. Of these the largest number will be discrimination learning trials. Video recordings of the trials allow for detailed analysis of arm movement inside the maze, and can yield information such as arm movement strategies (Gutnick et al. 2011, 2020), duration of movements, number of insertions or redirects in a single trial, and identity of the arm that is inside the maze (Figure 5). These details

can then be used to examine movement strategy shifts to allow for stimulus interaction, online interaction between the CNS and the PNS in sensory stimulus collection and decision making, and distinguishing between arm level vs. CNS level learning. Additionally, behavior of the animal can also be examined, such as sitting positions or location and movement of arms that are not in the maze. Such information can be used for analyzing arm preference and potential reflex recruitment of neighboring arms toward the arm in the maze (these were not discussed in Gutnick et al., 2020). Depending on choice of analysis software, detailed tracking of motion and stimulus interaction can also be assessed.

The total number and the sequence of side 1 and side 2 choices can be used to assess innate side preference. The sequence of correct vs. incorrect choices can be used to follow the learning process.

The basic maze form can be adapted to test additional sensory modalities (Gutnick et al., 2020), as well as similar topics such as multimodal learning, the sensitivity of the tactile discrimination sense and detailed sucker-stimulus interaction.

QUANTIFICATION AND STATISTICAL ANALYSIS

Our analyzed data was exported from The Observer XT for further analysis in Microsoft Excel and SPSS.

For nominal data we used non-parametric statistics:

1. Analyze successes (marked as 0 or 1) within each stage of the experiment using $\chi 2$ tests.
2. Calculate arm preference on the 4 most frequently used arms using $\chi 2$ tests, assuming an equal probability of use if no preference exists. If more than 4 arms are used with the same frequency, all should be added to the $\chi 2$ test.
3. Using binomial tests, compare the frequency of arm movements strategy utilized by the animal.

For numeric data (movement durations):

4. We used a Kolmogorov-Smirnov test with the null hypothesis that the durations measured come from a normal distribution. We then compared the durations of the arm movement strategies using a Mann-Whitney U test (2 sided).

Observer reliability:

5. We used Cohen's kappa for inter-rater reliability (2 sided)-
   a. Comparing results recorded during the experiment with results of video analysis.
   b. Comparing initial video analysis results with video analysis results of 20% of trials conducted by an additional observer.

LIMITATIONS

In our experience for complex arm manipulation tests such as these, automation of the maze apparatus, to remove potential experimenter influence on the behavior of the animal is unfeasible. The experimenter needs to react quickly to any attempts of the octopus to open the goal boxes directly, insert multiple arms into the maze, insert arms between the maze and front glass, and otherwise break the maze. Therefore, the presence of the experimenter is necessary.

There is a potential effect of bias due to the presence of experimenters in the room.

If possible, the same experienced experimenters should conduct the entire process, from animal selection to experiment, to ensure comparable results. Because the experiment requires the animal to

choose one of two sides, the location of the experimenters is important. The location of the experimenters on each side of the camera should regularly change, and apart from necessary operations, movement should be minimal. It is best to perform all sessions at the same time of the day.

It is important, that if octopuses are housed in a common use room, all access to the experiment area should be restricted during testing sessions. Visible movement around the room, as well as loud vibrations, or changes in water values (temperature, chemicals etc.) can distract the animals, and affect performance.

The general learning behavior of octopuses, as found in many studies, is not identical to the classical learning curves that have been observed in other animals. Octopuses will rarely reach over 75%–80% reliability even in simple discrimination tasks. There is also very limited information of what the effects of overtraining on octopuses' performance might be.

A clear end criterion should be selected. Since this is a complex manipulation task for octopuses an 8 out of 10 correct choices in a single session criterion was used (a statistically significant difference between correct and incorrect choices), but it must be compared both with side preference results, and behavior during the initial pre-learning, naive, stages of the experiment.

TROUBLESHOOTING
Problem 1
Obtaining research animals (before you begin: Experimental Animals)

Potential solutions
A major limitation of octopus studies is the availability and comparability of subjects. Unlike studies with lab bred animals, the age of wild-caught octopuses cannot be accurately determined without dissection. Animal size is often due to amount of food consumption rather than age. Additionally, many captured animals will not have all 8 arms intact.

In locations with easy sea access, local registered fishermen are a good potential source for animals. They can also be provided with equipment for appropriate collecting and temporary housing of animals.

There are several European marine animal wholesalers that list available species online.

When trying to obtain *O. vulgaris* for areas outside Europe, Northern Africa and the Mediterranean coasts, verify that your facility has the permissions to house non-local species.

Problem 2
Selecting responsive octopuses (before you begin: Experimental Animals)

Potential solutions
Handling the animals (Feeding and tank maintenance) prior to the experiment helps in selecting animals that are responsive to humans and reliable eaters. It also helps in determining the general health and condition of the arms. Animals that do not respond or eat consistently should be excluded.

It is worthwhile to wait to start an experiment until an appropriate number of similar sized, intact, octopuses are collected, ideally with a good male/female ratio.

The sex of the octopus can be determined by the presence of a characteristic male spermiduct on the web between R3 and R4 and a groove for spermatophores leading to the tip of the hectocotylus R3

Problem 3

Octopus inserts arm between maze and glass (step-by-step method details: Pretraining, side preference and tactile discrimination experiment)

Potential solutions

The flat surface of the maze is bent. At the end of each day lay the maze down on a flat surface and place weights on all 4 corners. If the problem persists, consider building replacement mazes with a thicker acrylic glass.

Problem 4

Changes in octopus' responsiveness during the experiment (step-by-step method details: Pretraining, side preference and tactile discrimination experiment)

Potential solutions

Sometimes during training octopuses will stop responding. This often happens when there is a shift between stages of the experiment. Ending a session after 3 unresponsive trials and continuing training on the next training day will often be enough.

General responsiveness can also be enhanced if the octopuses are fed only by the experimenters, and not by any maintenance or care staff.

When an octopus responds during the first few trials of the day, but is less responsive in the last trials, it can help to reduce the size of each reward piece, or to split the daily sessions in two with a longer interval between trials 5 and 6.

Problem 5

General changes in octopus behavior (step-by-step method details: Pretraining, side preference and tactile discrimination experiment)

Potential solutions

If the octopus in is both unresponsive during the experiment and not eating regularly, it is possible that the changes are related to the life cycle.

The age of octopuses cannot be reliably assessed. There is very little information about senescence in octopuses. However, there are several external signs, that together with unresponsiveness might suggest that the animals should be removed from the experiment: (1) relaxed musculature around the eye, revealing a white area surrounding the pupil; (2) development of skin lesions; and (3) a female octopus that refuses to leave the den but continues to take offered food might have reached the reproductive stage and will need to be removed from the experiment.

Problem 6

Single octopus failing to reach learning criterion (Tactile discrimination experiment)

Potential solutions

Not all animals will succeed in learning a task, especially when the task is reward motivated and complex. In these cases, the animal must be reported as not learning, and if necessary, an additional animal must be trained.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Tamar Gutnick (tamar.gutnick@mail.huji.ac.il).

Materials availability

The materials used for animal keeping and handling are available commercially. We are happy to provide husbandry advice upon request. The mazes are custom designed, and we are happy to advise about construction upon request.

Data and code availability

This study did not generate any new code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101192.

AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, T.G., L.Z., B.H., and M.J.K.; Writing, T.G. and M.J.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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