Report

Current Biology

Octopus bimaculoides can learn to utilize a mirror to localize a reward outside the line of sight

Highlights

- Cephalopods can interpret mirror reflections for spatial navigation
- Travel duration to reward site decreases significantly across trials
- Octopuses climbed a barrier suggesting spatial mapping of mirror cues to tank layout
- These abilities may reflect convergent evolution of spatial cognition

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In brief

Kieseler et al. show that three *Octopus bimaculoides* learned to move to a reward site using a mirror. Octopuses climbing the start-chamber wall indicates integration of spatial mapping of mirror cues to tank geometry. This extends mirror-use capabilities to invertebrates and suggests convergent evolution of underlying cognitive principles.

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Report

Octopus bimaculoides can learn to utilize a mirror to localize a reward outside the line of sight

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SUMMARY

Mirror-mediated localization of hidden objects is well documented in vertebrates[1–12] but has never been demonstrated in invertebrates. Using mirrors to locate otherwise occluded objects is a form of mediated perception, linking a visible reflection to an occluded location[13,14] and is seen by some as a precursor to self-recognition.[15] Cephalopods offer a fascinating test case of convergent cognition, having independently evolved sophisticated perceptual and cognitive abilities that are similar to mammals,[16–18] after diverging from a common ancestor over 520 million years ago.[19] In addition, they react to mirror images as though they were conspecifics.[20–23] We projected a virtual crab that was visible only via mirror reflection onto a tank wall. Three *Octopus bimaculoides* were trained to navigate to the projection site instead of the mirror. All three octopuses learned this task, successfully choosing the correct side in 73% of trials. Critically, octopuses sometimes moved away from the visible reflection and climbed over the side walls of the start chamber to reach visually occluded locations that were spatially aligned with the reflected prey location. This behavior suggests (1) the ability to inhibit a direct approach to salient visual stimuli, and (2) a spatial representation that integrates mirror information with knowledge of 3D tank geometry. These findings extend mirror-use capabilities to invertebrates, demonstrating that cephalopods can employ mirror reflections for spatial navigation. The independent evolution of cognitive capacities underlying mirror use across diverse taxa suggests that common solutions may have evolved to solve spatial navigation challenges.

RESULTS

To test if *O. bimaculoides* (henceforth octopuses) can learn to use mirror reflections to localize targets outside of their direct line of sight, we proceeded in three steps. First, three wild-caught octopuses were individually habituated to a mirror in the experiment tank (Figure 1B; STAR Methods). Once habituated, the octopuses began mirror-use learning trials in which a live crab in a glass jar was visible only via a mirror reflection (Figure 1C). The octopuses were already familiar with extracting crabs from jars through daily enrichment puzzles, thus isolating mirror use as the novel challenge.

On the first mirror-use learning trial, all the octopuses moved toward the crab's reflection in the mirror, as if approaching prey at that apparent location, while showing no reaction to the live crab when it entered view. After reaching the mirror, the octopuses redirected toward the jar and extracted the crab.

Learning to consistently approach the crab directly without first approaching the crab's reflection in the mirror required 10–12 trials per animal (see Table S1). This learned inhibition of the initial approach response to the visible reflection suggests that the octopuses' predatory behavior is subject to executive control rather than being an obligate stimulus-response reflex.

In the testing phase, a virtual crab stimulus was projected from the outside onto the left or right side of a translucent screen attached to the outside of the tank's back wall. A mirror spanning the tank's width was positioned at the tank's midline, facing the back wall. A start chamber—open to the top and the mirror-facing front—was placed in the sand at the center of the back wall. The chamber's walls blocked direct line of sight to the projected stimulus, ensuring that only its reflection in the mirror was visible to the octopus while in the start chamber (Figure 1D). We used a virtual crab to ensure that the octopuses relied exclusively on visual cues to locate the crab, as octopuses respond to virtual crabs with natural predatory behaviors.[24,25]

At the start of a trial, an octopus was placed in the start chamber. After ~30 s, the virtual crab stimulus appeared on the left or right side of the screen. To receive a live crab reward, the octopus had to move to the back wall on the side matching the virtual crab's location and cross the imaginary line parallel to the mirror at the start-chamber opening (see Figure S1A), rather

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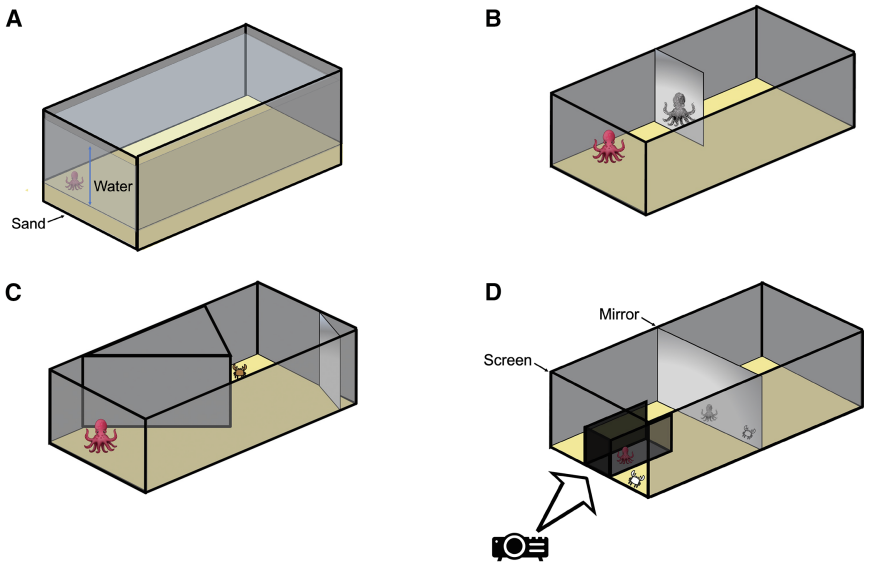

Figure 1. Schematics of tank setups during the different stages of the experiment

(A) The tank setup with a proportionately sized octopus in red, sand depth (5–6 cm), and water depth (52 cm).

(B–D) Omitted sand and water depth to improve visibility.

(B and C) Exaggerated octopus and stimulus size.

(D) Exaggerated stimulus size; other items are proportionately sized.

(B) shows the setup for mirror habituation: a mirror covering half of the tank's width was placed in the middle of the tank allowing the octopus to freely navigate the tank. (C) shows the tank setup for mirror-use learning trials with the occluder on the left and the mirror on the right side (setup was reversed for half of the trials): a live crab in a glass jar (not displayed here) is hidden from direct view by an occluder made from opaque black corrugated plastic sheets and is only visible in the mirror placed in the corner opposite the crab. (D) shows mirror-use testing: the octopus is placed in a start chamber with an open front and top positioned opposite a mirror that covers the entire width of the tank. A virtual crab is projected either to the left or right side of the enclosure and is only visible via mirror-reflection to the octopus in the start chamber. The octopus is shown in red; the mirror image of the octopus is gray. The real live crab is shown in brown, the projected virtual crab is shown in white.

See also Figure S1.

than approaching the mirror where the reflection was visible. No reward was given for incorrect side choices.

Trials were recorded from above and independently scored as either conclusive trials (the octopus moved to either side of the back wall without touching the mirror) or inconclusive trials when the octopus either (A) did not leave the start chamber or (B) touched the mirror after stimulus presentation.

Mirror-guided locomotion in octopuses

We report task performance for both a base dataset (n = 30) excluding mirror-touch trials, and an expanded dataset (n = 41) including mirror-touch trials.

The octopuses performed significantly above chance in both the expanded and the base data set (Figure 2). In the expanded dataset, the octopuses chose correctly significantly more often than incorrectly across 41 trials (correct/incorrect; O1, 15/4; O2, 8/5; O3, 6/3; mean accuracy, 69.1%; with 95% confidence interval: [58.9%, 79.2%]). Three statistical tests confirmed that their performance was significantly above chance (Fisher's combined probability test: χ^2 (6, n = 3) = 14.51, p = 0.024; Stouffer's Z score method, *sumz* = 2.05, p = 0.020; binomial simulation, z = 2.33, p = 0.010, all one-sided; see STAR Methods). In the base dataset, octopuses performed similarly across 30 trials, making more correct than incorrect side choices (correct/incorrect; O1, 10/3; O2, 6/3; O3, 6/2; mean accuracy, 72.9%; with 95% confidence interval: [62.7%, 83.0%]; Fisher's combined probability test: χ^2(6, n = 3) = 12.76; p = 0.047; Stouffer's Z score method, *sumz* = 1.97, p = 0.025; binomial simulation z = 2.45, p = 0.007; see Figure 2). These results show that octopuses in this study were able to extrapolate the location of a future reward that was not along the line of sight based on what they saw in the mirror.

The initial travel direction largely predicted each trial's outcome: in 27/30 trials the octopuses' initial movement toward a side was maintained (19/27 toward the correct side, 8/27 toward the incorrect side). In three trials (trials 1, 9, 10 from octopus 1), course corrections were observed after an initial ambiguous movement forward toward the mirror followed by redirection to the correct side of the back wall, resulting in a correct trial. After

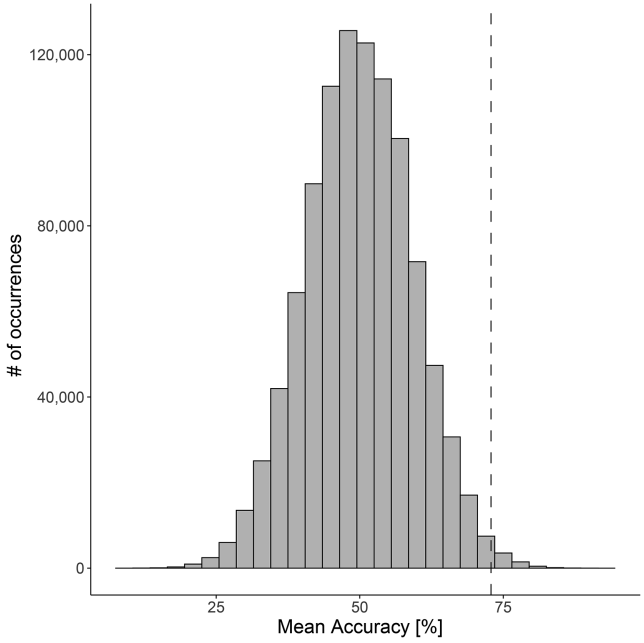

Figure 2. Histogram showing the result of 1,000,000 simulations of the mirror trial experiment in the base dataset without including mirror-touch trials, with three animals randomly guessing

The dashed line shows the actual mean accuracy of the three animals in our study, indicating that our animals were unlikely to have been guessing.

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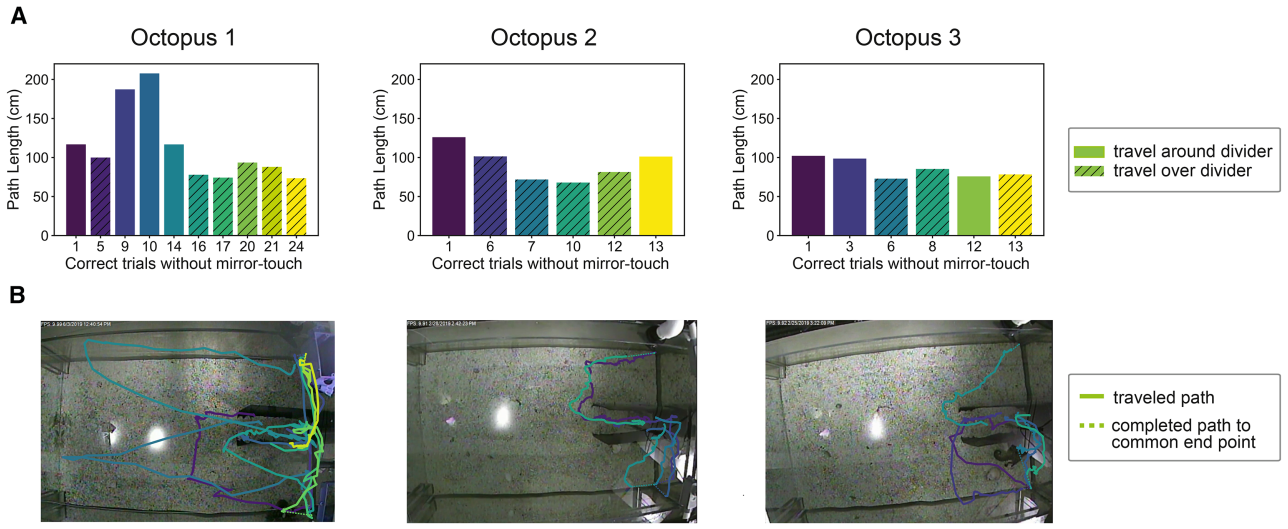

Figure 3. Distance traveled per correct trial by each octopus

(A) Bar graphs show the distance traveled to the reward location plus distance to a common endpoint from the reward location (completed distance), solid bars indicate travel around the divider, striped bars indicate climbing over divide, and the *x* axes show trial numbers of correct trials without mirror touching.

(B) Tank photos show tracked paths superimposed over a photo of the experiment tank, with trials colored as in (A); the solid line shows the actually traveled path, the dotted line shows added distance to a common end point.

moving toward a side, octopuses never reversed to the other side during the trial, indicating that side-selection mostly occurred before leaving the start chamber and that navigation reflected their initial choice, not trial and error.

The octopuses showed a slight rightward bias in their choices, though this varied by individual. In the expanded dataset ($n = 39$ because the octopuses did not make a side choice in two trials), the side choice distributions were relatively balanced in octopus 1 (9R/10L) and octopus 2 (7R/5L) but biased in octopus 3 (7R/1L). A similar bias was observed when restricting analysis to the base dataset (30 trials): O1 (6R/7L), O2 (5R/4L), and O3 (7R/1L). Permutation analyses for all animals confirmed that performances exceeded what side bias alone could produce in both the base dataset ($z = 2.31$, $p = 0.010$, real animals outperformed the simulation in 98.3% of the simulated experiments) and the expanded dataset ($z = 2.69$, $p = 0.004$, real animals outperformed the simulation on 99.5% in simulated experiments), demonstrating that performance reflected stimulus-dependent choices rather than side preference alone. Assessing side choices regardless of stimulus presentation with individual one-sided binomial tests revealed that only O3 exhibited a significant bias to the right side (base and expanded datasets, $p = 0.035$) whereas O1 (base, $p = 0.710$; expanded, $p = 0.676$) and O2 (base, $p = 0.500$; expanded, $p = 0.387$) did not.

Navigation efficiency increases with experience

To evaluate whether navigation became more efficient with experience, travel duration and path length were assessed across trials. Two datasets were analyzed: all conclusive trials ($n = 30$, excluding mirror-touches) and correct trials ($n = 22$) only. Linear mixed-effects models were fitted to log-transformed (log-normal) data, including trial number as fixed effect, the octopus as random intercept, and the trial rank as a predictor in secondary analyses. Duration was measured from when the octopus started moving after stimulus onset to its arrival at the back of the tank. The path length was measured from the starting position to the standardized endpoint at the back corner on the chosen side.

Travel duration decreased over the correct trials (χ^2 [1, $n = 22$] = 6.72, $p = 0.01$) and all the conclusive trials (χ^2 [1, $n = 30$] = 6.05, $p = 0.01$). Including trial rank for the correct trials (χ^2 [1, $n = 22$] = 7.12, $p = 0.007$) and the conclusive trials (χ^2 [1, $n = 30$] = 5.29, $p = 0.02$) also revealed a significant decrease in travel time, showing that the octopuses' mirror-guided navigation became faster and more efficient with experience (see Table S2).

Path length analyses revealed a more nuanced pattern and showed a significant decrease in path length across correct trials (χ^2 [1] = 4.38, $p = 0.036$, see Figure 3) but not the conclusive trials (χ^2 [1] = 1.09, $p = 0.29$). Including trial rank showed a trend toward shortening paths on correct trials (χ^2 [1, $n = 22$] = 3.73, $p = 0.053$) but did not show an effect in the conclusive trials (χ^2 [1, $n = 30$] = 0.81, $p = 0.37$). These results suggest learning-related path optimization in the correct trials, although the basis for increased variability in the incorrect trials remains unclear (see Table S2).

DISCUSSION

We find that octopuses can learn to use a mirror as a source of information about events occurring outside of their direct line of sight. Three *Octopus bimaculoides* demonstrated mirror-guided navigation, moving toward a hidden prey stimulus (i.e., a projected image of a crab) visible only as a reflection in a mirror from their starting location. All three octopuses were able to move to the correct location—the left or right side at the back side of the tank where the virtual crab was projected—significantly more often than if they were moving randomly, providing evidence that the visual information from the mirror guided their

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movement toward one side of the tank. Our results demonstrate that this visual system can be extended beyond direct lines of sight through mirror use. Specifically, this study establishes the feasibility of mirror-mediated foraging in *O. bimaculoides*, representing the first evidence of this capability in octopuses, and, to our knowledge, in invertebrates.

Across trials, octopuses became significantly faster at reaching the reward location, yet they did not consistently select the shortest available pathway, despite some path shortening over correct trials. Upon leaving the start chamber, octopuses maintained directional choice to a side (left/right) of the tank. Directional corrections were observed only when initial movement was directed toward the mirror before redirecting to the chosen side at the back wall. These patterns raise the question whether learning could have involved improved decision-making speed or confidence rather than spatial route optimization. Future studies with larger sample sizes could assess this by examining early directional commitment and whether path optimization involves path length and duration.

Mirrors create a unique perceptual situation, and the process of learning to use a mirror would appear to involve some understanding of the function of a mirror as a reflection of their surroundings.[14] *O. bimaculoides* are visual hunters[26] and prey for other predators. Therefore, they must have a clear understanding of their surroundings[27] to aid in their hunting efforts and increase their chances of survival. The octopuses had substantial experience of moving around the tank prior to trials during the habituation periods (including above and around the start chamber), allowing them to become familiar with the testing tank.

The performance of the octopuses in this study could be interpreted as a form of associative learning, where animals learn that a visible position X on the mirror is associated with reward at a non-visible position Y elsewhere in the tank. Indeed, the data across the training and testing phases show an increase in correct attempts over time, consistent with learning through reinforcement. Alternatively, the octopus' movement toward the correct side could result from approaching the perceived or expected location of the crab, using the mirror for spatial referencing within an internal representation of the tank. Several observations suggest that spatial referencing may better explain the data. First, octopuses frequently climbed over the side walls of the start chamber, moving away from the salient mirror reflection to reach locations that were visually occluded but spatially congruent with the reflected crab. They climbed over the correct side in 59% of correct trials, and they moved to the bottom of the tank, where the image of the crab was projected, even though a live crab was dropped in from above the tank. Second, octopuses demonstrated early directional commitment, selecting and maintaining a direction upon leaving the start chamber with few mid-journey corrections. These never involved crossing to the other side of the tank, suggesting that they linked the image location in the mirror with its projected position at the back of the tank. Associative learning in the absence of any representation of the tank's space might instead predict that octopuses would go to the water's surface where the crab release box was, just above the water level. Third, the experimental setup differed substantially between mirror-use learning and testing. During mirror-use learning, the octopuses had to perform a 90° turn close to the mirror, whereas in mirror-use testing octopuses had to leave the start chamber, perform a 180° turn, and move away from the mirror to the back wall of the tank, or climb over the side wall of the start chamber, which also involved a 180° change in path trajectory. Yet, all three octopuses succeeded on their first testing trial, suggesting that they understood the underlying spatial principle rather than simply associating specific visual features with reward. Whether the mechanism of learning was purely associative or involved referencing to an internal representation of space will require further research. But the main point remains that octopuses can learn to use mirrors as tools to locate a rewarded location outside the line of sight.

As octopuses respond to virtual images of crabs as if they were actual prey,[24,25] it is conceivable that octopuses would be motivated to move to the perceived location of the crab image. If true, their performance would suggest that octopuses are capable of mirror-mediated spatial localization of objects and can use a mirror as a perceptual device to gain information about their surroundings. However, the current study was not optimally designed to definitively distinguish between associative and spatial-learning mechanisms. Future studies employing generalization tests, novel mirror configurations, and larger sample sizes would be necessary to conclusively determine whether octopus learning involved spatial reasoning or associative learning.

An alternative explanation for the octopuses' high success rates in this experiment could be that the octopuses approached the mirror reflection initially, then upon exiting the start chamber directly saw the projected crab and redirected their motion toward it. This account is inconsistent with the fact that in 59% of correct trials, octopuses climbed over the side wall of the start chamber near the back wall. Unless the octopus started out at the back wall, this required movement away from the mirror reflection before gaining visual access to the crab projection site after climbing the side wall. This behavior indicates that the octopuses were not simply detecting the actual crab location upon exiting the chamber, but rather were using mirror information to guide their navigation to a location they could not see directly. Our data suggest that the path taken is subject to executive control rather than being an obligate behavior forcing motion toward the perceived location of a prey stimulus.

Although our data reveal that octopuses can learn to use a mirror as a tool for the localization of prey outside the line of sight, their behavior was not always consistent, even in later trials. This variability suggests that other potential factors may have influenced the octopus' behavior, whether internal, such as mood or degree of hunger, or external, such as trial variability. Sample size in this study was limited by resource constraints and animal senescence. Nonetheless, our findings reveal octopus mirror competencies and open new questions about their behavioral and cognitive capacities. Specifically, it raises the question as to whether octopuses might possess an internal map of the 3D terrain that can be used to navigate, and whether octopuses possess more complex mirror competencies, such as those associated with self-recognition. *Octopus vulgaris* has been found capable of inter-individual discrimination between conspecifics for at least 1 day.[28,29] This kind of social discrimination implies a representation of others as distinct agents and may provide the cognitive scaffolding necessary for recognizing the self as a unique individual. Indeed, a preliminary protocol has

recently been developed to adapt the Gallup mirror test for use in *O. vulgaris*,[23] enabling further investigation.

Mirror use has been demonstrated across several distantly related animal groups, from mammals[1–10] to birds[11,12] and now cephalopods. The independent evolution of this ability in octopuses and mammals—lineages that diverged at least 520 million years ago[19]—raises the possibility that underlying cognitive processes represent universal solutions to spatial cognition challenges. If true, such convergent evolution suggests that certain cognitive capacities may be fundamental to how animals process and represent spatial information, regardless of evolutionary history.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to the lead contact, Mary Kieseler (marie-luise.kieseler@dartmouth.edu).

Materials availability

This study did not generate any new, unique reagents.

Data and code availability

- Manuscript data have been deposited Zenodo and are publicly available as of the date of publication at 10.5281/zenodo.17552606.
- All original code has been deposited at Zenodo and is publicly available at 10.5281/zenodo.17604739 as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Designed the research, M.K., M.R.M., K.R.F., C.H., Z.H., N.D., J.F., S.G., J.M., J.V., M.R., D.E., and P.U.T.; performed the research, M.K., Z.H., N.D., J.F., S.G., J.M., J.V., M.R., J.O.F., M.A., and J.M.V.; analyzed the data, M.K., M.R.M., K.R.F., C.H., J.O.F., and M.A.; wrote the paper, M.K. and P.U.T.; created the stimuli, M.R.M.; revised the paper, M.K., M.R.M., K.R.F., D.E., P.U.T., and J.M.V.; conceived the experiment, M.K., S.G., J.M., and P.U.T.

DECLARATION OF INTERESTS

The authors report no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used Claude in order to improve the readability and clarity of some text in the manuscript. After using this tool or service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2026.05.012.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Behavioral data – trial overview	This paper	Database: 10.5281/zenodo.17552606
Behavioral data – duration and distance	This paper	Database: 10.5281/zenodo.17552606
Analysis code	This paper	Database: 10.5281/zenodo.17604739
Experimental models: organisms/strains		
Octopus bimaculoides	Aquatic Research Consultants, Long Beach, California, USA	wild type
Octopus bimaculoides	Dr. Lisa Abbo and Bret Grasse, Marine Biological Laboratory, Woodshole, Massachusetts, USA	laboratory raised
Software and algorithms		
R	https://www.r-project.org/	RRID: SCR_001905
MATLAB	The MathWorks, Inc.	RRID: SCR_001622
Python	http://www.python.org/	RRID: SCR_008394

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This study was approved by the Dartmouth College Institutional Animal Care and Use Committee. Octopuses were tested in a room in which no humans were present during a trial to rule out the possibility that the presence of humans might influence octopus behavior.[30] In the adjacent room, video cameras permitted experimenters to see all events in the experiment tank in the testing room in real time. Experimenters could control events in the octopus testing room, such as releasing crabs for reward, by remotely manipulating aspects of the test tank via fishing tackle lines running between the testing and observation rooms. All octopuses were tested individually.

Seven wild-caught California two-spot octopuses (*O. bimaculoides*) were purchased from Chuck Winkler, Aquatic Research Consultants, Long Beach, California. Octopuses were caught at San Pedro pier near Santa Monica and Newport Beach the day before their arrival in the lab and were delivered via overnight FedEx. Three laboratory-raised octopuses were acquired from Dr. Lisa Abbo and Bret Grasse, Marine Biological Laboratory, Woods Hole, Massachusetts. Octopuses were given a week to acclimate to the lab surroundings before experimentation commenced; longer acclimation periods may reduce stress-related variability.[31]

Three wild-caught animals completed the study; the other animals were either participating in pilot experiments during study development or started senescence before completing the study (see Table S3). Two of the three animals that completed the study were procured in September 2018, one in March 2019. Upon arrival, all octopuses' mantles were about four centimeters long. Upon death, the mantle of octopus 1 was 10 centimeters long, the mantles of octopuses 2 and 3 were 9 centimeters long. Sex was not confirmed by dissection. Visual identification of the hectocotylus initially suggested that all ten animals were male; however, three animals (none of the study completers) subsequently laid eggs.

Housing

All octopuses inhabited a ~3400-liter closed water system across two rooms. The experiment room can be seen here: https://sites.dartmouth.edu/peter/octopus-research/. Octopuses were housed in individual tanks supplied with artificial seawater prepared by mixing Instant Ocean salt with reverse osmosis deionized water (salinity: 1.026 specific gravity; ~31.1 ppt or 35.11 g/L). Water quality was maintained at pH 8.0, with ammonia, nitrite, phosphate, and chlorine levels undetectable, and nitrate levels < 10 ppm. The water in the tank system was pumped by two Marathon S100SEQ23 pumps that pumped up to 22,000 liters per hour each. Water returned passively to the sump tank via overflow and gravity. Water temperature was maintained between 20–21 °C (68–70 °F) throughout the study, falling within the range of water temperatures measured at the capture site. Lighting was controlled via a Current USA Ramp Timer Pro programmable controller, which simulated gradual sunrise and sunset transitions and provided low-intensity "moonlight" illumination during the dark phase. A 12-hour light/12-hour dark cycle was maintained. During the light phase, ambient LED lighting in the indoor laboratory (no windows) provided 98 lux at the water surface and during the dark phase, moonlight LEDs were maintained at < 1 lux. Illuminance values were measured using a Minolta CL-100 Chroma meter, which was pointed directly upward and positioned approximately 3″ above the water's surface near the center of the block of four aquariums that the octopuses in this study

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inhabited. Four aquariums (182.8 x 61 x 61 cm [LWH]; 680 l) were divided in half by translucent plastic dividers with slits allowing water circulation, creating eight housing units (91.4 x 61 x 61 cm; 340 l). Each unit contained ~5 cm of black sand substrate, an inverted terracotta flowerpot den, 1–3 pieces of live rock, and seashells. Octopuses were offered 5 fiddler crabs (*Uca pugilator*) per day, two of which were dropped into the housing unit. The remaining crabs were obtained by solving food puzzles for enrichment and participating in experimental trials. Food puzzles included extracting crabs from glass jars, familiarizing octopuses with this foraging technique prior to mirror-use learning. The back wall of the housing tanks was occluded with blue patterned fabric to screen piping and electrical lines from the animals' view.

Octopus laboratory acclimatization

After delivery via overnight FedEx from California, the animals were each given a week to acclimate to their new surroundings in their individual 91.4 x 61 x 61 cm home tank containing 340 liters of saltwater. This was half of a single large tank (182.8 x 61 x 61 cm, 680 liters), separated by a divider in the middle.

Octopus 1 quickly accepted food from metal tongs, left its flowerpot den to approach the side of the tank when staff entered the room, and started to investigate food puzzles shortly after the puzzles were placed in the tank. Octopuses 2 and 3 showed less approachable behavior in the beginning and did not accept food from metal tongs initially. Over time, octopus 2 consistently approached the side of the tank when staff entered the room, octopus 3 frequently but selectively left its hideout to approach the side of the tank near staff depending on which person entered the room.

During week 2, octopus 1 was acclimated to the 91.4 x 61 x 61 cm foot experiment tank by placing it in the tank with a flowerpot and a rock every other day for 45 minutes each, for a total of three sessions. When starting mirror habituation, octopus 1 approached the mirror for the first time during trial 2 and first pounced on and subsequently ate a crab during trial 3.

Octopuses 2 and 3 were not habituated to the experiment tank ahead of mirror habituation. Octopus 2 hid under rocks or in its flowerpot for the first 3 mirror habituation trials and showed burrowing behavior during trials 6, 7, and 9. Octopus 2 approached, touched, and swam back and forth in front of the mirror for the first time during habituation trial 6, and first pounced on a crab on trial 8. Octopus 3 hid for the first 3 trials and first approached the mirror on trial 4. It first pounced on a crab in front of the mirror during habituation trial 6. Considering octopus 1's three tank habituation session before habituation started, all three animals showed a similar timeline for approaching the mirror.

All three octopuses consistently approached, touched, and moved all GoPro cameras mounted on rocks and at the top of the mirror which led to the GoPros not being used during experiment trials, in favor of cameras mounted outside the experiment tank.

Octopus Senescence and end of study participation

Senescence is characterized by various morphological and behavioral markers, such as white skin lesions, lack of feeding, undirected or uncoordinated behavior, and skin retraction around the eyes.[32] Research has shown that male *Octopus vulgaris* exhibit increased but undirected activity, such as movement not associated with hunting or foraging, compared to non-senescing animals.[33] A recent study by Roura et al.[34] reported that both male and female *Octopus vulgaris* show chromatophore degeneration, with males also exhibiting necrosis at all arm tips except the hectocotylus. Since data collection concluded before the publication of Roura et al., these criteria were not used in our study. Octopuses were excluded from the study due to onset of senescence when all three of the following criteria were observed for at least seven consecutive days: (a) refusal to feed for one week, (b) display of distressed behavior in response to routine activities carried out in the octopus' vicinity that was not observed after acclimation to the laboratory, such as hiding when feeding tongs holding crabs were introduced to the aquarium, hiding when food puzzles were placed in the aquarium, or showing distressed reactions toward multiple familiar experimenters, and (c) extensive undirected activity.

METHOD DETAILS

Experiment aquarium setup

The experiment tank (182.8 x 61 x 61 cm [LWH]; 680 l) was located in an adjacent room with dark blue wall color and two LED light sources (2520 lm): one directly above the experiment tank, and one 2.5 meters away from the experiment tank. The bottom of the tank was covered with 5–6 cm of white sand for improved contrast during video recording. The water depth was 52 cm and water level was maintained 3 cm below the tank rim. The tank contained a bubbler that was removed during experiments. Learning phases of the experiment made use of the entire tank. During the testing phase of the experiment, a mirror covering the entire width and height of the tank (59 x 61 cm [WH]) was inserted in the midline, dividing the experiment tank in half (91.4 x 61 x 61 cm; 340 l).

Transport between home and experiment tank

Octopuses were transferred from their home tank to the experiment tank in the adjacent room using a white plastic transport container (height: 25 cm, diameter: 22 cm) filled with tank water. A net was used to gently guide each octopus into the transport container, which was then covered with the net to prevent escape and limit visual stimulation during transit. Duration of transit was less than half a minute as the experiment tank was in the adjacent room. Upon arrival, the container was submerged in the

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experiment tank, allowing the octopus to exit freely. Each octopus was then given 30 minutes to acclimate and explore the experiment tank. Octopuses were returned to home tanks using the same transfer protocol.

Study design

Prior to mirror habituation, octopus 1 was given a 45-minute session every other day for a total of three sessions to habituate to the experiment tank. This pre-habituation protocol was implemented for O1 after observing that O2 and O3, which began mirror habituation without tank pre-habituation, hid extensively (under rocks or in flowerpots) during initial mirror sessions. O1 exhibited exploratory behavior during pre-habituation and subsequently required fewer mirror habituation sessions to reach baseline activity (O1: 7 sessions; O2: 16 sessions; O3: 10 sessions).

The experiment consisted of three phases: habituation, learning mirror-use, and mirror-use testing.

During the mirror habituation phase, the entire experiment tank was used. A mirror (27.9 x 40.6 cm [LH]) strapped to a black plastic board (61 x 83.8 cm) was inserted in the midline between both connected tanks as shown in Figure 1 B. The mirror was placed either in the leftmost position, the rightmost position, or directly in the middle of the plane at the intersection of both halves of the experiment tank. The location of the mirror varied across trials based on a predetermined pseudorandom order. The side of the tank facing the mirror contained a plastic sand dollar (*Clypeasteroida*) 10 cm away from the mirror, a live-rock with a GoPro camera mounted on top of it 40 cm away from the mirror, and a GoPro camera placed in the sand 10 cm from the mirror. Octopus 1's affinity for in-tank cameras resulted in the animal interacting almost exclusively with the cameras. To avoid this behavior, GoPros and the live rock that one was mounted on were removed after mirror habituation session #3 for this octopus. For all octopuses, the side of the tank facing the back of the mirror contained a piece of live-rock in either corner 50 cm from the back of the mirror. Approximately 20 seashells were scattered around the tank floor.

To facilitate octopuses' understanding of the mirror as an object rather than an extension of the tank,[10] octopuses were allowed to interact freely with the mirror during habituation sessions. Each session concluded after 60 minutes with a live fiddler crab reward dropped near the mirror. Habituation continued until octopuses successfully hunted the reward crab in at least 3 of 5 consecutive sessions, after which one additional habituation session was conducted (O1: 7 total sessions; O2: 16 sessions; O3: 10 sessions). Octopuses remained in the tank for an additional 15–20 minutes after catching the crab.

Mirror-use learning

Five octopuses completed this phase. The mirror from habituation was positioned in the left or right corner (Figure 1C), the occluder was placed on the opposite side. A live crab in an open glass jar was placed in the other corner behind a trapezoidal occluder made from corrugated black plastic, blocking direct sight. The mirror was angled so that the crab was visible as a reflection in the mirror from the octopus' starting position, opposite the crab and diagonal to the mirror. After camera verification of mirror angle, octopuses were placed at the starting position and allowed to explore until locating the crab. After successful retrieval, octopuses remained in the tank for 15 minutes before return transport to their home tank. On training days, octopuses participated in one trial per day (see Table S1). A correct trial required the octopus to go to the crab in the jar without touching the mirror. On an incorrect trial, the octopus touched the mirror before touching the crab in the glass jar. Training concluded once octopuses had participated in at least ten trials, of which a minimum of three trials needed to be correct. O1 performed ten trials, O2 performed 12 trials, and O3 met the criteria for advancement after ten trials but performed three additional trials for a total of 13 trials due to miscounting of successful trials. An additional pilot paradigm – Mirror-use training 2- with O2 and O3 yielded inconclusive results and was not used with O1 (see Figure S1B).

Main experiment: mirror-use testing

To ensure that octopuses located the crab solely visually rather than by using confounding non-visual cues, such as water movement, vibrations, electro- or chemoreception, we used a virtual moving crab for the main experiment. Octopuses are known to respond to virtual representations of physical objects,[35] including displaying approach or attack behavior towards virtual crabs.[24,25]

A mirror (59 x 61 cm [WH]) was inserted at the tank's midline dividing the tank in half and restricting the experiment space to 91.4 x 61 x 61 cm (340 l, Figure 1 D). The back wall of the tank (61 x 61 cm), opposite the mirror, was covered with a translucent projection screen (Rose Brand, 55 in IFR Rear Grey) that served as the projection plane for the virtual crab. A projector (Epson Home Cinema 2045) positioned 127 cm from the screen displayed a virtual crab – a white silhouette comparable in size and shape to a fiddler crab (28 mm width) – moving horizontally at sand level at 4 cm/s on a dark background. Crab movement was restricted to the left or right side of the screen. Opaque matte black corrugated plastic sheets along the tank's long sides minimized external distractions and reflections. GoPro footage taken from inside the tank established that there were no spurious reflections of the crab on the tank walls or on the water surface, as viewed from the starting location of the octopus (see Figures S2 and S3 for details). An opaque black plastic start chamber (21.6 x 25.4 x 11.4 cm) was placed in the sand, centered along the back wall. The start chamber was open on top and on the front (mirror-facing) side; the back and side walls blocked the octopus' view of the projection screen, ensuring octopuses saw only the mirror side of the tank when inside the chamber. Two opaque plastic enclosures, each containing a live crab, were positioned 10 cm above the water line on the left and right sides of the tank at the projection screen end. Both enclosures were remotely operated via fishing line from the adjacent room.

The octopus was transported to the experiment tank and given a 20-minute habituation period during which it could move freely around the tank. The animal was then gently moved into the start chamber with a net. The net was used to keep the octopus inside the

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start chamber until it stopped moving. Once the octopus remained still with the net removed, the experimenter left the room and closed the door. Approximately 30 seconds later, the virtual crab was projected on a pseudo-randomly predetermined side. If the octopus exited the start chamber before projection onset, the experimenter re-entered the room and guided it back into the chamber. After ten unsuccessful attempts to retain the octopus in the chamber, the trial was aborted.

Octopuses were rewarded with a real crab for moving to the correct side of the projector end of the tank. The crab was released directly above the octopus from one of the two boxes 10cm above the water line via a pulley system from the adjacent control room. A trial ended when: (1) the octopus made a side choice (see Trial Categorization for scoring criteria), (2) the octopus touched the mirror, or (3) the octopus did not exit the start chamber within one hour of stimulus onset. Following outcomes 1 or 2, octopuses remained in the tank for 30 additional minutes, or until five minutes after dropping and moving away from the crab carcass without returning to pick it back up, whichever took longer. Dates and outcomes of training and testing trials can be found in Table S1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Trial categorization

Trials were categorized as conclusive, inconclusive, or excluded. Six trials (10.9%) were excluded because the octopus was inactive or asleep, four trials (7.3%) due to equipment failure, and four because the octopus repeatedly exited the start chamber before stimulus onset (>10 exit attempts). Inactive/sleeping trials were excluded rather than scored as failures because sleep behavior does not reflect waking cognitive abilities.

Forty-one of 55 trials (74.5%) were included in the analyses. Of these, 30 trials (73.1%) were conclusive, comprising 22 correct and 8 incorrect trials. In conclusive trials, the octopus moved its entire mantle beyond an imaginary line parallel to the mirror at the start chamber opening without touching the mirror. This occurred by either exiting frontally, turning toward the back of the tank, and crossing toward the projection screen, or by climbing and moving its entire mantle over the start chamber side walls. Trials were scored as correct if the octopus chose the side matching the virtual crab's location, or incorrect otherwise. Rewards were dispensed immediately when the octopus' mantle fully crossed the imaginary line on the correct side (see Figure S1A for visual illustration) or moved its entire mantle over the start chamber walls, with a slight delay between the crab's release and it falling through the water to the floor of the aquarium. No reward was given for incorrect choices, or aborted trials.

Eleven of 41 trials (26.8%) were categorized as inconclusive when the octopus either (a) remained in the start chamber throughout the trial or (b) touched the mirror after the virtual crab appeared. Mirror touches were inconclusive because octopuses frequently explored the mirror regardless of stimulus presence. For analytical transparency, mirror touch trials were additionally analyzed as if conclusive, scored based on the octopus' final position. In two mirror-touch trials, one from O2 and O3, respectively, the octopuses touched the mirror but did not cross the line toward the back of the tank. These trials were included as failures.

Trials were independently categorized by MA and JOF, and verified by MK and KRF, with 100% inter-rater agreement. Coders were non-blind to experimental conditions.

Trials in which the octopus touched the mirror prior to moving to a side at the back wall pose a challenge to the analysis. To assess whether mirror-touch differed from conclusive trials, we used a generalized linear mixed effects model with binomial distribution. We found that the time between stimulus onset and the octopus leaving the start chamber is highly predictive of mirror-touch, with longer delays increasing the probability of touching the mirror. Including the delay between stimulus onset and octopus' first movement from the chamber as a predictor significantly improved model fit compared to an intercept-only model (likelihood ratio test: $\chi^2(1) = 23.15$, $p < .001$). This suggests that mirror-touch trials differ systematically from non-touch trials thereby justifying exclusion from subsequent analyses. For transparency, we report task performance for both the base dataset ($N = 30$) excluding mirror-touch trials, and an expanded dataset ($N = 41$) including mirror-touch trials.

Statistical assessment of testing trials

All statistical analyses were carried out in R[36] using various packages.[37–45] Figures were created with R and Python[46] using various Python packages.[47–49]

A generalized linear model from the binomial family revealed that mirror-touch probability was predicted by delay between stimulus onset and exit from the start chamber. Statistical analyses were conducted on two datasets: a base dataset with 30 conclusive trials only, and an expanded dataset with a total of 41 trials.

Data from both the base and expanded data sets were analyzed using Fisher's combined probability test, Stouffer's Z-score method, and binomial simulation. For the simulation in the base dataset, 1,000,000 simulation experiments were generated in which three animals completed 13, 9, and 8 trials respectively, with each trial having $p = 0.5$ probability of correct choice. For each simulated experiment, the number of correct trials per animal were recorded. The proportion of simulations in which all three simulated animals achieved performance equal to or exceeding actual performance (correct/incorrect: O1: 10/3, O2: 6/3, O3: 6/2; see Figure 2 in main text) was calculated to assess the probability of observed results occurring by chance. For the expanded dataset, the simulation experiment was set up in the same way (correct/incorrect: O1: 15/4, O2: 8/5, O3: 6/3).

Side bias assessment

To assess whether side preferences alone could account for above-chance performance, data were analyzed with permutation resampling without replacement. For each octopus, actual right/left choice distributions and stimulus locations were preserved, but

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which trials received which choices were randomly reassigned. Across 100,000 simulations per dataset (base: $N = 30$; expanded: $N = 39$ because octopuses did not make a side choice in two trials), the distribution of simulated performance was compared to actual mean performance using one-tailed z-tests. Individual binomial tests of each octopus' side choice compared with side choice on chance-level (0.5) were used to assess octopuses side preferences.

Tracking path length and travel duration

Quantitative video analyses were performed in Matlab2020a.[50] The octopus' position was manually tracked frame-by-frame using the point between its two eyes. Path endpoints were defined as the back corner of the tank on the chosen side. For climb-over trials, the start chamber height was added to path length to account for vertical distance traveled, allowing comparison with trials where octopuses moved around the floor of the chamber. Path lengths were converted from pixels to centimeters using known tank dimensions. Travel durations were measured in seconds from when the octopus began moving after stimulus onset until it reached the back of the tank. Path lengths and travel durations showed log-normal distributions and were therefore log-transformed prior to statistical analysis.

Linear mixed-effects models with log-transformed data were used to assess changes in path length and duration across trials, with trial number as a fixed effect and random intercepts for each octopus to account for individual baseline differences. Analyses were conducted separately for correct trials only and for all conclusive trials. For each dataset, models were run with and without trial rank as an additional predictor. See Table S2 for detailed results.

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Supplemental Information

Octopus bimaculoides can learn to utilize a mirror to localize a reward outside the line of sight

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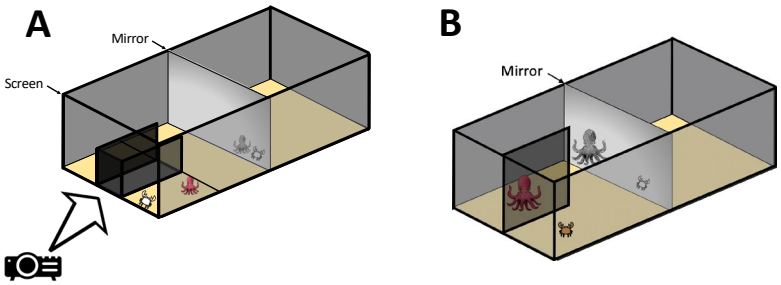

Figure S1. Tank setup clarification. Related to Figure 1 and STAR Methods. A) Tank during mirror-use testing showing the imaginary line the octopus' mantle had to fully cross to count a trial as conclusive. The octopus is shown in red; the mirror image of the octopus is gray. The projected virtual crab is shown in white, and its mirror reflection in light gray. Crab size exaggerated for visibility. B) Tank setup for mirror-use training 2. Octopuses 2 and 3 underwent this additional step of training where a live crab was placed on the other side of a divider and the octopus had to go around the divider to reach the crab. The octopus is shown in red, while the mirror image of the octopus is gray. The real live crab is shown in brown, while the mirror image of the crab is gray. Sand and water level not depicted. Octopus, crab, and reflections are enlarged to improve visibility.

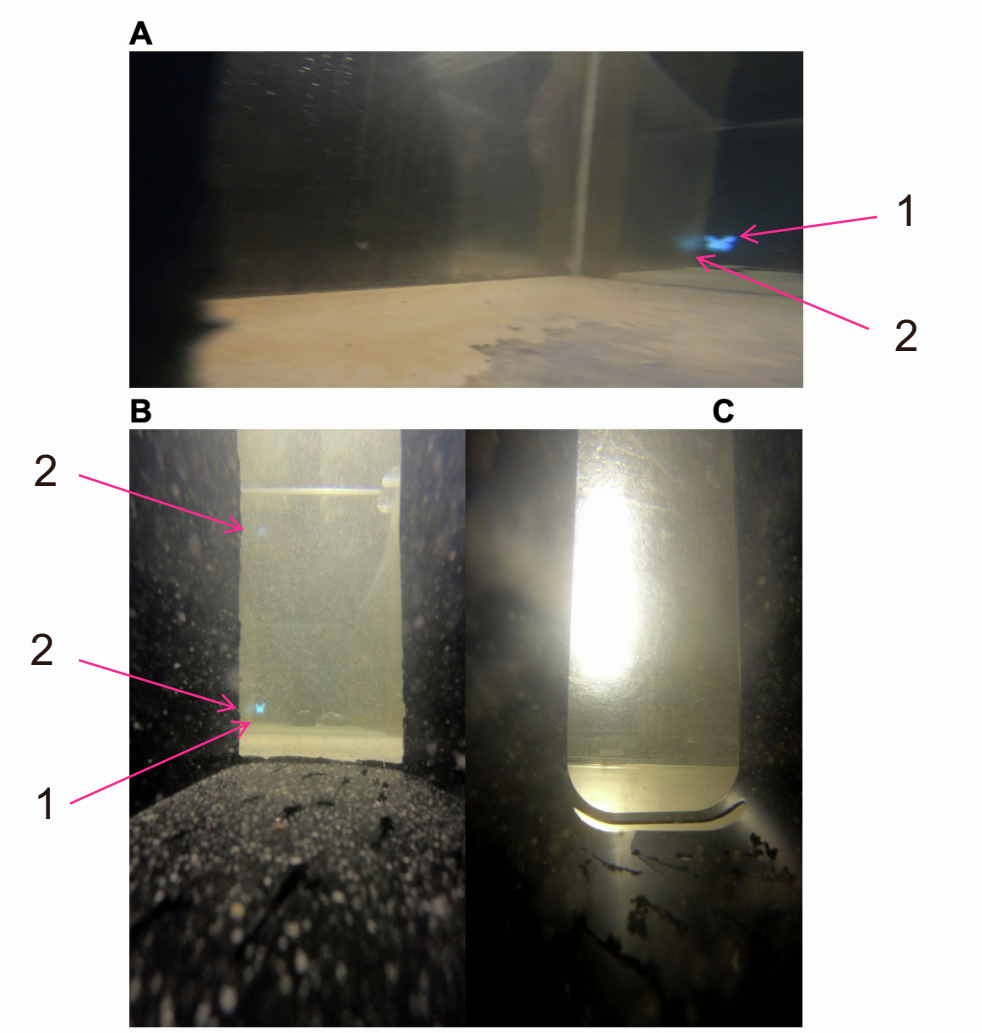

Figure S2. Assessment of reflections visible from the octopus's starting box using a stimulus with higher luminosity to increase visibility in photos. **Related to STAR Methods.** The octopus' visual field contained a complex array of reflections, which in a plane mirror are technically defined as virtual images (images that appear to be located behind the mirror's surface). The most prominent of these were the primary virtual images formed by a single reflection, indicated by number 1. These included the target crab stimulus, the octopus's own reflection, and the surrounding tank environment. A much fainter secondary reflection of the stimulus (indicated by number 2), formed by a two-reflection path (Stimulus → Side Wall → Mirror → Eye), was also visible. To document these reflections, photographs were taken with a GoPro camera from the octopus's perspective. The crab stimulus used for these photographs had a higher luminosity than in the behavioral trials to enhance visibility. A) View from the front of the starting box, angled left. This view shows that while no reflection was visible directly on the side wall itself, a faint secondary reflection of the stimulus appeared in the mirror. This secondary reflection is superimposed on the reflection of the side wall and is located adjacent to the bright, primary reflection of the virtual crab stimulus. The black object spanning the entire height of the left side of the image is the left wall of the starting box. The faint virtual reflection of the crab always appeared on the same side as the primary reflection, as viewed by the octopus. B) View from the back of the starting box. From this perspective, the bright, primary reflection is observed in the mirror. Additionally, two faint, secondary virtual images of the stimulus are visible: one appears in the far mirror adjacent to the primary virtual image, and the other appears as a reflection on the water's surface near the mirror. C) View looking directly upward from within the starting box. No reflection of the stimulus was visible on the water's surface from this vantage point.

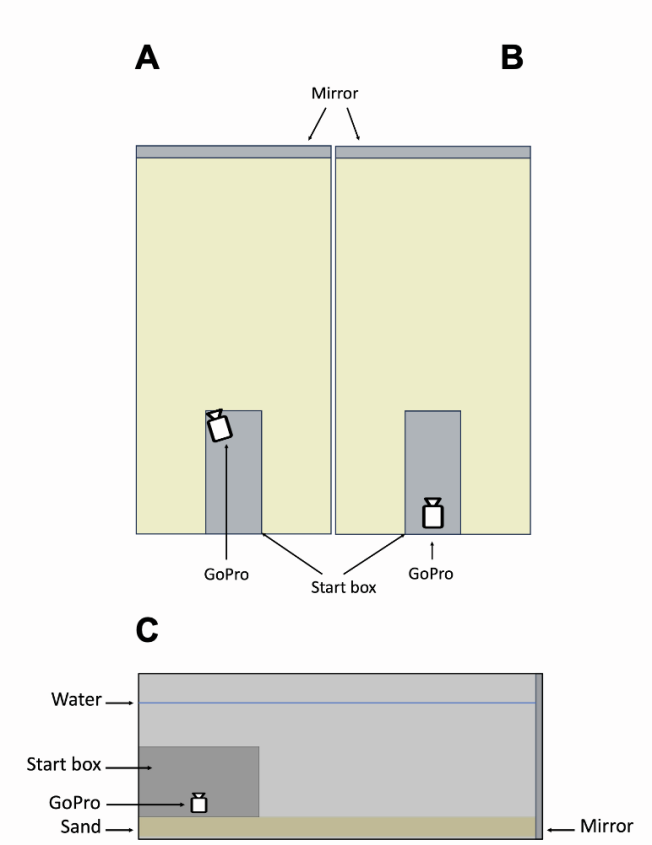

Figure S3. Schematics for tank setup to assess potential stimulus reflections off tank walls or off the water surface. Related to STAR Methods. A) Schematic shows tank setup for Figure S2, panel A from above with the GoPro camera placed at the front of the start box and angled to the left to assess stimulus reflections off the side walls when the octopus was about to leave the start box. B) Schematic shows tank setup for Figure S2, panel B from above with the GoPro camera placed at the back of the start box. C) Schematic shows tank setup for Figure S2, panel C from a side view with the GoPro camera placed in the start box and pointed toward the water surface.

Octopus name	Sex	Cause of departure	Wild	Mirror Experiment Stages	Other experiments
Ada Lovelace	male	euthanized	no	Mirror Hab + Mirror 1st tool stage	no
Archimedes	female	laid eggs and passed	yes	Pilot: Mirror Habituation	no
Elizabeth Blackwell (Octopus 2)	male	euthanized	yes	completed study	no
Emmanuelle Charpentier (Octopus 1)	male	euthanized	yes	completed study	no
Lawrence of Arabia	male	euthanized	yes	Pilot: Mirror Hab. + training stages	yes
Marco Polo	female	euthanized due to senescence after laying eggs	no	Pilot: Mirror Hab	yes
Marian Diamond	male	euthanized	yes	Pilot: Mirror Hab + Mirror training + Mirror test	yes
Odysseus	female	moved to aquarium after laying eggs	yes	Pilot: Mirror Hab + training stages	yes
Rosalind Franklin (Octopus 3)	male	euthanized	yes	completed study	no
Wilhelm Wundt	male	euthanized	no	Mirror Hab + Mirror training + 7 trials of Mirror testing stage (4 conclusive)	no

Table S3. List of octopuses that participated in the study. Related to STAR Methods. The table provides an overview of the octopuses that participated in the study, their probable sex (determined visually, not by dissection), cause for departure, if they were wild-caught or laboratory raised, which stages of the experiment the octopus participated in, and whether they participated in other experiments. Octopuses that completed the study are shaded in gray.