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Fully aquatic adaptation generally leads amniotes to change sensory modalities drastically. Terrestrial snakes rely heavily on chemical cues to locate and recognize prey, but little is known about how sea snakes find prey fishes underwater. Sea snakes of the genus *Hydrophis* are fish-eating marine elapids which adapted from land to water approximately 5–10 million years ago. Here, using two species of captive *Hydrophis* snakes, we show that they can recognize and discriminate their preferred fish species solely by using olfactory cues. However, they locate places where their preferred fishes may hide without relying on chemical cues. These findings indicate that *Hydrophis* snakes find prey in water as follows: they use visual cues to locate a place where their prey fishes are likely to hide, and then use chemical cues to find and attack prey. As is the case for other aquatic amniotes, snakes also modified their sensory modalities upon becoming aquatic.

Key words: *Hydrophis melonocephalus*, *Hydrophis ornatus*, aquatic adaptation, olfaction, vision

INTRODUCTION

Transition between sea and land is one of the most striking types of evolutionary event in the history of life, and how vertebrates profoundly modified their sensory modalities upon terrestrial/aquatic adaptation has been studied widely. This is especially true in the case of chemoreception because vertebrates' chemosensory receptors such as olfactory receptors (ORs) can functionally be divided into two groups; receptors for airborne molecules and those for water-soluble molecules (Niimura and Nei, 2006; Nei et al., 2008). In fact, the OR gene repertoire had been changed drastically in our ancestors during their transition from water to land (Niimura and Nei, 2005), and the OR genes possessed by terrestrial amniotes are prone to be lost from genomes of fully aquatic amniotes (Kishida et al., 2007; Hayden et al., 2010; Kishida et al., 2015). As an extreme case, several species of extant whales possess no nervous system components to mediate olfaction (Oelschlager and Oelschlager, 2008).

Terrestrial snakes, especially those without heat-sensitive pits such as elapids, rely heavily on chemical cues to locate and recognize prey (Halpern, 1992; Schwenk, 1995). However, like other aquatic amniotes, the OR gene repertoires in fully aquatic sea snakes (Hydrophiini, Elapidae, Serpentes) are also suggested to be degenerated compared to that in terrestrial elapids (Kishida and Hikida, 2010). This raises a question: how do sea snakes find prey underwater?

Shine et al. (2004b) reported that, similar to terrestrial snakes, turtle-headed sea snakes (*Emydocephalus annulatus*) locate prey underwater by scent rather than visual cues. However, as the authors pointed, turtle-headed sea snakes are highly specialized for eating fish eggs and their foraging mode is an extreme exception among sea snakes (Shine et al., 2004b), and no other detailed studies have been reported to date mainly due to the difficulty of observing sea snakes underwater.

Sea snakes of the genus *Hydrophis* are fully aquatic hydrophiins with more than 30 species distributed widely around the coasts of tropical/subtropical west Pacific (Wallach et al., 2014). There are two species of this genus, *H. ornatus* and *H. melanocephalus* (Supplementary Figure S1), distributed around the Ryukyu Archipelago sympatrically, but stomach contents suggest that these species prefer different preys. *Hydrophis ornatus* is a generalist eating broad taxa of fishes (Rasmussen, 1989), while *H. melanocephalus* is specialized for small anguilliforms which generally live on the bottom in burrows or in cracks and crevices (Glodek and Voris, 1982; Voris and Voris, 1983). In this study, we presented several chemical and visual stimuli to captive individuals of these two species in order to test how they respond to these stimuli, and to answer the question raised above.

MATERIALS AND METHODS

Study site and subjects

This study was conducted at the Suma Aqualife Park, Japan, with four *H. ornatus* and five *H. melanocephalus* kept in this aquarium. All nine snakes, which originated around the Ryukyu Archipelago, are kept together in a tank (Supplementary Figure S2). These snakes are fed once a week on average, and all experiments described below were conducted during the daytime at least two days after last feeding. Each snake individual was identified based mainly on tail shape. All procedures performed in this study were approved by the Animal Experiment Committee of Kyoto University.

Chemical preference test

Mucus of five fish species (Table 1) were extracted by wiping surface of freshly-dead fish using sterilized cotton gauze. These fish were provided by commercial suppliers that provide feed for breeding animals in aquariums. Extracted mucus were dissolved into filtered seawater, and each dissolved water was dispensed into aliquots of 15 ml each and stored in −25°C. Aliquots of 15 ml filtered seawater without fish mucus stored in −25°C were also prepared for control. For each trial, a sheet of sterilized cotton gauze soaked with a thawed aliquot of mucus of a fish or control was presented to a snake individual (Supplementary Figure S3), and the duration of tongue-flicking behavior against the gauze was recorded using a GoPro HERO3+ camcorder (Supplementary Figure S3A). The duration of tongue-flicking was measured instead of the number of times tongue-flicking occurred, because it is difficult to count tongue-flicking precisely due to the small and color-less tongues of these snakes. Each trial was judged to be finished when the subject removed from the gauze for more than 3 s. At least two-hour intervals were taken between each trial in order to flow through residual odor stimuli. A *H. melanocephalus* individual was excluded from this test because it tended to escape from the tool used for this test. Except for this individual, 12 trials (five fish mucus and a control (six stimuli in total), two trials per a stimulus) were conducted for each snake (*i.e.*, 12 trials × 8 individuals = 96 trials in total). The order of stimuli and examinee subjects were randomized. Statistical analyses were conducted based on the results of these trials, and *P*-value for each stimulus compared with the control was calculated using the single-tailed unpaired t-test.

Visual preference test

Three ringed blue wires (*r* = 11 cm) were put on the bottom of the tank, and each encircled region visually imitated a complex-structured rocky bottom where many fishes but no prey of *H. melanocephalus* are expected to stay (rock model: bricks, PVC pipes and male screws were put on the bottom), or a sandy bottom with several holes where small anguilliforms are expected to hide (hole model: PVC pipes and female screws were implanted in the bottom sand). A control region (control model: no additional objects were installed) was also prepared (Supplementary Figure S4). These models were set up at 9:30 of the experiment days, and the number of pecking at these models by each snake individual was counted during 10:30–16:30 (six hours). The behavior of a pecking is defined as striking or indenting an object or bottom sand with the beak. Pecking at the edge of each model (i.e., a blue wire) was excluded from counting. This experiment was conducted three days (18 hours) in total, and the position of each model was rotated day by day. Statistical tests were conducted based on the total number of pecking instances by all individuals, and *P*-values between models were calculated using the two-tailed exact binomial test. Preference of each model was also analyzed for each individual, and *P*-values for each model compared with the control were also calculated for all individuals using the single-tailed exact binomial test.

RESULTS

Chemical preference test

Figure 1 shows average duration of tongue-flicking against each odor stimu-lus. As this figure indicates, *H. ornatus* performed tongue-flicking against all fish stimuli longer than that against the control (although moray eel stimulus was statistically not significant compared with control), but *H. melanocephalus* did so only against garden eels and congers.

Visual preference test

Figure 2 shows average numbers of pecking at each model per hour by two snake species. Both rock and hole models are significantly preferred compared with the control model for both species. However, *H. ornatus* prefers both models equally, whereas *H. melanocephalus* significantly prefers the hole model compared with the rock model. This tendency is also confirmed from the individual-based analyses. Most *H. ornatus* individuals pecked frequently at both rock and hole models, but more than half of *H. melanocephalus* individuals pecked frequently only at the hole model.

Table 1. Fish species used as chemical stimuli.

Perciformes	*Ammodytes personatus* (Pacific sand lance)[1]
	Parajulis poecilepterus (wrasse)
Anguilliformes	*Gymnothorax minor* (lesser moray eel)
	Heteroconger hassi (spotted garden eel)[2]
	Conger myriaster (whitespotted conger)

[1] Feeding to *H. ornatus* evely week
[2] Feeding to both species evely week
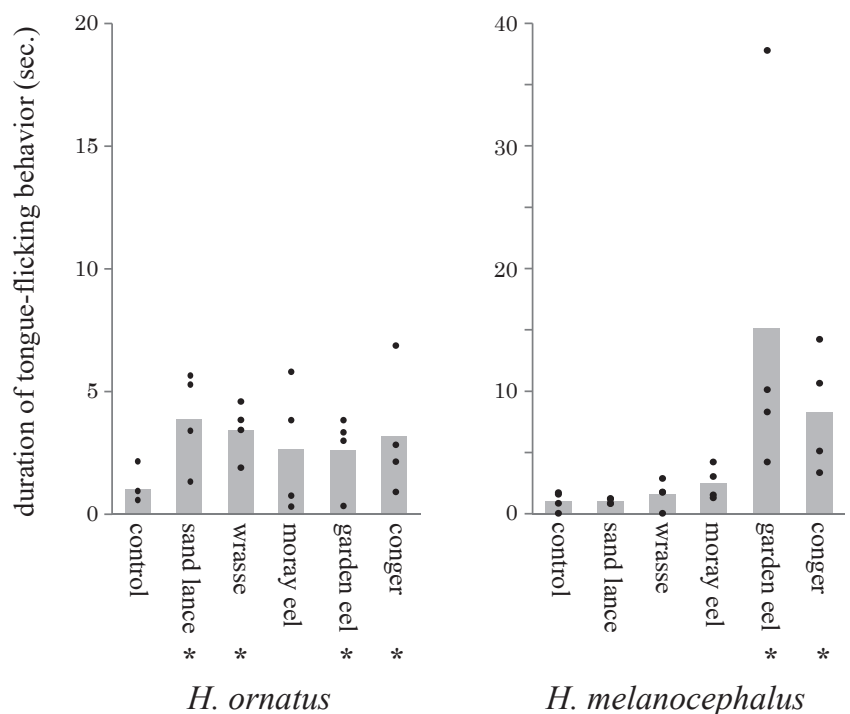
Fig. 1. Mean duration of tongue-flicking behavior against each odor stimulus. Each plot on each odor stimulus shows duration of each individual tested in this study (average of two trials). *P*-value for each stimulus compared with the control was calculated using the single-tailed unpaired t-test, and significantly (*P* < 0.05) preferred stimuli are shown with asterisks. Actual *P*-values for all stimuli are shown in Supplementary Table S1.
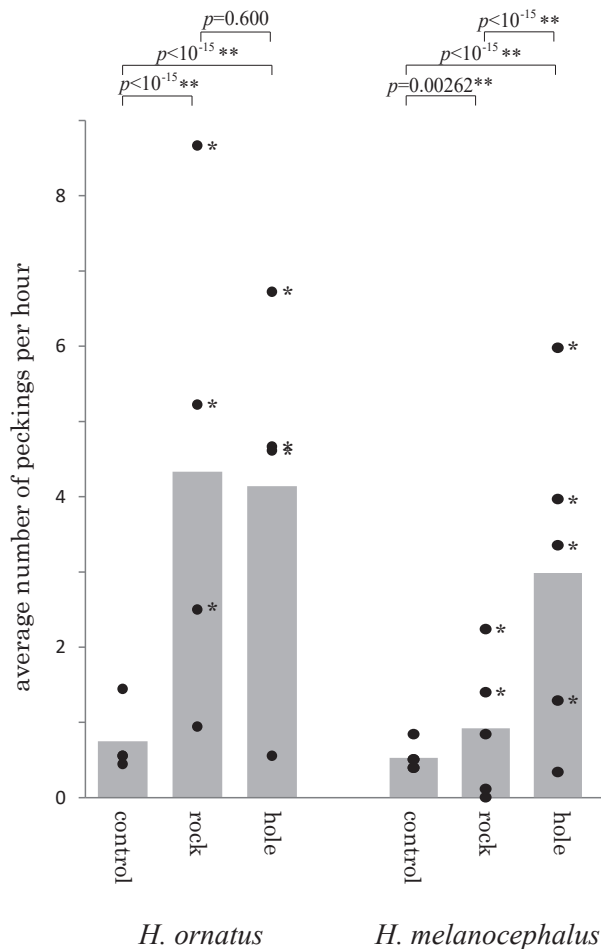
Fig. 2. Average number of pecking at each model per hour by two snake species. Each plot on each visual model shows number of pecking by each individual. *P*-values between models were calculated using the two-tailed exact binomial test, and *P*-values < 0.01 are shown with double-asterisks. Preference of each model was also analyzed for each individual. *P*-values for each model compared with the control were also calculated for all individuals using the single-tailed exact binomial test, and significantly ($P < 0.05$) pecked models are shown with asterisks. Actual *p*-values for all individuals are shown in Supplementary Table S2.

DISCUSSION

Results of the chemical test suggest that *H. ornatus* prefers mucus of all fishes, while *H. melanocephalus* prefers only garden eels and congers. It is consistent with their prey repertoires: *H. ornatus* eats broad taxa of fishes, while *H. melanocephalus* eats only small anguilliformes. *Hydrophis melanocephalus* did not perform tongue-flicking against stimulus of lesser moray eel (Anguilliformes) significantly, but this eel is too large for sea snakes to eat, and predation of this eel by *H. melanocephalus* has not been reported to date. This test provides evidence that *Hydrophis* snakes can recognize and discriminate prey fish solely by using chemical cues through tongue-flicking. Snakes deliver odor molecules to their vomeronasal organs using tongues, suggesting that tongue-flicking is involving in olfaction rather than in gustation (Halpern, 1992). Indeed, taste buds are absent on the tongue of these species (Young, 1997; Schwenk, 2008) but snakes, including fully aquatic hydrophiins, possess well-developed vomeronasal organs (Schwenk, 2008; Shichida et al., 2013).

It has widely been considered that terrestrial olfaction does not function in water, and therefore fully aquatic adaptation generally causes profound degeneration of the sense of smell. For example, all modern whales have lost the vomeronasal organs during their transition from land to water (Pihlström, 2008; Kishida et al., 2015). Some modern whales still possess highly degenerated main olfactory systems, but they can smell only in air, not underwater (Thewissen et al., 2011). Loss of the vomeronasal organ is also documented in sirenians (Pihlström, 2008). To our knowledge, no fully aquatic amniotes can smell underwater and sea snakes are the only exception from this rule.

However, through this test, we realized that sea snakes recognize presence of fish stimuli only within short distance (approx. < 10 cm) as subject individuals do not respond to the mucosa-soaked cotton gauze until the gauze is moved close to its head. Shine (2005) pointed out that mate-searching male turtle-headed sea snakes use visual cues to search for snake-shaped objects, although snakes do not have good eyesight, because they cannot locate females using chemical cues over long distance. In addition, snakes studied here often peck around rocks and/or sandy bottom as if they are searching for foods. These report and observation led us to hypothesize that fish-eating hydrophiins use visual cues to locate places where their prey may hide. Results of the visual test clearly support this hypothesis. *Hydrophis* snakes locate places where their prey may be present without chemical cues. Several amphibious snakes are also reported to use visual cues for underwater foraging (Drummond, 1985; Shine et al., 2004a; Vincent et al., 2005). It is speculated that olfaction is insufficient for them to find prey underwater because there are limitations to modify their olfactory capability for smelling underwater due to their reliance on terrestrial habitat. On the other hand, fully aquatic hydrophiins do not have to maintain terrestrial olfaction and thus there are no ecological limitations for them to adapt their olfactory capability to the aquatic environment. Nevertheless, *Hydrophis* snakes also have to rely on vision for underwater foraging.

Based on these findings, we speculate that fish-eating *Hydrophis* snakes find prey underwater using the following strategy: first, they use visual cues to locate a place where their preferred fishes are likely to hide, and then they use chemical cues to locate prey exactly and attack. Actually, during a trial of the chemical test, we observed that a *H. melanocephalus* individual attacked cotton gauze soaked with mucus of garden eels (Supplementary Video), suggesting that chemical stimuli are sufficient for *Hydrophis* to trigger an attack behavior. Although hydrophiins still possess well-developed vomeronasal olfaction which function underwater, snakes also changed their sensory modalities for foraging on becoming aquatic.

COMPETING INTERESTS

We have no competing interests.

AUTHOR CONTRIBUTIONS

All authors designed this study. RK and TS conducted experiments. RK and TK analyzed data.

TK drafted manuscript. All authors gave final approval for publication.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: http://www.bioone.org/doi/suppl/10.2108/zs180059).

Supplementary Figure S1. Two species of *Hydrophis* sea snakes used in this study, *H. ornatus* (upper) and *H. melanocephalus* (lower). It is noted that these snakes are also described recently as *Chitulia ornata* and *Leioselasma melanocephala*, respectively (Wallach et al., 2014).

Supplementary Figure S2. A picture of the sea snake tank in the Suma Aqualife Park, Kobe, Japan. Nine *Hydrophis* snakes used in this study are kept together in this tank. This tank is approximately 2.5 m × 1.8 m × 1.2 m (width × depth × height) and filled with 5400L of filtered seawater. The filtration pump used in this tank treats approx. 6900L of water per hour.

Supplementary Figure S3. (A) A tool used for the chemical preference test. **(B)** A picture taken during a trial of the chemical preference test.

Supplementary Figure S4. A picture taken during the visual preference test. Upper circle, control; middle, rock model; lower, hole model.

Supplementary Table S1. *P*-values calculated in the chemical preference test. Note that all trials were treated independently (i.e., $n = 8$). It is assumed that multiple observations from each individual will not be independent of one another. Therefore, we performed an 'unpaired' t-test to account for this by using a model where snakes were chosen randomly with replacement. *P*-values calculated using a 'paired' t-test ($n = 8$, Table S3) and a non-parametric test based on mean value of observations from each individual ($n = 4$, Tables S4) also show essentially same results.

Supplementary Table S2. *P*-values calculated in the visual preference test. For *p*-value calculation, alternative hypothesis is given as "true probability of pecking at the control model is less than 0.5".

Supplementary Table S3. *P*-values calculated using paired single-tailed t-test ($n = 8$).

Supplementary Table S4. *P*-values calculated using single-tailed asymptotic Wilcoxon-Pratt signed rank test ($n = 4$).

Supplementary Reference
Supplementary Movie

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