

OLFACTION AND PREY SEARCH IN THE CARNIVOROUS LEECH *HAEMOPIS MARMORATA*

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Summary

Haemopsis marmorata, the green horse leech, is carnivorous and readily eats earthworms. Using a Y-maze with flowing water, we show that specimens of *H. marmorata* are attracted to live earthworms. Ablating the dorsal lip, the presumed site of the chemoreceptors that this species uses in prey search, disrupts the ability of the leeches to find the earthworms in the Y-maze. Earthworm wash, a preparation of the collagen coating of earthworm skin, shock-induced earthworm secretion, mammalian

blood and a salt–arginine mixture are not attractive to the green horse leech. The tails of freshly killed earthworms are attractive to the leeches, but tails from worms killed 8–12 h previously and stored cold are not. Our conclusion is that the earthworms produce a metabolite that attracts the leeches.

Key words: olfaction, prey search, Y-maze, evolution, feeding behavior, leech, *Haemopsis marmorata*.

Introduction

The present understanding of the effects of evolution on nervous systems remains rudimentary. Behavior is the output of nervous systems, and it is behavior upon which selective forces act. Both the function and structure of particular neural elements might be subject to change by adaptation (Arbas *et al.* 1991). A comparison between two closely related species that have evolved different behavior for the same adaptive task might prove instructive as a model for the study of neural evolution (e.g. Dumont and Robertson, 1986). Leeches possess a variety of species-specific feeding behavior patterns and may provide such a model.

The ancestral arhynchobdellid leech is believed to have been a free-living predator of invertebrates, possibly preferring earthworms. Sanguivorous parasitism of vertebrates has been an evolutionary trend in leeches, culminating in such parasitism of mammals by the medicinal leech *Hirudo medicinalis* and the North American sanguivore *Macrobdella decora*. Macrophagous predation was retained or secondarily re-acquired by other hirudiniform leeches such as the green horse leech *Haemopsis marmorata*, a macrophagous distichodont that prefers earthworms as prey (Sawyer, 1986).

The green horse leech feeds at night by generalized predation and opportunistic scavenging (Karrer and Sahley, 1988a; Sawyer, 1986). Oligochaetes, gastropods and chironomid larvae make up 80% of this leech's diet (Riggs, 1980). This leech forages slightly above the shoreline, using a series of exploratory head movements interrupted by vermiform crawling (Sawyer, 1986). Upon coming into contact with an earthworm, the leech seizes it with the anterior sucker

and mouth and proceeds to swallow it whole. The leech frequently attaches its rear sucker to the worm as a prehensile appendage. The worm is swallowed whole in 5–30 min (T. W. Simon, unpublished observations).

On the basis of the reported exploratory head movements of the green horse leech (Sawyer, 1986) and our initial observations, we suspected that these head movements might be olfactory explorations, each analogous to a 'sniff' in mammalian species (Wellis *et al.* 1989) or the antennular flick of decapod crustaceans (Daniel and Derby, 1991). Therefore, we hypothesized that the green horse leech would use chemoreception to find its earthworm prey.

Here, we show that the green horse leech can distinguish its earthworm prey in a Y-maze test with flowing water. The ability to locate prey under these conditions appears to depend on the olfactory capability of the animal: surgically ablating the array of chemoreceptors presumed to be located on the dorsal lip of the prostomium destroys the leeches' ability to find the worm. A variety of olfactory cues related to possible prey items were presented to the green horse leech in the Y-maze, and the leeches displayed a preference only for live or freshly killed earthworms. We discuss possible implications for future evolutionary studies comparing the nervous system of the green horse leech with those of sanguivorous leeches.

Materials and methods

Animals

Haemopsis marmorata Savigny (green horse leeches or 'mud

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leeches') were obtained from bait distributors in Atlanta, Georgia, USA, or directly from collectors in Minnesota, USA, by overnight mail. Leeches were maintained in either soft mud or artificial spring water (Instant Ocean artificial sea water, 0.5 g l^{-1} , having a NaCl concentration of approximately 7 mmol l^{-1}) at 15°C and were not fed. Leeches were used only once so that any effect of learning could be ruled out. Earthworms *Lumbricus terrestris* were either collected locally or obtained at bait shops. These experiments were performed at several locations in the Atlanta, Georgia, area during the summers of 1991, 1992 and 1994.

Apparatus

A Y-maze was constructed from acrylic and dental periphery wax. The angle between the two arms of the Y was $40\text{--}45^\circ$. The stem of the Y was 15.2 cm long and each arm was 12.7 cm long. The stem and the arms of the maze were 2.5 cm wide and the walls were 3.0 cm high. A removable solid acrylic starting gate was placed 3 cm from the base of the stem to restrict the leech's movements prior to the commencement of a trial. The starting gate was almost as wide as the stem of the maze (2.3 cm) and served to isolate the leech from water movement caused by placing a prey item in the maze. Once the starting gate was removed at the commencement of a trial (see below), the leech was not isolated from water movement produced by the moving earthworm.

During each trial, artificial spring water was fed by gravity into both arms of the Y-maze at the same rate and removed by suction through a small standpipe at the foot of the stem. The rate of flow of the spring water was $8.6\pm 0.37\text{ ml min}^{-1}$ (mean \pm S.E.M., $N=5$) with half this flow going into each arm. The standpipe was adjusted so that the depth of water in the maze was approximately 1 cm .

Dye flow experiments using Methylene Blue were performed prior to the leech experiments and periodically throughout the course of the study. The dye front took $101.8\pm 5.85\text{ s}$ (mean \pm S.E.M., $N=35$) to reach the starting gate near the foot of the stem. We concluded each dye test when the dye reached the standpipe at the foot of the maze.

In most of the dye experiments, we observed unidirectional laminar flow and, as shown by the dye trail, a distinct separation of the water coming out of the left and right arms. The water from the left arm continued to flow on the left side of the stem and *vice versa* (e.g. Teyke *et al.* 1992). Hence, placing the prey in one of the arms should result in a large difference in odor stimulus intensity between the two arms.

In 24% (6/25) of the dye flow experiments, we observed an eddy and backwash at the fork of the Y-maze. The backwash caused dye (and presumably odor in a similar fraction of the other experiments) to flow into the non-prey arm of the maze. Hence, backwash might tend to decrease the fraction of leeches choosing the arm containing the prey item. However, we believe that the leeches were able to perceive the difference between the arms of the maze even when backwash occurred (see Results).

Between trials, the Y-maze was washed briefly with detergent (Alconox), then thoroughly rinsed twice with tap water and once with distilled water.

All experiments were performed in dim red light to minimize photic stimuli. We assumed that the leeches could not use visual cues under these conditions, but we did not test this explicitly. All trials were video-taped with a Canon video camera with a long infrared charge-coupled device and an infrared illuminator. The camera had a 20 mm manually focused lens and was placed on a tripod situated 1.5 m directly above the maze.

Prey items

Whole earthworms and the rear portions of earthworms were used as prey items. Control experiments were performed with an artificial worm made of clean foam rubber and soaked in distilled water for 4 h. In addition, a variety of chemical signals were prepared and used to make gelatin blocks. As a second control stimulus, distilled water was also used to make gelatin blocks ('water gelatin'). The gelatin plus chemical signal (or water gelatin control) was poured into a plastic Petri dish and allowed to solidify overnight. Small blocks of gelatin ($1\text{ cm}\times 1\text{ cm}\times 0.5\text{ cm}$) were cut and used as prey items for the leeches. The volume of a gelatin block was approximately 25–30% that of a typical earthworm and had a mass of $0.5\text{--}1\text{ g}$.

Earthworm wash (EWW; Wilde, 1938; Burghardt and Pruitt, 1975; Kirschenbaum *et al.* 1986) was prepared, and the solvent water was evaporated using a rotary evaporator to a final concentration of $0.2\text{ mg protein ml}^{-1}$. The EWW solution was then used to make gelatin blocks ('EWW gelatin').

The electric-shock-induced secretion of earthworms (SIEWS; Ressler *et al.* 1968; Rosenkoetter and Boice, 1975; Halpern *et al.* 1987; Jiang *et al.* 1989, 1990) was collected in a 100 ml plastic beaker. Silver wire electrodes were placed in the bottom of the beaker. A small hole in the center of the bottom permitted the collected secretion to drain. Intermittent d.c. current (pulse duration 0.25 s , frequency 1 Hz) at 30 V , $100\text{ }\mu\text{A}$ was used as a stimulus. We diluted the SIEWS with distilled water to a protein concentration of 1 mg ml^{-1} and used it to make gelatin blocks ('SIEWS gelatin').

Hemolyzed sheep blood diluted to a protein concentration of 1 mg ml^{-1} was used to make gelatin blocks ('blood gelatin'), as was a solution of 150 mmol l^{-1} NaCl and 1 mmol l^{-1} arginine, the components in blood shown to evoke feeding behavior in the medicinal leech (Galun and Kindler, 1966; Elliot, 1986) ('NaCl-Arg gelatin').

A commercial Biuret test kit (Sigma Chemical Co.) was used to measure the amount of protein in the various preparations.

In order to confirm that chemicals were diffusing from the gelatin blocks, we measured the amount of protein in 1 ml of distilled water following a 20 min incubation with a gelatin block at 20°C . Blood gelatin released $13.4\pm 3.0\text{ ng protein g}^{-1}\text{ gelatin min}^{-1}$ (mean \pm S.E.M., $N=5$). SIEWS gelatin released $17.6\pm 3.4\text{ ng protein g}^{-1}\text{ gelatin min}^{-1}$ ($N=5$). Both of these released significantly more protein than water gelatin, which

released $7.0 \pm 1.2 \text{ ng protein g}^{-1} \text{ gelatin min}^{-1}$ ($N=5$) (SIEWS *versus* water, $t_{(5,5)}=3.31$, $P<0.025$; blood *versus* water, $t_{(5,5)}=2.42$, $P<0.05$). The amounts of protein released from the SIEWS and blood gelatin were not significantly different (SIEWS *versus* blood, $t_{(5,5)}=1.13$, $P>0.1$). Hence, the SIEWS gelatin and the blood gelatin, respectively, released $11 \text{ ng g}^{-1} \text{ min}^{-1}$ and $6 \text{ ng g}^{-1} \text{ min}^{-1}$ more protein than the water gelatin.

We did not explicitly test the amount of protein produced by a worm or portion of a worm under the same conditions.

Performance of trials and behavioral scoring

We defined the choice of an arm of the maze as the leech moving into that arm and spending at least 5 s within 2 cm of the prey item (earthworms, foam rubber worms, or gelatin blocks containing chemical attractants) or the empty mound of wax. We recorded whether the leech chose the arm of the maze containing the prey, the time spent in various parts of the maze, the number of head turns and whether the leech put its mouth onto the prey item in an attempt to eat it. In some trials, the leeches did not move and, hence, did not make a choice. If an animal did not make a choice of arms within 5 min, the trial was excluded from the data set used for subsequent analysis. Nonetheless, we noted the numbers of animals that did not make a choice of arms within 5 min in response to the various chemical stimuli (see Table 1).

We attempted to remove non-olfactory sensory cues in these experiments. When the prey item was an earthworm, it was pinned through the tail onto a small dish prior to the experiment to allow the worm to become quiescent. For some earthworms, several other pins were placed around, but not through, the worm to hold it in position and to minimize movement and any consequent mechanical stimuli that might have served as a cue for the leeches. When we pinned the worm, we were careful to minimize water disturbance. At this point in an experiment, the solid acrylic starting gate was between the leech and the remainder of the maze and, as indicated above, it mitigated any inadvertent water movement. If an earthworm began to struggle during the course of a trial, thus providing possible mechanosensory cues to the leech, the trial was discarded. Our aim was to ensure that the only water movement in the Y-maze was due to the slow non-turbulent flow of spring water.

Leeches were placed behind the starting gate for at least 1 min prior to each trial. The flow of spring water through the Y-maze occurred continuously. At the start of a trial, the prey item was introduced into one of the two arms of the maze and fixed to one of the two mounds of wax placed at the end of each arm. The selection of the arm of the maze into which to place the prey was random. A coin toss was used to decide which arm to use in the first few trials prior to beginning each day's experiments; however, the choice of arms was balanced for each day's trials by specifying use of the left and right arms an equal number of times. The starting time of the trial was recorded as the time the prey was fixed to the wax mound. Immediately upon fixing the prey, the starting gate was removed without disturbing the leech.

In over 95 % of trials, the leech's first movement was an exploratory head turn. With the rear sucker planted, the green horse leech extended its prostomium and turned its head from side to side (see Fig. 1A). We believe these head turns are identical to those described by Sawyer (1986). Head turns were the most frequently observed behavior.

The chemoreceptors are located on the prostomium in hirudinid leeches (Elliot, 1986, 1987; see below). Presumably, prostomial extension is associated with sampling of the odors in the water. Turning the head together with prostomial extension is a means of determining the direction of the odor source, possibly by sampling at two spatially and temporally distinct points. We believe a series of such exploratory head turns constitutes searching behavior (Sawyer, 1986). The number of head turns per 10 s period was used as a measure of the leeches' interest in the prey, and we termed this measure the 'interest score'.

The leeches generally travelled up the stem of the maze by looping using a series of front and rear sucker plants. In less than 10 % of trials, the leech swam up the stem of the maze.

Chemoreceptor ablation procedure

Leeches were anesthetized using 8 % ethyl alcohol in leech saline solution containing (in mmol l^{-1}): 115 NaCl, 4 KCl, 1.8 CaCl_2 , 10 glucose, 10 HEPES, pH 7.4. Fine iridectomy scissors were used to cut away the band of dorsal lip organs. The assumption was made that the lip organs visible with a low-power ($<40\times$) microscope were analogous to those observed by Elliot (1987) in *Hirudo medicinalis*. Sham ablations were performed as controls by removing a strip of skin adjacent and dorsal to the lip organs.

The leeches were allowed to recover for 2 days in leech saline solution and between 3 and 7 days in artificial spring water before testing. All of the ablation experiments were conducted blind, i.e. the experimenter conducting the behavioral tests did not know the surgical status of the individual leeches.

Statistics

Statistical differences between the various sets of experiments were determined using a χ^2 -test to determine whether a particular result was different from that predicted by chance (i.e. 50 % choosing the arm of the maze containing the prey item), a log-likelihood ratio (G) test for heterogeneity, a Student's t -test or a Mann-Whitney U -test for independent unpaired samples (Sokal and Rohlf, 1981). Because we chose not to use any leech twice, we performed no paired trials. Because unequal proportions of animals failed to make a choice under the various test situations (see Table 1) and different numbers of trials made up the various experimental groups, statistical tests were performed on the assumption of independent samples and unequal sample numbers.

Results

Experiments with live earthworms and controls

When a live earthworm was used as the prey, the leeches

making a choice selected the arm of the maze containing the prey item in 65 % (34/52) of trials (Table 1; Fig. 1B). This proportion is significantly different from that predicted by chance ($\chi^2=5.45$, $P<0.025$). In all successful trials, after reaching the worm, the leeches attached both front and rear suckers to the worm and started to eat it, as described above and in Sawyer (1986). 11 % (7/59) of the all leeches tested with earthworm prey failed to make a choice (Table 1).

In 26 % (9/34) of experiments in which the leeches chose the arm containing the worm, they initially investigated as far as 4 cm down the empty arm. All these animals then made several head turns, contracted their bodies back into the stem of the maze, turned into the arm containing the earthworm and eventually found and ate the worm. This percentage (26 %) is quite similar to the percentage of dye experiments in which we observed backwash (24 %). We interpret the leeches' behavior as indicating that an eddy was present in those experiments. However, we did not add dye to the spring water flowing through the maze on the side of the prey to correlate the leeches' behavior with the presence of an eddy or backwash because of concern that the dye (Methylene Blue) could possibly provide an inappropriate chemical signal or interfere with any chemical signal from the prey item.

In experiments using the artificial foam rubber worm, the leeches making a choice showed no statistically significant preference, choosing the arm containing the piece of foam rubber in only 30 % (3/10) of trials ($\chi^2=1.9$, $P>0.1$; Fig. 1B; Table 1). When water gelatin was used as the prey item, the leeches making a choice also showed no preference (50 %; 8/16) for this prey item ($\chi^2=0.0$, $P>0.9$; Fig. 1B; Table 1).

In trials with the foam rubber worm, the leeches made a choice of arms in 45 % (10/22) of attempted trials, whereas in trials with water gelatin as prey, the leeches made a choice of

Table 1. Results from choice tests for *Haemopsis marmorata* presented with various prey items

Prey item	Percentage choosing prey item	Percentage making any choice at all
Earthworm	65* (34/52)	89 (52/59)
Foam rubber worm	30 (3/10)	45 (10/22)
Water gelatin	50 (8/16)	80 (16/20)
EWG gelatin	42 (8/19)	43 (19/44)
SIEWS gelatin	48 (15/31)	100 (31/31)
Blood gelatin	42 (8/19)	95 (19/20)
NaCl-Arg gelatin	55 (10/18)	90 (18/20)

*Indicates a significant preference above random (50%) ($P<0.025$).

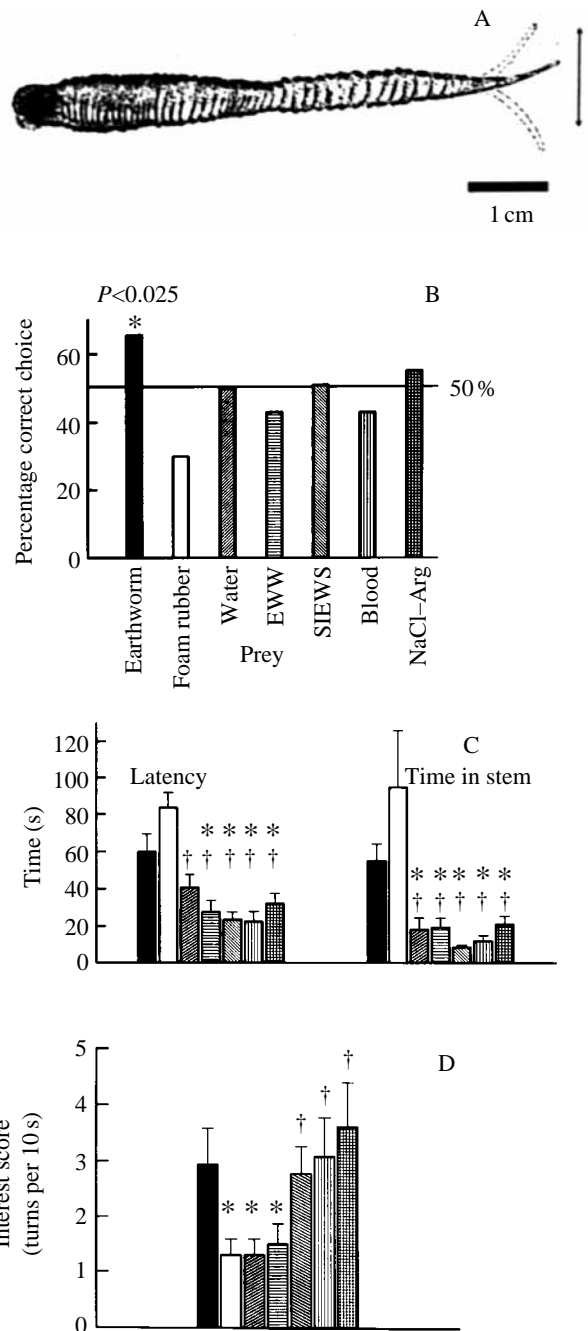


Fig. 1. Y-maze responses of *Haemopsis marmorata* to live earthworms, chemical stimuli in gelatin blocks and foam rubber and water gelatin controls. (A) Drawing of *H. marmorata* during searching behavior. Note the prostomial extension. Head turns are shown by the vertical arrow and the dotted outlines. (B) Percentage correct choice versus type of prey. The horizontal line indicates the null hypothesis of no preference. The asterisk and probability value above the bar indicate a statistically significant difference from this value. (C) Latency to first movement and time in the stem of the maze. (D) Interest score (number of head turns per 10 s period) for the prey items. The identity of the shaded bars is indicated in B. In C and D, an asterisk (*) above the bar indicates a statistically significant difference from the value for the earthworm, and a dagger (†) indicates a statistically significant difference from the value for the foam rubber worm. Values are means + S.E.M., N , see Table 1.

Table 2. Results from t-tests comparing latency to first movement of the leech *Haemopsis marmorata* presented with various prey items

Latency (s) mean \pm S.E.M.	Foam rubber worm	Water gelatin	EWW gelatin	SIEWS gelatin	Blood gelatin	NaCl-Arg gelatin
Earthworm (59.7 \pm 9.6) (N=52)	$t_{(60)}=1.87$ NS	$t_{(66)}=1.59$ NS	$t_{(69)}=2.78^*$ $P<0.01$	$t_{(81)}=3.44^*$ $P<0.005$	$t_{(69)}=3.36^*$ $P<0.002$	$t_{(68)}=2.47^*$ $P<0.02$
Foam rubber worm (83.4 \pm 8.3) (N=10)		$t_{(24)}=3.98^*$ $P<0.001$	$t_{(27)}=5.39^*$ $P<0.001$	$t_{(39)}=6.47^*$ $P<0.001$	$t_{(27)}=6.19^*$ $P<0.001$	$t_{(26)}=5.19^*$ $P<0.001$
Water gelatin (40.6 \pm 6.8) (N=16)			$t_{(33)}=1.39$ NS	$t_{(45)}=2.10^*$ $P<0.05$	$t_{(33)}=2.05^*$ $P<0.05$	$t_{(32)}=0.96$ NS
EWW gelatin (27.3 \pm 4.3) (N=19)				$t_{(48)}=0.54$ NS	$t_{(36)}=0.60$ NS	$t_{(35)}=0.53$ NS
SIEWS gelatin (23.2 \pm 5.3) (N=31)					$t_{(48)}=0.14$ NS	$t_{(47)}=1.21$ NS
Blood gelatin (22.2 \pm 5.3) (N=19)						$t_{(35)}=1.22$ NS
NaCl-Arg gelatin (31.8 \pm 5.5) (N=18)						

Comparisons showing statistically significant differences are marked with asterisks; NS, not significant.

arms in 80 % (16/20) of trials (Table 1). Some protein leached from the water gelatin into the flowing spring water (see Materials and methods), possibly serving as a chemical cue and accounting for the greater responsiveness of the leeches than when the prey was a foam rubber worm.

There were no significant differences in the latency to first movement between the earthworm and the two control prey items (Fig. 1C; Table 2). There was no significant difference in the time spent in the stem between the earthworm or the foam rubber worm. However, the time spent in the stem by the leeches when the prey was a piece of water gelatin was significantly less than with either a real earthworm or a foam

worm (Table 3; Fig. 1C). In addition, leeches showed a significantly greater interest score when the prey was a real worm than for either of the two controls (Table 4; Fig. 1D).

Ablation experiments

In *Hirudo medicinalis*, both large (35 μ m in diameter) and small (10 μ m in diameter) ciliated mounds are present on the dorsal lip (Elliot, 1987) and are suspected to be chemosensory organs (Elliot, 1986). Similar mounds were observed on the dorsal lip of *Haemopsis marmorata* during the ablation procedures in the present study. It is reasonable to suspect that these mounds are therefore chemoreceptive organs. We

Table 3. Results from t-tests comparing the time spent in the stem of a Y-maze of the leech *Haemopsis marmorata* presented with various prey items

Time in the stem (s) mean \pm S.E.M.	Foam rubber worm	Water gelatin	EWW gelatin	SIEWS gelatin	Blood gelatin	NaCl-Arg gelatin
Earthworm (54.6 \pm 9.2) (N=52)	$t_{(60)}=1.22$ NS	$t_{(66)}=3.24^*$ $P<0.005$	$t_{(69)}=3.32^*$ $P<0.002$	$t_{(81)}=4.94^*$ $P<0.0001$	$t_{(69)}=4.39^*$ $P<0.0001$	$t_{(68)}=3.25^*$ $P<0.005$
Foam rubber worm (94.5 \pm 31.2) (N=10)		$t_{(24)}=2.40^*$ $P<0.05$	$t_{(27)}=2.38^*$ $P<0.05$	$t_{(39)}=2.75^*$ $P<0.02$	$t_{(27)}=2.64^*$ $P<0.02$	$t_{(26)}=2.35^*$ $P<0.05$
Water gelatin (18.1 \pm 6.2) (N=16)			$t_{(33)}=0.11$ NS	$t_{(45)}=1.48$ NS	$t_{(33)}=0.90$ NS	$t_{(32)}=0.34$ NS
EWW gelatin (19.0 \pm 5.2) (N=19)				$t_{(48)}=1.93$ NS	$t_{(36)}=1.19$ NS	$t_{(35)}=0.25$ NS
SIEWS gelatin (8.5 \pm 0.9) (N=31)					$t_{(48)}=0.98$ NS	$t_{(47)}=2.58^*$ $P<0.02$
Blood gelatin (11.6 \pm 3.0) (N=19)						$t_{(35)}=1.64$ NS
NaCl-Arg gelatin (20.8 \pm 4.5) (N=18)						

Comparisons showing statistically significant differences are marked with asterisks; NS, not significant.

Table 4. Results from *t*-tests comparing the interest score (number of head turns per 10 s period) of the leech *Haemopsis marmorata* presented with various prey items

Interest score (turns per 10 s) mean \pm S.E.M.	Foam rubber worm	Water gelatin	EWG gelatin	SIEWS gelatin	Blood gelatin	NaCl-Arg gelatin
Earthworm (2.93 \pm 0.65) (<i>N</i> =52)	$t_{(60)}=2.24^*$ <i>P</i> <0.05	$t_{(66)}=3.23^*$ <i>P</i> <0.01	$U_{(69)}=338.51^{1,*}$ <i>P</i> <0.05	$t_{(81)}=0.93$ NS	$t_{(69)}=1.09$ NS	$t_{(68)}=1.59$ NS
Foam rubber worm (1.33 \pm 0.92) (<i>N</i> =10)		$t_{(24)}=0.08$ NS	$t_{(27)}=0.31$ NS	$t_{(39)}=2.51^*$ <i>P</i> <0.02	$t_{(27)}=2.29^*$ <i>P</i> <0.05	$t_{(26)}=2.94^*$ <i>P</i> <0.01
Water gelatin (1.30 \pm 0.29) (<i>N</i> =16)			$t_{(33)}=0.42$ NS	$t_{(45)}=2.60^*$ <i>P</i> <0.02	$t_{(33)}=2.45^*$ <i>P</i> <0.05	$t_{(32)}=2.79^*$ <i>P</i> <0.01
EWG gelatin (1.50 \pm 0.37) (<i>N</i> =19)				$t_{(48)}=2.02^*$ <i>P</i> <0.05	$t_{(36)}=1.96$ NS	$t_{(35)}=2.34^*$ <i>P</i> <0.05
SIEWS gelatin (2.77 \pm 0.49) (<i>N</i> =31)					$t_{(48)}=0.35$ NS	$t_{(47)}=0.89$ NS
Blood gelatin (3.08 \pm 0.69) (<i>N</i> =19)						$t_{(35)}=0.69$ NS
NaCl-Arg gelatin (3.6 \pm 0.79) (<i>N</i> =18)						

Comparisons showing statistically significant differences are marked with asterisks; NS, not significant.

¹The Mann-Whitney *U*-test was used for this comparison.

hypothesized that these presumed chemoreceptors would be necessary for the leeches to find their earthworm prey.

This hypothesis was confirmed: ablation of the presumed chemoreceptors on the dorsal lip of the prostomium had a striking effect on the leeches' ability to find earthworm prey successfully (Fig. 2A). Because prostomial mechanoreceptors and other types of receptors were ablated along with the chemoreceptors, we can claim only that the presence of prostomial receptors, rather than chemoreceptors specifically, is necessary for the successful location of the earthworm.

Of the leeches that made a choice, 43% (6/14) of the ablated animals chose the arm of the maze containing the earthworm whereas 86% (12/14) of the sham-ablated animals making a choice selected the arm of the maze containing the earthworm ($G_{H(2,2)}=5.86$, $P<0.025$). There were no statistically significant differences between the ablated and sham-ablated animals in latency to first movement, time spent in the stem or the interest score (Fig. 2B,C).

We performed 22 experiments with ablated animals but discarded eight (36%) of these because the animals did not make a choice within 5 min. Seven of these did not move away from the starting area. The other leech did not move as far as the junction between the two arms within 5 min. We discarded

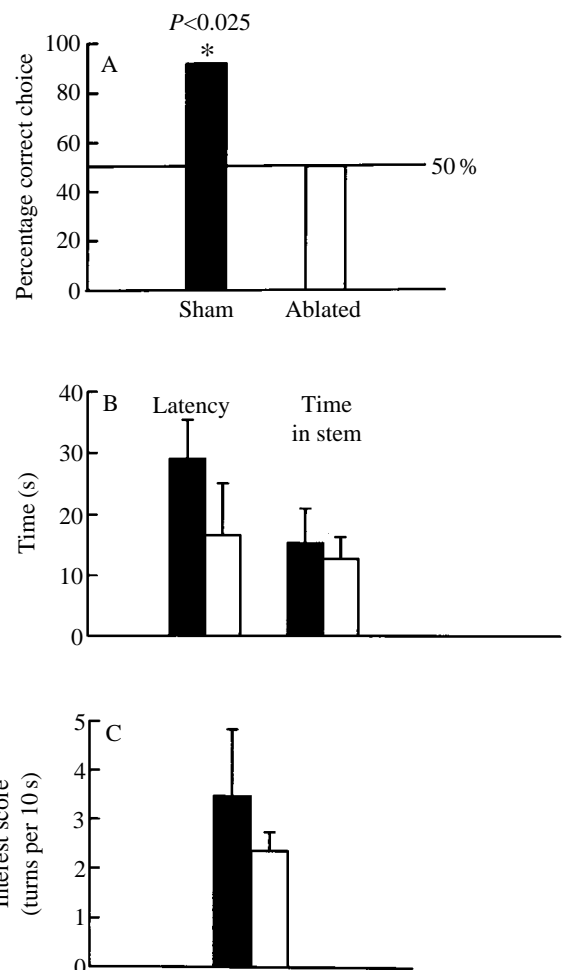


Fig. 2. Responses of chemoreceptor-ablated and sham-ablated leeches (*Haemopsis marmorata*) to live earthworms. (A) Percentage correct choice versus ablation status. The horizontal line indicates the null hypothesis of no preference. The asterisk and probability value indicate a statistically significant difference from this value. (B) Latency to first movement and time in the stem of the maze versus ablation status. (C) Interest score (number of head turns per 10 s period) versus ablation status. Values are means \pm S.E.M., *N*=14 for both ablated and sham-ablated leeches.

three of the 17 experiments (17%) with sham-ablated animals because these leeches did not move from the starting area.

The prostomial receptors are required for the leeches to find their prey in our test situation. The sham-ablated leeches were able to find the earthworm prey in the Y-maze and, hence, the failure of the ablated leeches is not due to non-specific surgical trauma or the presence of scar tissue near the prostomium.

Only nine of the 12 sham-ablated leeches making the correct choice attempted to eat the worm. The failure of the other three animals to attempt to eat the worm may have been due to non-specific surgical trauma.

Possible chemical stimuli for prey search

The importance of prostomial receptors to successful prey location suggests that the leeches identify the arm of the maze containing the worm *via* a chemical stimulus. The experiments using different chemical stimuli in gelatin blocks were therefore designed (1) to determine the possible identity of the chemical signal from the earthworms and (2) to determine whether chemical signals that trigger feeding in *Hirudo medicinalis* (Galun and Kindler, 1966; Elliot, 1986; Dickinson and Lent, 1984) could cause *Haemopsis marmorata* to display a preference in the Y-maze. We were interested in the identity of the chemical cue because an anecdotal report (Rupp and Meyer, 1954) indicates that other members of the genus *Haemopsis* are possibly attracted to blood.

Possible chemical stimuli from earthworms

We used two previously prepared chemical preparations from earthworms, earthworm wash (EWW) and shock-induced earthworm secretion (SIEWS), to make gelatin blocks. Both of these have been shown to be chemoattractants for garter snakes *Thamnophis sirtalis* (Kirschenbaum *et al.* 1986; Halpern *et al.* 1987). We hypothesized that *Haemopsis marmorata* might also use these earthworm-specific substances as chemical cues.

EWW contains a garter snake chemoattractant similar in amino acid composition and carbohydrate content to earthworm cuticle (Kirschenbaum *et al.* 1986). SIEWS is a thick yellow mucus and causes earthworms to exhibit aversive behavior, i.e. it seems to contain an 'alarm' pheromone (Ressler *et al.* 1968; Rosenkoetter and Boice, 1975). The alarm pheromone in SIEWS is a small fat-soluble molecule with a molecular mass less than 1 kDa (Jiang *et al.* 1989). SIEWS also contains a garter snake chemoattractant, a glycoprotein of about 20 kDa (Jiang *et al.* 1990).

Chemical signals triggering feeding in sanguivorous leeches

Mammalian blood has repeatedly been shown to trigger feeding in the medicinal leech (Galun and Kindler, 1966; Elliot, 1986). We hypothesized that *Haemopsis marmorata* would show a preference for whole blood or a component thereof in our test situation. The chemical signal in blood that elicits biting and feeding in the medicinal leech is very specific: 150 mmol l⁻¹ NaCl and 1 mmol l⁻¹ arginine (Galun and Kindler, 1966; Elliot, 1986). Hence, we also used a mixture of 150 mmol l⁻¹ NaCl and 1 mmol l⁻¹ arginine in our study.

Haemopsis marmorata did not display a significant preference for the arm of the maze containing a block of gelatin made with distilled water, EWW, SIEWS, whole blood or a NaCl-arginine mixture (Fig. 1B; Table 1). An earthworm was the only prey item for which a statistically significant preference was displayed ($\chi^2=5.45$, $P<0.025$).

When the prey was an earthworm, the latency was significantly longer than for any of the chemical stimuli used (Table 2; Fig. 1C). When the prey was a block of either SIEWS gelatin or blood gelatin, the leeches responded with a significantly shorter latency than when the prey was a 'control' block of gelatin made with distilled water (Table 2). The latency of the leeches' responses to the water gelatin, EWW gelatin and NaCl-Arg gelatin were not significantly different from each other (Table 2).

Once they had begun to move, the leeches spent a significantly longer time in the stem when the prey was an earthworm than for any gelatin block used (Table 3; Fig. 1C).

The interest score displayed by the leeches in the stem was significantly greater for earthworms, SIEWS gelatin, blood gelatin or NaCl-Arg gelatin than when the prey was a foam rubber worm, EWW gelatin or water gelatin (Table 4; Fig. 1D).

Freshly killed worms are the preferred prey

From the lack of a preference for either EWW or SIEWS in the gelatin preparations, we hypothesized that earthworms produce a metabolite that was detected by the leeches. We tested the leeches using pieces of freshly killed earthworms and pieces from worms that had been killed between 8 and 12 h previously. We killed the worms by cutting them in half, and used the posterior portion as the prey item because it moved very little and we wanted to minimize possible mechanosensory cues. Immediately after cutting the worm in half, we used absorbent paper to remove as much as possible of the thick yellow mucus and any blood that leaked out of the worm. Presumably, this mucus was emitted from the skin of the worm and is similar or identical to SIEWS. The posterior portions of worms were prepared either just prior to a trial or at the end of a day and stored covered at 4 °C overnight to use in the next day's trials.

The leeches showed a significantly greater preference for freshly killed worms (72%; 13/18) than for worms killed and left overnight (53%; 8/15) ($G_{H(2,2)}=12.7$, $P<0.01$, Fig. 3A). There was no difference in either the latency or the time spent in the stem of the maze for the freshly killed worms or the worms left overnight (Fig. 3B). The interest score displayed by the leeches was significantly greater when the prey was a freshly killed worm (2.85 ± 0.33 turns per 10 s; mean \pm s.e.m.) than when it was a worm left overnight (1.8 ± 0.46 turns per 10 s) ($t_{(31)}=2.44$, $P<0.05$, Fig. 3C). 10% (2/20) of the leeches failed to make a choice when the prey was a freshly killed worm and 6% (1/16) of the leeches failed to make a choice when the prey was a worm left overnight.

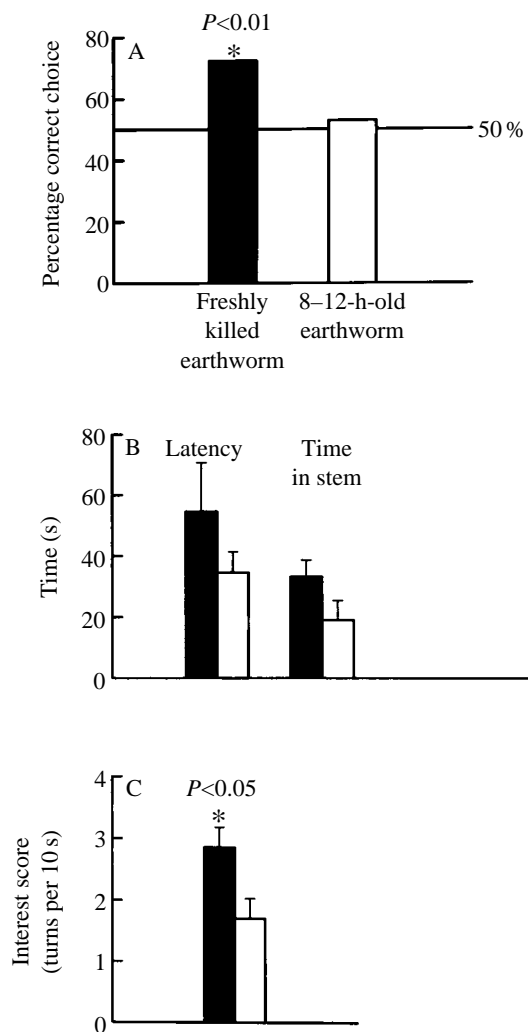


Fig. 3. Responses of leeches to freshly killed worms and to worms killed and stored for 8–12 h. (A) Percentage correct choice *versus* prey type. The horizontal line indicates the null hypothesis of no preference. The asterisk and probability value above the bar indicate a statistically significant difference from this value. (B) Latency to first movement and time in the stem of the maze *versus* freshness of the prey. (C) Interest score (number of head turns per 10 s period) *versus* freshness of the prey. The asterisk indicates a statistically significant difference between the responses to the two prey types. Values are means + S.E.M., $N=18$ for freshly killed worms, $N=15$ for worms 8–12 h old.

Discussion

Haemopsis marmorata can find its prey primarily by smell

Our primary conclusion is that *Haemopsis marmorata* can use olfaction to find its earthworm prey in water. Although we were not able to address the possibility that the leeches detected their earthworm prey by electroreception, it seems unlikely because (1) electroreceptive organs have not been discovered in leeches and (2) ablating the dorsal lip organs, the site of presumed chemoreceptors in *Hirudo medicinalis*, disabled the leeches' ability to find the prey in the Y-maze.

We did attempt to keep mechanical stimuli to a minimum

by reducing the earthworms' movements. Although we cannot completely rule out an effect of mechanoreception, leeches possess mechanoreceptors on every segment (Muller *et al.* 1981) not just the prostomium, so it is unlikely that the mechanoreceptive capability of the ablated leeches would have been completely disrupted. Nonetheless, mechanosensory neurons in the brain and subesophageal ganglia innervate the regions that were ablated (Yau, 1976), and it is probable that peripheral mechanoreceptors as well as chemoreceptors were removed from the ablated animals. If these prostomial mechanoreceptors relate to prey search and feeding specifically, then ablated animals would have diminished capabilities in both modalities, and we cannot rule this out as a reason for the ablated animals' failure to find prey in the Y-maze.

However, we believe that *Haemopsis marmorata* use olfactory rather than mechanosensory cues to find their earthworm prey for three reasons: (1) their positive response to the unmoving freshly prepared earthworm tails; (2) their correct responses when the presence of backwash was suspected; and (3) their failure to move when the prey was a piece of clean foam rubber, a situation with a dearth of chemical cues.

(1) The posterior portions of the freshly killed worms that were used as prey moved very little. The posterior portions left overnight did not move at all; the blood at the site of the cut had dried and the worm tail appeared 'dead'. In all these experiments, the tail of the worm would trail 'downstream' in the flowing spring water. That the leeches chose these barely moving tails as prey is consistent with the idea that the leeches are using chemoreception as opposed to mechanoreception for prey location.

(2) From the dye experiments, we know that eddies and backwash were present in the Y-maze in 24% of experiments. Although we do not know the particular leech trials in which eddies were present, the behavior of the leeches in a similar fraction of trials, moving into the empty arm and then the arm containing the prey item when the prey was an earthworm, also supports the conclusion that the leeches use chemoreception to find the earthworms.

(3) When the prey item was a piece of clean foam rubber, 45% (10/22) of intact leeches failed to move within 5 min. This was significantly different from the 11% (7/59) of intact leeches that failed to move when the prey was an earthworm ($G_{H(2,2)}=14.4$, $P<0.001$). With a clean foam rubber worm as the prey, there was an absence of information from chemical cues and, even though this comparison does not exclude mechanoreception as a cue, it suggests that *Haemopsis marmorata* finds the earthworms by olfaction.

In addition to these three points, we have also noticed that the green horse leech is relatively insensitive to handling and mechanosensory input. Although this observation is unquantified, it is clear that the green horse leech does not respond to handling as vigorously as the more mechanically sensitive medicinal leech (T. W. Simon, unpublished observations).

The preference for live earthworms is specific and possibly adaptive

Blood, earthworm tails stored overnight, SIEWS or EWW evoked no preference from the leeches. Presumably, the leeches were able to detect a labile metabolite released by live or recently killed worms or were responding to mechanical stimuli.

In the Y-maze, the leeches displayed similar levels of searching behavior, as indicated by the interest scores, for all prey items except for foam rubber worms, water gelatin blocks (controls) and EWW gelatin blocks. The presence of odors in the water may serve to arouse or alert the animals, but only the earthworm elicited a statistically significant number of correct choices in the Y-maze.

The situation in the Y-maze is quite unlike a natural situation in still water. When *Haemopsis marmorata* and earthworms are placed together in still spring water in a 100 cm×100 cm square flat dish 2.5 cm deep, the leeches become quite active and begin to swim, pausing periodically to execute prostomial extensions and head turns. Eventually, a few leeches will find a worm and begin to eat (T. W. Simon, unpublished observations). In this situation, the leeches appear to search at random for the worms. *Hirudo medicinalis* also appears to search at random for an unmoving blood source in still water (T. W. Simon and J. S. Sagan, unpublished observations). Both these situations appear to be similar to that of the marine mollusc *Aplysia californica*, whose movements during food searching in still water can be predicted by random search theory (Teyke *et al.* 1992).

Earthworms constitute 14% of the diet of *Haemopsis marmorata*, and another member of the same genus, *Haemopsis lateromaculatum*, consumes oligochaetes exclusively (Riggs, 1980). The three major prey items of *Haemopsis marmorata* are earthworms, snails and small insect larvae (Riggs, 1980); solely on the basis of size, one would expect earthworms to be the most desirable prey for many carnivorous leeches. Although we were not able to identify the specific chemical or chemicals released by the earthworm, a preferential response to a specific prey may be adaptive. One would expect that considerable energy expenditure is required for leeches to engage in random searching to find prey in still water. Presumably, when the leeches are in a natural situation in still water, e.g. a pond, detection of a specific chemical signal from earthworms would enable them to detect the presence (but not the location) of this prey and would prevent the waste of energy in random searches for less desirable prey such as tiny chironomid larvae. One would expect that it would take much less energy for the leech to remain stationary or to perform an exploratory head turn and thus sample the odors in the water than to crawl or swim even a short distance. Consistent with this explanation is the observation that the time spent in the stem when the prey was an earthworm was significantly greater than with any of the chemical attractant gelatin blocks (Fig. 1C; Table 3).

Karrer and Sahley (1988a) showed that *Haemopsis marmorata* would readily eat beef, chicken or liver and could be trained to distinguish between these food items. The

preference of this leech for earthworms in our experiments may possibly be an example of food attraction conditioning, a preference conditioned by experience in nature due either to prolonged exposure to earthworms as a food item or to brief exposure to earthworms as a food item during a critical period in juvenile animals (Teyke, 1995).

The lower interest score when EWW was the prey item may be due to the fact that this gelatin preparation contained only 20% of the protein concentration of either the blood gelatin or the SIEWS gelatin. Indeed, the EWW preparation may even have contained a small amount of the earthworm metabolite attractive to leeches, but the concentration may not have been sufficient to be attractive. Alternatively, the EWW preparation may not contain the metabolite or it may be labile in this chemical preparation.

Is SIEWS an 'alarm pheromone' for leeches as well as earthworms?

The repellent action of SIEWS on earthworms is well documented (Ressler *et al.* 1968; Rosenkoetter and Boice, 1975; Jiang *et al.* 1989). The leeches spent significantly less time in the stem of the maze when the prey was SIEWS gelatin than when the prey was an earthworm or NaCl–Arg gelatin (Fig. 1C; Table 3). However, if the leeches had been trying to 'escape' from the alarm pheromone, it is likely that the animals would have chosen the arm of the maze without the prey in a significant fraction of trials. However, the level of choice was no different from chance (15/31, $\chi^2=0.032$, $P>0.5$; Fig. 1B) and was not significantly different from that for any of the other prey items, with the exception of earthworms (Fig. 1B). Like earthworms, leeches produce copious mucus when injured or stressed (T. W. Simon, unpublished observations), but whether this mucus contains an 'alarm pheromone' for conspecifics or other annelids is not known. In these experiments, there is no clear evidence that the leeches are repelled by SIEWS.

Chemoreception and prey search in other species of leech

Olfactory responses of the medicinal leech are quite specific. *Hirudo medicinalis* will bite and feed on a mixture of 150 mmol⁻¹ NaCl and 1 mmol⁻¹ arginine (Galun and Kindler, 1966; Elliot, 1986). However, arousal and food searching in the medicinal leech are initiated by water movement and photic stimuli (Dickinson and Lent, 1984).

Haemopsis plumbea has been shown to reject all food items except live specimens of the leech *Erpobdella punctata* (Riggs, 1980). Introduction of live *Erpobdella punctata* into an aquarium containing *H. plumbea* induced searching behavior consisting of head turns similar to those we have described for *H. marmorata*. When an erpobdellid approached within 5–8 cm of *H. plumbea*, the haemopid struck and caught the other leech (Riggs, 1980). Clearly, the response of *H. plumbea* to the erpobdellids could have a chemosensory basis.

An anecdotal report has described *Haemopsis grandis* feeding from the wounds on trout made by *Macrobdella decora* (Rupp and Meyer, 1954). *H. grandis*, a distichodont leech, lacks teeth and cannot penetrate skin, but was evidently

attracted to the wounds from which *M. decora* had detached. On the basis of this report, we were surprised that *Haemopsis marmorata* was attracted neither to blood nor to a NaCl/arginine mixture.

Possible differences in the nervous systems of the green horse leech and the medicinal leech

A variety of chemical signals evoke searching behavior, as indicated by the interest scores, in *Haemopsis marmorata* (Fig. 1D). The nerves innervating the dorsal lip organs in this species enter the brain through the pharyngeal ganglion (Livanow, 1904; Karrer and Sahley, 1988b). The pharyngeal ganglion in the leech is thought to control the movements of the jaws and pharynx, perhaps analogous to the stomatogastric ganglion of crustaceans (J. Jellies, personal communication; T. Karrer, personal communication).

The presence of serotonin within the nervous system is obligatory for *Hirudo medicinalis* to feed (Lent *et al.* 1989, 1991). Recently, depletion of serotonin in the nervous system of *H. marmorata* has been shown to decrease but not eliminate feeding behavior (Goldburt *et al.* 1995). In *H. marmorata*, substance P has been shown to alter the activity of neurons associated with feeding behavior (Karrer and Sahley, 1988b). It would be interesting to examine the effect of altering the neuromodulatory state of this leech on its ability to find prey in the Y-maze.

A number of neurosecretory cells have been identified within the supraesophageal ganglion of the sanguivorous gnathobdellid leech *Macrobdeella decora*. These cells can be identified by their Tyndall blue-white appearance under reflected light (Webb and Orchard, 1979; Orchard and Webb, 1980). Cell bodies with a similar appearance can be observed in the supraesophageal ganglia of *Haemopsis marmorata* and *Hirudo medicinalis* (T. W. Simon, unpublished observations).

It seems likely from the behavior of *H. marmorata* in our experiments that neural signals from the lip chemoreceptors will eventually affect motor neurons mediating crawling, prostomial extension and head turns. However, at present, the identity of the motor neurons in the head ganglia of this species and of *Hirudo medicinalis* remains to be discovered. The lip chemoreceptor signals may be wired to a different neural target in *Hirudo medicinalis* from that in *H. marmorata*. Alternatively, neuromodulation of neural connections may account for the different responses to food stimuli found in the two leeches.

Possible evolutionary comparisons between carnivorous and sanguivorous leeches

The medicinal leech and the green horse leech are believed to have evolved from a common ancestor. These two leeches have distinctive species-specific feeding behavior that may permit exploration of how phylogenetic differences in behavior are represented by changes in the underlying neuronal architecture.

The medicinal leech is an obligate sanguivore and consumes a blood meal every 3–6 months. A hungry leech remains at the

water surface with both suckers attached to the substratum or bank. When aroused by mechanical or photic stimuli characteristic of water movement, the leech releases the anterior sucker and orients towards the source of the movement. After 10–20 s, the posterior sucker is released and the leech swims towards the source of water movements (Dickinson and Lent, 1984; Lent *et al.* 1989; Elliot, 1986). The natural hosts for *H. medicinalis* include aquatic birds and amphibians in addition to mammals (Sawyer, 1986).

The green horse leech is likely to employ chemical signals to find its prey at a distance, as shown in this study. Hence, the set of stimuli indicating the distant (greater than several body lengths) presence of food to a hungry animal seems to be primarily mechanical for the medicinal leech and primarily olfactory for the green horse leech.

The divergence of carnivorous and sanguivorous forms of predation is believed to be fairly recent in the evolution of leeches (Mann, 1962; Sawyer, 1986). Differences in the nervous systems of various leeches have been noted in the past (Lent, 1977; Keyser and Lent, 1977; Macagno, 1980); however, none of these differences has been related to species-specific behavior. The demonstration of the link between olfaction and prey searching in the green horse leech presented in this study provides part of the basis for further investigations into the neural differences between the two species that underlie their different feeding strategies.

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