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RESEARCH ARTICLE

Multiplexed modulation of behavioral choice

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ABSTRACT

Stimuli in the environment, as well as internal states, influence behavioral choice. Of course, animals are often exposed to multiple external and internal factors simultaneously, which makes the ultimate determinants of behavior quite complex. We observed the behavioral responses of European leeches, *Hirudo verbana*, as we varied one external factor (surrounding water depth) with either another external factor (location of tactile stimulation along the body) or an internal factor (body distention following feeding). Stimulus location proved to be the primary indicator of behavioral response. In general, anterior stimulation produced shortening behavior, midbody stimulation produced local bending, and posterior stimulation usually produced either swimming or crawling but sometimes a hybrid of the two. By producing a systematically measured map of behavioral responses to body stimulation, we found wide areas of overlap between behaviors. When we varied the surrounding water depth, this map changed significantly, and a new feature – rotation of the body along its long axis prior to swimming – appeared. We found additional interactions between water depth and time since last feeding. A large blood meal initially made the animals crawl more and swim less, an effect that was attenuated as water depth increased. The behavioral map returned to its pre-feeding form after approximately 3 weeks as the leeches digested their blood meal. In summary, we found multiplexed impacts on behavioral choice, with the map of responses to tactile stimulation modified by water depth, which itself modulated the impact that feeding had on the decision to swim or crawl.

KEY WORDS: Leech, Behavioral choice, Feeding, Environmental context, Behavioral response map

INTRODUCTION

Animals must constantly make choices among multiple alternative behaviors – to search for food or a mate, to escape from a threat or to fight – based on external stimuli and internal states (Palmer and Kristan, 2011). How these choices are made depends crucially upon the context the animal finds itself in: its physical and social environments and its internal/behavioral state. Animals often must make decisions in the face of multiple contexts, each of which can have possibly conflicting effects on behavioral choice. In this study, we tested the behavioral responses to tactile stimulation of leeches in simultaneously presented contexts in an effort to understand how their effects can be multiplexed.

The leech is a useful animal for elucidating the cellular mechanisms underlying behavioral choice because leech behaviors such as swimming (Brodfuehrer et al., 1995), crawling (Eisenhart et al., 2000), shortening (Shaw and Kristan, 1995) and local bending

(Lockery and Kristan, 1990a; Lockery and Kristan, 1990b) are well characterized and have been utilized to study decision-making at the neuronal circuit level (Briggman et al., 2005; Kristan and Gillette, 2007; Gaudry and Kristan, 2009).

A leech's choice of locomotory or avoidance behavior is affected by many external stimuli including chemical, thermal and mechanical cues (Galun and Kindler, 1966; Dickinson and Lent, 1984; Elliott, 1986; Gaudry and Kristan, 2009). However, the primary factor for determining which behavior the leech expresses in response to an external stimulus, such as touch, is the location of the stimulus. The leech is a segmented annelid with four head segments, 21 somatic segments and seven tail segments (Fig. 1A). In general, touch to the head and anterior somatic segments elicits shortening, touch to the middle somatic segments elicits local bending, and touch to the tail and posterior somatic segments elicits either of two locomotory behaviors: swimming or crawling (Kristan et al., 1982; Kristan et al., 2005). However, the exact distribution of behavioral responses resulting from stimulation to individual body segments along the entire length of the body is not known. Also, the neural circuitry underlying the connection between stimulus location and behavioral response has just begun to be described (B. Migliori, C.R.P. and W.B.K., unpublished).

One candidate for making choices between modes of locomotion is R3b1, a bilateral pair of neurons located in the subesophageal ganglion in the head of the leech. They are 'command-like' neurons for locomotion (Esch et al., 2002; Puhl et al., 2012) that respond in a distinctly location-specific manner: stimulating the posterior of the leech (which gives rise to swimming or crawling) activates them and anterior stimulation (which gives rise to shortening) inhibits them. The effect of activating R3b1 cells is influenced by the animal's environment: stimulating an R3b1 cell usually elicits crawling rather than swimming when a nearly intact leech is in shallow water, but the same R3b1 cell activation elicits mostly swimming when the leech is in deep water (Esch et al., 2002; Puhl and Mesce, 2010). So, both stimulus location and surrounding water depth modulate the choice between swimming and crawling, but how these stimuli interact to affect behavioral choice has yet to be tested.

Denervating a leech's head increased its likelihood of swimming in both deep and shallow water (Esch et al., 2002), suggesting that receptors in the head sense water depth, which selectively inhibits swimming. Other results suggest that additional factors influence the decision to swim or crawl. For example, during the consumption phase of feeding [which is at the top of the leech's behavioral hierarchy (Misell et al., 1998)], leeches ignore tactile stimuli, even noxious ones (Lent et al., 1988; Misell et al., 1998), because serotonin released during feeding inhibits the terminals of pressure-sensitive mechanosensory neurons (P-cells), the primary sensory neurons responsible for triggering swimming, crawling, local bending and shortening (Gaudry and Kristan, 2009). On a longer time scale, body distention from a meal [which can bloat a leech to 10–15 times its pre-fed mass (Dickinson and Lent, 1984; Lent et al., 1988; Gaudry and Kristan, 2009)] will tip the balance toward crawling and away from swimming (Lent et al., 1988). When well-

List of symbols and abbreviations	
CS	choice system
P-cell	pressure-sensitive cell
R3b1	a bilateral pair of neurons located in the subesophageal ganglion in the head of the leech
T-cell	touch-sensitive cell
x	leech diameter

fed leeches do swim, they make slower progress because of both reduced cycle frequency and reduced distance covered per locomotor cycle ('stride length' in biomechanical terminology) (Claflin et al., 2009). The feeding-mediated distention of the leech body appears to inhibit swimming by activating stretch receptors in the body wall, which inhibit swim gating neurons or swim central pattern generator neurons (Gaudry and Kristan, 2010).

In the present study, we tested interactions between feeding-induced body distention and water depth by feeding European leeches (*Hirudo verbana* Carena) to satiation with two solutions that trigger feeding: either blood or an arginine–saline solution (Galun and Kindler, 1966; Elliott, 1986; Lewis and Kristan, 1998). The

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Fig. 1. Anatomical and behavioral drawings of medicinal leeches performing behaviors in response to tactile stimulation. (A) Schematic of a leech illustrating four head segments, 21 somatic segments and seven tail segments. The white ovals on the dorsal midline are beads sewn onto the skin in the middle of each somatic segment. For our experiments, we confined stimulation to somatic segments 3–19. (B) Schematic of leech behaviors: whole-body shortening (a rapid bilateral shortening along the body length); local bending (a localized – fewer than three segments – unilateral shortening on the side of the body stimulated, combined with a passive lengthening on the opposite side that results in a localized 'U-shaped' bend of the body away from the stimulus); swimming (detachment of front sucker followed by dorsoventral undulatory cycles that moved backward along the leech's body, propelling the animal forward); and crawling (detachment of the front sucker, followed by an elongation of the body, reattachment of the front sucker, contraction of the body, followed by detachment of the rear sucker and its reattachment at a more forward site).

arginine–saline solution served as a control to test whether a blood meal affected locomotory choice as a result of the chemical constituents of blood or simply because of the volume of liquid ingested. To determine the detailed effects of distention and water depth on all touch-evoked leech behaviors, we made the first systematic map of the distribution of behavioral responses resulting from tactile stimulation of each body segment. We repeated this exercise for a number of water depths. We found a previously unreported effect of water depth: in shallow water, leeches are more likely to twist, or rotate, their bodies along the long axis as they start to swim. We also documented how the behavioral effects of distention waned over time since the most recent feeding: the probabilities of locomotory behaviors (swimming and crawling) were strongly changed during the initial days following feeding and returned to control levels many days after feeding with blood, and after only a few days after feeding with arginine. In summary, this study demonstrates several interactions among external stimuli and internal states in the selection of locomotory behaviors.

RESULTS
Mapping behavioral responses

We set out to determine which behaviors resulted from stimulation to each of the body's segments. We therefore video-recorded the behavioral responses of 24 leeches (freely moving on the moist surface of a plexiglass arena) to electrical stimulation in the middle of somatic segments 3–19 and analyzed the responses offline. We also stimulated the leech at the boundaries between segments, which we are calling the 'half-segments'. For each leech, there were three to five trials per segment and half-segment. Each behavioral response was classified as one of the following: (1) whole-body shortening: a quick bilateral contraction of longitudinal muscles along the length of the leech that rapidly pulls the anterior end away from the site of stimulation (Fig. 1B, top left); (2) local bending: a localized (fewer than three segments) unilateral shortening on the side of the body stimulated, combined with an elongation on the opposite side, producing a localized 'U-shaped' bend of the body away from the stimulus (Fig. 1B, top right); (3) swimming: detachment of both suckers, followed by repeated dorsoventral undulatory cycles that move backward along the leech's body, propelling the animal forward (Fig. 1B, bottom left); (4) crawling: a detachment of the front sucker, followed by an elongation of the body, reattachment of the front sucker, then shortening of the body, followed by detachment of the rear sucker and its reattachment at a more forward site (Fig. 1B, bottom right); (5) hybrid swimming/crawling: detachment of front sucker followed by swim-like body undulations but with the rear sucker attached; then the front sucker reattaches, the animal shortens, and the rear sucker reattaches, completing a crawling step (not illustrated); (6) other: an obvious response to stimulation that could not be categorized as any of the previously described behaviors; or (7) no response.

Fig. 2 (top panel) shows the map of behavioral responses to tactile stimulation of different body locations when a leech was placed in a moist arena (water depth=0). Whole-body shortening was the dominant behavior when segments 3–5.5 were stimulated [at each segment and half-segment, we performed paired *t*-tests on the mean frequencies at which each leech engaged in each behavior; for example, at segment 5, the mean frequency with which 17 leeches engaged in whole-body shortening (64%) was significantly higher than the frequency of local bending (6.5%; *P*=0.003)]. Local bending was the dominant behavior when segments 7.5–12.5 were stimulated, and crawling was the dominant behavior when segments 14.5–19 were stimulated. Stimulating segments 6–7 produced
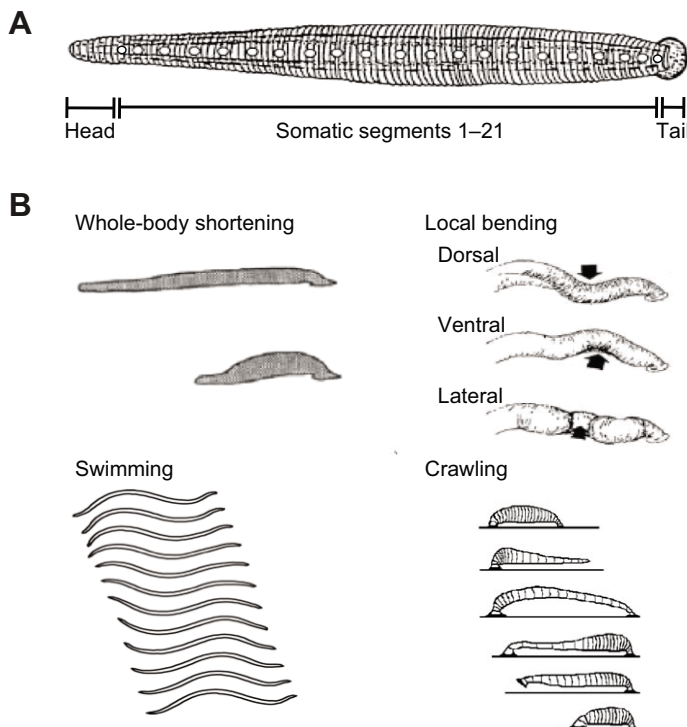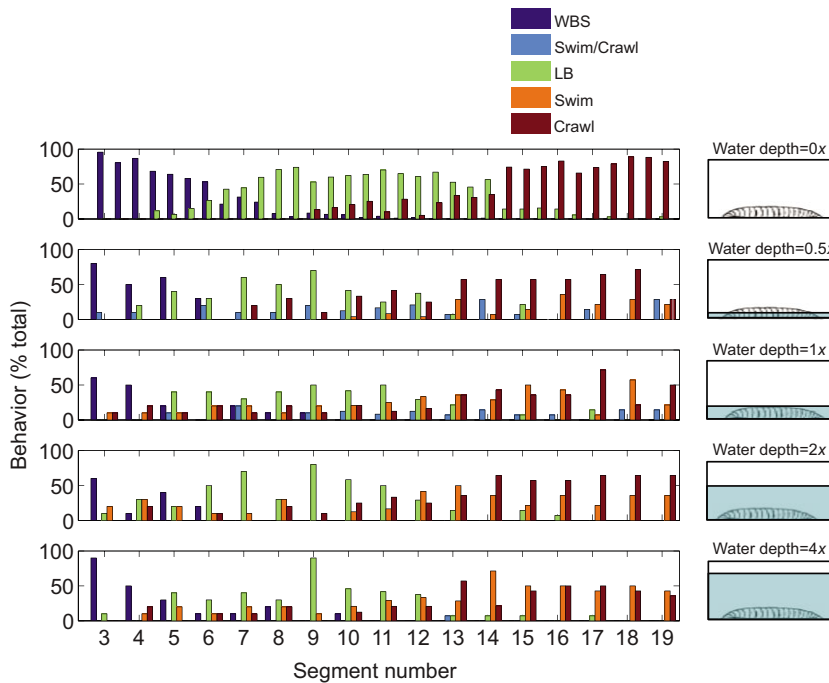
Fig. 2. Map of the behavioral responses to tactile stimulation along the length of the leech body in various water depths. Top panel: responses on a moist plexiglass surface (water depth=0x) to tactile stimulation on the dorsal surface, in the center of somatic segments 3–19 and at the midpoints between the segments. Bottom panels: responses to tactile stimulation at somatic segments 3–19 while immersed in water at four depths (0.5x, 1x, 2x and 4x) relative to the leech's dorsoventral thickness at its most relaxed length (n=14, mean=2.4±0.53 mm). Because there were usually a few 'other' responses (Cornford et al., 2006) or no response to some stimuli, the percent totals for each segment may not add up to 100. In the four water depths of 0.5x, 1x, 2x and 4x, we stimulated in the middle of each segment but not between segments. LB, local bending; WBS, whole-body shortening.

whole-body shortening and local bending with no significant difference in their probabilities of occurrence, and stimulating segments 13–14 produced local bending and crawling with no significant difference in their probabilities of occurrence. Neither swimming nor hybrid swimming/crawling were ever seen in these experiments when there water depth=0 in the leech's behavior arena.

Behavioral effect of varying both water depth and stimulus location

Fig. 2 (bottom four panels) shows the behavioral responses produced by leeches in an arena filled with water of various depths relative to the leech's body thickness. We observed several differences in the behavioral maps at different water levels. Most noticeably, swimming emerged as a more prominent behavior both in response to posterior stimulation (unpaired t-test collapsed across water depths comparing probability of swimming in response to stimulation of segments 3–10 versus segments 11–19, $P<0.001$) and in deeper water (unpaired t-test comparing probability of swimming in water depths of 0.5x and 1x, where x is the leech's diameter, with swimming at 2x and 4x, $P<0.001$).

Although local bending remained the most probable response when the body was stimulated between segments 3 and 15 when the leech was immersed in water, the probability of local bending decreased when the leech was placed in water [from 32% of all 0x trials (across all segments) to between 21 and 25% for trials in water], being replaced by an increased probability of swimming and

hybrid swimming/crawling (Fig. 2). However, in the cases where water depth was >0, the probability of local bending did not depend upon water depth, but did depend on stimulus location, occurring significantly more often (unpaired t-tests, $P<0.05$) with stimulation to the middle of the body (segments 9–13) than with stimulation to the anterior of the body (segments 3–7). Local bending occurred rarely in response to stimulation to the rear of the body (segments 15–19). Conversely, hybrid swimming/crawling did not depend upon stimulus location, but did depend upon water depth, occurring at a rate of 5–15% in shallow water (0.5x and 1x) but <1% in deeper water (2x and 4x), a difference that was significant (unpaired t-tests, $P<0.05$).

The effects of water depth on elicited locomotory behaviors were more complex. Stimulating the anterior segments (3–7) produced a low level of both swimming and crawling, independent of water depth (Table 1). At all water depths, stimulation to segments 9–13, as well as segments 15–19, induced more swimming and crawling than stimulation to segments 3–7, but for the most part the differences were not significant. At a depth of 0.5x, crawling occurred more often than swimming when the middle (segments 9–13; unpaired t-test, $P=0.004$) and posterior (segments 15–19; unpaired t-test, $P=0.009$) regions of the leech were stimulated. The probability of swimming and crawling was not significantly different in depths of 1x, 2x and 4x when the middle of the leech (segments 9–13) was stimulated, whereas stimulating the posterior region (segments 15–19) produced a significantly greater probability

Table 1. Relationship between the probability of eliciting swimming and crawling (expressed as a percentage of the total stimuli presented) by stimulating three different body regions as a function of water depth (normalized to the dorsoventral height of the leech at rest, x)

Water depth	Segments 3–7		Segments 9–13		Segments 15–19	
	Swim	Crawl	Swim	Crawl	Swim	Crawl
0.5x	0	4	9	28*	27	61*
1x	12	16	28	21	31	57*
2x	7	18	24	28	33	64*
4x	11	8	25	24	52	48

*Significant difference (unpaired t-test, $P<0.05$) between swim and crawl within a given water depth.
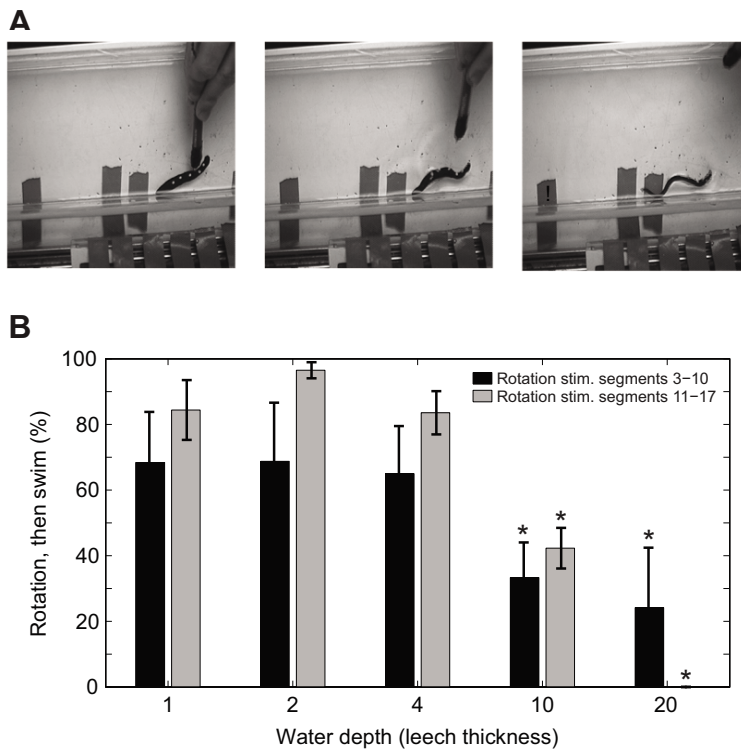
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Fig. 3. Rotation prior to swimming depends on water depth.
(A) Three video frames, spanning 1.2 s, looking down on a leech as it rotates its body prior to swimming in response to stimulation at segment 7 in a water depth of 4*x*. The leech has beads at segments 4, 6, 8, 10 and 12. In this example, in response to the stimulus (left frame), the leech rotates toward the side of body stimulated (middle frame) as it begins to swim (right frame). (B) A plot of the percentage of times that leeches rotated by 90 deg before beginning to swim in response to stimuli in the front half (segments 3–10) and back half (segments 11–17) of their bodies at different water depth shows that leeches rotated their bodies prior to swimming significantly less often in deeper water (10*x*) than in more shallow water (1*x*–4*x*). Percentages for water depths 1*x*, 2*x* and 4*x* came from re-analyzed data from Fig. 2. Percentages for water depths 10*x* and 20*x* came from separate experiments using a different set of five leeches. Asterisks indicate significant differences (*P*<0.01) in rotation rate for shallow versus deep water, according to unpaired *t*-tests. The *t*-tests compared the rate of rotation computed for each animal tested at water depths of 10*x* and 20*x* (*n*=5) with that computed for each animal tested at 1*x*, 2*x* and 4*x* depths (*n*=14). The *t*-tests were run separately for anterior (segments 3–10) and posterior (segments 11–17) body locations. Error bars are s.e.m.

of crawling at the shallower depths (unpaired *t*-tests; 0.5*x*, *P*=0.009; 1*x*, *P*=0.02; 2*x*, *P*=0.008), a difference that disappeared at a water depth of 4*x* (unpaired *t*-test, *P*=0.75). These results indicate that the choice of locomotion type depended upon both stimulus location and water depth.

Water depth and stimulus location also had an impact on a previously unreported aspect of swimming. At each water depth above 0.5*x* we observed many trials in which the leech turned on its side by rotating its body 90 deg along its long axis just prior to swimming (Fig. 3), presumably because the dorsal-ventral undulations in the shallow water would lift their body out of the water and make swimming impossible. The probability of pre-swimming rotation in all water depths was not significantly higher (*P*=0.13) when the leech was stimulated in its posterior half (segments 11–17) than when it was stimulated in its anterior half (segments 3–10; Fig. 3B). Although there was no difference in the probability of pre-swimming rotation in depths of 1*x*, 2*x* and 4*x*, in a water depth of 4*x* the leech would generally swim on its side for a few cycles, then rotate back to its normal dorsal-side-up position and continue swimming, whereas in the shallower depths (1*x* and 2*x*), the leech remained on its side for the full duration of the swimming episode.

We ran experiments on another five leeches at water depths of 7*x*, 10*x* and 20*x* and found that the probabilities of swimming and crawling were not significantly different than for the 4*x* depth (*t*-tests; 7*x*, *P*=0.09; 10*x*, *P*=0.82; 20*x*, *P*=0.42). However, at these depths, the probability of rotating prior to swimming was significantly lower (*P*<0.001, at both anterior and posterior stimulus locations) than in the relatively shallower water (1*x*, 2*x* and 4*x*). So, like hybrid swimming/crawling, rotation prior to swimming did not depend upon stimulus location, but did depend upon water depth.

Multiplexed behavioral effect of water depth and time since last feeding
A previous study (Misell et al., 1998) informally observed that leeches that appeared well fed crawled more and swam less than

hungry leeches. To quantify the effect of feeding on swimming and crawling, we fed seven leeches to satiation with blood, and then monitored their swimming and crawling behaviors for the next 60 days. Immediately after feeding, their behavioral performance changed dramatically, both qualitatively and quantitatively. When they did crawl, they took short steps with a longer cycle period. These blood-engorged leeches did not often attempt to swim, but when they did, their undulatory movements were slow and labored and the swimming episodes were short, consistent with previous observations (Claflin et al., 2009).

In addition to the differences in the quality of their movements, feeding also changed the relative percentages of elicited swimming and crawling episodes. The probability of crawling was higher at all water depths (except for the lowest depth, 0.5*x*, which was high at the start and did not significantly increase after feeding) for at least 4 days after feeding, then slowly returned to pre-fed levels at approximately 3 weeks (*t*-tests comparing mean probability of crawling in pre-fed leeches to post-feeding leeches, *P*<0.05; Fig. 4A). The probability of swimming showed the opposite pattern: it dropped significantly after feeding and returned to pre-fed levels by day 22 (*t*-tests comparing mean probability of swimming in pre-fed leeches to post-feeding leeches, *P*<0.05; Fig. 4B). The percentage of hybrid swimming/crawling episodes in fed leeches did not change significantly over this time: it was low at shallow water depths initially (and non-existent in deeper water) and remained low throughout the 60-day experiment (data not shown).

To see whether the behavioral changes were caused by the chemical composition of blood or by its volume, we performed the same set of experiments except we fed the animals a solution containing only arginine and saline, a mixture that leeches ingest as enthusiastically as blood but from which they gain little nourishment (Galun and Kindler, 1966; Elliott, 1986; Lent et al., 1988). Unlike the blood-fed leeches, the swimming and crawling behaviors of arginine-fed leeches returned to pre-fed levels by 1 day after feeding (Fig. 5). Again, the hybrid swimming/crawling behavior remained
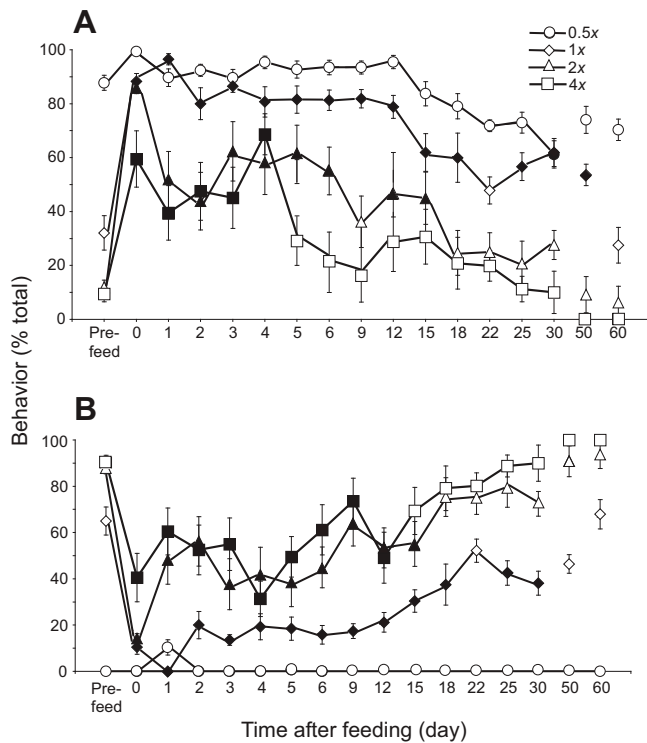
Fig. 4. Long-term effects of a blood meal on the pattern of locomotory behaviors used to traverse a behavioral arena at different water depths. We tested seven leeches (fed to satiation with blood on day 0) at four different depths – 0.5*x*, 1*x*, 2*x* and 4*x*. Percentage of (A) crawling steps and (B) swimming episodes before (pre-feed) and for 60 days after feeding while immersed at the four water depths. Error bars represent s.e.m. Filled data points differed significantly (*P*<0.05) from the corresponding pre-feeding data points.
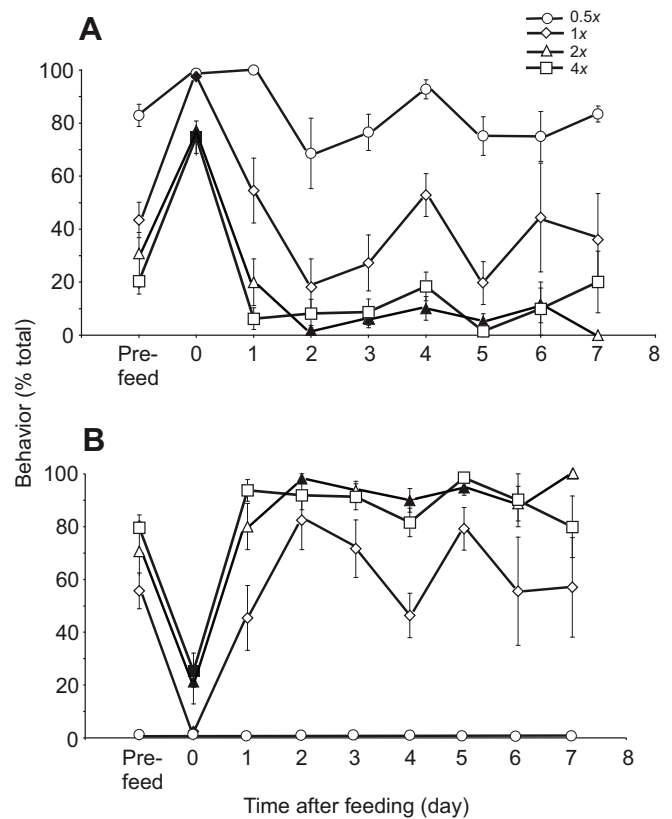
Fig. 5. Effects of an arginine–saline meal over time on the pattern of locomotory behaviors used to traverse a behavioral arena at different water depths. We tested 11 leeches [fed to satiation with a solution of arginine (1 mmol l^{-1}) and NaCl (150 mmol l^{-1}) on day 0] in four different depths – 0.5*x*, 1*x*, 2*x* and 4*x*. Percentage of (A) crawling steps and (B) swimming episodes before and after feeding while immersed at the four water depths. Error bars represent s.e.m. Filled data points differed significantly (*P*<0.05) from the pre-feeding data points.

infrequent throughout the test period (data not shown). This experiment was terminated on day 8 both because the behaviors had returned to pre-fed control levels and because some of these leeches died and many of the others were making abnormal, jerky movements. These difficulties were not apparent during the first 4–6 days, so it appeared that the arginine–saline meal produced toxicity approximately 1 week after ingestion.

Mass gains and losses after blood and arginine–saline ingestion

The differences in behaviors between the two types of meals (blood versus arginine–saline) could be caused by differences in the size of the meals (mass gain) or by the rate of utilization and/or excretion of the meals (mass loss). To test for these possibilities, we measured the mass of each leech on each day of testing and plotted these masses as a percentage of the pre-feeding mass (Fig. 6). Consistent with prior reports (Dickinson and Lent, 1984; Lent et al., 1988), our hungry leeches increased their body mass by an average of nearly 10-fold in a single feeding. The mass gain on the day of feeding (day 0) for arginine-fed leeches was high, but significantly lower than blood-fed leeches (unpaired *t*-test, *P*=0.04). One day later (i.e. day 1), both groups of leeches had lost nearly 40% of the mass gain (a significant decrease; paired *t*-tests, blood-fed, *P*<0.001; arginine-fed, *P*<0.001), probably through the excretion of fluid (Nieczaj and Zerbst-Boroffka, 1993). The mass loss was not different in the two groups (unpaired *t*-test, *P*=0.21). However, by day 2 post-feeding, the absolute masses in the two groups had significantly diverged (unpaired *t*-test, *P*<0.001). The blood-fed leeches slowly lost mass

over the next 2 months, so that at day 60 they remained over six times heavier than before feeding (Fig. 6A), whereas the arginine-fed leeches continued their day-2 rate of mass loss, so that by day 5, they were only approximately twice their pre-fed mass (Fig. 6B).

It is likely that even though the leeches did not lose much mass from day 2 to day 60 (Fig. 6A), they became less bloated and distended as their blood meals were being digested and converted into body mass – i.e. muscles, internal organs and neural tissue. We inferred the animals' gain in body mass over time by measuring the masses of individual leeches before the one-time feeding and then after removing all the blood from their guts at various times following the feeding (Fig. 7A) (see Materials and methods). These data indicate that the blood meal does not begin to be converted to body mass for several days following the meal, a result supported by previous studies (Dickinson and Lent, 1984; Gaudry and Kristan, 2010). We then calculated body distention as a ratio of the mass of the blood remaining in the body divided by the total mass of the blood meal (Fig. 7B). Distension remains fairly steady for the first several days, and then drops steeply around day 10, when it plateaus.

A previous study (Gaudry and Kristan, 2010) showed that distention of a leech's body inhibits the amount of swimming, measured in swimming cycles per minute. In the present study, we observed that swimming behavior steadily increased as days passed form the initial feeding (Fig. 7C, data replotted from Fig. 4). We
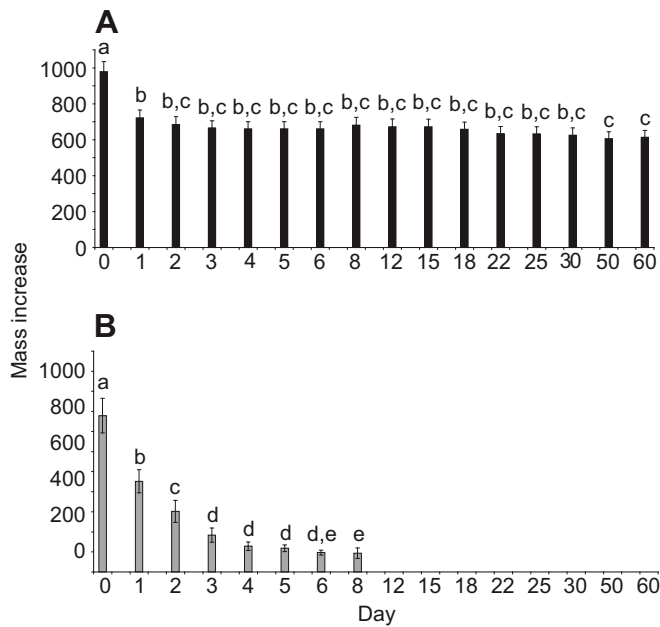
Fig. 6. Changes in body mass for up to 60 days following feeding on blood or on an arginine–saline solution. (A) Mass gains, and subsequent losses, of the seven blood-fed leeches, expressed as the average percentage of mass for each leech relative to its pre-feeding mass for the 60 days after feeding to satiation. Leeches were fed on day 0. (B) Mass gains, and subsequent losses, of the 11 arginine-fed leeches, expressed as the average percentage of mass for each leech relative to its pre-feeding mass for the 8 days after feeding to satiation. Error bars represent s.e.m. The letters above the data points represent classes of points that are statistically different from one another according to t-tests ($P<0.01$).

were then curious whether the progressive changes in probability of swimming after feeding could be caused by a progressive decrease in distention. So we replotted the swim probability (swim behavior as percentage of total responses) from Fig. 4 against body distention (Fig. 7D). We found a significant negative correlation ($R=-0.65$, $P=0.03$) between swim probability and distention. This result suggests that the major cause of the restoration of swimming

behavior in the 2 months following a large blood meal is the progressive decrease in body distention.

DISCUSSION

In this study, we have demonstrated that contextual factors – both external (e.g. stimulus location and water depth) and internal (e.g. the effects of feeding) – can interact to modulate behavioral choice. Such interactions illuminate the difficulties of measuring the effect of a single independent variable; the exact effect is likely to depend on several additional factors.

A major conclusion from this study is documenting the importance of touch location in determining the leech's behavioral response to a touch stimulus. Though the approximate touch locations along the body that produce different behavioral responses have long been known (Kristan et al., 1982), we created highly precise maps of the leech's behavioral responses to tactile stimulation, both in moist and water-filled environments. We found multi-segmental regions where individual behaviors dominated (e.g. whole-body shortening in segments 3–5.5), as well as more restricted regions where two behaviors occurred with equal probability (e.g. whole-body shortening and local bending in segments 6–7).

Our behavioral response maps will be useful in guiding future studies where it is vital to consistently induce a single behavior of interest. Also, given that each somatic segment contains its own ganglion with a full complement of sensory neurons, interneurons and motor neurons, knowing which segments (and by extension, which ganglia) of the body to stimulate to reliably produce two behaviors with equal probability can be helpful for physiological or optical imaging studies (e.g. Briggman et al., 2005) that examine the overlap in the neural circuits within individual ganglia that are dedicated to producing those behaviors (C.R.P. and W.B.K., unpublished).

As previously observed (Esch et al., 2002), when stimulated at the posterior end, the leech's decision to swim or crawl is significantly influenced by water depth: swimming was rare at shallow depths but became as likely as crawling as the water became deeper. We extended those findings by determining how the effect of water depth interacted with the effect of stimulus location. We found that
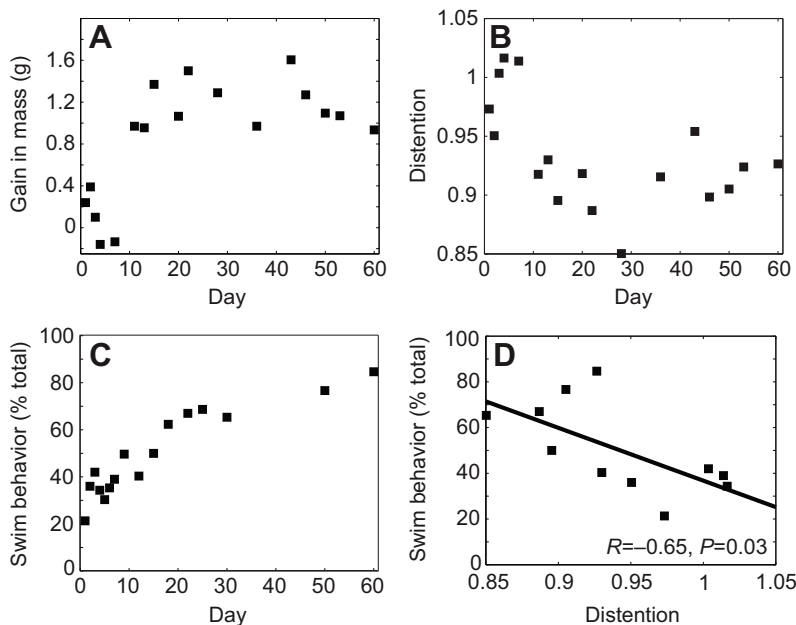

Fig. 7. Comparison of body distention following a blood meal with the increase in the probability of swimming following feeding. (A) Gain in mass, essentially a conversion of the blood meal into body mass, was measured by weighing individual leeches before the one-time feeding and then again after removing all the blood from their guts at various times following the feeding. (B) Body distention (mass of blood remaining after a meal/total mass of blood ingested during a meal) as a function of days since feeding. (C) The rate of swimming (collapsed over all water depths; replotted from Fig. 4B) that occurs following feeding to satiation on blood as a function of days since the feeding. (D) Swim behavior, as a percentage of the total number of behaviors, plotted against distention. Line is best linear fit to the data ($R=-0.065$, $P=0.03$).

the map of behavioral responses produced under conditions where water depth=0 was shifted in a number of interesting ways by increasing water levels. In a deeper-water environment, leeches produced less whole-body shortening and local bending and more swimming (primarily instigated by posterior stimulation) and hybrid swimming/crawling (instigated in shallow water by stimulation anywhere along body), as well as a moderate increase in crawling. We found that the effects of water depth and stimulus location were multiplexed in some behaviors, but not others: the probability of local bending depended on stimulus location, but not water depth; the probability of hybrid swimming/crawling depended on water depth, but not stimulus location; and the probabilities of swimming and crawling depended on both stimulus location and water depth.

Water depth and stimulus location not only had a significant impact on the decision to swim or crawl, they also impacted the leech's decision of whether to rotate its body 90 deg prior to swimming. Prior to swimming in relatively shallow water (water depth set to 1x–4x leech thickness), the leech rotated its body 65% to 85% of the time, whereas it rotated only approximately 50% of the time prior to swimming in deep water (10x). One obvious possible explanation for the increased probability of pre-swim body rotation in shallower water was that the leech needed to give itself more room to go through its full dorso-ventral swimming undulations, whose amplitudes can reach up to 20 mm (Chen et al., 2011) or, given our leeches' mean thickness of 2.4 mm, roughly equivalent to eight times the mean leech thickness. Another reason the leech may rotate prior to swimming in shallower water may be that the dorso-ventral undulations of swimming can lead to the leech's ventral surface hitting the bottom of its environment, activating touch-sensitive cells (T-cells) and pressure-sensitive cells (P-cells) (Nicholls and Baylor, 1968) that can potentially inhibit swimming altogether (Gray et al., 1938).

We further extended the findings of Esch et al. (Esch et al., 2002) by observing the likelihood of swimming and crawling as a function of water depth after the leeches were fed to satiation (resulting in an average of seven to 10 times increase in body mass). Feeding amplified the effect of water depth on behavioral choice (Dickinson and Lent, 1984; Misell et al., 1998; Gaudry and Kristan, 2009). As was the case with the unfed leeches, fed leeches crawled more than they swam in shallow water, but the differences were more severe in the just-fed leeches. The interaction of water depth and body

distention from feeding in the modulation of locomotory behavioral choice continued over the course of the next 2 to 3 weeks post-feeding as the distribution of behavioral responses reverted back to baseline.

Previous studies from our laboratory suggested that decreased incidence of swimming in fed leeches could be due to stretch receptors that may inhibit swim gating neurons or swim central pattern generator neurons (Gaudry and Kristan, 2010). The prolonged activation of these stretch receptors may explain our observations that the likelihood of swimming is relatively suppressed over many days and weeks after feeding. Over time, the body becomes less distended as the blood meal is digested and converted into body mass, a suggestion first made by Dickinson and Lent (Dickinson and Lent, 1984), who observed a return to baseline levels of behavior months after leeches were fed to satiation. In support of this idea, we found that the swim behavior, as a percentage of the total number of behaviors, we observed over the period of 60 days in our experiments was significantly correlated with the decrease in the leeches' body distention (Fig. 7). A similar initial decrease in the likelihood of swimming was observed in the leeches fed on the arginine–saline solution, but the leeches' behavior reverted to baseline much sooner (within 3 days of feeding), possibly because the body mass – and, hence, the strain of the stretch receptors – decreased much faster in these animals than in the blood-fed leeches (Fig. 6).

Our experiments extend and update a previous model (Esch et al., 2002) of the circuit underlying the choice to swim or crawl (Fig. 8), including the possibility that stretch receptors detect body distention that is due to feeding (Gaudry and Kristan, 2010). The previous model proposed that stimulated P-cells in the posterior of the animal activate a pair of bilateral neurons in the subesophageal ganglion called R3b1. Our updated version of the model additionally proposes that activation of P-cells in the anterior of the animal results in inhibition of the swim/crawl choice system (CS), which could involve neurons such as R3b1 in the anterior brain (Esch et al., 2002), trigger and gating neurons in most segmental ganglia (Brodfuehrer et al., 1995), and cell E21, a pair of neurons found only in the most posterior segmental ganglion (Mullins et al., 2011). Inhibition of the CS prepares the leech for whole-body shortening (Esch et al., 2002). Our model also notes that stimulation to the middle somatic segments – resulting primarily in local bending –
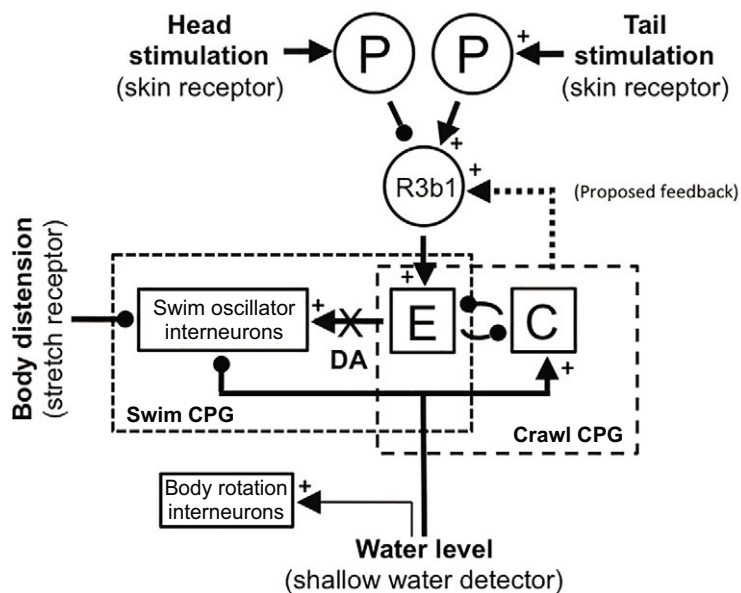

Fig. 8. Working model of the behavioral impacts of water depth, gut distention due to feeding, and location of tactile stimulation. P, pressure-sensitive mechanosensory cells; R3b1, neuron in the subesophageal ganglion that is involved in the choice between swimming and crawling; E, elongation network that is a component of both the swim and the crawl central pattern generators (CPGs); C, contraction network that is a component of the crawl CPG. Filled circles indicate inhibitory interactions; filled arrowheads with plus signs indicate excitatory interactions. DA indicates bath-application of dopamine; the X indicates that DA inhibits the pathway to the swim CPG; the DA also activates the crawl CPG (Puhl et al., 2012). Modified from Esch et al. (Esch et al., 2002).

has no impact on the CS (an effect not shown in Fig. 8). Activation of the CS leads to activation of circuits for body elongation (always the first stage of a locomotory behavior), which would then activate either swim oscillator interneurons or a contraction network that would produce crawling behavior. Based on their observations in experiments in which they denervated all head–brain nerves, Esch et al. (Esch et al., 2002) hypothesized that, though the default locomotory behavior is swimming, signals carried by head–brain nerves from yet-to-be-identified 'shallow water detectors' can bias the system towards activating the crawl network and inhibiting the swim network.

Though no 'shallow water detectors' have been identified in the leech, it is thought that *Caenorhabditis elegans*, which also bases its decision to swim or crawl in part based on water depth (Pierce-Shimomura et al., 2008), may encode water level using a class of dopamine-releasing mechanoreceptors (found in the head), which have sensitivity to substrate texture (useful for distinguishing immersion in water versus laying on a solid surface or suspended in air) (Sulston et al., 1975; Sawin et al., 2000). Dopamine, as well as serotonin, is known to play a role in the decision to swim or crawl. In the leech, dopamine has been found to bias behavioral choice toward crawling (Crisp and Mesce, 2004; Puhl and Mesce, 2008), whereas serotonin makes swimming more likely (Friesen and Kristan, 2007). *Caenorhabditis elegans* also uses dopamine and serotonin to set the balance between these two locomotory behaviors (Vidal-Gadea et al., 2011). Recently it has been discovered that, in the leech, systemically applied dopamine induces R3b1 to burst in a manner consistent with crawling and constrains the entire central nervous system to crawl only (never swim) when R3b1 is electrically stimulated (Puhl et al., 2012). A strategy of employing neuromodulators to encode an environmental context that would naturally be relatively unchanging, such as water depth, would be consistent with our observation [also seen by Esch et al. (Esch et al., 2002)] that leeches poke their heads above water when first introduced to a new environment (and only occasionally after that). A future experiment assaying the relative concentrations of serotonin

and dopamine in the blood of leeches upon exposure to different depths of water would support the existence of such an encoding strategy.

Our experiments with leeches fed to satiation require that our updated behavioral choice model take into account another factor: whether the animal is distended from feeding. Including results from another recent study related to the effect of feeding on behavioral choice (Gaudry and Kristan, 2009), we now know of at least four contextual factors that determine whether the leech will respond to tactile stimulation by swimming (Fig. 9). There are two external contextual factors (site of stimulation and water depth) and two internal contextual factors (whether the leech is currently feeding and whether its body is distended from having recently fed).

The multiplexed impact of these various contextual factors on behavioral choice may be akin to multimodal integration, where multiple stimulus inputs are integrated by neurons whose activity is correlated with discrete behavioral choices. This type of multimodal integration is seen in a variety of systems and in a variety of combinations of modalities (Palmer and Kristan, 2011). For instance, neurons in the dorsal medial superior temporal cortex of monkeys integrate visual and vestibular cues to discriminate headings (Gu et al., 2008) and neurons in the superior colliculus of cats integrate visual and auditory cues to inform eye movement decisions (Meredith and Stein, 1986). Pit vipers integrate infrared information from pit organs with visual information from the eyes in their optic tectum to orient predatory strikes (Newman and Hartline, 1981). Similarly, the contextual factors studied here in the leech (and summarized in our model) are each encoded by stimulus receptors – pressure receptors in the skin, as well as stretch receptors in the body wall (Gaudry and Kristan, 2009) and proposed shallow water detectors in the head (Esch et al., 2002) – whose output may be integrated to activate either the swim or the crawl central pattern generator.

Our results are also consistent with the notion of sequential decision-making (Esch and Kristan, 2002), whereby a given stimulus acts merely as a 'go' signal (e.g. tactile stimulation to the
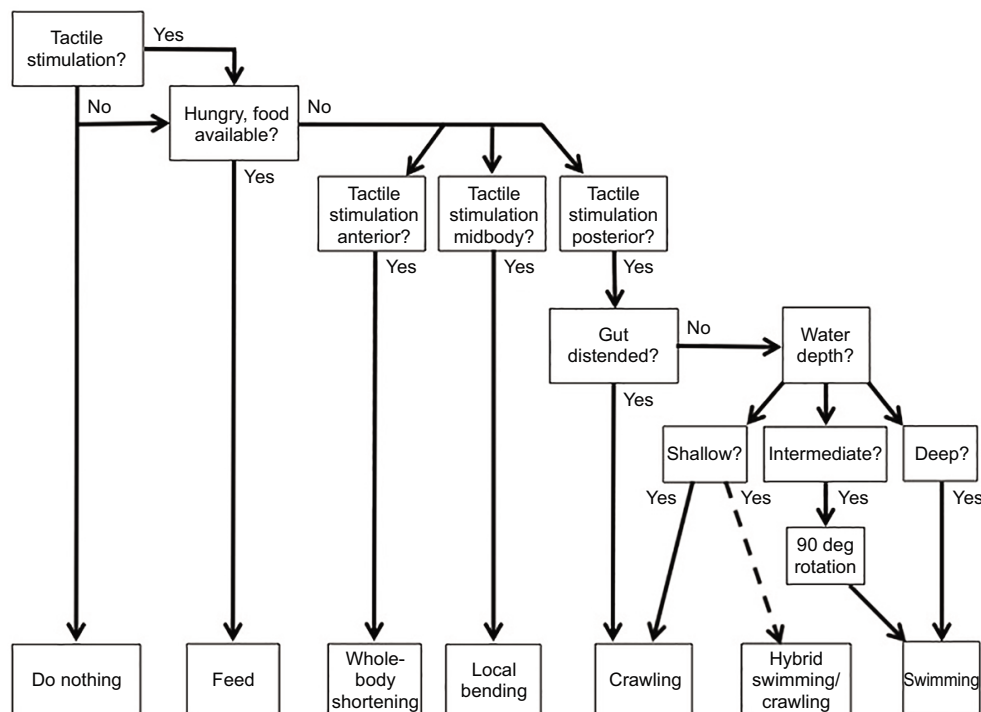

Fig. 9. Behavioral choice flow chart that incorporates all the stimuli and behaviors studied in this paper. This chart shows that the response of a leech to a moderate tactile stimulus depends upon its state of hunger, the location of the stimulus on the body, the degree of gut distention and the depth of the water. It also suggests a hierarchical set of decisions that the nervous system makes in producing a behavioral response. The dashed line indicates that hybrid swimming/crawling occurs infrequently.

posterior portion of the body) to initiate locomotion (e.g. swimming) (Fig. 9). Once the 'go' signal occurs, additional sensory input about external and internal context (e.g. body distention due to feeding, water depth), can then act to bias the choice between alternative behaviors (e.g. swim versus crawl). In the absence of additional sensory input, each alternative behavior will occur at chance levels. This phenomenon has been observed in many versions of the two-alterative forced-choice task (Fechner, 1889) – in a variety of taxa such as birds (Klump et al., 1986; Nelson and Suthers, 2004), mice (Busse et al., 2011), monkeys (Britten et al., 1993) and humans (Green and Swets, 1966) – where, in absence of compelling input, the likelihood of each behavior hovers at chance. Under our model's assumption, leeches have sensors that are activated by shallow water, but have no sensors that are activated by deep water. Consequently, in sufficiently deep water, the choice to swim or crawl would be equivocal. Also supporting this hypothesis is the observation that electrical stimulation applied to mechanosensory neurons in the posterior third of an isolated leech nerve cord produced roughly equal probabilities of fictive swimming and crawling (Briggman et al., 2005). Two possible mechanisms for the choice between swimming and crawling were proposed in this study. First, the choice may depend on the leech's pre-stimulus rest state. Spontaneous 'ongoing activity' has been shown to bias perception in mammals. Ongoing activity, represented in one experiment as coherent, large-scale fluctuations across the cat visual cortex, can explain the variability in cortical responses to repeated presentations of the same stimulus (Arieli et al., 1996). A second possibility is that the leech has no systemic bias prior to stimulation and the choice of locomotory behavior may depend on noise in the system accumulating stochastically until a decision threshold is reached. One prediction of such a mechanism is that the latency between stimulation and behavior onset should be longer in deeper water, where the decision is close to chance (Palmer et al., 2005). It is difficult to precisely determine this decision time based on behavioral observations alone, so future experiments examining the neural circuits responsible for this decision (Briggman et al., 2005) could test the prediction about latency.

MATERIALS AND METHODS
Animals
We obtained adult European medicinal leeches, *Hirudo verbana*, weighing 0.8–2.5 g, from three commercial suppliers: Leeches USA (Westbury, NY, USA), Zaug (Biebertal, Germany) and Carolina Biological Supply (Burlington, NC, USA). Leeches were maintained at 15°C in artificial pond water. Newly arrived leeches were very hungry; they responded strongly to food-related chemicals and the gut sacs proved to be nearly empty in sample animals that we dissected. We used leeches for the feeding studies within a week of their arrival. A different set of leeches was used 2 months after receiving them, when they were even more ravenous.

Animal preparations
Leeches used for mapping behavioral responses
To mark locations on intact leeches, we first anesthetized them in a dish of artificial pond water containing 8% ethanol at 20°C for up to 30 min. [We made the ersatz pond water from Instant Ocean Sea Salt (Aquarium Systems, Mentor, OH, USA) diluted 1:1000 with deionized water.] We then sutured small white or yellow beads onto the dorsal surface of the anesthetized leeches at segments 4, 6, 8, 10 and 12 (for one set of experiments) or 10, 12, 14, 16 and 18 (for a second set of experiments). After 30 min of recovery time in the artificial pond water without ethanol, leeches were transferred to a 49×4.5×7 cm rectangular behavior arena filled with artificial pond water to various depths relative to the leech's diameter, x (0x, 0.5x, 1x, 2x and 4x), to study their responses to mechanical stimulation at various body sites.

Blood-fed leeches
We warmed bovine blood (Animal Technologies, Tyler, TX, USA) to 38°C and placed it into sausage casings made from sheep intestines (obtained from a local delicatessen). We washed the brine from the casings by dousing them several times in deionized water. We placed the leeches on the blood-filled casings in a 10 gallon aquarium filled to approximately 1 cm of artificial pond water. We weighed each leech before and immediately after feeding. It took the leeches 20–30 min to feed to satiation. We then placed the leeches in individual containers for 1 h prior to the first round of behavioral testing.

Arginine-fed leeches
We fed a solution of 150 mmol l^{-1} NaCl and 1 mmol l^{-1} arginine, warmed to 38°C, to a different group of leeches, which were otherwise fed, handled and tested exactly as the blood-fed leeches.

Mapping behavioral responses
To map the dependence of behavioral responses on stimulus location, leeches with bead markers at discrete body segments were placed in a moist rectangular plexiglass behavior arena where water depth=0. Using a hand-held electrode (made from two Teflon-coated 0.05 inch silver wires glued to a stick) triggered with a foot pedal, the leeches were stimulated with a 0.5 s long, 10 Hz train of 1 ms pulses of 6 V. Control experiments (data not shown) indicated that behavioral responses were robust across a wide range of stimulus frequencies and intensities. We stimulated at a point midway between the dorsal and lateral edges, so as not to obscure the top-down view of the camera recording the exact segment stimulated and the onset of the behavioral responses. In other control experiments (data not shown), we stimulated at a variety of locations including the dorsal midline and the lateral edge, observing no differences in behavior. Likewise, for the rotational swims, we performed some stimulation trials on the dorsal midline and observed a similar rate of rotational swimming. Leeches were stimulated in the center of segments 3 through 12 (first set of experiments; 17 leeches tested) or 10 through 19 (second set of experiments; seven leeches tested), as well as at the midpoint between individual segments (Fig. 1A); segments 1–2 and 20–21 were too small to accommodate bead markers. Control experiments (data not shown) suggested that the leeches would only tolerate 100 or so stimulation trials. Because we aimed to perform five trials at each segment and half segment (33 total locations, 165 total trials), we decided to stimulate one set of leeches only at posterior locations and another set only at anterior locations. For similar reasons, we used two sets of leeches in the rotational swim experiments.

Stimulus pulses were produced by a Grass S88 stimulator, and were run through a Grass SIU5A stimulus isolation unit (Grass Instruments, Quincy, MA, USA). Three to five stimulation trials were performed at each site. The order of the trials was randomized. For each trial, leeches were stimulated only when they were near the middle of the behavior arena, and only after they were quiescent for a period of at least 5 s. A black and white video camera (Hitachi HV-C20; Hitachi Denshi, Tokyo, Japan) running at 30 Hz was used to record each trial (minimum 8 s long). Video recordings were saved onto a PC using MATLAB (MathWorks, Natick, MA, USA). The camera was mounted on a track with roller bearings to follow the progress of the leech in the behavior arena. Video records were used to verify the location of the stimulus and to characterize the behavioral responses as one of the following: whole-body shortening, local bending, crawling, swimming (Fig. 1B), hybrid swimming/crawling, other or none (see Results for descriptions of each behavior).

Behavioral effects of water depth on stimulus location
To study the interaction of water depth and stimulus location on the leech's behavioral responses, we repeated the mapping procedure described above with the behavior arena filled with water to various depths. For each animal, depths relative to the leech's dorsoventral diameter were tested: 0.5x, 1x, 2x and 4x (Fig. 2). For these experiments, the leeches were stimulated at segments 3 through 12 (first set of experiments; seven leeches) or 10 through 19 (second set of experiments; seven leeches). For each depth, two trials were performed at each of 10 segments, for a total of 80 stimulations per leech. Before placing a leech in the arena, we manually agitated the

leech to promote activity (we placed it between our cupped hands and shook it vigorously for approximately 3 s). Leeches typically continued to move in one direction; if they changed course in the middle of the chamber, we manually turned them back to the original direction. We removed the leech being tested from the chamber before changing the water level and we randomized the order of water level changes for each leech.

Behavioral interactions between water depth and time since last feeding

The experiments to determine the behavioral responses of blood-fed and arginine-fed leeches varied from the previous experiments only in that these leeches were stimulated manually rather than electrically, at a location along the posterior third of the animal, a region previously shown to elicit either swimming or crawling. Neither the exact location nor its intensity was a concern; we stimulated just strongly enough to produce a locomotor response, tallying whether the response was swimming or crawling. We tested the responses of seven blood-fed leeches every 1–4 days for 30 days, then at days 50 and 60, when their behavior had returned to control levels. We tested 11 arginine-fed leeches every day for a 7-day period, by which time their behavior was erratic and several of them had died. Because each leech lost mass throughout the 60 days post-feeding, the water levels ($0.5x$, $1x$, $2x$ and $4x$) in the behavior arena were adjusted to the leech's dorsoventral thickness on each day of the experiment. Two trials were performed per day at each water depth, presented in a randomized order. We calculated the percentage of behaviors in each category for each water level independently for each leech on each day of testing.

Measurement of body distention

After leeches feed, their bodies swell to approximately 10 times their pre-fed size. Over time, the blood is converted into body tissue, so the leeches grow larger and are thereby less distended. To measure this change in body distention, baseline masses were obtained for 40 leeches, which were then fed to satiation and reweighed. Every day or two for 2 months, all leeches were reweighed and two of them were dissected, drained of the blood in their gut pouches, and weighed again. (Because this method of measuring stored blood volume necessarily killed the animal, the number of leeches decreased over the 60 days of the study.) The decreased distention observed reflected the blood volume in the leech at a given time relative to the amount of blood immediately after a feeding. More specifically, distention (D) was calculated at these later time points as:

$$D = M_d - M_{pre}/M_{fed} - M_{pre}, \qquad (1)$$

where M_d is the leech's mass after being drained of blood, M_{pre} is the pre-fed mass and M_{fed} is the mass immediately after feeding.

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