Behavioral choice across leech species: chacun à son goût

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SUMMARY

At any one time, animals are simultaneously bombarded with many sensory stimuli, but they typically choose to respond to only a few of them. We used multidimensional analysis to determine the behavioral responses of six species of leeches to stimulation, as the responses are affected by species identity, diet, behavioral state and stimulus location. Our results show that each of the species tested while not feeding displayed remarkably similar behaviors in response to tactile stimulation of the surface of the body. When not feeding, stimulus location was the most reliable factor in determining behavioral response. While feeding, the three sanguivorous (bloodsucking) species tested ignored stimulation, whereas the three carnivorous leeches abandoned feeding in favor of locomotory responses, regardless of phylogenetic relationships. In the sanguivorous leeches, feeding abolished all mechanically elicited responses and mechanical stimulation in turn had no effect on feeding. We also show that the behavioral hierarchy of leeches was fixed and unchanging even in species that can consume both a carnivorous and a sanguivorous diet.

Key words: behavioral choice, behavioral hierarchy, leech, evolution, decision making.

INTRODUCTION

Over the past several decades the organization and function of decision-making circuits has been established in a wide range of species (Briggman et al., 2005; Calabrese, 2003; Dickson, 2008; Glimcher, 2003; Heekeren et al., 2004; Yang et al., 2008). However, little attention has been given to the evolution of these circuits within a phylogenetic clade. For instance, two species might show similar responses to the same stimulus either because they share ancestry or because they fill the same ecological niches. Feeding is a necessary function for animals of all kinds, but when presented with potential predation risk, a feeding animal must decide whether to continue feeding and risk being eaten or discontinue feeding and attempt to escape. Phylogenetic relatedness may contribute to similarities among species in making such a decision, but other ecological factors, such as diet, may produce convergent evolution between clades. Consequently, superficially similar behavioral patterns between two distantly related species may result from drastically different processes.

Invertebrate preparations are ideal for addressing this question. Their reduced number of neurons and simple decisions make it possible to determine the role of individually identified neurons in the decision-making process (Kristan and Gillette, 2007). One of the simplest decision-making tasks is to choose an appropriate response to conflicting stimuli. Because animals typically express a single behavior at a time (Sherrington, 1906), they must choose which stimulus to pay attention to and which to ignore. This paradigm has been used extensively to probe the priority that an animal places on a specific behavior (Carr et al., 2002; Edwards, 1991; Jing and Gillette, 1995; Misell et al., 1998; Norekian and Satterlie, 1996). Such studies can establish behavioral hierarchies by presenting an animal with two stimuli simultaneously that induce distinct behaviors when presented alone. The behavior elicited by the dual stimulus task is said to be dominant over the suppressed behavior (Davis et al., 1974a; Davis et al., 1974b).

Leeches are particularly suitable for studying the evolution of decision-making circuits. Many of the networks governing leech behaviors have been characterized from their sensory receptors, through complex layers of interneuronal networks, to motor outputs (Kristan et al., 2005). This rare luxury makes it possible to ask directly how two circuits interact at the cellular level to suppress one another (Shaw and Kristan, 1997; Briggman et al., 2005; Gaudry and Kristan, 2009). The behavioral hierarchy of the medicinal leech, *Hirudo*, has been well documented (Dickinson and Lent, 1984; Misell et al., 1998). In this obligate sanguivore, feeding reduces or abolishes the expression of all mechanically elicited behaviors. What remains unknown, however, is the prioritization of behaviors in other leech species, particularly carnivorous species, which occupy a drastically different ecological niche. The phylogenetic relationship between the major groups of leeches is well characterized (Borda et al., 2008; Borda and Siddall, 2004), and carnivory and sanguivory are both distributed throughout leech phylogeny. The repeated independent evolution of these feeding patterns makes it possible to test the relative importance of phylogenetic relationships and feeding ethology on establishing a behavioral hierarchy. Additionally, homologous neurons have been identified across numerous species (De-Miguel et al., 2001; Elsas et al., 1995; Kramer et al., 1985; Lent et al., 1989; Lent and Frazer, 1977; Nusbaum and Kristan, 1986), so that the evolution of homologous circuits can be distinguished from the evolution of non-homologous ones. The aim of this study was to establish the behavioral hierarchy of several leech species representing multiple families. We began by characterizing the basic feeding properties of each species and then looked at the complex interactions between diet, stimulation site and phylogenetic relationship in governing behavioral output. This behavioral framework will enable the future analysis of the evolution of decision-making circuits within a well-established experimental model system.

MATERIALS AND METHODS
Acquisition and maintenance of leech species

Leech species were acquired from various sources. *Hirudo verbana* L. was purchased from Carolina biological (Burlington, NC, USA) under the name *Hirudo medicinalis* (Siddall et al., 2007). All *Haemopis marmorata* Say and some *Macrobdella decora* Say were acquired from Vados Bait (Spring Lake Park, MN, USA). *Hirudinaria manillensis* L. was collected in Thailand; *Semiscolex* sp. were collected in Uruguay. *Erpobdella obscura* Verrill, *Placobdella rugosa* Verrill and additional *M. decora* were collected in ponds near Lake Itasca (MN, USA). *Hirudo verbana* and *H. manillensis* were housed in dilute Instant Ocean synthetic sea salts, made up as full-strength seawater that was diluted 1:1000 with deionized water (Gaudry and Kristan, 2009; Briggman et al., 2005; Misell et al., 1998). *Erpobdella obscura*, *P. rugosa*, *M. decora*, *Semiscolex* sp. and *H. marmorata* were all maintained in an artificial pond water: 47.9 mmol l^{-1} NaCl, 0.67 mmol l^{-1} KCl, 0.34 mmol l^{-1} Ca(NO$_3$)$_2$·4H$_2$O, 0.1 mmol l^{-1} MgSO$_4$·7H$_2$O, 100 mmol l^{-1} Hepes, pH 7.4, and diluted 1:100 with deionized water. To this was added calcium and magnesium salts: 20 ml of a stock solution of CaCl$_2$·2H$_2$O (13.2 g) and MgCl$_2$·6H$_2$O (13.2 g) in 1 l of deionized water was added to 200 ml of artificial pond water and the final volume was raised to 20 l. We found that these leeches could not be successfully maintained in the diluted Instant Ocean water. Tanks housing *H. verbana*, *E. obscura*, *P. rugosa*, *M. decora* and *H. marmorata* were all kept at 15°C. *Hirudinaria manillensis* and *Semiscolex* sp. were kept at room temperature (~24°C).

Feeding experiments

All leeches were individually fed in separate containers and weighed before and after every feed for each experiment. Prior to feeding experiments, all sanguivorous leeches displayed behavior typically associated with hungry leeches, such as raising of the head and flaring the dorsal lip in response to agitating the water surface in their aquaria. Dissection of similar tank mates confirmed that the gut of these animals had no residual blood from a previous meal. Sanguivorous leeches were all fed on fresh bovine blood (Animal Technologies, Tyler, TX, USA) in a sausage casing (purchased from delicatessens), warmed to 38°C. This blood contained the anti-clotting agent heparin and was used within 24 h. The duration of a bout of feeding in sanguivorous leeches was defined as the attachment of the front sucker to the blood sausage until the release of the front sucker after blood ingestion. The percentage body mass increase was calculated by the following equation:

% increase in body mass = [(post-mass/pre-mass) – 1] × 100 .

Carnivorous leeches received weekly meals to ensure that they would not starve. *Erpobdella obscura* was fed on fresh bovine liver purchased from a grocery store and *Semiscolex* sp. was fed on live black worms purchased from a tropical fish distributor. To ensure their motivation to feed, all carnivorous leeches were food deprived for 1 week prior to pretesting and deprived of food for another week between the prefeeding and feeding sessions. Feeding in carnivorous leeches was measured as the number of bites and mass gain during a 30 min exposure to food. To determine the effects of starvation on the behavioral repertoire of a carnivorous leech, we also deprived the carnivore *E. obscura* of food for 4 months before experiments (data not shown; see Discussion).

Stimulation protocol

We used a previously established protocol (Misell et al., 1998) to measure the responses to tactile stimulation of all leech species.

Trains of electric shocks were delivered to the body wall using a hand-held electrode. The terminals of the electrode were made from two teflon-coated 0.008 in (0.2 mm) diameter silver wires with the metal tips exposed and separated by approximately 1 mm. We touched the tips of the wires to the leech's skin and held them in place before the electrical stimulus was given. This ensured that the response of the animal was due to the electrical stimulation of the mechanosensory neuron terminals and not to the placement of the stimulating electrode. Electrical stimulation of the skin activates the terminals of pressure-mechanosensory (P) cells and is thus directly equivalent to a mechanical stimulus (Kristan et al., 1982; Nicholls and Baylor, 1968; King-Wai, 1976). This stimulation method also allowed us to maintain consistent stimulus intensities between trials. We refer to all electrically mediated behaviors as mechanosensory responses. Stimulation was applied until the animal responded or for a maximum of 2 s, whichever occurred first. Shocks consisted of trains of 1 ms 8 V pulses at 10 Hz delivered through a Grass S88 Stimulator (Grass, Quincy, MA, USA) with a Grass Stimulus Isolation Unit. Stimuli were delivered randomly to the anterior, midbody or posterior of the leech. In pre-feeding sessions, stimuli were delivered every 3 min for 45 min. During feeding, stimuli were delivered to sanguivorous leeches every 3 min for the duration of the feeding bout. In carnivorous leeches, stimuli were delivered only if the leech was actively biting a food item although never more frequently than once per 2 min period. Responses by leeches to stimulation were characterized according to Table 1.

Statistical analysis

Comparisons of stimulated trials *versus* unstimulated trials and mass gain during feeding were done with paired Student's *t*-tests and contingency tables, using either Matlab statistical toolbox (MathWorks, Natick, MA, USA) or GraphPad online calculator. Paired Student's *t*-tests were used in experiments where the data were non-percentage based. Contingency tables were used on all percentage-based data using the total number of trials within an experiment. Thus, these data do not contain error bars reflecting the differences across test subjects. Contingency table analysis allowed us to calculate population percentages without having to rely on percentages drawn from animals with too few trials (such as the carnivore feeding experiments).

Behavioral response data were treated as a multivariate set, and were analyzed using canonical correspondence analysis (CCA) (Ter Braak, 1986) in R statistical software (http://www.r-project.org/), with the Vegan extension (http://vegan.r-forge.r-project.org). CCA seeks to find coordinated patterns of change across multiple response categories simultaneously, in response to a set of predictor variables (such as stimulus location). It has been applied to a variety of problems, principally in ecology, but has been used successfully to analyze experimental behavioral data in the leech as well (Cornford et al., 2006). CCA allows one to identify trends in expansive data sets by revealing these patterns graphically in an easy to interpret manner. Because behaviors are not independent, and in many cases are mutually exclusive, multivariate analysis is the best approach to understanding behavioral responses because it characterizes responses in suites of behaviors, and accounts statistically for a lack of independence among responses. Additionally, the total variance that CCA can explain in a multivariate data set is a quantitative indicator of how well all of the chosen predictors relate to the observed outcomes, accounting for correlations among them. For example, one could use a variety of *t*-tests or contingency tables on the data set to identify differences in behavioral responses. However,

Table 1. Behavioral response categories

Behavior	Description
Front sucker release	Detachment of the front sucker from the substrate
Back sucker release	Detachment of the back sucker from the substrate
Tense	Slight contraction of the body muscles
Local bend	Local shortening of the body on the side of the stimulus and elongation on the opposite side
Bend	Bending of the whole body away from the stimulus site
Crawl	Release of back sucker, shortening, reattachment of back sucker, and release and extension of the front sucker; half steps (back sucker release, shortening and reattachment of the back sucker) were also included in this category
Swim	Release of both suckers and a dorsal–ventral undulation of the entire body
Shorten	Withdrawal of the anterior end away from the stimulus, toward the posterior end
Curl	Rolling the anterior end ventrally to form a tight spiral; flattener muscles rigid and leech remains tightly curled in ball for tens of seconds to minutes
Other	Various uncategorized responses including the release of both suckers when stimulus was applied and writhing (these behaviors were so rare that we removed them from our analyses)
No response (NR)	No response to stimulus

this analysis would fail to reveal how much of the variance in behavioral output is actually caused by our predictors (species, feeding status, stimulus location), and would treat each significant effect as a separate, independent result, rather than as part of a multivariate suite of responses. In other words, CCA can reveal whether an important predictor has been ignored in the analysis, whereas a non-multivariate approach either could overlook this important fact or could overstate the strength of response by counting each positive effect as a separate, independent result. The variance in such a data set can be calculated with CCA and is termed 'inertia' (described below).

CCA is best applied to discrete data (in our case the number of observed behaviors) taken from individual observations (such as our trials). This analysis is sensitive to both linear and unimodal responses. This is important for our data set as some behaviors were observed to vary in either a linear fashion (such as an increase in locomotory responses with stimuli presented farther from the anterior end of the animal) or a unimodal manner (for example, observed local bending peaks in response to midbody stimulation and decreases with either anterior or posterior stimulation). CCA analysis is based on identification of 'axes' that each represent coordinated patterns of change in behavior across multiple responses, but that are independent of one another. The first CCA axis represents the strongest pattern of response, and subsequent axes decline in importance. Axes are constrained to align with a set of predictor variables, in this case represented by the stimulus location (anterior, midbody, posterior), leech species or diet (sanguivore or carnivore). CCA requires numeric predictors, so categorical predictor variables were dummy coded (i.e. levels of categorical variables, such as the carnivore and sanguivore levels within the diet variable, were each given a variable, and assigned a 1 when the leech belonged to the level and a 0 when it did not).

CCA axes are linear combinations of the raw behavioral variables, and both the raw variables and the individual leeches were assigned 'scores' on these linear combinations that are used for interpretation of patterns. The sign (negative or positive) of the CCA score is arbitrary, although once determined all related variables will receive scores with the same sign, and only the distance between groups along the CCA axes is important for interpretation. The measure of variance in CCA analysis (and many other multivariate analyses) is commonly referred to as inertia. Inertia is a multivariate measure of variance across all of the responses, with covariances between responses deducted; consequently, the variance explained by a

predictor in several correlated responses is not counted multiple times. We typically report the percentage of the total inertia that our analysis explains. This value, the explained inertia, represents how well our predictors (stimulus location, diet, etc.) can reliably predict the behavioral output of stimulated leeches, and is thus analogous to the regression sums of squares in a simple linear regression. Any residual inertia (or unexplained inertia) is likely the result of predictors that we could not or did not measure, or of unexplainable random variation in response. Residual inertia is thus analogous to residual sums of squares in a regression analysis. For example, if the internal state of the animal impacts its behavioral choice, this would lead to additional variance in our data set that cannot be accounted for by our measurements. The percentage of inertia explained by our set of predictors is, by extension, analogous to the coefficient of determination (R^2) from regression analysis. The sampling distribution for CCA models does not follow a simple parametric sampling distribution (such as the t or F), and thus statistical significance of CCAs is determined with a randomization test, in which the behavioral responses are randomly shuffled relative to the predictors, and CCA is conducted on each of these pseudo-data sets. The overall and explained inertia for each randomized data set is calculated, and F-values are calculated as observed divided by total inertia. The F-ratio from the observed data is then compared with the distribution of F-ratios for randomized data, and the number of F-ratios that are greater than or equal to the observed F-ratio is used to calculate a P-value. We used 999 iterations for all of our CCA analyses.

The primary method for displaying the results of a CCA is the biplot. Scores for behaviors on the first two CCA axes are plotted, such that behaviors that are close together on the biplot were typically exhibited during similar conditions, and behaviors that are far apart on the biplot typically did not occur during trials with similar conditions. Independent variables are plotted over the behaviors as vectors projecting from the origin. The direction of these vectors points to the behaviors they best helped to explain, and the length of the vectors represents the ability of that given independent variable to separate behaviors. Consequently, the behaviors with the largest distances between them in CCA space are those that change the most in response to the independent variables.

All leech species were stimulated in the same body locations, but we expected that responses to stimulation would differ by species. This difference could be due either to species-specific

characteristics or to differences in feeding mode (sanguivore or carnivore). However, because most leech species have only one feeding mode, it was not possible to simultaneously assess the relative importance of species or feeding mode in determining response to stimulation. Consequently, we conducted two sets of CCA analyses, one in which feeding mode was a predictor and another in which species was a predictor; stimulus location was included in both sets. The relative importance of species or feeding mode in explaining behavioral responses was assessed by comparing the proportion of deviance explained by each set of predictors.

RESULTS

We first determined the behavioral hierarchies across leech species. Specifically, we investigated the relative priorities of feeding and avoidance responses within a variety of leech species. Before studying interactions between behaviors across leech species, however, we first characterized the basic feeding properties of each species.

Mechanosensory stimulation does not affect feeding in either sanguivores or carnivores

Fig. 1A shows the phylogenetic relationship among the species tested in our study. A dashed line denotes a carnivorous species and a solid line indicates a sanguivorous leech. The asterisk represents an ancestor that could have either diet. Carnivorous and sanguivorous species are distributed throughout the clade, allowing us to test the relative importance of phylogenetic relationships and feeding ethology on the decision to perform behaviors.

We measured the percentage increase in body mass during feeding for six different species of leeches. The sanguivorous leeches, *H. verbana*, *M. decora* and *H. manillensis*, were fed on bovine blood warmed to 38°C, and *E. obscura* and *H. marmorata* were fed on fresh raw beef liver. *Semiscolex* sp. would not feed on liver but readily consumed live black worms and would either take individual bites from worms or swallow them whole over a period of minutes. All sanguivorous leeches showed a significant mass increase during feeding (Fig. 1B) while none of the carnivorous species gained appreciable mass.

Feeding in the sanguivorous leech *H. verbana* suppresses all mechanically elicited behaviors (Misell et al., 1998), making feeding a dominant behavior in this leech's behavioral hierarchy. However, whether the relationship between feeding and mechanosensory behaviors is absolute or reciprocal is unclear. In the absolute case, feeding would suppress mechanosensory-elicited responses and be unaffected by mechanosensory stimulation. In the reciprocal case, mechanosensory-elicited responses would be decreased during feeding and feeding behavior would be diminished by mechanosensory stimulation. We thus wanted to determine which was the case.

Fig. 2A shows the duration of single feeding episodes across the sanguivorous leech species. Stimulation of the body wall during feeding was found to have no effect on the duration of feeding in any of the sanguivorous species tested. Carnivorous leeches are intermittent feeders and did not engage in long continuous feeding bouts so we could not define durations for their feeding episodes. We were able, however, to measure the body mass in all species and determine whether stimulation affected the total amount consumed. We allowed carnivorous leeches to feed for 30 min, then delivered tactile stimuli only when they were in mid-bite, and no more often than once every 2 min. Stimulation during feeding did not significantly alter the increase
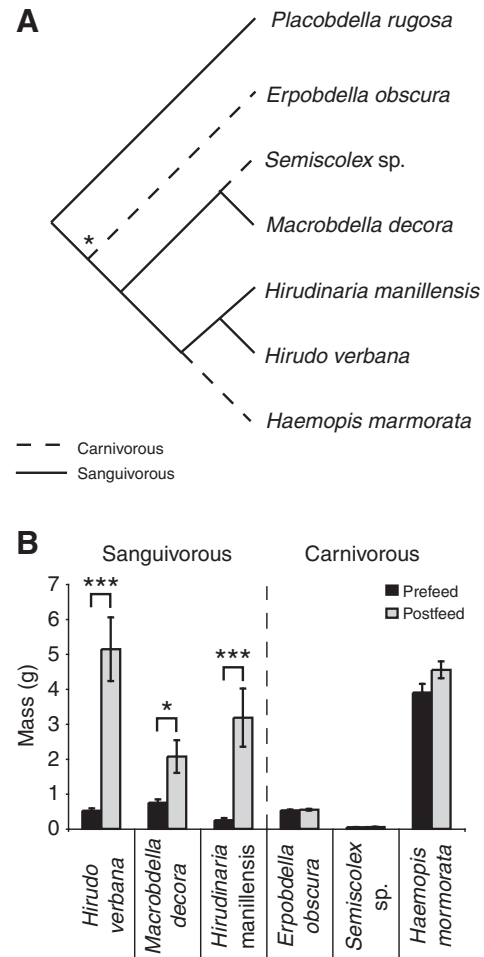

Fig. 1. Leech phylogeny and feeding behavior. (A) The phylogenetic relationships among the leech species used in this study. The asterisk indicates an ancestral state with an unknown diet. (B) Average mass of each species before and after feeding. Sanguivores were fed on warmed bovine blood until sated. Carnivorous leeches were fed for 30 min on either raw bovine liver (*Erpobdella obscura* and *Haemopis marmorata*) or live black worms (*Semiscolex* sp.). ***P<0.001, **P<0.01, *P<0.05. Error bars represent s.e.m. *N*=8 *Hirudo verbana*, *N*=8 *Macrobdella decora*, *N*=8 *Hirudinaria manillensis*, *N*=6 *Erpobdella obscura*, *N*=9 *Semiscolex* sp. and *N*=10 *Haemopis marmorata*.

in percentage body mass across all leech species, whether they were sanguivores or carnivores (Fig. 2B). This analysis was an insensitive measure of food intake for the carnivores because their food intake was insignificant even during unimpeded feeding. Thus, we also measured the number of bites taken by the carnivores in the first 30 min after food was presented. Even with this more sensitive measure, mechanosensory stimulation did not significantly affect the number of bites taken by the three carnivorous species (Fig. 2C). In each of the six species tested, stimulation did not significantly affect feeding behavior.

Feeding in one of the leech species (*H. verbana*) strongly inhibits mechanosensory-elicited responses (Misell et al., 1998) [note, Misell et al. refer to the species as *H. medicinalis*, although it was later reclassified as *H. verbana* (Siddall et al., 2007)], while mechanosensory stimulation has no effect on feeding. Thus feeding and mechanosensory-elicited responses have an absolute relationship in *H. verbana*, not a reciprocal one. To determine
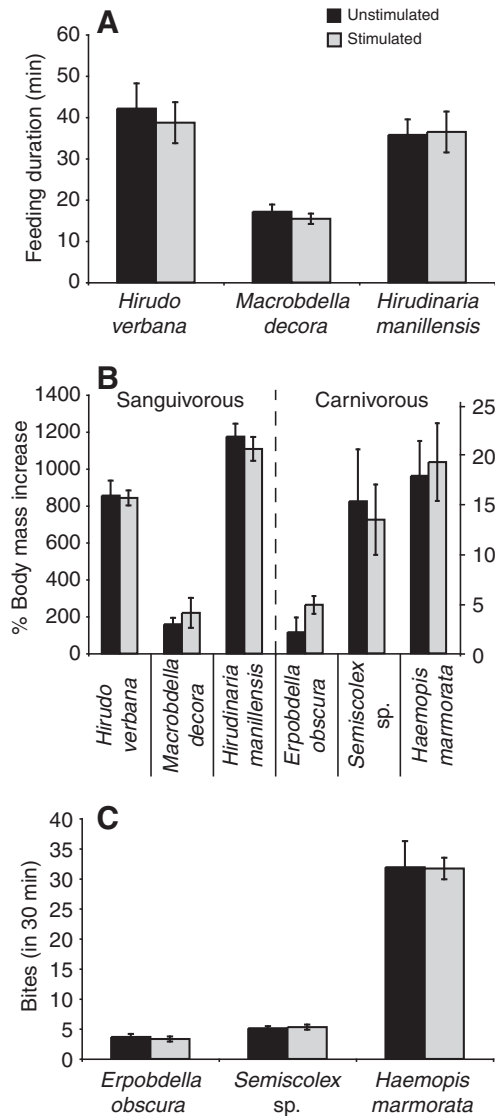
Fig. 2. The effect of mechanosensory stimulation on leech feeding. (A) Sanguivorous leeches were fed on warmed bovine serum through a feeding apparatus and either stimulated every 3 min or left to feed undisturbed. The stimulus location was pseudo-randomly varied between the anterior, middle and posterior regions of the animal. The duration of feeding was measured from the start of pharyngeal peristalsis after the front sucker was attached until the leech released its front sucker from the feeding apparatus. No statistical differences were observed between unstimulated and stimulated trials. (B) The percentage increase in body mass was measured after each feeding episode in both the stimulated and unstimulated case. The left scale applies to sanguivorous leeches and the right scale applies to carnivores. Carnivores were allowed to feed for a period of 30 min and stimulated in the same manner as described for the sanguivores. No differences between unstimulated and stimulated trials were found. (C) The number of bites taken by carnivorous leeches was quantified for each 30 min feeding trial in both stimulated and unstimulated animals. No statistical difference between stimulated and unstimulated trials was found. N values for unstimulated data are given in Fig. 1B; for stimulated data: $N=10$ *Hirudo verbana*, $N=7$ *Macrobdella decora*, $N=9$ *Hirudinaria manillensis*, $N=6$ *Erpobdella obscura*, $N=6$ *Semiscolex sp*. and $N=9$ *Haemopis marmorata*.

whether this relationship held for all the leech species, we determined whether feeding affected their responses to mechanosensory stimulation.

Feeding diminishes all mechanosensory-elicited behaviors only in sanguivores

Each time we stimulated a leech as it fed (see experiments above), we monitored its behavioral response. We compared these data with data from trials that we had conducted on the same animal 2 weeks previously, when it was not feeding. We plotted the 11 behavioral responses to mechanosensory stimulation at three sites in the six species when they were not feeding (Fig. 3A) and during feeding (Fig. 3B).

There are clear differences between feeding and non-feeding animals: all of them mostly shortened in response to stimulation of the anterior end, produced bending (both local and whole body) in response to stimulation of the midbody, and showed mostly locomotory responses (swimming or crawling) to stimulation of the posterior end when not feeding (Fig. 3A), and these responses tended to be less likely during feeding (Fig. 3B). There were some notable differences, however. For instance, all the sanguivores (*Hirudo*, *Macrobdella* and *Hirudinaria*) increased the number of local bending responses in all regions while feeding, whereas the carnivores (*Erpobdella*, *Semiscolex* and *Haemopis*) produced other behaviors instead. To quantify such differences, we compared the data from just these behaviors during feeding and non-feeding (Fig. 4).

Fig. 4A shows that the percentage shortening produced by anterior stimulation was similar prior to feeding in all species and decreased during feeding. During feeding, the sanguivores never shortened, whereas the carnivores did shorten and abandoned feeding significantly more frequently than their sanguivorous counterparts. Bending and local bending are the most prominent behaviors elicited in response to midbody stimulation in non-feeding leeches. Both sanguivores and carnivores showed a significant decrease in these behaviors during feeding (Fig. 4B). However, unlike shortening, bending and local bending did not interfere with feeding; both were still observed in all species as they fed. Stimulation of the posterior body wall typically resulted in locomotion (crawling and swimming) in sanguivores (~88% of trials across the three sanguivorous species) when the animals were not feeding (Fig. 4C). Non-feeding carnivores responded to posterior stimulation by either locomoting away from the stimulus (Fig. 4C) or curling into a sustained rigid tight ball (Fig. 3A). This type of curling was qualitatively different from the curling sometimes seen in *Hirudo* during strong nociceptive stimulation (such as pinning the animal prior to dissections). The curling described here involved a rigid and maintained contraction of the flattener muscles. *Erpobdella* would often assume this posture upon gentle handling and remain curled for tens of seconds to minutes at a time without any of the additional writhing responses that can be observed with *Hirudo* during strong nociceptive stimulation. Another clear difference between the two types of leeches was that carnivores often abandoned a feeding episode by either swimming or crawling, while sanguivores never locomoted in response to stimulation during feeding.

Rather than comparing each behavior independently to analyze the data in Fig. 3, we used CCA to highlight major differences in the behavioral hierarchies of the two types of leeches. Fig. 5 shows a biplot of the first two CCA axes for our analyses of the data in Fig. 3 using feeding status (green), stimulus location (blue) and species (red) as predictors. The first CCA axis (the horizontal axis) is dominated by stimulus location with negative CCA1 values being most correlated with a stimulus to the anterior end of the leech (i.e. it has the largest – in this case, negative – value along the CCA1 axis). Trials that elicited shortening also have negative CCA1
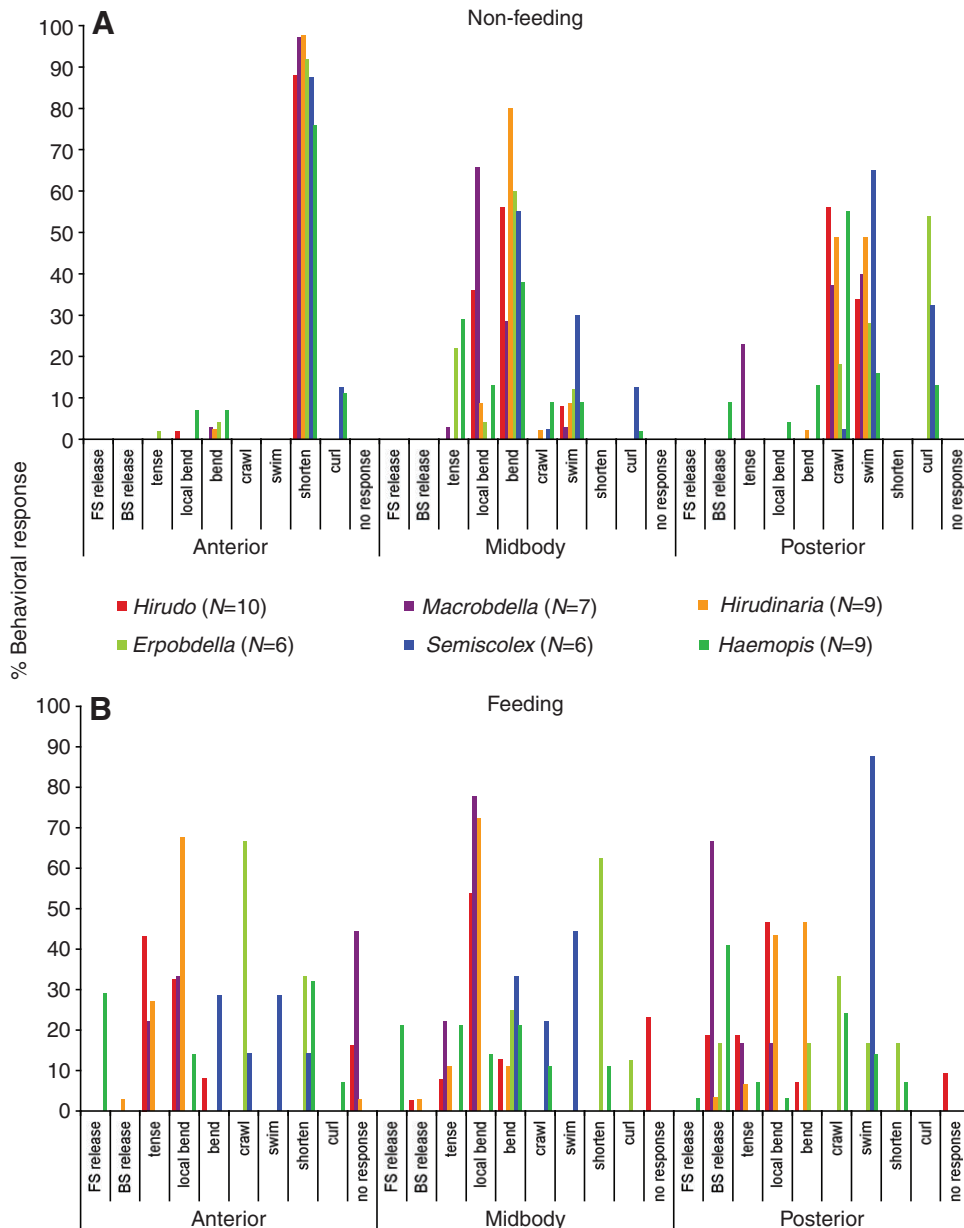
Fig. 3. Behavioral responses to stimulation across leech species. Each leech was stimulated five times at three different locations for a total of 15 trials per leech. (A) The stimulation-induced behavioral responses of leeches in the non-feeding state. *N* values correspond to the number of individual animals tested. The responses listed are defined in Table 1. Trials and responses at each stimulation site were grouped for each species and the behavioral response recorded is the percentage of trials showing a particular behavior out of all trials on that species. (B) The responses to stimulation of each leech species while the animals are engaged in feeding behavior. The number of trials across individual animals varied with feeding duration in the case of sanguivores and with the number of bites for carnivores.

values, which shows that shortening behavior often resulted from trials with anterior stimulation. In contrast, feeding behavior and species identity made more minor contributions to CCA1. CCA2 separates trials resulting in curling, swimming and crawling from trials leading to local bends, tensing, front sucker release and no response. CCA2 is most influenced by the status of feeding and whether midbody or posterior stimuli where presented to the leech. Overall, species identity had only a minor impact on the inertia in our data set; stimulus location and feeding status at the time of stimulation had major impacts. This CCA explained 66.2% of the total inertia in our data set ($P<0.001$), and the first two CCA axes explained 24.3%. Thus, by knowing only the stimulus location and feeding status of the animals (which dominate CCA1 and CCA2), we can explain more than 24% of the variability in their behavioral responses during stimulation. Additional variability (the 41.9% accounted for by the remaining CCA axes) can be explained by taking into account stimulus location, feeding status and species identity. Note that the contribution of a predictor to one CCA axis does not preclude it from contributing to additional axes.

The differences in pattern evident between feeding and non-feeding behaviors (compare Fig. 3A with B) required more detailed explanation, and thus we next analyzed the feeding and non-feeding trials separately using CCA. Stimulus location is the major determinant of the response to stimulation when leeches are not feeding, with species identity once again being a minor contributor to the overall inertia (Fig. 6A). As was apparent in the data summaries (Fig. 3), shortening was near to the anterior stimulation site, bending and tensing were close to the midbody site, and swim, crawl and back sucker release were near the posterior stimulation site. This CCA explained 50.6% of the inertia in the data set overall ($P<0.001$), and the first two CCA axes explained 41.3%. These data show that regardless of the species, the responses of non-feeding leeches were generally similar and dependent on stimulus location. Anterior stimulation led to shortening (negative CCA1 values), midbody stimulation led to local bending, bending and tensing (positive CCA2 values), and posterior stimulation led to crawling, curling, swimming and release of the back sucker (negative CCA2 values). In contrast with the non-feeding results, during feeding species identity becomes
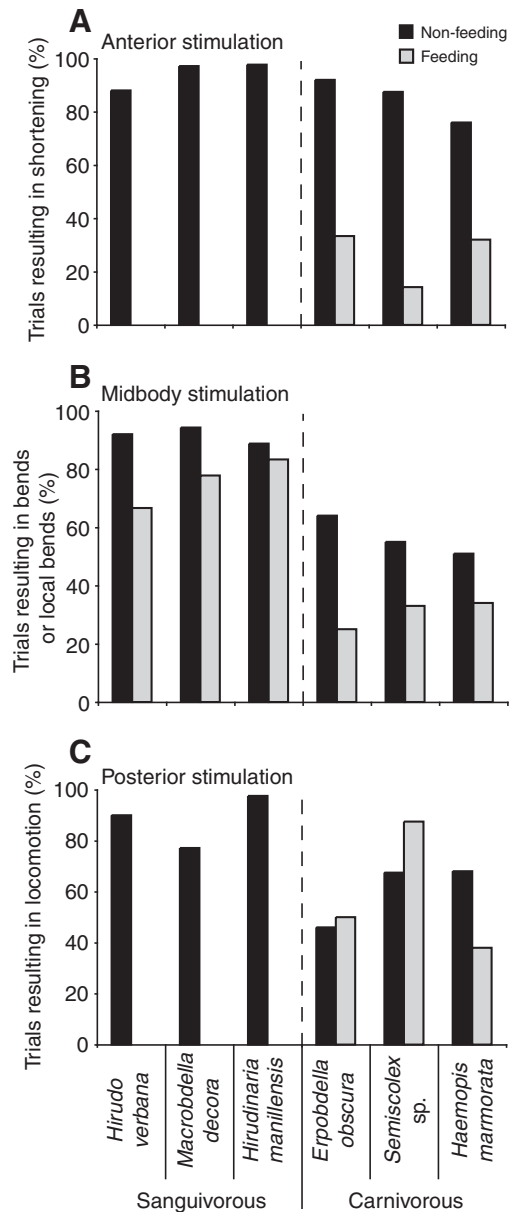
Fig. 4. Interspecies differences in behavioral responses. (A) The percentage of trials resulting in shortening in response to stimulation of the anterior end of leeches during feeding and non-feeding. The dashed vertical line separates sanguivorous and carnivorous species. Carnivorous and sanguivorous leeches show indistinguishable amounts of shortening in the non-feeding state, but carnivores show significantly more shortening during feeding than do sanguivores ($P<0.0001$; 38 carnivore trials and 81 sanguivore trials). (B) The percentage of midbody stimuli that elicited either local bending or bending. During feeding, both sanguivores and carnivores significantly decreased their percentage of local bending and bending responses ($P<0.05$ for carnivores and $P<0.01$ for sanguivores; carnivore trials: 105 non-feeding and 45 during feeding; sanguivore trials: 130 non-feeding and 85 during feeding). (C) The percentage of trials resulting in locomotion (either swimming or crawling behavior) in response to posterior stimuli across leech species. Carnivores showed a significantly higher percentage of locomotory responses compared with sanguivores ($P<0.0001$; 43 carnivore trials and 77 sanguivore trials).
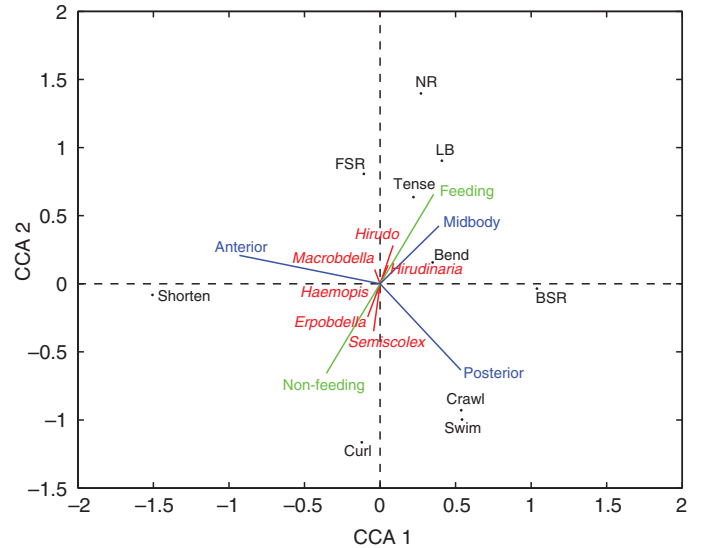

Fig. 5. Canonical correspondence analysis (CCA) biplot for all behavioral data. Behaviors observed during trials are plotted against synthetic CCA axes as points in the biplot. These are the total combined data for Fig. 3A, with feeding status included as a predictor variable. CCA axes are ranked with the first axis explaining more variance in the data set than each subsequent axis. Behaviors farthest from the origin of the plot were most easily distinguished from each other by the independent variables (stimulus location, blue; feeding status, green; and species, red). Independent variables are plotted as vectors projecting from the origin. The direction of each vector indicates which behaviors it most effectively sorted and which CCA axis it contributed most highly to. Vector magnitudes indicate the importance of that experimental condition in distinguishing among behaviors. Stimulus location, feeding status and species identity explained 66.2% of the inertia in the data set ($P<0.001$), while these two axes explained 24.3%. LB, local bend; BSR, back sucker release; FSR, front sucker release; NR, no response.

the dominant determinant of behavioral response to stimulation (Fig. 6B), as species becomes the greatest source of explained inertia and makes the greatest contribution to CCA1. This CCA explained

a total of 72.1% of the inertia in the data ($P<0.001$), and the first two CCA axes explained 20.4%. The carnivorous species, *Haemopis*, *Erpobdella* and *Semiscolex*, all have negative CCA scores and cluster with behaviors that cannot happen during ingestion. These behaviors include shortening, front sucker release, crawling and swimming. The sanguivores all have positive CCA1 scores and cluster with local bending, tensing and no response. Stimulus location is still very important in determining the resulting behaviors and is a large component of the second CCA axis.

These data show a clear difference in the change in behavioral responses observed between carnivorous and sanguivorous species during feeding. This distinction can be further illustrated by grouping each species by diet and performing CCA on this condensed data set (Fig. 6C). The diet of each leech species has a large impact on determining its behavioral response to stimulation during feeding. This revised data set and CCA confirm that a carnivorous diet is strongly correlated with the expression of swimming, crawling and other behaviors incompatible with feeding, whereas a sanguivorous diet correlates only with the expression of behaviors that do not interfere with ingestion (the CCA in Fig. 6C explains 81.2% of the inertia in the data set, $P<0.001$, and the first two CCA axes explain 18.2%).

Effects of feeding on a mixed sanguivore/carnivore

Macrobdella decora are unique among the leeches we tested because they can feed either on blood or on meat. To test whether

the food that *Macrobdella* was eating affected its behavioral repertoire during feeding, we tested the responses of this species to tactile stimuli while it was consuming meat, then while it was
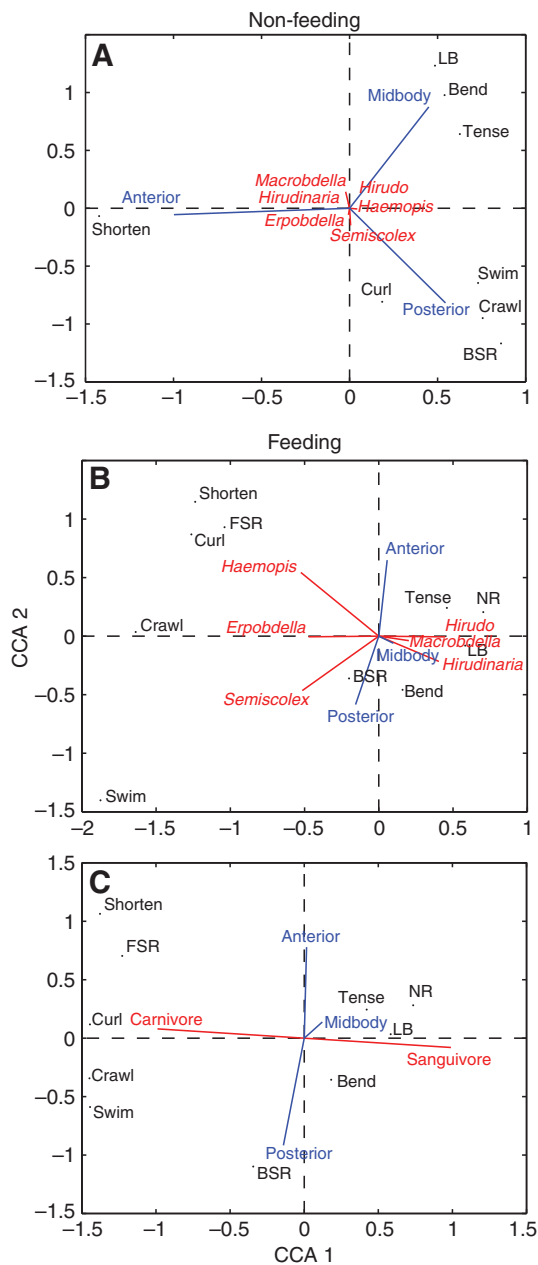

Fig. 6. CCA biplots of non-feeding and feeding behavioral responses. (A) Biplot shows the influence of species and stimulus location on behaviors in the non-feeding state. Data taken from Fig. 3A. Blue vectors indicate stimulus locations and red vectors show the effect of species identity. Only behaviors that were observed at least once are plotted. Stimulus location and species identity explain 50.6% of the total inertia in the data set ($P<0.001$), while these two axes explained 41.5%. (B) Biplot shows the effect of stimulus location and species on behaviors during the feeding. Colors are as in A. CCA axes for B are independent from those for A and cannot be directly compared. Stimulus location and species identity explain 72.1% of the total inertia in the data set ($P<0.001$), while these two axes explained 20.4%. Data taken from Fig. 3B. (C) CCA biplot of the data from Fig. 3B with all carnivore and sanguivore trials combined. Colors are as in A. Diet and stimulus location account for 81.2% of the inertia in the data set ($P<0.001$), and these two axes explain 18.2%.

sucking blood. We fed a group of *Macrobdella* on fresh liver and stimulated them during feeding, and compared their responses with those obtained previously from blood-fed *Macrobdella* (Fig. 3). No significant difference was found between the liver- and blood-fed leeches in the amount of shortening elicited in response to anterior stimulation (Fig. 7A). Midbody stimulation during feeding resulted in a similar amount of local bending and bending between both groups of animals regardless of diet (Fig. 7B). Likewise, posterior stimulation produced the same responses whichever food they were eating (Fig. 7C). These data suggest that the dominance of feeding behavior over conflicting behaviors is fixed in these leeches and is not affected by variations in their diet.

DISCUSSION

We have characterized the behavioral responses of several species of leeches across their phylogenetic clade. Our findings show the similarities in behavioral choice between specific species are highly determined by diet and less dependent on sharing a common ancestor. Each of the species of leech tested had a remarkably similar behavioral repertoire while not engaged in feeding behavior. In this state, stimulus location is the most influential factor in shaping the behavioral response of these animals. During feeding, however, we found that sanguivorous leeches will endure mechanosensory stimulation and continue feeding rather than withdrawing from a food source. Carnivorous leeches reliably abandoned a food source in favor of locomotion when presented with stimulation during feeding.

Feeding as a dominant behavior in sanguivorous leeches

While it has previously been shown that feeding is at the top of the behavioral hierarchy in *Hirudo* (Misell et al., 1998), we have revealed two key aspects about this behavioral choice paradigm. First, the dominance of feeding over mechanically elicited behaviors is absolute and not reciprocal. Specifically, while feeding efficiently suppressed these behaviors, tactile stimulation had no significant effect on the duration of feeding or the weight gain accompanied by ingestion in sanguivorous leeches. This observation is in contrast to hierarchies described in several other species. In the marine mollusk *Pleurobranchaea*, feeding is dominant over tactile stimulation. However, repetitive stimulation can decrease feeding responses (Davis et al., 1974a) and strong stimulation can elicit escape swimming during feeding (Davis et al., 1974b). In addition, male moths display a non-absolute hierarchy where avoidance of bat calls dominates tracking of female pheromones but increased pheromone concentrations increase the behavioral threshold of the avoidance behavior (Skals et al., 2005). We did not test a wide range of stimuli strengths so it is possible that higher stimulus intensities could have resulted in an early termination of feeding behavior in favor of escape responses. This is highly unlikely, however, because a feeding leech may be severed in half or dissected for extended periods of time without terminating a feeding bout (Lent and Dickinson, 1987) (Q.G. and W.B.K., Jr, personal observation). In fact, there are only two stimuli that have previously been shown to prematurely terminate feeding in the sanguivore *Hirudo*. The first is artificial distention of the animal's crop, which would affect the perceived quantity of food ingested by the leech (Lent and Dickinson, 1987). The second stimulus is to manipulate the food source being ingested through the addition of quinine or by replacing it with water (Kornreich and Kleinhaus, 1999). This switch will affect the quality of food being consumed by the leech. Interestingly, applying bitter substances to the external surface of the leech's chemosensitive dorsal lip (Kornreich and Kleinhaus, 1999) does not terminate feeding. Thus only sensory cues that directly affect feeding

terminate the behavior in *Hirudo* and mechanoreception may not be the only sensory modality affected by feeding. The absolute nature of feeding as a dominant behavior in sanguivorous leeches may be rare and a consequence of its unique ethology as an episodic feeder and obligate sanguivore.

The second key aspect of the behavioral choice that we found is that the dominance of feeding over all other behaviors in sanguivorous leeches is independent of diet. *Macrobdella decora* is unique in that it is a predominantly sanguivorous leech that will also

feed as a carnivore. We demonstrated that either feeding mode in *Macrobdella* will suppress locomotory behaviors. It thus appears that once a species of leech evolves to either a sanguivorous or carnivorous lifestyle, the nervous system governing this particular behavioral choice becomes hard-wired and unchanged even when the animal is coaxed to feed on a less preferred food item. It is always possible that another aspect of the leech behavioral hierarchy is plastic or that other dietary modifications could alter the ranking of behavioral responses in the leech. Many behavioral hierarchies and sensory-motor responses can be altered by behavioral or hormonal states. For example, in the toad *Bufo*, large moving objects signal the presence of a potential predator and thus elicit a cryptic posture in which the toad remains motionless and hunkered down. During the mating season, however, the same sensory stimulus now evokes exploration in the toad since the same stimulus may now represent a potential mate. The dominance of cryptic behavior over exploration is thus highly plastic and dependent on the hormonal and behavioral state of the animal (Carr et al., 2002).

Role of ecology and phylogeny in determining behavioral hierarchy

Why is the behavioral hierarchy of sanguivorous leeches absolute, fixed and so highly dominated by feeding behavior? In many species, including both invertebrates (Gillette et al., 2000) and vertebrates (Pitcher et al., 1988), the decision to feed and avoid risk appears to be calculated as a cost–benefit analysis dictated by hunger cues that oppose immediate (nociception) and more delayed (risk of predation) risks. Sanguivorous leeches appear to respond only to appetitive cues and to ignore all mechanical stimulation in their decision to feed. Because this behavior was observed in all lineages of sanguivorous leeches tested, and not carnivorous ones (even those more closely related phylogenetically), we believe that convergent evolution among the sanguivores is the most likely cause of their similarities in behavioral choice. As episodic feeders with highly specific diets, sanguivorous leeches may be required to endure extended periods of starvation. The dominance of feeding over escape responses may have convergently evolved in other taxa that share this distinct feeding ecology. For example, hard ticks (Ixodidae) are also episodic feeders and may have to wait an entire year for a blood meal (Sonenshine, 1991). Without this meal they cannot advance to the next developmental stage and thus place a high priority on their feeding behavior. Like leeches, ticks will endure harsh physical abuse and not abandon a blood meal. Feeding ticks may be covered in nail polish, doused in 70% isopropanol or pressed against a fire-hot match and still not withdraw their mouth parts from a host (Needham, 1985). These findings suggest that episodic feeders with highly specialized diets minimize the risk of death by starvation at the cost of increased risk by predation or infection due to trauma. This extreme circumstance may be represented in the nervous system by a total suppression of behaviors incompatible with feeding in these species.

The carnivorous leeches in our study displayed a more typical pattern of behavioral choice where the animals chose to locomote away from aversive tactile stimulation even during feeding. Surprisingly, in preliminary experiments, Erpobdellid leeches starved for 4 months did not change their responses to mechanosensory stimulation despite increasing their food-biting behavior. These results might be explained by a static behavioral hierarchy with a very low priority for feeding, possibly because the availability of prey is not an issue in their natural habitat. Interestingly, *Semiscolex* sometimes swam away from stimuli while holding their food in their mouths. In these few cases, the decision to feed or escape was avoided by doing both behaviors.
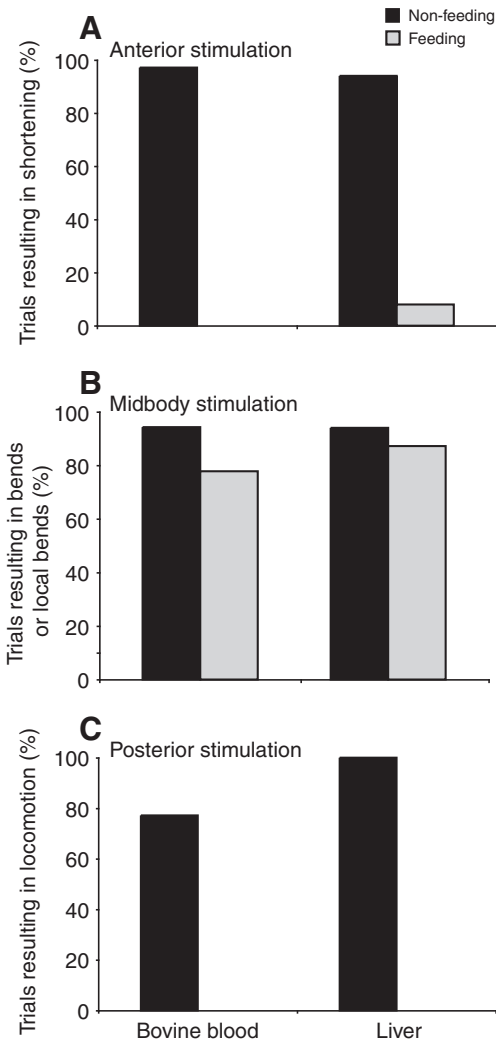
Fig. 7. Behavioral choice is innate in *Macrobdella decora*. (A) *Macrobdella* were fed on either fresh warmed bovine blood (data from Fig. 3) or fresh beef liver. Mechanosensory stimuli were applied to the anterior end of the leech and the percentage of stimuli that elicited shortening is plotted during non-feeding trials and feeding trials. No significant difference in the amount of shortening elicited during feeding was observed between the two groups of animals (P=0.5, 50 liver feeding trials in N=10 leeches and 13 blood feeding trials in N=7 leeches). (B) *Macrobdella* were stimulated in the midbody region during feeding and non-feeding trials. The percentage of trials resulting in bending and local bending is shown. No statistical difference in bending and local bending was observed between the two groups during feeding (P=0.6, 47 liver feeding trials in N=10 animals and 9 blood feeding trials in N=7 animals). (C) *Macrobdella* were stimulated in the posterior region during non-feeding and feeding trials. No statistical difference was observed in the percentage of trials resulting in locomotion across the two groups of animals (P=1, 48 liver feeding trials in N=10 animals and 9 blood feeding trials in N=7 animals).

Mechanisms for the evolution of behavioral choice

It is of great interest to know how a circuit underlying behavioral choice has evolved within a group of animals. What circuit features distinguish the responses of sanguivores and carnivores and why do *Macrobdella* behave differently from other carnivores while feeding on liver? The circuit underlying the suppression of mechanically elicited behaviors has recently been reported in *Hirudo* (Gaudry and Kristan, 2009). During ingestion, serotonin release onto the presynaptic terminals of P cells decreases the probability of synaptic release and results in decreased synaptic excitation in downstream networks. Two plausible and testable hypotheses regarding the decision-making networks in carnivorous leeches may be (1) the absence of such a serotonin receptor on the presynaptic terminals of the P cells in carnivorous leeches, or (2) the lack of serotonin release during feeding by carnivores. Either or both of these mechanisms may be found to have occurred since both blood feeding and feeding carnivorously have evolved multiple times within the leeches. The convergence upon a single mechanism may suggest an ideal solution for converting a circuit that ignores tactile stimulation during one behavior into a circuit that favors the generation of escape responses. However, the convergence onto a single mechanism could also reflect the constraints imposed by the central nervous system's need to generate many behaviors and to make various decisions regarding how to process tactile information. Alternatively, two carnivorous species, such as *E. obscura* and *H. marmorata*, could have evolved separate mechanisms to regain their ability to respond to tactile stimulation during feeding. This result would be interesting and could reflect further differences between the behaviors of the two species beyond just their tendency to abort feeding in the presence of tactile stimulation.

Although the systemic disruption of the serotonergic system in intact leeches *via* the toxin 5,7-dihydroxytryptamine (Lent and Dickinson, 1984) or various antagonists (Q.G., personal observation) can interfere with feeding behavior (at least in *Hirudo*), semi-intact preparations should prove useful in blocking local serotonin signaling and revealing the underlying circuitry (Gaudry and Kristan, 2009). This work will require establishing homology between neurons and their functions across the leech species. However, it should help reveal how a distinct decision-making circuit has evolved within a phylogenetic clade. Fortunately, the similarity of the leech ganglion across species should simplify the task of identifying homologous neurons (Lent and Fraser, 1977; Kramer and Goldman, 1981; Nusbaum and Kristan, 1986; Elsas et al., 1995).

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