Statistical characterization of social interactions and collective behavior in medicinal leeches

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Abstract

In the present manuscript we analyze the behavior and interactions among leeches in the same observation tank. Colored beads were glued onto their skin, so that their behavior could be followed and quantified. When 2 or 3 leeches were present in the observation tank, they searched around for a maximum of two hours and their motion and behavior were independent from those of their conspecifics. When the number of leeches in the tank was increased to 10, leeches were attracted to each other and exhibited episodes of highly correlated behavior. Solitary leeches injected with serotonin or dopamine increased the portion of time spent pseudo-swimming and crawling, respectively. The behavior of 3-5 leeches injected with serotonin was not statistically independent and leeches were attracted to their conspecifics and exhibited episodes of correlated behavior. Therefore, serotonin not only induces pseudo-swimming in leeches but also promotes social interactions, characterized by a mutual attraction and by episodes of correlated /collective behavior.
Introduction

Animals - from simple invertebrates to higher primates - not only interact with the environment but are also able to establish, keep and maintain social interactions with their conspecifics (Sokolowski, 2010). Social interactions are the basis of species survival and are fundamental for establishing societies (Parrish and Edelstein-Keshet, 1999). In birds, fish, and in invertebrates such as *C. elegans*, *Drosophila* and locusts, social interactions lead to group decision making, characterized by the formation of aggregates, where several tens and even thousands of conspecifics adopt the same behavior (Allee, 1927). Underlying biological mechanisms are largely unknown, but specific genes, such as the *npr-1* gene in *C. elegans* (De Bono and Sokolowski, 2007) and well-known neuromodulators, such as serotonin (5-HT) in locusts (Anstey et al, 2009) seem to play a major role.

The emergence of social interactions has not yet been investigated in leeches, but their stereotyped nervous system provides an ideal preparation to relate behavior to underlying neuronal networks (Kristan et al., 2005). The nervous system of the leech *Hirudo verbana* is composed of 21 segmental ganglia, consisting of about 400 neurons each (Macagno, 1980; Muller et al., 1981). Two pairs of roots formed by axons of sensory neurons and motoneurons, emerge from each ganglion and project into the corresponding body segment. Sensory neurons have been identified and extensively characterized (Nicholls and Baylor, 1968; Muller et al., 1981). Most motoneurons have also been identified and their role in behavioral responses has been determined (Stuart, 1970; Ort et al., 1974; Muller et al., 1981; Kristan et al., 1982; Lockery and Kristan, 1990). Indeed, neuronal circuits controlling swimming (Kristan and Calabrese, 1976; Friesen, 1989; Mangan et al., 1994) and crawling (Eisenhart et al., 2000; Friesen and Kristan, 2007; Puhl and Mesce, 2008) have already been identified and also those involved in feeding and mating are beginning to be unraveled (Dickinson and Lent, 1984; Tan et al., 2004; Gaudry and Kristan, 2009; Wagenaar et al., 2010). Specific neuromodulators, such as serotonin (5-HT), dopamine (DA) and octopamine trigger or control these behaviors (Willard, 1981; Puhl and Mesce, 2008).

In the present manuscript we quantify leech behavior (Mazzoni et al., 2005; Kristan et al., 2005) and analyze how they are modified by the presence of other leeches. We glued colored beads onto the skin, so that the leeches’ motion could be tracked for several hours and their behavior quantified with a high statistical accuracy. When more than 5 leeches were present in the observation tank, we quantified their collective behavior by an appropriate analysis of the image sequences. In this way, it was possible to quantify and characterize the emergence of social interactions in leeches. These social interactions are characterized by two features leading to group decision making: firstly a mutual attraction among conspecifics resulting in conspecifics aggregation and secondly in an imitative behavior consisting in episodes of collective swimming (Sokolowski, 2010).
Methods

Animals and preparations

Adult leeches (Hirudo verbana) obtained from Ricarimpex (Eysines, France) were kept at 10°C in tap water dechlorinated by aeration for 24 h. Leeches were anesthetized with an 8% ethanol in leech saline solution (in mM: 116 NaCl, 1.8 CaCl₂, 4 KCl, 1.5 MgCl₂, 10 glucose, 10 Tris maleate buffered to pH 7.4) and two colored beads with a diameter of ~3mm were glued onto the dorsal side of the leech with 3M(TM) Vetbond Tissue Adhesive (World Precision Instruments, Berlin, Germany) near their head and tail. Leeches were allowed to recover from anesthesia and then were transferred in the observation tank where their motion was tracked in real time. Leeches were kept in either a large (top diameter: 28 cm; bottom diameter: 18 cm, height: 15 cm) or a small (top diameter: 18 cm; bottom diameter: 14 cm, height: 10 cm) circular tank, filled with dechlorinated tap water kept at 10-15°C. Observation tanks were illuminated with a dim, diffuse light with no abrupt spatial and/or temporal gradients. No additional sensory stimuli, such as chemical, mechanical or visual inputs were intentionally applied. A transparent, plastic disc was used to keep leeches inside the tank. We used two leech populations, referred to as “hungry” and “satiated”: satiated leeches were those arrived from the supplier and kept in the laboratory unfed for less than two months; hungry leeches were those kept unfed for more than 8 months.

Delivery of neuromodulators

Some leeches were injected with specific amounts of dopamine (DA) or serotonin (5-HT). A 4 mM DA (Sigma-Aldrich) containing saline solution was freshly prepared every day, stored in dark conditions at 4°C and used within 1 h. A 1 mM 5-HT (Sigma-Aldrich) containing saline solution was stored at -20°C in 500 μl aliquots, and defrosted in 30 minutes at room temperature. Immediately before use, an aliquot of the DA or 5-HT stock solution was diluted in leech saline solution to its final concentration. We injected 300 μl of 5-HT or DA solutions at different concentrations, using 1 ml insulin syringes with 29 GA and 15 mm long needles. Each leech was gently held in one hand and the needle was inserted under the skin of the dorsal side, along its dorsal axis at a depth of about 10% of the body thickness. We assume that the injected solution was diluted into the extracellular space roughly 25-30% of the leech volume, usually 3-4 ml. Therefore, the final drug concentration injected in the leeches is approximately one fourth of the injected drug concentration.

The body volume of an adult leech is 3-4 ml, while the overall blood volume is about 8-9% of the body mass, corresponding to 240-360 μl (Wenning and Meyer, 2007). To determine the relation between the internal level of a neuromodulator and the associated behavior we dissolved different amounts of 5-HT or DA varying from 50 to 800 μM in the same amount of leech saline (300 μl). As a control for the effects of diluting the leech’s own neuromodulators, we also monitored the behaviors of leeches injected with 300 μl of leech saline without any added drug. Changes of body volume are well tolerated by leeches; because they can increase their initial weight by 8-11 times during feeding (Dickinson and Lent, 1984), it is likely that an increase of the body volume (about 10% of its body mass) caused by the injection did not impair the animal’s movements.
Imaging

We used a color CCD camera (640x480 pixels of image size; model DFK 21BF04; The Imaging Source Europe) to image leeches from above to monitor their movements (Fig. 1A). The camera was connected via the FireWire-output to a frame grabber (PCI-1394; Texas Instruments) installed on a personal computer, able to process images in real time. Colored beads glued onto the backs of the leeches were tracked at 15 Hz using software developed in Matlab language (MathWorks, Natick, MA). Images were directly acquired in the hue/saturation/lightness color space (Fig. 1B) and converted into red, green and blue (RGB) values (Fig. 1C).

(Figure 1 near here)

The real-time algorithm is able to track up to 6 beads of different colors. Because the tank and the leeches have almost the same dark blackish color, the algorithm easily detects the colored beads. Dark pixels corresponding either to the background or to the leech bodies were first detected by a simple threshold procedure. The RGB color distribution of used beads was measured before the experiment. As beads are homogeneously colored, their color distribution is Gaussian and each bead is characterized by its mean color vector and the correspondent covariance matrix. Subsequently, a Bayesian classification of colored pixels was performed, in the RGB feature space. If \( \mathbf{v}_i = [r,g,b] \) is the color vector of the i-th pixel, \( \mathbf{m}_k \) is the mean vector, and \( \Sigma_k \) is the covariance matrix respectively of the k-th detected colored region (\( k=1...L; \ L=N+1; \) where \( N \) is the number of the beads). Each colored bead represents a single class that can be visualized as a covariance ellipsoid in the feature space (Fig. 1C). The most probable class or color to which the i-th pixel belongs, is obtained by minimizing the Mahalanobis distance (Mahalanobis, 1936)

\[
d_{ik} = (\mathbf{v}_i - \mathbf{m}_k)^T \Sigma_k^{-1} (\mathbf{v}_i - \mathbf{m}_k).
\]

This criterion represents a quadratic Bayesian classifier that can be implemented to work in real-time. After classification, the algorithm produces \( N \) binary images, one for each bead, in which the ON bit represents the pixel belonging to it. Then the algorithm searches for a cluster of at least 10 ON pixels, and calculates the center of mass of its coordinates (Fig. 1D). The software stores only the coordinates of detected beads so that it is possible to monitor leech behavior for several hours. In experiments requiring the use of a gold fish, the algorithm was tuned to recognize the color of the fish body and the color of the leeches’ body.

Computation of velocity

From the time series describing the coordinates of the head and tail bead we computed the head \( V_{\text{head}}(n) \) and tail \( V_{\text{tail}}(n) \) velocity. In other occasions we computed the leech center of mass, as the midpoint between the head and tail beads (see Fig. 2) and its velocity was computed. Center of mass, head and tail velocities were computed by the convolution of the corresponding time series of the x and y coordinates with the derivative of a Gaussian function. Similarly, in experiments requiring the use of a gold fish, the fish velocity \( V_f \) was calculated by convolving the x and y coordinates of the center of mass with the derivative of a Gaussian function and the average velocity vector from the leech population \( <v> \) was calculated by convolving the x and y coordinates of the center of mass of each detected leech – or group of leeches – with the derivative of a Gaussian function. In all the calculations we used a Gaussian function with a standard deviation of 1-2 s.
Leeches were monitored for 3 to 12 h. The corresponding time series usually comprised more than 100,000 samples. We adopted an automatic classification method to analyze behavior, based on the kinematics and spectral content of the recorded time series as previously described (Mazzoni et al., 2005). Briefly, leech movements were classified into five different categories (Fig. 1): exploratory movements (Fig. 1E), crawling (Fig. 1F), swimming (Fig. 1G), pseudo-swimming (which is likely to be ventilation) (Fig. 1H) and stationary (i.e., not moving). At each time \( n \) of the recorded time series, different criteria were used to discriminate each behavior. The stationary state was detected by computing the speed of the head \( V_{\text{head}}(n) \) and tail \( V_{\text{tail}}(n) \). If both speeds were less than 1 pixel per second (pixel/s), then the state was considered stationary. The other behavioral categories were identified by computing the following parameters: (1) elongation \( e(n) \), i.e. the beads distance from head to tail; (2) maximal speed \( V_{\text{max}}^T(n) \) of the head over a time window of width \( T \) centered in \( n \) (with \( T \) ranging from 20 to 40 s); (3) dominant frequencies \( f_{T}^d(n) \) of the spectrogram \( S(t,f) \) of \( e(n) \), computed using a Hamming window of length 30 s, and with 96.7% overlap. Oscillatory behaviors (such as swimming, pseudo-swimming, and crawling) could be reliably identified by analyzing the pairs of values \([V_{\text{max}}^T(n), f_{T}^d(n)]\): swimming episodes were characterized by \( f_{20}^d(n) \sim 1 \) Hz and \( V_{\text{max}}^{20}(n) \sim 20 \) pixels/s, whereas crawling was associated to \( f_{50}^d(n) \sim 0.08 \) Hz and \( V_{\text{max}}^{50}(n) \sim 30 \) pixels/s. Note that all definitions of behavior are based on units of pixels; in our recording system, 1 pixel corresponds to 0.4 mm.

Test for independence between behavioral processes

In the time domain, transitions among behaviors can be described by a Markov process, the structure of which (allowed states and transitions) is highly conserved across individuals (Garcia-Perez et al., 2005). We thus estimated the transition matrix of a hidden Markov model by the maximum likelihood estimator implemented in Matlab (function hmmestimate), on 20 minutes time-windows. These windows were used to ensure stationarity. Let \( \{X_n\}_T \) and \( \{Y_n\}_T \) be two Markov chains modeling the leech X and Y behavior in a time-window of 20 minutes, with their transition matrices \( \Pi^X \) and \( \Pi^Y \), with \( T = 66 \) ms, and \( n \) positive integer. Let be \( P^X \) and \( P^Y \) the maximum likelihood estimates \( \Pi^X \) and \( \Pi^Y \), respectively. The statistical test for independence (H0) between two binary Markov chains is obtained by a likelihood-ratio test, or a \( \chi^2 \)-test (Anderson and Goodman, 1957; Sandland, 1976). By adopting a simplifying procedure (Solow et al., 1995), the final result consists of a 5x5 matrix reporting the conditional significance level for all possible pairs of behaviors (Fig. 6).

In experiments with more than 5 leeches, only one bead was attached to each animal; under these conditions it was possible to measure and quantify their relative distance but not their behavior. In order to quantify the emergence of collective behaviors we focused our attention on the swimming behavior because it is easy to recognize and not very frequent (Fig. 3C). By visually inspecting acquired movies, we identified all the times \( t_n^B \) and \( t_n^T \) when \( k \) leeches begin and stop swimming, respectively. In the time interval between \( t_n^B \) and \( t_n^T \) \( k \) leeches swim with \( k \) varying from 1 to \( L \), i.e. the total number of leeches in the observation tank. We then estimated the probability \( p_{sw} \) that a leech swims as:

\[
p_{sw} = \sum_{n=1}^{N} \frac{[t_n^T - t_n^B] \cdot R_n}{(LT)}
\]
where $N$ is the total number of observed swimming episodes and $T$ is the duration of the experiment. We have computed the probability of observing the simultaneous swimming of $k$ leeches (regardless the behavior of the other $L-k$ leeches) $f_k$ for $k = 1, \ldots, L$. If leeches swim independently of each other, $f_k$ is expected to be proportional to $f_1^k$ (where $f_1 = p_{sw}$). The probability $f_k^*$ that exactly $k$ leeches swim, follows a Binomial distribution. Experimental probability density estimates were compared with the Binomial equation, and a Kolmogorov-Smirnov test was performed to determine the statistical significance of the deviation. A statistically significant deviation indicates the emergence of a collective behavior and social interactions.
Results

Leeches, like many other invertebrate species, exhibit both solitary behavior and also a more social attitude when conspecifics are present (Lang, 1974). Social behavior is broadly defined as an interaction between members of the same species that changes their subsequent behavior (Sokolowski, 2010). The present manuscript aims at analysing the behavior of leeches and in particular the transition from a solitary behavior to a more gregarious, social behavior. We attached two colored beads to the skin of a small number (typically 2 or 3) of leeches, to tag individual leeches and follow their behavior for long periods of time.

The searching behavior of leeches

Three leeches tagged with colored beads were kept in a tank in darkness for at least 2 hours. In some experiments, the light source illuminating the tank was switched on and their behavior was observed (Fig. 2A). In these experiments, leeches started to move around exploring the environment for some minutes (1.3 ± 1.8 min; n = 9). In other experiments leeches were moved into a different tank (Fig. 2B) and their behaviors were similarly monitored; in this case, leeches were active for longer periods (54 ± 13 min; n = 13). To quantify these movements, we computed the leech center of mass and its velocity (see Methods). Leeches were assumed to search the environment if the velocity of the center of mass was larger than 3 pixels /s. The duration of the searching phase (Fig. 2D, red background) was calculated: we compared how long leeches searched in a known environment (Fig. 2E, first bar) to the duration of searching a new environment (Fig. 2E, second bar). Leeches searched the new tank for 54 ± 13 minutes and then stopped moving and remained stationary for a time varying from tens of minutes up to 1 or 2 hours. The duration of this initial searching phase in 13 leeches had a Gaussian distribution (Jarque–Bera test; p = 0.14) (Fig. 2F). From the center of mass trajectories, we determined the time spent in each location (x, y) of the tank (Fig. 1G): leeches preferentially explored the external edge of the bottom of the tank and its upper rim. When the explored location was determined in polar coordinates (R, ϕ) no radial preference was observed neither during the searching phase (Fig. 1H) nor during the following stationary phase (Fig. 1I).

Behavior during the searching phase

By using the procedures described in the Methods section we classified the behavior during the searching phase and the following stationary phase (Fig. 3A). During the searching phase (Fig. 3B), leeches exhibited all five stereotyped behaviors (Mazzoni et al., 2005): they alternated swimming (blue), pseudo-swimming (dark green), crawling (red), episodes where they explored the environment with their head (pink) and brief periods where they were static (light green). During the early searching phase (Fig. 3C), the most common behavior was crawling (44.5 ± 17.3 % of the total searching phase), followed by static behavior (30.2 ± 17.9 %), swimming (8.9 ± 11.4 %), exploration (8.9 ± 8.5 %) and pseudo-swimming (6.1 ± 7.9 %). A different static behavior percentage was observed during the stationary phase: leeches were static for most of the time (89.5 ± 11.4 %), but moved their head (5.9 ± 9.7) with a percentage very similar to what observed during the initial searching phase, and very rarely exhibited crawling (2.3 ± 2.5 %), pseudo-swimming (1.8 ± 2.4 %), or swimming (0.6 ± 1.4 %).
We also investigated whether the size of the tank and the state of the animal affected the searching phase. We compared the statistics of the searching phase of leeches moving in a small and in a large tank, and the same analysis was performed on fed leeches (shipped no more than two months earlier) and on hungry ones (kept unfed for at least 8 months). The duration of the searching phase was statistically longer (Student’s t-test; \( p<0.05 \)) in the larger tank (48 ± 25.4 minutes, 5 leeches) than in the smaller tank (21 ± 7.8 minutes, 7 leeches) (Fig. 3D). The fraction of time spent in swimming, pseudo-swimming, crawling, resting and moving the head was not different when leeches were in a large or small tank (Fig. 3E). As shown in Figs. 3F and G, the duration of the searching phase was longer for hungry (53.6 ± 13.1 minutes, 13 leeches) than for fed leeches (11.4 ± 6.4 minutes, 18 leeches) (Student’s t-test, \( p<0.01 \)) and the behavioral percentages during the searching phase was similar for fed and hungry leeches. Therefore, hungry leeches search a new environment for a longer period of time but the specific behaviors adopted during the searching phase is independent from the size of the tank and on the state of the animal.

The effect of 5-HT and DA

Leech behavior is affected by specific neuromodulators such as 5-HT and DA: a high blood concentration of 5-HT promotes swimming in intact leeches, and in isolated nerve cord preparations (Willard, 1981). Similarly, DA induces fictive crawling in isolated nerve cord preparations or in single isolated ganglia (Puhl and Mesce, 2008). To test the effects of these modulators on intact leeches, we determined the percentage of time leeches injected with different amounts of 5-HT (Figs. 4A,B) and DA (Figs. 4D,E) spent either pseudo-swimming or crawling during the initial search of a new environment. Leeches injected with 300 \( \mu \)l of a saline solution – with no neuromodulator added – spent a small amount of time pseudo-swimming (see Fig. 4A), whereas uninjected leeches rarely exhibited pseudo-swimming (Fig. 3).

Uninjected leeches pseudo-swam for about 6% of the searching phase, suggesting a low level of 5-HT in the blood (<50 nM) (Willard, 1981): a subsequent saline injection is expected to lower further the endogenous 5-HT concentration in the blood (Fig. 4A, saline control value). Injection of 5-HT concentrations higher than 100 \( \mu \)M promotes pseudo-swimming: leeches injected with 150-200 \( \mu \)M 5-HT spent almost half of their searching time pseudo-swimming (Fig. 4A). The modal duration of pseudo-swimming episodes is also increased (Fig. 4B,C). Under the same conditions leeches hardly swam: the time spent swimming was 0.5 ± 0.3 %. 5-HT is known to control the swimming behavior through two different mechanisms: Retzius cells exert a neurohormonal action whereas cells 21 and 61 act synaptically (Kristan and Nusbaum, 1982), but the mechanisms underlying the generation of two similar behaviors such as swimming and pseudo-swimming are still unknown because previous studies have been carried out on isolated nerve cord preparations, where these two behaviors cannot be distinguished.

Uninjected leeches crawl for about 45% of the searching phase, but leeches injected with saline solution crawl for only 9.9 ± 3.0 % of the searching phase. These observations suggest that a significant level of DA is present in the blood and that the injection of 300 \( \mu \)l of saline solution may significantly lower the endogenous DA concentration in the blood. This is supported by experiments in which we measured the percentage of crawling after
the injection of increasing saline volumes (Fig. 4F). Higher volumes reduce the crawling percentages (Student’s t-test, p<0.01), suggesting an increasing dilution of DA in the blood. When DA was added to the injected saline, over a broad range of DA concentrations, leeches exhibited the usual amount of crawling behavior and the modal duration of crawling episodes is unchanged (Fig. 4 D,E). When either 5-HT or DA was injected at a high concentration (larger than 500 μM), neither pseudo-swimming nor crawling was observed. The resulting dose-response curves relating the amount of injected neuromodulator and the percentage of pseudo-swimming and crawling were "bell-shaped". This kind of shape could originate from two distinct mechanisms. Firstly, receptor desensitisation occurring at higher agonist concentrations (Lopez-Garcia and King, 1996; Wilkie et al., 1996) and secondly because of the existence of two different types of 5-HT receptors, as in leech mechanosensory P cells (Henderson, 1983; Drapeau and Sanchez-Armass, 1988) and S cells (Burrell et al., 2001). In leech P-cells, application of low levels of 5-HT onto the soma produces an increase in CI− conductance and a consequent hyperpolarization, possibly mediated by one type of 5HT3 receptor (Ali et al., 1998), whereas a higher level of 5-HT induces depolarization resulting from the activation of one type of 5HT2 receptor causing an increase of a nonspecific cationic conductance (Sanchez-Armass et al., 1991; Catarsi and Drapeau, 1997). Ionic mechanisms for 5-HT-mediated inhibition and excitation are unknown in the S-cell, but could be explained by different 5-HT receptors and receptor desensitization (Burrell et al., 2001).

The body concentration of 5-HT clearly influences the percentage and the duration of pseudo-swimming episodes (compare black and gray lines in Figs. 4B and C). The change in the modal duration suggests that other modulatory effects could be present in different regions: for example it is known that the head brain contributes not only to swimming initiation, but also to the quality (duration and rhythmicity) of these episodes (Crisp and Mesce, 2006).

The percentage of crawling was clearly influenced by the body concentration of DA, but their duration was not: crawling episodes had similar duration in the non-injected and injected leeches (compare black and gray lines in Figs. 4E). Similar results were observed in isolated nerve cord preparations with DA (Puhl and Mesce, 2008).

**Social interactions among conspecifics: experiments with 3 leeches**

Having characterized searching behaviors in solitary leeches in a new environment we asked whether their behavior was influenced by the presence of other conspecifics. To test this possibility, we analysed the distribution density of distances (Dleeches) between pairs of leeches (Fig. 5). The presence of other conspecifics could also influence their behavior: swimming or crawling in one leech might induce similar behaviors in other leeches, so that their behavior would become correlated. This possibility was tested by analyzing whether pairs of leeches adopted a similar behavior in the same time windows (Fig. 6). Deviation from statistical independence would indicate social interactions.

(Figure 5 near here)

We first investigated social interactions among leeches in groups of 3. For each leech we determined its center of mass (see Methods) and computed the distribution density (pdd) of the pair-wise distances (Dleeches) among all pairs of leeches in the observation tank. In Fig. 5A (gray bars), the pdd of Dleeches was expressed in relation to the normalized radius of the recording arena (equal to 1), so that two diametrically opposite leeches are at a distance of 2 normalized units. The pdd was calculated from data collected
during the initial searching phase (i.e. the first 100 minutes). The pdd obtained from the same experiments, after shuffling the data points, had a similar probability density (blue curve) suggesting independent behaviors of the three leeches.

To verify that the 3 leeches searched the tank in an independent fashion we simulated leech trajectories with built-in reciprocal independence. From 15 experiments with solitary leeches we constructed their radial and angular densities in the tank (blue data in Figs. 5 A and B). The small peak at 0.6 normalized radius of the radial density $f_r(r)$ corresponds to the external edge of the bottom of the tank (Fig. 2H). $f_r(r)$ was modelled as the sum of a log-normal and a normal distribution

$$
\begin{align*}
    f_r(r) &= \frac{\pi_1}{\sqrt{2\pi\sigma_1^2}} \exp \left( -\frac{(1 - r - \mu_1)^2}{2\sigma_1^2} \right) + \frac{\pi_2}{(1 - r)\sqrt{2\pi\sigma_2^2}} \exp \left( \frac{\log (1 - r) - \mu_2^2}{2\sigma_2^2} \right),
\end{align*}
$$

(2)

The six parameters of the radius density were identified with a nonlinear least-squares method based on the Levenberg-Marquardt algorithm (Marquardt, 1963), implemented in Matlab ($\pi_1 = 0.123; \mu_1 = 0.428; \sigma_1 = 0.061; \pi_2 = 0.134; \mu_2 = -1.982; \sigma_2 = 1.422$) ($R^2 = 0.967$) giving the red curve in Fig. 5B. The angular density $f_\theta(\theta)$ was assumed to be uniform, producing the red circle in Fig. 5C. From these quantities we were able to obtain simulations of the leech trajectories satisfying the experimentally observed radial and angular distribution densities (Figs. 5B and C). A comparison between the experimental and simulated trajectories is shown in Fig. 5D. From these simulated trajectories we determined the corresponding pdd (red curve in Fig. 5A). The pdd of experimentally observed pair-wise distances $D_{\text{leeches}}$ is well fitted by those obtained from independence hypotheses using the data-shuffling technique ($R^2 = 0.963$) or the stochastic point process model ($R^2 = 0.922$).

(Figure 6 near here)

To test whether the presence of other leeches influenced the behavior of each leech, we compared the behavior percentage of each leech (Fig. 6A) while one was crawling (dark gray bars) or was static (light gray bars). The percentage of a given behavior was very similar in control conditions (black bars) and in groups of 3 leeches while one was crawling (dark gray bars) or was static (light gray bars).

A more accurate analysis relies on the fact that the time structure of leech behavior is well described as a first-order Markov process with transitions among five different behavioral categories (Garcia-Perez et al., 2005): swimming, pseudo-swimming, crawling, static and exploratory (Fig. 6B). To determine if behaviors were performed independently, we implemented a conditional test for independence of two Markov chains as described in detail in the Methods section. In a temporal window of 20 minutes we performed a conditional significance test for an independent Markov process (Fig. 6C). The final result is a 5x5 matrix with entries showing the conditional significance level for all the possible behavior couple and the fraction of samples for which statistical independence is observed with a $p > 0.01$. This matrix (Fig. 6D; $N = 351$ windows, 9 animals) indicates a substantial statistical independence when 3 leeches are positioned in the observation tank. These leeches move independently and do not show any evident social interaction.

**Emergence of social interactions in leeches: the effect of a number of individuals and of 5-HT**
The 3 leeches observed were in a tank with a volume of 6 liters, corresponding to a density of 0.5 leeches/dm³. Because the absence of social interactions (Figs. 5 and 6) could result from the low leech density, we increased the number of leeches in the observation tank. Our CCD camera was not able to reliably distinguish the color of more than 6 beads, so we adopted a different experimental approach. We developed an algorithm to follow in real time the contour of individual leeches and to keep track of the number of leeches that had aggregated in a group. Also, a red bead was placed onto the skin of up to ten leeches, in the mid-body. The distance between leeches was measured as the distance between the red beads. A shallow tank (8 cm high) was used, to ensure that leeches belonging to the same group were not at different depths, yet allowing the performance of all possible behaviors.

The distribution density of pair-wise distances (D\text{leeches}) among leeches was markedly different in the case of 3 (example in Fig. 7A) and 10 leeches (example Fig. 7B). With 10 leeches in the observation tank, many times leeches congregated in a group (13 out of 15 experiments), so that the distribution density of pair-wise distances (D\text{leeches}) had a peak close to 0 (Fig.7B); such peak was not present when only 3 leeches were observed (Fig. 7A, peak value at a normalized distance of 1.8). When the 3 leeches have all been injected with 150-200 μM of 5-HT, the distribution density of pair-wise distances (D\text{leeches}) had a large peak corresponding to a normalized distance of 0.7 (Fig. 7C, average distribution, N=5) which was different from the independent behavior observed among 3 non-injected leeches (Fig. 7A). The injection of 650-700 μM DA produced similar but less marked changes (peak at a normalized distance of 1.40) from the independent behavior (Fig. 7D, average distribution, N=5).

(Figure 7 near here)

The median pair-wise normalized distance (D\text{leeches}) when 3 and 10 leeches were in the observation tank was 1.29 and 1.09 respectively. Therefore, the median value of D\text{leeches} is lower when 10 leeches are present in the tank (Mann-Whitney U-test; p<0.01). In leeches moving independently in the tank, the median value of D\text{leeches} does not depend on the number of conspecifics, therefore social interactions emerge when the leech density in the tank is increased. To determine whether the neuromodulators affected social interactions, we measured inter-leech distances after injecting leeches with 5-HT or DA. The median pair-wise normalized distance D\text{leeches} when 3 leeches were injected with 5-HT was 0.89. The same quantity when leeches were injected with DA was 1.01. Therefore, 5-HT (Mann-Whitney U-test; p<0.01) and at a lower extent DA (Mann-Whitney U-test; p<0.01) promotes the emergence of social interactions also in groups of 3 leeches. As shown in (Figs.5-7) the behavior of 3 leeches in the same tank was not significantly affected by the presence of conspecifics.

In the presence of 10 leeches in the observation tank, the emergence of collective behavior was observed (Fig.7B). Indeed, leeches could explore the environment (Fig.8A), be stationary (Fig.8B) but could also exhibit a gregarious behavior (Fig.8C). We observed also episodes in which all 10 leeches swam at the same time (Fig.8D). (Figure 8 near here)

The probability f_k* that exactly only k leeches swim, follows a Binomial distribution. As shown in Fig.8E, the binomial distribution (black line) is in agreement with the experimentally observed percentages (gray line) only for values of k less than 5 (Two-sample Kolmogorov-Smirnov test – p<0.01) and are different for k larger than 5. Indeed, groups of leeches equal or less than 5 behave almost independently and social
interactions characterized by the occurrence of collective behaviors occur with more than 5 conspecifics. In 6 out of the 9 experiments analyzed (Fig. 8F), the percentage that at least 5 leeches swim simultaneously is above the theoretical value (where the percentage of swimming is ∼10%) expected in the case of independent behavior (Fig. 8F, horizontal black line). The statistical analysis shown in Fig. 8E is performed on a single experiment. As the behavior of leeches injected with 5-HT (Fig.7) deviates from statistical independence for groups of only 3 conspecifics, we investigated whether we could observe collective behavior in groups of 5 injected leeches. Indeed, 5 leeches injected with 200 µM 5-HT (volume 300 µl) initially explored the tank (Fig.9A) and could come to rest in a solitary manner, but more often aggregated (Fig. 9C) and more notably showed collective swimming episodes (Fig. 9D). The observed collective behavior occurred with a percentage significantly higher (Fig. 9E, gray line) than that expected from independent behavior (Fig. 9E, black line) (Two-sample Kolmogorov-Smirnov test – p<0.01, statistical analysis performed on a single experiment). 5-HT injection increases the percentage of spontaneous pseudo-swimming, but also of swimming, if a mechanical stimulation is applied on the tail (data not shown), as reported also in (Willard, 1981). In the presence of several conspecifics there are more mechanical perturbations in the water, leading to mechanical stimulations possibly inducing the leeches to swim.

We analysed the emergence of social interactions induced by 5-HT in a series of other experiments. In experiments with several leeches (such as 10), we waited the formation of a large group formed by at least 5 conspecifics (Fig. 9F). We then introduced in the observation tank a leech injected with 5-HT (300 µl of 200 µM 5-HT) which had a colored bead attached to the skin on its back. Therefore, the motion of this leech could be followed and we could verify whether this leech was attracted by the large group of conspecifics. The injected leech explored the tank for about 10-20 minutes (see light gray line indicating its trajectory in Fig. 9F) and then invariably (N=7) joined the group of conspecifics not injected with 5-HT (dark gray circle in Fig. 9F). In other experiments (N=3), the injected leech was introduced in a tank where a black large object was positioned, roughly mimicking a large group of leeches or a black stone. Under these circumstances, the injected leech explored the tank and the object, but neither rested in its proximity, nor took shelter underneath it (Fig. 9G). In all 7 analyzed experiments, the percentage that at least 4 leeches swim simultaneously is above the theoretical value (where the percentage of swimming is ∼10%) expected in the case of independent behaviour (Fig. 9H, horizontal black line). Moreover, the percentage of time spent in a group of at least 4 conspecifics by a leech injected with 300 µl of saline (N=5) is significantly lower than the time spent by a leech injected with 5-HT (N=7) (Fig. 9I, Student’s t-test, p<0.01). The same can be observed in experiments with fake group (N=3). These results suggest that leeches injected with 5-HT are attracted by their conspecifics and have a clear tendency to establish social interactions.

To test whether the collective swimming behaviour is the mere result of the presence of an object moving in the water or is truly a social behavior, we introduced a gold fish in the tank (Fig.10A). The gold fish swam most of the time, producing an almost continuous periodic, water movement leading to mechanical stimulations of the leech bodies (Fig. 10A).
Our real-time algorithm allowed a precise tracking of the fish motion (as its color is red) and of the leeches (as they are black), as shown in Fig.10B. By studying the movies we observed several episodes in which the fish apparently chased a leech (Fig. 10B). This observation is also confirmed by the fact that the scalar product (Fig.9C) between the velocity vector $v_f$ of the gold fish and the average velocity vector of all leeches $<v>$ shows more positive than negative peaks (Fig. 10D). The scalar product $v_f \cdot <v>$ is positive when the two vectors have the same direction, implying that the gold fish and some leeches are swimming in the same direction. When the gold fish is present in the tank, its continuous swimming produces mechanical waves, certainly reaching the leech body, and the movement is likely to produce a mechanical stimulation to their tail, known to initiate swimming. Nevertheless, we observed a decrease in the swimming percentage, and very rarely more than 3 leeches swam simultaneously (Fig.10E). This analysis suggests that leeches are able to sense the presence of a non-conspecific organism in the neighborhood, which could be a predator. As a consequence, leeches could adopt a quieter behavior, in order to minimize the risk of being predated.
Discussion

In the present manuscript we present the first analysis of the occurrence of social interactions in medicinal leeches. Social interactions consist in group decision making (Sokolowski 2010), characterized by a mutual attraction leading to aggregation of conspecifics and an imitative behavior causing episodes in which conspecifics adopt the same behavior. This study provides three major results: firstly, when the leech density is low (less than 3 leeches in 6 litres) leech behavior is unaffected by the presence of other conspecifics; secondly, when the leech density is increased (more than 8-10 leeches in 3 litres) leeches no longer move in a statistically independent way but are attracted to each other and exhibit episodes of collective behavior; thirdly, the leech density at which social interactions emerge is lowered by injection of 5-HT into their body. Let us now discuss in detail the emergence of social interactions in leeches.

Leech ethology

Leech ethology has been extensively studied and several excellent books describe natural leech behavior in great detail (Müller et al., 1981; Sawyer, 1986a, 1986b). In their daily lives, leeches perceive and are affected by a broad range of stimuli; those with the highest biological relevance are light, odorants, water movements, touch, and temperature.

Leeches are negative phototactic, which means that leeches crawl and move away from light sources, seeking protection under a stone or other shelter, where they then remain. Different leech species show different degrees of phototaxis, in response to the dynamics of environmental light, leading to the regulation of their reproductive cycle during the year (photoperiodism), inactivity during the daytime and increased activity at night (diurnal rhythmicity) and alerting posture following sudden changes in light intensity (shadow reflex). The diurnal rhythm can be reversed by reversing the lighting regime and it is abolished under constant dark or light conditions (Elliott, 1973). Leeches are very sensitive to touch, and in Hirudo four behaviors – local bending, whole-body and local shortening, crawling, and swimming – can be elicited by tactile stimulation of the leech. At a threshold level of mechanosensory stimulation, the predominant response elicited depends upon the location of the stimulus: stimulating the anterior end produces shortening, stimulating the posterior end produces crawling or swimming, and stimulating midbody sites produces local bending (Kristan et al., 1982). In addition to localized tactile stimulation, leeches also show a generalized response to touch, as they are often found under rocks, leaf axils of aquatic vegetation or other tight-fitting places which offer maximum physical contact with the substrate (positive thigmotaxis) (Lang, 1974; Sawyer, 1986a). Therefore, it is likely that positive thigmotaxis and negative phototaxis contribute to the deviation of the statistical independence here observed: the former could favour the physical contact between conspecifics, while the latter can be achieved by mutual covering. Nevertheless we speculate that conspecifics recognition may occur as well, as suggested by the data showed in Fig.9.

Searching behavior, deviation from independence and emergence of social interactions

When placed in a new environment, leeches begin to explore it, with particular interest for its boundaries. This “searching behavior” lasts for an amount of time dependent on the size of the tank (Fig. 2-3). This initial searching phase was not described in a previous study (Mazzoni et al., 2005), because in that study data collection and behavioral characterization began 30-60 minutes after positioning leeches in the new environment.
Therefore this initial phase was not observed. Our new Bayesian classifier of pixels color made it possible to track six or more beads in real time so that we could follow the position and the behavior of three leeches placed into an unexplored water tank, and to follow the emergence of social interactions.

When a small number of leeches are in the same environment they hardly notice one another and their motion and behavior are independent with a high statistical significance (Figs. 5, 6). When a higher number of leeches are in the same tank (Fig. 7), they spend more time closer to one another and assemble in groups (peak at short distances in Fig. 7B). This attraction could be in part due to positive thigmotaxis and negative phototaxis: the former leading the leeches to stay in contact, the latter leading the leeches to mutually cover each other. However, the deviation from independence observed in small groups of leeches injected by 5-HT cannot be explained by positive thigmotaxis and negative phototaxis.

In some cases, we have observed 10 leeches swimming simultaneously. Because solitary leeches swim at a low probability \( p_{sw}=0.12 \), seeing 10 independently-acting leeches swimming simultaneously is extremely unlikely and it is a clear indication of the emergence of social interactions among leeches. Therefore, leeches as other lower invertebrates, exhibit a collective behavior, where conspecifics adopt the same behavior. Leeches are also able to sense the presence of a non-conspecific and are able to collectively modify their behavior (Fig.10) accordingly. Locusts can switch from a solitary to a gregarious behavior in different conditions such as dispersal and migration (Buhl et al., 2006; Anstey et al., 2009). Neurobiological mechanisms underlying social interactions in animals are largely unknown, but several reports indicate that well-known neuromodulators, such as serotonin (5-HT) can play a major role. Indeed, the internal level of 5-HT, a conserved mediator of neuronal plasticity, mediates the shift towards a gregarious behavioral pattern in locusts (Anstey et al., 2009). Thus, our results (Figs. 7, 8 and 9) suggest the emergence of social interactions among leeches when their density is increased and/or the level of endogenous 5-HT is elevated.

The effect of 5-HT and DA

In the leech, each segmental ganglion contains a network of serotonergic neurons (Lent and Frazer, 1977; De-Miguel and Trueta, 2005) that acts on different targets to modulate the swimming motor program (Ort et al., 1974; Kristan and Nusbaum, 1982; Friesen, 1989); these cells are the pair of large Retzius cells, one pair of dorso-lateral (DL) and ventro-lateral (VL) interneurons, and two pairs of medial interneurons (cells E and M) (Lent et al., 1991). The neuropil of the supraesophageal ganglion is densely filled with serotonergic arbors, and a number of 5-HT-immunoreactive fibers were found in a region previously identified as a neurohemal release site (Webb, 1980; Crisp et al., 2002); these 5-HT fibers contained varicosities and represent possible sites of neurohemal secretion. The subesophageal ganglion is a fusion of four neuromeres, each of these contains a pair of large Retzius cells, and two pairs of serotonergic interneurons (cells E and M); in addition, the large serotonin effector cells (LL) are contained in neuromere 1 (Lent et al., 1991; Crisp and Mesce, 2006). It has also been shown that fine 5-HT-immunopositive fibres are associated to the lateral blood vessels close to the dorso-ventral and the longitudinal body wall muscles (Leake et al., 1985). Moreover, there is also evidence that Retzius cell stimulation, which causes mucus secretion from the skin, can be mimicked by exogenous 5-HT, injected on the dorsal side (Lent, 1973). These observations support the notion that 5-HT could act peripherally and hormonally, by circulating in the blood (Willard, 1981).
Dopaminergic neurons have a different distribution than the serotonergic ones: two pairs of cells are present in the supraesophageal ganglion, seven pairs in the subesophageal ganglion and in the tail brain, while each segmental ganglion receives dopaminergic inputs from a peripheral cell located in the anterior root ganglion (Crisp et al., 2002). Thus the combined action of neurons able to secrete DA and 5-HT and those with the corresponding receptors affects and modulates several aspects of the leech behavior. Indeed, the biological mechanisms of behavioral selection of the leech have been gradually uncovered during the last decade. The decision to swim is distributed across populations of swim-activating and swim-inactivating neurons descending from the subesophageal ganglion of the head brain (Crisp and Mesce, 2006). The modulatory effect of 5-HT on the generation of the swimming motor program has been quantified using isolated nerve cord preparations (Willard, 1981). The choice between swimming and crawling in leeches is also mediated by DA, which represses swimming and evokes crawling (Friesen and Kristan, 2007; Puhl and Mesce, 2008). In our work we extend these findings, by demonstrating that in intact animals - and not only in isolated nerve cords 5-HT made the swimming motor program more likely, leading to an increased percentage of pseudo-swimming episodes (Fig. 4). Leeches injected with 5-HT were also more prone to swim after tail stimulation (data not shown) and although the swimming probability did not change significantly (Figs. 8 and 9) leeches were more prone to swim collectively. Similarly, DA increased the percentage of crawling episodes, but no collective behavior has been observed. These two amines are released locally into the leech ganglia by peripheral and central neurons, and may be released into the blood by a putative neurochemical complex in the supraesophageal ganglion of the head brain (Webb, 1980; Crisp et al., 2002), or by the ganglia themselves (Glover and Lent, 1991). In this work we observed that 5-HT and - to a lesser extent - DA, affect social leech behavior and indeed these two neuromodulators have a role in determining how leeches alter their behavior in the presence of their conspecifics: injection of these neuromodulators decreases the pair-wise average distance between conspecifics promoting the emergence of social interactions. These observations are very similar to what already observed in other simple nervous systems such as C. elegans, Drosophila, and other invertebrates (Sokolowski, 2010). The effect of 5-HT, here described, is similar to what observed in desert locusts Schistocerca gregaria (Anstey et al., 2009) becoming gregarious following injection of 5-HT. The neurobiological mechanisms generating and modulating these social interactions in the leech are not yet known; therefore such studies are appropriate subjects for the newly developing field of Social Neuroscience (Adolphs, 2010).

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Figure Legends

Figure 1. A: Diagram of the experimental set-up. A color CCD camera imaged from above the observation tank containing one or several leeches. Two colored beads were glued onto their skin near their head and tail (right side of panel D). The locations of the colored beads were detected at a rate of 15 frames per second. A real-time algorithm performed a Bayesian classification of the beads color in the RGB feature space (B). Each color is represented by the covariance ellipsoid in the RGB feature space (C). After classification, bead coordinates were saved on a hard disk for later off-line analysis (D). From the analysis of bead trajectories five different behaviors were classified and characterized. Exploratory movements (E) were recognized as non-periodic movements of the head while the tail bead was stationary; crawling (F) and swimming (G) episodes were detected by considering their characteristic frequency, and pseudo-swimming events (H) where detected as those episodes in which the head bead oscillates but the tail bead remains stationary. The stationary state corresponds to periods of time in which the velocity of the head and tail beads is below a given threshold. Leech length was normalized to the maximum measured length.

Figure 2. A and B: Schematics of the experiments; before turning on the light and beginning recording leech motion, leeches were kept in the same tank (A) or were transferred in a new tank (B). In the latter case, we observed an initial phase, during which leeches explored and searched the new tank, followed by a second phase where leeches were not moving. C: Time evolution of the displacement – in pixels - of a leech center of mass from the axis origin (corresponding to the lower left corner of the camera acquired frames). To define the duration of the initial searching phase, we calculated the center of mass displacement (C), and considered episodes where its velocity exceeded 3 pixels/s (D, red frames): the length of this episode - occurring during the first 90 minutes - is taken as the duration of the searching phase. E: comparison of the duration of the searching phase in the two experimental conditions illustrated in A and B. F: Histogram of the duration of the searching phase in the new tank for experiments illustrated in B. Data from 13 solitary leeches. G: Joint density of the normalized Cartesian coordinates P(X, Y), estimated from the trajectory of solitary leeches during their searching phase. G-I: Joint density of the radial and angular coordinates P(R, ϕ), both during the searching phase (G) and the following phase in which leeches were not moving (H). The radial density is in essence flat, but the radial density has two peaks corresponding to the upper and lower rim. During the post-exploratory phase, no spatial preference is evident (I).

Figure 3. Identification of the different leech behaviors. A: The leech length was recovered from the bead coordinates and normalized to its maximal observed length. Leech behavior was classified into the five stereotyped behaviors most commonly observed: swimming, pseudo-swimming, crawling, static and exploratory. B: Enlarged view, showing the first ten minutes of the trace in A. C: Percentage of behavior. Each bar represents the percentage of each behavior during the searching (colored bars) and static phases (colored textured bars). During the searching phase the leech crawls along the rims of the tank, with sporadic swimming bouts, whereas in the following static phase it does not move, or probes the environment with exploratory head movements. D: Comparison of the duration of the searching phase for leeches in the small and large tank. The searching phase duration is longer in the large tank (Student’s t-test, p = 0.037). E: Comparison of behavior percentage of leeches in the small and large tank. F: Comparison of the duration of the searching phase for well fed and hungry leeches. The searching phase duration is longer for hungry leeches.
(Student’s t-test, \( p = 4.5 \times 10^{-9} \)). G: Comparison of behavior percentage of well fed and hungry leeches. The small circular tank had a volume of 2 litres (lower diameter: 14 cm, upper diameter: 18.2 cm) and the large circular tank had a volume of 6 litres (lower diameter: 18 cm, upper diameter 27.7 cm).

**Figure 4.** Effect of body injection of 5-HT and DA on leech behavior. A: Percentage of pseudo-swimming after the injection of varying 5-HT concentrations (black dots) and comparison with saline (dark gray square) and not injected (light gray triangle) controls. 5-HT concentrations of 150-200 µM elicited the highest percentage of pseudo-swimming. B: Comparison of duration of uninterrupted bouts of pseudo-swimming in leeches injected with 150-200 µM 5-HT (black line) and in not injected leeches (gray line). C: Cumulative distribution plot (same data as panel B): 5-HT injected leeches show longer pseudo-swimming bouts (Kolmogorov-Smirnov test, \( p<0.01 \)). D: Percentage of crawling after the injection of varying DA concentrations (black dots) and comparison with saline (dark gray square) and not injected (light gray triangle) controls. DA concentrations of 650-700 µM elicited the highest percentage of crawling. D: Comparison of duration of uninterrupted bouts of crawling in leeches injected with 650-700 µM DA (black line) and in not injected leeches (gray line). A statistical test between the cumulative distributions did not show a significant difference (Kolmogorov-Smirnov test). Reported amounts of 5-HT and of DA were dissolved in 300 µl of leech saline and were injected in leeches as described in the Methods Section. F: Percentage of crawling after the injection of increasing volumes of saline. Higher volumes have the effect of reducing the crawling percentages (t-test \( *= p<0.01 \)), suggesting an increasing dilution of DA in the blood.

**Figure 5.** Independent motion in 3 leeches. A: The probability density (pdd) of pair-wise distances (\( D_{\text{leeches}} \)) from 5 experiments with 3 leeches (gray vertical bars); pdd obtained from the same experiments but with shuffled data (blue trace), and pdd from simulated data using the independent model (red trace). The similarity between the three pdds indicates independent motion in groups of 3 leeches. B-C: The radial and angular densities of the location of solitary leeches obtained from 15 experiments (blue data in A and B); the radial density was fitted with the sum of a log-normal and a normal distribution (see eq. 1) providing the red line in B along with 0.95 confidence bounds (dotted red lines), the angular density was assumed to be uniform and is shown as a red line in C. By using these theoretical densities we have simulated independent spatial point processes describing leeches, moving independently from each other. D: Comparison between 3 experimental trajectories and 3 simulated trajectories.

**Figure 6.** A: Comparison of behavior percentage as in Fig. 3C (black bars) and when one other leech was crawling (dark gray bars) or was static (light gray bars). These behavior percentages do not depend on whether another leech is static or is crawling suggesting that the behavior of the 3 leeches in reciprocally independent. B: Model of the dynamics of the leech behavior as a 5 five states homogeneous Markov chain (not all transitions are shown) C: Statistical analysis of the independence of leech behavior. The behavioral sequences of pairs of leeches were divided in windows of 20 minutes and each couple of windowed sequences was tested for the null hypothesis that the two sequences form a couple of independent Markov chains. D: The 5x5 matrix with entries reporting the fraction of samples for which statistical independence is observed with a p-value > 0.01 (N=12 couples of leeches).
Figure 7. Effect of the different leech number and of injected neuromodulators on the probability density of normalized pair-wise distance $D_{\text{leeches}}$. A: the distribution density of $D_{\text{leeches}}$ among 3 non-injected leeches (black bars) and the theoretical distribution expected in the case of 3 independently moving leeches (gray line), B: the distribution density of $D_{\text{leeches}}$ among 10 non-injected leeches (black bars) and the theoretical distribution expected in the case of 10 independently moving leeches (gray line). The peak at a small distance indicates the formation of a group of leeches in physical contact. C: the distribution density of $D_{\text{leeches}}$ among 3 leeches injected with $150-200\ \mu M$ of 5-HT (black bars) and the theoretical distribution expected in the case of 3 independently moving leeches (gray line). D: the distribution density of $D_{\text{leeches}}$ among 3 leeches injected with $650-700\ \mu M$ DA (black bars) and the theoretical distribution expected in the case of 3 independently moving leeches (gray line).

Figure 8. Emergence of collective behavior when the number of conspecifics is increased. 10 leeches explored the tank (A) and came to rest either in a scattered configuration (B) or in a gregarious configuration (C). All 10 leeches could also swim (D) in a collective manner. Beads are also shown in gray. E: comparison of the experimental percentage of collective swimming in $k$ leeches (gray line) and the binomial distribution where the probability of swimming is 0.10 (black line). The lines are different according to the two-sample Kolmogorov-Smirnov test – $p<0.01$. F: comparison of the estimated probability that at least 5 leeches swim contemporarily and the theoretical value (black horizontal line) that would be expected in the case of independent motion where the probability of swimming is 0.10.

Figure 9. Emergence of collective behavior in leeches injected with 200 $\mu M$ 5-HT. 5 injected leeches explored the tank (A) and could come to rest in a scattered (B) or a gregarious configuration (C). Sometimes all 5 leeches swam (D) simultaneously. Beads are also shown in gray. E: Same as in Fig.8E, but for 5 injected leeches. F: Attraction of conspecifics in leeches injected with 5-HT. 10 not injected leeches were positioned in the observation tank. After the formation of a group of 5 conspecifics (large black shape in the upper portion of the observation tank), a leech injected with 200 $\mu M$ 5-HT was placed in the tank. A coloured bead was glued on the back of this leech so that it was possible to follow its trajectory (light gray line). The final position of the injected leech is indicated by the dark gray circle. G: Trajectory of a leech injected with 200 $\mu M$ 5-HT and placed in a tank where a black plastic object (rectangular black shape, regarded as a fake group) was positioned. The injected leech did not come to rest near the black object. H: Comparison of the probability that at least 4 leeches swim contemporarily and the theoretical value (black horizontal line) that would be expected in the case of independent motion where the probability of swimming is 0.10. I: Percentage of time spent in a group of at least 4 conspecifics under different treatments (*=Student’s t-test, $p<0.01$).

Figure 10. Effect of the presence of a non-conspecific on collective swimming behavior. A: A gold fish (red) was placed in the tank along with 10 leeches (black). B: The real-time tracking algorithm allowed for the coordinate extraction of the centers of mass of the gold fish and of the group of leeches. Here, three panels show the gold fish chasing one swimming leech. C: Scalar product between the velocity vector $v_f$ of the gold fish and the average velocity vector from the leech population $<v>$ (see Methods). D: Positive-over-negative ratio of scalar product values calculated in 5 experiments. The higher fraction of positive peaks reflects the occurrence of episodes in which at least one leech and the gold fish swim in the same direction. E: Comparison of the probability that at least 4 leeches swim contemporarily and the theoretical value (black horizontal line) that would be
expected in the case of independent motion where the probability of swimming is 0.10. The gold fish presence could potentially inhibit the occurrence of collective swimming episodes.
References


**Figure A**

Bar graph showing the percentage of time spent in different behaviors: Swimming, Pseudoswimming, Crawling, Static, and Exploratory. The graph compares the control group and the group when crawling or static.

**Figure B**

A flowchart illustrating the movement between behaviors: Swimming, Pseudoswimming, Static, Crawling, and Exploratory.

**Figure C**

A Markov chain model for Leech 1 and Leech 2 behaviors. Leech 1 behavior is represented as a sequence of numbers, and Leech 2 behavior is represented similarly. It includes a significance level and a conditional test of independence between two Markov chains.

**Table D**

A confusion matrix for Leech 1 and Leech 2 behaviors.

- **Leech 1**
  - Swimming: 99.15, Pseudoswimming: 97.58, Crawling: 98.15, Static: 96.15, Exploratory: 98.86
  - Pseudoswimming: 97.58, 92.88, 94.87, 91.74, 97.01
  - Crawling: 98.15, 94.87, 98.58, 94.44, 97.72
  - Static: 96.15, 91.74, 94.44, 90.03, 95.73
  - Exploratory: 98.86, 97.01, 97.72, 95.73, 97.44

- **Leech 2**
  - Swimming: 99.15, Pseudoswimming: 97.58, Crawling: 98.15, Static: 96.15, Exploratory: 98.86
  - Pseudoswimming: 97.58, 92.88, 94.87, 91.74, 97.01
  - Crawling: 98.15, 94.87, 98.58, 94.44, 97.72
  - Static: 96.15, 91.74, 94.44, 90.03, 95.73
  - Exploratory: 98.86, 97.01, 97.72, 95.73, 97.44