Earthworm individualities when facing a conflict between turn alternation and aversive learning

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Received May 10, 2018; accepted July 6, 2018

An individual’s personality develops through a combination of experiences and parental inheritance. When faced with a conflict, will an individual take an innate behavior or a learned one? In such situations, individuality will manifest itself. Here, we focused on turn alternation behavior, which is a habitual tendency to turn in the direction opposite the preceding turn, in earthworms (Eisenia fetida) and examined how this behavior is affected by an aversive stimulus. Of 10 earthworms, 3 were affected by the stimulus. Turn alternation deteriorated in two worms, one of which showed anti-turn alternation behavior, whereas the remaining worm showed an enhanced tendency toward turn alternation. Earthworms have a relatively simple nervous system. This study opens the door to investigate the neuronal basis for individuality that emerges between nature and nurture.

Key words: Earthworm, turn alternation, conditioned stimulus, innate behavior, learned behavior

We investigated whether an aversive stimulus affects turn alternation (TA) in earthworms. TA is a tendency to turn in the direction opposite the preceding turn. The apparatuses used in TA studies are similar to those used in conditioning studies. However, there are no studies in invertebrates that combine these two types of studies. Each earthworm was tested in a sufficient number of trials, to allow us to evaluate its unique response. Two of ten earthworms stopped making TAs after aversive stimulation, while one worm increased the TA rate after stimulation. Earthworm individualities become obvious at the conflict between alternation behaviors.

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two behaviors have never been compared in the same individual earthworm.

Therefore, in this study, we examined whether an unconditioned stimulus would affect earthworms’ turn-alternation behavior in a J- or τ-shaped maze. To evaluate individuality, each subject was tested in a sufficient number of trials to permit statistical analyses, and changes resulting from stimulation were tracked for each worm. This approach revealed individual variation in behavior. Specifically, we found that although many worms showed turn alternation before and after the aversive stimulus, two ceased the behavior after the stimulus, while one performed it more frequently after stimulation.

Methods

We used commercially available live earthworms, Eisenia fetida. Worms were actively mobile, and were gently cleaned to remove excessive soil. The mud covering their bodies was cleaned off. The body length of each worm was measured before the experiment (mean body length 7.3±1.1 cm, n=15).

We tested the turn-alternation behavior of earthworms in standard acrylic mazes, that is J-shaped and τ-shaped mazes (Fig. 1A). Each maze had specified starting (S), forced turning (F), and choice (C) points. The distances between S and F and between F and C were set to 5 and 10 cm, respectively. The width and depth of the groove were 4 and 5 mm, respectively. The maze in which the earthworm was forced to turn left at F, namely J-shaped maze was also referred to as the “forced-left” or “L-maze.” Conversely, the τ-shaped maze, which forces the worm to turn right, is referred to as the “forced-right” or “R-maze”. Turn-alternation behavior at point C was defined as turning in the opposite direction to the turning at point F. The turn-alternation rate was calculated as the ratio of the number of trials in which turn alternation was observed divided by the total number of trials.

The experiment consisted of three sessions: the pre-stimulus session of 11 trials, the stimulus session of a single trial, and the post-stimulus session of 11 trials (Fig. 1B). Half of the test subjects started the first trial of the pre-stimulus session in the forced-left apparatus (‘L’-first test), while the other half of the test group started in the forced-right apparatus (‘R’-first test). The test apparatus type was then alternated between the forced-left and forced-right mazes for the remaining trials in the pre-stimulus session. In the stimulus session, a single aversive stimulus was applied by poking the earthworm with a needle (TERUMO 27G × 3/4, φ=0.40 mm) forcefully enough to induce a contraction response, taking great care not to cause flows of blood or body fluid or movement disorders. The poke was applied at 5, 20, and 40 s after the worm began to turn at C. At the point where the stimulation was applied in each trial, the distance between the mouth of the worm and C was measured. The post-stimulus session started with a trial using the same apparatus type as in the stimulus session to confirm whether the earthworm avoided turning in the same direc-
tion as in the stimulus session. As in the pre-stimulus session, apparatus types were alternated in subsequent trials. All trials were recorded on video (Panasonic HDC-TM90).

To prevent earthworms from escaping from the groove, the apparatus was covered with a sheet of plastic wrap. The pain stimulus was applied with a needle through this wrap. The apparatuses were washed after each trial to remove any body fluids from the previous trial. The earthworms were moistened before each trial. The room was kept at a constant moderate temperature and humidity, and the room light was dimmed to prevent it from acting as an additional aversive stimulus.

**Results**

In all, 15 earthworms were used in the experiment. It took several hours to test one worm on the three sessions. Ten completed all three sessions. The results were analyzed only for test subjects that completed all three trial sessions.

As a population (n = 10), the mean turn-alternation rates in the pre- and post-stimulus sessions did not differ significantly (paired t-test: t10 = 0.844, n = 110, P = 0.401; Fig. 2A). There was also no significant difference in turn-alternation occurrence between L-first and R-first sessions for the entire experimental period (t-test: t10 = 1.591, nL = 11, P = 0.113). The mean turn-alternation rate of the post-stimulus session was slightly lower than that of the pre-stimulus session. However, this was not due to the short-term effects of pain stimulation, as the population exhibited significant turn alternation (binominal test: 9/10, P < 0.05) even in the first trial of the post-stimulus session.

The majority of the earthworms (n = 6) exhibited significant turn-alternation behavior in both the pre- and post-stimulus sessions. One earthworm did not display significant turn-alternation behavior in either binominal test: pre-stimulus session, n = 11, P = 0.73; post-stimulus session, n = 11, P = 0.11). The remaining three earthworms displayed significant turn-alternation behavior in only one session, either pre- or post-stimulus.

One worm (#12–26–1) did not display significant turn alternation in the pre-stimulus session (binominal test: n = 11, P = 0.34) but did in the post-stimulus session (binominal test: n = 11, P < 0.05; dotted line in Fig. 2A and Fig. 2B). During the pre-stimulus session, this individual appeared to have a directional preference, turning to the right in 10 of 11 trials.

By contrast, two earthworms (#12–22–1 and #12–25–1) had significant turn-alternation rates in the pre-stimulus session (binominal test: #12–22–1, n = 11, P < 0.001; #12–25–1, n = 11, P < 0.05; dashed lines in Fig. 2A and Fig. 2C and D) and non-significant rates in the post-stimulus session (binominal test: #12–22–1, n = 11, P = 0.27; #12–25–1: n = 11, P = 0.99). This reduction in the turn-alternation rate was significant (paired t-test: #12–22–1, t10 = 2.391, n = 11, P = 0.05; #12–25–1: t10 = 2.609, n = 11, P = 0.05). Subject #12–22–1 received the aversive stimulus while it was turning right.

![Figure 2](image-url) Experimental effects of applying an aversive stimulus on turn alternation responses in earthworms. A. Mean turn-alternation rates (averaged over 10 worms and 11 trials) for the pre- (Pre-stim.) and post-stimulus (Post-stim.) sessions. O, individual worms with significant mean turn-alternation rates for the session; X, nonsignificant. Dotted line, the performance of #12–26–1. Dashed lines, the performances of #12–22–1 and #12–25–1 respectively. n.s., non-significant. B. Subject #12–26–1 showed no significant turn alternation before stimulation, but significantly displayed turn alternation after stimulation. Subjects C. #12–22–1 and D. #12–25–1 displayed significant turn alternation before, but not after, stimulation. O, turn alternation response; X, anti-turn alternation response in each trial. Other labels are as represented in Figure 1B.

Subsequently, it appeared to avoid turning right in half of the post-stimulus trials (three of six trials) using the ‘L’ apparatus. As for Subject #12–25–1, it exhibited significant anti-turn alternation during the post-stimulus session, turning in the same direction as its previous turn in 9 of 11 trials (binominal test: n = 11, P < 0.05).

We examined the relationships of turn-alternation rates in the post-stimulus sessions with both the timing of the stimulus application (Fig. 3A) and the distance from C to the earthworm’s mouth (Fig. 3B). However, no significant correlations were detected (timing: r = –0.057, n = 10, P = 0.88; distance: r = 0.026, n = 10, P = 0.94). We also examined turn-alternation rates between the two sessions (Fig. 3C). Subjects that displayed low turn-alternation rates in the pre-stimulus session seemed to have low turn-alternation rates in...
the post-stimulus session as well, although this correlation was not significant ($r=0.20$, $n=10$, $P=0.58$).

**Discussion**

We examined whether the turn-alternation behavior of earthworms was affected by an aversive stimulus. Each worm performed sufficient trials to allow us to evaluate its unique response. We identified individuals that maintained turn alternation before and after an aversive stimulus, one that exhibited an enhanced rate of turn alternation after the stimulus, and others that stopped the behavior after the stimulus.

The structure of the apparatus was appropriate for examining turn-alternation behavior, as it is known to influence the emergence of this particular behavior [21]. However, our research did not focus on which parameters affected turn alternation. Therefore, we selected the optimal apparatus size for facilitating turn alternation [22]. The appropriateness of our apparatus was confirmed by the frequency of turn alternation during the experiment.

Here, our results are interpreted as reflecting each worm’s individuality, rather than a particular experimental condition tested in this study. We tested different timings of the stimulus and also measured the distance between the choice point and the stimulation site in the apparatus. As indicated in Figure 3, these two values were unrelated to performance in the post-stimulus session. These results indicate that the lengths of the timings and distances examined in our study had no effect on the post-stimulus turn-alternation behavior. We also found no correlation between behavior before and after stimulation (Fig. 3C). If, instead, we had observed a significant correlation, we would have concluded that the individuals exhibiting high rates of turn alternation before stimulation could clearly recognize that turn-alternation behavior was punished. On the other hand, worms with low rates would have initiated the turn-alternation behavior to heighten their awareness of danger after the stimulus alerted them. In conclusion, the behaviors observed in the present study cannot be predicted from simple tasks and behavioral parameters; instead, they should be attributed to each worm’s individuality. This conclusion then begs the question of how each individual earthworm makes the decision.

In our experiment, the innate behavior of turn alternation was predominant in the majority of earthworms. Many behavioral experiments examining learning in earthworms have also used T-mazes (e.g. [17–19]). The apparatuses used in this study of turn-alternation behavior, namely, J-shaped and τ-shaped mazes, can be considered modified versions of a T-maze. Therefore, it would have not been surprising if aversive learning had been observed in our experiment.

The fact that behavioral changes were not observed for most of our test subjects presumably implies that learning is context-dependent and specific to the situation. The view that turn alternation is an innate behavior is also supported by the fact that it has also been observed even in *Paramecium* [23] and sperm [24]. Innate behavior has evolved over long periods of time to facilitate the survival of individuals and species; thus, it should not be readily changed by a single experience. For example, even when an individual has suffered greatly from ingesting a specific food or interacting with another individual of the opposite sex, the individual cannot survive if it avoids eating all food or interacting with all members of the opposite sex. This idea is supported by observations that aversive learning in earthworms occurs more frequently by repetitive stimulation or by associations with other sensory stimuli [17], both of which appear to facilitate the recognition of that particular situation as one in which to learn. The earthworms that did not exhibit turn alternation in the present experiment possibly did not regard the stimulus session as a situation in which to learn something.

Defining the range of a situation is also important in reinforcement learning theory [25], which explains conditional learning [26]. The observation that many earthworms were unable to learn from aversive stimulation in this study may have resulted from the experimental procedure that conditioned the earthworms with the sense of self-motion. From
the viewpoint of reinforcement learning theory, earthworms may not have an action-value function that includes the agent’s own action [27]. Instead, they may have a state-value function, as they are known to be responsive to conditioning by sensory stimuli. In other words, earthworms can learn associations involving a given sensory stimulus (sandpaper) and an aversive stimulus (electrical shock) [11]. Our method of combining turn alternation and aversive stimuli could be employed to investigate the existence of a “sense-of-self” in animals.

How did the three individual earthworms in which turn alternation was affected by the stimulus recognize the state of the environment they were in? Turn alternation is advantageous for escaping [28]. When an animal tries to escape from an enemy or a harmful place, it is more advantageous to flee in a linear manner. Turn alternation makes it possible to escape almost linearly even in the presence of obstacles. For example, a previous study demonstrated that turn alternation appeared more frequently in situations where the test subject was exposed to danger than in other situations [29]. Similarly, in our experiment, significant turn-alternation responses were observed in earthworm #12–26–1 after pain stimulation. This selection of the turn-alternation response was likely due to the earthworm’s recognition of an unsafe situation derived from having received a strong aversive stimulus. By contrast, the deteriorated turn alternation in #12–22–1 and #12–25–1 suggests that they recognize that going straight ahead is risky. In earthworms, turn alternation may be affected by recognition of the danger levels in the situation or the environment.

To investigate the neuronal basis for the individuality of earthworms observed in this study, interactions among several mechanisms discussed below should be examined. The first mechanism is turn alternation as an innate behavior. Hughes [5] concluded that thigmotaxis or tactile stimuli contributes predominantly to turn alternation in earthworms. The second mechanism is learning. In recent years, electrophysiological and imaging experiments of earthworms have been conducted to reveal their mechanism for learning and memory [12–14]. The third mechanism is perception of the state of environment. Sensory inputs modulate the rhythm generated by the central pattern generator (CPG) within each segmental ganglion that controls earthworm locomotion [30,31]. More fundamentally, we have to consider the sensory mechanisms for aversive stimuli and the locomotion mechanisms for turning left or right. Earthworms show rapid withdrawal reflexes mediated by giant nerve fibers when subjected to unfavorable stimuli. Enkephalin and β-endorphin-like substances have been found in earthworms [32], and injections of the opiate receptor antagonist naloxone have shown to inhibit the worms’ touch-induced escape responses [33], suggesting that the opioid substances may play a role in sensory modulation. On the other hand, the strength and rhythm of the muscle contraction of each body segment, the basis of earthworms’ locomotion, is generated by the nerve that controls it, and its amplitude and rhythm are regulated by octopamine [30]. In addition, cell population called CPP plays an important role in generating the rhythm [14]. Thus, which direction to go is presumably decided by controlling the difference of the amount of octopamine release between the left and the right sides within each segment. Based on these perspectives, it is necessary to examine how tactile and other sensory modulation of the CPG in earthworms are changed by aversive stimuli to reveal their individuality at a microscopic level.

Acknowledgements

We thank M. Takahashi for technical helps and advices. This research was supported by a Grant-in-Aid for Scientific Research on Innovative Areas ‘Non-linear Neuro-oscillology: Towards Integrative Understanding of Human Nature (15H05879)’ of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Conflicts of Interest

The authors declare no competing financial interests.

Author Contributions

T.N. and K.S. designed the study. T.N. performed the experiments. T.N. and K.S. analyzed the data. K.S. performed the statistical analysis. H.M. supervised the experiments. K.S. prepared the figures. K.S. wrote the manuscript.

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