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Developmental Changes in Conditioned Taste Aversion in *Lymnaea stagnalis*

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ABSTRACT—As the first step to study relationships between development and learning in the molluscan central nervous system, we examined developmental changes in acquisition and retention of a conditioned taste aversion (CTA) in the pond snail, *Lymnaea stagnalis*. We found that snails developed ability of a CTA as a long-term memory through three critical stages. Embryos in veliconcha started to respond to appetitive sucrose at the first critical stage. This response was in good agreement with morphological observations that embryos at this developmental stage seemed to be physically ready to eat. However, they could not associate this appetitive stimulus (conditioned stimulus: CS) with an aversive stimulus of KCl (unconditioned stimulus: UCS). At the second critical stage, embryos just before hatching acquired the CTA, but the conditioned response did not persist. Through this stage, they may acquire learning ability to safely seek out food in an external environment. At the third critical stage, immature snails with a 10 mm shell could use a long-term memory to maintain the conditioned response. This memory persisted for at least a month, showing that now they are able to maintain a long-term memory so that they can safely eat a variety of food when they cover wide territory to search for a mate. The present findings indicate that the development of learning ability in snails, which secures acquisition of better survival ability, is coincident with the major changes in their life cycle.

INTRODUCTION

Interest in the learning abilities of gastropod molluscs has been stimulated by the progress in which their relatively simple central nervous systems provided useful models for studies on the neuronal basis of learning (e.g. Ito *et al*., 1994). Associative learning has been studied particularly in the pond snail, *Lymnaea stagnalis* (Whelan and McCrohan, 1996; Kemenes *et al*., 1997; Kobayashi *et al*., 1998; Kojima *et al*., 1998; Lukowiak *et al*., 1998; Sakakibara *et al*., 1998). We could demonstrate that *L. stagnalis* can acquire a "conditioned taste aversion (CTA)" (Yamanaka *et al*., 1995; Kojima *et al*., 1996). When an appetitive stimulus (i.e. a conditioned stimulus: CS) was paired with an aversive stimulus (i.e. an unconditioned stimulus: UCS) in snails, significantly fewer feeding responses were elicited by the test appetitive stimulus (CS) when compared to control snails. The persistence of this CTA response was maintained for at least 30 days. The neural pathways (Sadamoto *et al*., 1998) and the neuronal plasticity (Kojima *et al*., 1997) in the CTA were analyzed by us at the cellular level.

Recently, the relationships between development and learning were strongly emphasized in gastropod molluscs (Marcus *et al*., 1994), because neurobiologists have long speculated that growing processes involved in the development of the central nervous system may persist into the adult where they could subserve learning and memory. To examine whether this speculation is true, *L. stagnalis* has a suitable system, since it can be studied from both developmental and learning perspectives. The metamorphosis of *L. stagnalis* is completed inside the egg mass, differing from other popular gastropods, such as *Aplysia californica* (Kriegstein, 1977a,b) and *Hermissenda crassicornis* (Avila *et al*., 1996). The culture of *L. stagnalis* in laboratories thus can be performed in the same condition throughout their life history without difficulty. This advantage of the culture in *L. stagnalis* enabled many works which studied the developmental changes

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in the central nervous system, such as those in the distributions of neurotransmitter-immunoreactive neurons (Croll and Chiasson, 1989; Marois and Croll, 1992; Voronezhskaya and Elekes, 1993, 1996; Elekes *et al.*, 1996; Serfözö *et al.*, 1998).

In the present study, as the first step to study the relationships between development and learning in a simple experimental system, we examined the developmental changes in which *L. stagnalis* acquired the CTA and became to retain the CTA as a long-term memory. The voluntary activity of developing snails was also observed to examine whether there were any changes in behavioral activity, other than the feeding behavior.

MATERIALS AND METHODS

Snails

Locally-reared pond snails, *L. stagnalis*, originally derived from the stocks of Vrije Universiteit in Amsterdam were used. Snails were fed with lettuce and turtle food (Tetra ReptoMin, TetraWerke, Germany), and were maintained on a 12:12 light-dark cycle at 20°C. We used embryos of developmental stages 24-29, juveniles with a 2 mm shell, immatures with a 10 mm shell, and adults with a 20 mm shell (see Fig. 1 to see outlines of the developing snails). The embryonic stages were morphologically classified using Meshcheryakov's criteria (Cumin 1972; Meshcheryakov 1990). The stage 24 embryo is a late veliger with a shell length of 0.4 mm, a height of 0.6 mm, and a width of 0.3 mm. The stage 25 embryo is a veliconcha with a shell length of 0.7 mm, a height of 0.6 mm, and a width of 0.6 mm. The shells of the stage 26 and 27 embryos are 1.0 mm long, 0.6 mm high, and 0.6 mm wide, and the shell of the stage 28 embryo is 1.1 mm long, 0.6 mm high, and 0.7 mm wide. Because the last three embryonic stages are difficult to discriminate, we considered them as one group. The shell of the stage 29 embryo is 1.2 mm long, 0.8 mm high, and 0.8 mm wide. When a snail hatches, it has an adult form, but is smaller in size. When a snail becomes sexually matured, its shell grows to a length of approximately 15 mm. We therefore referred to the snails with a 2 mm shell as juveniles, those with a 10 mm shell as immatures, and those with a 20 mm shell as adults.

An embryo in its individual egg capsule is protected by the capsular membrane and the cocoon envelope. Therefore, to apply solutions of taste substances to embryos, we removed the embryos from these surrounding materials using fine forceps under a stereoscopic microscope. The embryos isolated from their capsules were placed in a modified Jockush saline containing 10.3 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, and 20 mM HEPES-NaOH (pH 6.7) (see Jockush, 1968). The juveniles, immatures and adults were removed from their home aquaria and placed in distilled water (DW) one day before experimentation. None of them had access to food for this adaptation period. All experiments were performed in the light period.

Examination of chemosensitivity

Kojima *et al.* (1996) used a 10 mM sucrose and a 50 mM KCl solution as a conditioned stimulus (CS) and an unconditioned stimulus (UCS), respectively, to obtain a reliable CTA in adult snails. We, however, experimentally determined the minimum concentration of solution of sucrose (CS) which induces a reliable feeding response (biting) and that of KCl (UCS) which elicits a withdrawal response of pulling its body into the shell in snails at different developmental stages. A perfusion container, which was a set of mesh pot in a center hole of a silicon stage, was used for the examination of chemosensitivity of embryos (Fig. 2A). Figure 2B is a different container for juveniles, immatures, and adults. The number of bites in the snails that did not exhibit the spontaneous feeding responses before the application of sucrose was counted in a period of 1 min following the sucrose application, if necessary using a stereoscopic microscope or a magnifying glass. Note that the most snails set in a perfusion container did not show the spontaneous feeding responses. We also observed the

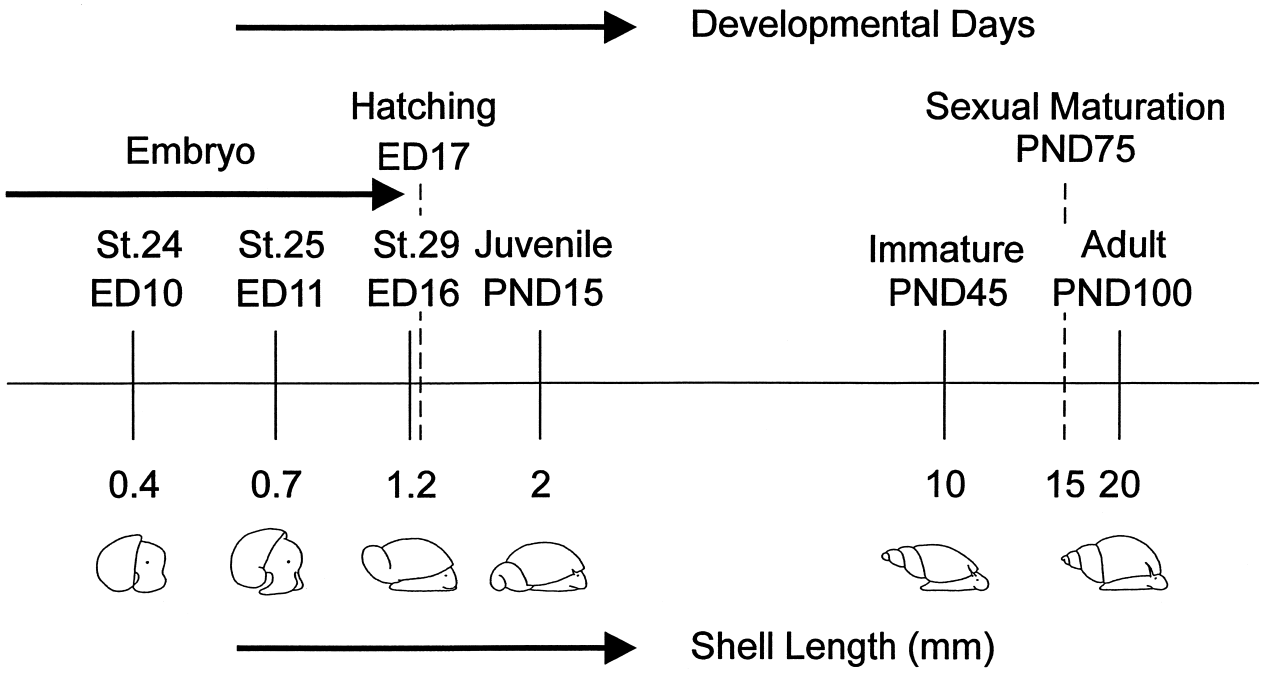

Fig. 1. Outline of developing *L. stagnalis* kept at 20°C. The shell length is expressed in a logarithmic scale. ED and PND indicate embryonic days and postnatal days, respectively. St.: embryonic stage according to Meshcheryakov's criteria (1990). Note that the developmental days are synchronized well in the early stages of embryos, but not in the late stage embryos (time lag of a few days), juveniles and immatures (a few weeks) and adults (a few months).

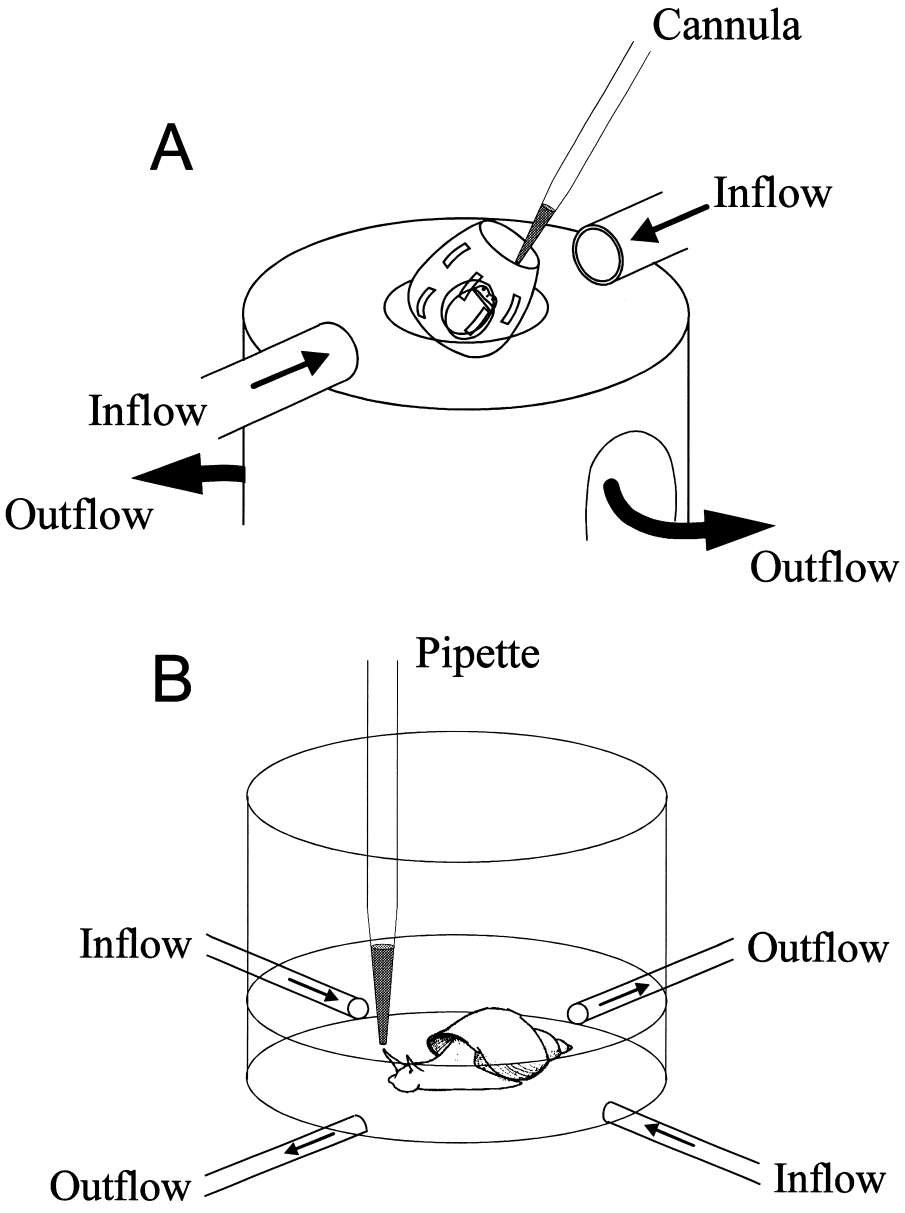

Fig. 2. Schematic drawings of training and test containers. (A) Container for embryos. The mesh pot was constantly perfused with embryo saline. Solutions of taste substances were applied with a cannula. (B) Container for juveniles and adults. The container was constantly perfused with DW. Solutions of taste substances were applied with a cannula or a pipette.

magnitude of the withdrawal response following the KCl application. We examined whether osmotic changes within the tested range of the applied solutions caused a feeding or withdrawal response. When a 100 mM solution of D-cellobiose (a disaccharide which is not perceived as sweet by humans) was applied to stage 25–29 embryos, juveniles, and immatures, neither increase of feeding responses nor withdrawal responses was observed, indicating that the effects of osmotic changes were negligible. See Kojima *et al.*, 1996 for the data for adults.

CTA paradigm

To induce CTA learning in snails, sucrose and KCl solutions were applied as a CS and an UCS, respectively. The concentrations of these solutions were determined from the results of the previous experiments. A 0.05 ml solution of 100 mM sucrose and a 0.05 ml solution of 100 mM KCl were used for embryos; a 0.3 ml solution of 10 mM sucrose and a 0.3 ml solution of 50 mM KCl were for juveniles; a 1 ml solution of 10 mM sucrose and a 1 ml solution of 50 mM KCl were for immatures. The application volume, the application period, and the apparatus for the training were identical to those for the chemosensitivity examination. The CTA paradigm is shown in Fig. 3. Prior to the training session, the CS was applied to the snails, which did not exhibit the spontaneous feeding responses before the application of the CS, to check the feeding response as a pre-test following a 10 min adaptation period. Non-responsive snails in this pre-test were removed from the consecutive training procedure. Ten min later, pairs of the CS and UCS were repeatedly applied to the lips of snails (Fig. 3A, C). The application periods of the CS and UCS, the interstimulus interval (ISI) between the onsets of the CS and UCS, the intertrial interval (ITI) between paired presentations of the CS-UCS, and the number of paired trials were as follows. For embryos, the CS and UCS were applied for 30 sec and 3 sec, respectively; the

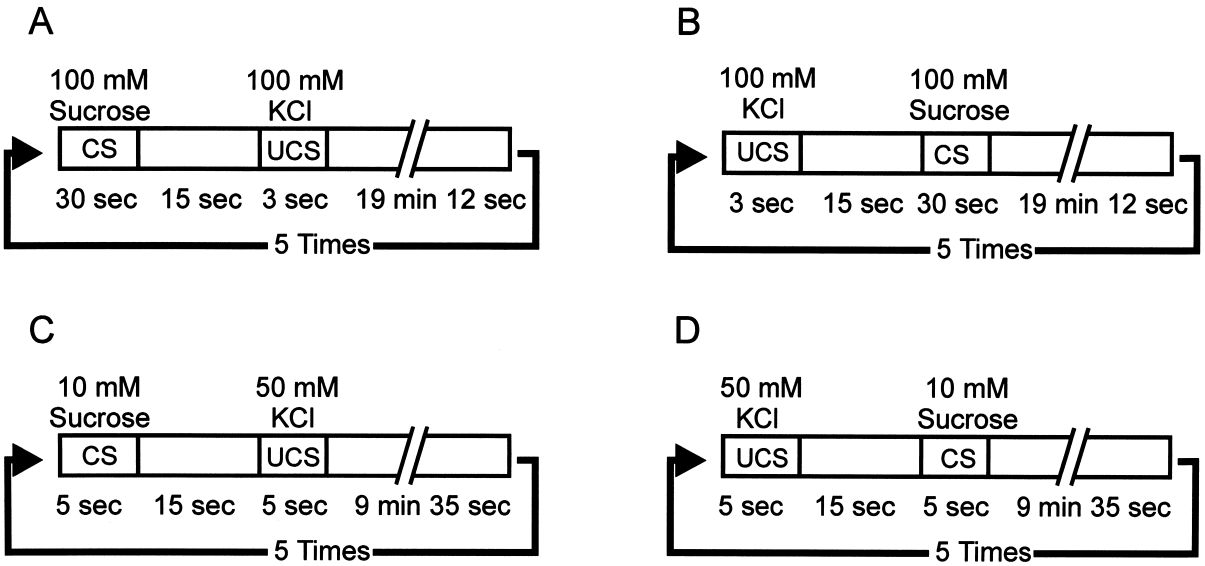

Fig. 3. Paradigm for learning of conditioned taste aversion (CTA). (A) CTA procedure for embryos. (B) Backward-conditioning procedure for embryos. (C) CTA procedure for juveniles and adults. (D) Backward-conditioning procedure for juveniles and adults. CS, conditioned stimulus; UCS, unconditioned stimulus.

ISI and ITI were 45 sec and 20 min, respectively; the number of paired trials was five. For juveniles and immatures, the CS and UCS were applied for 5 sec each; the ISI and ITI were 20 sec and 10 min, respectively; the number of paired trials was also five. Note that these parameters were set after trying many variations (see Kojima *et al.*, 1996 for the details of this parameter determination). As can be seen in RESULTS, only the stage 29 embryos developed a CTA. Therefore, we fixed the most suitable parameters, which could produce the CTA learning in the stage 29 embryos, for the CTA paradigm in all embryos. After the training session, the feeding response to the CS was counted for 1 min in the embryos and for 1.5 min in the juveniles and immatures. The difference in the periods for counting will be discussed later. Note that no spontaneous feeding responses were observed after the training session.

A backward (UCS-CS) conditioning control (Fig. 3B, D) and a random control procedure were also employed. The backward conditioning control procedure and the random control one applied CS and UCS in a contrary and a random order, respectively, but kept the total number of stimuli and the training period as the same as in the learning procedure for CTA. To determine the persistence of learned behavior, the response to the CS was tested 24 hr and 7 days later in the embryos, and 10 min, 3 hr, 24 hr, 3 days, 7 days and 30 days later in the juveniles and immatures. The conditioned embryos, juveniles, and immatures were kept with lettuce and turtle food in embryonic saline or in tapwater.

The learning experiments were performed with a blind protocol. That is, the experimenters who trained the CTA learning in snails and those who counted the feeding responses of the same snails achieved their own work without knowing the data obtained by the others.

Voluntary activity

To examine whether there are any changes in activity other than the feeding response in developing snails, we observed their voluntary activity for 30 min after an adaptation period of 10 min. We put an individual snail into a straight tube, which was 15 times as long as the shell and contained DW. The width and height of the tube were determined to be large enough for snails to turn around. To make clear the relationship between the voluntary activity and the size of snail, the overall length of the tube was divided into eight equal parts, and the number of parts passed by the snails was used to express the unit of voluntary activity.

Statistical analyses

Data were expressed as means ± SEM. The feeding responses (Fig. 5A-C) were evaluated for statistical significance ($p<0.05$) with one-factor ANOVA followed by Scheffé's F test. The long-term memory (Fig. 5D) and the voluntary activity (Fig. 6) were evaluated using Student *t*-test.

RESULTS

Responses to chemical stimuli

We applied 0.1, 1, 10 and 100 mM sucrose solutions to the snail lips in a perfusion system using a cannula or a pipette (Fig. 2). The perfusion rate, the volume of this perfused container, and the volume and period of the application for the sucrose solution were varied for snails at different developmental stages to satisfy the following three conditions: (1) snails must not be washed away by the perfusion; (2) the solution must be presented until a first biting is evoked; (3) the solution must be removed within 15 sec after the application period so that the sucrose would not mix with a next KCl solution, when the same conditions were employed for the CTA procedure. The conditions (1) and (2) were confirmed by careful observation. The condition (3) was confirmed by examination whether or not a 1 ml solution that was taken from the container in 15 sec after the application period induced the feeding responses in different snails. Thus, for embryos, 0.05 ml of sucrose solution was applied for 30 sec using a cannula in a container with 0.1 ml of embryonic saline constantly perfused at 0.5 ml/min. For juveniles, a 0.3 ml sucrose solution was applied with a cannula for 5 sec directly in front of the snail lips. DW was constantly perfused at 4 ml/min in a container that keeps 4.5 ml DW. For immatures and adults, a 1 ml solution was applied for 5 sec with a pipette. DW was con-

stantly perfused at 25 ml/min in a container with 15 ml DW.

Embryos up to stage 24 (veliger) did not respond to any tested concentrations (≦100 mM) of sucrose (n=10). Figure 4 shows feeding responses to sucrose in snails from stage 25 embryo (veliconcha) to adult (n=20 each). The concentration of sucrose sufficient to induce a reliable feeding response, in which ≧70% of tested snails that had expressed no spontaneous feeding responses before the sucrose application started the responses by this sucrose stimulation, was 100 mM for stage 25 to 29 embryos and 10 mM for juveniles, immatures, and adults (data not shown).

The solutions of 0.1, 1, 10, 50 and 100 mM KCl were tested as well, except for a variance in the application period. The appropriate period was determined to be 3 sec, which was adequate to produce a reliable withdrawal response.

The KCl concentration that elicited a reliable withdrawal response (≧70% of tested snails) was 100 mM for stage 26-29 embryos and 50 mM for juveniles, immatures, and adults (n=20 each, data not shown). Although the shell of a stage 25 embryo is too small to withdraw its body into the shell, ≧70% of tested embryos at stage 25 (n=20) exhibited avoidance behavior such as head waving with 100 mM KCl.

CTA Learning

Based on the above data, we decided to use a 100 mM sucrose and a 100 mM KCl solution as the CS and UCS for testing CTA learning in embryos (Fig. 3A). Although the first critical stage in the development to CTA, i.e. beginning of biting, was at embryonic stage 25, CTA learning did not occur at this stage (Fig. 5A). This was because no differences were found between the conditioned (CS-UCS), the backward-conditioning control (UCS-CS), and the random control groups at stage 25. In other words, all these training conditions resulted in a decrease in feeding. To search out the reason for this decrease in feeding in the control snails, we examined the CS only group using stage 25 embryos. When a 100 mM sucrose solution was applied repeatedly to this group in a similar way as the conditioning paradigm, no decrease in the feeding response was observed (data not shown). That is, there was no desensitization to sucrose in stage 25 embryos. Therefore, the decrease in feeding in the control snails was due to the fatigue by use of aversive KCl in the training, even though the concentration of the presented KCl solution was minimum to induce a reliable withdrawal response. The same situation persisted for CTA learning until the embryos reached stage 28. The feeding-response data from stage 26 to 28 are summarized as follows: Pre, 13.2 ± 0.7 (n=30); Conditioned, 7.1 ± 2.3 (n=10); Backward, 4.9 ± 1.8 (n=10); Random, 4.8 ± 2.2 (n=10).

The number of feeding response to sucrose was fewer in the conditioned embryos than those in the control animals at stage 29 (one-factor ANOVA, $F=38.47$, $p<<0.0001$; Scheffe's F test, $p<0.05$ for Conditioned; Fig. 5B). Appetitive sucrose was thus associated with aversive KCl owing to the CTA learning, indicating that stage 29 embryos can acquire the aversive response to sucrose. This is the second critical stage in the CTA in *L. stagnalis*. This conditioned response in stage 29 embryos disappeared within a week (Conditioned response 7 days later: 21.1 ± 2.3 (n=22)). When juveniles were conditioned, we also confirmed the formation of a CTA (feeding response of Pre, 23.5 ± 1.0 (n=40); Conditioned, 6.4 ± 1.3 (n=15); Backward, 16.3 ± 2.6 (n=15); Random, 20.7 ± 2.6 (n=10); one-factor ANOVA, $F=61.95$, $p<<0.0001$; Scheffe's F test, $p<0.01$ for Conditioned), but the CTA persisted only for several days (Conditioned response 7 days later: 16.4 ± 2.8 (n=12)).

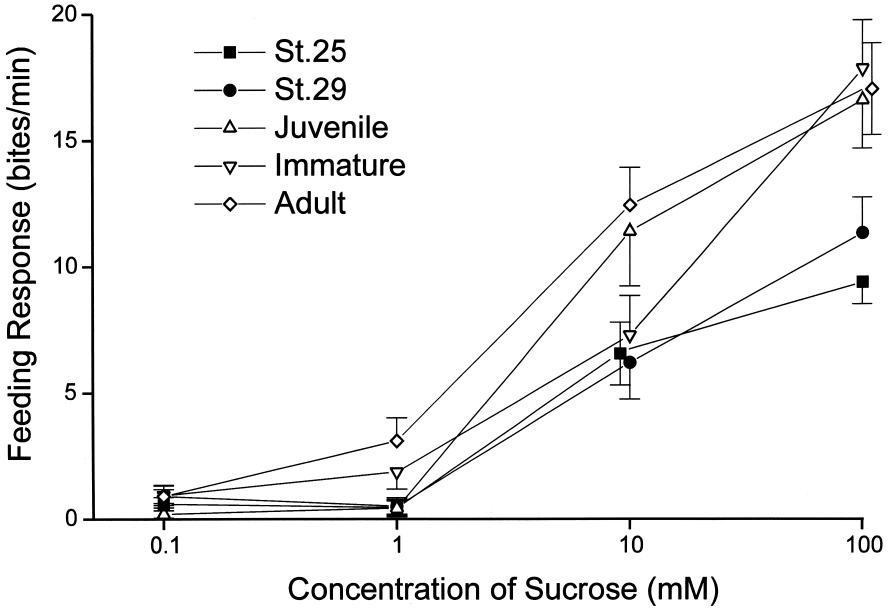

Fig. 4. Feeding responses to sucrose. Means ± SEM (n=20). The abscissa is indicated in a logarithmic scale.

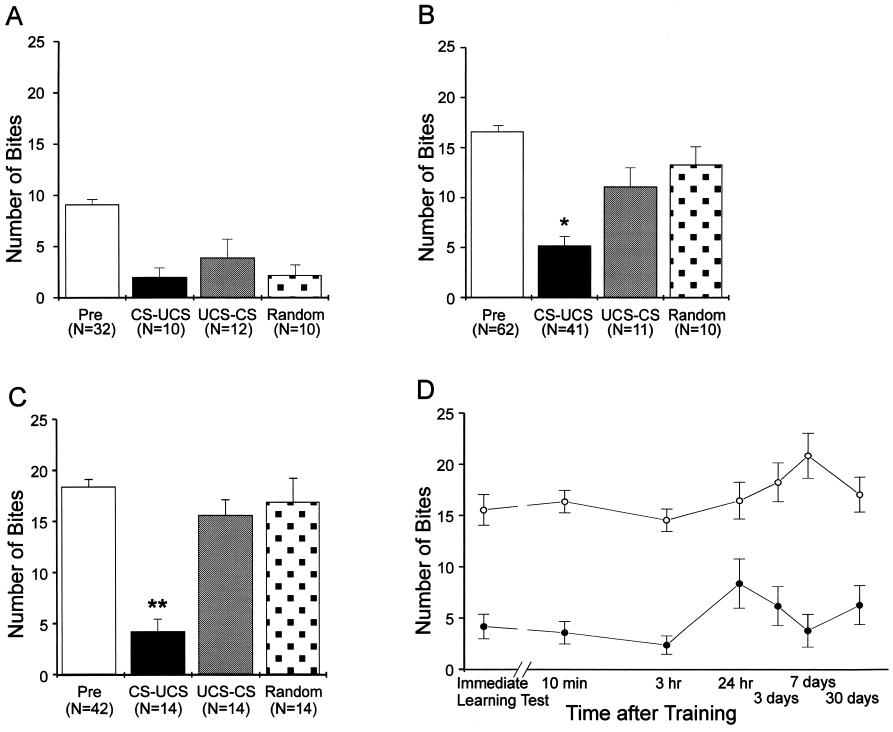

Fig. 5. CTA learning and time dependence of persistence. Pre is the result of a pre-test, the data in which excluded non-responsive snails. CS-UCS, UCS-CS, and Random indicate the results of a conditioned, a backward-conditioning control, and a random control group, respectively. (A) The number of bites for 1 min in stage 25 embryos stimulated by 100 mM sucrose. These embryos could respond to sucrose but there were no differences between the results of the conditioned and the control groups. (B) The number of bites for 1 min in stage 29 embryos stimulated by 100 mM sucrose. The test CS elicited significantly fewer feeding response in the conditioned group (Scheffé's F test, ★$p<0.05$) than in the control groups, but the conditioned response did not persist for a week. Note that the number of snails for CS-UCS was very large because the survival rate was so small that a large number of snails were needed to test the persistence. It has been statistically confirmed that a small number of snails were sufficient to verify the formation of the CTA. (C and D) The number of bites for 1.5 min in immatures stimulated by 10 mM sucrose. The conditioned response (Scheffé's F test, ★★$p<0.01$) was maintained for a month (at least $p<0.02$). In (D), the open and the closed circles represent the backward-conditioning control and the conditioned data; the abscissa is in a logarithmic scale. Only 13 conditioned snails were used on 7 days and later because one of the first 14 subjects died between the 3rd and the 7th day. Means ± SEM.

When immatures were conditioned, the feeding response to the CS was suppressed in the conditioned group but not in the control groups (one-factor ANOVA, $F=23.08$, $p<<0.0001$; Scheffe's F test, $p<0.01$ for Conditioned; Fig. 5C). This conditioned response lasted for at least a month (Fig. 5D), indicating that the immatures used a long-term memory to maintain the conditioned response. This is the third critical stage to the CTA in *L. stagnalis*.

The data for CTA in adults were reported in our previous work (Kojima *et al.*, 1996, 1997). The formation and persistence of CTA learning in adults appear to be similar to those occurred in immatures.

Voluntary activity

Adult snails voluntarily moved 12.0 ± 1.9 (arbitrary unit), which was equivalent to translocation of 448 ± 72 mm, in 30 min (Fig. 6, n=20). Embryos (stages 24 to 29) showed much less activity ($p<0.0001$) than juveniles, immatures, and adults, although these embryos did show some peristaltic movement of their pedal epidermis. This peristaltic movement appears to be the same as that in adults. The most important point was that while the activity of juveniles was similar to that of adults, immatures showed vigorous activity ($p<0.0001$ vs. others).

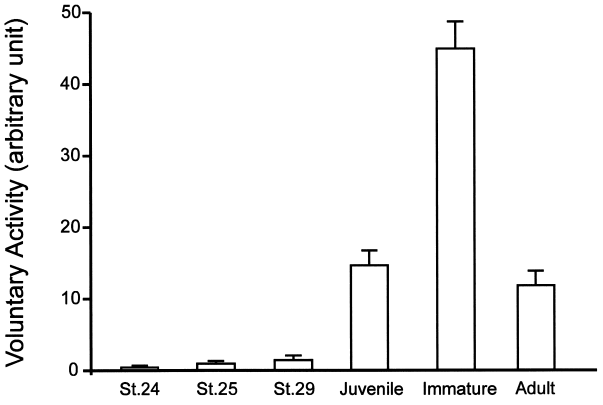

Fig. 6. Voluntary activity of *L. stagnalis*. All data (means ± SEM) were obtained for 30 min from 20 snails each. See MATERIALS AND METHODS for determination of the activity and RESULTS for the p values.

DISCUSSION

We found that *L. stagnalis* developed a conditioned taste aversion (CTA) as a long-term memory through three critical stages. At the first critical stage, stage 25 embryos started to respond to the appetitive sucrose (Fig. 4). However, they could not associate this appetitive sucrose (CS) with the aversive KCl (UCS) (Fig. 5A). At the second critical stage, stage 29 embryos and small juveniles could acquire the CTA (Fig. 5B), but the conditioned response did not persist. At the third critical stage, 10 mm and more developed immatures could acquire the CTA which would become a part of long-term memory. The conditioned responses persisted for at least a month (Fig. 5D).

Our result that the feeding response was initiated at embryonic stage 25 is in good agreement with morphological observations by Meshcheryakov (1990), that is, "The radular cavity is connected to the oral cavity. The jaw has formed completely. Salivary glands have been laid down as diverticula of the oral cavity walls." This description suggests that stage 25 embryos are physically ready to eat as is shown in our CTA experiments.

The embryos (stage 24 to 29) showed very low levels of voluntary activity (Fig. 6), even though the same peristaltic movement seen in juveniles and adults was observed. Since Meshcheryakov (1990) described that the foot showed spontaneous movements for the first time at stage 23, we can suppose that the basic network for this foot movement had already been formed in the stage 24 embryos tested here. In any case, they do not need to move widely, because the embryos exist only in the egg.

The last two critical stages, the formation of CTA and its long-term memory, appear to correspond well to the life cycle of snails. The CTA learning was acquired just before hatching (stage 29 embryos), and its long-term memory began to be formed just before sexual maturation (immatures). The stage 29 embryos may become prepared to safely seek out food in an external environment by acquiring learning ability. After hatching, the voluntary activity in snails increased to accompany their somatic development with sexual maturation (Fig. 6). The immatures just before sexual maturation showed strong voluntary activity (Fig. 6) and acquired a CTA which was memorized for a long term (Fig. 5D). This result suggests that snails must become able to maintain a long-term memory so that they can safely eat a variety of food when they cover wide territory to search for a mate.

We should add two comments on the present method and findings. First, we used two different periods for counting the feeding response in embryos, juveniles, and immatures. Embryos, which undertook the training session but seemed not to acquire the CTA, quickly started the feeding response to the test CS. On the other hand, juveniles, immatures, and adults after the training tended to hesitate the feeding response to the test CS. For example, some of them kept closing the mouth for tens of seconds, and suddenly started the feeding response. As a result, these snails were judged to be the poor learners in this study. The others, good learners, did not open the mouth at all. Therefore, we determined that the sufficient time for counting the feeding response in embryos was 1 min and that it for juveniles, immatures, and adults was 1.5 min to judge the formation of CTA. In the future work, this hesitation time will be a useful index to analyze the CTA. Second, the activity level of adults was lower than that of the immatures (Fig. 6). After the snails reached sexual maturation, its movement slowed. These effects are considered to be caused by "aging'' and are out of the range of this study.

As shown in the present study, it is interesting that the development of learning ability in snails seems to coincide with the major changes in their life cycle, possibly allowing them to live safely. The detailed relationships between the development of central nervous system and the formation of CTA in *L. stagnalis* will be examined in our next work (Yamanaka *et al.*, in preparation).

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