

Forgetting and the extension of memory in *Lymnaea*

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Summary

Aerial respiratory behaviour in *Lymnaea stagnalis* was operantly conditioned using a procedure that results in long-term memory (LTM) persisting for 1 but not 3 days. By manipulating the snails' post-training environment, i.e. preventing *Lymnaea* from performing aerial respiratory behaviour, memory persistence was significantly extended. Memory retention, however, is only extended if snails are prevented from performing aerial respiration in the same context in which they were trained. Snails trained in the

'standard' context but prevented from performing aerial respiration in the 'carrot-odor' context (and vice versa) did not extend their memory. These data are consistent with the hypothesis that forgetting is due to interfering events, that occur following learning and memory consolidation.

Key words: associative learning, *Lymnaea stagnalis*, forgetting, operant conditioning, memory.

Introduction

The pond snail, *Lymnaea stagnalis*, exhibits associative learning and long-lasting memory (Benjamin et al., 2000; Ito et al., 1999; Taylor and Lukowiak, 2000). We have chosen to study operant conditioning of aerial respiration primarily because a 3-neuron central pattern generator (CPG), whose sufficiency and necessity has been established, mediates this behaviour (Syed et al., 1990, 1992). In the operant conditioning procedure snails are placed in a hypoxic environment and receive a tactile stimulus to their respiratory orifice, the pneumostome, every time they attempt to open the pneumostome. Snails associatively learn not to perform aerial respiration and, depending on the training procedure used, long-term memory (LTM) persists from 1 day to several weeks (Lukowiak et al., 1998, 2000). Since *Lymnaea* are bi-modal breathers, satisfying their respiratory needs *via* cutaneous and/or aerial respiration, we are able to perform experiments such as preventing aerial respiratory behaviour without harming them.

Learning and memory are two distinct but related processes, each with its own forms and rules (Milner et al., 1998; McGaugh, 2000). We define learning as the acquisition of a skill while memory is the ability to retain that skill. Forgetting, or memory transience, is the loss of the learned response (Squire, 1987; Schacter, 2001). While forgetting is often *correlated* with the passage of time, the passage of time alone does not *cause* forgetting (Jenkins and Dallenbach, 1924).

Memory persistence depends in part on the training procedure used. For example, 'massed-training' and 'spaced-training' result in similar behavioural phenotypes; however,

'spaced-training' results in a much longer-lasting memory (i.e. less forgetting; Rowe and Craske, 1998; Carew et al., 1972; Hermitte, 1999; Lechner et al., 1999; Lukowiak et al., 2000). Memory persistence is also dependent, among other things, on the number of training sessions, the previous history of the animal, and the schedule of reinforcement used (Mackintosh, 1974). So too, is the effect that stress has on memory retention; it can positively or negatively affect the persistence of the memory (de Quervain et al., 2000).

In dealing with the subject of forgetting we have to be specific about the form of memory we are discussing. Memory can be categorized into two forms: declarative and non-declarative (Milner et al., 1998). The form of memory examined in this paper is non-declarative, and is stored within the same neural circuit that mediates aerial respiration (Milner et al., 1998; Scheibenstock et al., 2002). We thus avoid the problem of whether memory is forgotten or rather just inaccessible (McGeoch, 1932; Capaldi and Neath, 1995; Schacter, 2001), because if the snail can perform the behaviour (i.e. access the neural circuit) the memory *cannot* be inaccessible. In declarative memory, different neural circuits from those that mediate the learning subservice memory storage. We will not venture into the realm of how forgetting might occur within the structures necessary for declarative forms of memory (for a thoroughly enjoyable exposé of memory and forgetting, see Schacter, 2001). At least five theories have been proposed to explain forgetting: (1) Decay, (2) Consolidation, (3) Interference, (4) Retrieval failure and (5) Repression (Reed, 2000). While each of the theories has their particular strengths,

all suffer from failure of mechanistic explanation at the neuronal level. Moreover, since we are studying non-declarative memory in *Lymnaea*, at least two of these theories are inappropriate (e.g. retrieval failure implies the memory is there but not accessible, and Freud's theory of Repression). We have hypothesized, as have others (Jenkins and Dallenbach, 1924; McGeoch, 1932; Minami and Dallenbach, 1946) that memory transience is due to 'interfering events', which occur after memory formation and result in the loss of the memory. By manipulating the snails' post-training environment in a way that prevents 'interfering events' from occurring, it may be possible to extend the persistence of memory.

The results we report here on the post-training extension of LTM are consistent with the hypothesis that forgetting is due to interfering events (i.e. the theory of interference) and that decreasing the occurrence of these events improves memory retention.

Materials and methods

Lymnaea stagnalis L., the animal model we used for all of our experiments, were bred and raised in the snail facility at the University of Calgary. All snails used (2.5–3.0 cm shell length) were maintained at room temperature and had continuous access to lettuce in their home eumoxic aquaria.

Operant training and memory testing procedures

The reinforcing tactile stimulus to the pneumostome

Individually labeled snails were placed in a 1 liter beaker containing 500 ml of water made hypoxic by bubbling N₂ through it 20 min prior to and during training. We refer to this as the 'standard' hypoxic training procedure. We also utilize a 'different context' training procedure, which we will refer to as the 'carrot context'. To create the 'carrot context', N₂ was first bubbled through a 750 ml Erlenmeyer flask with chopped carrots and water before being bubbled into the training beaker (for complete details, see Haney and Lukowiak, 2001). The term 'change of context test' means that snails were tested in the context that they were **not** trained in. This test is used as a control to show that following a given procedure, which may extend memory, snails are still as responsive as they were in the initial training session.

In all of the training, memory test and change of context test sessions, a gentle tactile stimulus (a sharpened wooden applicator) was applied to the pneumostome area (the respiratory orifice) every time the snail began to open its pneumostome to perform aerial respiration. This tactile stimulus only evoked pneumostome closure; it did not cause the animal to withdraw its foot and mantle area (i.e. the whole-animal withdrawal response), nor did pneumostome stimulation cause the snails to sink to the bottom of the beaker. The time of each attempted opening was recorded and tabulated.

In all experiments, the snails were first given a 10 min acclimatization period, where they could perform aerial respiration without receiving reinforcement. The onset of

operant conditioning training was initiated by gently pushing the snails beneath the water surface. Between the training sessions and between the training and the memory test sessions, as in all our previous experiments, snails were placed in eumoxic pond water where they were allowed to freely perform aerial respiration. We did not monitor the snails' breathing behaviour during the periods they were in their eumoxic home aquaria.

The 30 min associative training procedure

In this operant conditioning training protocol (Figs 1–4), snails received two 30 min training sessions separated by a 1 h rest interval. A 30 min memory test session was given to separate cohorts of snails either the next day or 3 days later (the 1- and 3-day memory tests, respectively).

Submersion experiments

A 30 min associative training procedure including both the standard and carrot-odorant contexts was used in these experiments. Immediately following the last training session, half of the snails were placed in an uncovered eumoxic aquarium. The other half was placed in a eumoxic aquarium containing a plastic barrier. Snails were placed beneath the barrier, thus preventing them from reaching the water's surface and performing aerial respiration. The barrier had small holes in it, so that air bubbles could not accumulate on its undersurface. Atmospheric air, to create eumoxia, was continuously bubbled while the snails were maintained under the barrier. Routing the air first through a 750 ml Erlenmeyer flask with chopped carrots created the carrot-odor context. All groups had continuous access to food (lettuce) during the intervals between training and testing. Snails placed beneath the barrier were never observed to escape nor were they observed to perform aerial respiratory behaviour. 3 days after training, both control and submerged snails were given a memory test. In some experiments, 2 h later, the submerged group received a test in the other context to control for unresponsiveness.

Breathing behaviour observations

Naïve snails were placed in a 1-liter beaker filled with 500 ml of water made hypoxic by bubbling N₂ through it 20 min prior to and during observations. Animals were allowed a 10 min acclimatization period before being gently poked under the water to signify the beginning of the observation period. Total breathing time and the number of pneumostome openings were measured during a 30 min period.

Yoked control experiments

To show that the changes in behaviour resulting from operant conditioning training are due to associative processes, we performed yoked control experiments, as previously described (Lukowiak et al., 1996, 2000; Spencer et al., 1999). Yoked controls were used for the 30 min (Table 1) training procedure. Briefly, yoked animals received a tactile stimulus to their pneumostome area whenever the animal to which they

Table 1. Mean number of attempted pneumostome openings for yoked controls before and after training (30 min session)

Session	N	Number of attempted pneumostome openings (mean \pm S.E.M.)
Pre-test	10	6.8 \pm 1.28
Post-test	10	7.6 \pm 6.27

Pre-test and Post-test were not significantly different, $P>0.05$.

were yoked attempted to open its pneumostome. That is, there was not a contingency between pneumostome opening and application of the tactile stimulus in the yoked animals.

We made use of the fact that a single 30 min training session does not result in LTM to demonstrate that yoked control animals do not form an association (i.e. do not exhibit associative learning) (Lukowiak et al., 2000). The 'responsiveness' of the yoked control snails was first found in a 'pre-test' hypoxic session (30 min). In this session the snails received a tactile stimulus to the pneumostome whenever they attempted to open it (i.e. contingent stimulation), but the single 30 min session does not have an effect on the next day's session (Lukowiak et al., 2000). On the following day(s) the yoked control snails were again placed in the hypoxic test beaker but now received the tactile stimulus to the pneumostome area whenever the snail to which they were yoked attempted to open its pneumostome. 24 h after the last yoked control session, the yoked control snails were again placed in a hypoxic test beaker, and received the 'post-test'. In the 'post-test' session a tactile stimulus was again applied to the pneumostome area whenever they attempted to open their pneumostome. If the yoked-control procedure had an effect on how the snails responded in the hypoxic environment, then the number of attempted pneumostome openings in the post-test session should be significantly different than in the pre-test session. On the other hand, if the yoked procedure did not result in an associative effect then there should be no difference in the number of attempted pneumostome openings between the 'pre-test' and the 'post-test' sessions. The data in Table 1 show that there was not a significant decrease or increase in the number of attempted pneumostome openings between the 'pre- and 'post-test' sessions (paired t -test, $P>0.05$), and thus we concluded that the significant changes seen in the operant training procedures were genuine examples of associative learning.

Blind testing of snails

With the exception of the experiments in Figs 1 and 2, all experiments were performed blindly. That is, the experimenter performing the memory test had no knowledge of the previous training, context, whether the snail was submerged, etc. Only after all the results were tabulated did we know the outcome of the various experiments.

Operational definitions of learning and memory

We used the same criteria to define learning and memory

as in previous studies (Lukowiak et al., 1996, 2000; Spencer et al., 1999). Associative learning is defined as a significant effect of training on the number of attempted pneumostome openings [one-way analysis of variance (ANOVA), $P<0.05$; followed by a *post-hoc* Fisher's LSD protected t -test, $P<0.05$, within each separate group]. The number of pneumostome openings in the final training session has to be significantly less than the number of attempted openings in the first session. The criteria for long-term memory (LTM) are: (1) the number of attempted pneumostome openings in the memory-test session is not significantly different from the number of attempted openings in the last training session; (2) the number of attempted openings in the memory-test session is significantly less than the number of attempted openings in the first session.

Statistics

A paired student t -test was used to compare differences in breathing time and number of pneumostome openings between cohorts of snails tested following the submersion experiments as well as for the yoked-control experiments.

Results

A cohort of snails ($N=24$) received an operant conditioning training procedure consisting of two 30 min training sessions separated by a 1 h interval. This procedure was sufficient to produce learning (Fig. 1). That is, as the number of attempted openings in Session 2 was significantly different from Session 1, we conclude, based on our operational definition of learning, that learning had occurred. We randomly selected 12 of these snails and tested (Session 3) their memory retention 1 day later, while the other 12 snails were tested for memory 3 days later. In the group of snails tested 1 day later there was memory. That is, the number of attempted openings in the memory test session (Session 3) was not different from Session 2 but was significantly different from Session 1, satisfying the criteria for memory. We found, however, that the memory did not persist for 3 days. That is, the number of attempted openings in the 3-day memory test session (Session 3) was significantly different from the last training session (Session 2) but was not different from Session 1 (i.e. the criteria for memory were not met). Table 1 shows data for animals given two 30 min yoked control training sessions. The pre-test was not significantly different from the post-test, again demonstrating that the significant change in breathing behaviour (Fig. 1) are true examples of associative learning. We conclude that two 30 min training sessions separated by a 1 h interval is sufficient to produce associative learning and LTM that persists for 1 but not for 3 days. Hence, loss of memory occurs during the time the snails are in their eumoxic home aquaria.

We next asked whether the disappearance of the memory between the last training session and the 3-day memory test session was due to the occurrence of un-reinforced aerial respiratory behaviour (i.e. the hypothesized 'interfering event') that occurs when the snails are in their home aquaria. If our

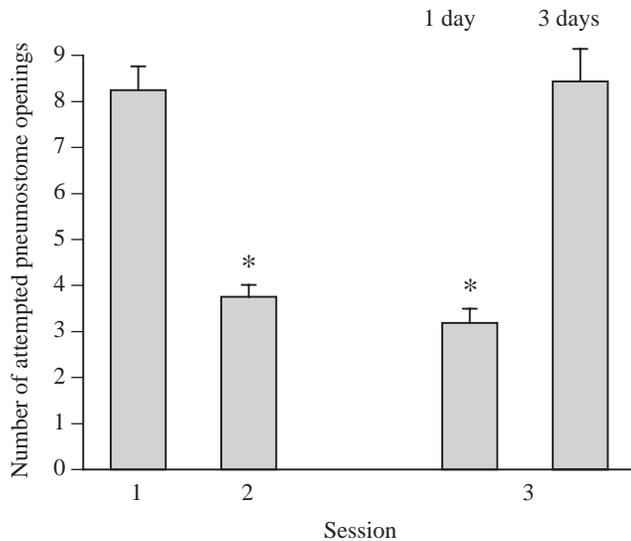


Fig. 1. The 30 min training procedure results in a 1-day, but not a 3-day memory. A cohort of snails ($N=24$) received operant conditioning training of two 30 min training sessions separated by a 1 h interval (Sessions 1 and 2). In the first randomly picked group ($N=12$), we tested for long-term memory (LTM) 1 day later (Session 3). There was a significant effect between Sessions 1 and 2 (ANOVA; Fisher's LSD protected t -test, $P<0.01$), demonstrating learning (asterisk). As can be seen, LTM was also demonstrated, i.e. there was no significant difference (NS; Fisher's LSD protected t -test, $P>0.05$) between Session 2 and the 1-day memory test Session 3, and the significant difference from Session 1 remained (asterisk). The second group of snails, was tested 3 days later (3 days) and LTM was not demonstrated, i.e. there was a significant difference (Fisher's LSD protected t -test, $P<0.01$) between Session 2 and the 3-day memory-test Session 3, but not between this session and Session 1.

hypothesis is correct, preventing aerial respiration (precluding an 'interfering event') should extend memory persistence.

However, before proceeding with those experiments, we first had to show that preventing aerial respiration for 3 days does not significantly alter subsequent aerial respiratory behaviour. Aerial respiratory behaviour was therefore monitored before and after snails were submerged underneath a barrier for 3 days that prevented them from coming to the air-water interface to open their pneumostomes. We found that this submerging/preventing aerial respiration did not alter their subsequent aerial respiratory behaviour (Fig. 2).

With this finding we were able to test our hypothesis regarding memory extension. A cohort of naïve snails ($N=14$; Fig. 3A) was operantly conditioned using the training procedure in Fig. 1 (i.e. two 30 min sessions separated by a 1 h interval). Immediately following the last training session they were placed below the barrier in a eumoxic aquarium and prevented from performing aerial respiration for 3 days. When we tested these snails for memory, it was still present 3 days after the last training session. That is, the number of attempted openings in the memory test session was not significantly different from the number in Session 2 but was significantly different than the number in Session 1 (i.e. the criteria for

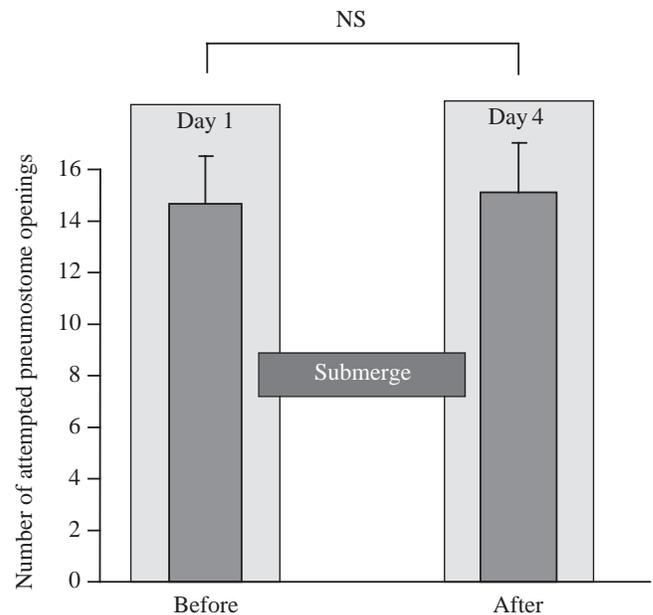


Fig. 2. Preventing aerial respiration for 3 days does not alter subsequent aerial respiratory behaviour in the hypoxic training/testing apparatus. 12 snails were tested (Before) in the hypoxic testing apparatus as described in Materials and methods. 1 day following training they were placed for 3 days in a eumoxic aquarium with a barrier that prevented them from reaching the surface of the water, so that they could not perform aerial respiration. After 3 days the snails were tested (After) in the hypoxic training/testing apparatus and responded no differently (paired t -test; NS, $P>0.05$) than they did before being placed in a situation where they were not able to perform aerial respiration.

memory were met). As a further control to show that preventing aerial respiration in trained snails did not result in 'abnormal' activity, we changed the context (CC) of the test session. As can be seen, the snails responded as if they were naïve (i.e. there was no significant difference between Session 1 and the change of context test). We conclude that memory, which in control snails only persisted for 1 day (Fig. 1), can be extended for at least 3 days by preventing un-reinforced behaviour.

To show that this extension of memory is not a 'trivial' finding we performed two further sets of experiments utilizing the fact that learning and LTM are context dependent in *Lymnaea* (Haney and Lukowiak, 2001; McComb et al., 2002). In the first of these experiments (Fig. 3B) a cohort of naïve snails ($N=14$) was trained in the 'carrot-odor' context with the procedure that results in LTM persisting for 1 day. These snails were then submerged for 3 days in a eumoxic 'carrot-odor' context. Memory for the 'carrot-odor' context was maintained when tested 3 days later, but if challenged in a standard context test, the snails responded as they did in the first session. We conclude that submerging snails for 3 days in the context that they were trained in extends the persistence of memory for that context.

We could now ask the question, would submerging snails in

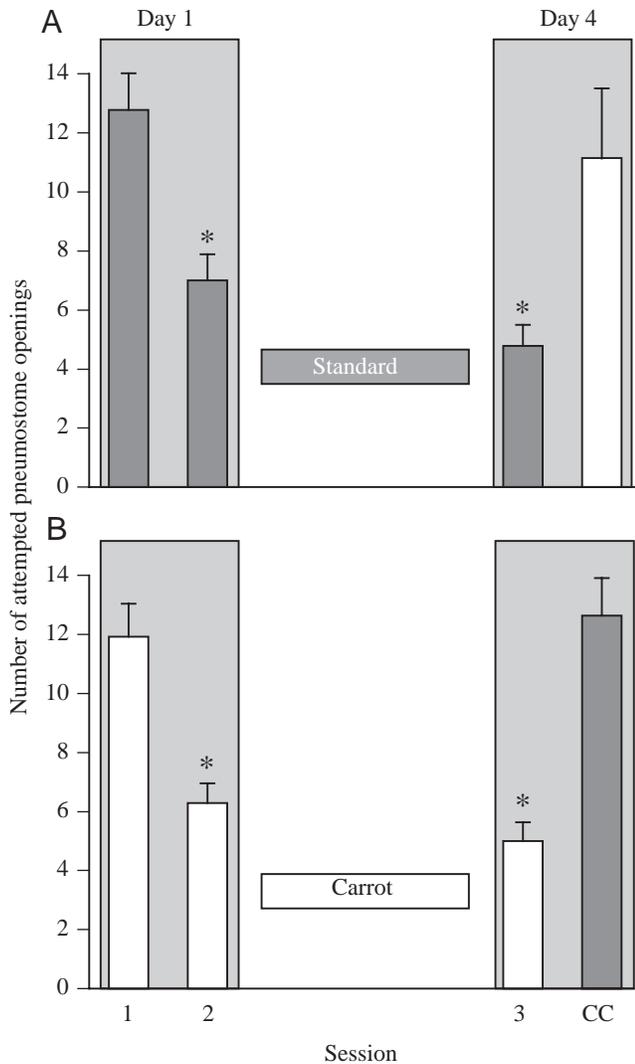


Fig. 3. Submerging in the same context after operant training prolongs the memory for operant conditioning. (A) Two 30 min sessions separated by 1 h in the standard context were followed by 3 days of submersion in the standard context. The test session in standard context was significantly different (asterisk; Fisher's LSD protected t -test, $P < 0.01$) from Session 1, demonstrating a 3-day memory. A change of context test (carrot, CC) was not significantly different from Session 1 (Fisher's LSD protected t -test, $P > 0.05$, $N = 14$). (B) Two 30 min sessions separated by 1 h in carrot context were followed by 3 days of submersion in carrot context. The test session in carrot context was significantly different (asterisk; Fisher's LSD protected t -test, $P < 0.01$) from Session 1, demonstrating 3-day memory. A change of context test (standard; CC) was not significantly different from Session 1 (Fisher's LSD protected t -test, $P > 0.05$, $N = 14$).

a *different context* to the one in which they were trained also extend memory? If memory extension was *solely* due to the prevention of aerial respiration, then memory even for a different context should be extended. These data are presented in Fig. 4. A naïve cohort of snails ($N = 14$; Fig. 4A) was operantly conditioned in the 'standard' context. Immediately after the last training session the snails were submerged in a

'carrot-odor' context for 3 days and were then given the memory test. As can be seen no memory was shown. That is, the number of attempted openings in the memory test session was significantly greater than in the last training session (Session 2) and was not different from the number in Session 1. Likewise, training snails ($N = 14$) in the carrot-context and submerging them in the 'standard-context' (Fig. 4B) produced similar results (i.e. memory was not extended). Since the criteria for memory were not met, we conclude that prevention of aerial respiration *alone* was not sufficient to extend memory retention.

Discussion

A number of different factors affect, in both positive and negative fashions, the persistence of memory; and these factors may occur *before* or *after* the learning and memory consolidation processes. Previously, our thinking was that the training procedures used *before* memory consolidation were the major factor in determining how long memory persisted. For example, 'massed' training and 'spaced' training in *Lymnaea* result in a similar level of performance; yet 'spaced' training results in a memory that is significantly longer lasting (Carew et al., 1972; Rowe and Craske, 1998; Lukowiak et al., 2000). A greater number of training sessions in *Lymnaea* result in a longer lasting memory. Thus, two 30 min training sessions separated by a 1 h interval produce LTM that persists for 1 but not 3 days (Fig. 1), whereas three 45 min training sessions spread over a 1.5-day period results in LTM that persists for up to 5 days (McComb et al., 2002). Even more impressive is the finding that associative training over a 2-week period results in LTM that persists for at least 1 month (Lukowiak et al., 1998). LTM memory persistence was also prolonged following the formation of intermediate-term memory (ITM), which only persists for 3–4 h even if the ITM was no longer observable (Smyth et al., 2002). Finally, memory retention is prolonged following the switch to a partial reinforcement schedule after learning acquisition (Sangha et al., 2002). How all of these pre-memory consolidation procedures/factors, and others not mentioned here, alter memory persistence are not known since it is not clear what the underlying neuronal mechanisms of forgetting are. None of the proposed theories of forgetting explains all facets of memory transience, especially at the neuronal level (Reed, 2000). Since it has been argued (Squire, 1987; Squire and Kandel, 1999; McGaugh, 2000) that learning and its consolidation into memory are due to specific changes in neuronal morphology (e.g. change of synaptic architecture, etc.) we suggest that forgetting would have to be due to alterations (i.e. returning back towards their original state) in those specific changes.

We show here that procedures *after* the consolidation process can also significantly alter memory persistence. Our working hypothesis is that forgetting of the learned behaviour in the snail (a non-declarative memory) is the result of 'interfering events', specifically the occurrence of unreinforced aerial respiratory behaviour that happens when the

snails are placed back into their home, eumoxic aquaria, between the training and the memory test session. These interfering events result in memory loss. Therefore the longer they are maintained in the eumoxic aquarium (increased number of interfering events) the greater the probability of memory deterioration. The finding that preventing aerial respiration between the last training and memory test session prolongs memory is consistent with our hypothesis that 'interfering events' explain forgetting. However, (Fig. 4) preventing un-reinforced aerial respiration *per se* does not necessarily extend memory. Snails prevented from performing aerial respiratory behaviour in a *different context* over the same time period **do not** have their memory extended (Fig. 4). Only snails trained and submerged in the *same* context have their memory extended (Fig. 3). Thus it is not just the physical prevention of un-reinforced aerial respiratory behaviour that prolongs memory retention. Forgetting was delayed only if aerial respiratory activity was prevented in a context that was the same as training. Perhaps the reason animals forget when submerged in a different context to that which they were trained in is that the switch in context may be perceived as an 'interfering event' and thus lead to forgetting.

The hypothesis that forgetting is due to interfering events is not new (Jenkins and Dallenbach, 1924) and has been tested before in both vertebrates and invertebrates. Jenkins and Dallenbach (1924) found in human subjects that after periods of sleep, retention of nonsense syllables was superior than after corresponding periods of normal waking activity in the same subjects. McGeoch (1932), reviewing his and other work, also proposed that forgetting was due to 'interfering events' that occurred *post-learning*. Similarly, Minami and Dallenbach (1946) demonstrated using cockroaches that after intervals of inactivity in which the cockroaches were immobilized in small boxes filled with tissue paper, memory retention and relearning was far superior to those insects that received corresponding intervals of normal rest. In addition, this same study illustrated that forced activity following learning led to savings scores that were much poorer than after corresponding intervals of normal rest. Together, these studies demonstrate that it is not the passage of time that results in memory decay; rather it is a result of interference from new events (Minami and Dallenbach, 1946).

Our data likewise suggest that forgetting is not due to the 'decay' of memory occurring with the passage of time. If decay with time was the primary source of forgetting, then the rates of forgetting should be similar in the submerged and control groups, and we showed they were not (Figs 1, 3). Moreover, even in the submersion experiments, if decay were the cause

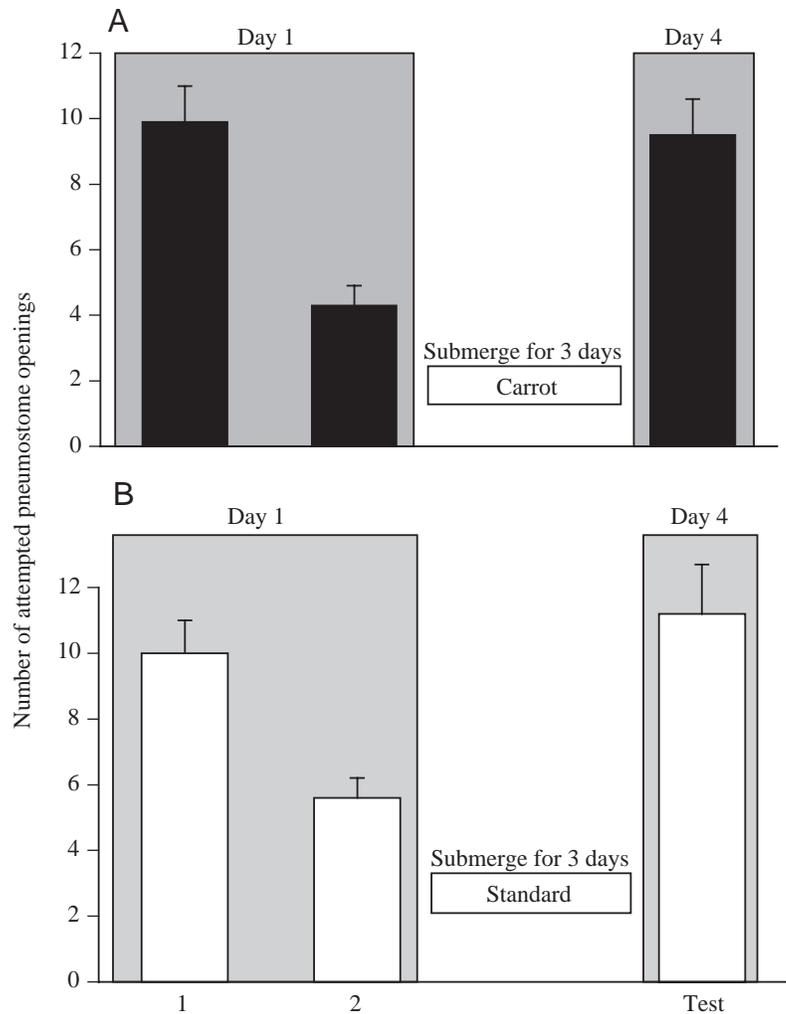


Fig. 4. Submerging snails in a different context does not prolong the memory for operant conditioning. (A) Two 30 min operant conditioning training sessions separated by 1 h in standard context were immediately followed by 3 days of submersion in carrot context. The test session in standard context was significantly different (paired *t*-test, $P < 0.01$, $N = 14$) from Session 2 but not from Session 1, showing that memory was no longer present. (B) Two 30 min operant conditioning training sessions separated by 1 h in the carrot context were immediately followed by 3 days of submersion in the standard context. The test session in the carrot context was significantly different (paired *t*-test, $P < 0.01$, $N = 14$) from Session 2 but not from Session 1, showing that memory was no longer present.

of forgetting, it should make no difference in which context the submerged snails were maintained. As we found (Fig. 4), this was not the case; only when the context of the submersion was similar to the context of training would memory be extended. It is more likely that memory transience is the result of interference or the elimination of the 'old' memory by a new memory that resembles the naïve state. The 'new memory' is the result of an active process in which the snail associatively learns and remembers that opening of the pneumostome does not result in tactile stimulation of the pneumostome.

Associative learning can be defined as two events being

linked to each other due to past experiences (Kimble, 1961; Dudai, 1989; Milner et al., 1998; Kapp et al., 1998). The data presented here support the idea that forgetting is a form of learning as new associations are being made, specifically, there is an association between the behaviour and **no** reinforcement. As a consequence, if the animals are not allowed to perform the behaviour in the proper context then there is memory extension, because there is no new association made. Since forgetting appears to involve two 'linked' events, it is reasonable to view forgetting as a process that involves learning new associations. These new associations should therefore lead to the reworking of the neuronal changes that occurred in neurons during the initial learning and memory consolidation. Future experiments will determine if this is indeed the case.

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