

# Long-Term Habituation in the Marine Mollusc *Tritonia diomedea*

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**Abstract.** *Tritonia diomedea* is one of several gastropod molluscs used to study cellular mechanisms of learning and memory. Previous studies in this organism have focused on short-term habituation and sensitization. This report presents the first detailed description of long-term habituation in *Tritonia*. Experimental animals were given 11 swim sessions, each consisting of 10 trials, over 6 days, during which they typically displayed an initial sensitization, followed by short-term, within-session habituation. Responses were compared to controls, which were given a single stimulus per day. Cycle number habituation steadily accumulated over the days of training, and then persisted for at least 2 days after the end of training. These findings will permit comparative studies of the cellular mechanisms of short- and long-term memory in this highly tractable model system.

## Introduction

Habituation is a form of nonassociative learning, common to all animals, in which behavioral responses to repeated stimuli decrease over time. Habituation's importance lies in its ability to allow organisms to avoid responding unnecessarily to the many repetitive innocuous stimuli in the environment. Many invertebrates, such as the marine mollusc *Aplysia californica* (Pinsker *et al.*, 1970; Carew and Kandel, 1973; Kandel and Schwartz, 1982) and the nematode *Caenorhabditis elegans* (Rose and Rankin, 2001), have been used to investigate the cellular mechanisms of both short- and long-term habituation.

The marine mollusc *Tritonia diomedea* exhibits an escape swim response, consisting of a series of alternating ventral

and dorsal body flexions. This escape swim is normally triggered by contact with predatory sea stars, but it can also be triggered artificially by applying an aversive salt stimulus to the skin. Previous studies have characterized short-term modifications of this response, including habituation (Abraham and Willows, 1971; Brown *et al.*, 1996; Frost *et al.*, 1996), dishabituation (Mongeluzi and Frost, 2000), and sensitization (Frost *et al.*, 1998), as well as the sensory gating phenomenon prepulse inhibition (Mongeluzi *et al.*, 1998; Frost *et al.*, 2003). Here we provide the first evidence for long-term learning in *Tritonia*, long-term habituation, in which the animal's reduced responsiveness to a repeated stimulus persists for several days after training.

## Materials and Methods

Individuals of *Tritonia diomedea* were collected by divers from the waters of Puget Sound, Washington, and maintained in running seawater tables (11–12 °C) at Friday Harbor Laboratories, Friday Harbor, Washington. During the experiment, animals were housed individually in 12-inch-diameter plastic-mesh pens. Animals used in the experiment had minimal prior experimenter-elicited swimming experience. None had been made to swim more than two times before it began, and no such swims had occurred within the 4 days before the start of the experiment. The swim stimulus, 0.15 ml of 4 M NaCl colored with fast green dye for visibility, was applied, using a handheld syringe fitted with a blunted 18-gauge needle, to the dorsal surface of the animal's tail, over a period of about 5 s. Before the start of the experiment, all animals were pretested with a single swim stimulus and rejected if they failed to swim at least five cycles ( $n = 3$ ). Animals passing this pretest ( $n = 24$ ) were placed in individual mesh pens and left undisturbed for a minimum of 3 h before the start of training.

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Animals remained in their individual pens for the entire 12-day experiment.

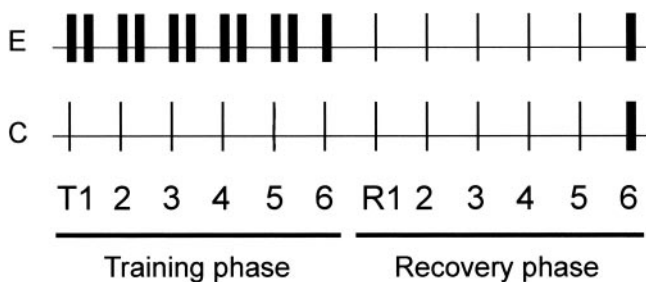
During the experiment, one person delivered the stimulus and counted the number of swim cycles. A second experimenter used a stopwatch to measure swim onset latency, defined as the number of seconds from stimulus onset to the maximum point of the first ventral flexion of the swim. Because the tail stimulus could not reliably be delivered to an animal that was positioned head-up on the pen wall or lying on its back, such individuals were gently repositioned with the rounded end of a glass rod. No animals swam to this procedure, which was used in about 20% of all trials.

Data are presented as means  $\pm$  standard error. Statistical tests included repeated measures between-groups ANOVAs for independent measures (Statistica, ver. 5; StatSoft, Inc, Tulsa, OK), and *F*-tests for comparing between-groups linear regression slopes fit to multiple trial data (GraphPad Prism; ver. 4).

## Results

The experiment consisted of a 6-day training period, followed by a 6-day recovery period (Fig. 1). The control group ( $n = 11$ ) was given a single stimulus per day at 0900 during both the training and recovery periods. The experimental group ( $n = 13$ ) was given two 10-trial training sessions (at 0900 and 1600, 2-min intertrial interval) per day for 11 total sessions. During the recovery period, both groups were treated identically, receiving a single stimulus per day on the first 5 days, and a 10-trial session on the 6th day.

We first consider training-induced alterations occurring within the individual 10-trial sessions (short-term learning), then alterations persisting across sessions (across-session



**Figure 1.** Training and testing protocol. During the 6-day training phase, experimental animals (E) received two sessions per day of 10 trials each, except for the 6th day, when they received just one session (11 total sessions, 110 total trials). During this period, control animals (C) received a single trial per day (thin vertical bars) for a total of 6 trials. Each trial produced a maximum of one swim. During the 6-day recovery phase, both groups received one trial per day for 5 days, beginning 24 h after the last training session. On the 6th recovery day, both groups received a standard 10-trial training session, to test for residual memory effects on subsequent habituation training. Ten-trial sessions appear as thick vertical bars; single trial sessions appear as vertical lines.

learning), and finally those persisting 24 h or more beyond the end of training (long-term learning).

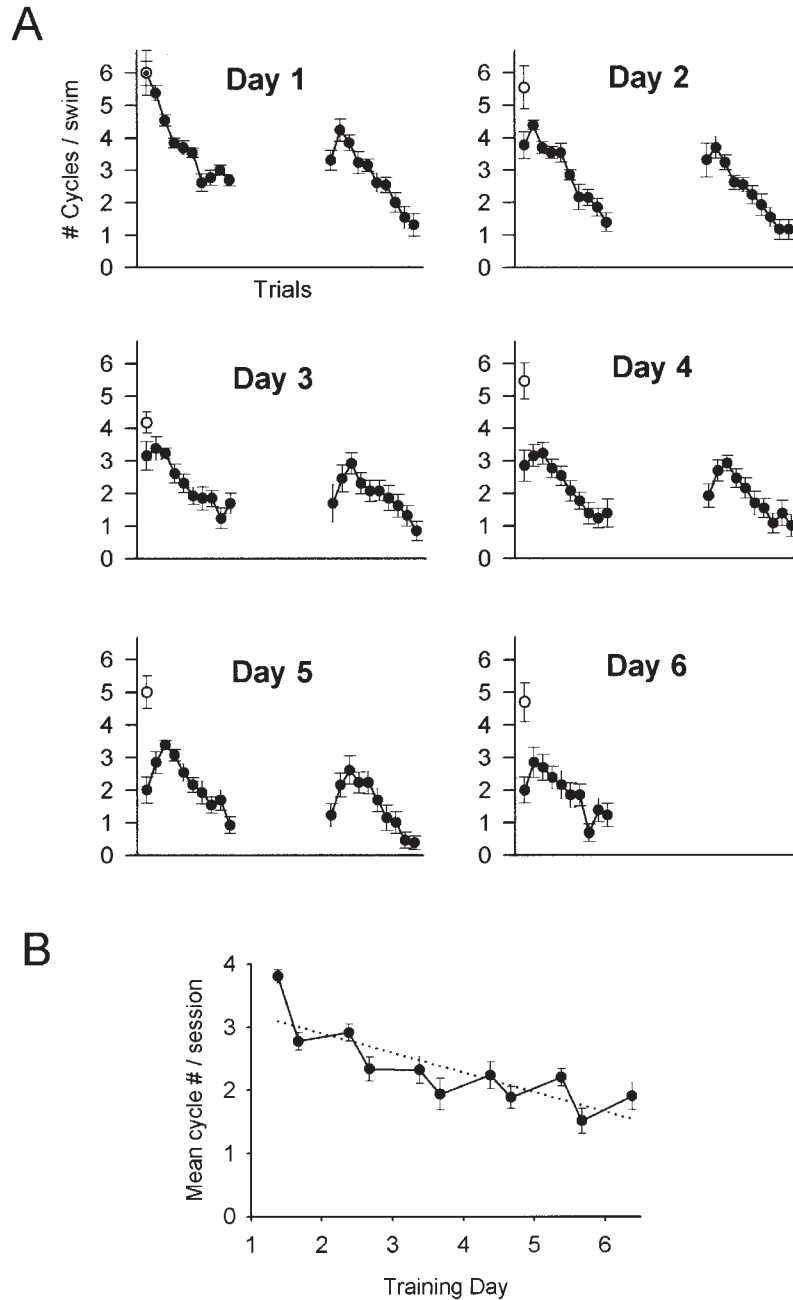
### Short-term (within-session) learning

**Cycle number.** Cycle number scores for all trials and sessions during the 6-day training period are shown in Figure 2A. All 11 experimental group training sessions displayed a decrement in cycle number from the 1st to the 10th trial, demonstrating short-term, within-session habituation, consistent with results from many previous studies. Although cycle number consistently decreased across the 10 trials of each session, in all but the first session it initially increased or stayed high before declining, a phenomenon often observed in habituation studies (Groves and Thompson, 1970; Stopfer *et al.*, 1996; Ezzeddine and Glanzman, 2003). Our prior studies have shown that an initial stimulus induces an hour-long short-term sensitization, involving the enhancement of several features of the animal's escape response, including increased swim cycle number, reduced swim onset latency (see below), decreased threshold, and reduced gill and rhinophore withdrawal latencies (Frost *et al.*, 1998). It therefore seems likely that the initial increase in cycle number in all but the first training sessions may be due to sensitization produced by the first trial of each session. Additionally, the fact that the within-session increase in cycle number is delayed until the second training session, when enduring habituation has begun developing, raises the possibility that it involves the dishabituation of developing long-term habituation. A similar profile of delayed increase in cycle number was observed in a prior study using multiple training sessions (Mongeluzi and Frost, 2000).

**Onset latency.** Swim onset latency scores for all trials and sessions are shown in Figure 3A. As in previous studies, these showed an initial quickening, reflecting the sensitization produced by the initial stimulus (Abraham and Willows, 1971; Brown *et al.*, 1996; Frost *et al.*, 1998; Mongeluzi and Frost, 2000). Onset latency was typically shortest on the second or third trial, after which it climbed back toward its starting value during the remaining trials of the session.

### Across-session learning

**Cycle number.** Figure 2A also shows that swim cycle number decreased, not just within each individual session, but also steadily across the repeated sessions of the experiment. This latter effect can be seen in Figure 2B, which shows the mean cycle number for each 10-trial session for the 13 experimental animals. The slope of the linear regression line fit to the data was significantly different from zero, confirming that habituation occurred across sessions ( $F(1,9) = 18.50, P < 0.01$ ). Within each day, the mean cycle number of the second session was always lower than that of the first,

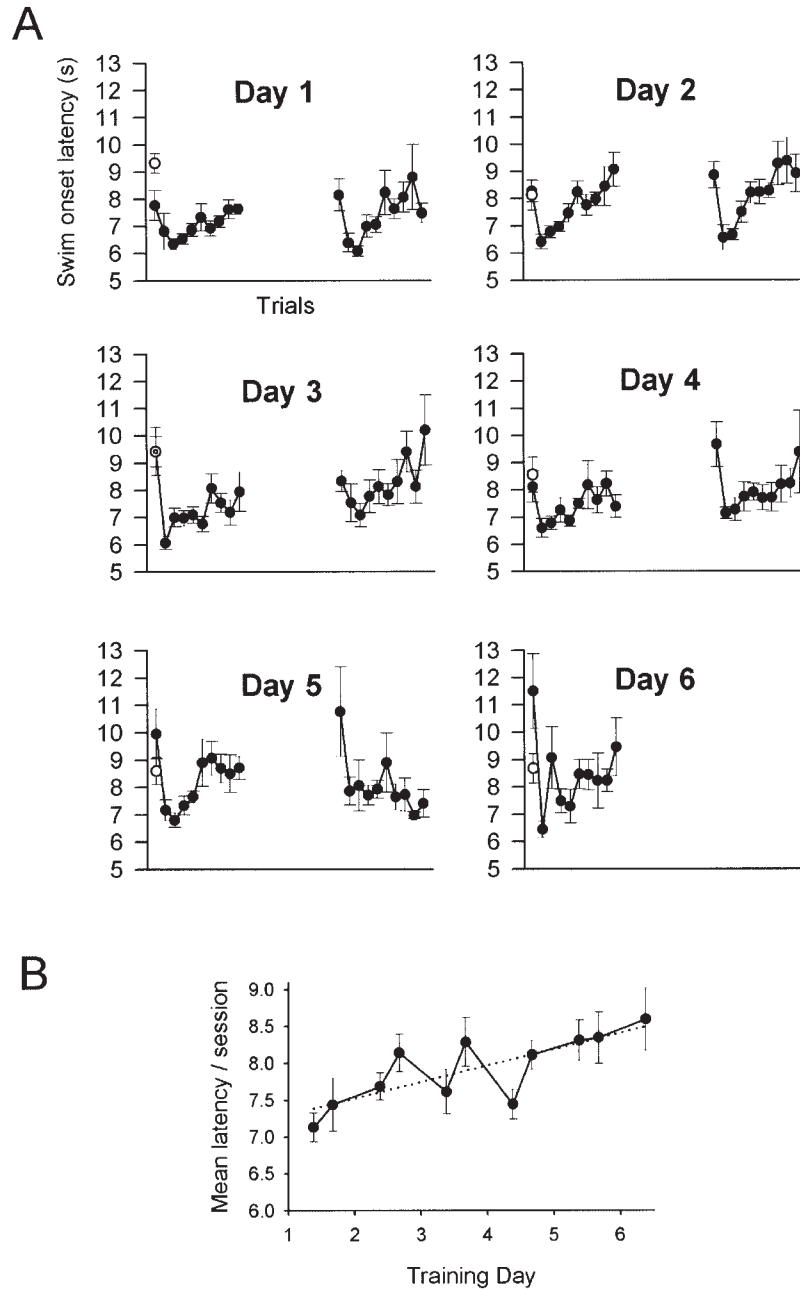


**Figure 2.** Number of swim cycles during the training phase of the experiment. Experimental animals (black) were given 110 total trials, delivered in two 10-trial sessions per day over a 5 1/2 day period. Control animals (white) were given a single trial per day over the same period. **(A)** Data for all trials. An initial sensitization followed by within-session habituation is evident. Trials that elicited no swim contributed a cycle number of 0. **(B)** Habituation across training sessions. Each point represents the mean number of cycles in all swims in that session for the experimental animals. The slope of the regression line fit to these data (dotted line) was significantly different from zero, indicating across-session habituation of cycle number.

indicating that the habituation produced by the first session lasted at least 5.5 h (mean reduction =  $22.0 \pm 3.1\%$ ,  $t = 7.15$ ,  $P < 0.01$ ). Furthermore, only modest recovery occurred during each 16.5-h overnight rest period, indicating

that the memory lasted at least that long as well (mean recovery =  $12.5 \pm 3.52\%$ ,  $t = 3.54$ ,  $P < 0.05$ ).

The control group was stimulated once per day to test whether the apparent progressive habituation across the 6

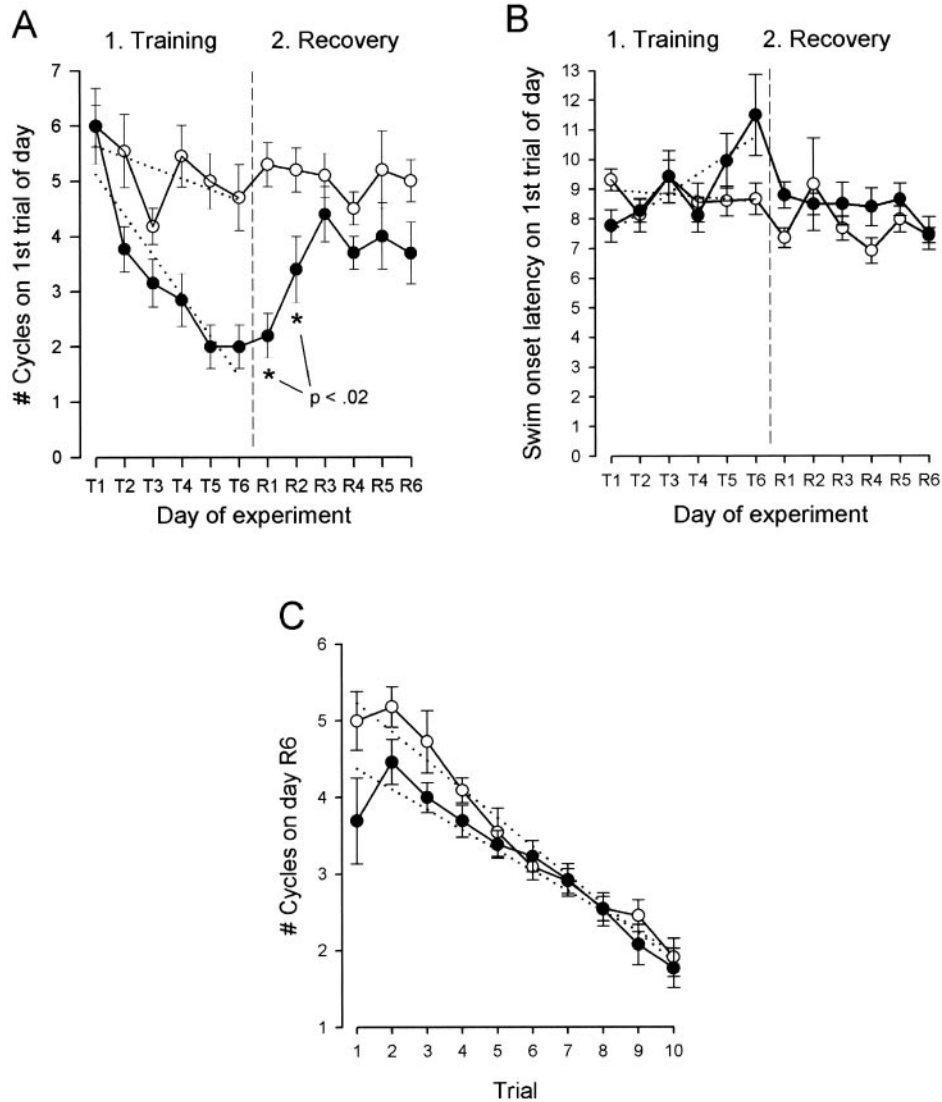


**Figure 3.** Swim onset latency during the training phase of the experiment. **(A)** Data for all trials that elicited swims (black = experimental animals, white = controls). An initial sensitization (quickening) of swim onset latency is evident from the first to the second trial of each session. Trials that elicited no swim had no latency, and thus are not represented in the data shown. **(B)** Habituation across training sessions. Each point represents the mean swim onset latency of the 10 trials in each session for the experimental animals. The slope of the regression line fit to these data (dotted line) was significantly different from zero, indicating across-session habituation of swim onset latency.

days of training was indeed due to learning, as opposed to other factors unrelated to training (*e.g.*, temperature effects, sickness), which would be experienced by both groups of animals. Because the controls were stimulated just once per day, we compared their responses to the first trial of each day in the experimental group (Fig. 4A1). We computed regression lines for these first trial data and found that the

slopes of the experimental and control group lines were significantly different ( $F(1,8) = 6.25, P < 0.05$ ), reflecting the accumulating cycle number habituation in the experimental group across sessions.

*Onset latency.* Figure 3B shows the mean swim onset latency for each 10-trial session for the 13 experimental



**Figure 4.** Between-groups comparison of experimental and control groups during the training and recovery phases of the experiment. Each point represents the mean response of the experimental (black) or control (white) animals for the first trial of the day. **(A)** Cycle number data. A1. Training phase. The slopes of the regression (dotted) lines fit to the E and C groups were significantly different, indicating that across-session habituation of cycle number occurred in the experimental group. A2. Recovery phase. Long-term habituation of cycle number. A between-groups repeated measures ANOVA applied to the recovery phase (days R1–R6) followed by *post hoc* between-groups Duncan tests at each time point indicated that cycle number habituation lasted 2 days following the end of training. **(B)** Swim onset latency data. B1. Training phase. The slopes of the regression (dotted) lines fit to the E and C groups were significantly different, indicating that across-session habituation (progressive slowing) of swim latency occurred in the experimental group. B2. Recovery phase. No long-term habituation of swim latency. A between-groups ANOVA applied to the recovery phase (days R1–R6) detected no long-lasting effect of training on swim latency. **(C)** Evidence for memory lasting several days. On recovery day 6, both groups were given a 10-trial habituation training session (see Fig. 1). The cycle number responses of the experimental group were consistently lower in the initial trials, causing a lower overall slope, indicating the persistence of memory until day 6.

animals. Although onset latency initially decreased within each session, the mean session latency progressively increased over the several days of training, reflecting the development of across-session habituation: the slope of the regression line fit to the data was significantly greater than

zero ( $F(1, 9) = 13.95, P < 0.01$ ). Examining the data more closely, on any given day the mean latency of the second session was longer than that of the first, with some recovery often occurring overnight. This zig-zag structure in the mean session curves was similar for the cycle number and

latency data (compare Figures 2B and 3B). One notable feature of swim latency was that, although the initial latency in each session progressively increased over the days of training, the latency on the second trial tended to drop to a consistent time of about 6.5 s.

As with the analysis of cycle number, we next compared the slopes of regression lines fit to the experimental and control latency data during the 6-day training period. This between-groups analysis, limited by the control protocol to the first trial of each day, revealed that the slopes were significantly different (Fig. 4B1,  $F(1,8) = 9.21, P < 0.02$ ), reflecting the progressive increase in across-session latency habituation in the experimental group over the 6 days of training.

**Threshold.** Another parameter that changed with repeated sessions was the proportion of animals that swam at all to the stimulus. In the first session, animals failed to swim on just 2 of 130 trials, and just 5 of these had latencies of 10 s or higher. By the last session, animals failed to swim on 38 of 130 trials, with latencies of 10 s or higher on 18 trials. This result is interpreted here to indicate an accumulating across-session effect of habituation training on swim threshold over the 6 days of training. Because the experiment had a fixed training protocol, we did not probe animals with higher strength stimuli to test the possibility that their failure to swim stemmed from their having temporarily lost the ability to swim, rather than because they had undergone a change in threshold. Such tests have demonstrated threshold changes in prior studies of short-term habituation of the *Tritonia* swim (Brown *et al.*, 1996).

#### Long-term ( $\geq 24$ h post-training) learning

**Swim cycle number.** The progressive, across-session changes described above were consistent with a developing long-term habituation of swim cycle number and latency, but this could not be confirmed because the longest inter-session interval during training was 17 h (overnight). To address whether the memory was indeed long term ( $>24$  h), we carried out the recovery phase of the experiment in which, after the end of training, both control and experimental groups continued to receive a single test stimulus once per day for 6 consecutive days (Fig. 4A2). A two-way repeated measures between-groups ANOVA for independent groups applied to this recovery period (days R1–R5 plus the first stimulus of R6) indicated a persistent effect of training on swim cycle number ( $F(1, 22) = 17.30, P < 0.001$ ). *Post hoc* Duncan tests comparing control and experimental animals indicated that significant habituation persisted for the first 2 days of the 6-day recovery period ( $P < 0.02$ ). Although the *post hoc* tests did not reveal a significant difference on recovery days 3–6, the consistently lower scores in the experimental group during all 6 recovery

days suggested that a low level of residual habituation memory might exist throughout this period. To test this possibility, both the control and experimental groups were given a standard 10-trial session on recovery day 6 (Fig. 1). We found that the slopes of regression lines fit through the control and experimental data for these 10-trial sessions were significantly different ( $F(1,16) = 6.95, P < 0.02$ ), with the experimental group having a lower slope (Fig. 4C). The two slopes were still significantly different when trials 2–10 were analyzed separately ( $F(1,14) = 6.15, P < 0.03$ ). This altered short-term habituation slope in the experimental group, while admittedly influencing just the first few trials, supports the possibility that residual long-term habituation memory persisted for at least 6 days after the end of long-term habituation training.

**Swim onset latency.** A two-way repeated measures ANOVA for independent groups applied to the corresponding latency data (days R1–R6) found no persistent long-term effect of training on this swim feature (Fig. 4B2,  $F(1,15) = 2.07, P > 0.05$ ). Thus, in spite of a progressive increase in swim latency across training sessions (Figs. 3B and 4B1), the habituation apparently did not to persist longer than 24 h, unlike the memory for cycle number habituation.

**Threshold.** Whereas the experimental animals failed to swim on 38 of the 130 trials on the 6th day of habituation training, they returned to a normal level of just four failures on the first recovery day (24-h rest). Thus, like swim onset latency, swim threshold showed no evidence of long-term habituation.

## Discussion

Long-term habituation is phylogenetically widespread, having been demonstrated in invertebrates, including the nematode *C. elegans* (Rose and Rankin, 2001), the marine mollusc *Aplysia* (Carew and Kandel, 1973; Stopfer *et al.*, 1996; Ezzeddine and Glanzman, 2003), and the crab *Chasmagnathus* (Tomsic *et al.*, 1998), as well several vertebrates, including rats (Leaton and Supple, 1986), mice (Plappert and Pilz, 2005) and humans (Ornitz and Guthrie, 1989; Maschke *et al.*, 2000). Prior studies of habituation in *Tritonia* suggested a slow rate of recovery, consistent with a long-lasting memory (Abraham and Willows, 1971; Frost *et al.*, 1996; Brown, 1998). The present study was the first to focus on this issue, however, and to deliberately evaluate the duration of the learning. We here demonstrated long-term habituation in cycle number lasting at least 2 days after the end of training, with evidence suggesting the possibility of a residual memory lasting 6 days. By contrast, habituation of swim latency and threshold lasted hours, not days, suggesting that these different components of memory may

be mediated by at least partly separate mechanisms in the swim network.

Long-term memory for habituation is significant from multiple perspectives. First, studies in both vertebrates and invertebrates have shown that the memory for long-term habituation requires gene expression and protein synthesis for its consolidation (Squire and Becker, 1975; Pedreira *et al.*, 1996). In this respect, habituation shares mechanistic features with other forms of learning. Second, long-term habituation is deliberately induced in therapeutic procedures used to treat anxiety (Marks and Tobena, 1990) and post-traumatic stress disorder (Vaughan and Tarrrier, 1992; Rothbaum and Davis, 2003). Third, a number of studies support the hypothesis that chronic anxiety (Lader and Wing, 1964; Raskin, 1975) and post-traumatic stress disorder (Rothbaum *et al.*, 2001; Rothbaum and Davis, 2003) result in part from a decreased ability of certain individuals to habituate to stressful stimuli. This latter possibility has prompted efforts to use habituation as a screening method for developing and comparing anxiolytic drugs (Quermonne *et al.*, 1993). Clearly the cellular mechanisms mediating long-term habituation are of clinical interest.

In addition to its potential usefulness for the study of short- versus long-term memory mechanisms, *Tritonia diomedea* is well-suited for exploring a variety of network-level issues involving memory storage. Habituation, dishabituation, and sensitization are well characterized in this system, and share many features with their counterparts in vertebrates (Brown *et al.*, 1996; Frost *et al.*, 1996, 1998; Brown, 1998; Mongeluzi and Frost, 2000). The neural circuitry mediating the behavior modified by learning is well understood (Willows *et al.*, 1973; Getting, 1976, 1983; Hume *et al.*, 1982; Frost and Katz, 1996; Frost *et al.*, 2001). The swim's rhythmic nature makes its underlying motor program easy to recognize in isolated brain preparations, and the neural correlates of several features of behavioral habituation have been identified (Frost *et al.*, 1996; Brown, 1997). This ability to study the network elements responsible for habituation-related changes in swim cycle number, onset latency, threshold, and cycle period provides a rich environment for studies of how the information acquired in learning is fragmented among several distributed sites of network modification—a well-known but poorly understood feature of vertebrate memory. Because *Tritonia* can simultaneously show habituation of swim cycle number and sensitization of swim onset latency (Brown *et al.*, 1996; Mongeluzi and Frost, 2000), this preparation also offers the opportunity to study how multiple memories manage to share space in the same network without interfering with one another (Wang and Frost, 2002). Finally, the fact that the *Tritonia* swim network is multifunctional, with roles in withdrawal, swimming and crawling (Getting, 1989; Popescu and Frost, 2002), makes it possible to study the degree to which memories residing in multifunctional net-

works may selectively influence subsets of the behaviors or processes mediated by those networks.

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