

# Extinction memory in the crab *Chasmagnathus*: recovery protocols and effects of multi-trial extinction training

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Received: 21 July 2009 / Revised: 16 September 2009 / Accepted: 17 September 2009  
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**Abstract** A decline in the frequency or intensity of a conditioned behavior following the withdrawal of the reinforcement is called experimental extinction. However, the experimental manipulation necessary to trigger memory reconsolidation or extinction is to expose the animal to the conditioned stimulus in the absence of reinforcement. Recovery protocols were used to reveal which of these two processes was developed. By using the crab contextual memory model (a visual danger stimulus associated with the training context), we investigated the dynamics of extinction memory in *Chasmagnathus*. Here, we reveal the presence of three recovery protocols that restore the original memory: the old memory comes back 4 days after the extinction training, or when a weak training is administered later, or once the VDS is presented in a novel context 24 h after the extinction session. Another objective was to evaluate whether the administration of multi-trial extinction training could trigger an extinction memory in *Chasmagnathus*. The results evince that the extinction memory appears only when the total re-exposure time is around 90 min independently of the number of trials employed to accumulate it. Thus, it is feasible that the mechanisms described for the case of the extinction memory acquired through a single training trial are valid for multi-trial extinction protocols. Finally, these results are in agreement with those reports obtained with models phylogenetically far apart from the crab. Behind this attempt is the idea that in the domain of

studies on memory, some principles of behavior organization and basic mechanisms have universal validity.

**Keywords** Extinction memory · Reinstatement · Reacquisition · Spontaneous recovery · Crab · Invertebrate

## Introduction

A decline in the frequency or intensity of a conditioned behavior following the withdrawal of the reinforcement is called experimental extinction (Dudai 2002). This phenomenon has been demonstrated in a large number of animals through several experimental paradigms (Berman and Dudai 2001; Pedreira and Maldonado 2003; Sandoz and Pham-Delegue 2004; Sangha et al. 2004). While some investigators have conjectured that extinction represents the destruction of the original learning—i.e. the association between the conditioned stimulus, CS, and the unconditioned one, US—(McClelland and Rumelhart 1985; McCloskey and Cohen 1989; Rescorla and Wagner 1972); other results indicate that extinction is not due to forgetting or destroying the primary association. Extinction may be an active learning process that exhibits many of the same properties as the original memory, but its meaning is distinct from the original acquisition (Kornorski 1948; Pearce and Hall 1980; Wagner 1981). Similar to a classical associative memory, extinction requires a consolidation phase (Berman et al. 2003; Suzuki et al. 2004) and the resulting memory is itself subject to forgetting (Bouton and Nelson 1994). This model is supported by behavioral experiments showing that the extinguished response may be replaced by the conditioned behavior again as a consequence of one of four so-called “recovery protocols” (Bouton 2004; Myers and Davis 2002). These recovery protocols include: (1)

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re-exposing the animals only to the US, called reinstatement (Bouton and Bolles 1979b; Rescorla and Heth 1975; Vianna et al. 2001), (2) switching the extinction context results in re-emergence of the original behavior, termed renewal (Bouton and Bolles 1979a; Bouton and King 1983), (3) recovering the conditioned response with the passage of time without any additional training, termed spontaneous recovery (Brooks and Bouton 1993; Pavlov 1927; Robbins 1990), (4) pairing again the CS–US results in a facilitation of relearning in the retraining, denominated reacquisition (Bouton 1993; Ebbinghaus 1885; Napier et al. 1992). In fact, it is broadly accepted that the recovery protocols represent diagnostic tools to characterize the extinction memory (Davis and Myers 2002; Duvarci and Nader 2004; Eisenberg et al. 2003).

Until recently we studied the extinction of a contextual memory in the crab *Chasmagnathus*. In brief, our behavioral paradigm involves the quantification of the crab's escape response, which is elicited by the presentation of a visual danger stimulus (VDS). Following repeated presentation of the VDS, the crab's escape response declines and the animal begins to freeze in response to the VDS; this freezing persists over time and is expressed as the predominant response to VDS during a test period (Pereyra et al. 1999, 2000). This long-term memory appears only when the context—the environmental features of the training session—is equal in all respects to the context of the test session (Tomsic et al. 1998). Based on these results, we have proposed that this memory consists of an association between the context and the features of the VDS (the signal). Therefore, this memory is termed context-signal memory (CSM; Tomsic et al. 1998).

We have previously shown that a crab's re-exposure to the training context, 24 h after acquisition, in the absence of the VDS over a short time period (5–40 min) induces a process of destabilization (labilization) and restabilization of the original memory trace (i.e. CSM) called reconsolidation (Nader et al. 2000). On the other hand, re-exposure for over an hour using a single re-exposure period to the training context, a single extinction trial, induces extinction. Thus, the total exposure time to the context acts as a switch between memory reconsolidation and extinction memory (Pedreira and Maldonado 2003). Moreover, through close examination of this extinction process we have determined that non-reinforcement during context re-exposure, defined as an expectation time, is necessary for the switching mechanism to be operative (Pedreira et al. 2004); and the acquisition of the extinction memory is completed in a minimum of 45 s after the end of re-exposure (Pérez-Cuesta et al. 2007).

It is well-known that the experimental manipulation necessary to trigger memory reconsolidation or extinction is the exposure of the animal to the CS in the absence of the

reinforcement. Some authors have considered that the reminder presentation might operate as an extinction trial (Myers and Davis 2002; Fischer et al. 2004). Consequently, it is argued that, after a post-labilization treatment with protein synthesis inhibitors, the performance at testing may be the result of an enhancement of the extinction memory consolidation instead of an interference with the reconsolidation process (Myers and Davis 2002; Fischer et al. 2004).

Given that extinction involves a new learning process (Pavlov 1927), it seems to be counterintuitive to argue that blocking protein synthesis would facilitate the consolidation of this memory. But, if the memory deficit induced by protein synthesis inhibition after memory retrieval was caused by enhanced extinction memory, then the recovery protocols should cause the resurgence of the old memory.

Thus, when in the same paradigm it is possible to induce either memory labilization–reconsolidation or extinction memory, it is necessary to use the recovery protocols as a powerful tool to reveal which of these two processes was developed when the animal was confronted with the CS without reinforcement (Fischer et al. 2004; Merlo and Romano 2008; Duvarci and Nader 2004).

On these grounds, two aspects have driven us to study whether these protocols restore the original memory in *Chasmagnathus*. First, the possibility of determining the presence of these diagnostic features to fulfill the characterization of this memory in this invertebrate, showing the maintenance of the basic properties of the different memory phases throughout evolution (Carew 2000; Maldonado 2002). Second, when these tools have been delineated, their use could reveal whether the effect of treatment, given near context re-exposure, has affected memory labilization–reconsolidation or the acquisition/consolidation of a new extinction memory.

As shown in previous reports, we proposed an interpretative model that attempts to explain the mechanisms underlying extinction memory in crabs using extinction training that consisted of a single context-exposure trial that lasted over an hour (Pedreira and Maldonado 2003; Pedreira et al. 2004; Pérez-Cuesta 2007; Merlo and Romano 2008). However, since the pioneering experiments of Pavlov, the vast majority of extinction studies have been performed using multi-trial designs. Considering that an important and unresolved enigma in the neurobiology of memory is whether learning mechanisms (and forgetting) are conserved throughout the animal kingdom, we were interested in the extent to which our extinction model may also cover the case of multi-trial extinction. Knowing this would allow us to compare crab extinction memory with vertebrate extinction memory acquired through multi-trial extinction training. In spite of the extensive research on the experimental extinction memory in *Chasmagnathus*, this is the first attempt to transform a single extinction trial

paradigm into a multi-trial one. Therefore, this transformation opens up two new possibilities. First, to draw comparisons between the crab extinction memory and the classical vertebrate extinction models, such as rats (Myers and Davis 2002); and second, this kind of protocol represents a powerful tool to make pharmacological and behavioral interference during the extinction training session.

Here, we report the efficacy of multi-trial extinction training in crabs, estimating the trial duration and number of trials necessary to achieve extinction memory. With regard to this new protocol, one interesting point was to evaluate the total exposure time required for extinction in a multi-trial training protocol with that of the previously used single-trial training protocol. Finally, we discuss these results in light of our previous interpretative model.

## Methods

### Animals

Animals were adult male crabs (*Chasmagnathus granulatus*) 2.7–3.0 cm across the carapace, weighing around 17.0 g, collected from water <1-m deep in the rías (narrow coastal inlets) of San Clemente del Tuyú, Argentina. Once transported to the laboratory, they were lodged in plastic tanks (35 × 48 × 27 cm) filled with 2 cm of diluted marine water at a density of 20 crabs per tank. Tanks and other containers for the experiments were filled with water prepared using Marinemix Cristal Sea (USA), salinity 10–14‰ and pH 7.4–7.6. The holding and experimental rooms were maintained on a 12-h light–dark cycle (light on 07:00–19:00 h) and maintained within a range of 22–24°C. Animals were fed on rabbit pellets (Nutrientes S.A., Argentina) every 3 days and the water was changed after feeding. Experiments were carried out within 10 days after the animals' arrival, from January to August, and between 08:00 and 18:00 h.

### The experimental device

The apparatus (Maldonado 2002), referred to as the training context, consisted of a bowl-shaped opaque container with a steep concave wall 12-cm high (23-cm top diameter and 9-cm floor diameter) covered to a depth of 0.5 cm with artificial seawater. During each trial of 9 s, an opaque rectangular screen (25 × 7.5 cm), termed the visual danger stimulus (VDS), was moved horizontally above the animal. A VDS consisted of a left to right movement of the screen, followed by a right to left movement which returned the stimulus to its original position. The VDS provoked an escape response from the crab contained in the bottom of the container. The consequent container vibrations were

converted into electrical signals through a piezoelectric transducer placed on the external wall of the container. These signals were amplified, integrated during each 9-s trial, and translated into arbitrary numerical units ranging from 0 to 6,000, before being processed by a computer. The activity of every crab was recorded during the entire trial period. The experimental room had 40 such devices, separated from one another by partitions. During the experiments, crabs were illuminated with a 5 W bulb, either from above or below the container (Pérez-Cuesta et al. 2007). Throughout this paper, data were only recorded during the trial period; that is, during the 9-s VDS. No spontaneous activity was recorded during context exposures.

### Data analysis

Our data analysis was designed to test a basic prediction drawn from our extensive work on the crabs' context-signal memory (CSM, Maldonado 2002). At a test trial, animals that were given 15 or more training trials with 3 min of inter-trial interval (trained crabs, T) show a level of escape response that is lower than that of animals that remained in the training context without being trained (untrained crabs, U). A statistically significant U–T difference ( $p < 0.05$ ) is invariably found, even when crabs are injected with saline pre- or post-training. Therefore, a trained group is said to show context-signal memory retention when the basic assumption is confirmed. Based on the  $U > T$  prediction, data are analyzed using a priori Fisher-LSD planned comparisons following a significant main effect in a one-way ANOVA,  $\alpha < 0.05$  (Howell 1987; Rosenthal and Rosnow 1985). The experiments described in this paper used different numbers of untrained–trained pairs (U–T groups). However, equal numbers of crabs (from 30 to 40) were included in each group within the same experiment. The number of pairs assigned to each experiment was dependent on the particular experimental factors being tested. For each experiment, which included two or three U–T pairs of groups, three or five comparisons were carried out: between the two or three U-groups, and within the U–T pairs for each treatment. Considering the expected variation of the response levels in a natural population, all the values were represented as the normalized mean  $\pm$  the standard error with respect to the main-control group (100%). We analyzed the data using Statistica '99 Edition Windows 6.1 software package.

## Results

### Part 1: recovery protocols

Previous results have shown that crabs exposed for a single 12-h period to the training context, in the absence of the

VDS, exhibited extinction retention at the test session (Tomsic et al. 1998). More recently, we demonstrated that when animals were re-exposed for more than an hour to the training context 24 h after CSM training, the extinction memory was expressed in a test session given one day later (Pedreira et al. 2004; Pedreira and Maldonado 2003). Thus, an extinction memory acquired by a single-trial protocol was characterized from both behavioral and physiological standpoints (Pedreira and Maldonado 2003; Pedreira et al. 2004; Pérez-Cuesta et al. 2007). However, these reports did not include an analysis of the parameters that define extinction as an active new learning process (Bouton 2004; Rescorla 2004). Therefore, the purpose of the first series of experiments was to analyze the effect of spontaneous recovery, reacquisition and reinstatement protocols on the extinction memory in the crab *Chasmagnathus*.

#### Experiment 1: spontaneous recovery

Pavlov's early experiments (1927) demonstrated that the loss of behavior that results from presenting the un-reinforced CS was not permanent and that the conditioned response (CR) was recovered after a given post-extinction time interval. Moreover, it was shown that longer intervals between extinction training and the test session produced greater spontaneous recovery of the initially learned behavior (Bouton 2002). Thus, the goal of this experiment was to explore the existence of the spontaneous recovery phenomenon after one-trial extinction training in *Chasmagnathus*.

#### Experimental protocol

The experiment lasted 5 days (Fig. 1a) and included three untrained–trained pairs of groups (U–T): the context-signal memory pair (CSM), the extinction memory 24 h before test pair (EXT<sub>24</sub>) and the extinction memory 72 h before test pair (EXT<sub>72</sub>). *Day 1* (training session): Untrained animals (U) of each pair were kept in the training context (container with white walls) during the entire training session (circa 50 min) as controls; i.e. without receiving the visual danger stimulus (VDS). After 5 min without stimulation, trained animals (T) of each pair received 15 training trials, each consisting of a 9-s VDS presentation, separated by a 3-min inter-trial interval (ITI). Immediately after the training session, both U and T crabs were moved from the training context to be housed individually in the resting containers. These containers were plastic boxes filled with 0.5 cm of water and kept inside dimly lit drawers. *Day 2*: crabs were exposed to the novel context for 2 h, which consisted of a container with black-and-white striped walls instead of white ones (CSM pair), or to the training context (EXT<sub>72</sub> pair), both without VDS presentation. All the animals were maintained in the

rest containers until the test session. *Day 4*: subjects from the EXT<sub>24</sub> pair were confronted with the training context that did not include the VDS for 2 h. *Day 5* (test session): all crabs were again placed in the training context for 5 min, which was followed by the test trial, i.e. the VDS presentation.

#### Results

Figure 1b shows the test results of each group normalized to the mean response of the CSM-U group on Day 5 [ANOVA  $F_{5,222} = 6.38$ ,  $p < 0.001$ ]. Fisher-LSD planned comparisons performed on the data revealed a significant difference (CSM retention) for the CSM pair ( $p = 0.007$ ). This memory could be evoked 96 h after training and the exposure to a novel context, done 24 h after training, did not affect retention as we had observed in previous studies (Pedreira et al. 2004; Pedreira and Maldonado 2003). In turn, the pairs that received the extinction training at different intervals from the CSM training session showed opposite effects. Thus, the EXT<sub>24</sub> pair exhibited extinction retention (Fisher-LSD planned comparisons:  $p = 0.33$ ), but when the inter-session interval was prolonged to 72 h (EXT<sub>72</sub>), the context-signal memory reappeared (Fisher-LSD planned comparisons:  $p = 0.004$ ).

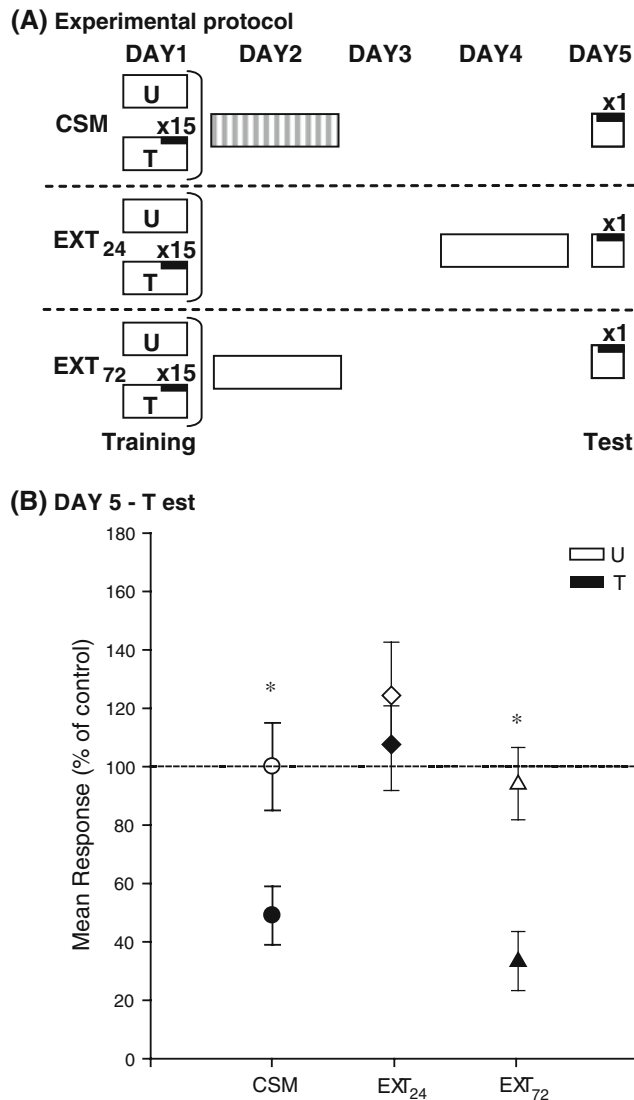
This data show that when the test session is separated from extinction training by 72 h, the expression of the CSM memory reemerges spontaneously. Indeed, the crucial difference is the moment at which extinction took place, presenting the possibility of observing the spontaneous recovery phenomenon as a consequence of the interval between the one trial-extinction training and the test session (Rescorla 2004).

#### Experiment 2: reacquisition

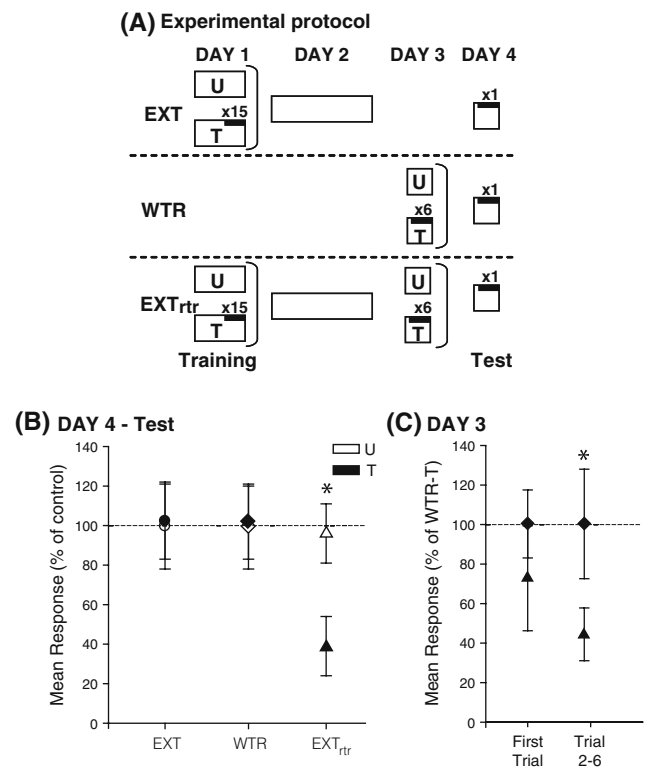
In other vertebrate models it was demonstrated that when new CS–US pairings were introduced after extinction, the reacquisition of the conditioned response could be faster than during acquisition. Moreover, this process could be evaluated during the new CS–US pairings or 24 h after this presentation (Ebbinghaus 1885; Napier et al. 1992). Therefore, this experiment was aimed at studying the effect of reacquisition on the extinction memory in crabs.

#### Experimental protocol

Experiment 2 lasted 4 days (Fig. 2a) and consisted of three paired groups: the extinction pair (EXT), the weak training pair (WTR) and the extinction retraining pair (EXT<sub>tr</sub>). *Day 1* (training session): EXT and EXT<sub>tr</sub> pairs went through a training session for CSM (15 trials, ITI = 3 min). *Day 2*: the



**Fig. 1** Spontaneous recovery. **a** Experimental protocol ( $n = 38$ ). *Open boxes* stand for time periods of training, treatment and test sessions spent in the training context. *Striped boxes* stand for time periods of exposure to the novel context. The *black horizontal bars* stand for the trials (each consisting of a 9-s VDS presentation) administered during the sessions. Experiment 1 included three untrained–trained pairs of groups (U–T pair): the context-signal memory pair (CSM), the extinction memory 24 h before testing pair (EXT<sub>24</sub>) and the extinction memory 72 h before testing pair (EXT<sub>72</sub>). Training session for CSM on Day 1: trained animals (T) received 15 trials (=x15), whereas untrained animals (U) of each pair were kept in the training context (container with white walls) without receiving the VDS during the entire session (circa 50 min) as controls. Day 2: crabs from the CSM and EXT<sub>72</sub> pairs were exposed to the novel or the training context for 2 h, respectively. Day 4: subjects from the EXT<sub>24</sub> pair were confronted with the training context without the VDS for 2 h. Day 5: animals were tested with a test trial (=x1). **b** DAY 5 - Test. Mean responses on Day 5, normalized with respect to the mean response of the CSM-U group. *Circles* stand for CSM pair, *rhombus* for EXT<sub>24</sub> pair and *triangles* for EXT<sub>72</sub>. *White symbols* stand for untrained (U) groups and *black* for trained (T) groups. *Bars* stand for S.E.M. \* $p < 0.05$



**Fig. 2** Reacquisition. **a** Experimental protocol ( $n = 39$ ). Experiment 2 consisted of three pairs of groups: the extinction training pair (EXT), the weak training pair (WTR) and the extinction retraining pair (EXT<sub>Trtr</sub>). Day 1: crabs from EXT and EXT<sub>Trtr</sub> pairs went through a typical training session for CSM. Day 2: these subjects were re-exposed to the training context for 2 h. Day 3: crabs from the EXT<sub>Trtr</sub>-T and WTR-T groups received a weak training protocol (6 trials), whereas untrained animals (U) of each pair were kept in the training context as controls. Day 4: animals were tested as in Experiment 1. **b** DAY 4 - Test. Mean responses on Day 4, normalized with respect to the mean response of the EXT-U group. *Circles* stand for EXT pair, *rhombus* for WTR pair, and *triangles* for EXT<sub>Trtr</sub> pair. *White symbols* stand for untrained (U) groups and *black* for trained (T) groups. *Bars* stand for S.E.M. Symbols and indications as in Fig. 1. **c** DAY 3. Mean responses for the first trial and the accumulated response for 2–6 trials on Day 3, normalized with respect to the mean response of the WTR-T group. *Black rhombus* stand for WTR-T group, and *triangles* for EXT<sub>Trtr</sub>-T group. *Bars* stand for S.E.M. Symbols and indications as in Fig. 1

EXT and EXT<sub>Trtr</sub> pairs were re-exposed to the training context for 2 h. *Day 3*: the EXT<sub>Trtr</sub>-T and WTR-T groups received a weak training protocol (6 trials, ITI = 3 min). During this session (circa 20 min) untrained animals (U) of each pair were kept in the training context as controls. *Day 4*: test session as in Experiment 1.

In this experiment an additional statistical comparison was made: for the data obtained during the weak training protocol on Day 3, a *t*-test was used to compare the mean response level of the first trial and of the following 2–6 accumulated trials.



## Results

The results from Experiment 2 are displayed in Fig. 2b. At the test on Day 4 [ANOVA  $F_{5,228} = 1.7$ ,  $p = 0.13$ ], Fisher-LSD planned comparisons showed extinction retention for EXT pair ( $p = 0.94$ ), failed to reveal CSM retention for WTR pair ( $p = 0.9$ ) and disclosed recovery of CSM expression for EXTrtr pair ( $p = 0.03$ ). Furthermore, we analyzed the performance of the animals during the reacquisition session on Day 3. Figure 2c shows the data normalized to the response of the WTR-T group for the WTR-T and EXTrtr-T during the first trial and the mean accumulated scores normalized to the accumulated score of WTR-T for trials 2–6 (retraining). Animals from both groups revealed a similar escape response for the first trial ( $t$ -test;  $t_{(60)} = -1.064$ ,  $p = 0.15$ ); however, during the retraining (trials 2–6) the EXTrtr-T group showed a lower level of response compared with the WTR-T group ( $t$ -test;  $t_{(60)} = -1.817$ ,  $p = 0.03$ ). Therefore, the results obtained for the EXTrtr-T group may be explained by the expression of the extinction memory in the first trial and by a rapid loss of the escape response through the retraining phase as a consequence of saving.

Thus, this profile of results is consistent with those obtained by this type of manipulation in other models (Napier et al. 1992; Ricker and Bouton 1996). The administration of a weak training protocol after extinction is enough for CSM re-appearance at testing 24-h later. Finally, the recovery of the original learning observed with this type of practice depends on additional trials, since no spontaneous recovery is disclosed with the sole one-day protracting of the intersession interval (i.e. EXT group).

### Experiment 3: reinstatement

In extinction paradigms, it was shown how the US presented on its own after extinction could cause the restoration of the extinguished response (CR; Bouton and Bolles 1979b; Rescorla and Heth 1975). However, in contextual fear conditioning, where the context represents the CS, it is necessary to present the US in a non-specific way (Vianna et al. 2001). Since the US presentation in the training context could represent re-training rather than reinstatement, the aversive US should be given after extinction in a different context (Bertotto et al. 2006). Therefore, the goal of this experiment was to evaluate the effect of the VDS presentation on its own in a different context 24 h after extinction training.

### Experimental protocol

Experiment 3 required 4 days and comprised three pairs of groups (Fig. 3a): The context-signal memory pair (CSM), the extinction novel context pair (EXTnct) and the

extinction novel context plus a VDS pair (EXTnct + vds). *Day 1* (training session): the three pairs received a training session for CSM. *Day 2*: the CSM pair was exposed to the novel context for 2 h. The other two pairs were placed in the training context without reinforcement for 2 h. *Day 3*: the EXTnct pair was exposed to the novel context for 5 min; the EXTnct + vds pair was exposed to the novel context for 5 min and received a trial in the last minute. *Day 4*: test session as in Experiment 1.

Considering the characteristics of the CSM-paradigm, where the danger stimulus was associated with the training context, we administered the VDS for reinstatement in a novel context, created by the incorporation of black and white bands to the container, producing a major visual change in the experimental device. However, the inclusion of this striped context had a detrimental effect on the escape response measurement.

## Results

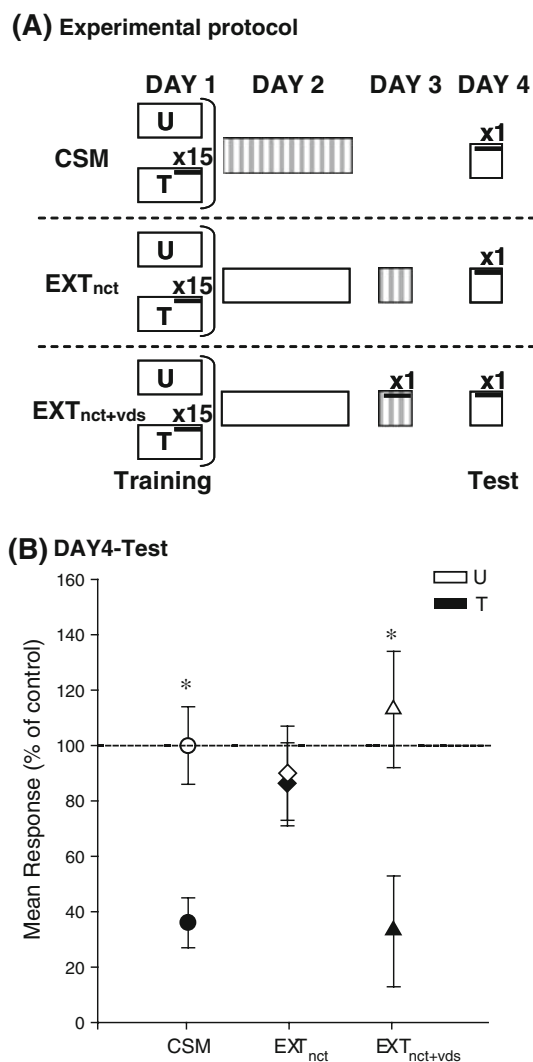
Figure 3b displays the results obtained with this experimental design [ANOVA  $F_{5,234} = 4.89$ ,  $p < 0.001$ ]. Fisher-LSD planned comparisons performed at the test trial on Day 4 revealed a significant difference (CSM retention) for the CSM pair ( $p = 0.002$ ). In turn, the performance of the pairs that remained in a novel context with a VDS after extinction was contrary to the ones that did not receive the VDS in a novel context. While the animals that were only exposed to the novel context (EXTnct pair) evinced extinction memory (Fisher-LSD planned comparisons:  $p = 0.85$ ), the EXTnct + vds pair that received a VDS in the last minute of the exposure, exhibited CSM at testing ( $p = 0.003$ ).

Thus, the extinguished conditioned response reappears when the unsignaled presentation of one VDS is interposed between extinction training and testing. Moreover, in preliminary experiments, the presentation of one VDS in the training context 24 h after the extinction training did not reinstate the CSM memory; and the performance of a EXTnct + vds pair on Day 3 failed to show retention as a consequence of the incongruence between the features of the training context and the context presented during the reinstatement session (data not shown).

On the whole, these results support the view that the reappearance of the CSM resulting from VDS administration in a new context after extinction could represent the relevance of the VDS in a novel situation without ambiguity. This protocol is a sufficient condition to allow old memory re-emergence at testing (Bouton 2002).

### Discussion of the recovery protocol experiments

As shown with vertebrate models in which the extinction memory represents an inhibitory learning mechanism, the



**Fig. 3** Reinstatement. **a** Experimental protocol ( $n = 40$ ). Experiment 3 consisted of three pairs of groups: the context-signal memory pair (CSM), the extinction and novel context pair (EXT<sub>nct</sub>) and the extinction and novel context plus a visual danger stimulus pair (EXT<sub>nct</sub> + vds). Day 1: crabs received a 15-trial training session for CSM. Day 2: animals from the CSM pair were exposed to the novel context for 2 h. The rest of the subjects were placed into the training context for 2 h. Day 3: animals in the EXT<sub>nct</sub> pair were exposed to the novel context for 5 min; whereas subjects in the EXT<sub>nct</sub> + vds pair were confronted to the novel context for 5 min and received a trial in the last minute. Day 4: animals were tested as in Experiment 1. **b** DAY 4 - Test. Mean responses on Day 4, normalized with respect to the mean response of the CSM-U group. Circles stand for CSM pair, rhombus for EXT<sub>nct</sub> pair and triangles for EXT<sub>nct</sub> + vds pair. White symbols stand for untrained (U) groups and black for trained (T) groups. Bars stand for S.E.M. Symbols and indications as in Fig. 1

extinction memory in crabs induced with single extinction training exhibited reinstatement, spontaneous recovery and reacquisition. Moreover, and supporting this classical extinction memory process, we demonstrated that consolidation of the extinction memory, but not acquisition requires de novo protein synthesis (Pedreira et al. 2004; Pedreira and Maldonado 2003; Pérez-Cuesta et al. 2007).

To sum up, it could be assumed that during acquisition of conditioned tasks, animals learn new relations among novel stimuli, but during extinction, the relations established through the conditioning could be overturned (Berman et al. 2003). This reversion establishes a new relation, and depending on the experimental manipulations, one association or the other could be expressed (Myers and Davis 2002).

Despite the evidence for recovery of the original learning, some reports suggest that the extinction may induce partial or complete erasure of the old memory trace (Lin et al. 2003a, b). More recently Myers et al. (2006) has suggested that extinction may induce erasure, inhibitory learning or both, depending on the maturity of the fear acquisition memory. Through a series of behavioral experiments, they tested the hypothesis that different neural mechanisms are recruited for extinction depending on the temporal delay between the original training and the extinction training. Consistent with extinction as an inhibitory learning mechanism in rats, the memory was extinguished 24–72 h after conditioning exhibited, in different degrees, reinstatement, renewal and spontaneous recovery. In contrast, and consistent with an erasure mechanism the memory was extinguished 10 min or an hour after the original acquisition exhibited little or no reinstatement, renewal or spontaneous recovery. However, subsequent experiments in both rats and humans that used variations of this protocol have met with limited success (Schiller et al. 2008).

The findings presented in this first series of experiments concerning the effect of recovery protocols on the extinction memory seem to be in line with Myers' report. In fact, the interval between the acquisition of the CSM and the extinction training in our experimental designs was not less than 24 h, so the original memory was already consolidated when it was extinguished (Maldonado 2002).

#### Part 2: multi-trial extinction training

In previous reports (Pedreira et al. 2004; Pedreira and Maldonado 2003; Pérez-Cuesta et al. 2007), we posited a functional model to explain the results obtained with one-trial extinction training in *Chasmagnathus*. We suggested that the acquisition of the extinction memory requires computing time of over 1 h during context-exposure from the beginning of the context presentation, which retrieves CSM memory, to the end, which represents the termination of the un-reinforced presentation. This context re-exposure for extinction is an expectation time, which depends on the time up to the context-offset; and the acquisition of the extinction memory being accomplished in a minimum of 45 s after context-offset (Pedreira et al. 2004; Pérez-Cuesta et al. 2007). As we pointed out before, one of the objectives

of this work was to determine if our interpretative model, attempting to explain the mechanisms underlying extinction memory using a single training trial, could be extended to an extinction memory obtained with a multi-trial training. For this purpose, the following series of multi-trial experiments with a different number of trials and 1-min inter-trial intervals were performed.

Experiment 4: series of experiments to evaluate the effect of successive un-reinforced re-exposures to the training context

### *Experimental protocol*

Prior to performing these experiments, it was necessary to overcome a practical drawback. With the present protocol, for the execution of multi-trial experiments, crabs would require to be removed from their training context at the end of each trial, moved to the respective rest container for 1 min and moved back to the training context for the following trial. These maneuvers of repeated handling, proved to affect the behavior to be evaluated. Thus, we created another way to finish the exposure to the context. The end was “signaled” without removing the animal, specifically, by only changing the illumination of the training context from above to below.

The assumption for this design is that the illumination represents a situation comparable to taking out the animal from the training context, and, what is more, the context illuminated from below is perceived as a different one. Two different lines of experiments demonstrated the efficacy of this change in the illumination. First, after the single extinction trial, a minimum of 45 s with the light entirely from below is the time interval necessary for the animal to take the illumination shift as equivalent to the end of training context presentation (Pérez-Cuesta et al. 2007). Second, putting the animals in the containers illuminated from below for 2 h failed to induce extinction memory (data not shown). Thus, a trial is defined here as a time interval during which the crab is illuminated from above (i.e. exposure to the training context) and an inter-trial as a time interval during which it is illuminated from below.

The experimental conditions and data analysis were similar to those described in Experiment 1. We used the same experimental device but with the addition of a lighting implement, which lights the apparatus from below. So, the context could be illuminated either from above or below.

In this series of experiments we increased the number of re-exposures to the training context with light from above (extinction trial), separated by 1-min expositions with light from below (ITI), until the acquisition of the extinction memory. Each version of the experimental design lasted 2 days (Fig. 4a) and consisted of 2 pairs of groups: the

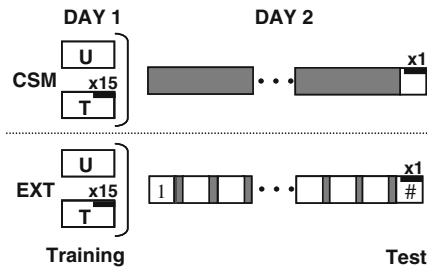
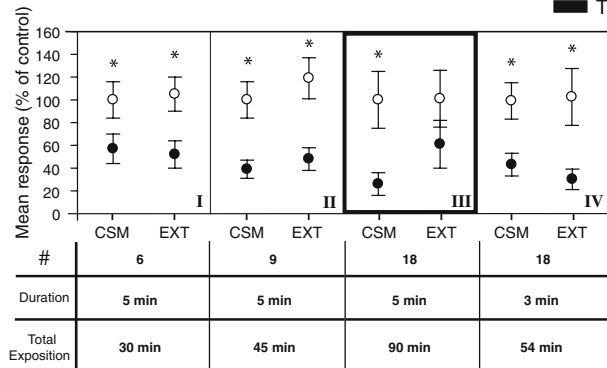
context-signal memory pair (CSM) and the extinction pair (EXT). *Day 1* (training session, with the light from above): each of the four experiments consisted of two pairs that received normal training for CSM, consisting of 15 trials of 3-min ITI for the T-groups and only exposure to the training context for the U-groups. *Day 2*: both groups (U–T) of the EXT pair were subjected to the iterative extinction training during the treatment phase with a specific number of 5-min extinction trials (6, 9 or 18 trials) of 1-min ITI (Fig. 4a). In the last experimental design each of the 18 extinction trials lasted 3 instead of 5 min. The groups of the CSM pair had light from below for the entire phase for an equal amount of time. The test phase began after the last context-offset and lasted 5 min. During this phase the illumination was from above and the test trial was given in the last minute.

### *Results*

Figure 4b shows results corresponding to a test trial given at the last trial of multi-trial extinction training for each of the performed experiments. For the first one with six 5-min trials each (30 min total context-exposure, Fig. 4b-I, [ANOVA  $F_{3,123} = 3.74$ ,  $p = 0.01$ ]), Fisher-LSD planned comparisons showed that the EXT pair failed to reveal extinction since a significant difference between groups was found ( $p = 0.01$ ) and as expected, the CSM pair discovered a significant difference ( $p = 0.04$ ) despite illumination from below. Similar results are shown in Fig. 4b-II [ANOVA  $F_{3,148} = 8.04$ ,  $p < 0.001$ ] when the EXT pair was given nine 5-min trials (45 min context-exposure: Fisher-LSD planned comparisons:  $p = 0.002$ ), while the respective CSM pair showed good retention ( $p < 0.001$ ). However, extinction retention was disclosed in the EXT pair after eighteen 5-min trials (Fig. 4b-III), that is, after 90 min of total context-exposure [ANOVA  $F_{3,116} = 2.58$ ,  $p = 0.04$ , Fisher-LSD planned comparisons, EXT pair:  $p = 0.18$ ], while the CSM pair revealed a good retention again after constant illumination from below ( $p = 0.015$ ).

Intriguingly, context-exposures for 30 or 45 min failed to produce extinction either when it was presented as one block in single training trial experiments (Pedreira and Maldonado 2003; Pérez-Cuesta et al. 2007) or as an accumulation of 6 or 9 trials 5 min each (Fig. 4b-I, II, respectively). In contrast, 90 min of context-exposure produced extinction either when it was presented as a single block in one-trial training (Pérez-Cuesta, personal communication) or as an accumulation of 18 trials of 5 min each (Fig. 4b-III). An alternative explanation could be given to explain the findings obtained in this experiment. At this point, and remembering that the termination of the context-exposure is a critical parameter in the model, it is possible that the number of context-endings is the necessary cue to trigger



**(A) Experimental protocol****(B) DAY 2 - Test**

**Fig. 4** Series of experiments to evaluate the effect of successive unreinforced re-exposures to the training context. **a** Experimental protocol. *Open boxes* stand for time periods of training, treatment and test sessions spent in the training context (with the illumination from above). *Gray boxes* stand for time periods of exposure to context with the illumination from below (intertrial interval = 1 min). The *black horizontal bars* stand for the VDS presentation during the sessions. Each experiment consisted of two pairs of groups: the context-signal memory pair (CSM) and the extinction pair (EXT). Day 1: crabs received a standard training for CSM with light from above the entire session. Day 2: animals from EXT pair were subjected to a discrete extinction training with 6, 9 or 18 extinction trials (#, each of them consist in the re-exposure to the training context without the VDS for 5 or 3 min with illumination from above), separated by 1-min illumination from below (ITI = 1 min). Crabs from the CSM pair stayed all this training session in the container without the VDS with the illumination from below. Test phase: subjects received the test trial (VDS) after 5 min with the light from above. **b** DAY 2 - Test. Mean responses on Day 2, normalized with respect to the mean response of the CSM-U group. Each panel corresponds to one experiment (I,  $n = 34$ ; II,  $n = 38$ ; III,  $n = 30$  and IV,  $n = 31$ ). The number and duration of the extinction trials for each experiment, and the total exposition to the training context, are shown in the table below. *White circles* stand for untrained (U) groups and *black circles* for trained (T) groups. *Bars* stand for S.E.M. \* $p < 0.05$

extinction memory independently of the time. To analyze this proposal, we carried out the last experiment of this series, in which the parameters were similar to Experiment 4b-III in all respects except one: for the EXT pair, the extinction trials lasted 3 min instead of 5 min, resulting in 54 min of context-exposure. During the test phase (Fig. 4b-IV), both pairs revealed CSM retention [ANOVA  $F_{3,120} = 8.3$ ;  $p < 0.001$ ; Fisher-LSD planned comparisons:

CSM pair  $p < 0.001$  and EXT pair  $p < 0.001$ ]. Therefore, the critical parameter to acquire the extinction memory seems to be the accumulated total time of context-exposure rather than the accumulation of context terminations only.

#### Experiment 5: long-term extinction memory acquired by multi-trial extinction training

Finally, after the determination of the multi-trial extinction training which induced the acquisition of an extinction memory tested near the training, the aim of the last experiment was to evaluate this memory after a long interval like 24 h.

#### Experimental protocol

The parameters of this experiment were similar to Experiment 4b-IV in all respects except one: the test trial was presented on Day 3. The experimental design lasted 3 days (Fig. 5a) and consisted of 2 pairs of groups: the context-signal memory pair (CSM) and the extinction pair (EXT). *Day 1* (training session, with the light from above): The two pairs received a normal training for CSM, consisting of 15 trials of 3-min ITI for the T groups and only exposure to the training context for the U groups. *Day 2*: both groups of the EXT pair were subjected to the iterative extinction training of 18 trials of 5 min. The groups of the CSM pair had light from below for the entire phase. *Day 3*: test session (with the light from above) lasted 5 min and the test trial was given in the last minute.

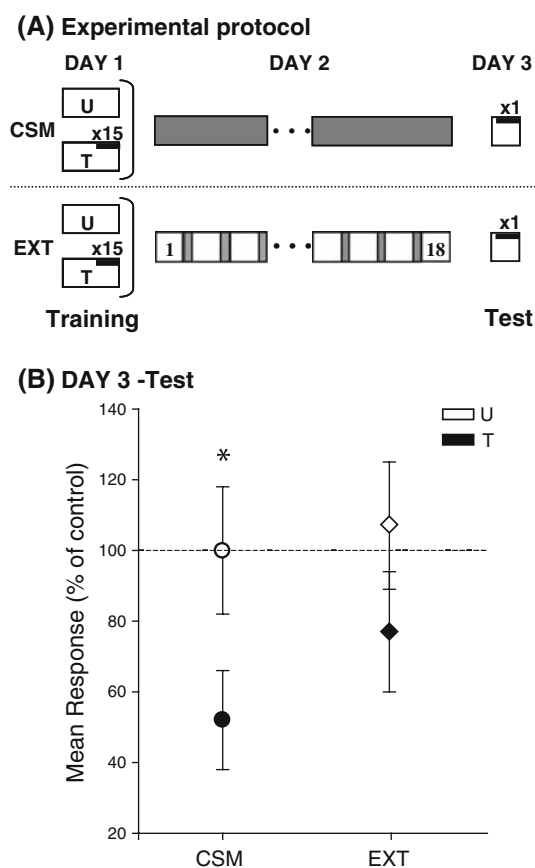
#### Results

Figure 5b shows results corresponding to a test trial, given on Day 3, 24 h after multi-trial extinction training. Extinction retention was discovered in the EXT pair after eighteen 5-min trials on Day 3, that is, after 90 min of total context-exposure [ANOVA  $F_{3,128} = 2.15$ ,  $p = 0.097$ ; Fisher-LSD planned comparisons, EXT pair:  $p = 0.21$ ], while the CSM pair revealed retention after constant illumination from below ( $p = 0.047$ ).

Thus, this last experiment completes the new picture of the extinction memory obtained with multi-trial extinction training. A first analysis of this remarkable result supports the above proposal that single-trial and multi-trial extinction share a common parameter of time re-exposure up to which the acquisition of the long-lasting extinction memory is triggered.

#### Discussion of the multi-trial extinction training experiments

A revision of previous reports analyzing the factors that influence extinction acquisition in vertebrates, such as trial



**Fig. 5** Long-term extinction memory acquired by multi-trial extinction training. **a** Experimental protocol ( $n = 33$ ). The experiment consisted of two pairs of groups: the context-signal memory pair (CSM) and the extinction pair (EXT). Symbols and indications as in Fig. 4. Day 1: crabs received a standard training for CSM with light from above the entire session. Day 2: animals from EXT pair were subjected to a discrete extinction training with 18 extinction trials (each of them consist in the re-exposure to the training context without the VDS for 5 min with illumination from above), separated by 1-min illumination from below (ITI = 1 min). Crabs from the CSM pair stayed all this training session in the container without the VDS with the illumination from below. Day 3: test phase. Subjects received the test trial (VDS) after 5 min with the light from above. **b** DAY 3 - Test. Mean responses on Day 3, normalized with respect to the mean response of the CSM-U group. Circles stand for CSM pair, and rhombus for EXT pair. White symbols stand for untrained (U) groups and black for trained (T) groups. Bars stand for S.E.M.  $*p < 0.05$

spacing of non-reinforced CS presentations, number of trials or duration of the trial stimulation, show that they are either ambiguous or contradictory (Amsel et al. 1971; Baum et al. 1990; Cain et al. 2003; Hill and Spear 1962; Martasian et al. 1992; Sandoz and Pham-Delegue 2004; Schiff et al. 1972; Shipley 1974; Stanley 1952; Pavlov 1927). Nevertheless, results obtained by Shipley et al. (1971) demonstrated that when keeping a constant ITI, the number and duration of CS exposures is completely interchangeable, since they combine multiplicatively to form total non-reinforced CS exposure. Consistent with this research, our results in these

experiments reveal that re-exposure to the training context induces extinction memory only after having accumulated an amount of non-reinforced context-exposure similar to that presented in one-trial training of 90 min.

## General discussion

The aim of this report was to characterize more profoundly the extinction memory in an invertebrate model. To reach this purpose we advanced in two different directions, establishing the two main conditions that allow us to compare our extinction model with the classical multi-trial extinction paradigms utilized with vertebrates.

The first condition is that the recovery protocols which are used to characterize the multi-trial extinction in rodents (Myers and Davis 2002) are also suitable concerning the single extinction trial in *Chasmagnathus*. We demonstrated that the extinguished response in the crab model is susceptible to a number of recovery treatments. The CSM came back 4 days after the extinction training (Experiment 1, Spontaneous recovery), or when a weak training protocol was administered later (Experiment 2, Reacquisition), or once the VDS was presented in a novel context 24 h after the extinction session (Experiment 3, Reinstatement). These results support the hypothesis that extinction is not unlearning and could represent the development of a new association between the context and the absence of the VDS (non VDS). It is a new memory that leaves the old memory, the CSM, intact though unexpressed. The demonstration that the recovery protocols improve the reappearance of the CSM, after the acquisition of the extinction memory with one-trial training, supports the idea that this memory shares the basic characteristics with the one acquired using multi-trial extinction training.

In a recent report using a classical fear conditioning paradigm, Monfils et al. (2009) illustrated the importance of the recovery protocols as a tool to determine whether the performance during testing is a consequence of an extinction memory or the weakening of the original memory during reconsolidation. They showed that presenting extinction training, within a reconsolidation time window opened up by an isolated CS, prevents renewal, reinstatement and spontaneous recovery from fear memory. Thus, they have suggested that a post-consolidation behavioral manipulation can render a memory labile and re-write and/or update, re-encoding the once fear-inducing CS as safe. Therefore, the recovery protocols represent a powerful tool to differentiate the outcome of an experimental design which could induce a labilization–reconsolidation phase or a new extinction memory.

The second condition is that the extinction memory can be acquired by using an iterative presentation of the context,

and that it depends on the total time of context-exposure irrespective of the fact that such total time was recruited in a single trial or in a succession of trials. Indeed, the results showed that re-exposure to the training context induced extinction memory only after having accumulated a total amount of time similar to that used in a single non-reinforced context-exposure, i.e. a sole trial of at least 90 min circa (Pérez-Cuesta personal communication), or 18 extinction trials of 5 min each separated by 1 min. It is worthwhile noticing that in the present experiments we tested the extinction memory acquired with multi-trial training a few minutes after acquisition (e.g. short-term extinction memory) and at a long-term test (e.g. long-term extinction memory), which allowed us to compare this extinction memory with that obtained with single training trial.

At this juncture, it is germane to analyze these results in the light of the interpretative scheme we proposed in previous works (Pedreira et al. 2004; Pérez-Cuesta et al. 2007). The crabs' re-exposure to the training context for a short time (5 to 40 min) without reinforcement induces memory labilization–reconsolidation, whereas re-exposure for a longer time (one or more hours using a single trial) induces extinction, both depending on *de novo* protein synthesis. In fact, the systematic study of the relationship between extinction and increasing periods of un-reinforced context presentation (Pedreira and Maldonado 2003; Pérez-Cuesta et al. 2007), confirmed the view that extinction requires a minimum duration of context re-exposure (>1 h), but, in addition, it supported the idea that extinction also requires the actual termination of the context re-exposure. Moreover, reconsolidation and extinction memory have shown to be mutually exclusive (Eisenberg et al. 2003; Pedreira et al. 2004; Pedreira and Maldonado 2003; Sangha et al. 2003a, b; Suzuki et al. 2004). This necessarily means that there is a key time point in the behavioral experience, where one process is triggered and the other is cast off. In our memory paradigm, this key point has shown to be the context-offset, since before this point CSM memory is always disclosed intact and consolidated (Pedreira et al. 2004) and, after it, extinguished (Pérez-Cuesta et al. 2007). Thus, we assume that it is the context-offset and not the mere retrieval which triggers the acquisition of extinction memory. Hence, we hypothesize that the entire period of context re-exposure is an expectation time, where the animal computes time up to the finalization of context re-exposure without triggering either memory process. Once the context-offset comes, extinction is triggered provided that the expectation time lasts >1 h and no VDS is presented. This model posits that one-trial extinction training may also cover the results of multi-trial extinction training shown here. We propose that each trial in the series of a multi-trial experiment should have all the essential features we described for the one-trial experiment. So, in a series of successive trials, the time

computation in each trial includes not only its own expectation time, but also those of prior trials. When the accumulation of expectation time surpasses a certain threshold, around 90 min, the context-offset triggers the acquisition of the extinction memory (Fig. 4).

Further, Pérez-Cuesta and Maldonado (2009) have shown that both memory labilization–reconsolidation and extinction are triggered after the end of context re-exposure and that reconsolidation and extinction processes are mutually exclusive when they are triggered by a single re-exposition. However, they can co-exist and develop in parallel when they are serially triggered by respective short and long context re-exposures.

Finally, in a novel insight into the molecular mechanisms involved in the switch between memory reconsolidation and extinction in crabs, Merlo and Romano (2008) have proposed the inhibition of the transcription factor NF- $\kappa$ B as the engaged mechanism underlying extinction. One of the outstanding findings of this work was to provide evidence about the role of the NF- $\kappa$ B in memory extinction, based on the lack of spontaneous recovery after the administration of sulfasalazine, a NF- $\kappa$ B inhibitor. The spontaneous reappearance of the original response normally occurs 2 or 3 days after the induction of extinction. In this case, however, the combination of a 2 h single trial and the administration of sulfasalazine enhanced the extinction memory. Moreover, it was necessary to apply a strong reinstatement protocol (10 trials in a different context) to induce the re-emergence of the first learning. The main difference with the experiment presented here is that the extinction memory was improved by pharmacological treatment.

It was said that this model for multi-trial extinction training hinges on the total expectation time accumulated through successive un-reinforced episodes presented with an inter-trial interval of 1 min. As we pointed out, this sum of time turns out to be the same as the time necessary to trigger the extinction memory with one trial. However, it is possible to assume that if we changed one of the variables used here (e.g. frequency, time of re-exposure and number of context-offsets), the algorithm could work in a different way. Therefore, we suggest that under these new conditions we would find a more intricate rule that would permit us to incorporate the effect of consecutive un-reinforced trials and, consequently, the time accumulated in this case may be less than an hour.

On the whole, the results of our recovery protocols and our hypothesis about the multi- versus one-trial extinction training are in agreement with those reports obtained with models phylogenetically far apart from the crab. Behind this attempt is the idea that in the domain of studies on memory, some principles of behavior organization and basic mechanisms have universal validity (Carew 2000; Pedreira and Maldonado 2003).

**Acknowledgments** This work was supported by FONCYT (Grant PICTR 00349) and by CONICET (Grant PIP2004/5466).

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