



## Extinction, spontaneous recovery and reinstatement in the garden snail, *Helix aspersa*



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Extinction and its related phenomena are central to the study and development of associative learning theory. For a better understanding of the processes involved in extinction, it is important to know how general these phenomena are in different species. Extensive evidence of extinction in invertebrate species would be necessary in order to test the generality of its current theoretical and physiological accounts. We carried out three sets of experiments using terrestrial snails *Helix aspersa*. The repeated nonreinforced presentation of the conditioned stimulus (CS) resulted in a decrease in responding (extinction); the introduction of a delay between the extinction of a CS and its test produced an increase in responding (spontaneous recovery); and re-exposure to the unconditioned stimulus after extinction also led to an increase in responding (reinstatement). The results are discussed in relation to current interference theories of extinction.

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Extinction processes are of great interest because of both their theoretical relevance and therapeutic use in human and nonhuman animals (e.g. Bouton, 1988). None the less, the mechanisms of extinction are not completely understood (e.g. Todd, Vurbic, & Bouton, 2014). In experimental psychology, most of the research on extinction has focused on two main aspects: the theoretical mechanisms involved in these processes (e.g. Bouton, 2004; Myers & Davis, 2002) and the neural and molecular substrates that may underlie extinction (e.g. Myers & Davis, 2002; Todd et al., 2014). However, there is another equally interesting aspect of extinction that does not receive as much attention as the two previously mentioned but provides a tool for better comprehension of extinction. The adaptive value of extinction and its evolution across the phylogenetic tree are relevant aspects of these processes that can help widen our understanding of extinction and the mechanisms involved in it. To achieve this goal, a comparative approach based on a systematic comparison between species' learning processes must be used (Prados et al., 2013).

The phenomenon of extinction takes place when a conditioned stimulus (CS), previously paired with an unconditioned stimulus (US), is presented in the absence of that US, producing a reduction in the conditioned response observed. Since its experimental description (Pavlov, 1927/2003), several accounts have tried to explain this decrease in responding to the CS. Currently, there are two main types of theory that try to account for extinction. One set of theories explains extinction based on changes in the initially acquired association (e.g. Rescorla & Wagner, 1972). The other set states that new learning takes place during extinction. Owing to this new learning, interference between inconsistent information occurs (see Bouton, 2004). Three experimental phenomena related to extinction show that certain manipulations can cause the recovery of the conditioned response that seemed to be lost, supporting the latter type of theory. When a long period of time elapses from extinction training to test, spontaneous recovery from extinction takes place (e.g. Pavlov, 1927/2003). Likewise, when extinction is tested in a different context to that in which it had been trained, an increase in responding or renewal effect is observed (e.g. Bouton & Bolles, 1979a; Bouton & King, 1983). Moreover, re-exposure to the US employed during conditioning after extinction produces a recovery in responding, the so-called reinstatement effect (e.g. Bouton & Bolles, 1979b; Rescorla & Heth, 1975). These three phenomena do not support the idea of

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extinction as a result of changes in a previously acquired association alone.

Here we tested whether extinction involves learning of new relationships between the CS and the US (Bouton, 1993, 2004). Bouton (1993) proposed a model based on this idea. He suggested that during the first phase of training in an extinction design, an excitatory association would be formed between the CS and the US, but that during the second phase, this association would be kept mostly intact, and a second inhibitory association would be created. However, the latter would become context dependent. This means that this association can be activated only to the extent to which the context in which it has been acquired or some of its features are present. This offers an explanation for the extinction-related phenomena. In the case of renewal (e.g. Bouton, 1988; Bouton & Bolles, 1979a), acquisition usually takes place in one context and extinction in another. According to Bouton's model, during the test trial, the excitatory association that was acquired in the first place will be engaged regardless of the context in which the test trial takes place. Thus, when the test context is the same as the extinction context, the inhibitory association acquired during the second phase will be activated. The sum of the activation of the associations would be low, which would lead to a small number of responses. On the other hand, if the test takes place in a different context, the second association would be activated to a lesser degree, leading to higher net activation and a higher number of responses. Equally, the passage of time (e.g. Bouton, 1993, 2004; Pavlov, 1927/2003) and the re-exposure to the US employed in conditioning (e.g. Bouton & Bolles, 1979b; see Bouton, 2004 for a review) has the same effect on the recovery of the response (spontaneous recovery and reinstatement, respectively). In fact, according to Bouton's model both passage of time and re-exposure to the US actually produce a context change similar to changes in the physical environment. Given that it can easily and parsimoniously explain extinction and its related phenomena, Bouton's model has become the most widespread explanation of extinction.

Besides the development of learning theories that try to account for these characteristics of extinction, the neural and molecular bases of these phenomena are receiving increasing attention (e.g. Corcoran, Desmond, Frey, & Maren, 2005; Lissek, Glaubitz, Uengoer, & Tegenhoff, 2013; see Milad & Quirk, 2012 for a review), and evidence of these phenomena in invertebrate species could offer more research possibilities to this aim, from the study of protein signalling pathways involved in extinction (e.g. Isiegas, Park, Kandel, Abel, & Lattal, 2006; Sossin & Abrams, 2009) to the neurotransmitters implicated in this learning phenomenon (e.g. Chhatwal, Davis, Maguschak, & Ressler, 2004; McPartland, Agraval, Gleeson, Heasman, & Glass, 2006). None the less, most of the studies mentioned above, from either behavioural or mechanistic approaches, have focused on a very small number of species, and almost exclusively in mammals. However, for better comprehension of the mechanisms involved in extinction, the study of such phenomena across different species is necessary, since, by means of a comparative study, the generality of the theoretical and physiological mechanisms of extinction can be tested. The study of learning phenomena in invertebrate species may thus shed some light on this issue. On the one hand, a cross-species study of these phenomena would permit researchers to test whether the theoretical mechanisms involved are constant along the phylogenetic tree, as well as to test whether the molecular bases that underlie these phenomena are at all similar or whether they differ from one species to another. On the other hand, a comparative study would offer a wider understanding of the origin and evolution of the extinction mechanisms and its adaptive value.

Extinction has been documented in several invertebrate species. In some of them only extinction has been reported. In *Aplysia*

*californica* an aversive procedure in which a tactile stimulus is paired with a shock (Carew, Walters, & Kandel, 1981; Colwill, Absher, & Roberts, 1988) and an appetitive discriminative conditioning procedure have been used to study extinction (Colwill, Goodrum, & Martin, 1997). In *Paramecium caudatum* extinction has been tested in aversive Pavlovian procedures in which vibrations (Hennessey, Rucker, & McDiarmid, 1979) and light or dark areas (Armus, Montgomery, & Gurney, 2006) have been paired with shocks during the conditioning phase. Aversive procedures have also been used to study extinction in planarians. Baxter and Kimmel (1963) reported extinction in *Dugesia dorotocephala* with an aversive procedure in which a light was paired with a shock. In *Caenorhabditis elegans* one study showed extinction of olfactory aversive learning (Amano & Maruyama, 2011) and, with an appetitive procedure Gomez et al. (2001) assessed the genetic and molecular bases of extinction.

As far as we know, there are only five invertebrate species in which additional phenomena apart from just extinction have been studied. In honeybees, *Apis mellifera*, an appetitive conditioning procedure of the proboscis extension was employed to study extinction (Takeda, 1961), spontaneous recovery (Bitterman, Menzel, Fietz, & Schäfer, 1983; Sandoz & Pham-Delègue, 2004; Stollhoff, Menzel, & Eisenhardt, 2005; Takeda, 1961) and reinstatement (Plath, Felsberg, & Eisenhardt, 2012). In the fruit fly *Drosophila melanogaster* extinction (Lagasse, Devaud, & Mery, 2009; Qin & Dubnau, 2010; Schwaerzel, Heisenberg, & Zara, 2002; Tully & Quinn, 1985) has been reported by means of a differential aversive conditioning paradigm in which an odour was paired with either an electric or a mechanical shock (CS+) and another odour was presented on its own (CS-). Extinction and spontaneous recovery of the habituation of the jump-and-fly response to a visual stimulus has also been studied in this species (Engel & Wu, 1996). There are also studies in crustaceans. For instance, Nathaniel, Panksepp, and Huber (2009) used a conditioned place preference (CPP) procedure in the crayfish *Orconectes rusticus* in which morphine was paired with a distinct visual and tactile environment. This CPP was then extinguished by pairing the context with saline and reinstated when subjects re-experienced morphine after extinction. In the crab *Chasmagnathus granulatus* habituation of the escape response was studied in a contextual fear conditioning task. Results showed extinction of the habituation response (e.g. Tomsic, Pedreira, Romano, Hermitte, & Maldonado, 1998), along with spontaneous recovery and reinstatement (Hepp, Pérez-Cuesta, Maldonado, & Pedreira, 2010; Merlo & Romano, 2008). These studies, taken together, provide evidence for a context-specificity explanation of extinction in the invertebrate species investigated. Experiments that showed renewal and reinstatement of the conditioning response especially support this contextual account of extinction.

In molluscs, besides those on *Aplysia*, no studies on extinction processes have been conducted with a Pavlovian appetitive procedure. Notwithstanding, operant conditioning procedures have been used to examine extinction, spontaneous recovery (e.g. Rosenegger & Lukowiak, 2013; Sangha, McComb, Scheibenstock, Johannes, & Lukowiak, 2002; Sangha, Scheibenstock, Morrow, & Lukowiak, 2003), renewal (McComb et al., 2002) and reinstatement (Carter, Lukowiak, Schenk, & Sorg, 2006) in the species *Lymnaea stagnalis*. However, as shown by Brembs and Plendl (2008), operant and classical conditioning, and their biological mechanisms, can be dissociated from each other in invertebrates. In their study, a double dissociation between both types of learning was described in the fruit fly by a genetic manipulation. When flies lacked *rut-AC* (i.e. a gene that affects a type I adenylyl cyclase regulated by Ca<sup>2+</sup>/Calmodulin and G protein) they did not show classical conditioning, but had no problems in solving an operant conditioning task. On the other hand, when the *PKC* gene had been

manipulated, flies showed classical conditioning but not operant learning. Thus, it seems that describing one type of learning in one species, either operant or classical, does not necessarily imply that the other would also be observed (Brembs & Plendl, 2008). Therefore, although operant aversive extinction has been found in *L. stagnalis*, whether Pavlovian extinction would be observed in snails remains unknown.

The general procedure that we used in the current study, developed from the experimental work done by Teyke (1995) and Ungless (1998, 2001), has already proven to be a good index of learning, as shown in previous studies on different learning phenomena such as acquisition, latent inhibition, overshadowing, sensory preconditioning, second-order conditioning (Loy, Fernández, & Acebes, 2006), blocking (Acebes, Solar, Carnero, & Loy, 2009) and conditioned inhibition (Acebes, Solar, Moris, & Loy, 2012). We carried out three sets of experiments testing extinction, spontaneous recovery and reinstatement on *Helix aspersa*, using an appetitive conditioning procedure. In the first set of experiments a stimulus was conditioned and then subjected to extinction, testing the conditioned response right after extinction (experiment 1A) or with 1 day of delay between extinction and its test (experiment 1B). The course of extinction was also described providing a measure of the trial by trial change in conditioned responding (experiment 1C). In experiment 2 we assessed spontaneous recovery by means of a within-subject design in which two stimuli were conditioned but extinguished at different points in time from conditioning. Finally, in experiments 3A and 3B we analysed the phenomenon of reinstatement using different control conditions.

## GENERAL METHODS

### *Apparatus and Stimuli*

The experimental setting used for all the experiments was a plastic perforated surface (390 mm × 360 mm; 5.5 mm diameter holes) placed 65 mm above the surface of a table, allowing a dish with pieces of fruit to be placed beneath the perforated surface. The experimental room was maintained at 22 °C and illuminated with a red light (60 W). The conditioned stimuli were the odours from the fruits placed underneath the perforated surface, and the unconditioned stimulus was a piece of food to which the animal was given access during the conditioning trials, in the presence of the odours.

### *Procedure*

Before the start of an experimental session, animals were placed on their sides and sprayed with fresh tap water in order to induce activity. Once the snails had emerged from their shells, they were placed onto the perforated surface and the experimental trial started. In all trials, the distance between the odour cue and the perforated surface was 65 mm. On conditioning trials a dish containing a few slices of fruit was placed below the perforated surface and a piece of carrot was placed in front of the snail. Animals were allowed to eat for a period of 120 s (the 2 min period began 5 s after the presentation of the food; by then the snails had usually started to eat). After this time animals were removed from the experimental setting and put back in their cages. This treatment was carried out six times with an intertrial interval (ITI) of 1 h. During extinction trials the same procedure was used, except that the CS was presented in the absence of the US for 120 s. As in conditioning, the treatment was carried out six times with an ITI of 1 h. In conditioning and extinction trials, all the snails belonging to the same experimental group were run

simultaneously in the same experimental setting unless otherwise stated. Note that only training trials were run with more than one snail in the experimental setting. Test trials were always run sequentially with only one snail each time as described below. Thus, data were always recorded from one isolated snail each time.

Test trials consisted of 120 s of presentation of the CS in the absence of the US. Following Ungless's procedure (see Figure 2 in Ungless, 2001, p. 99), each movement of the tentacle below an imaginary line over the top of the head was scored as one response throughout the 2 min session. The snails were randomly selected and tested individually in random order, and the observer was unaware of the group to which the subject belonged. To simplify the test procedure, only movement of the snail's left posterior tentacle was recorded, as described by Ungless (1998, 2001). Snails' tentacles are a multifunctional organ with visual, tactile and olfactory sensory capabilities. Concerning its olfactory function, tentacle lowering has been shown to be involved in food finding (e.g. Chase & Croll, 1981; but see Ungless, 2001). Although the role of olfaction in feeding behaviour is not completely clear (Ungless, 2001), it has been observed that naïve snails do not lower their tentacles in the presence of a food odour source with which they had not had experience. However, tentacle lowering is elicited by the odour-alone source after eating a given food (Peschel, Straub, & Teyke, 1996; Ungless, 1998).

### *Housing*

Subjects were snails taken from the wild. They were individually housed in plastic cages (50 mm × 50 mm and 100 mm high), which had air holes and contained a small amount of water. The boxes were placed in a dark room in which temperature was held constant at 22 °C. In the experiments reported, snails were deprived of food for 10 days prior to the start of the experiment. This is a short period of deprivation for this species since snails typically hibernate for up to 3 months during winter (or in summer when facing drought periods or high temperatures). During hibernation they seal themselves within the shell and live off of the stored fat. We used 10 days of deprivation to match the experimental procedure used in comparable experiments (e.g. Loy et al., 2006; Ungless, 1998). After the experiments, snails were given food ad libitum and placed back into the wild.

### *Analyses*

All the analyses were run using SPSS v21 (SPSS Inc., Chicago, IL, U.S.A.). The statistics of effect size reported were Cohen's  $d'$  (Cohen, 1992) and  $\eta_p^2$  (Cohen, 1973) for  $t$  tests and ANOVA respectively. Means are given  $\pm$ SEMs.

## EXPERIMENT 1A

The aim of this experiment was to assess whether extinction would occur in *H. aspersa*. Evidence of extinction would be provided if snails subjected to the extinction procedure showed a lower level of conditioned response to the CS than subjects in the control group.

### *Methods*

#### *Subjects*

Subjects were 16 snails with a shell diameter of 26 mm (range 24–29 mm) at the start of the experiment. They were divided into two groups: Experimental ( $N = 8$ ) and Control ( $N = 8$ ).

### Procedure

After 10 days of deprivation, snails of both Experimental and Control groups received six conditioning trials on the first day of the experiment. The next day, six extinction trials took place followed by a test trial for the experimental group. Snails in the Control group were awakened six times and put back into their home cages so as to equate experimental manipulations before the test trial. The test trial was conducted at the end of the second day.

### Results and Discussion

Two snails belonging to the Experimental group and one to the Control group could not be awakened at some point along training and thus were excluded from the experiment, resulting in a total of 13 subjects. As shown in Fig. 1a, animals that received nonreinforced presentations of the CS after conditioning showed a lower mean level of conditioned responding ( $2.33 \pm 0.73$ ) than animals that did not receive such training ( $4.71 \pm 0.67$ ). Statistical analysis indicated that the difference was statistically significant ( $t_{12} = 5.80$ ,  $P = 0.035$ ,  $d = 1.424$ ).

The results of the Experimental group are what would be expected after extinction training. Repeated presentations of an odour-CS alone that had been previously paired with food in snails led to a decrease in tentacle-lowering responding.

### EXPERIMENT 1B

A replica of experiment 1A was run, with one change in the procedure. One day was left between conditioning and extinction to test the level of conditioning that would be preserved. The purpose of this was twofold, first, to determine whether the results of experiment 1A could be substantiated, and second, to examine whether extinction would still occur if a delay was introduced between the acquisition and extinction phases. The goal of testing extinction with this procedure was to assess whether an experiment to test spontaneous recovery was also feasible, as long-term retention of conditioning is a prerequisite for this phenomenon to be observed. All procedural details were as in experiment 1A, except for the introduction of a rest day between the 2 days of conditioning. Thus, both groups received conditioning on Day 1. On Day 2 activity was induced in all animals and once they had emerged from their shells, they were replaced in the cages. On Day

3, subjects received the same treatment as described above: the Experimental group was subjected to extinction training whereas the Control group was only awakened. The test took place on Day 3 at the end of training.

### Subjects

Subjects were 20 snails with a shell diameter of 29 mm (range 28–32 mm) at the start of the experiment. They were divided into two groups: Experimental ( $N = 10$ ) and Control ( $N = 10$ ). One snail of each group could not be awakened at some point during the training and were removed from the experiment.

### Results

As depicted in Fig. 1b, snails that received nonreinforced presentations of the CS showed a lower mean number of conditioned responses ( $3.1 \pm 0.84$ ) than Control subjects ( $6.44 \pm 1.04$ ). A  $t$  test showed significant differences between the two groups ( $t_{17} = -2.53$ ,  $P = 0.022$ ,  $d = 1.159$ ) supporting the results obtained in the previous experiment.

### EXPERIMENT 1C

One characteristic of extinction is that it occurs gradually across training, and the conditioned response is reduced progressively as extinction trials occur. The aim of this experiment was to analyse the extinction process trial by trial so as to observe the development of the extinction curve across training.

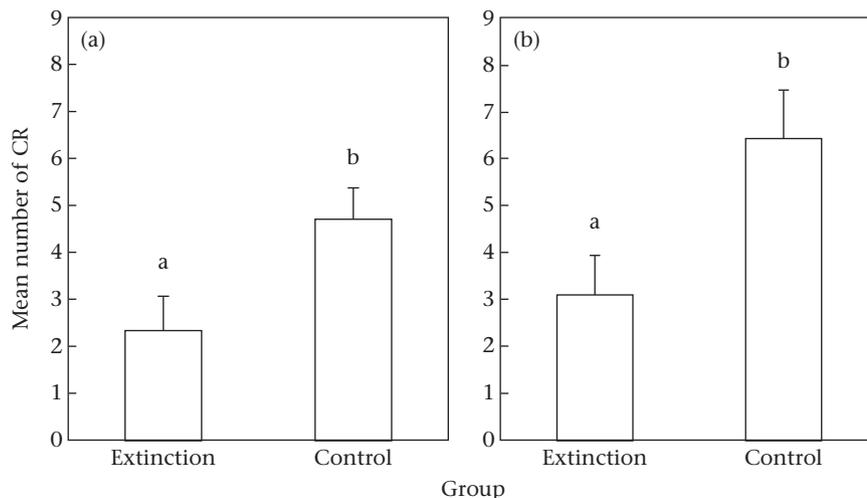
### Methods

#### Subjects

Subjects were nine snails with a mean shell diameter of 27 mm (range 22–30 mm) at the start of the experiment.

#### Procedure

After the 10-day deprivation period, all animals underwent six conditioning trials, as described above. On the second day of the experiment, six extinction trials took place. Each snail was tested individually in every trial in order to record the conditioned response across the six extinction trials. Since there was a single



**Figure 1.** (a) The mean number of tentacle-lowering responses (CR) made by Experimental (extinction) and Control (no extinction) groups in (a) experiment 1A and (b) experiment 1B. Vertical bars represent SEMs. Different lowercase letters above bars reflect significant differences ( $P < 0.005$ ) between groups.

condition in this experiment, the observer was not unaware of the treatment. However, subjects were tested in random order, the experimenter did not know what animal was being tested and was also unaware of the score of the previous trial.

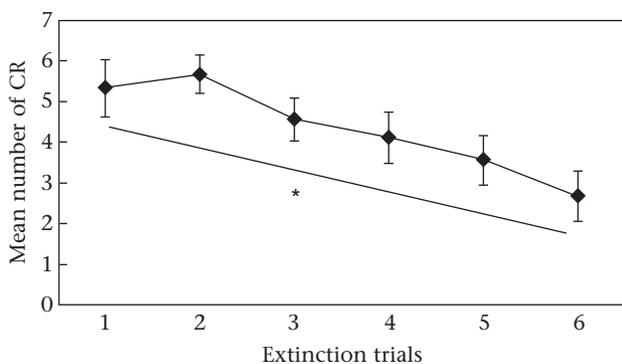
### Results and Discussion

The number of tentacle-lowering responses progressively decreased along the extinction trials (Fig. 2). A repeated measures ANOVA showed a statistically significant effect of extinction trial ( $F_{5,40} = 6.512, P < 0.001, \eta_p^2 = 0.45$ ). More specifically, there was a significant linear trend ( $F_{1,8} = 21.40, P = 0.002, \eta_p^2 = 0.73$ ), which indicated that with more extinction trials responding decreased progressively in a linear fashion. Results also indicate that six extinction trials are sufficient to find extinction in this species.

## EXPERIMENT 2

Spontaneous recovery refers to the re-emergence of the conditioned response to an extinguished CS that is observed with the passage of time when that CS is again tested following extinction training (Rescorla, 2004; Robbins, 1990; Sissons & Miller, 2009). After conditioning has led to an increase in responding, extinction would result in a reduction in the responding that had already been established. However, when time is allowed to elapse between the last extinction trial and the test, the presentation of the extinguished CS causes an increase in the conditioned response compared with a control situation in which the test trial takes place right after the last extinction trial.

Rescorla (2004) described how it is not possible to demonstrate spontaneous recovery completely as no experimental design could simultaneously rule out all possible confounds (e.g. elapse of time since original training, differences in the overall test context or age of the subjects owing to the passage of time). However, he showed how a within-subject design in which the two stimuli are conditioned and tested at the same time is the best way to test spontaneous recovery as it 'allows comparison of responding during the same test session, after the same number of extinction trials, to stimuli that share the time since original training but differed in the time since their extinction training' (Rescorla, 2004, p. 502). Therefore, a within-subject design in which two different stimuli are used seems to be the best option to control for most possible confounds. Despite its advantages, in invertebrates, none of the studies reviewed above used this experimental design to study spontaneous recovery. With this procedure, a lower level of responding to a first extinguished CS than to a recently



**Figure 2.** Mean number of tentacle-lowering responses (CR) along the extinction sessions (experiment 1C). Vertical bars represent SEMs. The line with the asterisk indicates a significant linear trend ( $P = 0.002$ ) across extinction trials.

extinguished CS would be consistent with a spontaneous recovery of the conditioned response.

### Methods

#### Subjects

Subjects were eight snails taken from the wild, with a mean shell diameter of 26 mm (range 22–29 mm) at the start of the experiment. The other parameters were kept as described above.

#### Procedure

An overview of the design is depicted in Table 1. Two different stimuli were paired with the presence of the US (access to carrot, + in Table 1) on 2 days. Extinction of CS<sub>1</sub> was carried out the day after conditioning and of CS<sub>2</sub> 3 days later. Two types of stimuli were used as CS<sub>1</sub> and CS<sub>2</sub>, apple odour and pear odour, with their role counterbalanced across subjects.

On Day 1 half of the animals were exposed to CS<sub>1</sub>+ on the first trial and to CS<sub>2</sub>+ on the second trial, and vice versa for the other half. This cycle of two trials was repeated three times on Day 1 and three times on Day 2, resulting in a total of six conditioning trials with each CS over the 2 days. On Day 3, six trials of extinction of CS<sub>1</sub> were carried out. On Days 4 and 5, animals were awakened and put back in their home cages after 5 min of activity. On Day 6 extinction of CS<sub>2</sub> was carried out. Finally, on Day 7, the level of tentacle-lowering response to both CS<sub>1</sub> (first extinguished CS) and CS<sub>2</sub> (second extinguished CS) was measured. The order in which the CSs were tested was fully counterbalanced and the ITI was 1 h.

### Results and Discussion

Figure 3 shows the mean number of tentacle-lowering responses in the presence of the CS extinguished immediately after conditioning (CS<sub>1</sub>) and the CS extinguished 3 days after conditioning (CS<sub>2</sub>). Snails responded more to CS<sub>1</sub> ( $4.88 \pm 0.22$ ) than to CS<sub>2</sub> ( $3.63 \pm 0.41$ ). A related-sample *t* test confirmed this description of the data, showing significant differences between treatments ( $t_7 = 3.04, P = 0.017, d = 1.154$ ).

When the test took place soon after extinction training, subjects showed lower levels of conditioned response. However, when tested 3 days after extinction training, more of the tentacle-lowering response was found. These results showed clear evidence of spontaneous recovery in *H. aspersa*.

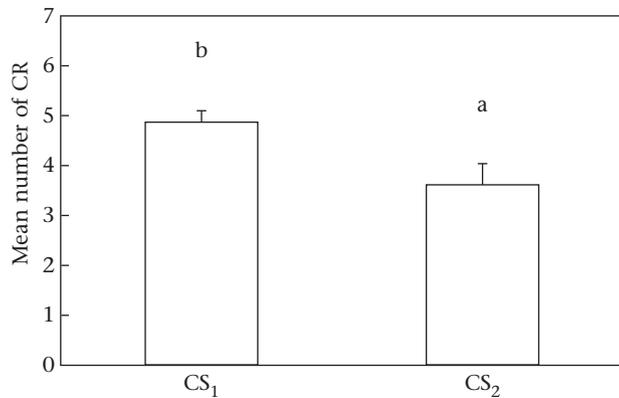
## EXPERIMENT 3A

Reinstatement is defined as the increase in the conditioned response to a CS that has been extinguished when the subject is re-exposed to the US that had been used during conditioning (Bouton, 2004). Re-experiencing the US that had been paired with a CS, after that CS has undergone extinction training, leads to higher levels of conditioned responding compared with the responding rate of those subjects that experienced a novel US after the same learning experience. Although reinstatement and its contextual dependency has been shown in invertebrate species such as the honeybee (Plath et al., 2012), a crab and a crayfish (Hepp et al., 2010; Merlo &

**Table 1**  
Experimental design of spontaneous recovery

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7 (Test)
3 CS <sub>1</sub> +	3 CS <sub>1</sub> +	CS <sub>1</sub> –	Awake	Awake	CS <sub>2</sub> –	CS <sub>1</sub>
3 CS <sub>2</sub> +	3 CS <sub>2</sub> +					CS <sub>2</sub>

The CSs employed were apple and pear odours. + indicates the presence of US and – indicates the absence of US.



**Figure 3.** Mean number of tentacle-lowering responses (CR) in experiment 2 to the CS extinguished immediately after conditioning (CS<sub>1</sub>) and to the delayed extinguished CS (CS<sub>2</sub>). Vertical bars represent SEMs. Different lowercase letters above bars reflect significant differences ( $P = 0.017$ ) between conditions.

Romano, 2008; Nathaniel, Panksepp, & Huber, 2009), none of these studies has tested the US specificity of reinstatement. In this experiment, we used a different US of the same modality and motivational properties as the original US (e.g. a different type of food).

Experiment 3A tested whether snails would show reinstatement and, if that were the case, whether this reinstatement effect would be specific to the US employed during conditioning. If subjects re-exposed to the conditioning US after extinction showed significantly more responding to the CS than those subjects exposed to a different US to that employed during conditioning, reinstatement in snails would be observed.

## Methods

### Subjects

Subjects were 24 snails taken from the wild, with a mean shell diameter of 23 mm (range 20–27 mm) at the start of the experiment. The other parameters were kept as described above. Subjects were divided into two groups: Reinstatement ( $N = 12$ ) and Control US ( $N = 12$ ).

### Procedure

Table 2 illustrates the trials in which the two groups of subjects participated across the experiment. Unlike previous experiments, two USs were employed, US<sub>1</sub> and US<sub>2</sub> (carrot and lettuce, counterbalanced across subjects). For both Reinstatement and Control US groups, apple odour worked as the CS and was paired with US<sub>1</sub> (referred to as + in Table 2), during conditioning training. Then the same CS was subjected to six extinction trials on the next day. To guarantee that US<sub>2</sub> had been experienced enough to prevent any neophobic response, it was presented on its own between conditioning and extinction trials.

**Table 2**

Experimental design corresponding to the first experiment on the phenomenon of reinstatement (experiment 3A)

Group	Day 1	Day 2		
Reinstatement	6 CS+/6 US <sub>2</sub>	6 CS–/6 US <sub>2</sub>	+	Test
Control US	6 CS+/6 US <sub>2</sub>	6 CS–/6 US <sub>2</sub>	US <sub>2</sub>	Test

Two USs were employed in the experiment, carrot and lettuce. They were counterbalanced across subjects. One of them was used in the conditioning trials (represented as + in the table), while the other was not (indicated as US<sub>2</sub>). The trials in which neither US was presented have the sign –.

Before the test trial, subjects of group Reinstatement were exposed to US<sub>1</sub> for 2 min, which was expected to reinstate responding to the CS, whereas subjects of the Control US were exposed to US<sub>2</sub>, which was not expected to increase responding to the CS as it had never been paired with the CS. The ITI was 1 h.

## Results and Discussion

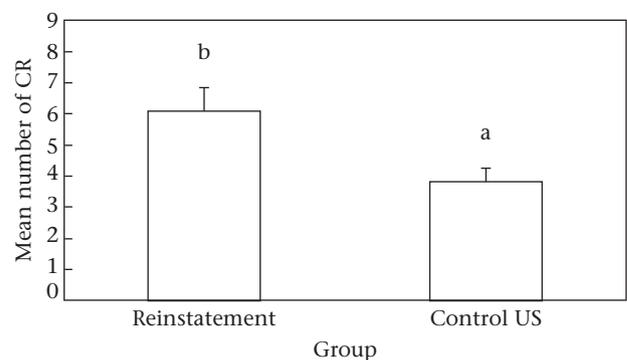
One subject in the Reinstatement group was excluded as it was not possible to awaken it during training. Thus, the Reinstatement group ended up with 11 snails whereas the Control US group remained intact. As depicted in Fig. 4, animals that were re-exposed to the US showed more responding to the CS ( $6.09 \pm 0.75$ ) than subjects that were presented with the control US ( $3.83 \pm 0.42$ ). A  $t$  test revealed this difference as significant ( $t_{21} = 2.66$ ,  $P = 0.015$ ,  $d = 1.139$ ).

These results provide evidence of reinstatement in snails. Nevertheless, there could be an alternative explanation based on a different effect. Possibly, the differences in experience with both USs were affecting the results. US<sub>2</sub> was presented during both conditioning and extinction and thus the experience with the USs was uneven. Before reinstatement, animals had more experience with US<sub>2</sub> (12 presentations) than with the US employed during conditioning (US<sub>1</sub>), which was presented six times. The greater experience might have led to higher habituation to the US changing somehow the behaviour of the snails in the last test. Although previous experiments in our laboratory on conditioning in *H. aspersa* (Loy et al., 2006; experiment 1A, Fig. 1b) do not reflect this habituation effect, a demonstration of the effect in which the experience with USs was equated would provide more straightforward evidence of reinstatement. Thus, a second experiment that ruled out any effect of habituation was conducted.

## EXPERIMENT 3B

In this experiment, experience with both USs was equated. Snails of both groups received six presentations of US<sub>1</sub> and six presentations of US<sub>2</sub>. To prevent any potential relation that could be learned between this second US and either the conditioning or extinction context, US<sub>2</sub> was presented on a different day: after conditioning and prior to extinction training. This treatment was given to all groups, including the two extra control groups added in this experiment.

The third group used in this experiment, Control noUS, was not exposed to any US during the reinstatement trial. To equate



**Figure 4.** Mean number of conditioned responses (CR) during the test of experiment 3A of the Reinstatement group, re-exposed to the US used during conditioning after extinction, and of the Control US group, exposed to a different US. Vertical bars represent SEMs. Different lowercase letters above bars reflect significant differences ( $P < 0.005$ ) between groups.

experiences and manipulations, it was exposed to the experimental context alone. A fourth group was included to test how much reinstatement occurred and to measure the magnitude of the effect. Subjects in this No Extinction group were not subjected to extinction training. Instead, activity was induced, as for the other groups, but they were placed back into their home cages. Like snails belonging to the Reinstatement group, they had access to the US used during conditioning in the reinstatement trial.

## Methods

### Subjects

Subjects were 56 snails taken from the wild, with a mean shell diameter of 23 mm (range 20–27 mm) at the start of the experiment. The rest of the parameters were as described above. Subjects were divided into four groups of 14 subjects each: Reinstatement, Control US, Control noUS and No Extinction.

### Procedure

The experimental design is depicted in Table 3. Apple odour was used as the CS and paired with access to a US (US<sub>1</sub>) during six conditioning trials on Day 1. On Day 2, US<sub>2</sub> was presented six times (so as to equate experience with both USs). Extinction of the CS took place on the third day for all groups but not for the No Extinction group, which was only awakened. Reinstatement and test trials followed extinction trials. For the Reinstatement and No Extinction groups, the reinstatement trial consisted of a 2 min trial of access to US<sub>1</sub> (referred to as + in Table 3) in the experimental setting. For the Control US group, US<sub>2</sub> was employed instead. The Control noUS group was placed in the conditioning context for 2 min without any US being present. As in experiment 3A, carrot and lettuce were counterbalanced as USs.

### Results and Discussion

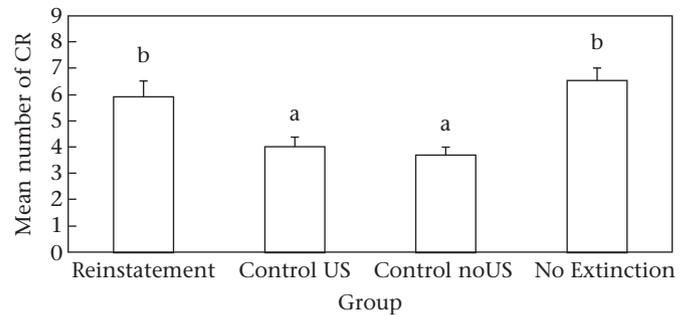
Two subjects of the Reinstatement group, one of the Control noUS and another of the No Extinction groups were excluded as activity could not be induced during training. Thus, this experiment ended with 52 subjects: Reinstatement ( $N = 12$ ); Control US ( $N = 14$ ); Control noUS ( $N = 13$ ); and No Extinction ( $N = 13$ ). As can be seen in Fig. 5, animals that, in the reinstatement phase, were re-exposed to the US used during conditioning showed more responding to the CS than animals that had been exposed to another US or to the context, and similar levels to those that had not received extinction training. The Reinstatement group showed a higher level of conditioned responding to the CS ( $5.91 \pm 0.60$ ) than the Control US ( $4.0 \pm 0.37$ ) and Control noUS ( $3.69 \pm 0.32$ ) groups, and a similar level of responding to the No Extinction group ( $6.53 \pm 0.48$ ). An ANOVA showed significant group differences

**Table 3**

Experimental design corresponding to the second experiment on the phenomenon of reinstatement (experiment 3B)

Group	Day 1	Day 2	Day 3		
			Extinction	Reinstatement trial	Test
Reinstatement	6 CS+	US <sub>2</sub>	6 CS–	+	Test
Control US	6 CS+	US <sub>2</sub>	6 CS–	US <sub>2</sub>	Test
Control noUS	6 CS+	US <sub>2</sub>	6 CS–	Context	Test
No Extinction	6 CS+	US <sub>2</sub>	6 Awake	+	Test

Two USs were employed in the experiment, carrot and lettuce. They were counterbalanced across subjects. One of them was used in the conditioning trials (represented as + in the table), while the other one was not (indicated as US<sub>2</sub>). The trials in which neither US was presented have the sign –. In trials marked as Awake, snails were only handled and awakened, without any stimulus presentation nor placement in the training context.



**Figure 5.** Mean number of conditioned responses (CR) of the Reinstatement group, re-exposed to the US used during conditioning after extinction, the Control US group, exposed to a different US, the Control noUS group, which received no US, and the No Extinction group, which did not receive extinction training during experiment 3B. Vertical bars represent SEMs. Different lowercase letters above bars reflect significant differences (all  $P$ s < 0.024) between groups.

( $F_{3,48} = 9.567$ ,  $P < 0.001$ ,  $\eta_p^2 = 0.374$ ). A Tukey post hoc analysis revealed significant between-group differences between Reinstatement and Control US groups ( $P = 0.023$ ), between Reinstatement and Control noUS ( $P = 0.007$ ), but not between Reinstatement and No Extinction groups. Differences were also found between No Extinction and Control US ( $P = 0.001$ ), and No Extinction and Control noUS groups ( $P < 0.001$ ). No differences were found between Control US and Control noUS groups.

These results show clear evidence of reinstatement. When the US used during conditioning was re-experienced after extinction, recovery of the conditioned response was observed. By contrast, experiencing a different US or the experimental context in which the experiment had been conducted did not lead to a recovery of the CR.

## GENERAL DISCUSSION

The present investigation aimed to assess extinction processes in the garden snail by means of an appetitive procedure. Little research on extinction has been carried out in invertebrate species and this lack of research impairs the development of a comparative approach to extinction processes. In snails, extinction had been studied only with an aversive operant conditioning procedure by Lukowiak and his collaborators, whereas appetitive Pavlovian conditioning had not been used before for the study of extinction learning. As described in Brembs and Plendl (2008), both types of learning can be dissociated and results obtained with one procedure may not be found in the other. Consequently, extinction of Pavlovian learning remained unexplored in this species. This work is the first demonstration of extinction in the species *H. aspersa* with an appetitive classical conditioning paradigm, and adds another instance of invertebrate extinction in the hope of a comparative approach.

The results showed that snails were subjected to extinction (experiments 1A and 1B) when the odour used as a CS was repeatedly presented in the absence of reinforcement. This treatment led to a decrease in the conditioned response compared with a control group that did not receive such training. When the conditioned response to the nonreinforced CS was individually recorded in every extinction trial, a typical extinction curve was observed (experiment 1C), endorsing the results found in experiments 1A and 1B. In experiment 2 spontaneous recovery of the conditioned response was observed: responses to a recently extinguished stimulus were lower than to a CS extinguished further in the past. The third set of experiments (3A and 3B) showed that

re-exposure to the unconditioned stimulus used during conditioning after extinction training reinstated responding to the CS.

Taken together, these experiments give support to the notion that also in *H. aspersa* extinction does not just involve changes in the previously acquired associations, since responding to the CS can be recovered with time after extinction and with re-exposure to the US employed during conditioning. The phenomena of spontaneous recovery and reinstatement in snails could be underlain by the same processes that are used to explain similar results in other species, such as context dependency or CS–no US memory retrieval by the context (Bouton, 2004; Myers & Davis, 2002). After extinction training, there would be two different associations and performance will depend on the association that is retrieved by the context. Consistent with the results shown in snails, temporal context could set the occasion for which of the two possible meanings of the CS acquired during both conditioning and extinction training is retrieved (Bouton, 2004). Another implication of these results is that if the same psychological processes affect extinction, spontaneous recovery and reinstatement in snails, interference phenomena could then be studied in invertebrate species. The study of these phenomena in snails and other invertebrate species may help broaden the understanding of extinction from a comparative approach, from which the development of theoretical models and therapeutic treatments could also benefit. These results, along with those reported in other invertebrate species, suggest that even though the nervous systems of all these species and mammals are different, the same extinction phenomena can be described by means of similar paradigms. As Plath et al. (2012, p. 547) stated, ‘although the ability for extinction learning seems to be evolutionary conserved in both vertebrates and invertebrates, their respective brain architectures are fundamentally different. This suggests that the ability for extinction learning is not rooted in the specific brain architecture [...]’. This leaves open the question of the evolution of these mechanisms from a neural point of view, but also from behavioural and ecological perspectives.

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