4 Chapter III

The influence of reward duration on consolidation of extinction memory

4.1 Introduction

In classical conditioning an animal learns that a previous neutral stimulus (conditioned stimulus, CS) acts as a predictor for the appearance of a biologically significant stimulus (unconditioned stimulus, US). After an association has formed animals display the conditioned response (CR) in anticipation of the US. In studies on aversive learning paradigms in crabs (Pedreira & Maldonado, 2003), snails (Sangha et al., 2003b; Sangha et al., 2003), fishes (Eisenberg & Dudai, 2004) and rats (Eisenberg et al., 2003; Suzuki et al., 2004), it has been shown that retrieval of a learned association by the presentation of the CS without reinforcement (CS-only) can lead to two opposing phenomena. Few or weak CS-only trials lead to a protein synthesis-dependent reconsolidation process, while a strong or many CS-only trials lead to the induction of a protein synthesis-dependent extinction process. This led to the hypothesis of trace dominance (Eisenberg et al., 2003). A retrieval trial involves at least two associations: the excitatory CS-US association, and a new inhibitory CS-noUS association. Both associations compete for the control of behavior. The dominant trace drives the animal's behavioral response and becomes sensitive to consolidation blocker (Eisenberg et al., 2003). This means that whenever retrieval does not induce significant extinction then reconsolidation of the initial excitatory CS-US association becomes the dominant protein synthesis-dependent process. In contrast if retrieval does induces significant extinction consolidation of inhibitory CS-noUS association becomes the dominant protein synthesis-dependent process (Eisenberg et al., 2003; Nader, 2003).

This negative correlation could not be demonstrated in PER conditioning (Chapter II). In bees one CS-only trial does not induce significant extinction, but neither an induction of a reconsolidation process nor consolidation of an extinction memory could be

observed as predict by the trace dominance theory. With the application of two CS-only trials the CS-noUs association becomes the dominant trace and therefore sensitive to the consolidation blocker. Five successive retrieval trials lead to spontaneous recovery and, therefore, to reconsolidation. These results pose the questions whether the same type of relationship between retrieved memory stability and trace dominance underlies both aversive and appetitive learning. Our work was aimed to further analyze the trace dominance hypothesis in the context of appetitive learning.

It has been shown, that a difference in the associative strength of the CS-US pairings can be the reason for the induction of different consolidation processes after memory retrieval. In a conditioned taste aversion paradigm retrieval of one-trial memory leads to consolidation of an extinction memory. Whereas two learning trials result in an acquisition memory, that is more resistant against extinction. Accordingly, retrieval of the two-trial memory results in its reconsolidation (Eisenberg et al., 2003). In this experiment the strength of retrieval was not changed, but the strength of the association had changed according to the Rescorla-Wagner model (Rescorla & Wagner, 1972): Rescorla and Wagner developed a trial based model to describe the growth of associative strength. The model is based on following assumptions. There is a maximum associative strength that can develop between CS and US. The US determines the limits of associative strength, which is equivalent with an asymptote level of the CR. The strength increases with each training trial and depends on the prior training. Hence every training trial results in an increase of the associative strength, as seen by the results of Eisenberg et al.

We hypothesized therefore that the differences in induction of a reconsolidation after one retrieval trial were due to differences in the strength of the CS-US association in the appetitive paradigm of the honeybee and the above mentioned aversive paradigms. This hypothesis is tested in the study presented here

To do so I attempted to manipulate the strength of the CS-US association It has been shown in a run alley paradigm that large amounts of reward results in a better performance during acquisition than a small amount of reward (Wagner, 1961). The amount of reward influences also the time the US is presented, since a larger reward takes longer to consume. Furthermore, different durations of US presentation influence the resistant to extinction in a color choice experiment with freely flying bees. Resistance is weak after a short reward (2 s) and strong after a long reward (15 s)

(Menzel, 1968). Accordingly it can be supposed, that the length of the US presentation has an impact on the strength of the CS-US association. Therefore I decided to change the length of the US presentation during conditioning to manipulate the associative strengths. The assumption was that a short presentation of the US during training (short conditioning protocol) results in a weaker associative strengths than a long US presentation (long conditioning protocol). The strength of the association should in turn influence the induction of the retrieval induced processes (e.g. consolidation of extinction memory or reconsolidation of the acquisition memory).

In the former conditioning protocol (Chapter II), one CS-only trial applied 24 h after conditioning does neither induce reconsolidation of the acquisition memory nor consolidation of the extinction memory. With a short conditioning protocol the acquisition memory should be less resistant to extinction and therefore consolidation of an extinction memory should be induced with one CS-only trial. An increased length of US presentation during acquisition should result in a stronger associative strength. Retrieving this memory by one CS-only trial should result in reconsolidation of the acquisition memory.

Two CS-only trials result in consolidation of an extinction memory with the former used conditioning protocol (Chapter II). Therefore, with a short US presentation two CS-only trials should also lead to consolidation of extinction memory. If bees were trained with a long US presentation, two CS-only trials could induce reconsolidation of the acquisition memory. It is also conceivable that both processes are equally strong induced. In this case neither consolidation of extinction memory nor reconsolidation of acquisition memory should be induced, similar to the results with one CS-only trial in the previous study in Chapter II.

4.2 Methods

Behavioral procedure

The experiments were conducted in autumn 2004 and summer 2005 in Berlin, Germany. The protocol was the same as described in Chapter II.

In brief, foraging bees leaving the hive were caught in the afternoon one day before the experiment started. They were immobilized by cooling and then harnessed in small plastic tubes and were fed up with sucrose (1M) to satiation in the evening. During the

rest of the experiment animals were fed with four droplets of sucrose (~ 15 µl) in the evening. Between experimental manipulations the bees were placed in a dark and humid box at room temperature.

Conditioning procedure

Acquisition consist of three pairings of odor (CS) delivered through a 20-ml-syringe with sucrose 1,25M (US) with an intertrial-interval (ITI) of 10 min. An acquisition trial starts with a 10 s placement of the animal in front of an exhauster. Subsequently the odor (carnation oil) was presented for 5 s. After 2 s the antennae were touched with a sucrose-moistened toothpick, depending on the experimental group the bees were allowed to lick for 2 s (short group) or 10 s (long group). In Figure 4-1 the conditioning scheme is illustrated. For the animals in the short group the offset of the CS and the US was isochronic. For the long group the offset of the US was 7 s after the offset of the CS. In order to equalize the time spent at the set-up, animals of the short group were left for 7 s after the offset of CS and US in front of the exhauster.

A bee scored positive if it extends its proboscis between the onset of the CS and the presentation of the US.

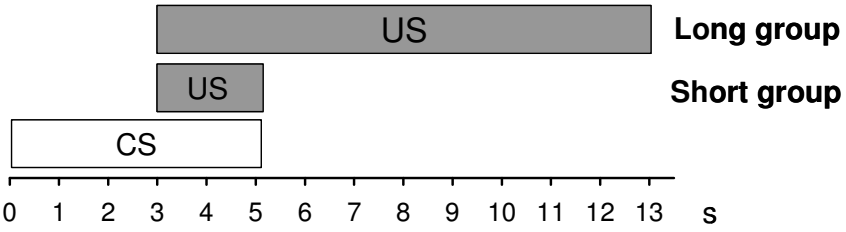

Figure 4-1: Conditioning scheme. Bees of both experimental groups receive a 5 s presentation of the odor (CS). After 2 s the antennae were touched with a sucrose moistened toothpick and subsequently the animal was allowed to lick for 2 s (short group) or 10 s (long group).

Retention test / Cs-only trials

CS-only trials were presented 24 h after acquisition and consisted of 5 s CS presentation without reinforcement. If more than one CS-trial was presented the ITI was 10 min. The final retention trial was performed 48h after acquisition in the same way as CS-only trials.

Injection

Emetine (Fluka Chemie, Switzerland; Cat No. 45160) was dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.2). One µl of emetine (10mM) or PBS was injected manually into the thorax using a calibrated glass capillary (capilettor tips and sticks, Selzer GmbH, Waghäusel, Germany; Cat No. 224804 and 224847, Chemie, Buchs Switzerland). Injections were applied 30 min before the first CS-Us pairing or the first CS-only trial.

Data analysis

Animals that survived the entire experiment showed the proboscis extension response (PER) elicited by sucrose at the end of the experiment and did not show a spontaneous extension of the proboscis to the odor (CS) at the first CS-US pairing were included in the analysis. For the statistical analysis differences were considered to be significant if $p < 0.05$.

Test of heterogeneity between groups

The experiments consist of three phases: acquisition, presentation of CS-only trials and retention test. The critical test is the retention test at the end of the experiments. To ensure that all groups within an experiment showed similar acquisition, the CRs at the second and third acquisition trial were tested for heterogeneity between groups (Zar, 1997).

Within group comparison

For the within group comparison between the CR at the last extinction trial and the CR at the retention test the McNemar-$\chi 2$ test was used.

Between group comparison

The differences of CR between differently treated groups were tested by the application of a G-test for contingency tables (=log likelihood ratio for contingency tables) (Zar, 1997)

4.3 Results

The length of the US presentation does not influence the performance during acquisition

First, we addressed whether or not US-length influences acquisition. To do so, harnessed honeybees were trained with three pairings of an odor (CS) with sucrose (US) with an inter-trial interval (ITI) of 10 min. The length of the sucrose stimulation was varied. One group received a 2 s presentation (short group) of the US on the proboscis, the other one a 10 s presentation of the US (long group). The CRs rose from 56% in the second trial to 76% at the third CS-US pairing in the short group (N = 106). There is no significant difference in comparison to the long group (N = 97), where the performance level increased from 59% to 77% (data not shown, G-Test: $G_{A2} = 0.10$, N.S.; $G_{A3} = 0.50$, N.S., df = 1). Hence, in appetitive olfactory conditioning with restrained animals the length of the US does not influence the performance during acquisition.

Acquisition memory is not susceptible to protein synthesis inhibitor 24 h after training

As shown in previous experiments (Chapter I)I, consolidation of the acquisition memory is not any longer susceptible to protein synthesis inhibitors 24 hours after conditioning. In the present study the conditioning protocol has been changed and it is therefore conceivable that the time course of consolidation has also altered. To test if memory is susceptible to protein synthesis inhibitors 24 hours after acquisition the following experiment was conducted. Bees were trained on day 1, either with a 2 s (S-group) or a 10 s US presentation (L-group). On the next day both groups were divided into two subgroups and received either an injection of 10 mM of the translation inhibitor emetine (S-Eme, L-Eme) or PBS (S-PBS, L-PBS). The animals were not subjected to a CS-only presentation. On day 3 retention of the memory was tested. CRs to the odor presentation of the animals during the experiment are shown in Figure 4-2. During acquisition the CRs increased from around 45 % at the second trial to nearly 80 % at the last trial. On the second trial less animals of the short groups reacted to the CS as compared to the CRs of the long group. But this difference is not significant and disappeared on the last acquisition trial (test for heterogeneity: $\chi^2_{A2} = 0.01$, N.S..; $\chi^2_{A3} = 0.02$, N.S., df = 1). During retention test on day 3 around 70 % of the bees in all groups showed a CR. There are no significant differences between all groups

(G_{Test_All} = 1.40, df = 3; N.S.). Therefore, independent of the length of US presentation during conditioning, the consolidation process is 24 hours after acquisition not longer susceptible to interference with protein synthesis inhibitor.

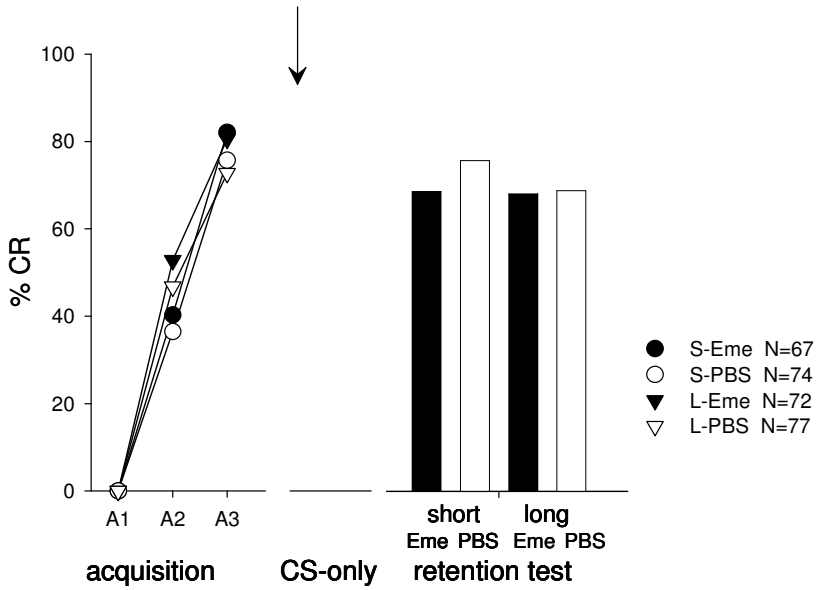

Figure 4-2 Acquisition memory is not susceptible to protein synthesis inhibitor 24 h after training. On day 1, animals were subjected to three CS–US pairings (A1–A3) with a short (2 s, circles) or long (10 s, triangles) US presentation (acquisition). After 24 h, on day 2, emetine (black symbols) or PBS (white symbols) was injected. On day 3, memory was tested (retention test). Presented are percentages of bees showing the CR to the CS at different phases of the experiment. Arrow, time of injection.

One CS-only trial applied 24 h after acquisition does not result in extinction nor reconsolidation

Independent of the conditioning protocol, one day after conditioning the acquisition memory is not susceptible to inhibitors of translation. With a 4 s presentation of the US no consolidation of extinction is initiated by one CS-only trial applied in a consolidated memory 24 hours after acquisition (Chapter II). Here the influence of the length of the US presentation on processes induced by one CS-only trial is analyzed. The experimental schedule was the same as in the previous experiment. Honeybees were trained on day 1 with a short US presentation of 2 s (S-group) or long US presentation of 10 s (L-group). On day 2 both groups were divided in two subgroups and subsequently were systemically injected with 1 µl emetine (10 mM) (Eme: S-Eme, L-Eme) or saline (PBS: S-PBS, L-PBS). 30 min afterwards all animals were subjected to one CS-only presentation. On the next day the memory retention was tested. Figure 4-3 shows performance of all groups throughout the experiment.

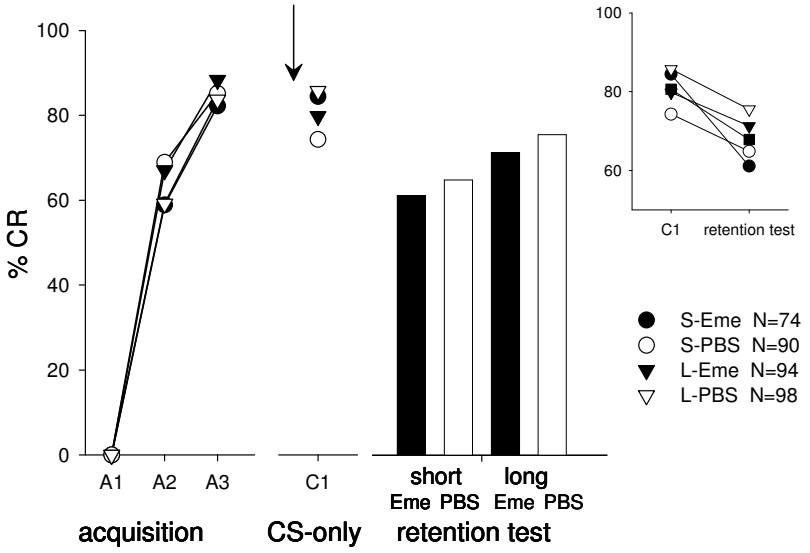

Figure 4-3 One CS-only trials does not induce extinction nor reconsolidation. On day 1, animals were subjected to three CS–US pairings (A1–A3) with a short (2 s, circles) or long (10 s, triangles) US presentation (acquisition). After 24 h, on day 2, emetine (black symbols) or PBS (white symbols) was injected 30 min before one CS-only trial (C1). On day 3, memory was tested (retention test). Presented are percentages of bees showing the CR to the CS at different phases of the experiment. Arrow, time of injection. Inlet: Only animals in the short and emetine-treated (S-Eme) group show a significant reduction of CR from retrieval (C1) to the retention test 24 h later.

During the course of acquisition the CRs of all groups increased homogenously from ~60% at the second trial to ~80% at the third trial (test for heterogeneity, $\chi^2_{A2} = 3.03$, N.S.; $\chi^2_{A3} = 1.03$, N.S., df = 1). At the CS-only presentation (C1) on day 2 around 80 % of the animal showed a CR to the odor. There are no significant differences in CRs between all groups. On the critical retention test on day 3 CRs of both short groups reached around 61%, whereas the long groups showed a response level of 73 %. A 4 x 2 G-test reveal this differences as non significant ($G_{Test\_All} = 5.32$, df = 3; N.S.). The CRs of all groups were reduced. By comparing CRs on the retrieval trial (C1) on day 2 with the CRs at the final retention test a McNemar-test signs them as non significant different for both long-groups (L-Eme, L-PBS) and for the PBS injected short-group (S-PBS) (McNemar: $\chi^2_{L\text{-}Eme} = 1.88$, N.S.; $\chi^2_{L\text{-}PBS} = 3.76$, N.S.; $\chi^2_{S\text{-}PBS} = 2.12$, N.S, df = 1). As illustrated in the inlet in Figure 4 - 3 the decrease of 23% in CR for the emetine treated short group (S-Eme) is significant (McNemar: $\chi^2_{S\text{-}Eme} = 13.79$, df = 1, $p < 0{,}001$).

Thus a long presentation of the US does not result in reconsolidation of the acquisition memory after one CS-only trial and a short presentation of the US during acquisition does not lead to extinction. This is in contradiction to the predictions stated above.

Two CS-only trials applied 24 h after acquisition result in extinction

In a previous study two CS-only trials applied in a consolidated memory result in the formation of a protein synthesis-dependent extinction memory (Chapter II). To investigate if induction of the extinction memory is dependent on the length of the US presentation during acquisition a second experiment was conducted. Honeybees were trained with a short or long conditioning protocol (S- and L-group) on day 1. The next day injections of emetine or PBS were applied (S-PBS, S-Eme, L-PBS or L-Eme) and 30 min afterwards all animals received two CS-only trials with a 10 min ITI. The retention tests took place on day 3.

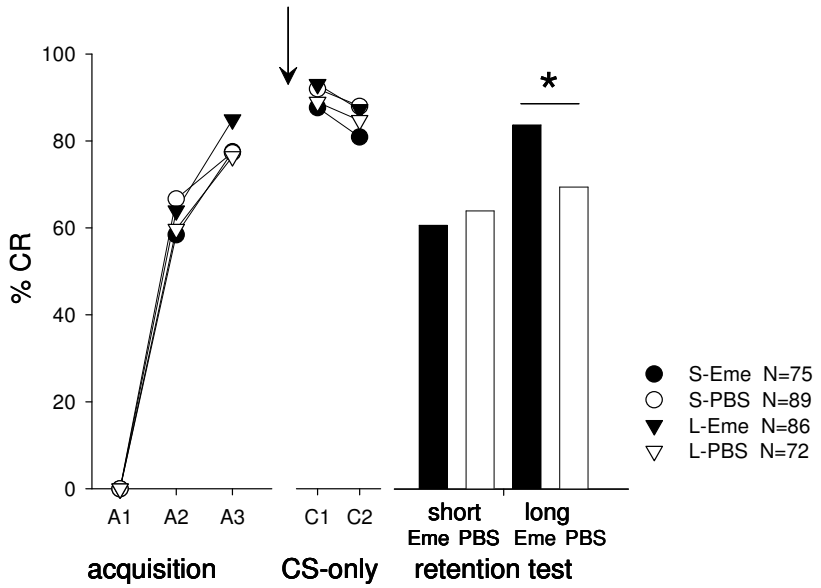

Figure 4-4 Two CS-only trials applied 24 h after acquisition result in extinction. On day 1, animals were subjected to three CS–US pairings (A1–A3) with a short (2 s, circles) or long (10 s, triangles) US presentation (acquisition). After 24 h, on day 2, emetine (black symbols) or PBS (white symbols) was injected 30 min before two CS-only trials (C1, C2). On day 3, memory was tested (retention test). Presented are percentages of bees showing the CR to the CS at different phases of the experiment. Asterisk indicates significant differences ($p < 0.05$); arrow, time of injection.

Figure 4-4 shows the conditioned response of all groups to the CS throughout the experiment. During conditioning on day 1, the response level increased from 60 % at the second trial to 80 % at the third one for all groups (test for heterogeneity, $\chi^2_{A2} = 1.32$ N.S.; $\chi^2_{A3} = 0.95$, N.S., df = 1). On day 2 animals were subjected to two CS only trials (C1, C2). The response level decreased for all groups around 5 %, from ~90 % to ~85 %. On the retention test 24 hours later both short-groups (S-PBS, S-Eme) and the L-PBS group show a reduced CR of 64, 60% and 69% respectively. In the

three groups a McNemar test yielded the reduction of CRs of ~ 17% from the last CS-only trial (C2) to the retention test 24 h later to be significant (McNemar test, $\chi^2_{S\text{-}PBS}$ = 2.04, $p < 0.01$; df = 1; $\chi^2_{S\text{-}Eme} = 9.03$, $p < 0.01$; $\chi^2_{L\text{-}PBS} = 5.26$, $p < 0.05$, df = 1). In contrast, the CRs on the retention test of the L-Eme group decreased only slightly to 83 %. This reduction of 5 % is not significant (McNemar test: $\chi^2_{L\text{-}Eme} = 0.36$, NS, df = 1). Furthermore, by comparing the CRs of all groups on the retention test a significant difference is revealed ($G_{all\ groups} = 13.39$, $p < 0.001$, df = 3). The difference in CR of 14 % between animals in the long groups (L-eme, L-PBS) is significant ($G_{L\_group} = 4.54$, $p < 0.05$, df = 1). Thus a long presentation of the US during conditioning does result in a consolidation of the extinction memory induced by two CS-only trials whereas this is not the case for a short US-presentation during conditioning.

4.4 Discussion

A correlation has been reported between the stability of retrieved memory and the control of behavior by that memory, indicating that the outcome of the competition between excitatory CS-US and inhibitory CS-noUS associations depends upon the intensity of the original training and the number of retrieval trials. This means that if the original training is highly robust and/or the number of extinction trials is too small, the 'inhibitory' trace may not gain control of behavior and, therefore, will become insensitive to consolidation blockers (Eisenberg et al., 2003). In order to evaluate these predictions in the context of appetitive learning, we manipulated the original training of animals that were subsequently exposed to retrieval. To do so, we varied the length of US presentation during PER conditioning in honeybees.

Associative strength is thought to be reflected during acquisition by its concurrent effects on the animal's CRs(Rescorla & Wagner, 1972). However, in the present context US lenght, which might be directly related to the prospective associative strength (Wagner, 1961), showed no effect on the animals CRs during acquisition. Moreover, when bees trained with different US duration were exposed to CS presentations 24 h later no differences in their CRs were observed (Figure 4-3,Figure 4-4). However, a consolidation blocker applied prior two CS-only trial revealed that CS-US associations underlying CRs of in these groups were probably different.

Thus, a single CS-only trial leads neither to consolidation of a new, extinction learning nor to reconsolidation of the acquisition memory, although a tendency was observed in the short-group indicating that emetine-treated animals decreased their CRs . Such tendency might indicate that reconsolidation of the acquisition memory is impaired in the short group. To test this hypothesis further experiments have to be conducted. Retention should be tested later on, since it has been shown that blocking reconsolidation of acquisition memory leads to impaired memory retention 48 h when bees were trained with 4 s presentation of the US (Chapter I).

With two CS-only trials the CRs decreases significantly in the long and short group from the last CS-only trial 24 h after conditioning to the retrieval trial after 48 h. This result are in close agreement to the experiments with two CS-only trials with a 4 s US presentation during training in chapter II. In those experiments a significant extinction was induced by the presentation of two CS-only trials and the induction of a protein synthesis- dependent extinction memory was inhibited. Surprisingly, however, in the present experiments application of the protein synthesis inhibitor only appeared to interfere with the consolidation of the extinction memory, when the animals had been exposed to a long US presentation. In contrast a short US presentation (2 s) results in significant extinction after two CS-only trials that is not disturbed by the application of emetine. Hence, a protein synthesis-dependent extinction was impaired by increasing US-length.

In PER conditioning , it has been shown that memory formation depends strongly upon satiation (Friedrich et al., 2004): bees fed 4 h before conditioning, show impaired acquisition performance and a lower response probability at the following retention test. In comparison to the short group, bees in the long group were allowed to lick the offered sucrose solution during seven additional seconds; therefore they receive a larger amount of sucrose, than animals in the short group. During such a short interval, however, harnessed bees may obtain only small amounts of sugar solution, which are, in turn, insufficient to significantly change their levels of satiation. Indeed, US-length did not affect learning performances during acquisition nor CRs at the CS-only presentation (Figure 4-3,Figure 4-4). Moreover, animals were daily fed up to satiation during the evenings. Hence, present results can not be explained by means of possible differences in satiation levels.

By testing predictions from the trace dominance hypothesis by manipulating the original training and the number of extinction trials the present results raises additional questions, since protein synthesis-dependent extinction appears to be impaired by decreased US-lengths. One might consider that different US-lengths lead to different reward expectations, irrespective of the level of retention observed during acquisition. Pavlovian learning is supposed to depend upon the unpredictability of the US (Wagner & Rescorla, 1972). According to this hypothesis an US becomes gradually less efficient to change (e.g. increase during acquisition, decrease during extinction trials) a behavioral response as the predictability of the US grows during learning. The difference between the actual occurrence and the prediction of the US is referred to as predictive error. Following this argumentation the mismatch between what is expected and what is experienced during the extinction trials is larger for animals of the long group than for animals of the short group. Even if this does not result in a difference in decrease of CRs, one might consider, for instance, that this is the 'force' to switch between protein synthesis-dependent consolidation of extinction memory and protein synthesis-independent formation of an extinction memory in this study.

Nevertheless it was demonstrated, that the length of the US in an appetitive learning paradigm is most likely critical for the state of the acquisition memory which most likely has an impact on the consolidation processes induced by retrieval. The nature of these US-dependent differences in the state of the acquisition memory is unclear and remains to be elucidated.