

Chapter 1

Forward and backward second-order Pavlovian conditioning in honeybees

Summary:

Second-order conditioning (SOC) is the association of a neutral stimulus with another stimulus that had previously been combined with an unconditioned stimulus (US). We used classical conditioning of the proboscis extension response (PER) in honeybees (*Apis mellifera*) with odors (CS) and sugar (US). Previous SOC experiments in bees were inconclusive and therefore, we attempted to demonstrate SOC in the following three experiments. Exp-1: After differential conditioning (pairing odor A with US and presenting odor B without US), the bees experienced two pairs of partially overlapping odors, either a new odor C followed by a previously reinforced odor A (C-A) or a new odor C followed by a previously non-reinforced odor B (C-B). Exp-2: After differential conditioning, bees were presented with C-A or A-C. Exp-3: Bees were first presented with C-A or A-C before differential conditioning and were tested with odor C. We observed; Exp-1: 40% of the bees showed PER to the C-A presentation, but only 20% showed PER to the C-B presentation. Exp-2: 40% of the bees showed PER to the C-A presentation, while only 20% showed PER to the reversed sequence A-C. Experiments 1 and 2 showed that a previously reinforced odor can be a secondary reinforcer for excitatory SOC only with forward-pairing. Exp-3: PER towards C was lower (15%) in bees presented with A-C than with C-A (25%). This showed that backward SOC is not as effective as forward SOC (Exp-2). These results help to delineate different conditions that are critical for the phenomenon of SOC.

Introduction

Associative learning enables organisms to internally represent correlations among environmental events. Animals and humans associate one cue with another when one of them is biologically relevant. This is the simplest form of association, called first-order conditioning. In addition, associative learning can be indirect or of second-order, as in the case of Pavlov's dog (Pavlov 1927). A dog - when put into a dark room and given food (primary reinforcer - US) just before switching on the light (conditioned stimulus - CS) - salivated (conditioned response - CR) every time the light was switched on (CS leads to CR). Then, in a separate event, the dog was presented with the tone of a bell (neutral stimulus - NS) followed by the light (CS, a secondary reinforcer). After several such combinations, the dog already salivated in the presence of the tone, although it never had been combined with the primary reinforcer food. Pavlov called the latter process "reflex of the second order" which is now known as second-order conditioning (SOC).

In nature, animals have to relate one stimulus with another consistently and precisely in order to acquire food, find mates, and escape predators. Insects such as honeybees have to come across certain stimuli that have no direct meaning. They have to decipher random stimuli such as trees, buildings, flowers, odors, etc. into meaningful information. For example, during foraging trips honeybees encounter several odors and not all odors lead to food source. But certainly some odors might serve as a clue to reach another odor which might be the source of food. Making secondary associations such as these towards biological relevant stimuli is a complex learning task requiring complex information processing of the brain.

The phenomenon of SOC has been studied in many animal species, e.g. monkeys (Findley *et al* 1966), rats (Kamil 1969; Holland and Rescorla 1975b), pigeons (Rescorla 1979, Stanhope 1992), zebra fish (Hall and Suboski 1995), and goldfish (Amiro and Bitterman 1980). It was also observed in invertebrates like mollusks (Sahley 1981), *Aplysia* (Hawkins *et al.* 1998), fruit flies (Brembs and Heisenberg 2001), and honeybees (Takeda 1961; Bitterman *et al.* 1983; Menzel 1990).

The experiments by Takeda (1961) and Bitterman *et al.* (1983) had already shown some evidence of SOC, but were inconclusive due to insufficient controls (like pairing

effect and backward SOC control) and a small number of animals (<10). The present three experiments (for overview see Table 1) not only attempt to overcome these insufficiencies, but also to delineate the conditions being critical for the occurrence of SOC. The exact sequence of stimuli which triggers SOC is not known and also which different learning processes are involved during SOC or reversal of SOC phases is not known. To understand this, starting from experiment 1 we manipulated different parameters such as sequence of stimuli (experiment 2) and the protocol sequence (experiment 3).

We used odors as CSs and sugar reinforcement – as US to train the honeybees in an appetitive Pavlovian conditioning paradigm called proboscis extension response (PER) (Kuwabara 1957; Bitterman et al. 1983). Our experiments show that bees learn second-order associations only under certain special conditions.

Materials and methods:

Foraging honeybees (*Apis mellifera*) were caught in a transparent box at the entrance of the outdoor hive 2 hours prior to training and were cold-anesthetized on ice. The immobile bees were harnessed in metal tubes such that only the mandibles, proboscis, and antennae could move freely (Bitterman et al. 1983). 10 minutes before training, bees were checked for PER (unconditioned response: UR) by lightly touching the antennae with 30% sucrose solution (US). Only bees which demonstrated the UR were trained (<5% were discarded). Citral, geraniol, 2-hexanal, nonanone, 2-octanol, and limonene were used as odor CSs, referred to as A, B, and C in later sections. A computer-driven odor-device, blowing a continuous stream of air over the antennae, was used to deliver the odors to the bees (Galizia et al. 1997, Komischke et al. 2002). Four 1-ml syringes, each containing 4 μ l of the desired odor on a half sq. inch filter paper, were fitted into the holes of the odor-delivering device (Galizia et al. 1997). Each bee was fixed in front of this device with its antennae facing the air-stream, and an exhaust system behind the bee removed the odor.

Experimental procedure:

The bees were kept within the air-stream of the odor-delivering device for a total of 60 s during each trial; 26 s before and 28 s after the stimulus and 6 s during the presentation of the test stimuli. During the 6 s stimulus time, stimulus-1 and stimulus-2 were presented for 4 s and 3 s, respectively, with 1 s overlap between the two. In all phases of all experiments we used 5 trials per stimulus and the inter-trial-interval (ITI) was always 12 min. The differential conditioning phase started with a presentation of odor A for 4 s that was subsequently reinforced with 30% sucrose solution (US) for 3s, with an overlap of 1s between A and US. In the next trial, a second odor B was presented, but without any US reinforcement. The percent proboscis extension response (% PER) of the bees as observed during the first two seconds of odor presentation was calculated for A and B.

In the SOC phase 2 odors were used. Throughout this phase, the bees were never reinforced with US. The first odor was presented for 4 s, followed by the second odor for

3 s. The overlap between these two odors was 1 s. The % PER to the first odor was recorded beginning at onset for 2 s. Thus the recording ended before onset of the second odor. In addition, in experiments 2 and 3 responses to the second odor were recorded for 2 s after the offset of odor 1. A computer beep signaled the timing for the presentation. Furthermore, onset and offset of an odor was indicated by audible clicks from the solenoid valves (a part of the odor-device).

Each of the three experiments consisted of two groups and at least two phases. The different phases followed each other immediately. All groups in all experiments experienced the same differential conditioning protocol (A – US; B – noUS). Experiments 1 and 2 studied SOC in the conventional manner i.e. differential conditioning in Phase 1 was followed by an SOC protocol in Phase 2. This we call **forward SOC**. In experiment 1, a new odor C was followed by odor A that was initially paired with the US in Group plus (C - A), while in Group minus, C was followed by B, the odor that was initially presented without the US (C - B). Successful SOC can be inferred from a between-group comparison of response to C at the end of Phase 2. Group forward of experiment 2 experienced exactly the same sequence of differential conditioning and SOC as group plus of experiment 1 (forward SOC with forward-pairing C – A). Group backward of experiment 2, however, received A – C pairings instead of C – A pairings in Phase 2 after the very same differential conditioning protocol as the other three groups (forward SOC with backward-pairing A – C). Hence, in experiment 2 the sequence of stimuli was reversed (forward- versus backward-pairing). In experiment 2, successful SOC can be evaluated by comparing response to the first and last presentation of C in Phase 2 within-groups and also by between-group comparison of 4th and 5th trials of each group.

Finally, experiment 3 is the same as experiment 2, but now the Phase 1 and Phase 2 protocols were reversed (**backward SOC**). In this experiment, differential conditioning took place in Phase 2, after C – A pairings (group forward: forward-pairing) or A – C pairings (group backward: backward-pairing) were administered in Phase 1. This backward SOC can also be looked at as two variants of sequential sensory preconditioning (see Müller et al., 2000). In order to evaluate SOC, response to C at the

first test trial in Phase 3 was compared to response to C in the last trial of Phase 1 within-groups.

Table 1. Experimental design:

Experiment no.	Group type	Phase 1	Phase 2	Test
Experiment 1	Group Plus	A-US; B-noUS	C-A	-
	Group Minus	A-US; B-noUS	C-B	-
Experiment 2	Group Forward	A-US; B-noUS	C-A	-
	Group Backward	A-US; B-noUS	A-C	-
Experiment 3	Group Forward	C-A	A-US; B-noUS	C
	Group Backward	A-C	A-US; B-noUS	C

Dependent Variable and Statistics:

The PER is a clear response which leads to either a full extension of the proboscis or no response. Therefore, publications based on PER scoring involve video recording only when quantitative measures need to be taken (e.g. latency of PER, duration of PER). This was not the case in our study. There was another reason why it was also not necessary to run blind experiments. The experiments involved a large number of animals which were marked beforehand by the experimenter with numbers. During this procedure the different experimental groups were shuffled in such a way that sequential animals belonging to the different groups were treated at the same intervals and spaced out equally through the experimental period. It was, therefore, impossible for the experimenter to keep track of animals belonging to the same experimental group.

Between-group comparisons were analyzed using the Kruskal-Wallis-Anova and chi²-tests. Within-group comparisons were performed with the Wilcoxon-matched-pairs-test and the McNemar-test. The alpha-level was set to 0.05 for all analyses.

Results

Experiment 1: Does olfactory SOC occur in honeybees?

The odors used as A, B, and C were citral, geraniol or 2-hexanal. They were equally balanced and used in all possible combinations to exclude odor-specific effects. The first column of Figure 1.1 shows that the acquisition curves for the differential conditioning protocol in Phase 1 were quite similar for both groups ($n = 60$ each). The middle column of Figure 1.1 shows response to the new odor C for forward-pairing with A (C – A) in group plus (upper quarter) and for forward-pairing with B (C – B) in group minus (lower quarter). Comparisons between groups by Kruskal-Wallis-Anova showed significantly higher response for forward-pairing with A compared to forward-pairing with B in trial 4 ($H=4.56$, $p<0.05$, $df=1$) and in trial 5 ($H=7.01$, $p<0.01$, $df=1$).

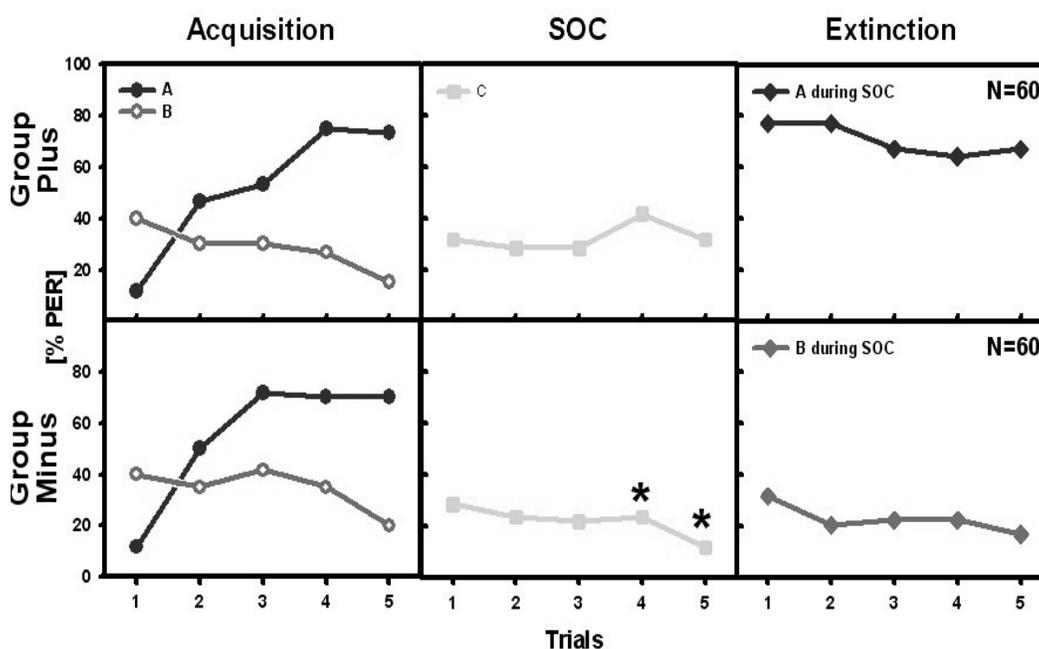


Fig 1.1. Second-order conditioning in honeybees. Acquisition of odors A and B (left panel) in a differential conditioning design (A-US, B-noUS). During SOC phase (right panel) group plus received pairings of a new odor C with an already conditioned excitatory odor A, while group minus received pairings of a new odor C with an odor that already had been presented without US. Bees in group plus showed more response to C at the end of SOC phase (trials 4 and 5). Significant differences are marked with an asterisk.

In both groups, the % PER to the stimuli was recorded in three parts: first stimulus alone, overlap, second stimulus alone. Therefore we were able to take a look at

the extinction of A in group plus and B in group minus (see Fig. 1.1 third column). Response to A (the secondary reinforcer) did not decrease from Trial 1 to Trial 5 ($\chi^2=0.75$, n.s., $df=1$) nor did response to B increase for group minus ($\chi^2=3.56$, $p<0.06$, n.s., $df=1$). Hence, within 5 SOC trials there was no extinction.

In order to replicate this finding, we repeated experiment 1 with different experimenters using different odors (nonanone, 2-octanol, limonene; data not shown). Again both groups (group plus: $n = 109$; group minus: $n = 108$) differed significantly in trial 4 ($H=5.40$, $p<0.05$, $df=1$) and in trial 5 ($H=5.94$, $p<0.05$, $df=1$; data not shown).

Because we obtained very similar results in two experiments, including the missing of extinction, we conclude that olfactory SOC in honeybees occurs when a new odor is forward-paired with an odor that had been conditioned earlier.

Experiment 2: Does the order of pairing of the two odors presented during the SOC phase influence performance?

The odors used as A and C were citral, geraniol or 2-hexanal, odor B always was nonanone. The three odors were equally balanced and used in all possible combinations to exclude odor-specific effects. The first column of Figure 1.2 shows that the acquisition curves for the differential conditioning protocol in Phase 1 were quite similar for both groups ($n = 70$ each). Bees from both groups showed nearly 80% PER towards A and <10% PER towards B after 5 trials. The panels in the middle column of Figure 1.2 show response to the new odor C for forward-pairing with A (C – A) in group forward (upper part) and for backward-pairing with A (A – C) in group backward (lower part). Comparisons within groups by means of McNemar-tests showed significantly increasing response (15% - 40%) from Trial 1 to Trial 5 for forward-pairing with A ($\chi^2=10.26$, $p<0.001$, $df=1$) and significantly decreasing response (55% - 20%) from Trial 1 to Trial 5 for backward-pairing with A ($\chi^2=12.71$, $p<0.001$, $df=1$).

Between-group comparisons (Kruskal-Wallis-Anova) revealed significant differences in Trial 1 ($H=26.04$, $p<0.001$, $df=1$) and Trial 5 ($H=9.47$, $p<0.01$, $df=1$). Thus, bees in group forward and group backward started into SOC from a different response level and they also ended up at different levels.

In both groups, the % PER to the stimuli was recorded in three parts: first stimulus alone, overlap, and then second stimulus alone. Therefore we were able to take a look at extinction of odor A in both groups (see Fig. 1.2 third column). Response to A did not decrease from Trial 1 to Trial 5, neither for group forward ($\chi^2=0.00$, n.s., $df=1$) nor for group backward ($\chi^2=0.66$, n.s., $df=1$). Hence, within 5 SOC trials there was no extinction of A.

The order of pairing of stimuli during the SOC phase had an influence on the performance during SOC. Forward-pairing (C - A) led to an increase in response to C. Backward-pairing (A - C) seemed to have had an inhibitory effect in response to C during SOC, although in both groups bees remained motivated by showing response to A.

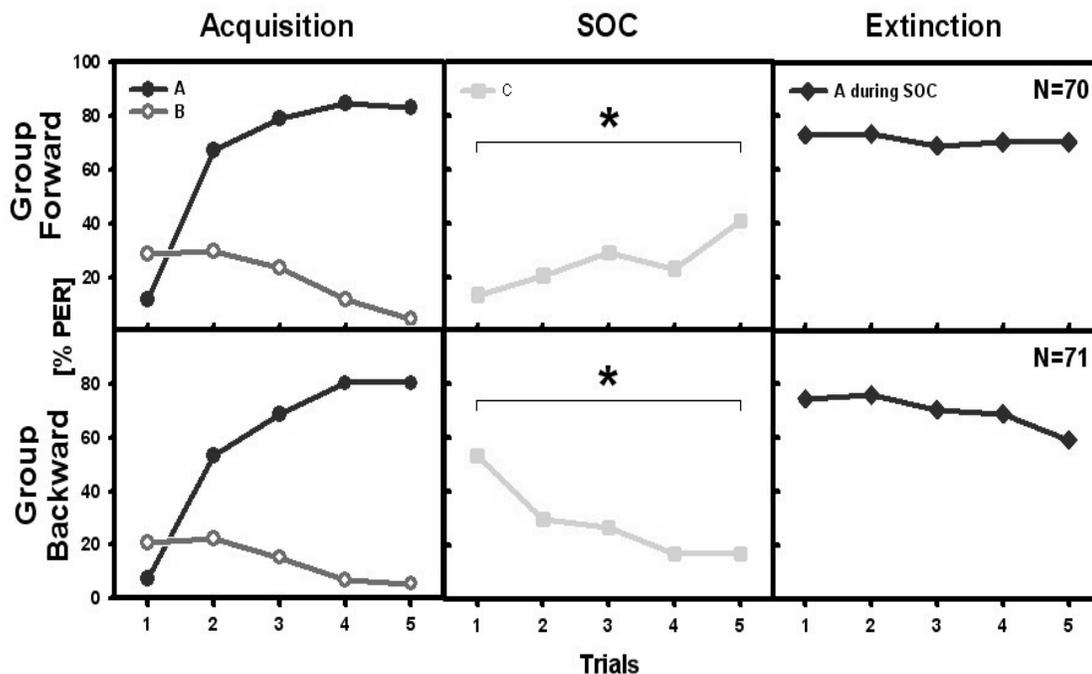


Fig 1.2. Contiguity and second-order conditioning in honeybees. Acquisition of odors A and B (left panel) in a differential conditioning design (A-US, B-noUS). During SOC phase (middle panel) group forward received a forward-pairing of new odor C with an already conditioned excitatory odor A, while group backward received a backward-pairing of A and C. Bees of group forward showed an increase in response to C during the SOC phase while group backward showed a decrease (comparing trial 1 and 5). With respect to responses to C during SOC, group forward and group backward differed significantly in trial 1 and trial 5. At the end of the SOC phase the group that received a forward-pairing showed more responses to C than the backward-paired group. The right panel shows the level of extinction of responses to A during the SOC phase. The tendency of extinction visible in group backward is not statistically different. Significant differences are marked with an asterisk.

Experiment 3: Backward SOC with forward- and backward-pairings

The odors used as A and C were citral, geraniol, or 2-hexanal. The three odors were equally balanced and used in all possible combinations to exclude odor-specific effects. For all bees, the % PER to the stimuli was recorded in three parts: first stimulus alone, overlap, and second stimulus alone. Since response during the overlap could be influenced by both stimuli and therefore is ambiguous, response during overlap is not shown in the figure.

The first column of Figure 1.3 shows response to odor C for forward-pairing with odor A (C – A) in group forward (upper part, $n = 71$) and for backward-pairing with A (A – C) in group backward (lower part, $n = 60$). The panels in the middle column show the acquisition curves for the differential conditioning protocol in Phase 2. The third column of Figure 1.3 shows response to stimulus C that was never directly paired with the US.

During the initial pairing phase (Phase 1) we found no significant decrease with respect to response to C in trials 1 and 5, neither for group forward ($\chi^2=0.80$, n.s., $df=1$) nor for group backward ($\chi^2=2.56$, n.s., $df=1$). Response to C differed in the last trial of the initial pairing phase between group forward and group backward ($\chi^2=6.96$, $p<0.01$, $df=1$).

In the differential conditioning phase (Phase 2) both groups showed an increase in response to A comparing trial 1 and 5 (group forward: ($\chi^2=40.20$, $p<0.001$, $df=1$); group backward: ($\chi^2=27.38$, $p<0.001$, $df=1$)). There was no difference between groups with respect to response to A in the 5th trial ($\chi^2=0.92$, n.s., $df=1$).

Comparisons within groups by means of Wilcoxon-matched-pairs-tests showed significantly increasing response to C from the last trial of Phase 1 to the first trial of the test phase for group forward with forward-pairing of C and A (C – A) in Phase 1 before differential conditioning of A in Phase 2 ($Z=3.62$, $p<0.001$). For group backward, with backward-pairing of C and A (A – C) in Phase 1 before differential conditioning of A in Phase 2, the response difference was not significant ($Z=0.30$, n.s., $df=1$).

The association between A and C should be strongest in animals which successfully mastered differential conditioning in Phase 2, because only these animals

learned that odor A is the only odor rewarded by a US during the whole experiment. Comparison of only those animals showed a significant difference between groups in the first test to C in the test phase (χ^2 -test: $\chi^2=3.98$, $p<0.05$, $df=1$).

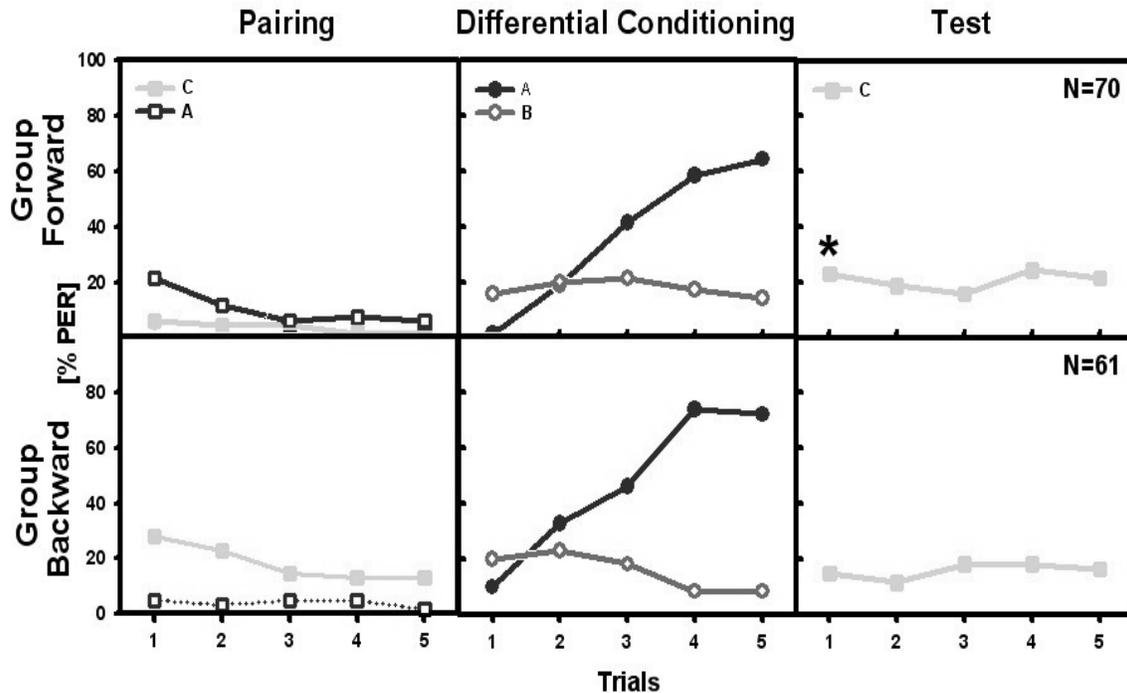


Fig 1.3. Backward second-order conditioning containing forward- and backward-pairing. The left panel shows response to A and C during the initial pairing phase. The second phase was differential conditioning (A-US, B-noUS; middle panel). In the last phase, responses to C were tested (right panel). Initially, forward-pairing (group forward) showed a strong increase in responses to C from the first to the last phase of the experiment. Backward-pairing (group backward) did not initially show any difference with respect to responses to C between the first and the last phase. Classical conditioned odors prevent other odors from being learned, if they have been backward-paired before classical conditioning. Significant differences are marked with an asterisk.

Discussion:

The experiments by Takeda (1961) and Bitterman et al. (1983) had already shown evidence of forward SOC, but Takeda's experiment did not exclude the possibility of sensitization as the cause for SOC, and in the Bitterman experiment very few animals (N=10) were tested and a control group with backward-pairing was missing. In the present series of experiments, a large number of animals were tested, the odors were balanced during training, which eliminated the possibility of odor-specific effects, and effects due to successive presentation of two odors were evaluated by including backward-paired controls. Furthermore, the impact of the sequence of the first-order and second-order protocol was looked at.

Experiment 1 showed that forward pairing of a new odor C with a previously reinforced odor A led to higher response to C than forward pairing of a new odor C with a previously un-reinforced odor B. This finding alone, however, cannot be interpreted as evidence for excitatory SOC. First, it might be argued that the mere presence of an excitatory CS in the second-order conditioning phase may have maintained motivation, alertness and so forth, accounting for maintenance of response to the second-order CS in Group Plus. Second, the backward procedure as a well-known inhibitory learning procedure might have led to inhibitory SOC in Group Minus. Hence, the interpretation of the observed difference between groups has to be postponed.

Experiment 2 therefore examined whether the mere presence of two odors (new odor C and reinforced odor A) leads to excitatory SOC per se or whether the order of pairing is crucial for SOC. We had two groups, C – A and A – C. Excitatory SOC was observed only with C – A pairing but not with A – C pairing. Although the already reinforced odor A was presented in the latter case, too, we there observed a decrease in response to C. The increase of response to C with C – A pairing together with the decrease of response to C with A – C pairing shows the following. First, the increase in response to C cannot be due to the mere presence of an excitatory CS. Second, the within comparison showing a significant increase with forward pairing strengthens the possibility that excitatory SOC took place in experiment 1, too. Third, the significant decrease with backward pairing might be interpreted as evidence for inhibitory SOC in

experiment 2. Excitatory SOC needs forward pairing with an already trained excitator, while backward pairing of a new stimulus with an already trained excitator might yield inhibitory SOC. With experiment 3, we show that in addition to the sequence of pairing the sequence of first-order and second-order protocol is also important. Unlike experiment 2, where A – US followed by C – A led to conditioning of a new odor, in experiment 3, C – A followed by A – US did not lead to conditioning of the new odor C.

Experiments 1 and 2 together clearly showed the existence of forward SOC (excitatory and/or inhibitory) in olfactory learning in honeybees. Bees are able to learn to associate an odor with an appetitive reinforcer (sugar) via a pairing with another odor which had been reinforced previously. Forward SOC effects could be seen in forward- and backward-pairings of odors (experiment 2). The SOC was stronger in experiment 2 compared to experiment 1 because the first-order conditioning response was higher than in experiment 1. This is supported by the fact that we found a positive correlation between first-order conditioning and SOC in group plus of experiment 1 (+0.43) and group forward of experiment 2 (+0.72). Odor A in experiment 2 had a higher reinforcing value than odor A in experiment 1 which was reflected in the subsequent SOC levels. Experiment 3 even showed successful backward SOC, at least for a stimulus that was followed by another stimulus (forward-pairing) before this other stimulus was paired with US.

In all differential conditioning experiments, training started with A – US and then B – noUS. Therefore, response towards B was always higher than towards A in the first trial. This is a generalization effect which is commonly observed in most of our differential conditioning experiments. Experiment 1 showed that SOC in olfactory PER conditioning in honeybees is a weak effect, because only about a quarter of the animals demonstrated that they had learned the new odor C. Nearly 75% of the bees do not change their learning pattern after SOC. SOC is much weaker than first-order conditioning, where the percentage of animals showing learning is about three times higher. One reason for this might lie in the higher reinforcement value of the US sugar solution, since the primary reinforcer obviously provides a much higher US strength than an acquired US. Thus, the transmission of associative strength to C is weaker. Another potential cause for the reduced strength of an acquired US is extinction occurring due to

repeated presentations of a previously reinforced odor without US (Bitterman et al. 1983, Menzel 1990, Hammer and Menzel 1998, Bonod et al. 2003, Stollhoff et al. 2005). In group plus of experiment 1 and group forward of experiment 2, however, there was no extinction during SOC. One would expect extinction of A in both groups, since A was repeatedly presented without US, and extinction has been frequently demonstrated in olfactory PER conditioning (see above). Since we did not include a control group which experienced an A-only stimulus in the second phase of conditioning, we cannot decide whether the lack of extinction in experiment 2 can be related to the occurrence of a preceding C.

We chose a second group (group backward) to prove that the mere presence of a previously reinforced odor (A) is not sufficient to cause SOC, but instead a special sequence of two odors is necessary for SOC to occur. In group backward of experiment 2, a previously reinforced odor A preceded the new odor C (A - C). The bees learned to extend their proboscis at a high rate towards A in Phase 1. In Phase 2 they continued to do so until the beginning of C (which overlapped with A). Remarkably, during Phase 2, the bees learned to retract the proboscis with the onset of new odor C (Fig. 1.2 lower middle panel). At Trial 5 the response to C in group backward was significantly lower than in Trial 1. Thus backward-pairing of A with C led to a termination of the learned response at the onset of C. While the same new odor C in group forward became a predictor for the occurrence of a learned odor, it developed an inhibitory property in group backward. Thus, SOC follows the rules of first-order conditioning: forward-pairing leads to excitatory conditioning and backward-pairing to inhibitory conditioning. This finding emphasizes the special inhibitory meaning of backward-pairing in classical conditioning, as has been shown in other invertebrates as well (Tanimoto et al. 2004). Group backward not only proved that the SOC effect cannot be explained as a generalization effect, but also that the sequence of pairing (C - A vs. A - C) is of relevance in SOC.

Experiment 3 showed that in Phase 1 honeybees acquired associations between odors even if no US occurred before or during acquisition. With respect to group forward, data showed that bees are able to acquire information about the relationship of different odors even if these odors are presented without the presence of an US. A subsequent

pairing with the US of one of the odors presented in phase 1 makes the former learned association visible within a behavioral response. The fact that our data do not show an effect with respect to group backward does not mean that no association was learned between the two odors in phase 1. It may only show that we did not choose the right testing procedure in order to show such associations. A summation test might possibly have been the proper test. Therefore one should have trained an odor X before the experiment only for the half of possible satiation (using only two learning trials). Afterwards it would be possible to show excitatory or inhibitory influences of C to X within a mixture test.

In the last trial of phase 1 response to C was higher in group backward than in group forward. This might be one reason that the increase in response from phase 1 to phase 3 failed to become significant in group backward and only became significant in group forward. The between groups analysis of learners only, however, showed significantly higher response in group forward compared to group backward in phase 3, although before learning in phase 2 had taken place response to C had been higher in group backward. Hence, there is some evidence that the increase in response is not due to a non-associative effect.

Experiment 3 is quite similar to a set of experiments which were related to sensory preconditioning (Müller et al. 2000). In Müller's work odors from phase 1 were presented in a purely simultaneous way and in a sequential way, presenting one odor after the other without an overlap. Both groups are different from our experimental design. Müller et al. found significant effects for the simultaneous group, but not for the sequential one. The experimental design used in our study is right in the middle of the groups used by Müller et al.: odors were presented in a sequential way but with an overlap and therefore with a simultaneous part of odor presentations. Thus odor stimulation might just have been "simultaneously enough" to lead to learning of an association between the two odors. In general, backward SOC, as used in our study, might offer the opportunity to uncover earlier hidden learning, as in phase 1 of experiment 3. Pairing of a stimulus with an US provides the "energy" or "biological strength" that is necessary to map the already learnt but so far hidden association between this stimulus and another one onto response.

The neural correlate of the reinforcing function in odor conditioning in bees, the neuron VUMmx1 (Hammer 1993, 1997; Menzel & Giurfa 2001) was found to require responses to the reinforced odor during acquisition, a property this neuron shares with dopamine neurons of the ventral tegmentum in the primate brain stem (Schultz 2006). Therefore, activation of the VUMmx1 neuron by the reinforced odor provides an internal reward signal under the control of this odor. Pairing a new odor with a previously reinforced odor in the SOC phase will, in fact, lead to a pairing of the new odor with an internal reward signal. Similarly, replacing the reward signal with a local injection of the putative transmitter of the VUMmx1 neuron (octopamine) leads to learning of the odor paired with the injection of the transmitter (Hammer & Menzel 1998). It is, therefore, reasonable to assume that SOC in bees is based on an internal reward signal which got under control during the initial acquisition phase.