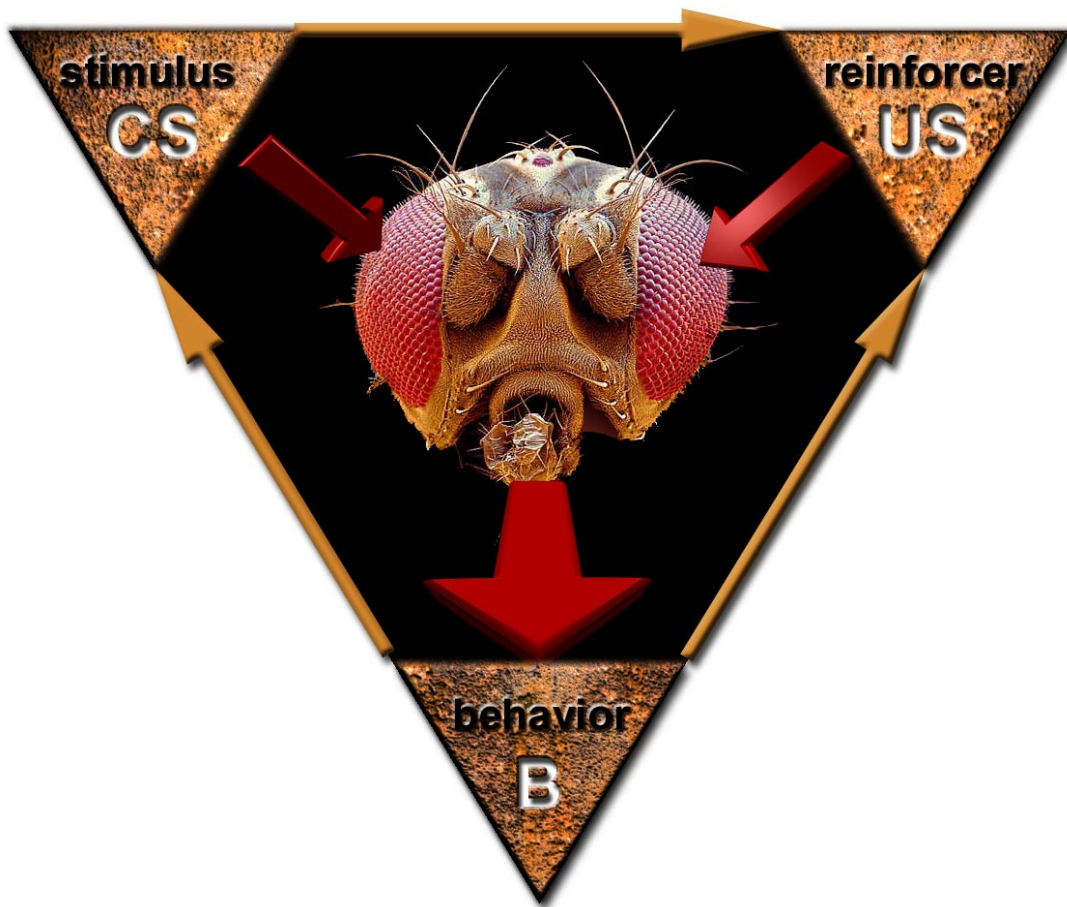


# An Analysis of Associative Learning in *Drosophila* at the Flight Simulator



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Doctoral thesis

“An adequate formulation of the interaction between an organism and its environment must always specify three things (1) the occasion upon which a response occurs, (2) the response itself and (3) the reinforcing consequences. The interrelations among them are the contingencies of reinforcement.”

*Burrhus F. Skinner*

# 1 Introduction

## 1.1 Associative learning

Ambulatory organisms are faced with the task of surviving in a rapidly changing environment. As a consequence, they have acquired the ability to learn. Most learning situations comprise one or more initially neutral stimuli (conditioned stimulus, CS), the animal's behavior (B) and a biologically significant stimulus (unconditioned stimulus, US). Freely moving animals experience a stream of perceptions that is crucially dependent on the animal's behavior. Opening or closing the eyes, directing gaze or ears, sniffing, biting or locomotion all have sometimes drastic effects on the animal's stimulus situation. The archetypal example of such a learning situation is a frog or toad trying to eat a bee or wasp. During the search for prey ( $B_1$ ), movement detectors in the anuran's eye detect the hymenopteran's locomotion ( $S_1$ ). The frog will perform a taxis or approach towards the prey ( $B_2$ ). There is no apparent difference in the frog's behavior whether the prey is colored brightly (CS) or dull, if the frog is naive, i.e. has not encountered bees before. If the bee continues to move and fits some other criteria to classify it as appropriate prey ( $S_n$ ), the frog will try to catch it using his extendable, adhesive glossa ( $B_3$ ). The sting of the bee (US) will lead to immediate rejection ( $B_4$ ). One can describe this sequence more generally as  $B_1$  leading to  $S_1$  which causes  $B_2$  which in turn is followed by the perception of the CS. The  $S_1$  does not only causes  $B_2$ , but in conjunction with other stimuli ( $S_1+S_n$ ) leads to  $B_3$  which makes the bee sting (US) which in turn leads to  $B_4$ . In other words, close temporal proximity can often be regarded as a clue for a causal relationship. This is a central insight for the understanding of associative learning. It becomes clear that stimuli can be both causes and consequences of behaviors. Therefore, the 'three term contingency' (Skinner, 1938) between B, CS and US is best described using feedback loops. The animal's brain chooses an action (B) from its behavioral repertoire which will have consequences on the animal's stimulus situation (CS, US), which will in turn enter the brain via



**Fig. 1:** *Drosophila* in a natural learning situation. The animal's CNS spontaneously generates motor patterns which manifest themselves in behavior (B). B either alters some of the stimuli currently exciting the sensory organs of the fly or leads to the perception of new stimuli. If one of these stimuli has a direct feedback onto the behavior, i.e. the perception of the stimulus persistently leads to the production of a particular behavior, this stimulus can be a reinforcer (US). If a certain behavior consistently leads to perception of the reinforcer, the animal can learn about this relationship in order to avoid aversive or obtain appetitive reinforcers (i.e. form a B-US association). Sometimes the US is consistently accompanied by an initially neutral stimulus (CS). In these cases, there is the possibility to learn about the relation between the CS and the US (i.e. form a CS-US association) in order to anticipate the appearance of the US. As CS and US share a temporal relationship and both are controlled by the B, in such a situation both B-US, B-CS and CS-US associations can form. Red arrows – neuro-physical or physico-neural interactions, brown arrows – physico-physical interactions. (Scanning electron micrograph courtesy of 'Eye of Science')

sensory organs and influence the next choice of B (Fig. 1). Eventually, the frog will continue to forage ( $B_1$ ) after some time and the whole sequence can start anew. It is common knowledge that if the prey exhibits the CS at a subsequent encounter, at least  $B_3$  will not occur. Often a behavior similar to  $B_4$  can be observed and sometimes  $B_2$  will also be left out.

## 1.2 Components of associative learning

Evidently, on occasions like the one described above the animal learns that the CS is followed by an aversive US. Such learning about relations between stimuli is referred to as Pavlovian or *classical conditioning*. Classical condi-

tioning is often described as the transfer of the response-eliciting property of a biologically significant stimulus (US) to a new stimulus (CS) without that property (Pavlov, 1927; Hawkins et al., 1983; Kandel et al., 1983; Carew and Sahley, 1986; Hammer, 1993). This transfer is thought to occur only if the CS can serve as a predictor for the US (Rescorla and Wagner, 1972; Pearce, 1987; Sutton and Barto, 1990; Pearce, 1994). Thus, classical conditioning can be understood as learning about the temporal (or causal; Denniston et al., 1996) relationships between external stimuli to allow for appropriate preparatory behavior before biologically significant events ("signalization"; Pavlov, 1927). Much progress has been made in elucidating the neuronal and molecular events that take place during acquisition and consolidation of the memory trace in classical conditioning (Kandel et al., 1983; Tully et al., 1990; Tully, 1991; Tully et al., 1994; Glanzman, 1995; Menzel and Müller, 1996; Fanselow, 1998; Kim et al., 1998).

On the other hand, the animal has learned that its behavior  $B_3$  caused (was followed by) the US and therefore suppresses it in subsequent encounters. Such learning about the consequences of one's own behavior is called instrumental or *operant conditioning*. In contrast to classical conditioning, the processes underlying operant conditioning may be diverse and are still poorly understood. Technically speaking, the feedback loop between the animal's behavior and the reinforcer (US) is closed. Obviously, a behavior is produced either in response to a stimulus or to obtain a certain stimulus situation (goal) or both. Thus, operant conditioning is characterized mainly by B-US but also by B-CS associations (see for a general model: Wolf and Heisenberg, 1991). Analysis of operant conditioning on a neuronal and molecular level is in progress (Horridge, 1962; Hoyle, 1979; Nargeot et al., 1997; Wolpaw, 1997; Spencer et al., 1999; Nargeot et al., 1999a; b) but still far from a stage comparable to that in classical conditioning.

Considering the example above, it becomes clear that more often than not operant and classical conditioning can not be separated as clearly as they are separated in the literature. As the appropriate timing is the key criterion for both types of learning to occur, both operant and classical conditioning can be conceptualized as detection, evaluation and storage of temporal relationships. One recurrent concern in learning and memory research, therefore, has been the question whether for operant and classical conditioning a common formalism can be derived or

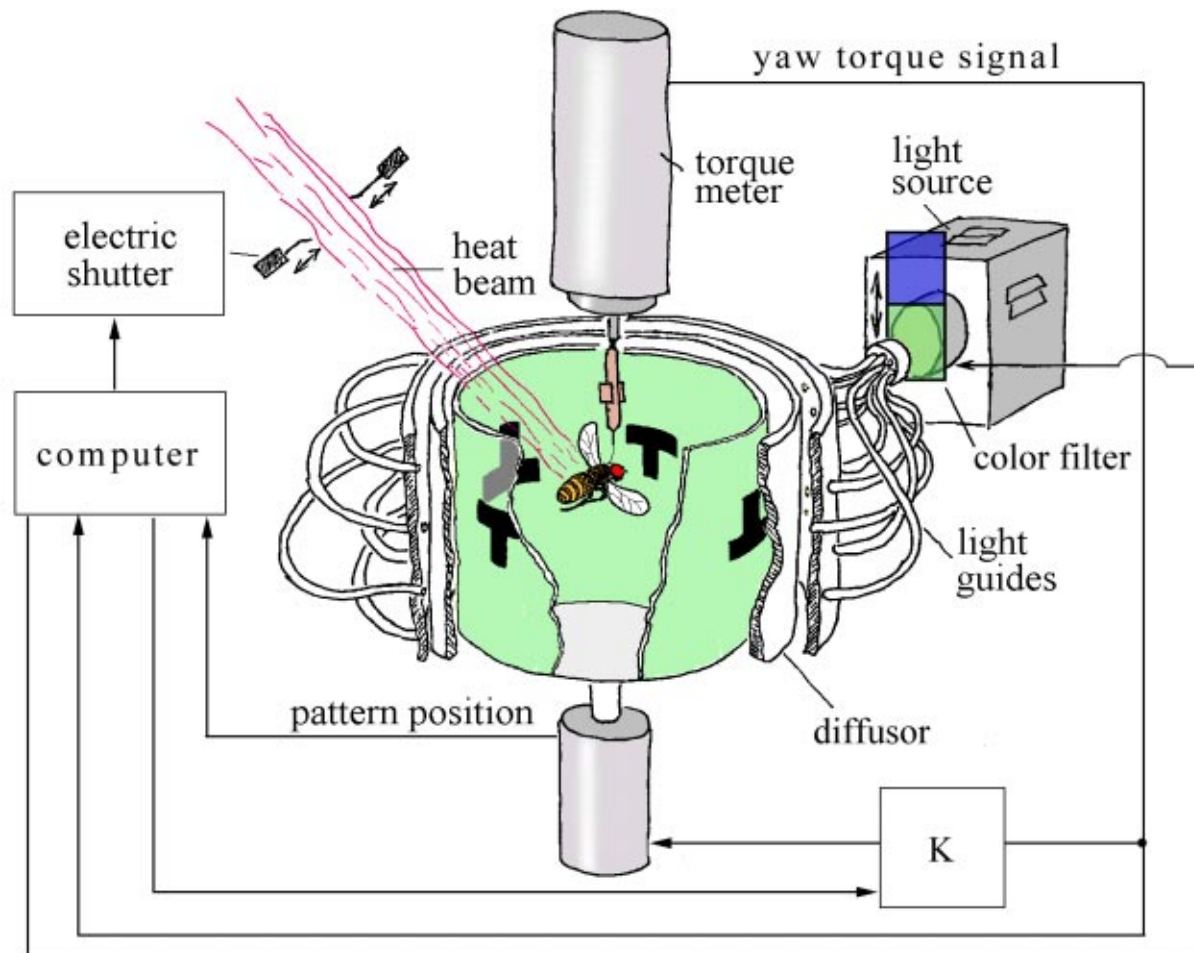
whether they constitute two basically different processes (Gormezano and Tait, 1976). Both one- (Guthrie, 1952; Hebb, 1956; Sheffield, 1965) and two-process theories (Skinner, 1935; Skinner, 1937; Konorski and Miller, 1937a, b; Rescorla and Solomon, 1967; Trapold and Overmier, 1972) have been proposed from early on, and still today the issue remains unsolved, despite further insights and approaches (Trapold and Winokur, 1967; Trapold et al., 1968; Hellige and Grant, 1974; Gormezano and Tait, 1976; Donahoe et al., 1993; Hoffmann, 1993; Balleine, 1994; Rescorla, 1994; Donahoe, 1997; Donahoe et al., 1997).

As exemplified above, often it is impossible to discern the associations the animal has produced when it shows the conditioned behavior. In a recent study, Rescorla (1994) notes: "...one is unlikely to achieve a stimulus that bears a purely Pavlovian or purely instrumental relation to an outcome". With *Drosophila* at the torque meter (Heisenberg and Wolf, 1984; Heisenberg and Wolf, 1988), this disentanglement of Skinner's now classic three term contingency has been achieved. Classical and operant learning can be separated with the necessary experimental rigor and directly compared in very similar stimulus situations to show how they are related.

### 1.2.1 *Drosophila* at the torque meter

In visual learning of *Drosophila* at the torque meter (Fig. 2; Wolf and Heisenberg, 1991; Wolf and Heisenberg, 1997; Wolf et al., 1998; Liu et al., 1999) the fly's yaw torque is the only motor output recorded. The fly is surrounded by a cylindrical arena that may be used for stimulus presentation. Most simply, yaw torque can be made directly contingent on reinforcement (infrared light delivering instantaneous heat) with none of the external stimuli bearing any relation to the reinforcer (*yaw torque learning*; Wolf and Heisenberg, 1991; Fig. 3I). The fly learns to switch the reinforcer on and off by producing yaw torque of a certain range without the aid of additional stimuli. Adding a CS (color or patterns) to this set-up brings about a new operant paradigm at the torque meter to be called *switch (sw)-mode* (Fig. 3II). The color of the arena illumination (or the orientation of patterns on the arena) is exchanged whenever the yaw torque of the fly changes from the punished to the unpunished range and vice versa.

More sophisticatedly, the angular speed of the arena can be made negatively proportional to the fly's yaw torque, enabling it to stabilize the



**Fig. 2:** Flight simulator set-up. The fly is flying stationarily in a cylindrical arena homogeneously illuminated from behind. The fly's tendency to perform left or right turns (yaw torque) is measured continuously and fed into the computer. The computer controls pattern position (via the motor control unit K), shutter closure and color of illumination according to the conditioning rules.

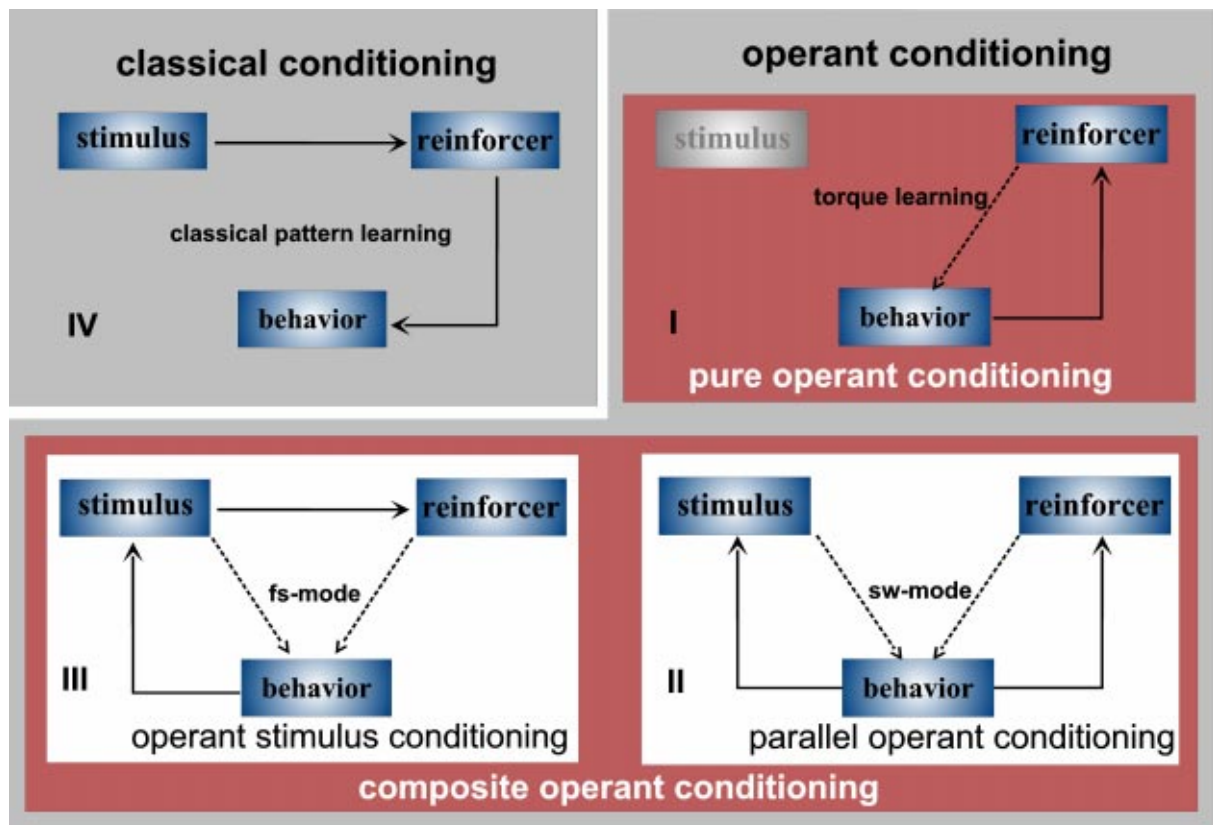
arena, i.e. to fly straight (closed loop; Wolf and Heisenberg, 1991; for detailed explanation see MATERIALS AND METHODS). In this *flight simulator (fs)-mode* (Fig. 3III), the fly can learn to avoid flight directions denoted by different patterns (*operant pattern learning*) or by different arena coloration (*operant color learning*; Wolf and Heisenberg, 1997). In the latter case, a uniformly patterned arena is used to allow for turn integration to occur. Using both colors and patterns as visual cues in *fs-mode* results in *operant compound conditioning*.

Finally, the fly's behavior may have no relation whatsoever with the appearance of the heat, but the reinforcer is contingent upon the presentation of a CS. Wolf et al. (1998) have first described *classical pattern learning* at the flight simulator (Fig. 3IV). The setup is identical to the

operant pattern learning paradigm, except for the training phase where the fly cannot interfere with pattern presentation (open loop). Again, this setup can also be used with identical patterns and different arena illumination (*classical color learning*). In all instances learning success (memory) is assessed by recording the fly's behavior once the training is over.

Thus, all components of the three term contingency are available: the behavior B (yaw torque), the reinforcer or US (heat) and a set of conditioned stimuli or CSs (colors or patterns). The flexible setup enables the establishment of virtually all possible combinations between the three components for later fine dissection of the associations the fly has formed during the training phase.





**Fig. 3:** Block diagram of the experiments used in this study. Solid arrows – feedforward relations; dotted arrows – feedback relations. Note that only the logical relationship between the components of the learning situation is depicted. Neither the way the experiment works, nor the possible associations nor any physical relationships are addressed.

### 1.2.2 Initial framework and assumptions

The components of the three term contingency B, CS and US can be arranged in at least the four different ways depicted in Fig. 3. The four situations can be grouped into single-association or monodimensional tasks (Fig. 3 I + IV) and composite or multidimensional tasks (Fig. 3 II + III). The monodimensional tasks require only simple CS-US or B-US associations in order for the animal to show the conditioned behavior. The multidimensional tasks are more complex and offer the possibility of forming a number of different associations, each of which may be sufficient to show the appropriate learning. Usually it is not clear, which of the associations are formed. Composite learning situations are always operant tasks as the feedback loop between the stimuli and the behavior is closed. They are of two types: (1) Situations in which the CS is only paralleling the appearance of the US, i.e. the change in a behavioral program primarily determines reinforcer presentation (B-US). In the sw-mode the CS parallels the appearance of the US during a 'pure' operant conditioning process and the CS-US association forms

in parallel to the concomitant motor program modulation. The fly learns to avoid the heat by restricting its yaw torque range and at the same time the heat can induce the pattern or color preference (CS-US association). One can thus refer to situations like this as 'parallel'-operant conditioning. Parallel-operant conditioning in essence is the additive combination of classical and pure-operant conditioning. (2) Situations in which the behavior controls the CS onto which the US is made contingent; i.e. there is no *a priori* contingency between a motor program and the reinforcer as in (1). Direct B-US associations can not occur, but the behavioral control of the CS may induce (maybe US mediated) B-CS associations. This type of situations may be called 'operant stimulus conditioning'. All types of learning have in common that either a behavior (Fig. 3 I) or a stimulus (Fig. 3IV) or both (Fig. 3 II, III) can in principle be used as predictors of reinforcement. From this formal point of view, behaviors and predictors can be treated as equivalent entities as long as the experimental design ensures equal predictive value. In other words, provided that both behaviors and stimuli in a composite conditioning experiment can be used equally well to predict reinforcement, both

B-US and CS-US associations should be formed. Similarly, if the formal description holds, the single-association tasks (Fig. 3 I + IV) should not differ, i.e. they should require similar amount of training.

### 1.2.3 Analyzing the components in *Drosophila* learning

Since operant pattern learning at the torque meter was first reported (Wolf and Heisenberg, 1991), the method has been used to investigate pattern recognition (Dill et al., 1993; Dill and Heisenberg, 1995; Dill et al., 1995; Ernst and Heisenberg, 1999) and structure function relationships in the brain (Weidtmann, 1993; Wolf et al., 1998; Liu et al., 1999). Dill et al. (1995) have started a behavioral analysis of the learning/memory process and others (Eyding, 1993; Guo et al., 1996; Guo and Götz, 1997; Wolf and Heisenberg, 1997; Xia et al., 1997a, b; Wang et al., 1998; Xia et al., 1999) have continued. Yet, a formal description of how the operant behavior is involved in the learning task is still in demand.

In contrast to operant pattern learning, the formal description for classical pattern learning seems rather straightforward: In order to show the appropriate avoidance in a subsequent closed-loop test without heat the fly has to transfer during training the avoidance-eliciting properties of the heat (US<sup>+</sup>) to the punished pattern orientation (CS<sup>+</sup>), and/or the 'safety'-signaling property of the ambient temperature (US<sup>-</sup>) to the alternative pattern orientation (CS<sup>-</sup>). As the fly receives no confirmation which behavior would save it from the heat, it is not able to associate a particularly successful behavior with the reinforcement schedule. In other words, it is assumed that classical conditioning is solely based on an association between CS and US and not on any kind of motor learning or learning of a behavioral strategy.

As both operant and classical pattern training lead to an associatively conditioned differential pattern preference, it is clear that also during operant training a CS-US association must form. Knowing that this association can be formed independently of behavioral modifications, one is inclined to interpret the operant procedure as classical conditioning taking place during an operant behavior (pseudo-operant). However, Wolf and Heisenberg (1991) have shown that operant pattern learning at the flight simulator is not entirely reducible to classical conditioning. In a yoked control in which the precise sequence of pattern movements and heating episodes pro-

duced by one fly during operant (closed loop) training was presented to a second fly as classical (open loop) training, no learning was observed.

Two interesting questions arise from these findings: (1) Why does this form of training not show a learning effect despite the fact that flies in principle are able to learn the patterns classically (Wolf et al., 1998)? Why do Wolf et al., (1998) find classical pattern learning but Wolf and Heisenberg (1991) do not? A more extensive yoked control is performed to find an answer to this question. (2) Why does the same stimulus sequence lead to an associative aftereffect if the sequence is generated by the fly itself (operant training), but not if it is generated by a different fly (classical replay training, yoked control)? What makes the operant training more effective? Two possible answers have been addressed. For one, the operant and the classical component might form an additive process. In other words, during operant conditioning the fly might learn a strategy such as: "Stop turning when you come out of the heat" in addition to the pattern-heat association. The operantly improved avoidance behavior would then amplify the effect of the CS-US association upon **recall** in the memory test. This question was tackled by Brembs (1996) and is thoroughly discussed and rejected there. As the alternative, the coincidence of the sensory events with the fly's own behavioral activity (operant behavior) may facilitate **acquisition** of the CS-US association. In this case, there would be no operant component stored in the memory trace (only operant behavior during acquisition) and thus the classical CS-US association would be qualitatively the same as in classical conditioning. A transfer of this CS-US association learned in one behavior to a new behavior would be compatible with such an hypothesis.

The approach just described compares a simple classical with a composite operant conditioning procedure in which both classical and operant components may occur, in order to find out more about the contribution of the operant (B-US, B-CS) component to pattern learning in *Drosophila*. In a second set of experiments a single association operant task (yaw torque learning, only B-US associations required) to compare with a second composite operant task (sw-mode) with both operant and classical components, in order to learn more about the classical (CS-US) contribution. The formal description of yaw torque learning is rather straightforward: once the fly has successfully compared the temporal structure of the heat with its motor output, it has to transfer the avoidance eliciting proper-

ties of the heat to the punished range of its yaw torque generating motor programs (i.e. it has to form a B-US association). In the subsequent test phase, these motor programs have to be suppressed (avoided) in favor of other programs in order to show a learning score. With classical pattern learning and yaw torque learning being 'pure' experiments where single associations are assumed to be formed, both fs-mode and sw-mode conditioning are composite forms of learning where the formation of two or more associations are possible. Assessment of the contribution of both classical and operant components to sw-mode learning is brought about by rearranging or separating behavior and stimulus. With this array of experiments, it should be possible to estimate the contribution of behavioral and sensory predictors to complex, natural learning situations. The hypothesis to be tested, derived from the formal considerations above, is the equivalence of B-US and CS-US associations: are both operant and classical associations formed?

Once the relations and interactions of the individual associations during memory acquisition within a complex learning task are elucidated, the next logical step is to analyze the single associations more closely. It was mentioned above that behavior is not produced for its own sake, but rather to achieve a certain stimulus situation or goal ('desired state'; Wolf and Heisenberg, 1991). Moreover, some behaviors occur more frequently upon perception of a given stimulus than other behaviors, i.e. certain stimuli have (or have acquired) the potential to elicit certain behaviors. Thus, stimulus processing is of outstanding importance for the understanding of learning and memory. Therefore, the acquisition of stimulus memory is subjected to closer scrutiny. Here, the *Drosophila* flight simulator offers a unique opportunity for studying the properties of the CS-US association (i.e. the associations formed if more than one CS-US association is allowed). First, there are, to my knowledge, no studies explicitly dealing with compound stimulus learning in a complex situation. As mentioned above, most experiments do comprise both operant and classical components regardless of the initial intent to separate them. However much the operant and classical components may vary, though, the degree to which the behavior controls the animal's stimulus situation in unsurpassed in the flight simulator. Second, the flight simulator in its restrictedness offers the experimenter exquisite control over the stimuli the animal perceives and thereby minimizes the

amount of variation between animals. Most confounding variables that complicate other learning experiments are eliminated in the flight simulator. Third, the recent development of operant compound conditioning in the flight simulator enables the experimenter to investigate into complex processes hitherto mainly studied in vertebrates.

### 1.3 Properties of associative stimulus learning

There can be no doubt that stimulus learning is not only of prevalent importance for the animal's survival, but the literature on associative learning is strongly biased towards this type of association as well. The vertebrate literature is dominated by both operant and classical experiments in a number of species dealing with the properties of the CS-US acquisition process. The results reveal a surprising generality across the varying degree of operant and classical influences as well as across species. This generality has led to the development of quantitative rules characterizing associative stimulus learning and hence the suggestion of common learning mechanisms across phyla (Pavlov, 1927; Skinner, 1938) and across traditionally distinct paradigms as classical and operant conditioning (Skinner, 1938; Trapold and Winokur, 1967; Trapold et al., 1968; Grant et al., 1969; Mellgren and Ost, 1969; Feldman, 1971; Hellige and Grant, 1974; Feldman, 1975; Williams, 1975; McHose and Moore, 1976; Pearce and Hall, 1978; Williams, 1978; Zanich and Fowler, 1978; Williams and Heyneman, 1982; Ross and LoLordo, 1987; Hammerl, 1993; Rescorla, 1994; Williams, 1994; Lattal and Nakajima, 1998). It would be interesting to know how far this generality can be stretched.

How can one formally conceptualize the acquisition of memory? Usually, the simple notion of pairing CS and US is formalized as the amount or increment of learning ( $\Delta V$ ) being proportional to the product of reinforcement ( $\lambda$ ) and the associability ( $\alpha$ ) of the CS (e.g. Rescorla and Wagner, 1972; Pearce and Hall, 1980).

$$\Delta V \approx \alpha \lambda \quad (1)$$

More typically, inequality (1) is refined as  $\Delta V$  being proportional to the difference between the actual level of reinforcement ( $\lambda$ ) and the amount of learning already acquired (i.e. the degree to which the US is signaled or predicted by the CS:  $\bar{V}$ ). Modifying the reinforcement

term yields an asymptotic learning rule – the so-called ‘delta rule’:

$$\Delta V = \alpha(\lambda - \bar{V}), \quad (2)$$

This class of learning theories has also been called “error correcting learning rules” because increments in learning lead to  $\bar{V}$  approaching  $\lambda$  and thereby correct the error between observation and prediction. Several such rules refining and extending the simple concept that temporal pairing of CS and US are necessary and sufficient to form an association between them have been found in vertebrates (Rescorla and Wagner, 1972; Mackintosh, 1975b; Pearce and Hall, 1980; Sutton and Barto, 1981; Pearce, 1987; Sutton and Barto, 1990; Pearce, 1994). The most commonly observed phenomena providing evidence for such rules are ‘overshadowing’ (Pavlov, 1927), ‘blocking’ (Kamin, 1968), ‘sensory preconditioning’ (Brogden, 1939; Kimmel, 1977) and second-order conditioning (Pavlov, 1927).

*Overshadowing* may occur in a conditioning experiment when a compound stimulus, composed of two elements, is paired with the reinforcer (CS1+CS2+US). If the elements of the compound differ in associability, the conditioned response is stronger for the more associable stimulus than for the other. Thus one stimulus ‘overshadows’ the other (Pavlov, 1927). Overshadowing is a well known phenomenon from classical (Pavlov, 1927) and operant (Miles, 1969; Miles and Jenkins, 1973) conditioning in vertebrates and from invertebrates (Couvillon and Bitterman, 1980; Couvillon and Bitterman, 1989; Couvillon et al., 1996; Pelz, 1997; Smith, 1998). The degree to which different stimuli can overshadow each other depends largely on their modalities and is usually correlated with their physical intensity (Mackintosh, 1976). As will become clear below, overshadowing may interfere with blocking, sensory preconditioning and second-order conditioning experiments.

*Blocking* implies that temporal CS-US pairing does not transfer the response-eliciting property of the US to the CS if the CS is presented together with another CS that already fully predicts the US. In a classical blocking design, a first (pretraining) phase consists of training one stimulus (CS1+US) until the subject shows a maximal learning response. Subsequently, a new stimulus (CS2) is added and the compound is reinforced (CS1+CS2+US). If afterwards CS2 is tested alone, the subject shows a learning score

below that of a control group that has not received any pretraining. Thus, the pretraining has ‘blocked’ learning about CS2 (Kamin, 1968). Part 2 of this procedure is very similar to an overshadowing experiment and hence it becomes clear that ideally the elements of the compound should not show overshadowing without any pretraining (but see Schindler and Weiss, 1985; Weiss and Panilio, 1999 for sophisticated two compound operant experiments with rats and pigeons, respectively, that can overcome strong overshadowing effects and produce blocking).

Often blocking is explained in terms of predictability or expectation: only if a US is ‘surprising’ (Kamin, 1968; Kamin, 1969), i.e. if it is not well predicted, can the stimuli having a predictive value for the US enter into the association. In a blocking experiment, the novel CS2 is compounded with the already well trained CS1 as a redundant predictor. Thus, CS2 accrues less associative strength than if no pretraining had occurred (Rescorla and Wagner, 1972; Pearce and Hall, 1980; Sutton and Barto, 1981; Sutton and Barto, 1990; Pearce, 1994). Blocking was initially developed in classical (Pavlovian) conditioning paradigms (e.g. Kamin, 1968; Fanselow, 1998; Thompson et al., 1998). It was later extended also to instrumental (operant) conditioning, using discriminative stimuli (SD; e.g. Feldman, 1971; Feldman, 1975) and is now widely generalized to operant conditioning together with other prominent concepts like ‘unblocking’ and ‘overexpectation’ (e.g. McHose and Moore, 1976; Haddad et al., 1981; Schindler and Weiss, 1985; Williams, 1994; Lattal and Nakajima, 1998; Weiss and Panilio, 1999). Operant SDs, however, only indicate during which time the B-US contingency is true and thus share a feature with ‘classical’ CSs: they are at most only partially controlled by the animal. While it seems that SDs are not entirely reducible to classical CSs (e.g. Holman and Mackintosh, 1981; Rescorla, 1994), they still are very different from the stimuli controlled *entirely* by the animal as in the flight simulator. I do not know of any study using this type of operant conditioning to produce blocking. It would be interesting to find out whether the high degree of operant control over the stimuli as in the flight simulator has any effect on blocking.

Even though our understanding of the ecological significance (Dukas, 1999) and neural mechanisms underlying blocking is still in its infancy (Holland, 1997; Fanselow, 1998; Thompson et al., 1998), it has become a cornerstone of modern learning theories (Rescorla and



Wagner, 1972; Pearce and Hall, 1980; Sutton and Barto, 1981; Wagner, 1981; Sutton and Barto, 1990; Pearce, 1994). The discovery of blocking by Kamin (1968), has had a large impact on research in many vertebrates (e.g. Marchant and Moore, 1973; Bakal et al., 1974; Mackintosh, 1975a; Cheatle and Rudy, 1978; Wagner et al., 1980; Schachtman et al., 1985; Barnet et al., 1993; Holland and Gallagher, 1993; Batsell, 1997; Thompson et al., 1998) including humans (e.g. Jones et al., 1990; Kimmel and Bevill, 1991; Levey and Martin, 1991; Martin and Levey, 1991; Kimmel and Bevill, 1996). The literature on invertebrates is more scarce. Reports include honeybees (Smith, 1996; Couvillon et al., 1997; Smith, 1997; Smith, 1998), *Limax* (Sahley et al., 1981) and *Hermissenda* (Rogers, 1995; Rogers et al., 1996). In all instances, however, confounding effects have been pointed out and remain to be solved (Farley et al., 1997; Gerber and Ullrich, 1999). To my knowledge, there is no unambiguous evidence in the literature that invertebrates exhibit blocking.

In *second-order conditioning* (SOC) a stimulus (CS1) is paired with a US until it has acquired a predictive function for the US. In the second part of the experiment the CS1 is paired with a CS2 but without reinforcement. Finally, the CS2 is presented alone to test whether by having been paired with the CS1 it has become a predictor of the US as well. One can perceive an SOC experiment as a blocking experiment where the reinforcement is omitted in the compound phase. However, in SOC a positive learning score indicates a successful experiment, whereas blocking would be indicated by a negative result (compared to control groups). Thus, SOC constitutes an important control of the blocking experiment: if blocking is not obtained, it might be due to SOC masking a potential blocking effect. As the analogy of a blocking experiment to SOC is striking, one can wonder how blocking can be observed at all. A pioneering study by Cheatle and Rudy (1978) suggests that reinforcement during compound training disrupts the transfer of the response eliciting properties from the pre-trained CS1 to CS2. This is compatible with newer neurobiological data (Hammer, 1993; Hammer, 1997; Fanselow, 1998; Kim et al., 1998) that imply a negative feedback mechanism to attenuate US effectiveness if reinforcement is well predicted (Fanselow, 1998; Kim et al., 1998) and instead lead to a US representation upon perception of the CS (Hammer, 1993; Hammer, 1997). SOC has been found in both vertebrates (Rizley and Rescorla, 1972; Holland

and Rescorla, 1975a; Holland and Rescorla, 1975b; Cheatle and Rudy, 1978; Rescorla, 1979; Rescorla and Cunningham, 1979; Amiro and Bitterman, 1980; Rescorla and Gillan, 1980; Rescorla, 1982; Hall and Suboski, 1995) and invertebrates (Takeda, 1961; Sekiguchi et al., 1994; Hawkins et al., 1998; Mosolff et al., 1998).

*Sensory Preconditioning* (SPC) is formally very similar to SOC. It again consists of three parts. In the first, the subject is presented with two stimuli (conditioned stimuli; CS1+CS2) without any reinforcement. Then, one of the stimuli (CS1) is reinforced alone. Provided the appropriate controls exclude alternative explanations, a significant learning score in the third phase testing the other stimulus (CS2) alone demonstrates that the response eliciting properties of the unconditioned stimulus (US) have been transferred to a CS with which it has never been paired. Compared to SOC the sequence of parts 1 and 2 is inverted. While thus SOC can be regarded as the temporally reversed analogue of SPC, there is one important difference between SPC and SOC: in vertebrates, extinction of the reinforced CS1 prior to testing of CS2 abolishes SPC but not SOC (e.g. Rizley and Rescorla, 1972; Cheatle and Rudy, 1978; Rescorla, 1983). Additional reported features of SPC comprise the dependence of the intensity of the non-reinforced but not of the reinforced CS (Tait and Suboski, 1972) and of the number of preconditioning trials (Prewitt, 1967; Tait et al., 1972), but see (Hall and Suboski, 1995 for zebrafish). Another especially noteworthy property of SPC is the less restrictive timing dependence in the CS1+CS2 compound phase: in rats, simultaneous pairings show stronger effects than sequential ones (Rescorla, 1980; Lyn and Capaldi, 1994) and backward pairing leads to excitatory, rather than inhibitory associations (Ward-Robinson and Hall, 1996; Ward-Robinson and Hall, 1998; see Hall, 1996 for a review). SPC may be perceived as a case of 'incidental learning' where CS1 becomes associated with CS2 (see DISCUSSION). There is one report on incidental learning at the flight simulator (novelty choice) by Dill and Heisenberg (1995). Flies can remember patterns without heat reinforcement and compare them to other patterns later.

Some of the above mentioned phenomena have warranted explanations that include cognition-like concepts of attention or expectation and prediction. The two types of CSs (visual patterns, colors) open the possibility to study the effects of compound CSs and, in particular, to investigate whether overshadowing, blocking, SOC and SPC

can be observed in flies. It is interesting to find out whether these phenomena are implemented in the fly and hence learning rules developed in vertebrates also apply to *Drosophila* visual

learning. Moreover, the recent discovery of context generalization in *Drosophila* at the flight simulator Liu et al. (1999) have shown that associative stimulus learning is still little understood.

## 2 Materials and Methods

### 2.1 Flies

The animals are kept on standard corn-meal/molasses medium (recipe see Guo et al., 1996) at 25°C and 60% humidity with a 16hr light/8hr dark regime. 24-48h old female flies are briefly immobilized by cold-anesthesia and glued (Loctite UV glass glue) with head and thorax to a triangle-shaped copper hook (diameter 0.05mm) the day before the experiment. The animals are then kept individually overnight in small moist chambers containing a few grains of sucrose.

### 2.2 Apparatus

The core device of the set-up is the torque meter. Originally devised by Götz (1964) and repeatedly improved by Heisenberg and Wolf (1984), it measures a fly's angular momentum around its vertical body axis. The fly, glued to the hook as described above, is attached to the torque meter via a clamp to accomplish stationary flight in the center of a cylindrical panorama (arena, diameter 58mm), homogeneously illuminated from behind (Fig. 2). Via the motor control unit K an electric motor can rotate the arena according to the experimental procedures described below. The light source is a 100W, 12V tungsten-iodine bulb. For green and blue illumination of the arena, the light is passed through monochromatic broad band Kodak Wratten gelatin filters (#47 and #99, respectively). Filters can be exchanged by a fast magnet within 0.1 sec.

The angular position of an arbitrarily chosen point of reference on the arena wall delineates a relative 'flight direction' of 0-360°. Flight direction (arena position) is recorded continuously via a circular potentiometer (Novotechnik, A4102a306) and stored in the computer memory together with yaw torque (sampling frequency 20Hz) for later analysis. The reinforcer is a light beam (diameter 4mm at the position of the fly), generated by a 6V, 15W Zeiss microscope lamp, filtered by an infrared filter (Schott RG780, 3mm thick) and focused from above on the fly. In all experiments the heat is life threatening for the fly: more than 30s of continuous irradiation are lethal. Heat at the position of the fly is switched on and off by a computer-controlled, magneto-electrical shutter intercepting the beam (Fig. 2). The maximum temperature at the point of the fly is measured separately after the experiments by a

blackened thermoelement of about 1mm<sup>3</sup> after 10s of continuous irradiation.

### 2.3 Experimental procedures

*Yaw torque learning.* The fly's spontaneous yaw torque range is divided into a 'left' and 'right' domain (approximately corresponding to either left or right turns; for a justification of this assumption see: Heisenberg and Wolf, 1993). Heat is switched on (input voltage 6.0V) whenever the fly's yaw torque is in one domain and switched off when the torque passes into the other (henceforth: yaw torque sign inversion). There are no patterns on the arena wall, but the illumination is spectrally restricted by a Schott daylight filter (BG18, glass, 3mm) as it was used by Liu et al. (1999) to allow for context generalization.

*Switch (sw)-mode:* As in yaw torque learning, the fly is punished whenever the fly's yaw torque passes into the punished range, but during yaw torque sign inversion not only temperature but also a visual cue is exchanged. Visual cues can be either colors (blue/green) or pattern orientations (up-right/inverted T in front). For color as visual cue, the panorama consists either of 20 evenly spaced stripes (pattern wavelength  $\lambda=18^\circ$ ; transfer experiments) or of no patterns at all (modified overshadowing) and the illumination of the arena is changed from green to blue or *vice versa*. For pattern orientation as visual cue, four black, T-shaped patterns of alternating orientation (i.e. two upright and two inverted) are evenly spaced on the arena wall (pattern width  $\psi=40^\circ$ , height  $\vartheta=40^\circ$ , width of bars= $14^\circ$ , as seen from the position of the fly). One of the pattern orientations is presented stationarily in front of the fly, the other at  $90^\circ$  and  $270^\circ$ . Whenever the range of the fly's yaw torque passes into the other half, the arena is turned by  $90^\circ$  to bring the other pattern orientation in front. For technical reasons, a hysteresis is programmed into the switching procedure: while pattern orientation requires a  $\pm 5.9 \cdot 10^{-10}$ Nm hysteresis during yaw torque sign inversion, a  $\pm 2.0 \cdot 10^{-10}$ Nm hysteresis is sufficient for color as visual cue if the striped drum is used. No hysteresis is necessary if the patterns are omitted altogether.

*Flight simulator (fs)-mode:* Closing the feedback loop to make the rotational speed of the arena proportional to the fly's yaw torque (coupling factor  $K=-11^\circ/s \cdot 10^{-10}$ Nm, Fig. 2) enables the fly to stabilize the rotational movements of the panorama and to control its angular orientation (flight direction). If pattern orientation is

used as visual cue, the same black, T-shaped patterns are used as in sw-mode (see above). For color as visual cue (Wolf and Heisenberg, 1997) the arena either consists of 20 evenly spaced stripes ( $\lambda=18^\circ$ ; transfer experiments) or of four identical vertical stripes (width  $\psi=14^\circ$ , height  $\vartheta=40^\circ$ ; compound experiments). A computer program divides the  $360^\circ$  of the arena into 4 virtual  $90^\circ$  quadrants. The color of the illumination of the whole arena is changed whenever one of the virtual quadrant borders passes the frontal midline (i.e. flight direction) of the fly. If a compound of colors and patterns is used as visual cue, the vertical stripes are replaced by the four T-shaped patterns and color is changed as described. Heat reinforcement (input voltage 6.0V) is made contiguous either with the appearance of one of the pattern orientations in the frontal quadrant of the fly's visual field or with either green or blue illumination of the arena.

*Transfer experiments.* Visual discrimination learning in fs-mode and sw-mode are carried out not only with patterns (upright and inverted T) but, in a second series of experiments, also with colors as visual cues. In each series six groups of flies were tested:

- (1) training and test in fs-mode;
- (2) training in fs-mode followed by test in sw-mode
- (3) training in fs-mode followed by familiarization training and test in sw-mode
- (4) training and test in sw-mode
- (5) training in sw-mode followed by test in fs-mode
- (6) training in sw-mode followed by familiarization training and test in fs-mode

*Modified overshadowing* (for an original overshadowing experiment see below). Groups (4) to (6) of the transfer experiments constitute one part of this experiment, yielding one group of flies that are trained with a behavior (yaw torque) and a stimulus (colors) as the elements of a compound (sw-mode). The 2x4 minutes of compound (composite) training follow a 4 minute preference test. Then, either color learning is assessed independently of motor learning in fs-mode (transfer experiment) or motor learning is assessed independently of color learning by replacing the blue and green color filters with the Schott daylight filter (BG18, glass, 3mm) known to allow for context generalization (Liu et al., 1999) or there is no change at all (control). In an approach to directly measure the associability of the behavioral vs. the sensory predictor, for one

group the contingencies between behavior and color are reversed in the final test (i.e. if 'right' turns lead to blue illumination during training, they will lead to green illumination during the final test phase and vice versa). To control for context generalization effects, one group of flies is subjected to yaw torque learning in which only the final test is carried out with the Schott BG18 filter, but in the preceding training the fly receives behavior independent color changes recorded from previously trained sw-mode flies (color replay). The control for this group was a yaw torque learning experiment as described above.

*Overshadowing.* To test whether the flies are able to separately process colors and patterns during compound (fs-mode) training, the animals are trained in the following sequence. Four minutes of unreinforced preference test are followed by 2x4 minutes of training, interrupted by a 2 min test period (Table 1a). After these 14 minutes of compound presentation, flies are either allowed to choose flight directions with the compound as visual cue (control) or with colors or patterns alone (experimental groups). A fourth group is presented a new compound in which the combination between patterns and colors is exchanged (e.g. if during training flying with an upright T in the frontal visual field led to green illumination of the arena, it now, during the 'exchanged' test phase, would lead to blue illumination).

*Blocking.* The two blocking experiments are designed as between groups experiments, each with one blocking and one control group. Both again consist of two half groups, one of which is presented with colors alone in the first training phase (CS1+US) and the other with patterns alone. The two experiments differ in the amount of compound training (CS1+CS2+US) and the choice of control procedures. In the first experiment (Table 1bI), flies receive equal amounts of first training and compound training. The control groups are provided with the same amount of CS1 and US experience as the blocking group. This is accomplished in two different ways: In the control group stimulated by colors as CS1 during the first conditioning phase flies are trained classically by recording the flight orientation traces and heating regime of the corresponding blocking group and playing them back to the naive flies (replay experiment; Wolf and Heisenberg, 1991). The other half of the control flies exposed to patterns as CS1 in white light are operantly trained. It was observed that pattern memory from training in white light is lost if

colors are added to generate compound stimuli (CS1+CS2). In the corresponding blocking group, a Schott BG18 3mm thick broad-band blue-green filter allows for generalization upon compounding the colors with the patterns (Liu et al., 1999). In this experiment as well as in the sensory preconditioning and second-order conditioning experiments, the BG18 filter is used throughout whenever patterns alone are presented, with the exception of the control group mentioned above. In the second experiment (Table 1bII), only half the amount of compound training is applied and the control groups do not receive any reinforcement before the compound phase.

*Second-order conditioning.* Two second-order conditioning experiments are conducted differing in the amount of second-order training (CS1+CS2). The first (Table 1cI) is modeled closely after the first blocking experiment (Table 1bI), except that the compound phase is shortened by 2 minutes. For the second experiment (Table 1cII) the second-order conditioning phase was shortened even more to only 2x2 minutes (matching the second blocking experiment most closely; see Table 1bII). Only colors are used as conditioned reinforcer.

*Sensory preconditioning.* Two groups of flies are allowed to fly without reinforcement using a compound of colors and patterns as orientation cues (CS1+CS2) for 10 and 16 minutes, respectively (Table 1dI-II). The groups are then further subdivided into two half experiments each, according to which stimulus (colors or patterns) is chosen as CS1 and is presented dur-

ing the subsequent single stimulus phase. This phase consists of 2x4 minutes of training (CS1+US), with an intermittent 2 minute test (CS1 alone). The final 2 min test is conducted with the alternative stimulus (CS2) alone (Table 1dI-II).

## 2.4 Analysis of Data

### 2.4.1 Arena position and yaw torque evaluation

The pattern, color or yaw torque range preference of individual flies is calculated as the performance index:  $PI = (t_a - t_b) / (t_a + t_b)$ . During training,  $t_b$  indicates the time the fly was exposed to the reinforcer and  $t_a$  the time without reinforcement. During tests,  $t_a$  and  $t_b$  refer to the times when the fly chose the situation designated as unpunished or punished, respectively.

### 2.4.2 Statistics

Tests for normal distribution of performance indices yield varying results. Therefore, where possible, non-parametric tests are used, i.e. a Kruskal-Wallis ANOVA to test the hypothesis that three or more samples were drawn from the same population, a Mann-Whitney U-test for comparing two independent samples and a Wilcoxon matched pairs test to test single performance indices against zero. For more complicated two-way designs, data are sufficiently close to being normally distributed to justify a repeated measures ANOVA whenever within and between group comparisons need to be carried out.



Table 1:  
a, “Overshadowing”

pt1	pt2	tr1	tr2	it	tr3	tr4	test
c o l o r s   a n d   p a t t e r n s							colors and patterns
							colors
							patterns
							c+p exchanged

b, “blocking”

I

pretraining phase									compound phase					test					
pt1	pt2	tr1	tr2	it1	tr3	tr4	it2	it3	tr5	tr6	it3	tr7	tr8	test					
block	colors	colors	colors	colors	colors	colors	colors	colors	c o l o r s   a n d   p a t t e r n s					patterns					
	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)						patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	colors	
control	colors (replay)	colors (replay)	colors (replay)	colors (replay)	colors (replay)	colors (replay)	colors (replay)	colors (replay)						patterns	patterns	patterns	patterns	patterns	patterns
	patterns (white)	patterns (white)	patterns (white)	patterns (white)	patterns (white)	patterns (white)	patterns (white)	patterns (white)						patterns (white)	patterns (white)	patterns (white)	patterns (white)	patterns (white)	colors

II

pretraining phase									compound phase			test	
pt1	pt2	tr1	tr2	it1	tr3	tr4	it2	it3	tr5	tr6	test		
block	colors	colors	colors	colors	colors	colors	colors	colors	c o l o r s   a n d   p a t t e r n s			patterns	
	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)				patterns (BG18)	patterns (BG18)
control	pt1	pt2	pt3	pt4	pt5	pt6	pt7	pt8	pt9	tr1	tr2	test	
	colors	colors	colors	colors	colors	colors	colors	colors	c o l o r s   a n d   p a t t e r n s			patterns	
	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	patterns (BG18)	colors

c, “Second-order conditioning”

I

pretraining phase								compound phase					test1	test2
pt1	pt2	tr1	tr2	it1	tr3	tr4	it2	so1	so2	so3	so4	so5	test1	test2
colors	colors	colors	colors	colors	colors	colors	colors	c o l o r s   a n d   p a t t e r n s					patterns	patterns

II

pretraining phase								compound		test1	test2
pt1	pt2	tr1	tr2	it1	tr3	tr4	it2	so1	so2	test1	test2
colors	colors	colors	colors	colors	colors	colors	colors	c o l o r s   a n d   p a t t e r n s		patterns	patterns

d, “Sensory preconditioning”

I

sensory preconditioning					single stimulus training					test
pc1	pc2	pc3	pc4	pc5	tr1	tr2	it1	tr3	tr4	test
c o l o r s   a n d   p a t t e r n s					colors	colors	colors	colors	colors	patterns
					patterns	patterns	patterns	patterns	patterns	colors

II

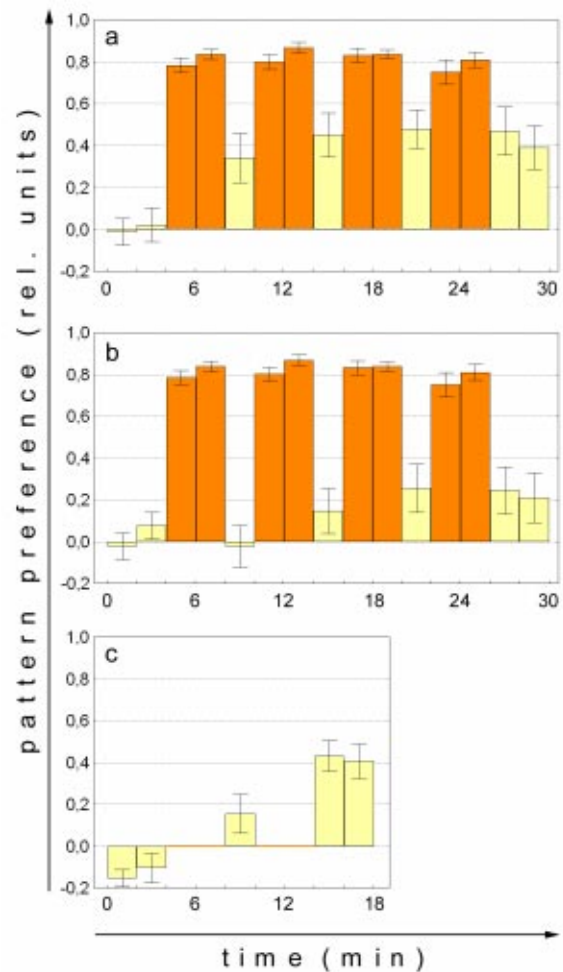
sensory preconditioning								single stimulus training					test
pc1	pc2	pc3	pc4	pc5	pc6	pc7	pc8	tr1	tr2	it1	tr3	tr4	test
c o l o r s   a n d   p a t t e r n s								colors	colors	colors	colors	colors	patterns
								patterns	patterns	patterns	patterns	patterns	colors

**Table 1:** Schematized sequence of training and test periods. Each block in the shaded rows represents one two minute period. pt – pretest; tr – training; it – intermittent test; c – colors; p – patterns; so – second-order training; pc – preconditioning phase.

### 3 Results

#### 3.1 Flies learn classically in the yoked control

Wolf and Heisenberg (1991) have shown earlier (see INTRODUCTION) that operant conditioning at the torque meter is more effective than a classical training procedure consisting of the same sequence of pattern orientations and heat/no-heat alternations (replay; yoked control). On the other hand, classical training with stationary pattern orientations yields learning scores comparable to those obtained in operant pattern learning (Fig. 4; Brembs, 1996; Wolf et al., 1998). The different effects of the two classical procedures require an explanation. In the latter experiments reinforcement is applied in a 3s 'hot' / 3s 'cold' cycle implying that the fly is heated during 50% of the training period (Brembs, 1996; Wolf et al., 1998). In the operant experiment the amount of heat the fly receives is controlled by the fly. In the experiment of Fig. 4a, for example, the fly manages to keep out of the heat for 80 to 90% of the time. If the amount of heat is taken as a measure of reinforcement the flies in the replay experiment receive substantially less reinforcement than the flies in the classical conditioning described above. The failure to learn under replay conditions may therefore be merely a matter of too little reinforcement. If this assumption were correct, prolonging the replay procedure should overcome this shortcoming. Fig. 4 shows that this apparently is the case. The first test after the final replay training shows a significant learning score ( $p < 0.04$ , Wilcoxon matched pairs test). Moreover, single learning scores cease to differ after three 4min training blocks (Test 1:  $p < 0.01$ ; Test 2:  $p < 0.05$ ; Test 3:  $p = 0.14$ ; Mann-Whitney U-Test). Nevertheless, a significant difference between master and replay flies remains if all five learning scores are compared ( $p < 0.02$ ; repeated measures ANOVA). In other words, it is possible for a classical (i.e. behavior-independent) component to be involved during operant conditioning, although without the operant behavior it is small. The fact that this classical component was not detected in Wolf and Heisenberg (1991) is due to the low level of reinforcement in that study. In the present replay experiment (Fig. 4) the memory score after the second 4min training block is not significantly different from that measured by Wolf and Heisenberg (1991). To investigate whether behavioral strategies are learned during operant training that



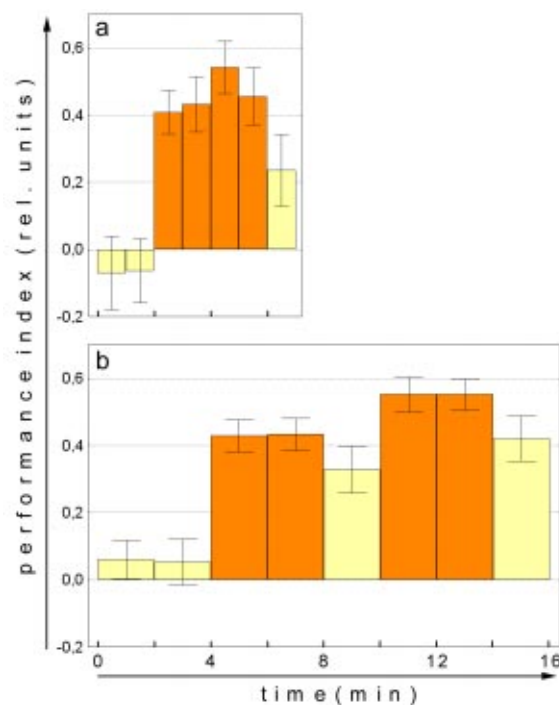
**Fig. 4:** Comparison of mean operant and classical pattern learning performance indices. a - Operant 'master' flies. N=30. b - Classical 'replay' flies. N=30. c - Classical pattern learning with rotating patterns (for illustration; from Brembs, 1996). Note that the training indices are not missing but zero due to the training procedure (see text and Brembs, 1996). N=36. Orange bars - training, yellow bars - test. Error bars (as in all figures) are S.E.M.s.

would facilitate retrieval of this memory trace, an extensive comparison of the microbehavior after operant and classical training, respectively, was performed (Brembs, 1996). These experiments strongly suggest that the operant conditioning does not modify the fly's behavioral strategies or motor patterns. Thus, there is no evidence that classical and operant components merely act additively on memory retrieval.

#### 3.2 Flies can transfer their visual memory to a new behavior

To gather positive evidence for the behavior-independence of the CS-US association occurring during operant conditioning, it was investigated whether flies could be trained in one operant learning paradigm and would subsequently display the pattern preference in a different one.

In addition to the standard operant procedure at the flight simulator (fs-mode) a new operant paradigm at the torque meter to be called switch (sw)-mode was used. It is based on yaw torque learning (Wolf and Heisenberg, 1991) in which the fly's spontaneous range of yaw torque is divided into a 'left' and a 'right' domain and the fly is conditioned by heat to restrict its range to one of the two. In the sw-mode two stationary orientations of the panorama (or two colors of the illumination) are coupled to the two domains. For instance, if the fly generates a yaw torque value that falls into the 'left' domain heat is on and the upright T is in frontal position; if the yaw torque changes to a value in the 'right' domain heat goes off and the arena is quickly rotated by 90° shifting the inverted T to the front (for further details see MATERIALS AND METHODS). The original experiment without visual cues is a case of 'pure' motor learning. In the sw-mode additionally a CS-US association may occur due to the pairing of the visual cues with heat and no heat during training. Time course and performance indices of two representative sw-mode experiments (one with patterns and one with colors as visual cues) are shown in Fig. 5.

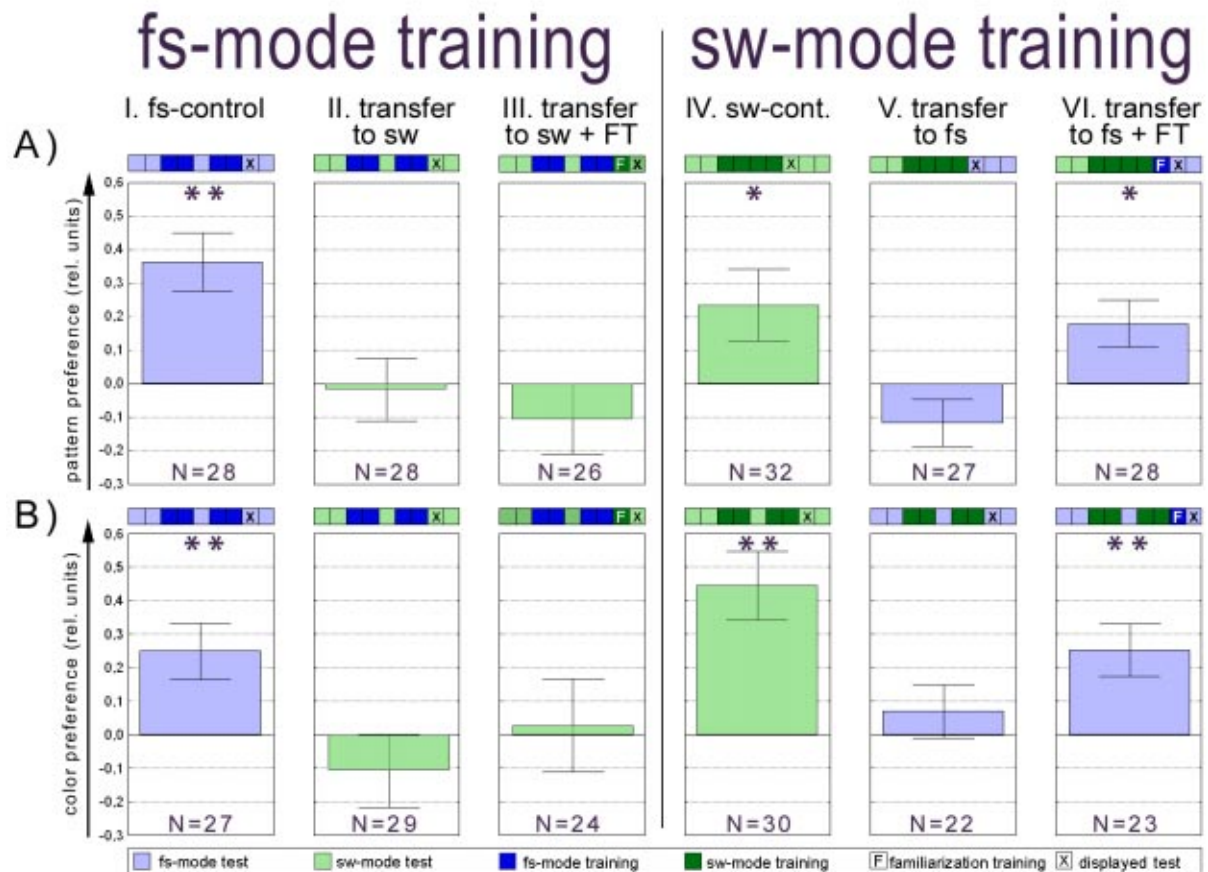


**Fig. 5:** Mean performance indices in two representative sw-mode experiments. a – Patterns as visual cue (one minute PI's), N=32. b - Colors as visual cue (2 minute PI's), N=70. Orange bars – training, yellow bars – test.

Although the two conditioning procedures take place at the torque meter in the same visual surround and both involve operant behavior, they

are entirely different. While in fs-mode the choice between different pattern orientations and the two temperatures depends on the ability to fly straight and, above that, upon a sequence of discrete, well timed orienting maneuvers, in sw-mode it is the actual value of the fly's yaw torque that controls this choice. With these two behavioral paradigms the possibility of training a fly in one mode and testing pattern preference in the other was examined. A significant learning score after a behavioral transfer would corroborate the hypothesis that the CS-US association formed during operant conditioning in the fs-mode does not rely on any motor or rule learning, but instead is a 'true' classical (i.e. behavior independent) association, the acquisition of which is facilitated by operant behavior.

The two forms of visual discrimination learning are tested not only with patterns (upright and inverted T) but, in a second series of experiments, also with colors as described by Wolf and Heisenberg (1997; and MATERIALS AND METHODS; Fig. 6). No direct transfer was observed when fs-mode and sw-mode were interchanged between training and test, neither with patterns nor with colors as visual cues (columns II and V in Fig. 6). Therefore, a short familiarization training was included because flies might not easily generalize across behavioral contexts (a similar but sensory effect was recently reported by Liu et al., 1999, who showed that flies in the fs-mode are unable to generalize between two monochromatic colors of illumination). Control experiments verified that the familiarization training alone is too short to sufficiently condition the fly (data not shown). With this modification significant transfer was found only from sw-mode training to fs-mode test for pattern and for color preferences (Fig. 6, column VI:  $p < 0.04$  pattern;  $p < 0.005$  color, Wilcoxon matched pairs test) but not in the opposite direction (Fig. 6, column III:  $p < 0.37$  pattern;  $p < 0.78$  color, Wilcoxon matched pairs test). This asymmetry is no surprise. The life threatening heat in sw-mode training enforces a behavioral modification that under natural conditions would be useless in expressing pattern, color or temperature preferences. After training in fs-mode the conditioned pattern or color preference does not have sufficient impact to also induce this strange restriction of the yaw torque range. It is considered more important that the memory template acquired during training in the sw-mode is sufficiently independent of the operant behavior by which it was mediated, to still be measurable in an entirely different behavior. Likely, the same



**Fig. 6:** Summary diagram presenting the results of all transfer experiments. A – patterns as visual cues. B – colors as visual cues. Experimental design is schematized by the 9 squares above each performance index. All experiments are divided in 2min test or training periods, except in A.IV-VI where 1 minute periods are used. Familiarization training is always 60s. Statistics were performed as a Wilcoxon matched pairs test against zero: \* -  $p < 0.05$ ; \*\* -  $p < 0.01$

process as in classical conditioning is at work in the operant sw-mode procedure. They both result in one or two memory templates with different ratings on an attraction/avoidance scale. The orientation behavior at the flight simulator has access to these templates. This result holds across different sensory cues (CSs: colors and patterns) and across slightly different training procedures (4 minutes of pattern vs. 8 minutes of color sw-mode training).

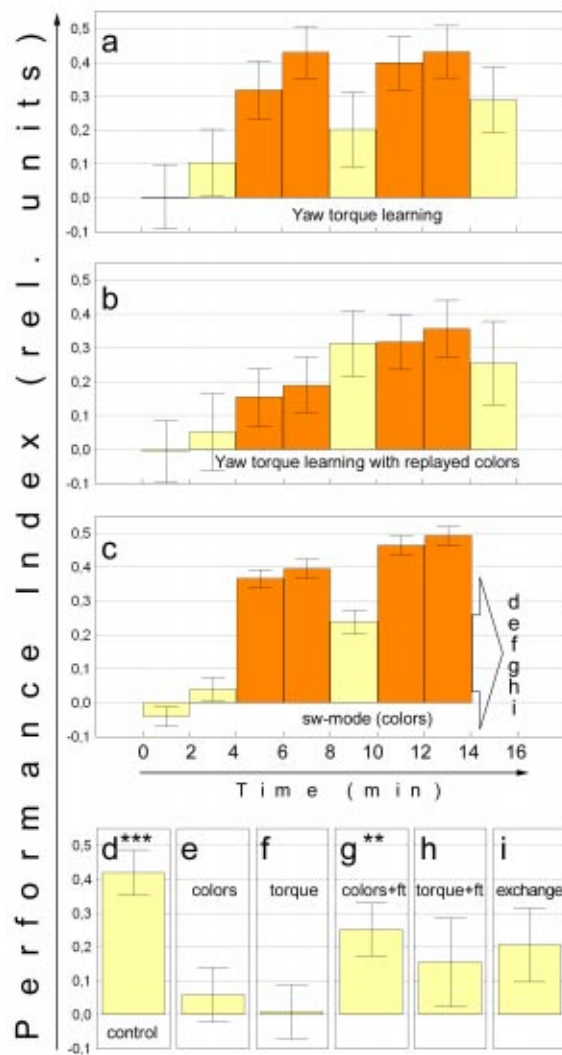
### 3.3 Flies do not learn motor patterns independently of color memory

In a three term contingency, there are always at least two predictors that can be used to predict reinforcement, the CS and the B. In operant pattern learning, yaw torque (B) does not have the same relation to the US as the patterns. While reinforcement is always switched on when the flight direction changes from the unpunished to the punished pattern, the same flight maneuvers (i.e. yaw torque modulations) may lead to both

US onset and offset. Therefore Brembs (1996) looked for higher-order behavioral strategies as evidence for B-US or B-CS associations. To find out whether the operant associations are still not formed when both B-US and CS-US are equilibrated for their predictive value and to maybe shed more light on the asymmetry of the results from the transfer experiments described above, a modified overshadowing (for an original overshadowing experiment see below and INTRODUCTION) experiment was performed. In sw-mode training, every switch from one yaw torque domain into the other changes both the color of the arena illumination and reinforcement. Thus both yaw torque domain (B) and colors (CS) are share the same predictive value for the US. Isolating the single B-US and CS-US associations after sw-mode training by either replacing the switching color filters with continuous arena coloration or by replacing yaw torque domain with flight direction (i.e. fs-mode), respectively, in the subsequent test phase will reveal the amount of associative strength accrued to the single predictors. Since one part



(the test for behavior-independent CS-US association) is also part of the transfer experiments, the data have been incorporated into Fig. 7: color



**Fig. 7:** A modified overshadowing experiment with yaw torque and colors as elements of the compound. a – Yaw torque learning, arena coloration BG18. N=30. b – Yaw torque learning with arena coloration recorded from the flies used in c and played back for the first 14 minutes of the experiment. The last test was performed using BG18 as constant color filter. N=30. c – Pooled sw-mode data of all flies tested for individual associations. The final 2 minute test periods of the sub groups in this experiment are depicted in d-i. N=250. d – Sw-mode control. N=70. e – Test for color learning in fs-mode. No familiarization training. N=22. f – Test for torque modulation. The color filters have been replaced by a BG18 filter. No familiarization training. N=73. g – Test for color learning. 60s of fs-mode familiarization training after sw-mode training prior to testing (not shown). N=23. h – Test for torque learning. 60s of familiarization training with BG18 after sw-mode training prior to testing (not shown). n=30. i – Conflicting predictors. Colors and yaw torque range contingencies have been reversed. Reversal was such that positive scores would indicate correct yaw torque modulation and negative scores correct choice of colors. N=32. Statistics were performed as a Wilcoxon matched pairs test against zero: \*\*\* - significant at  $p < 0.001$ ; \*\* - significant at  $p < 0.01$ . Orange bars – training, yellow bars – test.

memory can be retrieved in fs-mode after a 1 minute familiarization training (Fig 7e, g). In contrast, removing the color filters after sw-mode training abolishes yaw torque modulation completely (Fig. 7f,  $p=0.706$ , Wilcoxon matched pairs test) and cannot be restored by a 1 minute familiarization training (Fig. 7h,  $p=0.141$ , Wilcoxon matched pairs test). This effect is not due to a visual context generalization decrement, as the removal of switching colors without relation to the fly's behavior does not disrupt performance (Fig. 7b,  $p < 0.05$ , Wilcoxon matched pairs test). As this treatment does not lead to significantly different learning scores than regular yaw torque learning (Fig. 7a;  $p=0.871$ , Mann-Whitney U-test), these data were pooled and compared to the sw-mode control group (Fig. 7d). Although there is a tendency for sw-mode to yield higher PI's than yaw torque learning and the subjective impression upon observing the experiments is that sw-mode training is easier to accomplish than pure yaw torque training, this effect fails to reach significance ( $p=0.121$ , Mann-Whitney U-test). The reversal of the contingencies between yaw torque domain and color (Fig. 7i) is arranged such that positive learning scores indicate a dominance of yaw torque over colors and vice versa. The tendency for yaw torque to dominate over colors if both are arranged in a reversed contingency (as compared to the training), fails to reach statistical reliability ( $p=0.085$ , Wilcoxon matched pairs test).

### 3.4 Properties of associative stimulus learning in flies

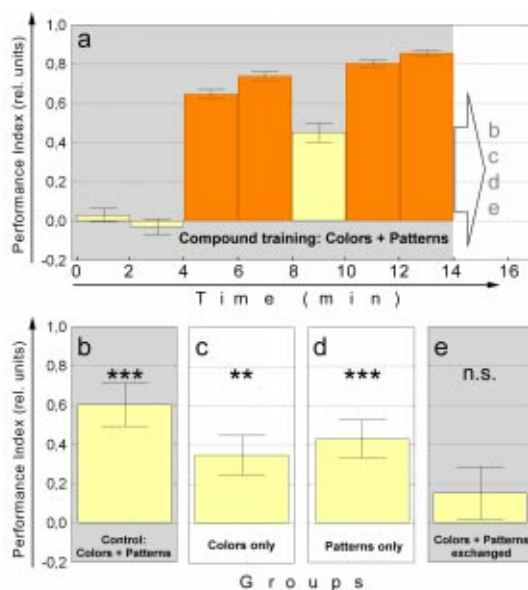
It has been shown earlier that *Drosophila* readily learns to distinguish different visual patterns in an operant learning paradigm (Wolf and Heisenberg, 1991). Using a similar setup, Wolf and Heisenberg (1997) later demonstrated that flies also can use switches between two colors at certain arena orientations as 'landmarks' and can be trained to avoid and prefer arena orientations associated with one or the other color. Not unexpectedly, *Drosophila* also learns colors and patterns if these are presented as compound stimuli (Fig. 8a, b). Again, the question is which of the two possible CS-US associations are formed? Is one association dominant over the other as in the modified overshadowing experiment described above, where the two associations were B-US and CS-US, respectively? This question is tackled in exactly the same way as in the experiment described above: isolation of the single associa-



tions by removing either one of the stimuli after compound training (CS1+CS2+US).

### 3.4.1 No ‘overshadowing’

If the fly during training is presented a compound CS of colors and pattern orientations and is subsequently tested with the component stimuli separately, four different results are theoretically possible. (1.) The fly might consider both components inadequate predictors of the US; it might use (2.) only the colors, (3.) only the pattern orientations, or (4.) colors as well as pattern orientations. Four groups of flies are arranged that all received 8 min of compound training during the first 14 min of the experiment (Fig. 8a). In a subsequent 2 minute test phase, the first (control) group is scored for the compound (Fig. 8b). The second and third groups are provided with colors alone (Fig. 8c) and patterns alone (Fig. 8d), respectively (experimental groups). The fourth group is presented a new compound



**Fig. 8:** Overshadowing experiment with patterns and colors as elements of the compound. a – Pooled compound training data of all flies used in this experiments. The last 2 minute test period of each subgroup is depicted in b-e. N=103. b – Compound control. N=25. c – Colors alone. T-Patterns have been replaced by 4 vertical bars. N=28. d – Patterns alone. Color filters have been removed. N=25. e – Nonsense compound. The contingencies between patterns and colors have been reversed such that positive scores would indicate correct color choice and negative scores correct pattern choice. N=25. Statistics were performed as a Wilcoxon matched pairs test against zero: \*\*\* - significant at  $p < 0.001$ ; \*\* - significant at  $p < 0.01$ . n.s. – not significant. Orange bars – training, yellow bars – test, shaded areas – compound stimulus, blank areas – single stimulus.

in which the contiguity between colors and patterns is reversed (Fig. 8e). This reversal is arranged such that positive learning scores would indicate a dominance of colors over patterns and a negative score the opposite.

A Kruskal-Wallis ANOVA test over all four groups reveals a significant difference between groups ( $p < 0.006$ ), encouraging a more detailed analysis. The control group (Fig. 8b) shows a large performance index. The difference between the control group and the group tested with the reversed pattern/color contiguity is highly significant (Fig. 8e;  $p < 0.003$ , Mann-Whitney U-test). Moreover, a Wilcoxon matched pairs test confirms that the learning scores for the control group and both experimental groups are significantly different from zero ( $p < 0.001$  – control,  $p < 0.005$  – colors alone,  $p < 0.001$  patterns alone), whereas the reversed color/pattern contiguity did not lead to a significant performance index ( $p = 0.23$ ). The two overshadowing groups do not differ significantly from each other ( $p = 0.47$ , Mann-Whitney U-test), but the group that was presented colors alone (Fig. 8c) differs significantly from the control group ( $p < 0.006$ , Mann-Whitney U-test). The difference between patterns alone and control just fails to reach significance ( $p = 0.07$ , Mann-Whitney U-test). One can thus conclude that presenting the individual stimuli alone after binary compound training of patterns and colors in the *Drosophila* flight simulator leads to intermediate, but nevertheless significant learning scores that do not differ from each other. Consequently, it is in principle possible for two predictors to accrue the same amount of associative strength and the results showing overshadowing when a stimulus is compounded with a behavior to form a composite predictor (see above) are not due to a general process always selecting only one out of the possible predictors. This result is also essential for obtaining interpretable results in the experiments below.

Once it is clear that none of the two CSs ‘overshadows’ (Pavlov, 1927) the other, one might assume that there never is any interaction between two stimuli at all, i.e. that the components of a compound stimulus gain or lose associative strength independently with reinforcement or nonreinforcement of the compound (Hull, 1929; Spence, 1936). To test this assumption, blocking, second-order conditioning (SOC) and sensory preconditioning (SPC) experiments were carried out.

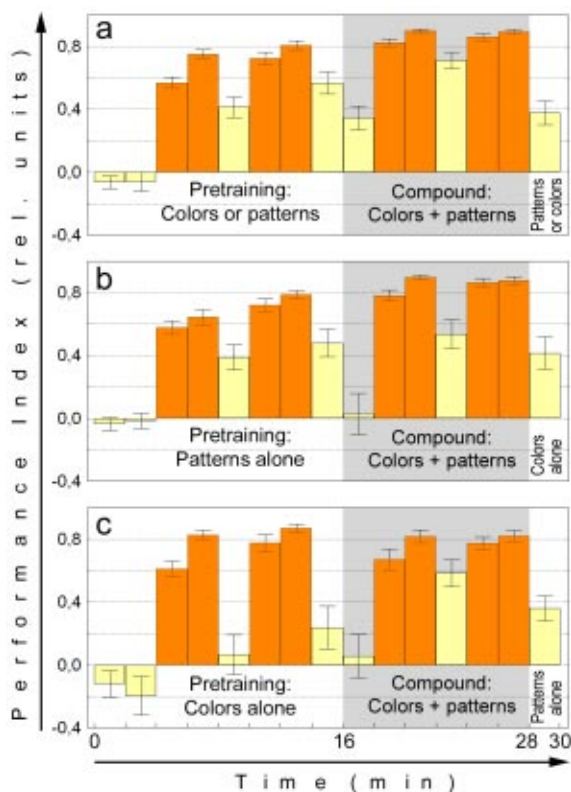
### 3.4.2 No blocking

Two blocking experiments were performed that differed in the amount of compound training and the choice of control procedures (see MATERIAL AND METHODS). As the outcome is essentially the same, only one of them is presented here in detail (Fig. 9). In this experiment, the final test during the pretraining phase and the carry-over in the first compound test phase of the blocking group do not differ between the two half experiments (pretraining colors and pretraining patterns) ( $p=0.08$ ; between groups effect in a repeated measures ANOVA over both periods and both half experiments). Therefore, these two half experiments are presented together (Fig. 9a). The same evaluation yields a significant within groups effect ( $p<0.008$ ), indicating the difference between the last test during pretraining and the carry-over in the first compound test phase to be statistically reliable. The corresponding control half experiments (Fig. 9b, c) were not pooled, because two different procedures were used for the first training phase (see

legend to Fig. 9 and MATERIAL AND METHODS).

In vertebrates several criteria have been found to be crucial for blocking. One is the equivalence of the two stimuli - i.e. no or little overshadowing should occur. This criterion was shown to be met in the present case (Fig. 8). Another essential criterion is the high predictive value of the pretrained stimulus. In operant conditioning it is not possible to verify a predictive value of 100% of a stimulus as there is no reflex-like relation of a response with a stimulus. Rather, the animal exhibits active behavior and controls its stimulus situation by trial and error (for a discussion of operant behavior and initiating activity see Heisenberg, 1983; Heisenberg, 1994). Therefore, pretraining is performed until an asymptotic level of performance is reached. Prolonged operant pattern learning determined this level to be reached after 4x2 minutes of training (see Fig. 4). This amount of training is used in the compound phase for the blocking experiment presented here (Fig. 9a-c).

The relevant difference between the experimental and control groups is the carry-over from the performance index in the last test period of the first training to the first test with the compound stimulus. In the experimental group (Fig. 9a) this carry-over should be large (i.e. the generalization decrement should be small), indicating that the reinforcer is well predicted by the compound. In contrast, there should be no significant performance index in the control groups (Fig. 9b, c). A Wilcoxon matched pairs test confirms that the control animals were naive to the compound ( $p=0.79$ ), whereas the performance index in the experimental group is highly significantly different from zero ( $p<0.0002$ ). Moreover, comparing the intermediate test period during the compound training phase between experimental and control groups, the experimental group still shows better avoidance than the control groups ( $p<0.045$ , Mann-Whitney U-test), indicating that the US was still better predicted in the blocking than in the control group. Despite the fact that all requirements for blocking seem to have been met, the final learning score is indistinguishable between the experimental and control groups ( $p=0.77$ , Mann-Whitney U-test) giving no indication of blocking. The same holds true for the second experiment in which the compound training phase was reduced to 4 minutes and the control groups were spared the first training phase with the single CS (CS1+US) in order to exclude any possible predictive value of US experience (data not shown).

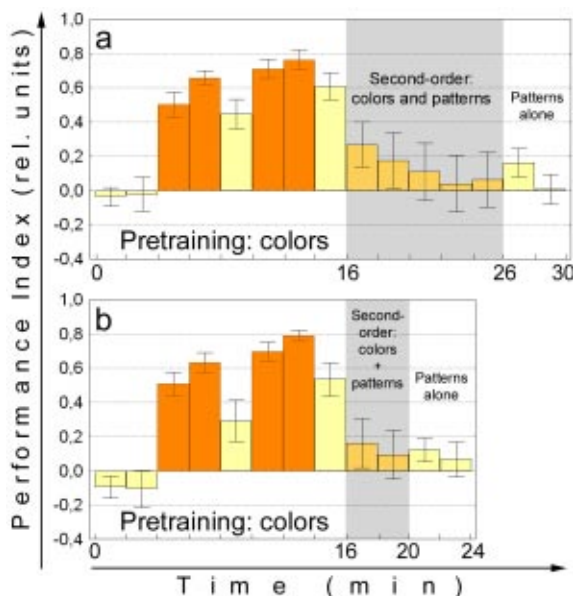


**Fig. 9:** A representative blocking experiment. a – Pooled half experiments with colors and patterns (with BG18 filter) during pretraining, respectively.  $N=53$ . b – Control group with patterns (white light) during pretraining.  $N=27$ . c – Control group with colors (replay) during pretraining.  $N=26$ . Orange bars – training, yellow bars – test, shaded areas – compound stimulus, blank areas – single stimulus.

### 3.4.3 Second-order conditioning

SOC is very similar to a blocking experiment. Again, after the training with the single stimulus (CS1+US) the compound is presented. However, compound presentation is not accompanied by reinforcement (CS1+CS2). In the training phase, CS1 is supposed to acquire the response eliciting properties of the US and might therefore be able to serve as a second-order US for CS2 during the compound presentation. However, the presentation of the compound without heat after the conditioning may lead to extinction of the learned association attenuating the CS1-US association. It has been shown above that in our paradigm operant learning needs considerably less training than classical learning (Fig. 4). Given the equivalence of the acquisition and extinction time constant, one might expect extinction to be very fast in our operant setup as well. Additionally, extinction might even be facilitated by the second stimulus (CS2) signaling non-reinforcement of the compound (CS1+CS2). Despite these considerations the test for SOC was carried out.

Only colors were used as CS1 assuming color might be a better second-order US than pattern orientation. The experiment was performed twice, with 10 and 4 minutes of second-order training. Both yield only small second-order learning effects that are statistically reli-

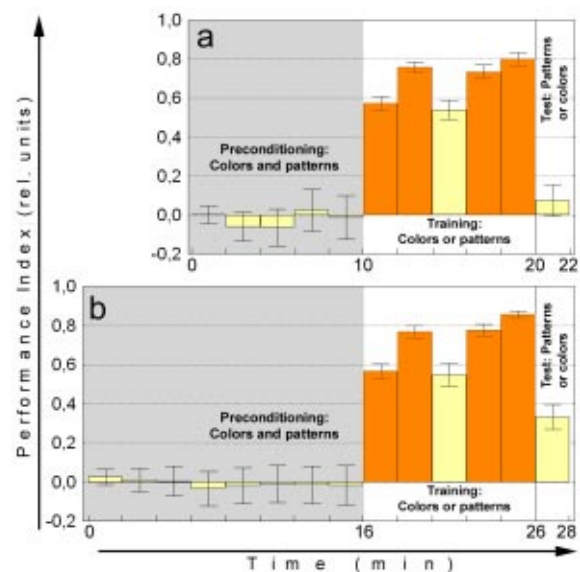


**Fig. 10:** Second-order conditioning. a – Second-order experiment modeled after the first blocking experiment (depicted in Fig. 7). N=20. b – Second-order experiment modeled after the second blocking experiment (not shown). N=22. Orange bars – training, yellow bars – test, yellow-orange bars – second-order training (no heat), shaded areas – compound stimulus, blank areas – single stimulus.

able only if the PIs of the two experiments are pooled ( $p < 0.02$ ; Wilcoxon matched pairs test;  $p = 0.08$  for both experiments separately). The steep extinction curve is the most likely explanation for the small second-order learning effect. Already in the first 2-min period of the second-order training phase avoidance of the color previously combined with heat is down to about  $PI = 0.2$ , from  $PI = 0.6$  for color alone after the initial training. Again only pooling the data of the two experiments (Fig. 10a+b) yields a statistically significant difference to zero ( $p < 0.02$ , Wilcoxon matched pairs test). For the second 2-min period, even pooling the two experiments fails to produce a statistically reliable performance index ( $p = 0.15$ , Wilcoxon matched pairs test). Taken together, a small SOC effect was found that is small presumably because the CS-US association is rapidly extinguished during the second-order conditioning phase.

### 3.4.4 Sensory preconditioning

Formally, SPC is the temporally reversed analogue of SOC. In SPC the exposure to the compound (CS1+CS2) precedes the training (CS1+US). Hence, no extinction can occur between training and test. Flies are exposed to 16 minutes of unreinforced flight at the flight simulator in an arena where flight directions are designated by compound stimuli consisting of colors and patterns (CS1+CS2). If immediately afterwards one of the stimuli is paired with heat (CS1+US), even the other one (CS2) is regarded



**Fig. 11:** Sensory preconditioning experiment. Each half experiments have been pooled. a – 10 minutes of preconditioning. N=56. b – 16 minutes of preconditioning. N=56. Orange bars – training, yellow bars – test, shaded areas – compound stimulus, blank areas – single stimulus.

as a predictor of safe and dangerous flight orientations in the subsequent test (Fig. 11b). No statistically significant learning score is observed in the final test with only 10 minutes of preconditioning (Fig. 11a). The difference between the learning scores after 10 and 16 min is statistically

significant ( $p < 0.01$ , Mann Whitney U-test). In each of the two experiments (Figs. 11a, b) the two half experiments (using colors or patterns as CS1, respectively) yielded statistically indistinguishable results, justifying the pooling of the corresponding data sets.

## 4 Discussion

Composite learning tasks consist of a three term contingency between the organism's behavior (B), the perceived stimuli (CS) and the reinforcer (US). In this study, the unique advantages of the *Drosophila* flight simulator allowed for a comprehensive investigation into the contributions of the single associations to a composite learning task. The central question in this study is: what associations are formed in *Drosophila* at the torque meter? In the replay experiment, the effect of operant behavior (B-US and B-CS associations) in operant pattern learning (a composite task) was assessed. In the transfer experiments, the possible formation of genuinely classical (i.e. behavior independent CS-US) associations during composite operant conditioning was examined. This was accomplished by developing a new composite learning paradigm, sw-mode. Using this new paradigm, the impact of the classical (CS-US) associations on learning performance in a composite learning situation was investigated. Finally, the question what associations are formed if more than one CS-US association is allowed (i.e. the properties of visual memory acquisition in *Drosophila*) was explored for the first time in an explicitly composite learning task.

### 4.1 Contributions of single associations to *Drosophila* visual learning

#### 4.1.1 The operant component: B-CS and B-US associations

It is conspicuous that operant pattern learning (B, CS and US) leads to a significant learning score already after 8 minutes of training, whereas

it takes 16 minutes of classical replay training (CS-US) for the flies to reach a significant performance (Fig. 4a, b). On the other hand, classical pattern learning (Fig. 4c; Brembs, 1996; Wolf et al., 1998) yields learning scores as high as operant pattern learning already after 8 minutes. Estimating the amount of reinforcement by multiplying the temperature of the IR-beam with the time the flies spent in the heat, it appears that classical learning roughly parallels energy uptake during training (Table 2). Most importantly, for similar learning scores this energy uptake is considerably larger during classical than during fs-mode operant learning. Compared to the total amount of heat the distribution and duration of hot and cold periods as well as the dynamics of pattern motion seem to be of minor importance for the learning success. Thus, to reach the same learning scores for the same pattern recognition task, composite operant conditioning requires less reinforcement than classical conditioning.

What makes the operant training more effective than the classical one? In principle, the operant behavior may either act during association *acquisition*, support memory *recall* or exert its effect in both phases. In the first case, only pattern memory (i.e. the CS-US association) is formed, if there are no behavioral (i.e. B-CS or B-US) associations detectable during retrieval. This genuinely classical association formation must then be facilitated by operant behavior as it takes more training to form during classical training. In the second case, there are additional operant (B-US or/and B-CS) associations formed that act additively on memory recall. A first investigation (Guo et al., 1996) found experience in the flight simulator prior to training (i.e. B-CS associations) to positively affect learning scores after training. In the present setup, however, some of these results could not be reproduced (data not shown), most likely due to the smaller

Type			Time in the heat	Est. Energy	PI	N
<b>composite</b>	operant	fs-mode	0.7min	41.1	0.45	30
	operant	ext. fs-mode	1.5min	85.7	0.47	30
	operant	sw-mode	2.0min	118.0	0.42	70
<b>pure</b>	operant	torque learning	2.4min	140.5	0.29	30
	classical	yoked control	0.7min	41.1	0.15	30
	classical	ext. yoked control	1.5min	85.7	0.25	30
	classical	rotating classical	4.0min	168.0	0.43	36

**Table 2:** Reinforcement times and estimated energy uptake in operant and classical conditioning at the flight simulator. Data have been pooled from both my diploma and my doctoral thesis. In the operant training, the time each fly spent in the heat was calculated from the individual avoidance scores. The amount of energy taken up by each fly (in relative units) was estimated using the temperature measured at the point of the fly and multiplying it with the time the fly spent in the heat. PI - learning test after the last training. N - number of flies.



coupling coefficient (see MATERIALS AND METHODS) between yaw torque and angular arena speed used here. Anyway, if there were any B-CS or B-US associations contributing additively to the CS-US association to improve pattern learning in fs-mode, a comparative analysis of the micro-behavior prior to training and both after operant and classical training should reveal any such effects. The evidence from Brembs (1996) provides not the least support for this alternative. Admittedly, these negative results do not entirely preclude that such differences still exist, hidden in the temporal fine structure of yaw torque modulations. However, as the advantage of the operant training is large (Fig. 4) one would expect the behavioral strategy providing it to show saliently in the torque traces.

#### 4.1.2 The classical component: CS-US associations

For the other option – facilitation of CS-US association acquisition – however, there is positive evidence from the transfer experiments (Fig. 6). The finding that the fly establishes pattern and color preferences while being engaged in one behavior (sw-mode) and later displays them by a different behavior (fs-mode) supports the notion that conditioned preferences are behavioral dispositions (central states) rather than modified motor patterns (for a general discussion of behavioral dispositions see Heisenberg, 1994). The necessity of a familiarization training slightly weakens this conclusion. In principle, the 60s familiarization training in the particular situation after the switch mode could be sufficient to generate the preferences anew, despite the fact that without the preceding sw-mode training it is not. This interpretation is considered unlikely and, instead, the view is favored that recall of the memorized ‘classical’ association is dependent not only on the sensory but also the behavioral context. In other words, an association might be easier to recall in the behavioral state in which it was acquired than in a different behavioral situation. The asymmetry in the transfer experiments between fs-mode and sw-mode is one of three conspicuous asymmetries that receive in-depth treatment below.

#### 4.1.3 Is the operant equivalent to the classical component?

Rescorla (1994) suggested that the behavior of the animal might compete with the sensory signals in the animal's search for a predictor of

the reinforcer. Unsuccessfully searching for temporal contingencies between motor output and the reinforcer could reduce the efficiency of the CS-US association formation in classical conditioning. Conversely, successful behavioral control of the CS and the reinforcer may increase the acquisition process. Could this be a symmetrical effect? Maybe the efficiency of B-US association formation is also reduced if the animal is searching unsuccessfully for a temporal contingency between a sensory stimulus and the reinforcer? In other words, does a composite operant experiment such as sw-mode yield better learning than a ‘purely’ operant one as yaw torque learning? Although there is a clear and repeated tendency to show lower learning and lower avoidance scores if color changes are not related to the fly's behavior (Fig. 7b) than when they are (Fig. 7c, d), large variation in these comparatively artificial and difficult experiments prohibits this tendency from being statistically reliable. This is the second of the conspicuous asymmetries that deserve special treatment (see below).

Thus, a facilitating effect of adding components to form a three term contingency has been shown only for the fs-mode (Fig. 4). A replay experiment for the switch mode is still in progress. Research in this direction has been hampered by the condition of our present fly stocks. For unknown reasons, the flies in our department to date show weakened classical learning while operant conditioning seems unaffected. It is probable, however, that also in sw-mode training there is a facilitating effect of operant behavior: in sw-mode training flies are exposed to the heat only for half as long as during classical conditioning and take up roughly 70% of the estimated energy, yet they reach about the same learning scores ( $PI=0.4$ ).

In fs-mode the fly can not modify a motor program (i.e. form a simple B-US association) according to experience - the motor programs used for choosing certain orientations are all the same whether the orientations are associated with the heat or not. In sw-mode, however, it explicitly has to do so in order to solve the learning task. Moreover, it can learn to modify its yaw torque even without the aid of external stimuli in yaw torque learning. Nevertheless, flies learn to discriminate the visual cues which is demonstrated by a transfer to fs-mode (Fig. 6). Even more surprisingly, this learning seems to block the B-US association that would be formed when the visual cues were not related to the fly's behavior (Fig. 7b). This is another finding corroborating the proposition of Wolf and Heisenberg

(1991) that it is important to distinguish between operant activity and operant conditioning. While operant activity controls biologically important stimuli, operant conditioning is an after-effect of operant activity that need not always follow operant behavior. The fact that the same operant behavior (controlling heat with yaw torque) in one case (Fig. 7b) leads to a lasting modulation of that behavior, whereas in the other (Fig. 7f) it does not is exemplary for this distinction. The effect of stimuli preventing B-US associations to form is also reminiscent of the ‘overshadowing/blocking of a response-reinforcer association’ found in vertebrates (Williams, 1975; Pearce and Hall, 1978; Williams, 1978; St. Claire-Smith, 1979; Williams et al., 1990) including humans (Hammerl, 1993). Those experiments trained rats in an experimental chamber to press a lever several times (B) to obtain a reward (US) after a certain delay. Barpressing (i.e. the B-US association) was found to be reduced when each reinforcement was signaled by a stimulus (CS). In these cases, however, the decrement in operant performance is not surprising as the stimulus always has better predictive value for the reinforcer than the behavior: while not every barpress leads to reinforcement, every stimulus presentation is followed by reinforcement. To my knowledge there is no vertebrate study where behavior and stimulus have been equilibrated for their predictive value. In sw-mode training, however, the contingencies are perfect: every yaw torque sign inversion leads to a change in arena coloration and in temperature. Thus, this profound difference between single and multiple association learning tasks was discovered here for the first time. Why does yaw torque seem to lose its associative strength when it can be used equally well as a predictor for reinforcement as the colors? Possibly the B-US association is not really absent, but maybe weaker and/or incorporated into a new association. One could imagine a sequential B-CS-US association or a hierarchical (B-CS)-US association. After reversing the contingencies between yaw torque domain and colors (i.e. inverting the B-CS relation), the behavior is not modified to avoid the punished color. Instead no yaw torque domain (or color) seems particularly preferred. If at all, a tendency to show the correct yaw torque modulation and to disregard the colors can be observed (Fig. 7i). This fact entices to speculate that the flies may use the instant where the arena illumination changes color as a ‘landmark’ to signal yaw torque sign inversion (and hence reinforcement), without memorizing any particular

color with the heat [i.e. a (B-CS)-US association with the color-switch as CS]. This consideration also sheds more light on the need for a familiarization training when the CS-US component is tested in fs-mode, as the flies might use the familiarization training as a signal for which ‘side’ of the color switch is reinforced in the new situation. Thus it seems that also the B-CS association contributes to the learning process. Guo et al. (1996) have shown that such processes can indeed occur at the *Drosophila* flight simulator and that they increase the performance indices. There is evidence that such B-CS learning occurs also in the absence of overt reinforcement (Guo et al. 1996; Brembs, 1996). Such reinforcer independent motor learning could be understood as a basic behavioral tuning mechanism that probably occurs continuously without being much noticed. As it seems not to be specific to the learning tasks examined in this study, it will not become a major focus of this work. In summary, it is conspicuous that behaviors and stimuli are apparently not treated as equivalent predictors of reinforcement (Fig. 7). This consideration will be discussed at length with the other two asymmetries:

#### 4.2 Three conspicuous asymmetries

It was mentioned in the INTRODUCTION that a formal analysis of the three term contingency suggests a symmetrical relation between the components of a composite learning task. Therefore, the three asymmetries in the association analysis after sw-mode and fs-mode training deserve special attention. (i) Why can a color or pattern memory be transferred from sw-mode to fs-mode but not vice versa (Fig. 6)? (ii) If a familiarization training can reveal a single CS-US association out of a seemingly combined association after sw-mode training, why can the same familiarization training not do the same with the B-US association (Fig. 7)? (iii) Why is a composite operant procedure more effective than a simple classical (Fig. 4), but not more effective than a simple operant task (Fig. 7)?

(i) Obviously, although both sw-mode and fs-mode take place at the torque meter in the same arena and involve operant behavior, they are entirely different. While in fs-mode the choice of flight direction and between the two temperatures depends on the ability to fly straight and, above that, upon a sequence of discrete, well timed orienting maneuvers, in sw-mode it is the actual value of the fly’s yaw torque

that controls this choice. Moreover, while in fs-mode the fly receives instantaneous feedback on the effect its behavior has on its stimulus situation in sw-mode it can only get this feedback at the point where the experimenter decides to invert the sign of the torque trace. Evidently, fs-mode is less artificial than sw-mode. It is thus easily appreciated that the CS-US association formed in classical pattern learning can be expressed in the fs-mode test without familiarization training (Brembs, 1996; Wolf et al., 1998). Judging from the transfer experiments, one would predict this to be more difficult when the test after classical conditioning were in sw-mode. If that were so, it would corroborate the conclusion from the transfer experiments that in principle operant pattern or color learning facilitates a behavior independent CS-US association and the familiarization training is necessary to overcome contextual effects. One might expect more familiarization training to bring out this association also in the fs-mode to sw-mode transfer (Fig. 6 column III).

(ii) Similarly, one might predict that more familiarization training might bring out the B-US association upon removal of the color filters after sw-mode training (Fig. 7i). The difficulty, on the other hand, to modify yaw torque without reinforcement (or without exactly the same three term contingency as during reinforcement, see Fig. 6 columns II, III, Fig. 7f, h, i) may also indicate that behaviors and stimuli can not be regarded as equivalent (i.e. equally salient) predictors of reinforcement but that there may be a preference to rather add stimuli to a predictor than a behavioral modification. Wolf and Heisenberg (1991) have shown operant behavior to flexibly and very quickly adjust the fly's stimulus situation according to its desired state. Reducing its behavioral options more permanently in anticipation of reinforcement may be an animal's last resort. In other words: The experiment depicted in Fig. 7 can be perceived as an overshadowing experiment (Fig. 8), where one of the elements in the compound is a behavior (yaw torque modulation) and the other is a stimulus (colors). In this case, stimulus learning (CS-US) overshadows behavioral learning (B-US). Overshadowing is usually described as the difference in the associabilities  $\alpha$  of the two components in equation (2) - the delta rule. Such a difference may be caused by different stimulus intensities. Alternatively, the animal may be predisposed by phylogenetic or individual experience to regard one component as a better predictor of the US than the other. One is inclined to generalize

asymmetry (ii) as a difference in associability between behavioral (B) and sensory (CS) predictors of reinforcement (US) if both are available.

(iii) It is surprising that a 'pure' operant conditioning task as yaw torque learning should be just as efficient as a composite task as sw-mode learning (Fig. 7), while classical pattern learning is less efficient than operant pattern learning (Fig. 4). Why should one single association task be less efficient than a composite task while the other is not? For one, from observing the animals one would strongly expect single (i.e. either only CS-US or only B-US) association tasks generally to be less efficient than composite experiments. Second, comparing the amount of heat uptake during training in the various procedures used in this study (Table 2) one can see that torque learning requires roughly the same amount of reinforcement as classical conditioning, while sw-mode training is slightly less efficient than fs-mode learning but still leads to less energy uptake than classical training. Thus, the relatively small difference between yaw torque learning and sw-mode learning can be attributed to the difficulty and artificiality of the sw-mode learning task.

### 4.3 A hierarchy of predictors

Apparently, once both stimuli and behaviors with similar predictive value are available during training, they are added to a three term predictor (operant and classical associations). Once one of the three relations is altered, it takes special treatment (familiarization training) to reveal the remaining associations. In contrast to the considerations above (see INTRODUCTION), the individual associations are not equivalent: The amount of familiarization training seems to vary with the component of the three term contingency. Components with high associability (i.e. easily learned) need less familiarization training than those with low associability. Compiling the data so far, one can postulate a hierarchy of predictors. Operant behavior occurring during composite operant conditioning should hardly be conditioned at all (Brembs, 1996, Fig. 7). Classical stimuli that bear no relation to the behavior of the animal should be of intermediate associability, as is operant behavior alone (Figs. 3, 5, Table 2). The relative associability of behaviors or stimuli alone most probably depends on the choice of stimuli/behaviors. Stimuli that are controlled by operant behavior should accrue associative strength most easily, whether the

direct B-US can be formed or not (Figs. 3, 5). It would be most interesting to test these predictions in other animals including humans.

#### 4.4 Properties of visual memory acquisition in *Drosophila*

Having established the paramount significance of the CS-US association in composite operant conditioning, the flight simulator is used for the first time to methodically examine the properties of this single association within an explicitly composite learning situation. Operant visual learning of *Drosophila* at the flight simulator (i.e. a composite task including B = choice of flight direction via yaw torque, CS = colors and patterns and US = heat) is explored using compound stimuli as CSs. Thus, there are two CS-US associations possible and again the question arises, which associations are formed and whether the relation between them is symmetrical. The overshadowing experiment shows that flies acquire, store and retrieve the two CSs 'colors' and 'patterns orientations' separately. They do not store them only as a compound. Whether they can distinguish the compound from the sum of the components ('configural learning') has not yet been investigated. In contrast to the similar experiment depicted in Fig. 7 this experiment did not reveal any differences in associabilities between the elements of the compound (Fig. 8c, d) and no familiarization training was necessary. Note that in discrimination learning each of the component CSs consists of a CS<sup>+</sup> and a CS<sup>-</sup> (blue and green; upright and inverted T). Dwelling time analysis seems to indicate that for colors and patterns both the CS<sup>+</sup> and the CS<sup>-</sup> are remembered (data not shown). This brings the number of simultaneously stored memory items up to four. Further investigating into the associations formed whenever more than one CS-US association is enabled, blocking, second-order conditioning (SOC) and sensory preconditioning (SPC) experiments are carried out. As these experiments were inspired by the successful development of quantitative learning rules in vertebrates, the results obtained here are compared to vertebrate conditioning data.

##### 4.4.1 Blocking and second-order conditioning

As the associabilities of the two stimuli CS1 and CS2 are generally equal in a blocking experiment (see INTRODUCTION), the difference in associative strength after conditioning has to be

due to the reinforcement term of the delta rule. If CS1 was trained to predict the reinforcer to 100%, the value for the delta rule equals zero and reinforcement is no longer effective ( $\lambda - \bar{V} = 0$ ). However, associability need not be a constant variable, but might change with conditioning experience as well. In the model proposed by Pearce and Hall (1980) the associability of a stimulus is proportional to  $\lambda - \bar{V}$ , while the reinforcement remains constant. In a blocking experiment  $\lambda - \bar{V} = 0$ , therefore, CS2 is not associated with the US. Both explanations have in common that the amount of blocking is crucially dependent on the degree to which the pretrained stimulus is recognized in the compound as a predictor of reinforcement. As there is evidence for both associability changes (Holland, 1997) and for changes in reinforcement processing (Schultz, 1995; Kim et al., 1998), one might suspect that both kinds of explanation are not mutually exclusive. Indeed, Holland (1997) suggests that processes modifying both associability and reinforcement are at work. The overshadowing experiment ensured that the two stimuli do not differ in associability ( $\alpha$ ) without prior conditioning (Fig. 8). Nevertheless, blocking could not be detected.

In the present experiments the key conditions allowing blocking to occur have been met. Control and experimental groups differed in the predictive value of the compound (Fig. 9). The first training phase caused neither overshadowing nor a large SOC (possibly masking a blocking effect), as the experiments in Figs. 8 and 10 show. Nevertheless, despite varying compound training and control procedures (see MATERIALS AND METHODS) no blocking effect could be detected. While this is one more piece of evidence that blocking might be absent in invertebrates, let us first consider potential other explanations why blocking was not found in this study.

There are two main basic reasons for blocking not to show up using the flight simulator design: (1) Either some components of the setup or the choice of stimuli principally interfere with an otherwise detectable blocking effect, and/or (2) blocking can not be obtained using the experimental time course used here.

(1) It is argued above that visual learning at the flight simulator is a case of classical learning in which the operant behavior facilitates CS-US acquisition. Although it is considered unlikely it can not be excluded that the operant aspect or any other property of the flight simulator paradigm interferes with blocking (see

INTRODUCTION). It could be that the high degree of operant control of the stimuli prevents blocking of redundant stimuli. The extreme behavioral restriction of the tethered animal or the particular choice of stimuli and feedback conditions could be prohibitive as well. Bitterman (1996) argues that blocking can only be shown within and not between modalities (Couvillon et al., 1997). Colors and patterns might be similar to two modalities. It can not be ruled out, but is also considered rather unlikely that any existing, small blocking effect could be masked by the equally small SOC effect.

(2) More importantly, though, the failure to obtain blocking could be due to a significant generalization decrement of the learning upon introduction of the second CS in the compound phase (Fig. 9a). The same rapid extinction of the generalized learning is observed in the SOC experiments (Fig. 10). This quick decay of the memory effect may continue in the presence of the US in the blocking experiment, attenuating the predictive value of the CS1 enough to make the flies near to naive even in the shorter (not shown) blocking experiment. In this case the compound stimulus (CS1+CS2) might be sufficiently 'surprising' (i.e. the value for the delta rule might be sufficiently large) for the new stimulus (CS2) to acquire associative strength. A more extensive (maybe spaced) pretraining (CS1+US) together with other technical measures should decrease the generalization decrement as well as minimize extinction. While it is reassuring that the SOC effect in this study is too small to mask any *significant* blocking, this fact may indicate that the associative strength of the CS1 after the standard training procedure is too weak to serve as a sufficiently 'safe' predictor in the compound. On the other hand, the larger learning score in the intermittent compound test in the blocking vs. the control group, and the (albeit small) final learning score in the SOC experiment are difficult to reconcile with these arguments. One would at least expect partial blocking, since the compound is, indeed, better predicted in the blocking than in the control groups. As a minimal conclusion, blocking in *Drosophila* is a less reliable and robust phenomenon than it appears to be in vertebrates.

Even if there is a number of reasons why blocking might be implemented in *Drosophila* but not detected in this study, the possibility remains that invertebrates do not exhibit blocking. Even though control and blocking groups differed in the predictive value of the compound (Fig. 9), this difference might have been insuffi-

cient to reveal blocking not in terms of stimulus generalization, but on principle grounds. Maybe in invertebrates the difference 'naive-conditioned' at the beginning of compound training is not sufficient to induce a difference between experimental and control groups after the CS1+CS2+US training. Evidence that this might be the case comes from a recent study in freely flying honeybees, which currently is the only undisputed case where blocking appears to have been detected (Couvillon et al., 1997). Couvillon and coworkers (1997) pretrain CS1 as conditioned inhibitor during a discrimination training in the control group, whereas it becomes a conditioned excitator in the blocking group. Transferred to the flight simulator paradigm, this would mean that compounding CS2 with CS1 would have to initiate reversal training (e.g. punishment on the upright T in pretraining and on the inverted T in the compound phase). In other words, the difference between blocking and control groups would be maximized by the control animals not being naive (i.e.  $PI=0$  as in the present study) but showing negative learning scores. This, however, would rather indicate an enhancement of associative strength to CS2 in the control groups (i.e. a particularly large value for the delta rule) than a reduction to CS2 in the blocking group (i.e. a particularly small value for the delta rule) and would thus still not show blocking. The necessary naive control group is not shown in the Couvillon et al. (1997) study.

If the still scarce data were to interpreted as a divergence in vertebrate vs. invertebrate learning mechanisms the question is imminent: What makes this elementary property of behavioral plasticity underlying blocking different in vertebrates and invertebrates? It has been argued before that blocking might involve attention-like processes (Mackintosh, 1975b) or some concept of expectation and prediction (Rescorla and Wagner, 1972; Sutton and Barto, 1990). In humans blocking has been implicated with causal judgement (Miller and Matute, 1996). However complex the explanatory concept may be, the proposed neural mechanism (Holland, 1997; Fanselow, 1998; Thompson et al., 1998) seems simple enough to be implemented also in the less complex invertebrate brains. However, vertebrate brains (especially in the intensively studied mammals) are considerably larger than those of invertebrates. Probably their ability to quickly discern essential from redundant or otherwise unimportant events is also much better than in invertebrates. While rats in an experimental chamber might learn that in this situation the

delivery of the reinforcer depends solely on one stimulus and nothing else (especially if trained in this chamber for weeks), it appears that for an invertebrate it is more difficult to reach this level of predictive value. One may even speculate that vertebrates may reach such a high level of confidence in the predictive value of a stimulus, that they can afford to ignore redundant stimuli albeit their relation to the reinforcer. In contrast, invertebrates may rely on redundancy to compensate for a larger error-proneness of their central nervous system. It would be very important for our understanding of general brain functioning if indeed different acquisition mechanisms had evolved due to different error rates in vertebrates and invertebrates. Until a satisfying concept of error-rate and reliability of sensory input is developed, however, this idea remains speculative. On the other hand, one needs not assume basically different acquisition processes at work in vertebrates and invertebrates. Indeed the added CS2 is correlated with the reinforcer and it is a matter of cost/benefit balancing whether it is taken into the association or not. This consideration and in particular the fact that in real life there is nothing like a 100% predictor of an event makes it easy to appreciate that the different outcome of the blocking experiment in vertebrates and invertebrates (if the few existing data can be generalized in this manner) may not necessarily reflect the difference in basic mechanisms of learning but rather a difference in the variables and thresholds determining whether a stimulus with a rather small predictive value is added to the predictor or not. The ambiguity in the invertebrate blocking literature supports this view.

#### 4.4.2 Sensory preconditioning

With no blocking and no overshadowing being observed in the present experiments, the only interaction of the two components in the compound stimulus is revealed by the fact that they form a reciprocal association if presented together without reinforcer (SOC, SPC). This is obvious in SOC where the CS1 assumes the role of the US, but also in SPC the preference and avoidance of CS2<sup>+</sup> and CS2<sup>-</sup> (respectively) in the final test reveals that CS1<sup>+</sup> and CS2<sup>+</sup> as well as CS1<sup>-</sup> and CS2<sup>-</sup> have formed specific associations during the preconditioning phase. There are some earlier reports of SPC in invertebrates (Couvillon and Bitterman, 1982; Suzuki et al., 1994; Kojima et al., 1998; Müller et al. submitted). SPC can most readily be perceived as a form of ‘inciden-

tal learning’ where two equally salient stimuli are associated in a symmetrical manner (as opposed to the asymmetric relation between CS or B and the US in regular associative learning). There is ample evidence for the symmetry in this association: Simultaneous pairings show stronger effects than sequential ones in honeybees (Müller et al., submitted) as well as in rats (Rescorla, 1980; Lyn and Capaldi, 1994). Also in zebrafish Hall and Suboski (1995) successfully used simultaneous light-odorant pairings. In mammals even backward pairing leads to excitatory, rather than inhibitory associations (Hall, 1996; Ward-Robinson and Hall, 1996; Ward-Robinson and Hall, 1998). In the flight simulator, the color of the arena illumination is changed exactly between two patterns, providing neither a forward nor a backward relationship between colors and patterns either. This difference between incidental learning (for a review see Hall, 1996) and regular conditioning is no surprise as the asymmetric dependence on the temporal arrangement of CS and US in regular conditioning is reflected by the difference in biological significance between CS and US (for a review on this timing dependence see Sutton and Barto, 1990).

Dill and Heisenberg (1995) have reported one case of incidental learning at the flight simulator called ‘novelty choice’. Flies without heat reinforcement remember patterns and compare them to other patterns later. Novelty choice learning seems to be considerably faster than the preconditioning effect observed in this study. In the novelty choice paradigm a one minute exposure already biases the subsequent pattern preference (Dill and Heisenberg, 1995) while in the present experiment a ten minute preconditioning phase is not enough for a significant association to be formed. Hence, establishing a memory template for a visual pattern is a fast process whereas associating different types of sensory stimuli takes more time. The fly probably links pattern orientations and colors during preconditioning because the sudden changes in the color of the illumination are firmly coupled to certain changes in pattern orientation. To detect such coincidences the fly has to compare the temporal structure of the various sensory channels. The same mechanism has recently been postulated also for regular associative conditioning because there too the animal needs to separate the CS from the context (Liu et al., 1999). In both instances, regular conditioning and sensory preconditioning, transient storage of the incoming sensory data, as in the case of novelty choice learning, is probably a prerequisite. This depend-

ence on the amount of preconditioning is observed in rats as well (Prewitt, 1967; Tait et al., 1972), but apparently neither in zebrafish (Hall and Suboski, 1995) nor in honeybees (Müller et al., submitted). In these reports, however, even the smallest amount of preconditioning used led to SPC. It might be that using even smaller amounts of preconditioning would also uncover a gradual increase of SPC with the amount of preconditioning in these animals. Alternatively, decreasing the associability of the stimuli until SPC is lost and subsequently increasing the amount of preconditioning in these altered conditions might reveal the dependence in question.

In summary, one can propose that incoming sensory data are briefly stored to allow for a search of temporal and spatial coincidences. Memory templates with similar temporal structure are bound together and kept in storage for an additional period of time.

#### 4.5 *What is learned in a composite learning situation?*

Natural learning situations most often comprise a wealth of stimuli that are at least partly under the control of operant behavior. The number of possible associations that can be made during reinforcement is proportional to the number of stimuli. However, the number of useful predictors is always less than the total number of stimuli present at the occasion. The difficult task lies in finding the behaviors and the stimuli that will lead to proper anticipation of the reinforcing events. In the frog-bee example from the INTRODUCTION it would be most fatal for the frog if it stopped flinging his tongue at all insects after the encounter with the bee. Likewise, it would not be very adaptive if it would cease using its glossa altogether and try to catch the bee with its mouth the next time. Apparently, it is entirely sufficient to memorize the coloration of the prey as being punishing (negative reinforcement) to keep the frog from trying to catch it – the CS has acquired the avoidance eliciting prop-

erties of the sting (US). Although an operant (B-US) association might have formed, it is not necessary. In most cases operant behavior will be flexible and fast enough to ensure proper preparatory behavior without or with only little aid of motor learning. Indeed, the results presented here suggest that the B-US associations are at least weaker than the CS-US associations in three term contingencies and may (if present) be better characterized as sequential B-CS-US or hierarchical (B-CS)-US associations (Fig. 7). Moreover, this study has substantiated the prevalence of stimulus learning by showing that it comes to dominate any other association in a complex learning task even though there are equally valid behavioral predictors present (Fig. 7). In contrast, once two stimuli share the same predictive value for the reinforcer both can accrue the same associative strength (Fig. 8), ruling out the possibility that in all learning situations one predictor comes to dominate all others. This seems even to be true if one of the two stimuli bears a weaker relation to the reinforcer (Fig. 9). This is either a difference between invertebrates and vertebrates or a particular property of the experimental design used here. More experiments are required to find out whether invertebrates rely on more predictors than vertebrates. Furthermore, the facilitating effect of operant behavior on this CS-US acquisition process has been shown here for the first time (Fig. 4). As expected, the more natural complex learning tasks are easier to solve than the more artificial, single-association tasks (Figs. 3, 5; Table 2). At the same time a new form of incidental learning was established for *Drosophila* (Fig. 11), showing that higher order learning forms developed in vertebrates can be successfully applied in invertebrates. Obviously *Drosophila* at the torque meter is a very good case study showing that no simple, symmetric notion of temporal proximity, but rather a more sophisticated, asymmetric set of rules is guiding the selection which of the predictors present in a composite learning situation are to be stored in memory for later use.



## 5 Abstract

Most natural learning situations are of a complex nature and consist of a tight conjunction of the animal's behavior (B) with the perceived stimuli. According to the behavior of the animal in response to these stimuli, they are classified as being either biologically neutral (conditioned stimuli, CS) or important (unconditioned stimuli, US or reinforcer). A typical learning situation is thus identified by a three term contingency of B, CS and US. A functional characterization of the single associations during conditioning in such a three term contingency has so far hardly been possible. Therefore, the operational distinction between classical conditioning as a behavior-independent learning process (CS-US associations) and operant conditioning as essentially behavior-dependent learning (B-US associations) has proven very valuable. However, most learning experiments described so far have not been successful in fully separating operant from classical conditioning into single-association tasks. The *Drosophila* flight simulator in which the relevant behavior is a single motor variable (yaw torque), allows for the first time to completely separate the operant (B-US, B-CS) and the classical (CS-US) components of a complex learning situation and to examine their interactions. In this thesis the contributions of the single associations (CS-US, B-US and B-CS) to memory formation are studied. Moreover, for the first time a particularly prominent single association (CS-US) is characterized extensively in a three term contingency. A yoked control shows that classical (CS-US) pattern learning requires more training than operant pattern learning. Additionally, it can be demonstrated that an operantly trained stimulus can be successfully transferred from the behavior used during training to a new behavior in a subsequent test phase. This result shows unambiguously that during operant conditioning classical (CS-US) associations can be formed. In an extension to this insight, it emerges that such a classical association blocks the formation of an operant association, which would have been formed without the operant control of the learned stimuli. Instead the operant

component seems to develop less markedly and is probably merged into a complex three-way association. This three-way association could either be implemented as a sequential B-CS-US or as a hierarchical (B-CS)-US association. The comparison of a simple classical (CS-US) with a composite operant (B, CS and US) learning situation and of a simple operant (B-US) with another composite operant (B, CS and US) learning situation, suggests a hierarchy of predictors of reinforcement. Operant behavior occurring during composite operant conditioning is hardly conditioned at all. The associability of classical stimuli that bear no relation to the behavior of the animal is of an intermediate value, as is operant behavior alone. Stimuli that are controlled by operant behavior accrue associative strength most easily. If several stimuli are available as potential predictors, again the question arises which CS-US associations are formed? A number of different studies in vertebrates yielded amazingly congruent results. These results inspired to examine and compare the properties of the CS-US association in a complex learning situation at the flight simulator with these vertebrate results. It is shown for the first time that *Drosophila* can learn compound stimuli and recall the individual components independently and in similar proportions. The attempt to obtain second-order conditioning with these stimuli, yielded a relatively small effect. In comparison with vertebrate data, blocking and sensory preconditioning experiments produced conforming as well as dissenting results. While no blocking could be found, a sound sensory preconditioning effect was obtained. Possible reasons for the failure to find blocking are discussed and further experiments are suggested. The sensory preconditioning effect found in this study is revealed using simultaneous stimulus presentation and depends on the amount of preconditioning. It is argued that this effect is a case of 'incidental learning', where two stimuli are associated without the need of reinforcement. Finally, the implications of the results obtained in this study for the general understanding of memory formation in complex learning situations are discussed.

## 6 Zusammenfassung

Die meisten Lernsituationen sind von komplexer Natur und bestehen aus einer engen Verknüpfung des Verhaltens eines Tieres (B) mit den wahrgenommenen Stimuli. Entsprechend der Reaktion des Tieres auf diese Stimuli werden diese als entweder biologisch neutral (konditionierte Stimuli, CS) oder signifikant (unkonditionierte Stimuli, US oder Verstärker) klassifiziert. Eine typische Lernsituation ist also durch eine Dreibegebeziehung zwischen B, CS und US gekennzeichnet. Eine funktionelle Charakterisierung der Einzelassoziationen während des Lernens in einer solchen Dreibegebeziehung war experimentell bisher kaum zugänglich. Operationell wird daher zwischen klassischer Konditionierung als *verhaltensunabhängigem* Lernvorgang (CS-US Assoziationen) und operanter Konditionierung als essentiell *verhaltensabhängigem* Lernen (B-US Assoziationen) unterschieden. In den meisten bisher beschriebenen Lernexperimenten ist noch nicht einmal diese Trennung in Einzelassoziationen vollständig durchzuführen gewesen. Im *Drosophila* Flugsimulator, in dem das relevante Verhalten eine einzelne Bewegungsvariable (das Gierungsdrehmoment) ist, können zum ersten Mal die operanten (B-US, B-CS) und die klassischen (CS-US) Bestandteile einer komplexen Lernsituation völlig getrennt und auf ihre Interaktionen hin untersucht werden. In der vorliegenden Arbeit wurden sowohl die Beiträge der Einzelassoziationen (CS-US, B-US und B-CS) bei der Akquisition der Gedächtnismatrix in komplexen Lernsituationen untersucht, als auch die Eigenschaften einer besonders prominenten Einzelassoziation (CS-US) während einer komplexen Lernsituation zum ersten Mal weitgehend charakterisiert. Mit einer gejochten (yoked) Kontrolle kann gezeigt werden, dass das klassische (CS-US) Musterlernen umfangreicheres Training als das operante Musterlernen erfordert. Außerdem kann die Fliege einen operant gelernten Stimulus von dem Verhalten mit dem er gelernt wurde, auf ein anderes Verhalten im Test übertragen. Dieses Resultat zeigt eindeutig, dass während der operanten Konditionierung klassische (CS-US) Assoziationen gebildet werden können. In einer Erweiterung dieses Ergebnisses zeigt sich, dass solch eine klassische Assoziation, wenn sie gebildet wird, die Bildung einer operanten Assoziation blockiert, die ohne operante Kontrolle der klassisch assoziierten Stimuli gebildet würde. Stattdessen scheint sich der operante Bestandteil weniger ausgeprägt zu entwickeln und ist eventuell in einer komplexen Drei-

wege-Assoziation eingebunden. Die Dreibege-Assoziation könnte entweder als sequentielle B-CS-US oder als hierarchische (B-CS)-US Assoziation implementiert sein. Der Vergleich einer einfachen klassischen (CS-US) mit einer komplexen operanten (B, CS und US) Lernsituation und einer einfachen operanten (B-US) mit einer anderen komplexen operanten (B, CS und US) Lernsituation, ermöglicht das Postulat einer Hierarchie der Prädiktoren für Verstärker. Operantes Verhalten während einer komplexen operanten Lernsituation wird wenig oder überhaupt nicht konditioniert. Die Assoziierbarkeit der klassischen Stimuli ohne Relation zum Verhalten des Tieres (CS-US) sind – wie operantes Verhalten alleine (B-US) auch – von mittlerer Assoziierbarkeit. Stimuli die von operantem Verhalten kontrolliert werden, erhöhen am schnellsten ihre assoziative Stärke. Sind mehrere Stimuli während des Lernvorgangs zugänglich, stellt sich erneut die Frage, welche von den CS-US Assoziationen gebildet werden. Eine Vielzahl verschiedenster Studien in Vertebraten wiesen erstaunlich übereinstimmende Ergebnisse auf. Diese Ergebnisse inspirierten dazu, die Eigenschaften der CS-US Assoziationen in der komplexen Lernsituation am Flugsimulator zu untersuchen und mit Ergebnissen in Vertebraten zu vergleichen. Es wird erstmals gezeigt, dass *Drosophila* zusammengesetzte Stimuli lernen und die Einzelkomponenten unabhängig voneinander und in etwa ähnlichen Proportionen wiedererkennen kann. Der Versuch „Lernen zweiter Ordnung“ mit diesen Stimuli zu erzielen, liefert einen relativ kleinen Effekt. Die Gegenüberstellung mit Daten aus Vertebraten liefert sowohl Abweichungen als auch Übereinstimmungen hinsichtlich der Lernregeln, die beim klassischen Konditionieren von Vertebraten gefunden wurden. Während es ein deutliches „sensorisches Präkonditionieren“ gibt, konnte kein „Blocken“ gefunden werden. Das sensorische Präkonditionieren in dieser Studie zeigt sich bei gleichzeitiger Stimuluspräsentation und ist vom Mass der Präkonditionierung abhängig. Es wird argumentiert, dass dieser Effekt ein Fall „beiläufigen Lernens“ ist, bei dem zwei Stimuli ohne die Notwendigkeit der Verstärkung assoziiert werden. Für das nicht gefundene Blocken werden mögliche Gründe diskutiert und weiterführende Experimente vorgeschlagen. Abschließend wird über die Implikationen der Resultate dieser Arbeit für das allgemeine Verständnis der Gedächtnisbildung in komplexen Lernsituationen nachgedacht.

## 7 Curriculum Vitae

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Björn Brembs

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## 9 References

- Amiro TW, Bitterman ME (1980)** Second-order appetitive conditioning in goldfish. *J. Exp. Psychol. Anim. Behav. Process.* **6**:41-48.
- Bakal CW, Johnson RD, Rescorla RA (1974)** The effect of change in US quality on the blocking effect. *Pavlov J. Biol. Sci.* **9**:97-103.
- Balleine B (1994)** Asymmetrical interactions between thirst and hunger in Pavlovian-instrumental transfer. *Q. J. Exp. Psychol. B.* **47**:211-231.
- Barnet RC, Grahame NJ, Miller RR (1993)** Temporal encoding as a determinant of blocking. *J. Exp. Psychol. Anim. Behav. Process.* **19**:327-341.
- Batsell WR (1997)** Retention of context blocking in taste-aversion learning. *Physiol. Behav.* **61**:437-446.
- Bitterman ME (1996)** Comparative analysis of learning in honeybees. *Anim. Learn. Behav.* **24**:123-141.
- Brembs B (1996)** Classical and Operant Conditioning in *Drosophila* at the Flight Simulator. Diplomarbeit. Würzburg: Julius-Maximilians-Universität. <http://brembs.net/diploma>
- Brogden JW (1939)** Unconditional stimulus-substitution in the conditioning process. *Am. J. Psychol.* **52**:46-55.
- Carew TJ, Sahley CL (1986)** Invertebrate learning and memory: from behavior to molecules. *Annu Rev Neurosci.* **9**:435-487.
- Cheatle MD, Rudy JW (1978)** Analysis of second-order odor-aversion conditioning in neonatal rats: implications for Kamin's blocking effect. *J. Exp. Psychol. Anim. Behav. Process.* **4**:237-249.
- Couvillon PA, Bitterman ME (1980)** Some phenomena of associative learning in honey bees. *J. Comp. Physiol. Psychol.* **94**:878-885.
- Couvillon PA, Bitterman ME (1982)** Compound conditioning in honey bees. *J. Comp. Physiol. Psychol.* **96**:192-199.
- Couvillon PA, Bitterman ME (1989)** Reciprocal overshadowing in the discrimination of color-odor compounds by honeybees further tests of a continuity model. *Anim. Learn. Behav.* **17**:213-222.
- Couvillon PA, Mateo E-T, Bitterman ME (1996)** Reward and learning in honeybees: Analysis of an overshadowing effect. *Anim. Learn. Behav.* **24**:19-27.
- Couvillon PA, Arakaki, L., Bitterman, M.E. (1997)** Intramodal blocking in honeybees. *Anim. Learn. Behav.* **25**:277-282.
- Denniston JC, Miller RR, Matute H (1996)** Biological significance as determinant of cue competition. *Psych. Sci.* **7**:325-331.
- Dill M, Heisenberg M (1995)** Visual pattern memory without shape recognition. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **349**:143-152.
- Dill M, Wolf R, Heisenberg M (1993)** Visual pattern recognition in *Drosophila* involves retinotopic matching. *Nature.* **365**:751-753.
- Dill M, Wolf R, Heisenberg M (1995)** Behavioral analysis of *Drosophila* landmark learning in the flight simulator. *Learn. & Mem.* **2**:152-160.
- Donahoe JW (1997)** Selection networks: Simulation of plasticity through reinforcement learning. In: *Neural-Networks models of cognition* (Donahoe JW, Packard-Dorsel V ed): Elsevier Science B. V.
- Donahoe JW, Burgos JE, Palmer DC (1993)** A selectionist approach to reinforcement. *J. Exp. Anal. Behav.* **60**:17-40.
- Donahoe JW, Palmer DC, Burgos JE (1997)** The S-R issue: Its status in behavior analysis and in Donahoe and Palmer's "Learning and Complex Behavior" (with commentaries and reply). *J. Exp. Anal. Behav.* **67**:193-273.
- Dukas R (1999)** Costs of Memory: Ideas and Predictions. *J. theor. Biol.* **197**:41-50.
- Ernst R, Heisenberg M (1999)** The memory template in *Drosophila* pattern vision at the flight simulator. *Vision Research* **39**:3920-3933.
- Eyding D (1993)** Lernen und Kurzzeitgedächtnis beim operanten Konditionieren auf visuelle Muster bei strukturellen und bei chemischen Lernmutanten von *Drosophila melanogaster*. Diplomarbeit. Würzburg: Julius-Maximilians-Universität.
- Fanselow MS (1998)** Pavlovian conditioning, negative feedback, and blocking: mechanisms that regulate association formation. *Neuron* **20**:625-627.
- Farley J, Reasoner H, Janssen M (1997)** Potentiation of phototactic suppression in *Hermisenda* by a chemosensory stimulus during compound conditioning. *Behav. Neurosci* **111**:320-341.
- Feldman JM (1971)** Added cue control as a function of reinforcement predictability. *J. exp. Psychol.* **91**:318-325.
- Feldman JM (1975)** Blocking as a function of added cue intensity. *Anim. Learn. Behav.* **3**:98-102.
- Gerber B, Ullrich J (1999)** No evidence for olfactory blocking in honeybee classical conditioning. *J. Exp. Biol.* **202**:1839-1854.
- Glanzman DL (1995)** The cellular basis of classical conditioning in *Aplysia californica* - it's less simple than you think. *Trends Neurosci.* **18**:30-36.
- Gormezano I, Tait RW (1976)** The Pavlovian analysis of instrumental conditioning. *Pavlov. J. Biol. Sci.* **11**:37-55.
- Götz KG (1964)** Optomotorische Untersuchung des visuellen Systems einiger Augenmutanten der Fruchtfliege *Drosophila*. *Kybernetik.* **2**:77-92.
- Grant DA, Kroll NE, Kantowitz B, Zajano MJ, Solberg KB (1969)** Transfer of eyelid conditioning from instrumental to classical reinforcement and vice versa. *J. Exp. Psychol.* **82**:503-510.

- Guo A, Götz KG (1997)** Association of visual objects and olfactory cues in *Drosophila*. *Learn. & Mem.* **4**:192-204.
- Guo A, Liu L, Xia SZ, Feng CH, Wolf R, Heisenberg M (1996)** Conditioned visual flight orientation in *Drosophila*; Dependence on age, practice and diet. *Learn. & Mem.* **3**:49-59.
- Guthrie ER (1952)** *The Psychology of Learning*. New York: Harper.
- Haddad NF, Walkenbach J, Preston M, Strong R (1981)** Stimulus control in a simple instrumental task the role of internal and external stimuli. *Learn. Motiv.* **12**:509-520.
- Hall D, Suboski MD (1995)** Sensory Preconditioning and Second-Order Conditioning of alarm Reactions in Zebra Danio Fish (*Brachydanio rerio*). *J. Comp. Psychol.* **109**:76-84.
- Hall G (1996)** Learning about associatively activated stimulus representations: Implications for acquired equivalence and perceptual learning. *Anim. Learn. Behav.* **24**:233-255.
- Hammer M (1993)** An identified neuron mediates the unconditioned stimulus in associative learning in honeybees. *Nature.* **366**:59-63.
- Hammer M (1997)** The neural basis of associative reward learning in honeybees. *Trends. Neurosci.* **20**:245-252.
- Hammerl M (1993)** Blocking observed in human instrumental conditioning. *Learn. Motiv.* **24**:73-87.
- Hawkins RD, Abrams TW, Carew TJ, Kandel ER (1983)** A cellular mechanism of classical conditioning in *Aplysia*: activity-dependent amplification of presynaptic facilitation. *Science.* **219**:400-405.
- Hawkins RD, Greene W, Kandel ED (1998)** Classical conditioning, differential conditioning, and second-order conditioning of the *Aplysia* gill-withdrawal reflex in a simplified mantle organ preparation. *Behav. Neurosci.* **112**:636-645.
- Hebb DO (1956)** The distinction between "classical" and "instrumental.". *Can. J. Psychol.* **10**:165-166.
- Heisenberg M (1983)** Initiale Aktivität und Willkürverhalten bei Tieren. *Naturwissenschaften.* :70-78.
- Heisenberg M (1994)** Voluntariness (Willkürfähigkeit) and the general organization of behavior. *L. Sci. Res. Rep.* **55**:147-156.
- Heisenberg M, Wolf R (1984)** *Vision in Drosophila. Genetics of Microbehavior*. Berlin, Heidelberg, New York, Tokio: Springer.
- Heisenberg M, Wolf R (1988)** Reafferent control of optomotor yaw torque in *drosophilamelanogaster*. *J. Comp. Physiol. A Sens. Neural. Behav. Physiol.* **163**:373-388.
- Heisenberg M, Wolf R (1993)** The sensory-motor link in motion-dependent flight control of flies. *Rev. Oculomot. Res.* **5**:265-283.
- Hellige JB, Grant DA (1974)** Eyelid conditioning performance when the mode of reinforcement is changed from classical to instrumental avoidance and vice versa. *J. Exp. Psychol.* **102**:710-719.
- Hoffmann J (1993)** *Vorhersage und Erkenntnis. Die Funktion von Antizipationen in der menschlichen Verhaltenssteuerung und Wahrnehmung*. Göttingen, Bern, Toronto, Seattle: Hogrefe.
- Holland PC (1997)** Brain mechanisms for changes in processing of conditioned stimuli in Pavlovian conditioning: Implications for behavior theory. *Anim. Learn. Behav.* **25**:373-399.
- Holland PC, Gallagher M (1993)** Effects of amygdala central nucleus lesions on blocking and unblocking. *Behav. Neurosci.* **107**:235-245.
- Holland PC, Rescorla RA (1975a)** The effect of two ways of devaluing the unconditioned stimulus after first and second-order appetitive conditioning. *J. Exp. Psychol. Anim. Behav. Process.* **104** :355-363.
- Holland PC, Rescorla RA (1975b)** Second-order conditioning with food unconditioned stimulus. *J Comp Physiol Psychol.* **88**:459-467.
- Holman JG, Mackintosh NJ (1981)** The control of appetitive instrumental responding does not depend on classical conditioning of the discriminative stimulus. *Q. J. exp. Psychol.* **33B**:21-31.
- Horridge GA (1962)** Learning of leg position by headless insects. *Nature* **193**:697-698.
- Hoyle G (1979)** Mechanisms of simple motor learning. *Trends. Neurosci.* **2**:153-155.
- Hull CL (1929)** A functional interpretation of the conditioned reflex. *Psych. Rev.* **36**:498-511.
- Jones SH, Gray JA, Hemsley DR (1990)** The Kamin blocking effect, incidental learning and psychoticism. *Br. J. Psychol.* **81**:95-109.
- Kamin LJ (1968)** Attention- like processes in classical conditioning. In: *Miami symposium on predictability, behavior and aversive stimulation* (Jones MR, ed), pp 9-32. Miami: Miami University Press.
- Kamin LJ (1969)** Predictability, surprise, attention, and conditioning. In: *Punishment and aversive behavior* (Church RM, ed), pp 279-296. New York: Appleton-Century-Crofts.
- Kandel ER, Abrams T, Bernier L, Carew TJ, Hawkins RD, Schwartz JH (1983)** Classical conditioning and sensitization share aspects of the same molecular cascade in *Aplysia*. *Cold Spring Harb. Symp. Quant. Biol.* **48**:821-830.
- Kim JJ, Krupa DJ, Thompson RF (1998)** Inhibitory cerebello-olivary projections and blocking effect in classical conditioning. *Science* **279**:570-573.
- Kimmel HD (1977)** Notes from "Pavlov's Wednesdays": sensory preconditioning. *Am. J. Psychol.* **90**:319-321.
- Kimmel HD, Bevill MJ (1991)** Blocking and unconditioned response diminution in human classical autonomic conditioning. *Integr. Physiol. Behav. Sci.* **26**:132-138.



- Kimmel HD, Bevill MJ (1996)** Blocking and unconditioned response diminution in human classical autonomic conditioning. *Integr. Physiol. Behav. Sci.* **31**:18-43.
- Kojima S, Kobayashi S, Yamanaka M, Sadamoto H, Nakamura H, Fujito Y, Kawai R, Sakakibara M, Ito E (1998)** Sensory preconditioning for feeding response in the pond snail, *Lymnaea stagnalis*. *Brain Res.* **808**:113-115.
- Konorski J, Miller S (1937a)** On two types of conditioned reflex. *J. Gen. Psychol.* **16**:264-272.
- Konorski J, Miller S (1937b)** Further remarks on two types of conditioned reflex. *J. Gen. Psychol.* **17**:405-407.
- Lattal KM, Nakajima S (1998)** Overexpectation in appetitive Pavlovian and instrumental conditioning. *Anim. Learn. Behav.* **26**:351-360.
- Levey AB, Martin I (1991)** Human classical conditioning. The status of the CS. *Integr. Physiol. Behav. Sci.* **26**:26-31.
- Liu L, Wolf R, Ernst R, Heisenberg M (1999)** Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature* **400**:753-756.
- Lyn SA, Capaldi ED (1994)** Robust conditioned flavor preferences with a sensory preconditioning procedure. *Psychon. Bull. Rev.* **1**:491-493.
- Mackintosh NJ (1975a)** Blocking of conditioned suppression: role of the first compound trial. *J. Exp. Psychol. Anim. Behav. Process.* **1**:335-345.
- Mackintosh NJ (1975b)** A theory of attention: Variations in the associability of stimuli with reinforcement. *Psychol. Rev.* **82**:276-298.
- Mackintosh NJ (1976)** Overshadowing and stimulus intensity. *Anim. Learn. Behav.* **4**:186-192.
- Marchant HG, Moore JW (1973)** Blocking of the rabbit's conditioned nictitating membrane response in Kamin's two-stage paradigm. *J. Exp. Psychol.* **101**:155-158.
- Martin I, Levey AB (1991)** Blocking observed in human eyelid conditioning. *Q. J. Exp. Psychol. B.* **43**:233-256.
- McHose JH, Moore JN (1976)** Expectancy, Salience and habit: an noncontextual interpretation of the effects of changes in the conditions of reinforcement on simple instrumental responses. *Psychol. Rev.* **83**:292-307.
- Mellgren RL, Ost JW (1969)** Transfer of Pavlovian differential conditioning to an operant discrimination. *J. Comp. Physiol. Psychol.* **67**:390-394.
- Menzel R, Müller U (1996)** Learning and memory in honeybees: from behavior to neural substrates. *Annu. Rev. Neurosci.* **19**:379-404.
- Miles CG (1969)** A demonstration of overshadowing in operant conditioning. *Psych. Sci.* **16**:139-140.
- Miles CG, Jenkins HM (1973)** Overshadowing in operant conditioning as a function of discriminability. *Learn. Motiv.* **4**:11-27.
- Miller R-R, Matute H (1996)** Biological significance in forward and backward blocking: Resolution of a discrepancy between animal conditioning and human causal judgment. *J. Exp. Psychol.* **125**:370-386.
- Mosolff, M, Müller D, Hellstern F, Hammer M, Menzel R (1998)** Effects of second order conditioning in honeybees are dependant on the inter stimulus interval. *Europ. J. Neurosci* **10**:358.
- Nargeot R, Baxter DA, Byrne JH (1997)** Contingent-dependent enhancement of rhythmic motor patterns: an in vitro analog of operant conditioning. *J. Neurosci.* **17**:8093-8105.
- Nargeot R, Baxter DA, Byrne JH (1999a)** In vitro analog of operant conditioning in *Aplysia*. I. Contingent reinforcement modifies the functional dynamics of an identified neuron. *J. Neurosci.* **19**:2247-2260.
- Nargeot R, Baxter DA, Byrne JH (1999b)** In vitro analog of operant conditioning in *Aplysia*. II. Modifications of the functional dynamics of an identified neuron contribute to motor pattern selection. *J. Neurosci.* **19**:2261-2272.
- Pavlov IP (1927)** Conditioned reflexes. Oxford: Oxford University Press.
- Pearce JM (1987)** A model for stimulus generalization in pavlovian conditioning. *Psychol. Rev.* **94**:61-73.
- Pearce JM (1994)** Similarity and discrimination: a selective review and a connectionist model. *Psychol. Rev.* **101**:587-607.
- Pearce JM, Hall G (1978)** Overshadowing the instrumental conditioning of a lever press response by a more valid predictor of the reinforcer. *J. Exp. Psychol. Anim. Behav. Process.* **4**:356-367.
- Pearce JM, Hall G (1980)** A model for pavlovian learning: Variations in the effectiveness of conditioned but not of unconditioned stimuli. *Psychol. Rev.* **87**:532-525.
- Pelz C, Gerber, B., & Menzel, R, (1997)** Odorant intensity as a determinant for olfactory conditioning in honeybees: Roles in discrimination, overshadowing and memory consolidation. *J. Exp. Biol.* **200**:837-847.
- Prewitt EP (1967)** Number of preconditioning trials in sensory preconditioning using CER training. *J. Comp. Physiol. Psychol.* **64**:360-362.
- Rescorla RA (1979)** Aspects of the reinforcer learned in second-order Pavlovian conditioning. *J. Exp. Psychol. Anim. Behav. Process.* **5**:79-95.
- Rescorla RA (1980)** Simultaneous and successive associations in sensory preconditioning. *J. Exp. Psychol. Anim. Behav. Process.* **6**:207-216.
- Rescorla RA (1982)** Simultaneous second-order conditioning produces S-S learning in conditioned

- suppression. *J. Exp. Psychol. Anim. Behav. Process.* **8**:23-32.
- Rescorla RA (1983)** Effect of separate presentation of the elements on within compound learning in auto shaping. *Anim Learn Behav* **11**:439-446.
- Rescorla RA (1994)** Control of instrumental performance by Pavlovian and instrumental stimuli. *J. Exp. Psychol. Anim. Behav. Process.* **20**:44-50.
- Rescorla RA, Cunningham CL (1979)** Spatial contiguity facilitates Pavlovian second-order conditioning. *J. Exp. Psychol. Anim. Behav. Process.* **5**:152-161.
- Rescorla RA, Gillan DJ (1980)** An analysis of the facilitative effect of similarity on second-order conditioning. *J. Exp. Psychol. Anim. Behav. Process.* **6**:339-351.
- Rescorla RA, Solomon RL (1967)** Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. *Psychol. Rev.* **74**:151-182.
- Rescorla RA, Wagner AR (1972)** A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. In: *Classical Conditioning II: Current research and theory* (Black A, W.F. P, eds), pp 64-99. New York: Appleton-Century-Crofts.
- Rizley RC, Rescorla RA (1972)** Associations in second-order conditioning and sensory preconditioning. *J. Comp. Physiol. Psychol.* **81**:1-11.
- Rogers RF, Schiller KM, Matzel LD (1996)** Chemosensory-based contextual conditioning in *Hermissenda crassicornis*. *Anim. Learn. Behav.* **24**:28-37.
- Rogers RF, Matzel LD (1995)** Higher-order associative processing in *Hermissenda* suggests multiple sites of neuronal modulation. *Learn. & Mem.* **2**:279-298.
- Ross RT, LoLordo VM (1987)** Evaluation of the relation between Pavlovian occasion-setting and instrumental discriminative stimuli: a blocking analysis. *J. Exp. Psychol. Anim. Behav. Process.* **13**:3-16. [retracted by **LoLordo VM, Ross RT**. In: *J. Exp. Psychol. Anim. Behav. Process.* 1990 **16**(4):402-6].
- Sahley C, Rudy JW, Gelperin A (1981)** An analysis of associative learning in a terrestrial mollusc. *J. Comp. Physiol. (A)*. **144**:1-8.
- Schachtman TR, KasproW WJ, Chee MA, Miller RR (1985)** Blocking but not conditioned inhibition results when an added stimulus is reinforced in compound with multiple pretrained stimuli. *Am. J. Psychol.* **98**:283-295.
- Schindler CW, Weiss SJ (1985)** Modification of a stimulus-reinforcer interaction by blocking. *Behav. Process.* **11**:123-130.
- Schultz W, Romo, R., Ljungberg, T., Mirenowicz, J., Hollerman, J.R., & Dickinson, A, (1995)** Reward-related signals carried by dopaminergic neurons. In: *Models of information processing in the basal ganglia*, (J.C. Houk DGB, ed), pp 233-248. Cambridge, MA: MIT Press.
- Sekiguchi T, Suzuki H, Yamada A, Mizukami A (1994)** Cooling-induced retrograde amnesia reflexes Pavlovian conditioning associations in *Limax flavus*. *Neurosci. Res.* **18**:267-275.
- Sheffield FD (1965)** Relation of classical conditioning and instrumental learning. In: *Classical Conditioning* (Prokasy WF, ed), pp 302-322. New York: Appleton-Century-Crofts.
- Skinner BF (1935)** Two types of conditioned reflex and a pseudo type. *J. Gen. Psychol.* **12**:66-77.
- Skinner BF (1937)** Two types of conditioned reflex: A reply to Konorski and Miller. *J. Gen. Psychol.* **16**:272-279.
- Skinner BF (1938)** The behavior of organisms. New York: Appleton.
- Smith BH (1996)** The role of attention in learning about odorants. *Biol. Bull.* **191**:76-83.
- Smith BH (1997)** An analysis of blocking in odorant mixtures: An increase but not a decrease in intensity of reinforcement produces unblocking. *Behav. Neurosci.* **111**:57-69.
- Smith BH (1998)** Analysis of interaction in binary odorant mixtures. *Physiol. Behav.* **65**:397-407.
- Spence KW (1936)** The nature of discrimination learning in animals. *Psych. Rev.* **43**:427-449.
- Spencer GE, Syed NI, Lukowiak K (1999)** Neural changes after operant conditioning of the aerial respiratory behavior in *Lymnea stagnalis*. *J. Neurosci* **19**:1836-1843.
- St. Claire-Smith R (1979)** The overshadowing of instrumental conditioning by a stimulus that predicts reinforcement better than the response. *Anim. Learn. Behav.* **7**:224-228.
- Sutton RS, Barto AG (1981)** Toward a modern theory of adaptive networks: Expectation and prediction. *Psychol. Rev.* **88**:135-170.
- Sutton RS, Barto AG (1990)** Time-derivative models of pavlovian reinforcement. In: *Learning and Computational Neuroscience: Foundations of Adaptive Networks* (Gabriel M, Moore J, eds), pp 497-537. Boston: MIT Press.
- Suzuki H, Sekiguchi T, Yamada A, Mizukami A (1994)** Sensory preconditioning in the terrestrial mollusk, *Limax flavus*. *Zool. Sci. (Tokyo)* **11**:121-125.
- Tait RW, Black M, Katz M, Suboski MD (1972)** Discriminative sensory preconditioning. *Can. J. Psychol.* **26**:201-205.
- Tait RW, Suboski MD (1972)** Stimulus intensity in sensory preconditioning of rats. *Can. J. Psychol.* **26**:374-381.
- Takeda K (1961)** Classical conditioned response in the honey bee. *J. Insect Physiol* **6**:168-179.
- Thompson RF, Thompson JK, Kim JJ, Krupa DJ, Shinkman PG (1998)** The nature of reinforcement in cerebellar learning. *Neurobiol. Learn. Mem.* **70**:150-176.
- Trapold MA, Lawton GW, Dick RA, Gross DM (1968)** Transfer of training from differential classical to differential instrumental conditioning. *J. Exp. Psychol.* **76**:568-573.

- Trapold MA, Overmier JB (1972)** The second learning process in instrumental conditioning. In: Classical Conditioning (Black AH, Prokasy WF, eds). New York: Appleton-Century-Crofts.
- Trapold MA, Winokur S (1967)** Transfer from classical conditioning and extinction to acquisition, extinction, and stimulus generalization of a positively reinforced instrumental response. *J. Exp. Psychol.* **73**:517-525.
- Tully T (1991)** Of mutations affecting learning and memory in *Drosophila* - the missing link between gene product and behavior. *Trends. Neurosci.* **14**:163-164.
- Tully T, Boynton S, Brandes C, Dura JM, Mihalek R, Preat T, Vilella A (1990)** Genetic dissection of memory formation in *Drosophila*. Cold Spring Harb. Symp. Quant. Biol. **55**:203-211.
- Tully T, Preat T, Boynton SC, Del-Vecchio M (1994)** Genetic dissection of consolidated memory in *Drosophila*. *Cell.* **79**:35-47.
- Wagner AR (1981)** SOP: A model of automatic memory processing in animal behavior. In Information processing in animals: Memory mechanisms. In: (N.E. Spear RPM, ed), pp 5-47. Hillsdale: Erlbaum.
- Wagner AR, Mazur JE, Donegan NH, Pfautz PL (1980)** Evaluation of blocking and conditioned inhibition to a CS signaling a decrease in US intensity. *J. Exp. Psychol. Anim. Behav. Process.* **6**:376-385.
- Wang X, Liu L, Xia SZ, Feng CH, Guo A (1998)** Relationship between visual learning/memory ability and brain cAMP level in *Drosophila*. *Sci. China C: L. Sci.* **41**:503-511.
- Ward-Robinson J, Hall G (1996)** Backward sensory preconditioning. *J. Exp. Psychol. Anim. Behav. Process.* **22**:395-404.
- Ward-Robinson J, Hall G (1998)** Backward sensory preconditioning when reinforcement is delayed. *Q. J. Exp. Psychol. B Comp. Physiol. Psychol.* **51**:349-362.
- Weidtmann N (1993)** Visuelle Flugsteuerung und Verhaltensplastizität bei Zentralkomplex - Mutanten von *Drosophila melanogaster*. Würzburg: Diplomarbeit.
- Weiss SJ, Panilio LV (1999)** Blocking a selective association in pigeons. *J. Exp. Anal. Behav.* **71**:13-24.
- Williams BA (1975)** The blocking of reinforcement control. *J. Exp. Anal. Behav.* **24**:215-225.
- Williams BA (1978)** Informational effects on the response-reinforcer association. *Anim. Learn. Behav.* **13**:6-12.
- Williams BA (1994)** Blocking despite changes in reinforcer identity. *Anim. Learn. Behav.* **22**:442-457.
- Williams BA, Heyneman N (1982)** Multiple determinants of blocking effects on operant behavior. *Anim. Learn. Behav.* **10**:72-76.
- Williams BA, Preston RA, DeKervor DE (1990)** Blocking of the response-reinforcer association additional evidence. *Learn. Motiv.* **21**:379-398.
- Wolf R, Heisenberg M (1991)** Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J Comp Physiol A.* **169**:699-705.
- Wolf R, Heisenberg M (1997)** Visual Space from Visual Motion: Turn Integration in Tethered Flying *Drosophila*. *Learn. & Mem.* **4**:318-327.
- Wolf R, Wittig T, Liu L, Wustmann G, Eydung D, Heisenberg M (1998)** *Drosophila* mushroom bodies are dispensable for visual, tactile and motor learning. *Learn. & Mem.* **5**:166-178.
- Wolpaw JR (1997)** The complex structure of a simple memory. *Trends. Neurosci.* **20**:588-594.
- Xia SZ, Feng CH, Guo AK (1999)** Temporary amnesia induced by cold anesthesia and hypoxia in *Drosophila*. *Physiol. Behav.* **65**:617-623.
- Xia SZ, Liu L, Feng CH, Guo A (1997a)** Memory consolidation in *Drosophila* operant visual learning. *Learn. & Mem.* **4**:205-218.
- Xia SZ, Liu L, Feng CH, Guo AK (1997b)** Nutritional effects on operant visual learning in *Drosophila melanogaster*. *Physiol. Behav.* **62**:263-271.
- Zanich ML, Fowler H (1978)** Transfer from Pavlovian appetitive to instrumental appetitive conditioning: signaling versus discrepancy interpretations. *J. Exp. Psychol. Anim. Behav. Process.* **4**:37-49.

## Publications:

- Brembs B (1996):** Chaos cheating and cooperation: potential solutions to the Prisoner's Dilemma. *Oikos* 76: 14-24
- Brembs B, Wolf R, Heisenberg M (1997):** Is operant behavior facilitating classical conditioning of *Drosophila* at the flight simulator? In: Elsner N, Waessle H (eds) Göttingen Neurobiology Report 1997. Georg Thieme Verlag Stuttgart New York:652
- Wolf R, Brembs B, Ernst R, Heisenberg M (1998):** Classification of learning in tethered flying *Drosophila*. In: Elsner N and Wehner R (eds) *New Neuroethology on the Move*. Georg Thieme Verlag Stuttgart New York: 111
- Brembs B, Wolf R, Heisenberg M (1998):** Operant and classical learning at the flight simulator: what is the role of the context? In: Elsner N and Wehner R (eds) Göttingen Neurobiology report 1998. Georg Thieme Verlag Stuttgart New York: 514
- Brembs B, Wolf R, Heisenberg M (1999):** Classical questions in an operant learning paradigm. In: Elsner N and Eysel U (eds) Göttingen Neurobiology Report 1999. Georg Thieme Verlag Stuttgart New York: 545
- Cutts CJ, Brembs B, Metcalfe NB, Taylor AC (1999):** Prior residence, territory quality and life-history strategies in juvenile Atlantic salmon (*Salmo salar* L.). *J. Fish. Biol.* 55: 784-794
- Brembs B, Heisenberg M (2000):** The operant and the classical in conditioned orientation of *Drosophila melanogaster* at the flight simulator. *Learn. Mem.* In Press.