

CONDITIONING WITH COMPOUND STIMULI IN *DROSOPHILA MELANOGASTER* IN THE FLIGHT SIMULATOR

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Accepted 31 May 2001

Summary

Short-term memory in *Drosophila melanogaster* operant visual learning in the flight simulator is explored using patterns and colours as a compound stimulus. Presented together during training, the two stimuli accrue the same associative strength whether or not a prior training phase rendered one of the two stimuli a stronger predictor for the reinforcer than the other (no blocking). This result adds *Drosophila* to the list of other invertebrates that do not exhibit the robust vertebrate blocking phenomenon.

Other forms of higher-order learning, however, were detected: a solid sensory preconditioning and a small second-order conditioning effect imply that associations between the two stimuli can be formed, even if the compound is not reinforced.

Key words: classical and operant conditioning, blocking, overshadowing, sensory preconditioning, second-order conditioning, *Drosophila melanogaster*, memory, learning.

Introduction

Animals can learn that initially neutral stimuli (conditioned stimuli, CSs) may predict biologically significant events (unconditioned stimuli, USs). They respond to the CS with behaviour anticipating the US, irrespective of whether the US is a consequence of their own behaviour (operant or instrumental conditioning; Skinner, 1938) or appears independently of it (classical or Pavlovian conditioning; Pavlov, 1927). During the investigation of associative learning, a number of phenomena have been found that are consistently observed across various experimental designs as well as across species (see e.g. Lattal and Nakajima, 1998; Mackintosh, 1990; Pearce, 1997; Williams, 1994, and references therein). This consistency has led to the conclusion that some 'learning rules' might be common to all animal species, at least among vertebrates (e.g. Mackintosh, 1975; McHose and Moore, 1976; Pearce, 1994; Rescorla and Wagner, 1972; Sutton and Barto, 1990). Most prominent among these phenomena are 'blocking' (Kamin, 1968) and 'sensory preconditioning' (Brogden, 1939; Kimmel, 1977).

Blocking implies that temporal CS–US pairing does not lead to a CS–US association if the CS is presented together with another CS that already fully predicts the US. In a classical blocking design, the first phase consists of training to 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 CS2 is then tested alone, the subject shows a learning score below that of a parallel group that received a control treatment instead of the

first training. Thus, the first training of CS1 has 'blocked' learning about CS2 in the second phase (Kamin, 1968). Most current models of associative learning (Pearce, 1994; Rescorla and Wagner, 1972; Sutton and Barto, 1990; Wagner, 1981) incorporate blocking as a critical constituent. Blocking is often explained in terms of predictability. Only if a US is 'surprising' (Kamin, 1968; Kamin, 1969) can new stimuli having a predictive value for the US enter into the association.

In sensory preconditioning, temporal CS–US pairing is not necessary for a CS to accrue associative strength. Sensory preconditioning consists of three parts. In the first, the subject is presented with two stimuli (conditioned stimuli; CS1+CS2) without any reinforcement. Second, one of the stimuli (CS1) is reinforced alone. In the third part, CS2 alone is tested. Provided that the appropriate controls exclude alternative explanations, a significant learning score for CS2 demonstrates that the response-eliciting properties of the US have been transferred to the CS2 with which the US has never been paired. Blocking and sensory preconditioning experiments have received much attention because they falsify the old idea that simple temporal pairing of a CS and a US is both a necessary and sufficient criterion for learning to occur: in blocking, CS–US pairings are shown to be insufficient and in sensory preconditioning they are not even necessary for memory formation.

In the flight simulator used here (Fig. 1), a tethered *Drosophila* can control, with its yaw torque, the angular velocity and orientation of a circular arena surrounding it. The

arena wall is decorated with different patterns (visual pattern learning; Wolf and Heisenberg, 1991), allowing the fly to choose its flight direction relative to these patterns. The fly can be conditioned by a beam of infrared light delivering instantaneous heat to avoid certain flight directions (i.e. angular orientations of the arena) and to prefer others. In a variant of this paradigm, the fly can identify arena orientations in a uniformly patterned arena if different orientations are combined with spectrally different arena illuminations (colour learning; Wolf and Heisenberg, 1997). Learning success (memory) is assessed by recording the fly's choice of flight direction once the training is over. In this study, we first establish that both patterns and colours are learned separately and symmetrically if both are presented as a compound during training. In an attempt to find blocking in *Drosophila*, two blocking groups are compared with five different control groups, four of which concern the amount of CS and US experience in the first training phase and one controls for confounding effects in the second training phase. Finally, we investigate the occurrence of sensory preconditioning in our paradigm.

Materials and methods

Flies

Drosophila melanogaster Meigen were kept on standard cornmeal/molasses medium (for details, see Guo et al., 1996) at 25 °C and 60% humidity with a 16h:8h light:dark regime. Female flies (24–48h) were immobilized by cold-anaesthesia and glued (Loctite UV glass glue) by the head and thorax to a triangular copper hook (diameter 0.05 mm) the day before the experiment. The animals were then kept individually overnight in small moist chambers containing a few grains of sucrose.

Apparatus

The *Drosophila* flight simulator (Fig. 1) is a computer-controlled feedback system in which the fly is allowed to control, by its yaw torque, the rotations of a panorama surrounding it. The core device of the experimental arrangement is the torque meter. Originally devised by Götz (Götz, 1964) and repeatedly improved by Heisenberg and Wolf (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 and performs tethered flight in the centre of a cylindrical panorama (arena, diameter 58 mm) homogeneously illuminated from behind (Fig. 1). The light source is a 100 W, 12 V tungsten-iodine bulb. For green and blue illumination of the arena, the light is passed through monochromatic broadband Kodak Wratten gelatine filters (nos 47 and 99, respectively). Filters can be exchanged magnetically within 0.1 s.

Via the motor control unit (K in Fig. 1), an electric motor rotates the arena, making its angular velocity proportional to, but directed against, the fly's yaw torque (coupling factor $K=11^\circ \text{ s}^{-1} 10^{-10} \text{ Nm}$). This enables the fly to stabilize the

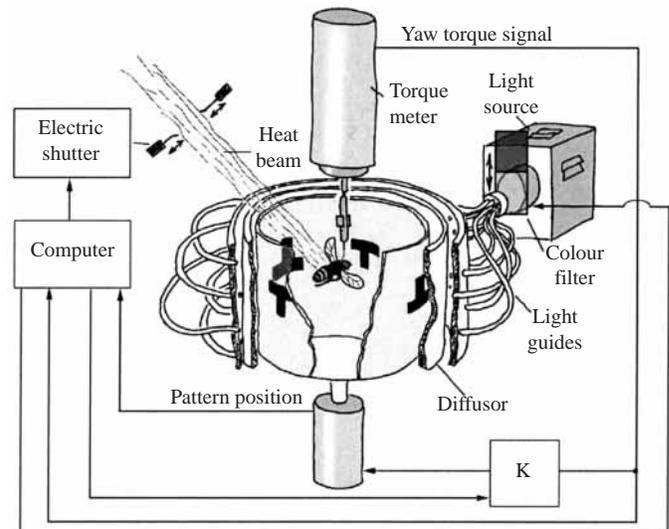


Fig. 1. The flight simulator. The fly is flying tethered in a cylindrical arena homogeneously illuminated from behind. The fly's tendency to perform left or right turns (yaw torque) during tethered flight is measured continuously and fed into a computer. The computer controls background pattern position, heat beam shutter closure and the colour of illumination according to the conditioning rules. K indicates the motor control unit.

rotational movements of the panorama and to control its angular orientation. The angular position of an arbitrarily chosen reference point 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 20 Hz) for later analysis. Reinforcement is achieved by applying heat provided by a light beam (diameter 4 mm at the position of the fly) generated by a 6 V, 15 W Zeiss microscope lamp, filtered by an infrared filter (Schott RG780, 3 mm thick) and focused from above onto the fly. Heat at the position of the fly is switched on and off by a computer-controlled shutter intercepting the beam (Fig. 1).

If patterns alone are used as visual cues (Wolf and Heisenberg, 1991), 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 $\theta=40^\circ$, width of bars 14° , as seen from the position of the fly). For colours alone as visual cues (see Wolf and Heisenberg, 1997), the patterns are replaced by four identical vertical stripes (width $\psi=14^\circ$, height $\theta=40^\circ$). A computer program divides the 360° of the arena into four virtual 90° quadrants, the centres of which are denoted by the stripes. The colour of the illumination of the whole arena is changed whenever one of the virtual quadrant borders passes a point in front of the fly. If a compound of colours and patterns is used as the visual cue, the four vertical stripes are replaced by the four T-shaped patterns, and colour is changed as described. During training, heat reinforcement (input voltage 6.0 V) is made contiguous

either with the appearance of one of the pattern orientations in the frontal visual field or with either green or blue illumination of the arena or with both. Reinforcement of each pattern/colour is always equalized within groups. During testing, the heat source is permanently switched off.

Experimental procedures: blocking

Two blocking experiments were performed. Both were designed as between-groups experiments, each with one blocking and one control group. Both again consisted of two half-groups, one of which was presented with colours alone in the first training phase and the other with patterns alone (CS1+US). Throughout this study (unless indicated otherwise), with patterns alone, the light of the arena illumination was passed through a 2 mm BG18 Schott 'daylight' (broad-band blue-green) filter which allows for generalization of pattern memory when switching from daylight to monochromatic blue or green light in the compound (Liu et al., 1999). The two experiments differed in the amount of compound training (CS1+CS2+US) and in the choice of control procedures. In the first experiment, flies received equal amounts of first training and compound training. In the second experiment, only half the amount of compound training was given.

The Kamin control

Four of the five control procedures concern the first phase of the experiment prior to the compound training. To test whether the flies learned colours and patterns well during compound training, 103 flies were trained omitting the first training phase. Four minutes of unreinforced preference testing with patterns and colours were followed by two 4 min training periods, interrupted by a 2 min test period. After these 14 min of compound presentation, the flies were allowed to choose the flight direction either with the compound as a visual cue (control) or with colours or patterns alone (experimental groups). A fourth group (exchange group) was presented with a new compound in which the combination between patterns and colours was exchanged (e.g. if, during training, flying towards an upright T led to green illumination of the arena, it would now, during the 'exchanged' test phase, lead to blue illumination). 'Overshadowing' (Pavlov, 1927) of one stimulus by the other would be indicated by a significant difference between the results of the two experimental groups (control 1).

Improved controls

Two additional control treatments in phase one provided the flies with the same amount of CS1 and US experience as in the first blocking experiment. After these treatments, the control and experimental groups differed only in the associative strength of CS1 – a clear advantage over another frequently used control that employs a novel third stimulus during the first training. This is accomplished in two different ways. In the control group stimulated by colours as CS1 during the first conditioning phase, flies were trained classically by recording their flight orientations and heating regime in the corresponding blocking group and playing them back to naive

flies (replay experiment; Wolf and Heisenberg, 1991). This implies that the control flies received the same sensory stimulation as the flies in the blocking group. However, it has been shown previously that this training is not sufficient for conditioning the flies (Wolf and Heisenberg, 1991). Thus, the flies received the same treatment as the blocking group, but were nevertheless 'naïve' when entering the second phase (control 2).

For the other half of the control flies trained to patterns as CS1, we took advantage of an effect that had been discovered independently of this study. We had observed that pattern memory from operant training in white light (no daylight filter; see above) is lost if monochromatic colours are added to generate compound stimuli (CS1+CS2; for details of the effects of colour changes on pattern memory, see Wiener, 2000). This effect was used for the second group of control flies trained to patterns as CS1. Training without the daylight filter in the first phase provided the animals with the same amount of CS1 and US exposure as the animals in the blocking group, but rendered the flies 'naïve' at the onset of compound training (control 3).

In the second experiment, only half the amount of compound training was given. In this experiment, the control groups did not receive any reinforcement before the compound phase. Instead, they perceived CS1 (either colours alone or patterns with a daylight filter) without reinforcement. If the control flies had developed a latent inhibition to CS1, reinforcement of the compound would have been even less expected, enhancing a potential blocking effect by increasing the control learning scores for CS2. A significant decrease in learning in the blocking *versus* any of the control groups for CS2 would be indicative of blocking (control 4).

Second-order conditioning control

The fifth control experiment addressed effects during the compound phase. Two second-order conditioning experiments were conducted differing in the amount of second-order training (CS1+CS2). The first was similar to the first blocking experiment, with the difference that the second phase, using the compound, was shortened by 2 min and included no reinforcement. For the second experiment, we shortened the second-order conditioning phase even more to only two 2 min periods (matching the second blocking experiment). The final test phase for pattern learning (CS2) was for two 2 min periods. Only colours were used as the conditioned reinforcer. Significant learning in the final test phase would indicate successful second-order conditioning that might mask a potential blocking effect (control 5).

Experimental procedures: sensory preconditioning

Two groups of flies were allowed to fly without reinforcement using a compound of colours and patterns as orientation cues (CS1+CS2) for 10 and 16 min, respectively. The groups were then further subdivided into two half-experiments each according to which stimulus (colours or patterns) was chosen as CS1 and was presented during the

subsequent single-stimulus phase. This phase consisted of two 4 min periods of training (CS1+US), with an intervening 2 min test (CS1 alone). The final 2 min test was conducted with the alternative stimulus (CS2) alone. Sensory preconditioning is said to have occurred if this final test shows a significant learning score.

Note that the control experiments commonly used in similar studies to rule out other effects such as generalization or sensitization are not necessary in our design because none of these effects could help the fly determine which of the two patterns or two colours it should avoid.

Data evaluation

The pattern or colour preference of individual flies was calculated as the performance index: $PI = (t_a - t_b) / (t_a + t_b)$. During training, t_b indicates the time for which the fly was exposed to the reinforcer and t_a indicates the time without reinforcement. During testing, t_a and t_b refer to the times when the fly chose the formerly (or subsequently) unpunished or punished flight direction, respectively. Thus, when $t_a = t_b$, $PI = 0$, when $t_a > t_b$, the learning score is positive and when $t_a < t_b$, the learning score is negative.

Statistical analyses

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

Results

In visual learning of *Drosophila* in the flight simulator (Fig. 1; Wolf and Heisenberg, 1991; Wolf and Heisenberg, 1997; Wolf et al., 1998), patterns and colours have been successfully used as CSs. Not unexpectedly, *Drosophila* also learn colours and patterns if these are presented as compound stimuli (Fig. 2A,B). This result constitutes the basis for testing blocking and sensory preconditioning in flies.

Blocking

Symmetrical stimuli

In his original design, Kamin (Kamin, 1968) used a simple control group that received no first training, but was otherwise identical to the blocking group. We have also tested a group of flies without a first conditioning phase (Fig. 2). This primarily ensures that both stimuli give reasonably high learning scores after compound training without prior conditioning history and serves as one of the comparisons with the blocking group. We used four groups of flies that all

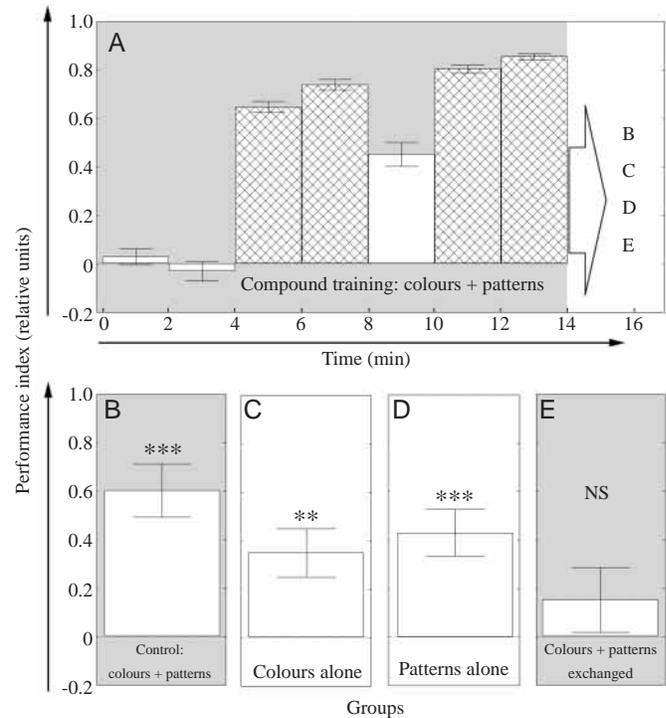


Fig. 2. Results from the 'Kamin control' experiment with patterns and colours as elements of the compound. (A) Pooled compound training data for all flies used in this experiment. The final 2 min test period (i.e. minutes 14–16) of each subgroup is depicted in B–E ($N=103$). (B) Compound control ($N=25$). (C) Colours alone. T-patterns were replaced by four vertical bars ($N=28$). (D) Patterns alone. Colour filters were removed ($N=25$). (E) Nonsense compound. The contingency between patterns and colours was reversed such that positive scores would indicate a correct colour choice and negative scores a correct pattern choice ($N=25$). Statistical analyses are the results of a Wilcoxon matched-pairs test against zero: ***significant at $P < 0.001$; **significant at $P < 0.01$; NS, not significant. Cross-hatched columns, training; open columns, test period; shaded areas, compound stimulus; unshaded areas, single stimulus. Values are means \pm S.E.M.

received identical compound training during the first 14 min of the experiment (Fig. 2A). In the subsequent test phase, the first (control) group was scored for the compound (Fig. 2B). The second and third groups were presented with colours alone (Fig. 2C) and patterns alone (Fig. 2D), respectively (experimental groups). The fourth group was presented with a new compound in which the contiguity between colours and patterns was reversed (exchange group, Fig. 2E). The learning scores were defined so as to indicate a dominance of colour over pattern when the score was positive.

A Kruskal–Wallis ANOVA for all four groups revealed a significant difference between groups ($P < 0.006$), encouraging a more detailed analysis. The control group (Fig. 2B) had a large performance index. The difference between the control and exchange groups was highly significant (Fig. 2B versus Fig. 2E; $P < 0.003$, Mann–Whitney *U*-test). Moreover, a Wilcoxon matched-pairs test confirmed that the learning scores

for the control group and both experimental groups were significantly different from zero (control, $P < 0.001$; colours alone, $P < 0.005$; patterns alone, $P < 0.001$), whereas the performance index for the reversed colour/pattern contiguity was not significantly different from zero ($P = 0.23$). The two experimental groups did not differ significantly from each other ($P = 0.47$, Mann–Whitney U -test), but the group that had been presented with colours alone differed significantly from the control group ($P < 0.006$, Mann–Whitney U -test). The difference between the group presented with patterns alone and the control group just failed to reach statistical significance ($P = 0.07$, Mann–Whitney U -test). We therefore conclude that presenting the individual stimuli alone after binary compound training of patterns and colours in the *Drosophila* flight simulator led to intermediate, but nevertheless significant, learning scores that did not differ from each other. This result is important for the interpretation of the experiments described below.

The blocking groups

Two blocking experiments were performed that differed in the amount of compound training and the choice of control procedures (see Materials and methods). As the outcome was essentially the same, the results of only one of the experiments are presented here in detail (Fig. 3). In this experiment, the final test during the first training phase and the carry-over (i.e. the amount of learning from the first phase still present in the subsequent phase) in the first compound test phase of the blocking group did not differ between the two half-experiments (first training colours and first training patterns) ($P = 0.08$; between-groups effect in a repeated-measures ANOVA over both periods and both half-experiments). Therefore, the results of these two half-experiments have been pooled (Fig. 3A). The same evaluation yielded a significant within-group effect ($P < 0.008$), rendering the difference between the last test during pretraining and the carry-over in the first compound test phase statistically reliable. We did not pool the corresponding control half-experiments (Fig. 3B,C) because two different procedures were used for the first training phase (see legend to Fig. 3 and Materials and methods).

In vertebrates, several criteria have been found to be crucial for blocking. One is the equivalence of the two stimuli. We have shown this criterion to be met in the present case (Fig. 2, Fig. 3B,C). Another essential criterion is the high predictive value of the stimulus trained first. In operant conditioning, it is not possible to verify a predictive value of 100% for a stimulus because there is no reflex-like relationship between a response and a stimulus. Rather, the animal exhibits active behaviour and controls its stimulus situation by trial and error (for a discussion of operant behaviour and initiating activity, see Heisenberg, 1983; Heisenberg, 1994). Therefore, the first training is performed until an asymptotic level of performance is reached. Prolonged operant pattern learning determined this level to be reached after four 2 min periods of training (Brembs and Heisenberg, 2000). Moreover, it has been shown that the level of performance reached after four 2 min periods of

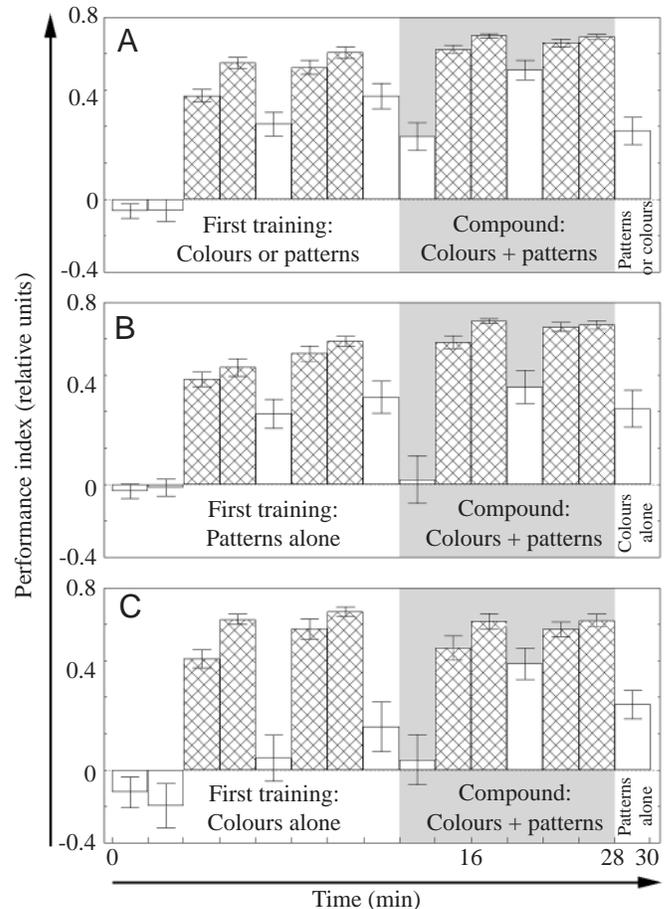


Fig. 3. Results of a representative blocking experiment. (A) Pooled half-experiments with colours or patterns (with BG18 filter) during the first training period ($N = 53$). (B) Control group with patterns (white light) during the first training period ($N = 27$); the switch from white light to monochromatic blue or green light erases the predictive value of the patterns. (C) Control group with colours (replay) during the first training period ($N = 26$). Cross-hatched columns training; open columns, test period; shaded areas, compound stimulus; unshaded areas, single stimulus. Values are means \pm S.E.M.

training was very stable, with little extinction over a period of at least 8 min (Guo et al., 1996; Liu et al., 1998; Wolf and Heisenberg, 1997; Xia et al., 1997b). Therefore, four 2 min periods of training should be optimal for producing a robust learning score during the first training phase for the first blocking experiment presented here (Fig. 3A–C). This will become very important when the blocking groups are compared with the various controls.

The Kamin control

Before we compare the first blocking group (Fig. 3A) with more rigorous control groups (Fig. 3B,C), it is interesting to compare it briefly with the 'classical' Kamin control (control 1), despite its apparent lack of control for the first training phase in the blocking group (data from the two groups in Fig. 2C,D are pooled for this comparison). Not surprisingly, the learning

score obtained for the compound stimulus was significantly higher in the blocking group (Fig. 3A) than in the control group (Fig. 2A,B) because the flies had already learned that one of the elements in the compound could be used to avoid heat ($P < 0.0002$, Mann–Whitney U -test between the results of the tests conducted with the compound stimulus prior to training the flies to the compound). This was still the case during training: the intermediate compound test score between the two training blocks was significantly higher in the blocking than in the Kamin control group ($P < 0.0001$, Mann–Whitney U -test). Although it seems that the compound was better predicted throughout the entire compound phase, the performance indices for the added stimulus in the blocking group were not significantly different from the corresponding performance indices in the Kamin control group ($P = 0.8$, Mann–Whitney U -test). On the contrary, the performance indices in the blocking group were just as high as after prolonged, asymptotic training (Brembs and Heisenberg, 2000).

Many blocking experiments control for the CS and US experience in the blocking group by first training, in the first phase, to a novel third stimulus that differs from both CS1 and CS2 prior to training the flies in the second phase to the compound (CS1+CS2+US). Therefore, we have designed more stringent control groups that not only encompass some of the variables controlled for by training a novel stimulus but also cover additional ones (see Materials and methods). Comparing any of these controls with the blocking groups, one might still find a significant difference.

An additional interesting result is revealed by the comparison of single-stimulus learning scores after compound (Fig. 2C,D) and after single-stimulus (Fig. 3A; ‘first training’) training. The significant difference ($P < 0.007$, Mann–Whitney U -test) indicates an interaction between the two stimuli because patterns and colours are learned better if trained and tested alone than if trained in a compound and tested separately. In other words, in principal, overshadowing does occur in *Drosophila* if stimulus intensities are chosen appropriately. In our design, however, either stimulus diminished the learning score of the other to the same extent. Thus, with our choice of stimulus intensities in the blocking experiment, overshadowing did not occur. With the appropriate choice of stimulus intensities/salience, a non-symmetrical overshadowing effect would, however, be expected.

Improved controls

Even though the ‘classical’ Kamin control experiment might be considered a sufficient control for the first blocking experiments (Fig. 3), we have addressed several possible confounding variables using four additional control procedures.

Similar to the ‘classical’ Kamin procedure, 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. 3A), this carry-over should be large (i.e. the generalization decrement should be small), indicating that the reinforcer is well predicted by the pretrained element

contained in the compound. In contrast, there should be no significant carry-over in the control groups (Fig. 3B,C). A Wilcoxon matched-pairs test against zero confirmed that the control animals were naive to the compound ($P = 0.79$), whereas the performance index of the experimental group was highly significantly different from zero ($P < 0.0002$). Thus, the application of heat is better predicted in the blocking group, satisfying the most important criterion for blocking to occur. Moreover, comparing the intermediate test period during the compound training phase between experimental and control groups, the experimental group still showed better avoidance than the control groups ($P < 0.045$, Mann–Whitney U -test), indicating that the US is better predicted not only at the beginning of the compound training, but also throughout the entire compound phase. Just as in the comparison with the Kamin control, there was again a significant difference between the blocking and the control group, demonstrating that the compound was predicting reinforcement better in the blocking group (controls 2 and 3).

Despite the fact that all requirements for blocking seemed to have been met, the final learning score was again indistinguishable between the experimental and control groups ($P = 0.77$, Mann–Whitney U -test), giving no indication of blocking. The same held true for the second experiment in which the compound training phase was reduced to 4 min and the control groups were spared the reinforcement in the first phase with the single CS to exclude any possible predictive value of US experience (data not shown; control 4).

The second-order conditioning control

Second-order conditioning is very similar to a blocking experiment. Again, after 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 expected 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. One can consider a second-order conditioning experiment to be a blocking experiment in which reinforcement is omitted in the compound phase. Thus, second-order conditioning constitutes an important control for the blocking experiment (control 5): if blocking does not occur, then this might be due to second-order conditioning masking a potential blocking effect (Dickinson et al., 1983). However, the presentation of the compound without heat after conditioning may attenuate the CS1–US association (extinction). In addition, extinction might even be facilitated by the second stimulus (CS2) signalling non-reinforcement of the compound (CS1+CS2; conditioned inhibition; see, for example, Gewirtz and Davis, 2000). Despite these considerations, we decided to control for second-order conditioning effects (Fig. 4). Arena illumination encompasses the patterns and constitutes a major portion of the fly’s visual field. Therefore, only colours were used as CS1 assuming that colour might be a better second-order US than pattern orientation. To match both blocking

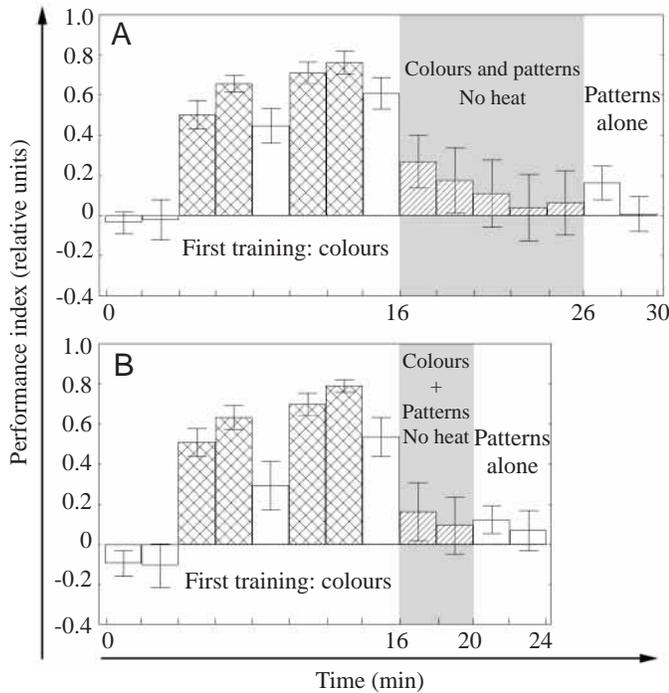


Fig. 4. Results from the second-order conditioning control experiments. (A) Experimental time course as in the blocking experiment depicted in Fig. 3 ($N=20$). (B) Time course as in A, but with the duration of the unreinforced presentation of the compound stimulus reduced from 10 to 4 min prior to testing ($N=22$). Cross-hatched columns, training; open columns, test period; hatched columns, second-order training (no heat); shaded areas, compound stimulus; unshaded areas, single stimulus. Values are means \pm S.E.M.

experiments, the experiment was performed twice, with 10 and 4 min of second-order training. Both yielded only small second-order learning effects that were statistically significant only if the performance indices of the two experiments were pooled ($P<0.02$; Wilcoxon matched-pairs test; $P=0.08$ for the two experiments considered separately).

A steep extinction curve (or conditioned inhibition) is the most likely explanation for the small second-order learning effect. By the first 2 min period of the second-order training phase, avoidance of the colour previously combined with heat was down to a performance index of approximately 0.2 from 0.6 for colour alone after the initial training. Again, only pooling the data from the two experiments (Fig. 4A,B) yielded a statistically significant difference from zero ($P<0.02$, Wilcoxon matched-pairs test). For the second 2 min period, even pooling the two experiments failed to produce a statistically reliable positive performance index ($P=0.15$, Wilcoxon matched-pairs test). Summing up, we could find only a slight second-order conditioning effect that was presumably too small to mask any strong blocking.

Sensory preconditioning

Formally, sensory preconditioning is the temporally reversed analogue of second-order conditioning. In sensory preconditioning, exposure to the compound (CS1+CS2) precedes

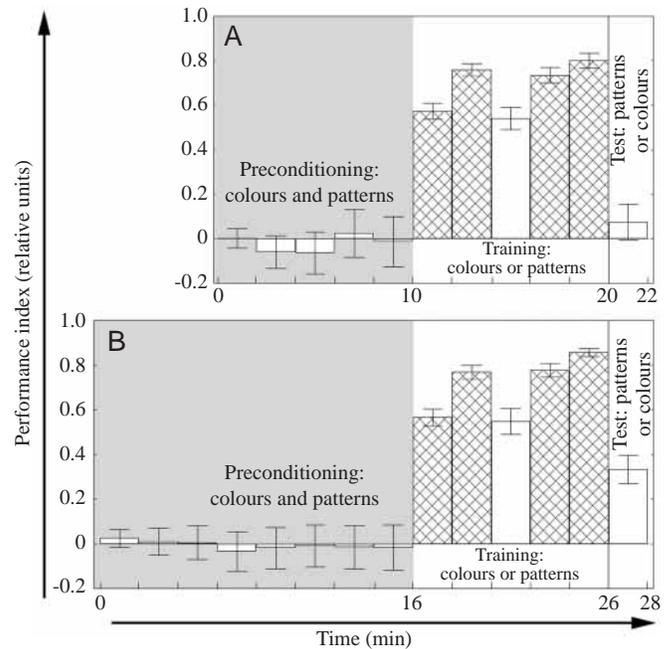


Fig. 5. Results from the sensory preconditioning experiments. In the preconditioning phase, the compound stimulus is presented without reinforcement. Results from corresponding half-experiments have been pooled. (A) 10 min of preconditioning ($N=56$). (B) 16 min of preconditioning ($N=56$). In the final test, the alternative stimulus to that used in the training period is used. Cross-hatched columns, training; open columns, test period; shaded areas, compound stimulus; unshaded areas, single stimulus. Values are means \pm S.E.M.

training (CS1+US). Hence, no extinction can occur between training and testing. Flies were exposed to 16 min of unreinforced flight in which flight directions were designated by compound stimuli consisting of colours and patterns (CS1+CS2). If, immediately afterwards, one of the stimuli is paired with heat (CS1+US), the other one (CS2) is regarded as a predictor of safe and dangerous flight orientations, respectively, in the subsequent test (Fig. 5B). No statistically significant performance index was observed in the final test with only 10 min of preconditioning (Fig. 5A). The difference between the results of these two experiments was statistically significant ($P<0.01$, Mann-Whitney U -test). This is in line with what has already been concluded on logical grounds (see Materials and methods), namely that other, non-associative effects such as generalization and sensitization cannot account for the significant sensory preconditioning effect found in Fig. 5B: presenting the compound stimulus for only 10 min is not sufficient to produce sensory preconditioning. In each of the two experiments, the two half-experiments (using colours or patterns as CS1, respectively) yielded statistically indistinguishable results, justifying the pooling of the corresponding data sets.

Discussion

We have explored operant visual learning of *Drosophila* in the flight simulator using compound stimuli as CSs. Several

conclusions can be drawn from our results. First, flies acquire, store and retrieve the two CSs, 'colours' and 'patterns', separately. They do not store them only as a compound. Whether they can distinguish the compound from the sum of its components ('configural learning') has not yet been investigated. We note that in discrimination learning each of the component CSs consists of a CS⁺ and a CS⁻ (blue and green; upright and inverted T pattern). Dwelling time analysis (Dill et al., 1995) indicates that, for colours and patterns, both the CS⁺ and the CS⁻ are probably remembered (data not shown). This would bring the number of simultaneously stored memory items to four.

Second, in all our experiments, reinforced compound presentation produced equally strong associations for both stimuli. We found no blocking and no overshadowing of colours over patterns or *vice versa* with our choice of stimulus intensities. We did, however, find reduced learning scores for either of the two stimuli after training with the compound stimulus compared with single-stimulus training. This implies, not unexpectedly, that overshadowing does exist in *Drosophila*. Overshadowing is a well-known phenomenon in classical (e.g. James and Wagner, 1980; Rauhut et al., 1999; Rubeling, 1993; Tennant and Bitterman, 1975) and operant (e.g. Farthing and Hearst, 1970; Miles and Jenkins, 1973) conditioning in vertebrates and invertebrates (e.g. Couvillon et al., 1996; Pelz et al., 1997; Smith, 1998). With our choice of stimuli, the finding that the overshadowing effect was symmetrical might be taken as a further indication of the absence of a significant difference in effectiveness between colours and patterns.

If prior conditioning history leads to the pretrained stimulus overshadowing the added stimulus, the effect is called blocking. Blocking is a cornerstone of modern learning theories (e.g. Pearce, 1994; Rescorla and Wagner, 1972; Sutton and Barto, 1990; Wagner, 1981). While its discovery by Kamin (Kamin, 1968) has had a large impact on vertebrate research (see Cheatele and Rudy, 1978; Holland and Gallagher, 1993; Jones et al., 1990; Kimmel and Bevill, 1996; Mackintosh, 1990; Pearce, 1997; Roberts and Pearce, 1999; Thompson et al., 1998), the ecological significance (Dukas, 1999) and neural mechanisms underlying blocking are little understood (Fanselow, 1998; Schultz and Dickinson, 2000). The literature on blocking in invertebrates is more scarce. Moreover, in the few instances where blocking has been reported (Couvillon et al., 1997; Rogers and Matzel, 1995; Rogers et al., 1996; Sahley et al., 1981; Smith, 1997; Smith, 1998), confounding effects have been pointed out and remain to be solved (Farley et al., 1997; Gerber and Ullrich, 1999). In particular, the case of honeybee proboscis extension learning has been intensely debated. Smith and co-workers first found blocking (Smith, 1996; Smith, 1997; Smith and Cobey, 1994). Gerber and Ullrich (Gerber and Ullrich, 1999) later identified confounding variables in the work of Smith that produced a blocking-like effect and showed that eliminating these variables also eliminated blocking. Most recently, Hosler and Smith (Hosler and Smith, 2000) have again reported blocking in the honeybee, but only with chemically similar odours. We could find no unambiguous or undisputed evidence in the literature that invertebrates exhibit blocking.

We conducted two blocking experiments that varied in the amount of compound training. The experimental design used for these experiments was derived from experiments showing asymptotic (Brembs and Heisenberg, 2000) and robust (Guo et al., 1996; Liu et al., 1998; Wolf and Heisenberg, 1997; Xia et al., 1997b) single-stimulus learning. Five different control procedures were carried out. Four of them qualitatively and quantitatively varied the CS, US and flight simulator experience of the flies prior to compound conditioning. One of the groups controlled for a second-order conditioning effect that might mask a potential blocking effect (Dickinson et al., 1983). In all experiments, the key conditions allowing the detection of a blocking effect have been met, namely, control and experimental groups differed in the predictive value of the compound (e.g. Fig. 3). Specifically, in the blocking group (Fig. 3A), each of the stimuli presented individually in the first phase of training retained its effectiveness in the second phase, i.e. in the training to the compound. The first training phase caused neither overshadowing of one stimulus over the other (Fig. 2) nor a large second-order conditioning (Fig. 4). Nevertheless, despite varying the 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 from invertebrates, let us consider other potential explanations for why it was not found in our experiments.

There are two types of reason for blocking not to occur in the flight simulator: (i) some qualitative property of the paradigm may interfere with it; and (ii) blocking might not be obtainable over the experimental time course used here.

First, blocking was initially shown to occur in classical (Pavlovian) conditioning paradigms (e.g. Holland, 1997; Kamin, 1968; Kimmel and Bevill, 1996; Marchant and Moore, 1973; Miller and Oberling, 1998; Tennant and Bitterman, 1975). It was later extended to instrumental (operant) conditioning, using discriminative stimuli (SDs; e.g. Feldman, 1971; Feldman, 1975). Operant SDs, however, share a feature with 'classical' CSs: they are at most only partially controlled by the animal. They are very different from stimuli controlled entirely by the animal, as in our approach. SDs can be perceived as 'setting the occasion' upon which behaviour-reinforcer contingency occurs. Their predictive relationship to the US is therefore only indirect (*via* the behaviour). We do not know of any study using our type of operant conditioning to produce blocking. It could therefore be that the high degree of operant control of the stimuli prevents blocking. We do not consider this explanation very likely, however, because we have evidence that visual learning in the flight simulator is a case of classical learning in which the operant behaviour facilitates CS-US acquisition (Brembs and Heisenberg, 2000).

Bitterman (Bitterman, 1996) argued that blocking in bees can only be shown within and not between sensory modalities (Couvillon et al., 1997). Colours and patterns might represent two modalities. Moreover, in honeybee proboscis extension conditioning, odours might have to be sufficiently similar to produce blocking (Hosler and Smith, 2000). In our case, the colours and patterns may be too dissimilar.

Second, and more likely, the failure to obtain blocking could be due to a significant generalization decrement of learning upon the introduction of the second CS in the compound phase (Fig. 3A). In addition, conditioned inhibition of generalized learning was observed in the second-order conditioning experiments (Fig. 4). This quick decay of the memory effect might continue in the presence of the US in the blocking experiment, attenuating the predictive value of the CS1 sufficiently strongly to render the flies almost naive even in the shorter blocking experiment (results not shown). In this case, the compound stimulus (CS1+CS2) might be sufficiently 'surprising' for the new stimulus (CS2) to acquire associative strength. The possible occurrence of this 'surprise' element may constitute the main difference between the blocking experiments conducted in invertebrates and vertebrates. Whereas, in our experiments, training in the first phase of the experiment lasted for no longer than 8 min, in the experiments on vertebrates it lasted for long periods, sometimes for a whole week.

Vertebrates may use this extensive training to explore the situation and to generate memory templates with much higher reliability than can ever be obtained with our design. In the flight simulator, in particular, the fly with a single degree of behavioural freedom has little opportunity to explore the situation and to increase its level of 'orientedness' (for an explanation of this term, see Heisenberg and Wolf, 1984). In addition, 8 min in the life of a fly might well be as long as several days in the life of a rat or a pigeon. Perhaps blocking occurs only if the initial training has not only rendered the CS1 a certain or almost certain predictor of the US, but has, in addition, been stored in the memory reliably enough to render CS1 particularly difficult to extinguish during further training. However, flies will show significant avoidance for at least 8 min if no changes are made to the experimental arrangement after training (Guo et al., 1996; Liu et al., 1998; Wolf and Heisenberg, 1997; Xia et al., 1997b).

Thus, while some conditioned inhibition was expected to occur, such a rapid decay of avoidance behaviour upon compounding the colours with the patterns is surprising. Maybe the standard procedure (Wolf and Heisenberg, 1991), even though it has been shown to produce asymptotic (Brembs and Heisenberg, 2000) and robust (Guo et al., 1996; Liu et al., 1998; Wolf and Heisenberg, 1997; Xia et al., 1997b) learning, is insufficient to produce the required CS1 processing. However, the significantly larger learning scores of both the carry-over and the intermediate compound test provide clear evidence that the compound becomes a reliable predictor of the US after only 6 min, rendering conditioned inhibition a less than obvious explanation. A more extensive first training (CS1+US; Xia et al., 1997a) could perhaps decrease the generalization decrement as well as minimize conditioned inhibition. If it were possible to obtain a clear second-order conditioning by attenuating the rapid extinction, this would open the possibility that the reinforcement during compound conditioning in a blocking experiment might have the same effect. In other words, reinforcement of the compound might decrease both the generalization decrement and extinction,

resulting in an augmented second-order conditioning that might, in turn, mask blocking that would otherwise be visible. Such effects remain to be discovered. However, Cheatle and Rudy (Cheatle and Rudy, 1978) showed that, in their study, reinforcing the compound blocked second-order conditioning. While it seems reassuring that the second-order conditioning effect in this study is too small to mask any substantial blocking, conditioned inhibition still needs to be completely excluded as an explanation for our failure to find blocking. For the reasons given above, however, one would expect at least partial blocking in the present experiments, since the compound is, indeed, better predicted in the blocking than in the control groups throughout the entire compound phase, despite conditioned inhibition.

While there are a number of reasons why blocking might exist in *Drosophila* but was not detected in the present study, the interesting possibility remains that flies, if not invertebrates in general, might lack blocking altogether in their learning performance. Why should blocking be a speciality of vertebrates? Clearly, whether to add further stimuli to the already existing CSs or not is a cost/benefit trade-off. There is no reason not to remember a stimulus, even if it is only vaguely predictive for the US, if this improvement of the memory template can be obtained at low cost. Apparently, for vertebrates, this cost exceeds the benefit: they show blocking. Since one would not assume the costs for a more elaborate memory template in invertebrates to be lower than those in vertebrates, one could argue that, for invertebrates, the benefit of improving memory templates is comparatively large because their reliability is generally low. In other words, blocking may not be implemented in invertebrates because their memory templates never convey the same high degree of reliability as those of vertebrates.

The experiments with reinforcement of the compound provide some evidence for complex stimulus processing in *Drosophila*. More clear-cut results than in the blocking experiment were obtained when the compound was experienced without reinforcement: we revealed a direct interaction between the two components in the compound stimulus because they formed a reciprocal association in our second-order conditioning and sensory preconditioning experiments. This is obvious in second-order conditioning, in which the CS1 assumes the role of the US. In sensory preconditioning, the preference of CS2⁺ and avoidance of CS2 (respectively) in the final test revealed that CS1⁺ and CS2⁺ as well as CS1 and CS2 have formed specific associations during the preconditioning phase. There are some earlier reports of sensory preconditioning in invertebrates (Couvillon and Bitterman, 1982; Kojima et al., 1998; Müller et al., 2000; Suzuki et al., 1994). Sensory preconditioning can most readily be perceived as a form of 'incidental learning' in which two equally salient stimuli are associated in a symmetrical manner (as opposed to the asymmetric relationship between the CS and US in normal 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., 2000) and in rats (Lyn and Capaldi, 1994; Rescorla, 1980). Also, in zebrafish (*Brachydanio rerio*), Hall and Suboski (Hall and Suboski, 1995) successfully used simultaneous stimulus presentations. In mammals, backward pairing with respect to the stimulus in the final test leads to excitatory, rather than inhibitory, sensory preconditioning associations (Hall, 1996; Ward-Robinson and Hall, 1996; Ward-Robinson and Hall, 1998). In the flight simulator, the colour of the arena illumination was changed exactly between two patterns, providing neither a forward nor a backward relationship between colours and patterns. This difference between incidental learning (for a review, see Hall, 1996) and normal conditioning is no surprise because the asymmetric dependence on the temporal arrangement of the CS and US in normal conditioning is reflected by the difference in biological significance between the CS and US (for a review on this timing dependence, see Sutton and Barto, 1990).

Dill and Heisenberg (Dill and Heisenberg, 1995) have reported one case of incidental learning in the flight simulator termed 'novelty choice'. Flies without heat reinforcement remember patterns and compare them with other patterns later. Novelty choice learning seems to be considerably faster than the preconditioning effect observed in the present study. In the novelty choice paradigm, even a 1 min exposure biased the subsequent pattern preference (Dill and Heisenberg, 1995), while in the present experiment a 10 min preconditioning phase was not enough for a significant association to be formed (Fig. 5A). 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 links patterns and colours during preconditioning because the sudden changes in the colour of the illumination are firmly coupled to certain changes in angular pattern position. To detect such coincidences, the fly must compare the temporal structure of the various sensory channels. The same mechanism has recently been postulated for normal associative conditioning because, to separate the CS from the context, the animal needs to compare the temporal structure of the various sensory stimuli present during training (Liu et al., 1999). In both instances, normal conditioning and sensory preconditioning, transient storage of the incoming sensory data is probably a prerequisite. In novelty choice learning, pattern storage might be a single step. In summary, one can propose that incoming sensory data are briefly stored to allow a search for temporal coincidences. Memory templates with a similar temporal structure are bound together and kept in storage for an additional period.

To conclude, reinforcing compounds of our choice of colours and patterns always produced symmetrical conditioning to the two CSs, regardless of previous conditioning history (no blocking). It would be premature, however, to conclude that simple temporal CS-US pairing is always sufficient to produce CS-US associations in *Drosophila*. We have demonstrated, however, that unreinforced presentation of the compound can lead to memory formation, proving that CS-US pairings are not necessary for a CS to accrue associative strength (sensory preconditioning).

As in vertebrates, associative learning in invertebrates requires complex processing of sensory stimuli during memory acquisition. Further research is needed to determine the extent to which these processes are shared across phyla.

We would like to thank Reinhard Wolf for his inspiration, the many invaluable discussions and his unselfish assistance in solving technical difficulties. We are also indebted to Robert Rescorla and Bertram Gerber for critically reading an earlier draft of this manuscript. One of the reviewers provided a thorough evaluation suggesting many improvements which we gratefully acknowledge. The work was supported by SFB554 and a grant (He 986/10) of the Deutsche Forschungsgemeinschaft.

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