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G. Wustmann · K. Rein · R. Wolf · M. Heisenberg

A new paradigm for operant conditioning of *Drosophila melanogaster*

Abstract A freely walking single fly (*Drosophila melanogaster*) can be conditioned to avoid one side of a small test chamber if the chamber is heated whenever the fly enters this side. In a subsequent memory test without heat it keeps avoiding the heat-associated side. The memory mutants *dunce*[1] and *rutabaga*[1] successfully avoid the heated side but show no avoidance in the memory test. Wildtype flies can be trained to successively avoid alternating sides in a reversal conditioning experiment. Every single fly shows strong avoidance and a positive memory score. The new conditioning apparatus has several advantages: (1) Statistically significant learning scores can be obtained for individual flies. (2) Learning scores are obtained fully automatically without interference of the experimenter. (3) The procedure is fast, robust and requires little handling. Therefore the apparatus is suitable for large-scale mutant screening. (4) Animals are not attached to a hook and thus can easily be used for breeding.

Key words Place learning · Reversal learning · Mutant analysis · Mutant screen

Abbreviations *dnc* *dunce* gene · PI performance Index · *rut* · *rutabaga* gene · S.E.M. standard error of mean

Introduction

Associative learning is classified either as classical (Pavlovian) or operant (instrumental). In classical conditioning the animal associates two sensory stimuli that occur simultaneously or in close succession. In operant conditioning the animal associates a stimulus and a motor program. A motor program that has an effect on a certain sensory stimulus is selected by trying out (Wolf and Heisenberg 1991). While considerable advances have been made recently in the analysis of classical conditioning (e.g. summarized in DeZazzo and Tully 1995; Kandel and Abel 1995), the cellular and molecular basis underlying operant learning is still little understood.

The fly, *Drosophila melanogaster*, provides the opportunity for a genetic dissection of learning and memory. Single gene mutations have been identified that specifically affect acquisition and various memory phases of odour discrimination learning (Tully et al. 1994; Heisenberg et al. 1985). Some of the respective genes may be involved modulating synaptic transmission (e.g. reviewed in Dudai 1988; Tully 1991; Davis 1993) others participate in setting up or maintaining certain neural circuits (review: Heisenberg 1989). Some of the mutants have been found in searches for genetic variants of brain structure others in behavioral screens. The two closely related learning paradigms used as assays are both olfactory conditioning tasks in which groups of flies are required to avoid an odorant coupled to an electric shock (Quinn et al. 1974; Tully and Quinn 1985). These experimental procedures require considerable experience, are capricious and time-consuming. Moreover, 100 to 200 flies are needed for a reliable learning score. Therefore, the number of mutant lines screened so far in this manner is still comparatively small.

Moreover, no direct effort towards obtaining mutants in operant conditioning has been reported. Genetic dissection of this type of learning would be of particular interest since it seems to consist of at least three independent components: endogenous activation of motor programs (initiating activity), correlation between efference copies of the motor programs and the relevant sensory stimulus (trying out) and the lasting modification of the successful motor program (operant

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Fig. 1 The apparatus and its connections (for explanations see text)
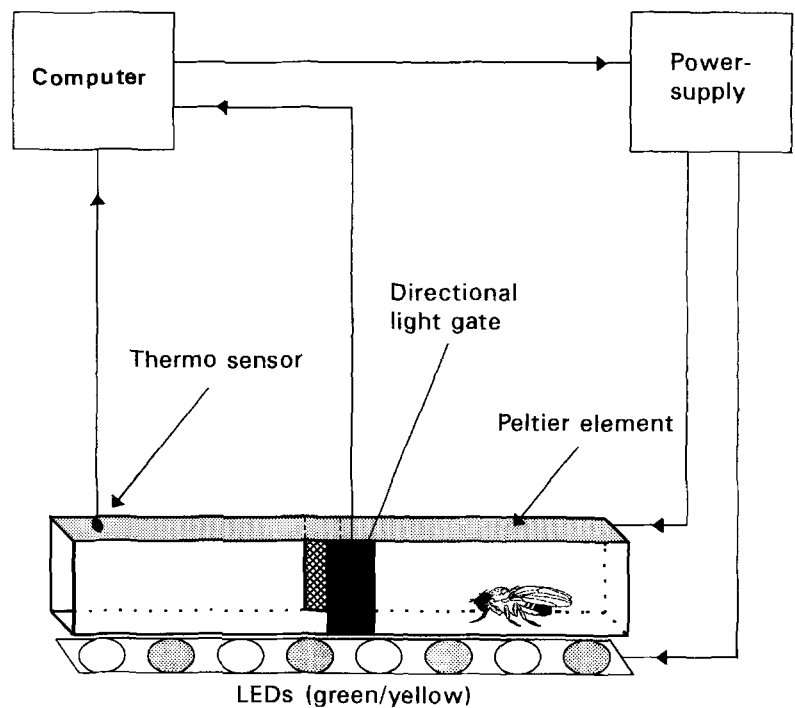
conditioning) (Wolf and Heisenberg 1991). A first step
towards such a screen has been made by Lofdahl and
Hirsch (1992) who describe an operant conditioning
procedure for single, freely walking flies which learn by
trying out to place their tarsi between the lines of an
electric grid in order to avoid receiving an electric
shock. Flies improve this behavior during the training
sessions but no aftereffect without reinforcement was
recorded. This type of avoidance behavior which may,
in principle, be immediately reversible as soon as the
reinforcement is over, is defined as "operant activity" to
distinguish it from the actual conditioning (Wolf and
Heisenberg 1991). Unfortunately, no successful mutant
screen has been reported so far with this device. We
describe here a new conditioning paradigm for
Drosophila, which allows to measure "operant activity"
as well as "operant conditioning" in single freely walk-
ing flies and we report the performance of existing
learning mutants in this apparatus.

Materials and methods

Flies

Canton-S (*CS*) and *Berlin* wildtype strains and the learning mutants
dunce[1] (*dnc*[1]) and *rutabaga*[1] (*rut*[1]) are used. Both mutations are in
a *CS* genetic background. All flies tested are between 2 and 6 days
old and are maintained at 25°C on standard cornmeal/molasses
medium in a 16 h light/8 h dark cycle at 60% humidity. Females as
well as males are studied (about 50% each). We have detected no
differences in the behavior of the two genders in the apparatus and
the respective data are pooled.

Conditioning apparatus

The test apparatus (Fig. 1) is a transparent rectangular chamber
$(40 \times 4 \times 2.5 \, \text{mm}^3)$. The upper surface $(4 \times 40 \, \text{mm}^2)$ consists of a
Peltier-element. A control circuit and a thermo-sensor in the
chamber keep the Peltier-element at a permissive "COLD" or non-
permissive "HOT" temperature. The chamber is virtually subdivided
perpendicular to its long axis into two halves ("LEFT" and
"RIGHT") by a directionally selective light gate. It consists of two
infra-red (IR) emitting diodes and two IR-sensitive photodedectors.
The dedectors are 3 mm apart (center to center).

The chamber is illuminated from below by 8 light emitting diods
(LEDs) alternating in color (green, yellow). Colors are switched by
the light gate so that the fly always sees 4 yellow diods while being
on one, and 4 green diods while being on the other side of the
chamber. [Originally it was assumed that the flies would modify
their behavior according to the colors but no such effect was found
(data not shown). However, since without illumination flies were
less active the switching of the LEDs was always included in the
procedure.]

Procedure

The experiment consists of a preference test, training and memory
test. A computer controls the experiment in the following manner:
During all 3 phases it continuously monitors the time and direction
of transitions at the light gate. During training, in addition, it turns
heat on when the fly enters one side and turns it off when the fly
leaves it again. Note that the Peltier-element covers the whole length
of the container. This ensures that the fly does not experience
a spatial but only a temporal temperature gradient. "HOT" is at
$(45 \pm 2)°C$ and "COLD" at $(28 \pm 2)°C$. The thermo-sensor responds
to the desired temperature within about 0.5 s and reaches the new
steady state in 3 to 4 s. During the preference and memory tests the
temperature is fixed at 28°C ("COLD"), independently of the posi-
tion of the fly. The chamber is used without cleaning for several
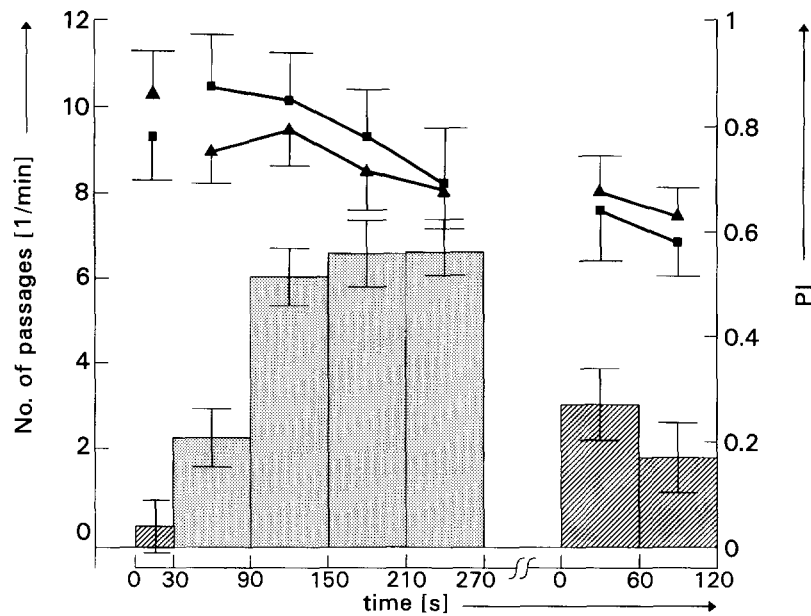
Fig. 2 *Upper part, scale on the left:* Average number of passages at the light gate per minute during a 390 s test session. In the experimental group (*squares*) heat avoidance conditioning is applied between 30 s and 270 s (see below). In the control group (*triangles*) no heat throughout. Data for experimental group are from experiment below. *Lower part, scale on the right:* Average Performance Index PI before, during and after avoidance training. The noxious stimulus is heat $(45 \pm 2)°C$. $PI = (A - B)/(A + B)$, where A is the time the fly spends on the side associated with no heat ("COLD") and B the time on the heat-associated side ("HOT"). Flies are trained to avoid the "LEFT" or the "RIGHT" side in an alternating sequence. Wildtype *Berlin*; number of flies in each group $n = 40$

hundred trials. Between consecutive trials sides for the permissive and non-permissive conditions are switched. No aftereffect from the previous experiment (e.g. alarm pheromone) is observed if this had been conducted with a different fly.

After training, the memory test does not start until the fly enters the other side. Since most flies efficiently avoid being heated, most of them are on the "COLD" side at the end of the training session. Therefore, most flies start the memory test on the "HOT" side. Because of this procedure we systematically underestimate the real memory score. For inactive flies that deviation may be large. However, as activity is monitored as well these flies can be evaluated separately.

Analysis of data

The performance of individual flies is calculated as Performance Index $PI = (A - B)/(A + B)$, with A indicating the time the fly spends on the side that during training is associated with low temperature ("COLD") and B indicating the time on the "HOT" side. The PI can vary between -1 and $+1$. A PI of zero indicates that the fly spends 50% of the time on the "HOT" side. In the preference test, PI is a measure for the flys spontaneous preference for one side of the chamber, during training it indicates heat avoidance and during the 3rd phase it is a memory score.

In most experiments, half of the flies are conditioned to avoid "LEFT" and the other half to avoid "RIGHT". In this way the effects of spontaneous preferences for one side and of slight asymmetries in the apparatus are largely eliminated from the data.

Results

The lower part of Fig. 2 shows the effect of heat conditioning on 40 wildtype *Berlin* flies. The following schedule is used: A preference test of 30 s is followed by a training phase of 240 s and a memory test of 120 s. No spontaneous preference for one side is apparent during the preference test (not shown in Fig. 2; $PI_{pref} = 0.049 \pm 0.05$; "LEFT" positive; note that the PI_{pref} in Fig. 2 shows the preference for the "COLD" side which, however, alternates between the "LEFT" and "RIGHT" side, and at that time is not yet revealed to the fly).

During the following 4 min of training flies manage to switch off the heat for about 75% of the time. Interestingly, avoidance improves rapidly during training. In the 1st minute flies seem still to be much confused since they reduce their heat exposure only slightly (from 50% (chance level) to 40% of the time). Already in the 2nd minute, however, they manage to stay out of the heat for over 75% of the time and remain at about that level for the rest of the training period. The difference in PI between the 1st and 4th minute of training is significant at a confidence level of $p < 0.01$. In the memory test, when heat is permanently switched off, the animals continue to prefer the side in the chamber that previously had not been heated ($p < 0.01$). The PI drops to about half the training value in the 1st minute. In the 2nd minute it declines even further but is still significantly positive. The rapid memory decay is not surprising considering that the memory test is at the same time an extinction training since the fly now experiences "COLD" on both sides of the chamber.

When first placed into the chamber most flies spontaneously start running back and forth between the two

Fig. 3 Average Performance Index PI before, during and after avoidence training of wildtype *Canton-S* flies (n = 40). For explanations see legend of Fig. 2
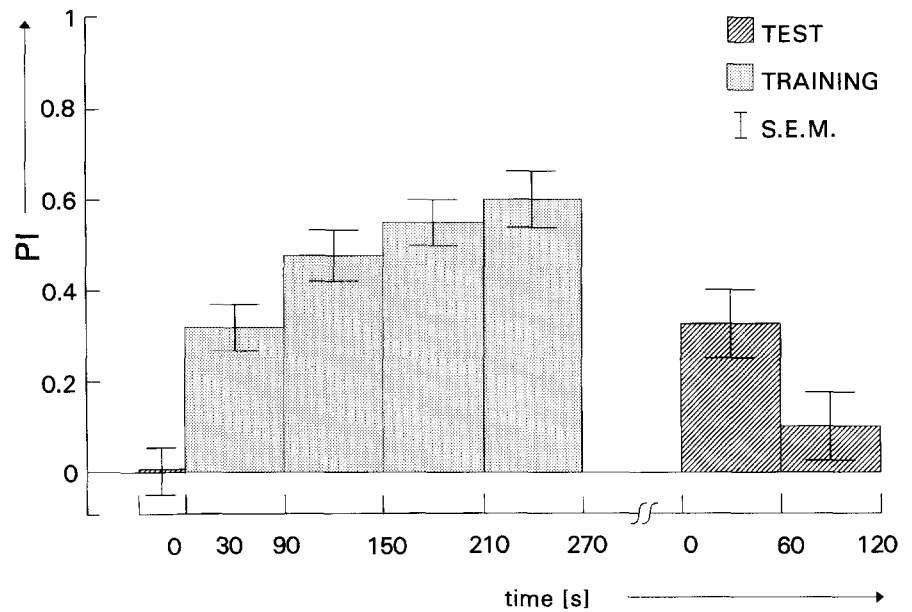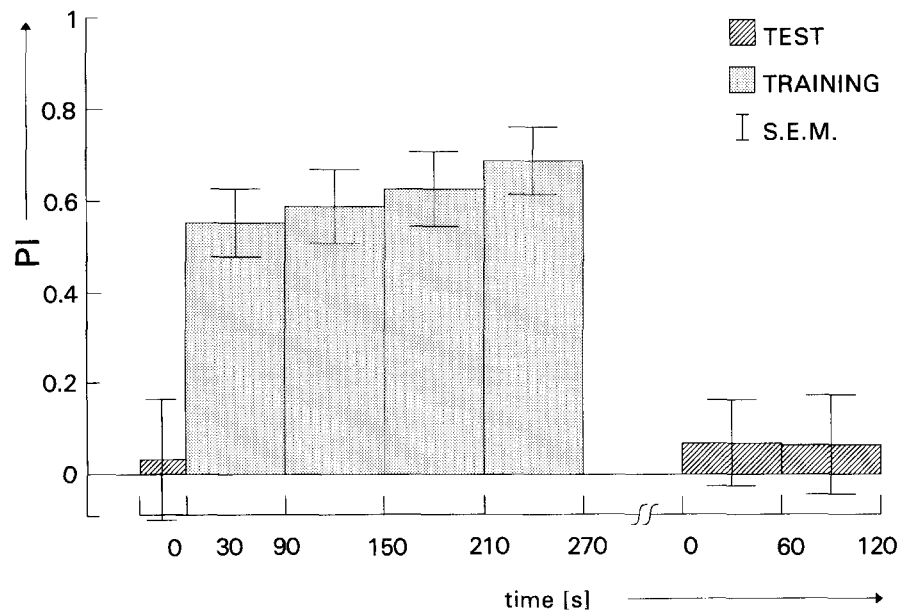

Fig. 4 Fast avoidance, no memory of cantonized *dunce*[1] flies (*n* = 40). Experimental conditions as in the experiment of Fig. 2

ends. This activity is interrupted by phases of rest which the flies may use for preening. Running can be interpreted as an attempt to escape or as exploratory behavior. In the present paradigm it is a prerequisite for operant conditioning. Running activity is estimated by counting the number of passages at the light gate. Flies showing no spontaneous running activity during the first minute (about 3%) are discarded since the performance of flies with low running activity can not be properly assessed. The results are shown in the upper part of Fig. 2. In the preference test at a continuous temperature of 28°C flies start out with about 10 passages per minute (p/min) and their activity declines to about 7 p/min at the end of the memory test. Surprisingly, under training conditions the activity profile seems to be little changed. The values lie on the same slope as those of the test phases and are not significantly different from the values of a control experiment without heat (triangles). Since during training flies spend about 25% of the total time at 45°C and in the mean temperature changes every few seconds between 45°C and 28°C, neither heat nor changes in temperature seem to have a major effect on running activity.

Fig. 5 Cantonized *rutabaga*[1]
mutant flies show poor
avoidance during training and
no memory. For explanations
see legend to Fig. 2
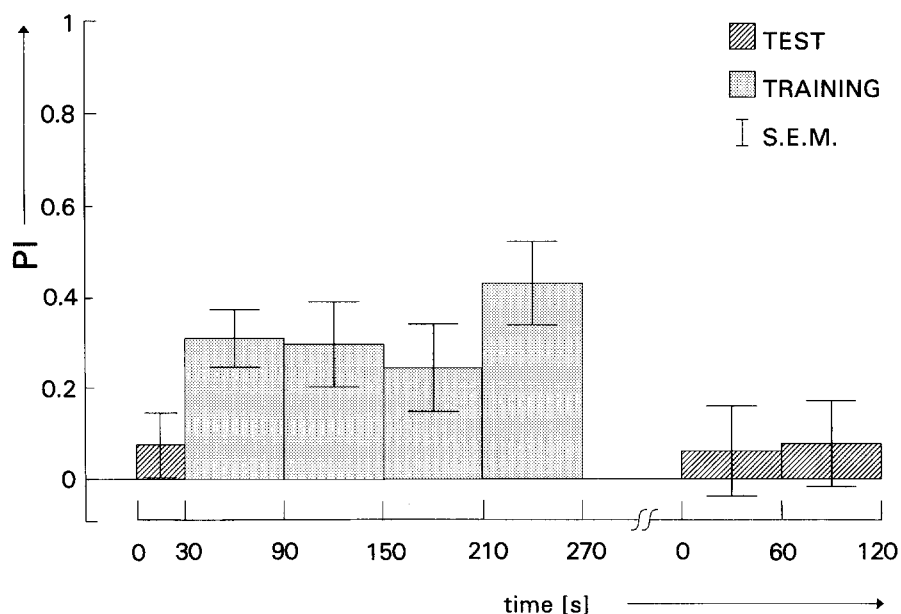
The corresponding performance of wildtype *Canton-S* flies is shown in Fig. 3. None of the data points differ significantly between *Canton-S* and *Berlin*. However, the increase in PI between the 1st and 2nd minute of training is not significant in *Canton-S* because the 1st-minute training value is already higher than in *Berlin*, indicating slightly faster learning. Also during the memory test *Canton-S* flies seem to learn faster. In the 2nd minute of the memory test the PI does not differ from zero ($p = 0.18$). Extinction is more pronounced than in *Berlin*.

Learning performance of mutants

The mutants *dnc*[1] and *rut*[1] which are deficient in classical odour avoidance conditioning (Dudai et al. 1976; Aceves-Pina et al. 1983), conditioned courtship suppression (Gailey et al. 1984) and visual pattern discrimination learning (Eyding 1993) were tested in the paradigm described here. The same procedure as above was used. The results are shown in Figs. 4 and 5, respectively. Because the *dnc*[1] and *rut*[1] stocks have the *Canton-S* genetic background, the mutants should be compared to this wild type. Both mutants avoid the heat during training but fail in the subsequent memory test. Interestingly, in contast to both wildtype strains, the two mutants show no improvement in their heat avoidance during the 4-min training phase. In *dnc*[1] flies the PI of the 1st minute is significantly higher than in *Canton-S*, implying that they learn faster than wild type. The avoidance performance of *rut*[1] flies starts at the same level as *Canton-S* but stays at that level for the rest of the training period. This defect may indicate a reduced perception of heat or a reduced association between sensory stimulus and behavior. Poor heat

avoidance during training of *rut*[1] has also been observed in pattern discrimination learning in the flight simulator (Eyding 1993). Running activity of the two mutants does not differ significantly from control flies. The frequency of passages at the light gate goes slighty and steadily down during the course of the experiment and the exclusion frequency is at about 3% (data not shown).

Reversal learning

As shown above wildtype *Drosophila* flies can learn to avoid heat by changing their position in a chamber and they keep avoiding the heat-associated side after the heat is switched off. This aftereffect, however, seems to fade quickly (Fig. 3). In order to assess how well in this paradigm new experiences can replace old ones, the following reversal learning schedule is applied: The fly is first trained for 3 minutes to one side of the chamber and then, after a memory test of 90 s, to the other. The second training is again followed by a memory test. This whole sequence is repeated. The mean performance of 12 flies is shown in Fig. 6. Half the flies start with heat on the "LEFT", the other half with heat on the "RIGHT" side. The memory score after the 2nd training session is significantly positive ($p < 0.05$) showing that flies prefer the side on which they had been safe for the last 3 min, despite bad experience there before. Nevertheless, it appears to be difficult for them to switch from one side to the other. During the 1st minute of the 1st and 3rd training they avoid the heat much better than in the same period of the 2nd and 4th training and also in the memory tests after the 1st and 3rd training they score higher than after the 2nd and 4th, indicating that in the new environment of the test

Fig. 6 Reversal learning of wildtype *Berlin* (number of flies: n = 12). Flies are trained to avoid one side of the container (duration of training: 180 s) and then tested for 90 s without heat. Immediately after the test training resumes but this time the fly has to avoid the other side. After a further memory test the whole sequence is repeated. Half the flies start with training to the "LEFT", the other half with training to the "RIGHT". The Performance Index PI is calculated as described in the legend to Fig. 2
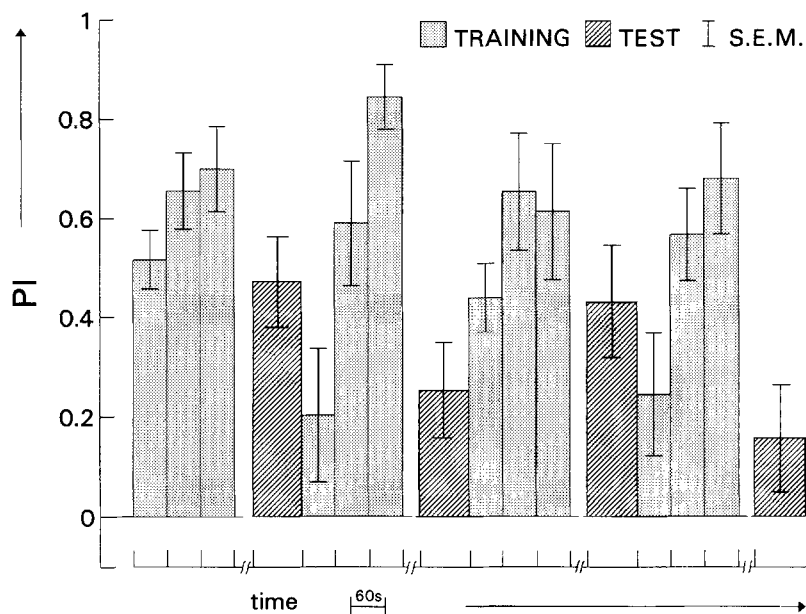

chamber the first experiences count more than subsequent ones. With 12 flies the memory score after the 4th training period is not significantly different from zero. Possibly, after repeated reversal conditioning flies rely more on immediate avoidance than on predictive strategies.

Single fly test

Fig. 7 shows training and memory scores for each of the 12 flies of the previous experiment. The four train-

ing and test phases are averaged for each minute of the training and the 90 s memory phase. Although differences among flies are large every single fly effectively avoids the heated side. Every one has a positive memory score and for 9 out of the 12 animals the memory score is $PI_{mem} > 0.2$. Furthermore, for 10 of the 12 flies avoidance is better in the 3rd than in the 1st minute of training. These results demonstrate that the new paradigm is well suited for single fly mutant screening. Note that the averaged memory score is only about 65% of that of the 1st memory score. Thus, without reversal memory scores are even more reliable.

Fig. 7 Reversal learning of individual wildtype *Berlin* flies. Performance indices for each minute of the training and the 90 s memory test from the four training/test periods are averaged (n = 4), irrespective of the side to which the flies were trained. Same data as shown in Fig. 6. See legend to Fig. 6 for further explanations
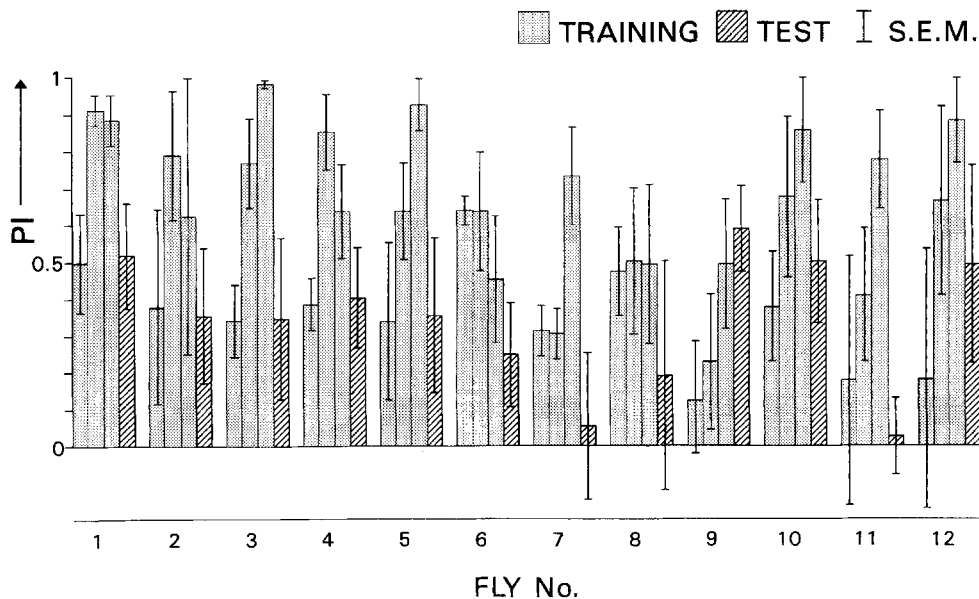
Discussion

Does the fly learn in the chamber?

In their natural habitat flies must be able to effectively avoid hot surfaces since even in temperate zones the sun heats dark stones to temperatures that may well be damaging. Do flies explore their environment when they are displaced to a new location? Can they remember, at least for a short while, where in their immediate surround the temperature is 'safe' and where not? We have interpreted the positive aftereffect in the memory test as evidence for learning and memory. As an alternative interpretation one has to consider the possibility that the fly might mark one of the sides with a pheromone and, in the so-called memory test, would discriminate between the two sides referring to this odour without having learned anything. We consider this explanation very unlikely. First, flies are tested in close succession. Hence, one would expect a bias for the "COLD" side of the previous experiment in the preference test. This is not observed. Also, we have tested naive flies immediately after an extensive training period of another fly and found no avoidance of the side on which the previous fly had been heated (data not shown). One might argue that the pheromone would decay fast but this does not fit with the positive PI in the 2nd minute of the memory test (Fig. 2). However, if each fly would avoid only its own odour traces, this would be difficult to distinguish from learning. Independent evidence for the aftereffect to be conditioned comes from the two memory mutants dnc^1 and rut^1. These have been shown to be disturbed in short-term memory but behave normally in at least some tests of spontaneous odour avoidance (e.g. Dudai 1988). We tentatively conclude that wildtype flies associate part of the chamber with heat and keep avoiding this part for a while even after they are not heated there anymore.

What are the essential cues in the chamber that are associated with heat? Vision seems to be of minor importance since the whole experiment can be run in complete darkness with the only disadvantage that running activity is much lower making the data less reliable. We assume that a combination of tactile and ideothetic information provide the flies with a 'spatial sense' telling it where in the chamber it is.

Is this learning operant?

Although in the new paradigm the fly switches the temperature by its walking behavior one can not tell whether this learning is operant or classical without going into the details of the fly's behavior in the chamber. In principle, the conditioning might be classical if the heat would cause an increase of running activity

and if, due to the training, the response to heat would be transferred to the coincident spatial cues in the chamber. This interpretation, however, is at odds with the data on running activity since this is not significantly increased during training. If an avoidance of $PI = 0.5$ were caused entirely by an increase of running activity in the heat the number of passages at the light gate should go up by 50%. One would have to postulate an equivalent reduction of running activity in the intermissions between the heating periods. Undocumented direct observation of the fly in the chamber does not support this assumption. Persistent differences in running speed or activity on the two sides are not apparent but one can often see the fly walking on the "COLD" side between the end and the middle of the chamber not crossing the (invisible) light gate. This behavior can hardly ever be observed in naive flies. Moreover, it would be difficult to reconcile the classical conditioning hypothesis with the improvement of the heat avoidance during training.

What, we believe, happens in this experiment is, that by trying out the fly discovers that its locomotion can switch the temperature. For trying out the fly correlates the efference copies of many or all of its possible motor activities with the temporal pattern of its thermoreceptors. In this way it can find out that temperature is influenced by its own position in the chamber. A significant correlation may reversibly change the respective motor program according to the sign of the correlation coefficient (operant activity) or the modification may even persist after the correlation coefficient is back to chance level (operant conditioning; Wolf and Heisenberg 1991) as in the case described here.

It should be mentioned on the side that the special construction of the chamber in which the whole length is simultaneously heated or cooled has nothing to do with the question whether the conditioning measured is classical or operant. Two separate Peltier-elements on the "LEFT" and "RIGHT" side, permanently adjusted to the "HOT" and "COLD" temperature respectively during the training period would probably be an excellent device making the learning task less confusing for the fly than the present apparatus and would provide similar data as shown here. The only reason we chose our design was that we had difficulties to measure how quickly after the training the temperature gradient in the chamber would disappear.

The two cAMP mutants dnc^1 and rut^1 mainly serve as controls in this study. Their memory defect in the new paradigm supports the notion that it is indeed memory that is recorded in this test. As was expected from visual pattern discrimination studies in the flight simulator (Eyding 1993) operant behavioral plasticity is not totally abolished in these mutants. Both still show operant activity. Avoidance is increased in dnc^1 and reduced in rut^1, as is the cAMP level (Dudai 1988). The deviations from normal in the two mutants are interesting for very different reasons.

In the case of *rut*[1] the low avoidance score may eventually be used to localize in mosaic animals that part of the circuitry involved in operant activity that requires the adenylate cyclase. The invariably high avoidance scores in *dnc*[1] question the interpretation of the increase in avoidance during the training period. We have proposed above that this increase reflects some learning performance and that *Canton-S* flies learn slightly faster than *Berlin* in this respect. Indeed, no increase is observed in the two mutants. However, if a high avoidance score in the 1st minute implies fast learning, *dnc*[1] flies learn even faster than *Canton-S*. It is not entirely impossible that the rate of this type of learning depends directly upon the cAMP level. Alternatively, one might speculate that when avoidance improves, the animal gradually reduces its scope of motor programs that it tries out and finally sticks to the successful one. In this case *dnc*[1] flies would try out fewer alternatives and would terminate their search process earlier than wild type.

Whatever the explanation may be, the experiments on *dnc*[1] and *rut*[1] prove that the new paradigm will effectively discriminate between wild type and learning/memory mutants in a screen for genetic variants. These should provide valuable insights into operant behavior and the relation between operant and classical conditioning.

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