Dissociation of visual associative and motor learning in *Drosophila* at the flight simulator

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Abstract

Ever since operant conditioning was studied experimentally, the relationship between associative learning and possible motor learning has become controversial. Although motor learning and its underlying neural substrates have been extensively studied in mammals, it is still poorly understood in invertebrates. The visual discriminative avoidance paradigm of *Drosophila* at the flight simulator has been widely used to study the flies' visual associative learning and related functions, but it has not been used to study the motor learning process. In this study, newly-designed data analysis was employed to examine the flies' solitary behavioural variable that was recorded at the flight simulator—yaw torque. Analysis was conducted to explore torque distributions of both wild-type and mutant flies in conditioning, with the following results: (1) Wild-type Canton-S flies had motor learning performance in conditioning, which was proved by modifications of the animal's behavioural mode in conditioning. (2) Repetition of training improved the motor learning performance of wild-type Canton-S flies. (3) Although mutant *dunce*[1] flies were defective in visual associative learning, they showed essentially normal motor learning performance in terms of yaw torque distribution in conditioning. Finally, we tentatively proposed that both visual associative learning and motor learning were involved in the visual operant conditioning of *Drosophila* at the flight simulator, that the two learning forms could be dissociated and they might have different neural bases.
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1. Introduction

Visual operant conditioning in *Drosophila* at the flight simulator has been extensively studied (for review, see Heisenberg et al., 2001). In this paradigm, the fly was trained to discriminate the flight direction with reference to two kinds of visual patterns where one pattern type was associated with heat punishment as a negative reinforcer (cf., Section 2). The paradigm has been used to investigate properties of the operant associative learning and memory in *Drosophila* (Wolf and Heisenberg, 1991; Guo et al., 1996; Xia et al., 1997; Brembs and Heisenberg, 2000), visual pattern recognition (Dill et al., 1993; Heisenberg, 1995; Ernst and Heisenberg, 1999), and functional interpretation of brain structures of flies (Wolf et al., 1998). Additionally, some cognition-like functions in *Drosophila* beyond simple associative learning, and their underlying neuronal substrates, have been recently revealed

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by this paradigm, such as context generalization (Liu et al., 1999), visual selective attention (Wu et al., 2000), and choice behaviour facing competing alternatives (Tang and Guo, 2001).

Since the paradigm was first reported (Wolf and Heisenberg, 1991), performance index (PI) has been introduced as a behavioural index to evaluate the flies' learning and memory abilities. In operant conditioning, the flight direction that the fly chooses determines the delivery of the reinforcer; the fly's own behavioural output is involved in conditioning. Thus, to explore how the animal's behavioural variable is modified with the learning course is as important as evaluation of PI. It was suggested that in operant conditioning there might be some motor skills or behavioural strategies involved (Wolf et al., 1998; Brembs and Heisenberg, 2000), but there was no evidence to provide the least support to explain flies' acquired motor programs in the visual discrimination conditioning. Therefore, further substaintial behavioural analysis of operant conditioning is needed, and the present study addresses to this issue.

Motor learning was defined as the acquisition of novel motor skills in motor tasks, and it was largely studied in mammals with a number of behavioural paradigms (Asanuma and Keller, 1991; Sanes, 2000). It has been demonstrated that motor cortex, cerebellum, and some other related brain regions are dedicated to various motor learning tasks. The present study used a torque analysis method to examine the flies' torque distribution in conditioning. After that, the flies' motor learning performance as related to modifications of the torque distribution was presented in the context of *Drosophila* visual operant conditioning. The ultimate goal of this study was to characterize flies' motor learning in such behavioural tasks and to reveal its relationship with visual associative learning.

2. Materials and methods

2.1. Flies

Drosophila melanogaster of wild-type Canton-S flies and mutant *dunce*[1] flies were used. Mutant *dunce*[1] flies have the same genetic background as Canton-S flies; behaviour–genetic analyses of the

dunce[1] fly have demonstrated that it is defective in olfactory associative learning and short-term memory but essentially normal in other behavioural respects (Dudai et al., 1976; Byers et al., 1981). Flies were maintained on a standard corn meal/molasses medium (Guo et al., 1996) at 25 °C and 60% humidity with a 14-h light:10-h dark photoperiod with light on at 08:00 h. Three- to four-day-old female flies were prepared according to standard procedures as described in earlier works (Heisenberg and Wolf, 1984). No sex-related difference was observed and it did not matter whether the female was virgin or not.

2.2. Apparatus and experimental procedures

The flight simulator (Fig. 1) was designed to set up an artificial closed feedback between the fly's yaw torque and angular velocity of the panorama surrounding the fly, which simulated circumstances for the fly's
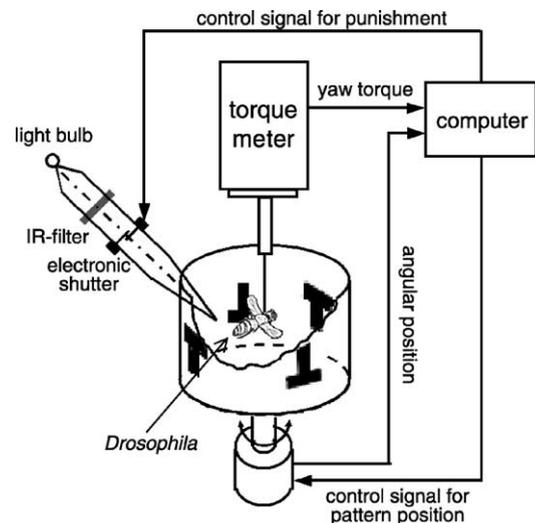

Fig. 1. Flight simulator setup for visual operant conditioning of *Drosophila*. Individual flies were fixed to a torque meter by glue at the head and thorax to complete stable flight. The torque meter measured each fly's yaw torque around its vertical axis, and the fly's only motor output was recorded. A rotating cylinder surrounding the fly served as the visual panorama and was conceptually divided into four quadrants with T-shaped patterns (two upright-T patterns and two inverted-T patterns) at the centres of the quadrants. An infrared (IR) beam was controlled by the computer and could be focused on the fly's rear to function as a negative reinforcer. Through a closed feedback the fly could control the rotation of the panorama with its yaw torque output.

stable flight (Heisenberg and Wolf, 1984; Wolf and Heisenberg, 1991). A torque meter was the core device of the flight simulator, on which individual flies were attached by a glued hook to the head and thorax. The torque meter and the tested single fly were placed in the centre of the cylindrical panorama that could turn around its vertical axis. The exclusive behavioural module of the tethered fly was the horizontal orientation, and the only recorded motor component of such flight behaviour was the fly's yaw torque around its vertical axis. The yaw torque was measured by the torque meter and stored in a computer. Two upright-T patterns and two inverted-T patterns on the cylinder were equally sized and located at the centres of the quadrants, and the same T-patterns were located in opposite quadrants. White light illuminated the panorama and the black visual patterns from behind. Through a feedback the fly could control the angular velocity of the panorama with its own yaw torque (coupling coefficient: $K = -11°$ (sec $\times 10^{-10}$ Nm)$^{-1}$, which enabled the fly to choose its flight orientation with reference to the two kinds of T-patterns on the panorama. The angular position of the cylinder was continuously recorded by the computer with the same sampling frequency (20 Hz) as the yaw torque variable. An infrared (IR) light beam was focused upon the fly's rear, which functioned as a negative reinforcer in conditioning and could be intercepted by a computer-controlled electronic shutter. Fig. 2 showed a fragment of the experimental traces for the recorded yaw torque and angular position of the panorama, with the shaded area indicating where the fly was heated by the IR beam.

Three kinds of experimental procedures were conducted (Table 1) and all of them were divided into 4-min long periods. The reinforcer was not introduced in the mass test procedure and the fly's spontaneous flight behaviour at the flight simulator was tested throughout. In experiments conducted with mass or standard training procedures, before each trial, one pattern type was appointed as the heat-associated punished object; during training, the heat was projected whenever the fly was heading towards quadrants containing the heated patterns and was intercepted when the fly oriented towards the other pattern type. In the mass training procedure, the negative reinforcer was presented throughout without test interval and the conditioned behaviour of the fly during successive training periods was checked. The standard training procedure was similar to that described in earlier works (Guo et al., 1996; Xia et al., 1997). In the pre-test period (PTE) before training, the IR beam was blocked and the fly's spontaneous preference for the two pattern types was tested; during the training periods (TR1 and TR2), the fly was trained to associate visual patterns with the negative reinforcer (heat punishment); the IR beam was blocked again in the test period (TE) and the memory retention of the fly was tested. Through the conditioning with mass or standard training procedures, the fly was trained to distinguish the four quadrants as conceptive 'HOT' domains (heat present) and 'COLD' domains (heat
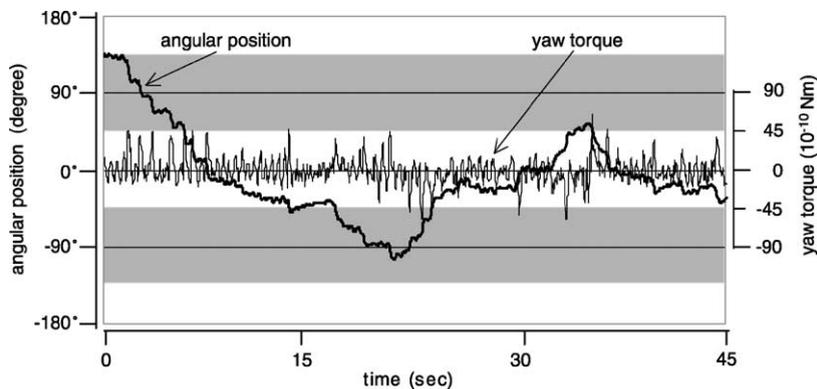

Fig. 2. Experimental traces from a 45-s experimental course. The variable of the arena's angular position varied between −180 to 180° and the variable of fly's yaw torque fluctuated around zero. The shaded area represented where the angular position was associated with the heat. Data was from a Canton-S fly during a training period with standard training procedure.

Table 1
Experimental procedures used to study the fly's associative learning and motor learning at the flight simulator

Time (min)	0–4	4–8	8–12	12–16
Mass test	Test (TE1)	Test (TE2)	Test (TE3)	–
Mass training	Training (TR1)	Training (TR2)	Training (TR3)	–
Standard training	Pre-test (PTE)	Training (TR1)	Training (TR2)	Test (TE)

absent) (Fig. 3A). To avoid the heat punishment, the fly learned to choose its flight pathways towards the COLD but not HOT domain. Data obtained with mass test and mass training procedures were used only for torque distribution analysis. Data obtained from standard training procedure were used for both torque distribution analysis and visual associative learning analysis.

2.3. Data analysis

2.3.1. Flight orientation and performance index
Flight orientation histogram and PI evaluation were previously introduced to estimate the fly's learning and memory performance (Wolf and Heisenberg, 1991). The times a fly spent at each angular position were counted out from the originally recorded angular
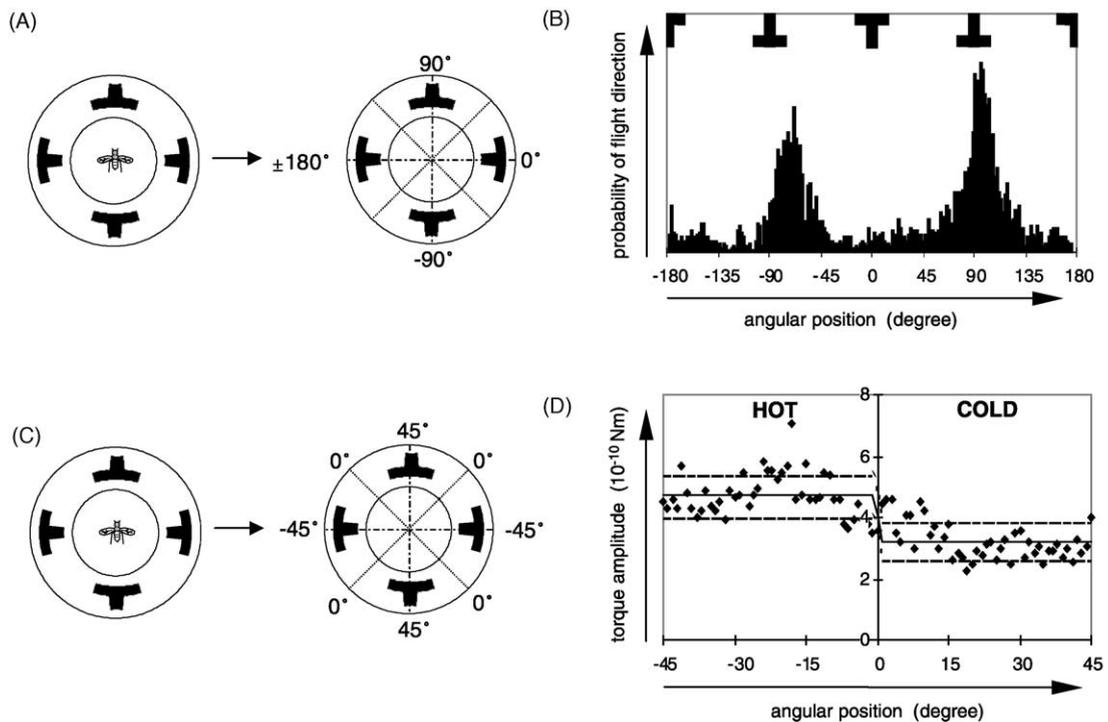

Fig. 3. Simplified diagrams showing the range of the angular position as used in PI analysis and torque-position map analysis. As an example, when the upright-T patterns were set as punished objects. (A) The 360° arena was divided into HOT domains containing the upright-T patterns and COLD domains containing the inverted-T patterns. (B) A flight orientation histogram showed the distribution of the probabilities of the fly's flight direction over angular positions with bin width of 1°; as the upright-T patterns were heat-associated, the fly showed a preference for inverted-T patterns and probabilities near the pattern type were high. (C) The variable of the angular position was transformed to range from −45 to 45° according to the heated pattern; places scaled as 45° indicate where the unpunished patterns were located and −45° indicate the punished patterns. (D) This diagram showed the torque-position map and torque distributions in HOT and COLD domains; the scores represent the average torque amplitude for each angular position; the horizontal "solid ± dashed lines" represent the average torque productions (mean ± S.D.) for each domain. The data shown were from one Canton-S fly in a TR2 period with standard training procedure.

position trace and were illustrated in the flight orientation histogram (Fig. 3B), in which the total relative time the fly spent in different flight directions was revealed. After that the times for the COLD and HOT domains were calculated out and were appointed as t_1 and t_2, respectively. During training, t_2 indicated the times that the fly was exposed to the negative reinforcer and t_1 the times without the negative reinforcement. During test, t_1 and t_2, respectively, referred to the times that the fly chose between the formerly unpunished and punished situations. The PI was computed as $PI = (t_1 - t_2)/(t_1 + t_2)$. $PI = 1$ meant the fly completely avoided the heat punishment, $PI = -1$ meant the fly was facing the punished patterns all the time, and $PI = 0$ meant the fly stayed in the HOT and COLD domains with equal probability. In standard training procedure, the PI was also regarded as the preference index in PTE, the avoidance index in TR1 and TR2, or the memory index in TE (Guo et al., 1996).

2.3.2. Torque-position map and torque distribution

The PI method only relied on the variable of the angular position of the arena, which did not take into consideration the flies' yaw torque variable. At the flight simulator, the yaw torque is the only motor output of flies. The yaw torque is mostly produced by the differentially beating wings of the fly. Yaw torque plays a role of 'operant' in the operant conditioning paradigm and has been regarded as one of the most important components in the conditioning feedback between the flies' behaviour and the negative reinforcer (see also, Wolf and Heisenberg, 1991; Heisenberg et al., 2001). Therefore, we did yaw torque analyses of the flies' conditioned behaviour. Specifically, data analysis was designed to take into account both the arena angular position of variable and flies' yaw torque variable recorded in the experiments.

The newly-designed torque-position map method was used to explore the torque that flies produced at each angular position of the arena in conditioning. The polarity of yaw torque is related to the turning direction of the flies in the flight simulator (positive torques represent clockwise turning actions and vice versa). Although earlier works have described the flies' flexibility to prefer one turning direction according to the association between torque polarity and heat punishment in an environment without any visual cue (Wolf and Heisenberg, 1991; Wolf et al.,

1998), our analysis revealed no significant correlation between the torque polarity and the angular position in the paradigm. Therefore, the torque-position map analysis only studied the relationship between the torque amplitude and the angular position of the arena. The analysis comprised a transformation with the angular position variable. The real angular position variable ranges from -180 to $180°$. Torque-position map analysis used the angular distances between the fly's orientation and the visual patterns to represent the fly's relative angular position (Fig. 3C). When the fly was heading towards the punished pattern type, the relative angular position of the fly was appointed as $-45°$; when heading towards another pattern type, the relative angular position was set as $45°$; and when heading towards the boundary between the HOT and COLD domains, the angular position was $0°$. The newly defined angular position enabled the torque-position map method to statistically delineate torque distribution of flies in conditioning. After the transformation with the angular position, the $360°$ panorama was equally divided into four domains (ranging from -45 to $45°$). The four domains could be superimposed, and the average torque amplitude over each angular position was obtained and plotted on the torque-position map (Fig. 3D).

In addition to the analysis of torque production at each angular position, torque distributions in the HOT and COLD domains were also considered and quantitatively compared with each other. In conditioned training, the reinforcer (heat) was presented throughout the HOT domain and cancelled throughout the COLD domain, and within the HOT domain the heat intensity did not depend on specific angular positions. Thus, all torque productions in HOT or COLD domain could be averaged to show the domain-related overall torque distribution, and torque distributions in HOT and COLD domains could be statistically compared to explore how the reinforcer affected the fly's torque distribution. As shown in Fig. 3D, the horizontal solid lines indicated the average torque amplitude levels in each domain and the dashed lines represented the deviations (S.D.) of torque distribution in each domain.

2.3.3. Statistics

A one-way ANOVA was employed to compare the fly's torque distributions between the two domains.

The two domains could be HOT and COLD domains of one experimental period, or two same domains (HOT or COLD domain) of two successive periods. When the torque distributions between HOT and COLD domains within one session were compared, the torque distribution was regarded as symmetric when ANOVA-test results showed no statistical significance, otherwise the torque distribution was defined as asymmetric. We also used the one-population *t*-test to test the significance of PIs from zero, or the two-population *t*-test to test the significance between different PI sessions.

3. Results

3.1. Behavioural modification and motor learning in Canton-S flies

The torque-position map analysis was first employed to reveal properties of torque distribution for wild-type Canton-S flies ($N = 13$) in experiments with a standard training procedure. In the PTE period (Fig. 4A), the flies produced approximately equal torque amplitude at different angular positions, and the torque distribution appeared to be symmetric
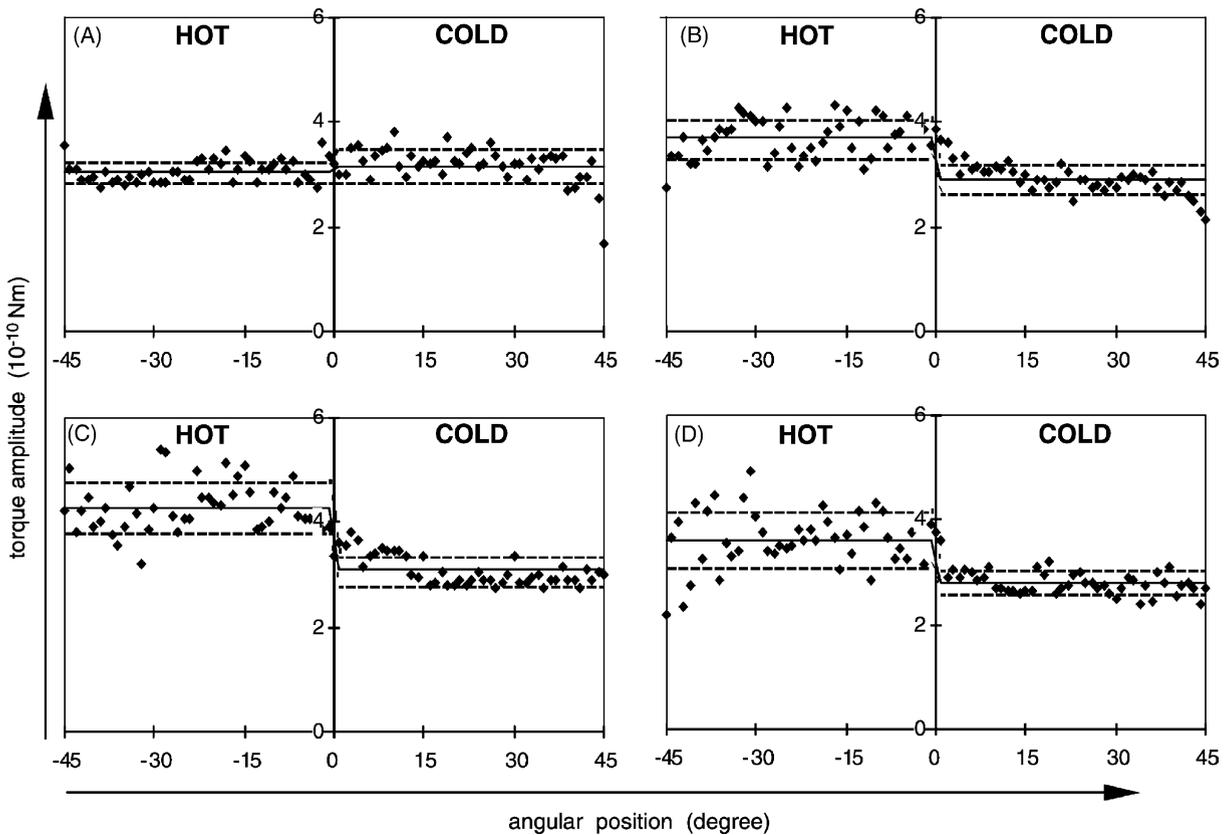

Fig. 4. Torque analysis for Canton-S flies ($N = 13$) in experiments with a standard training procedure. (A) In the PTE period before the operant training, Canton-S flies produced approximately equal yaw torque at each angular position and the torque distributions in HOT and COLD domains showed no difference. (B and C) In training periods, flies produced apparently higher level of torque amplitude in the HOT domain than in the COLD domain, and the modified behavioural mode indicated the flies' motor learning performance. (D) In the test period after the training, the flies' modified torque distribution still existed, which indicated the retention of motor learning. The horizontal lines represented overall torque distribution level for HOT or COLD domain in each session and were explained in texts. To eliminate the asymmetries in the setup or flies, 6 of 13 flies were trained with the upright-T patterns reinforced with heat and the other flies were trained with the inverted-T patterns reinforced.

between HOT and COLD domains. In contrast, in the training periods of TR1 and TR2 (Fig. 4B and C), the torque distribution came to be asymmetric and the torque amplitude level in the HOT domain was measurably higher than in the COLD domain. Finally, in the TE period (Fig. 4D), the torque distribution remained asymmetric and the torque amplitude level in the HOT domain still was higher than in the COLD domain.

Qualitative description of torque-position map analysis revealed the torque distribution modification of wild-type Canton-S flies. Obviously, the change of the flies' torque distribution could only be attributed to the operant training course in experiments. Furthermore, one-way ANOVA analysis was conducted to explore the torque distribution of Canton-S flies for the same conditioning tasks, which checked the difference of torque distributions between HOT and COLD domains for each session. In the PTE period, no significant difference of torque distributions between HOT and COLD domains was detected (means $\pm$ S.D. for HOT and COLD domains were 3.06 ± 0.21 and 3.18 ± 0.34 (10^{-10} Nm), respectively; $F = 3.75$, $P > 0.05$). In the training periods (TR1 and TR2), the averaged torque amplitude level in the HOT domain was higher than in the COLD domain, and the ANOVA analysis showed a significant difference in the torque distribution between the HOT and COLD domains (for TR1, means $\pm$ S.D. were 3.70 ± 0.37 and 2.92 ± 0.29 (10^{-10} Nm), respectively for HOT and COLD domains; $F = 122.94$, $P < 0.001$; for TR2, 4.29 ± 0.48 and 3.08 ± 0.28 (10^{-10} Nm), respectively, for HOT and COLD domains; $F = 209.86$, $P < 0.001$). In the TE, a significant difference in torque distributions

between the HOT and COLD domains was still detectable (3.62 ± 0.54 and 2.80 ± 0.23 (10^{-10} Nm) for HOT and COLD domains, respectively; $F = 86.00$, $P < 0.001$).

Thus the flies' torque distribution modifications in the training and test were found by quantitative analysis. The torque distribution modification actually indicated modification of the flies' behavioural mode in response to operant training. Such training-related behavioural modification was also manifested in experiments of Canton-S flies using the mass test and mass training procedures. As shown in Table 2, in experiments with mass test procedures, ANOVA-test results showed no significant difference between the torque distributions in HOT and COLD domains for all TEs (TE1, TE2 or TE3); the flies ($N = 8$) produced approximately equal torque in both domains. But in experiments with mass training procedures ($N = 7$), statistical significances of torque distributions between HOT and COLD domains were detected for all training periods (TR1, TR2 and TR3) ($P < 0.001$ for all), and the torque amplitude level in the HOT domain was higher than in the COLD domain, which was very similar to the situations in Fig. 4B and C.

The fly's behavioural modification related to the operant training was revealed, but functional interpretation of this phenomenon had to be done. In operant conditioning, the general rule is that the animal's motor output is involved in the feedback between the animal's behaviour and the reinforcer. Thus, the animal's implicit motor program has to be modified in response to the operant training. In the context of *Drosophila* visual operant conditioning, its completion depended critically on the fly's motor performance.

Table 2
Quantitative analysis of torque distributions for Canton-S flies in experiments with mass test and mass training procedures

Flies and procedures	Period	HOT domain	COLD domain	ANOVA-test results	
				F	P
Mass test ($N = 8$)	TE1	2.98 ± 0.18	2.91 ± 0.23	2.35	0.129
	TE2	3.02 ± 0.17	3.15 ± 0.24	9.36	0.003
	TE3	2.93 ± 0.18	2.87 ± 0.21	1.94	0.167
Mass training ($N = 7$)	TR1	3.30 ± 0.53	2.95 ± 0.38	12.38	<0.001
	TR2	3.55 ± 0.63	2.99 ± 0.31	27.63	<0.001
	TR3	3.83 ± 0.81	2.84 ± 0.33	56.36	<0.001

Score for torque distribution in 'HOT' or 'COLD' domain was mean $\pm$ S.D. (10^{-10} Nm). One-way ANOVA analysis was conducted to test the difference in torque distribution between 'HOT' and 'COLD' domains. N represents numbers of flies used.

Therefore, motor learning abilities were basically important for the fly to modify its spontaneous flying program in natural conditions and to adapt to the highly artificial circumstances in the flight simulator for stable flight behaviour. While the fly's motor output was restricted to the yaw torque at the flight simulator, the modification of the torque distribution objectively represented the fly's motor learning performance in the behavioural tasks. As mentioned in Section 2, although polarity of the yaw torque corresponding to the fly's turning direction was also an important factor in the behavioural task, the possible relationship between the yaw torque polarity and the flies' conditioning performances was not proved in our experiments.

According to the concept of motor learning, the torque distribution modifications of flies in training periods in experiments were regarded as motor learning performance. At the same time, they provided an understanding of the retention of modified torque distribution in the TE, which actually indicated the retention of the motor learning.

3.2. Repetition of training and motor learning in Canton-S flies

Since operant training resulted in the flies' motor learning performance in behavioural tasks, it could be expected that repetition of training would make such performance to be more salient. One-way ANOVA tests were also conducted to check the flies' torque distributions between successive training periods, which finally proved the positive effect of repetition of training on the flies' motor learning performance.

Comparisons of the torque distributions in HOT or COLD domain between two experimental periods were carried out for both standard training procedure (TR1 versus TR2) and mass training procedure (TR1 versus TR2 and TR2 versus TR3). For Canton-S flies in standard training procedure, ANOVA analysis results showed a statistical significance for torque distributions in HOT domain between TR1 and TR2 (3.70 ± 0.37, 4.29 ± 0.48 (10^{-10} Nm) for TR1, TR2, respectively; $F = 41.98$, $P < 0.001$). Whereas no significant difference was found for the COLD domain (2.92 ± 0.29, 3.08 ± 0.28 for TR1, TR2, respectively; $F = 7.50$, $P > 0.001$). In experiments with a mass training procedure, ANOVA tests also confirmed the change of torque distributions between successive training

periods in HOT domain but not in COLD domain (For TR1 versus TR2: $F = 4.12$, $P < 0.05$ for HOT domain and $F = 0.27$, $P > 0.05$ for COLD domain. For TR2 versus TR3: $F = 5.04$, $P < 0.05$ for HOT domain and $F = 3.24$, $P > 0.05$ for COLD domain).

Therefore, the torque distributions for the HOT domain in a subsequent training period always had a higher amplitude level than in the previous training period, but the distributions for COLD domain between two successive training periods did not demonstrate this change. These results indicated the positive effect of repetitive training on the flies' behavioural modifications, which meant that the flies' motor learning was enhanced by repetition of training. A phenomenon was noticed that in experiments using standard training procedure, the improvement in torque amplitude level for the HOT domain was large (from 3.70 ± 0.37 to 4.29 ± 0.48) from the first training session (TR1) to the second training session (TR2); while in experiments using mass training procedure, the increase from TR1 to TR2 was small (from 3.30 ± 0.53 to 3.55 ± 0.63) and the increase from TR2 to TR3 was large (3.55 ± 0.63 to 3.83 ± 0.81) again. It seemed that the training session in standard training procedure could more effectively improve the motor learning performance of Canton-S flies. Actually, the earlier study (Guo et al., 1996) had demonstrated that the standard training procedure and mass training procedure had different effect on the associative learning scores, that learning experiments without any pre-test session leaded to lower learning scores. It is likely that a test fly has to become familiar with the highly artificial environment at the flight simulator for stable flying, that is, to learn how to stabilize the panorama in the closed loop at the flight simulator. Here the torque analysis results demonstrated that this pre-test session could be also important to the wild-type flies' motor learning scores.

3.3. Motor learning in dunce[1] flies

Mutant *dunce*[1] fly has proved to be defective in aspects of olfactory associative learning and short-term memory (Dudai et al., 1976; Tully and Gold, 1993; Davis, 1996). Experiments have established that the *dunce*[1] fly is deficient in an isozyme of cAMP phosphodiesterase (Byers et al., 1981; Chen et al., 1986), hence in the catabolism of cAMP, a substance implicated in controlling elementary steps of memory

formation. Deficits of *dunce*[1] flies in visual associative learning at the flight simulator have also been demonstrated (Gong et al., 1998). In the present work the newly-designed torque analysis was employed to examine the motor learning performance of *dunce*[1] flies in comparison with the wild-type Canton-S flies. Behavioural data of *dunce*[1] flies were analysed with the same protocol as for Canton-S flies.

Torque-position maps for *dunce*[1] flies ($N = 15$) in experiments with a standard training procedure were shown in Fig. 5. The data indicated that the *dunce*[1] flies' motor learning performance was very similar to that of Canton-S flies. In the PTE period (Fig. 5A), *dunce*[1] flies also produced approximately equal torque amplitude at different angular positions and there was no measurable difference between HOT and COLD domains (2.20 ± 0.27, 2.14 ± 0.29 for HOT, COLD domain, respectively; $F = 0.97$, $P > 0.05$). In training periods (Fig. 5B and C), the torque amplitude in the HOT domain was relatively higher than in the COLD domain, and the difference was statistically significant (in TR1: 2.87 ± 0.43, 2.05 ± 0.28 for HOT, COLD domain; $F = 111.54$, $P < 0.001$. In TR2: 3.31 ± 0.51, 2.06 ± 0.35 for HOT, COLD domain; $F = 178.86$, $P < 0.001$). In the TE period (Fig. 5D), the difference remained statistically significant (2.32 ± 0.27, 1.94 ± 0.18 for HOT, COLD domain; $F = 60.40$, $P < 0.001$). The ANOVA test was also employed for *dunce*[1] flies in experiments with mass test and mass training procedures. As shown in Table 3, for the torque distributions in experiments with a mass test procedure ($N = 10$), ANOVA-test results showed no
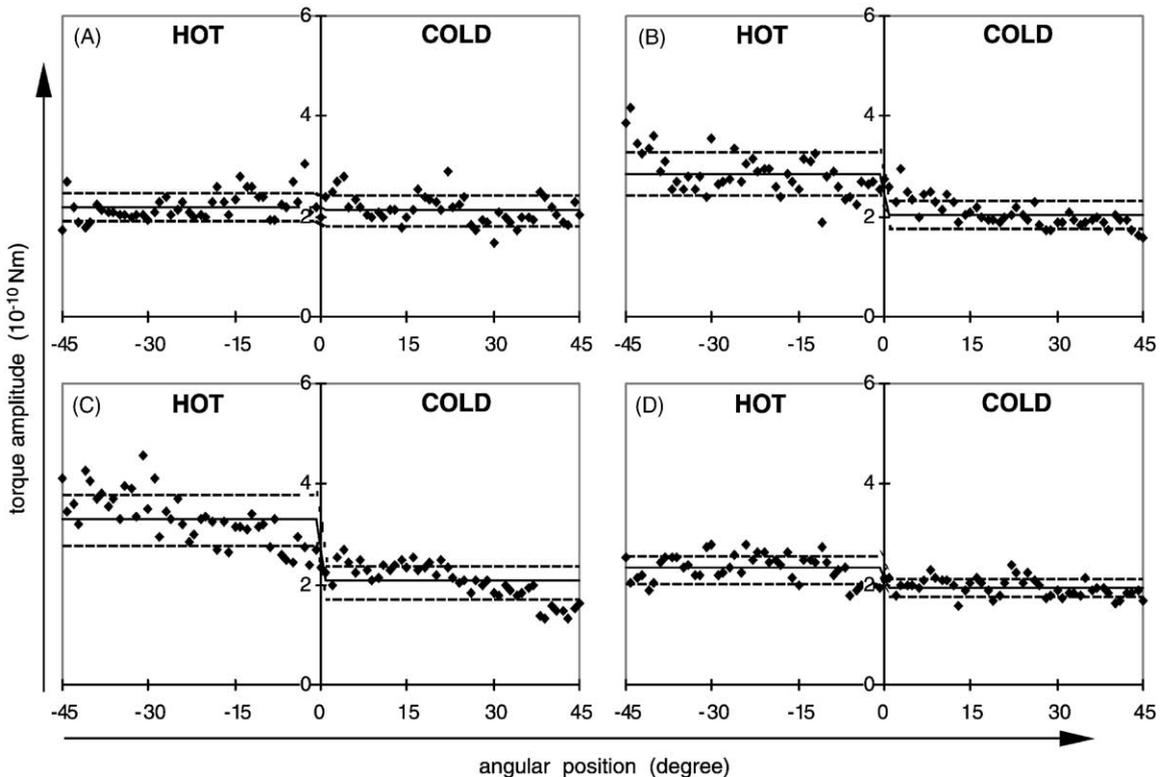

Fig. 5. Torque analysis for mutant *dunce*[1] flies ($N = 15$) in experiments with a standard training procedure. (A) In the PTE period, the yaw torques the mutant flies produced at each angular position were close to each other and the torque distributions in HOT and COLD domains showed no difference. (B and C) In training periods, the mutant flies produced significantly higher torque amplitude level in the HOT domain than in the COLD domain, which indicated the mutant flies also had normal motor learning performance as did the wild-type Canton-S flies shown in Fig. 4. (D) In the test period, the retention of motor learning was detectable. The horizontal lines represented overall torque distribution level for HOT or COLD domain in each experimental period and were explained in texts. Eight of 15 female *dunce*[1] flies were trained with the upright-T patterns associated with the heat; the others were trained with the inverted-T patterns as the heated pattern.

Table 3
Quantitative analysis of torque distributions for *dunce*[1] flies in experiments with mass test and mass training procedures

Flies and procedures	Period	HOT Domain	COLD Domain	ANOVA-test results	
				F	P
Mass test ($N = 10$)	TE1	2.18 ± 0.28	2.18 ± 0.26	0.01	0.925
	TE2	2.00 ± 0.14	1.94 ± 0.13	4.39	0.039
	TE3	1.96 ± 0.21	1.92 ± 0.12	0.78	0.380
Mass training ($N = 8$)	TR1	2.19 ± 0.48	1.78 ± 0.16	28.60	<0.001
	TR2	2.27 ± 0.57	1.88 ± 0.17	19.31	<0.001
	TR3	2.89 ± 0.70	1.87 ± 0.34	74.96	<0.001

Score for torque distribution in 'HOT' or 'COLD' domain was mean $\pm$ S.D. (10^{-10} Nm). One-way ANOVA analysis was conducted to test the difference in torque distribution between 'HOT' and 'COLD' domains. N represents numbers of flies used.

significant difference between HOT and COLD domains for all TEs (TE1, TE2 or TE3). In contrast, in experiments with a mass training procedure ($N = 8$), the torque amplitude level in the HOT domain was higher than in the COLD domain, and ANOVA-test results showed statistical significances for all training periods (TR1, TR2 and TR3; $P < 0.001$ for all). Thus, the analysis proved the modifications of the *dunce*[1] flies' torque distribution in response to the operant training, which indicated that the mutant flies also had similar motor learning performance as the wild-type Canton-S flies.

ANOVA-test analysis was also conducted to detect the effect of repetitive training on motor learning performance of the *dunce*[1] fly. For standard training procedure, in the latter training period (TR2) *dunce*[1] flies produced relatively higher torque amplitude level in HOT domain than in the previous training period (TR1) ($F = 19.04$, $P < 0.001$); whereas no such change happened in the COLD domain ($F = 0.02$, $P > 0.05$). For the mass training procedure, the results also demonstrated the increased torque amplitude level in HOT domain but not COLD domain (TR1 versus TR2: $F = 7.79$, $P < 0.05$ for HOT domain and $F = 0.62$, $P > 0.05$ for COLD domain. TR2 versus TR3: $F = 20.6$, $P < 0.01$ for HOT domain and $F = 0.02$, $P > 0.05$ for COLD domain). In the HOT domain *dunce*[1] flies always produced relatively higher torque amplitude level in the latter training session than in the previous training session, while in the COLD domain no such change was found. So, repetition of training reinforced the *dunce*[1] flies' motor learning performance, as it did for Canton-S flies. Comparison of torque amplitude level in the HOT domain be-

tween two successive training sessions also showed the instantly improved torque amplitude level when using standard training procedure (from 2.87 ± 0.43 in TR1 to 3.31 ± 0.51 in TR2), and the steady improvement when using mass training procedure (2.19 ± 0.48, 2.27 ± 0.57 and 2.89 ± 0.70 for TR1, TR2 and TR3, respectively).

3.4. Dissociation of motor learning and visual associative learning

As mentioned that a PI has been introduced to estimate flies' visual associative learning and memory abilities in operant conditioning paradigms at the flight simulator (Wolf and Heisenberg, 1991). In training courses, a positive PI means the fly spends a longer time in domain containing the unpunished visual pattern, which corresponding to the fly's successful avoidance from the negative reinforcer (heat). Higher PIs in training or test means the flies have a better capacity of acquiring or retaining the association between the negative reinforcer and visual patterns. In the present work, the learning and memory performance of both Canton-S ($N = 13$) and *dunce*[1] flies ($N = 15$) tested with the standard training procedure were evaluated (Fig. 6). In the PTE period, both strains had no significant PIs referred to zero (PIs $\pm$ S.E.M. were 0.03 ± 0.07, and -0.02 ± 0.04, respectively, for Canton-S, *dunce*[1]), and no significant difference existed between the two strains (t-test results: $t_{[1,26]} = 0.72$, $P = 0.24$). While, during or after the operant training, PIs of two groups came to be measurably different and *dunce*[1] flies' exhibited defective associative learning/memory abilities. Canton-S flies always
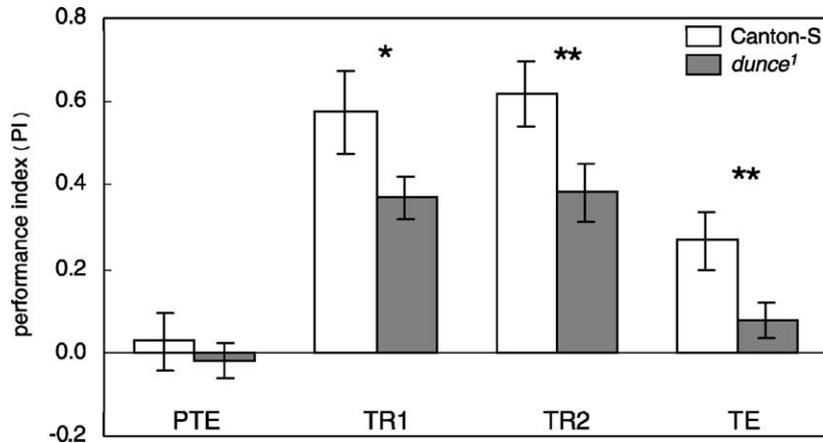
Fig. 6. PIs ± S.E.M. showed the visual associative learning and memory performance of both wild-type Canton-S and mutant *dunce*[1] flies in experiments with a standard training procedure. Difference of PIs between the two strains was tested with *t*-test analysis. "∗" and "∗∗" represent levels of significance $P < 0.01$ and $P < 0.001$, respectively. $N = 13$ and 15 for Canton-S and *dunce*[1] flies.

gained higher scores than *dunce*[1] flies in training and test: PIs ± S.E.M. were 0.58 ± 0.10, 0.37 ± 0.05 for Canton-S, *dunce*[1] flies in TR1 ($P < 0.01$); 0.62 ± 0.08, 0.38 ± 0.07 for Canton-S, *dunce*[1] flies in TR2 ($P < 0.001$); and 0.27 ± 0.07, 0.08 ± 0.04, respectively, for Canton-S, *dunce*[1] flies in TE ($P < 0.001$).

So, according to the context of visual operant conditioning at the flight simulator, PI analysis proved that *dunce*[1] flies had deficits in visual associative learning and short-term memory when compared to Canton-S flies. In previous sections, the torque analysis had demonstrated that both strains modified their behavioural mode in response to the operant training in the discriminative avoidance task, which finally indicated similarities in motor learning in experimental periods. Thus, it seemed that although the *dunce*[1] flies' visual associative learning was influenced by the mutant cAMP cascade, its motor learning was not disturbed. So, we proposed that the visual associative learning and motor learning were dissociated in the operant conditioned behaviour of *Drosophila* at the flight simulator, and the cAMP signal pathway might play different roles in flies' associative learning and motor learning processes.

4. Discussion

The main task of this study was to explore the flies' operant motor learning performance in the visual discriminative task at the flight simulator and to understand its relationship with visual associative learning. The newly-designed data analysis was carried out to assess the flies' only recorded motor variable—yaw torque, and the torque distribution analysis method was used to score the flies' motor learning performances, which was similar to PI defined in earlier works. The flies' yaw torque in the conditioned behaviour has been studied (Heisenberg and Wolf, 1984, 1988; Mayer et al., 1988; Brembs and Heisenberg, 2000), and yaw torque productions with a high amplitude level (higher than an arbitrary threshold) were revealed as torque spikes. The torque spikes were considered as a primary behavioural component by which the fly could adjust its flight orientation whether in fixed flight at the flight simulator or in natural free flight. In addition to the analysis of the discrete torque spikes, the torque distribution analysis was developed in the present work to examine the flies' distribution of yaw torque in angular positions within each experimental period. The motor learning of both wild-type Canton-S and mutant *dunce*[1] flies were disclosed by the torque distribution modifications, and the dissociation between visual associative and motor learning was exhibited. The discriminative avoidance paradigm has been extensively used to study flies' visual associative learning/memory and its underlying neural substrates; here the revealed motor learning and its features obviously enrich our understanding of the operant conditioned behaviour of *Drosophila*.

It was thought that in the operant conditioning of *Drosophila* at the flight simulator, the associative learning between 'behaviour' and 'reinforcer' or between 'visual pattern' and 'reinforcer' was the central task, while the flies' motor learning might be suppressed (Brembs and Heisenberg, 2000; Heisenberg et al., 2001). However, in this study the flies' torque distribution was extensively investigated and results revealed a modification in the flies' behavioural mode, which objectively indicated a new motor program of flies formed in the operant behavioural task. Furthermore, the analysis of the mutant *dunce*[1] showed that motor learning was less sensitive to the mutation of the cAMP cascade than associative learning was. Behavioural analysis suggested that the visual associative learning and motor learning at the flight simulator might be parallel processes involved in the visual discriminative avoidance behaviour of *Drosophila*. The cellular or molecular mechanism underlying the difference between visual associative learning and motor learning of *Drosophila* at the flight simulator are still less known, this study has attempted to understand their difference at the behavioural level. Other works (Wolf and Heisenberg, 1991; Wolf et al., 1998) have also described the flies' flexibility to perform motor learning in the flight simulator, in which no visual cue was presented and the flies were trained to form the association between torque polarities and heat punishment. Different from that work, the present study revealed the motor learning in the flies' visual discriminative avoidance behaviour and its relationship with visual associative learning.

While comparing the torque amplitude distributions between Canton-S and *dunce*[1] flies, we found that *dunce*[1] flies generally produced a lower torque amplitude level than Canton-S flies. Empirically, such a difference might be an outcome of the mutation, since mutant flies always exhibit less strength than wild-type flies. Nevertheless, this difference did not basically influence learning and memory performances in flies, considering that this phenomenon always exists among individuals within a wild-type or mutant strain while no correlated difference in learning/memory scores are found among the individuals. Actually, previous experiments with the same behavioural paradigm have demonstrated that the mutant *dunce*[1] flies had normal performance with regard to fixating ability, heat avoidance ability and other non-associative abilities (Gong et al., 1998). Thus, the difference in overall torque amplitude level between Canton-S and *dunce*[1] flies could not influence our conclusion that the *dunce*[1] flies showed normal motor learning performance as Canton-S flies did, in contrast to *dunce*[1] flies' defects in visual associative learning. It seemed that the *dunce*[1] flies' capacity in the motor learning process was not disrupted by the mutation of the cAMP pathway.

There is a common view that different learning tasks are related to different neural substrates and signal pathways (Milner et al., 1998; Guillou et al., 1999; Ghilardi et al., 2000). Studies on mammals have investigated neural substrates specifically assigned to motor learning in various motor tasks (Llinas and Welsh, 1993; Karni et al., 1995; Kleim et al., 1998; Doya, 2000). A question posed here is how different neural mechanisms could be responsible for associative learning and motor learning in *Drosophila* separately. Behavioural and molecular genetic analyses of *Drosophila* mutants have been widely employed to study the olfactory classical conditioning from genetic to behavioural levels. It is now clear that in olfactory classical conditioning (Tully and Quinn, 1985) the CS-US association mostly occurs within mushroom bodies (MBs) (Dubnau et al., 2001; McGuire et al., 2001). With the operant conditioning paradigm, the MBs were demonstrated to be dispensable for the visual associative learning (Wolf et al., 1998) but were necessary for some cognition-like functions (Liu et al., 1999; Tang and Guo, 2001). However, further cellular or molecular evidences related to the visual associative learning and related functions remain unknown. There is a complete lack of understanding of the underlying neural features of motor learning in *Drosophila*. Thus, although our results from studies of *dunce*[1] flies indicated that the cAMP pathway might exert different effects on visual associative learning and motor learning, we cannot delineate their different neural bases in detail yet. Visual associative learning underlies the fly's capacity to form the 'Visual Pattern–Reinforcer' association and is likely a neural reflection of the practiced conditioned behaviour, in which the fly is trained to avoid certain flight directions associated with the negative reinforcer and to choose the safer flight direction. The motor learning is likely more closely related to the fly's ability to modify its spontaneous flying program and to form

novel flying skills or patterns; thus it probably requires more involvement of activities of neuropils that relate to the animals' locomotion abilities. Actually, the conditioned behaviour of *Drosophila* at the flight simulator is so complicated that detailed neural substrates involved still remain unknown. But to some extent it can be inferred that visual associative learning has to be connected with the neural plasticity of the central structures of *Drosophila* brain, while it may not be the case for motor learning process. After all, it has been demonstrated that decapitated insects including *Drosophila* could show posture learning and plasticity of simple sensory motor responses (Horridge, 1962; Booker and Quinn, 1981; Corfas and Dudai, 1989). To determine where in the nervous system the visual associative learning or the motor learning is happening is still necessary and should be most interesting. In solving these problems behaviour–genetic analysis of *Drosophila* will be indispensable and featured mutants in behavioural aspects will be helpful.

Neuroscience studies in invertebrates always focuses on associative learning (classical or operant). In addition to the mentioned associative learning studies in *Drosophila*, classical and operant conditioned behaviour and their underlying neural structures in other invertebrates, such as Aplysia (Carew and Sahley, 1986; Cook and Carew, 1986; Nargeot et al., 1997) and honey bee (Hammer, 1993; Menzel and Müller, 1996; Erber et al., 2000), have also been extensively studied. But none of these studies have reported on motor learning in invertebrates so far. This study used newly-designed behavioural analysis to examine motor learning in *Drosophila*. The results were analysed to complement understanding of learning behaviour in *Drosophila* and even in vertebrates. Nevertheless, the in-depth neural mechanisms underlying the motor learning remain to be explored.

Acknowledgements

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