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CLASSICAL CONDITIONED RESPONSE IN THE HONEY BEE*

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Abstract—The classical olfactory conditioned response of the honey bee, involving the extension of the proboscis on tarsal contact with sucrose solution, has been investigated. The bee showed generalization between two similar aromas but distinguished the third. The bee clearly showed experimental extinction, differentiation, and conditioned inhibition, all of which were temporary and not retained until the following day. Thus, spontaneous recovery was observed. The experiments also suggest the occurrence of a conditioned response of the second order.

INTRODUCTION

THE occurrence of conditioning in invertebrates has been reported several times (THOMPSON, 1917; KREPS, 1925; PLAVILSTCHIKOV, 1928; COPELAND, 1930; COPELAND and BROWN, 1934; THOMPSON and MCCONNELL, 1955). MIKHAÏLOFF (1920a, b, c, 1921, 1922, 1923) reported various phases of conditioning, i.e. extinction, generalization, differentiation, and conditioning of the second order in Crustacea and Cephalopoda.

It is well known that many insects respond with extension of their proboscis on tarsal contact with sucrose solution (cf. MINNICH, 1921). In the honey bee, FRINGS (1944) established a conditioned response to the odour of cumarin, using the proboscis extension reaction, and KUWABARA (1957) established conditioned responses to coloured light and to water vapour.

It seems valuable to compare the characteristics of these responses in an invertebrate (which has a relatively simpler central nervous system) with the well-known phenomena in mammals, using classical conditioned reflex methods. The present investigation was carried out to determine the characteristics of the conditioned responses in the honey bee, using odour as the conditioning stimulus.

MATERIALS AND METHODS

All the experiments were performed in the summers of 1956 and 1957. Workers of the honey bee, *Apis mellifera*, were used. The bees were from the colony used in previous papers‡ (KUWABARA and TAKEDA, 1956; TAKEDA, 1957). They were

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‡ They were described there as *Apis mellifica*.

collected at a feeding dish containing a sucrose solution, placed several metres from the hive.

General methods for establishing the conditioned response are similar to those described in the previous papers. The workers caught were fastened on an experimental rack by holding their wings with a metal clip, after being anesthetized with ether for 20 sec. The experimental rack was divided by partitions into compartments for each bee to avoid, as far as possible, any visual or olfactory interference which might occur among bees during the experiments. Twenty-six bees could be used at one time.

Trials for establishing the conditioned response were initiated with the bees which were left fastened to the experimental rack 1 or 2 days without feeding.

Olfactory stimulus by aromatic compounds was used as the conditioning stimulus for the following reasons: (1) it was possible to use pure compounds of which the chemical structures were known; (2) the olfactory sense is well developed in the honey bee and plays an important role in its life; and (3) it was relatively easy to establish the olfactory conditioned response as compared with a visual one (KUWABARA, 1957).

The aromatic compounds used were citral (olefinic terpene aldehyde), hydroxycitronellal (olefinic terpene aldehyde), and *p*-isopropyl-α-methylhydrocinnamic aldehyde (aromatic aldehyde). Citral has a strong aroma to man in contrast to the latter two which have mild aromas. These aromatics were sucked into a glass capillary with a tapered tip of an inner diameter of about 0·2 mm. Under such conditions, these aromas were perceptible to me only when the capillary was brought near to my nose.

The glass capillary containing the aromatic was brought to within 1 cm of the antennae of the bee on the experimental rack. 5 sec after the presentation of the conditioning stimulus, the tarsi were touched with a 1·5 M sucrose solution as the unconditioned stimulus to cause extension of the proboscis. The animal was allowed to drink the solution for 2–5 sec according to the experiment.

These reinforcements were repeated at intervals of approximately 15 min. In most cases, the simultaneous conditioned response could be formed by several such reinforcements, but sometimes only by a single reinforcement. After the conditioned response was formed, the unconditioned stimulus was given as soon as the proboscis was extended by the presentation of the conditioned stimulus and the bee was thus reinforced. When non-reinforcement was needed, the conditioned stimulus alone was continued for 10 sec.

Approximately ten reinforcements were performed in a day. Generally, the bees used for further experiments were those in which the conditioned response was established during the reinforcements of one day.

All experiments were performed in an airy room and all windows were open. In every interval of the trials, aroma was eliminated by fanning. In practice, no aroma was perceptible to a human at the beginning of the next trial.

RESULTS

Compatibility of the proboscis extension reaction as an unconditioned response and of the olfactory stimulus used as conditioned stimulus

First of all, several points should be clear for the reliability of the results obtained. From the anatomical point of view (SNODGRASS, 1956), the proboscis extension reaction used is supposed to be a kind of reflex due to contraction of muscles.

In general, in our experiments, extension of proboscis was observed on tarsal contact with a sucrose solution. In only 4 per cent of the 2428 trials performed to establish the conditioned response did the bees fail to respond, probably due to satiety of food or to confusion. Therefore, the proboscis extension reaction could be regarded as an unconditioned response.

It has been shown by KENYON (1896) and VOWLES (1955) that sensory nerves from the antennae pass into the antennal lobes where they are connected with fibres extending into the *corpora pedunculata*, a pair of mushroom-shaped bodies. These bodies are regarded as co-ordinating centres of the brain, where the fibres might very probably connect with efferent motor fibres.

Since some insects react spontaneously by the extension of their mouth parts to appropriate olfactory stimulus (MINNICH, 1924; FRINGS, 1941), it is essential to determine whether the olfactory stimuli used were indifferent stimuli.

No extension of the proboscis occurred at the first presentation of the olfactory conditioned stimulus in any of the 296 bees used, although they extended their proboscis at the following presentation of the unconditioned stimulus. FRINGS (1944) reported a similar case in which seventy-six honey bees did not respond to the first presentation of cumarin aroma.

In addition, an experiment was performed to determine whether extension of the proboscis would not occur even in the worst nutritional condition. No extension of the proboscis at all was observed by presenting hydroxycitronellal for 20 sec to ten bees which had survived from forty-eight bees fastened for 52 hr without feeding. The same results were obtained when citral was presented to nine survivors 35 min thereafter, and also when *p*-isopropyl-α-methylhydrocinnamic aldehyde was presented to eight survivors 35 min after this.

It can therefore be concluded that the olfactory stimuli used are indifferent stimuli for the honey bee, thus could be used as a conditioned stimulus.

Experimental extinction

Extinction experiments were performed on honey bees conditioned to hydroxycitronellal with reinforcement on the preceding day. Only some of the typical results are shown in Table 1, because they are the same data as those cited in a previous paper (TAKEDA, 1957). Experimental extinction occurred clearly after ten or more repetitions of non-reinforcement. The fact that the unconditioned response was observed after the bee ceased to respond to the non-reinforced conditioned stimulus (at the last trial of the day in Table 1) means that this was not due to confusion but really due to the extinction of the conditioned response.

The individual No. 9 required more repetitions of the non-reinforcements before the occurrence of extinction. This might have been due to poor nutritional condition because this bee died soon after stable extinction had occurred.

TABLE 1—EXPERIMENTAL EXTINCTION

Date	Time	Conditioned stimulus and reinforcement	Conditioned response Animal No.	
			3	9
1	10.45 a.m.	H — S		
	11.00	H — S	+	+
	15	H — S		⊕
	30	H — S	+	⊕
	2.15 p.m.	H — S	+	+
	30	H — S	+	+
	45	H — S	+	+
	3.00	H — S	+	+
	15	H — S	+	+
	30	H — S	+	+
2	1.55 p.m.	H	+	⊕
	2.00	H	+	⊕
	5	H	⊕	⊕
	10	H	⊕	⊕
	15	H	⊕	⊕
	20	H	⊕	⊕
	25	H	⊕	⊕
	30	H	⊕	⊕
	35	H	+	⊕
	40	H	⊕	⊕
	45	H		⊕
	50	H	+	⊕
	55	H		⊕
	3.00	H	?	⊕
	5	H		⊕
	10	H		⊕
	15	H		+
	20	H		?
	25	H		
	30	H		+
	35	H		+
	40	H		
	45	H		?

Date	Time	Conditioned stimulus and reinforcement	Conditioned response Animal No.	
			3	9
2	3.50 p.m.	H		
	55	H		?
	4.00	H		
	5	H	No	+
	10	H	trial	
	15	H	+	
	20	H		
	27	H		
	36	H		
	47	H		
	5.01	H	±	Died
	16	H		
	31	H		
	38	S		
3	7.05 p.m.	H	⊕	
	10	H	⊕	
	15	H	⊕	
	20	H	⊕	
	25	H	⊕	
	30	H	⊕	
	35	H	⊕	
	40	H		
	45	H	⊕	
	50	H	⊕	
	55	H	⊕	
	8.00	H	+	
	5	H	+	
	10	H	+	
	15	H	?	
	20	H	+	
	29	S		
	34	H	?	
	36	H		

In the column of conditioned stimulus and reinforcement, H = hydroxycitronellal; − S = reinforcement by 1·5 M sucrose; S = feeding of the sucrose only. In the column of conditioned response, ⊕, +, ±, ? and no mark show the characteristics of the conditioned response, thus: ⊕ = complete proboscis extension with its vigorous movements; + = complete proboscis extension; ± = incomplete proboscis extension; ? = momentary extension or movement of proboscis; no mark = no reaction of proboscis at all (though there was complete proboscis extension when touching the tarsi with the sucrose). For further explanation see text.

Disinhibition was observed at the non-reinforcement after a pause of 15 min in No. 3 in which stable extinction had occurred by trials at 5 min intervals. However, extinction was observed even after a pause of 15 min by gradually lengthening the intervals of the non-reinforcements.

In this bee, a positive conditioned response appeared at the first presentation of the conditioned stimulus on the following day. More than ten non-reinforcements were required for the occurrence of extinction on that day. Thus spontaneous recovery of extinction occurred. The extinction is temporary in the honey bee as in mammals (KONORSKI, 1948).

Generalization

A series of experiments were conducted in which bees were conditioned to respond to one of the aromatics and then tested the following day with the other two. When conditioned to hydroxycitronellal bees would respond on the following day to *p*-isopropyl-α-methylhydrocinnamic aldehyde but not to citral. They were thus able to distinguish the latter but not the former from the conditioned stimulus, thereby indicating that the response was not due to a general 'sensitization' of the animal to olfactory stimuli. On conditioning to citral, however, no response could be elicited to either of the other two aromatics on the following day. It seems clear that the bee can discriminate between these two groups of odours.

TABLE 2—GENERALIZATION

Date	Time	Conditioned stimulus and reinforcement	Conditioned response Animal No.			
			1	11	21	23
19	10.00 a.m.	H — S				
	15	H — S				
	30	H — S	+	±		
	45	H — S		+		
	11.00	H — S	+	+		+
	15	H — S	+	+	New	±
	30	H — S	+	+	+	+
	45	H — S	+	+	+	+
	12.00 p.m.	H — S	⊕	+	⊕	⊕
	15	H — S	+	+	+	
	30	P			⊕	
	45	H — S	⊕	+	+	+
	3.45	P		+	+	
	4.00	H — S	+	+	+	+
20	4.05 p.m.	C		Died	Died	
	20	P	+			⊕
	35	H — S	+			+

In the column of conditioned stimulus and reinforcement, C=citral; P=*p*-isopropyl-α-methylhydrocinnamic aldehyde. In the column of conditioned response, 'New' means the bee was added for the experiment at that time. For further explanations of the abbreviations see Table 1.

A typical experiment is illustrated in Table 2 where bees conditioned to hydroxycitronellal could not distinguish *p*-isopropyl-α-methylhydrocinnamic

TABLE 3—DIFFERENTIATION

Date	Time	Conditioned stimulus and reinforcement	Conditioned response Animal No. 2
12	2.45 p.m.	H — S	
	3.00	H — S	
	15	H — S	
	30	H — S	
	45	H — S	
	4.00	H — S	
	15	H — S	
	30	H — S	
	45	H — S	
13	10.10 a.m.	C	
14	11.55 a.m.	H — S	+
	12.55 p.m.	H — S	+
	3.45	C	
	5.05	P — S	+
	20	H	+
	35	P — S	+
	50	H	+
	55	P — S	⊕
	6.00	H	+
	5	P — S	⊕
	10	H	+
	15	P — S	⊕
	20	H	+
	25	P — S	⊕
	30	H	
	35	P — S	⊕
	40	H	
	45	P — S	⊕
	50	H	
	55	P — S	
	7.00	H	
	5	P — S	
15	2.05 p.m.	H	⊕
	50	C	
	3.10	P — S	⊕
	15	H	⊕
	20	P — S	⊕
	25	H	⊕
15	3.30 p.m.	P — S	⊕
	35	H	⊕
	40	P — S	⊕
	45	H	
	50	P — S	⊕
	55	H	
	4.00	P — S	⊕
	10	H	
	15	P — S	⊕
	20	H	
	25	P — S	⊕
	30	H	
	35	P — S	
	40	H	
	5.30	P — S	⊕
	35	H	
	40	P — S	⊕
16	3.45 p.m.	H	⊕
	50	P — S	⊕
	55	H	⊕
	4.00	P — S	⊕
	5	H	+
	10	P — S	⊕
	15	H	+
	20	P — S	⊕
	25	H	
	30	P — S	⊕
	35	H	
	40	P — S	⊕
	45	H	
	52	P — S	⊕
	5.00	H	
	5	P — S	⊕
	10	H	
	15	P — S	
	20	H	
	25	P — S	⊕
	6.25	H	
	30	P — S	⊕

aldehyde but could distinguish citral. Bee No. 21 failed to discriminate *p*-isopropyl-α-methylhydrocinnamic aldehyde after only 15 min, bee No. 11 after 3 hr, but bees Nos. 1 and 23 did not do so until the following day. These results indicate that different periods are required by different bees for generalization to become effective.

Differentiation

Bees conditioned to respond to hydroxycitronellal were later tested alternately with hydroxycitronellal (non-reinforced) and *p*-isopropyl-α-methylhydrocinnamic aldehyde (with reinforcement) (Table 3) or with hydroxycitronellal (reinforced) and *p*-isopropyl-α-methylhydrocinnamic aldehyde (non-reinforced) (Table 4).

Table 4—Differentiation

Date	Time	Conditioned stimulus and reinforcement	Conditioned response Animal No.	
			7	8
3		H — S (4 times)		
4		H — S (12 times)		
5		H — S (15 times)		
6	9.48 a.m.	H — S	⊕	⊕
	10.30	P	⊕	⊕
	37	H — S	⊕	⊕
	48	P	⊕	⊕
	53	H — S	⊕	⊕
	1.01 p.m.	P	⊕	⊕
	20	H — S	⊕	⊕
	25	P	+	–
	30	H — S	⊕	⊕
	35	P		
	40	P		
	45	H — S	⊕	⊕
	52	H — S	⊕	⊕
	2.00	P		
	12	P		
	56	H — S	⊕	⊕

In each instance generalization occurred so that responses were given to both aromatics. However, as the tests were continued the response was only given to the reinforced stimulus and not to the non-reinforced, thus indicating clear differentiation. Occasional tests with citral showed no generalization.

Some features of the experiments are noteworthy. The response to hydroxycitronellal occurred at the first test 2 days after conditioning (Table 3); differentiation occurred after three to five pairs of tests and was retained for at least 1 hr but not until the following day, i.e. it is, like extinction, only temporary. Lack of response towards the end of days fourteen and fifteen was probably due to satiety as the bees were allowed to feed for 5 sec at each reinforcement: even with irregular presentation of stimuli (Table 4), differentiation was possible and indicates that the ability to differentiate represents a true differentiation of odours and is not a response to the rhythm of presentation.

Conditioned inhibition

Typical results of experiments on conditioned inhibition are shown in Table 5. Conditioned response to hydroxycitronellal was well established in this bee by reinforcements during 4 days. Conditioned inhibition experiments were then

TABLE 5—CONDITIONED INHIBITION

Date	Time	Conditioned stimulus and reinforcement	Conditioned response Animal No. 24
23		H — S (7 times)	
24		H — S (12 times)	
25		H — S (12 times)	
26	10.28 a.m.	H — S	+
	11.48	H — S	⊕
	1.24 p.m.	H — S	⊕
	45	CH	⊕
	52	H — S	⊕
	2.00	CH	±
	26	H — S	⊕
	50	CH	⊕
	55	H — S	⊕
	3.00	CH	⊕
	5	H — S	⊕
	10	CH	+
	15	H — S	⊕
	20	CH	+
	25	H — S	⊕
	30	CH	+
	35	H — S	⊕
	42	CH	
	47	H — S	⊕
	54	CH	
	4.00	H — S	⊕
	5	CH	
	12	CH	
	19	H — S	⊕
	26	H — S	⊕
	5.26	CH	
	36	H — S	⊕
27	10.50 a.m.	CH	⊕
	11.13	H — S	⊕
	2.39 p.m.	H — S	⊕
	45	CH	⊕
	53	H — S	⊕
	3.00	CH	±
	5	H — S	⊕
	14	CH	
	28	H — S	⊕
	37	CH	
	45	H — S	⊕
	52	CH	
	4.00	CH	
	7	H — S	⊕
	30	H — S	⊕

In the column of conditioned stimulus and reinforcement, CH = a mixture of equal volumes of citral and hydroxycitronellal.

performed by non-reinforcing a mixture of equal volumes of citral and hydroxycitronellal but reinforcing hydroxycitronellal. The mixture could be used as a conditioned inhibition stimulus because, as already shown, generalization does not occur to citral in a bee which has been conditioned to respond to hydroxycitronellal.

After eight paired repetitions of non-reinforcements and reinforcements, clear conditioned inhibition occurred in this bee. Conditioned inhibition also occurred regardless of the order of presentation of the stimuli, which indicated it

TABLE 6—CONDITIONED RESPONSE OF THE SECOND ORDER

Date	Time	Reinforcement	Conditioned response Animal No.							
			2	8	9	11	15	21	23	25
6	3.10 p.m.	C — S								
	35	C — S	+	+	+	+	+	+		
	50	C — S	+		⊕		⊕	⊕	+	+
	4.20	C — S					+	⊕		+
	50	C — S			⊕		⊕	⊕	+	⊕
	5.25	C — S			⊕		+	⊕		
7	10.40 a.m.	C — S	⊕	⊕	⊕		⊕	⊕		⊕
	11.48	C — S	⊕	+	⊕		+	⊕		
	2.25 p.m.	C — S	⊕	⊕	⊕	⊕	⊕	⊕		
	4.55	C — S	⊕	⊕	⊕	⊕	⊕	⊕		
8	10.50 a.m.	C — S	⊕		⊕	⊕		⊕	⊕	+
	11.15	C — S	⊕		⊕	⊕	⊕	⊕	⊕	
	4.25 p.m.	C — S	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	40	H								
		— C	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	55	H	⊕				⊕			
		— C	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	5.15	H	⊕				⊕			
		— C	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	30	H	⊕				⊕			
		— C	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	45	H	⊕				⊕		⊕	
		— C	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	6.00	H	+				±		+	
		— C	+	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	15	H	+	±			+		⊕	
		— C	⊕	⊕	⊕	⊕	⊕	⊕	⊕	⊕
	30	H	⊕				+		⊕	
		— C	⊕	⊕	⊕	⊕	⊕	+	⊕	⊕
	45	H							⊕	
		— C	+	+	⊕	±	+		⊕	⊕
	55	S								
9	11.45 a.m.	H	+				+			
		— C	+	⊕	⊕	⊕	⊕	⊕		⊕

In the formation of the second order conditioned response, non-reinforced citral was used instead of the unconditioned stimulus.

was due to a difference of the odour stimuli. The conditioned inhibition was preserved, as in differentiation, for at least 1 hr but not until the following day (Table 5). It thus appears that conditioned inhibition is also temporary in the honey bee.

In contrast to differentiation, the establishment of conditioned inhibition in the honey bee was difficult in the present experimental conditions. Some of the bees which showed a good response in the early stage of the experiments failed suddenly to respond.

From the present results on experimental extinction, differentiation, and conditioned inhibition, the existence of well developed internal inhibition in the honey bee is shown.

Conditioned response of the second order

Results are shown in Table 6. Bees were conditioned to citral for 3 days by reinforcement. Trials were then made to establish a conditioned response of the second order to hydroxycitronellal, to which generalization does not occur. In the trials citral without reinforcement was used. The procedure consisted of the presentation of hydroxycitronellal for 5 sec and the subsequent presentation of citral for 5 sec after an interval of 10 sec. In several bees, conditioned response of the second order was observed to occur after several trials. In some bees it was preserved until the following day.

The possibility that these results were due to 'sensitization' could not be excluded, since no check was made with a third group aroma to which a bee reinforced with either citral or hydroxycitronellal would not show generalization. However, the present experiments suggest the occurrence of a conditioned response of the second order in the honey bee, but final proof of this requires further experiments.

DISCUSSION

The conditioned response used in the present study seems to be comparable to the classical conditioned reflex. From this point of view, the proboscis extension reaction used is similar to that of the Bekhterev type in that it is a motor reaction evoked by the contraction of muscles, but like that of the Pavlov type in that it is an alimentary reflex.

The results obtained in the present experiments show that the conditioned response in the honey bee is more stable than those reported by MIKHAÏLOFF. Although there was a difference in the unconditioned reaction used (of preference in the present study but of avoidance in most experiments by MIKHAÏLOFF) many fewer reinforcements or non-reinforcements were needed to establish the various stable phases of conditioned response in the former. In addition, intervals between trials were much longer in the honey bee. Conditioning in the bee was so strong that the bees reacted 2 days after, even when they did not react apparently on the day of reinforcements and no reinforcement was performed on the following day (Table 3). In *Eledone* the conditioned response was extinguished on the following day, provided no further reinforcement was given.

It cannot be said at present whether or not the responses in the present experiments are specific to the honey bee or whether we were fortunate in choosing suitable material in which to identify otherwise hidden capacities of lower animals. At all events, the present experiments on olfactory conditioned responses showed the function of the CNS in the honey bee and also showed that the various phases of the conditioned reflex are very similar to those shown by higher mammals.

In higher animals the cerebral cortex plays an important role, and the conditioned reflex is definitely limited without this (cf. HILGARD and MARQUIS, 1940). On the other hand, the honey bee, which has no cerebral cortex, showed very rapid adaptive behaviour to the changes of environment. The question of homology of the underlying mechanisms of the conditioned reflex in mammal and insect is a subject for future research.

Results obtained in the generalization experiments showed that the honey bee can distinguish the groups of odours. As described, hydroxycitronellal and *p*-isopropyl-α-methylhydrocinnamic aldehyde have very similar aromas to a human, though they belong to different chemical groups. Citral has a strong aroma, although this belongs to the same olefinic terpen aldehyde group as hydroxycitronellal does. These facts agree with the assertion of VON FRISCH (1919) that it seems clear that odours which are similar to humans are also similar to the bees.

SUMMARY

(1) Various types of conditioned responses were investigated in the honey bee, *Apis mellifera*, using some of the aromas which were indifferent stimuli as conditioning stimuli, and extension of proboscis on tarsal contact with sucrose solution as the unconditioned response.

(2) Experimental extinction occurred after ten or more non-reinforcements, and during the experiments disinhibition was recognized. Extinction was temporary, as in mammals, and spontaneous recovery was observed on the following day.

(3) Generalization occurred between two aromas which are similar to humans but not between these two and a third aroma. Generalization required a certain lapse of time which differed in different individuals.

(4) Stable differentiation occurred after several repetitions of reinforcement of either of two stimuli and non-reinforcement of the other. Although the differentiation was preserved for at least 1 hr, it was not retained until the following day. Thus, differentiation was also temporary.

(5) Conditioned inhibition was established by repetitions of non-reinforcing a mixture of aromatics and reinforcing the conditioned stimulus. Conditioned inhibition was also temporary.

(6) The occurrence of a conditioned response of the second order seems quite probable.

(7) The results obtained are discussed and compared with what is known about mammals.

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