Associative learning in wild *Anastrepha obliqua* females (Diptera, Tephritidae) related to a protein source

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ABSTRACT. The aim of the present study was to determine whether wild adult *Anastrepha obliqua* (Macquart, 1835) females are able to associate a compound (quinine sulphate - QS) not related to their habitual diet with a protein-enriched food. Females were first fed on diets based on brewer yeast and sucrose containing or not QS. The groups were then allowed to choose between their original diets and a diet with or without QS, depending on the previous treatment, and between a diet based on agar and a diet containing agar and QS. When the nutritional value of the diets was adequate, the females did not show any preference for the diet with or without QS. With respect to the agar diet and the agar + QS diet, females previously fed on a nutritive diet containing QS preferred the diet containing QS, indicating an association between the compound and the nutritional value of the diet. The importance of this behavioral strategy is discussed.

KEYWORDS. Associative learning, fruit flies, *Anastrepha obliqua*, protein, feeding behavior.

RESUMO. Aprendizagem associativa em fêmeas selvagens de *Anastrepha obliqua* (Diptera, Tephritidae) em relação a uma fonte protéica. O objetivo do presente estudo foi determinar se fêmeas adultas selvagens de *Anastrepha obliqua* (Macquart, 1835) são capazes de associar um composto (sulfato de quinino-SQ) não-relacionado à sua dieta habitual com um alimento rico em proteínas. Primeiro, as fêmeas foram alimentadas com dietas à base de lêvedo de cerveja e sacarose contendo ou não SQ. Os grupos foram então colocados para escolher entre sua dieta original e dietas com ou sem SQ, dependendo do tratamento prévio, e entre uma dieta à base de agar somente e outra à base de agar e SQ. Quando o valor nutricional das dietas era adequado, as fêmeas não mostraram nenhuma preferência para a dieta com ou sem SQ. Em relação às dietas de agar e agar+SQ, fêmeas previamente alimentadas com uma dieta nutritiva contendo SQ preferiram a dieta contendo SQ, indicando uma associação entre o composto e o valor nutricional da dieta. A importância desta estratégia comportamental é discutida.

PALAVRAS-CHAVE. Aprendizagem associativa, moscas-das-frutas, *Anastrepha obliqua*, proteína, comportamento alimentar.

Insects display a wide range of stereotypic behaviors (fixed) and other variables depending on the ecology of the species (Hsiao, 1985). A species that presents high behavioral flexibility tends to have a greater probability to adapt to a new condition such as, for example, a new food source. One characteristic of polyphagous species is their high behavioral flexibility, since these species have to respond to the most variable stimuli related not only to feeding but also to the process of finding a host (Hsiao, 1985).

Many phytophagous insects select different nutrients in their diets that might act as phagostimulants (Bernays, 1995). Non-nutritive compounds with a characteristic odor or taste are also commonly selected to act as signaling substances for these insects (Prokopy *et al.*, 1996). These phagostimulants or signaling compounds are involved in a variety of processes which lead to significant differences in the acceptability of or preference for certain diets. Processes involving the development of a positive response to a diet include associative learning.

Associative learning can result in a change in feeding preferences if the taste of a diet is associated with satisfactory post-ingestion effects in such a way that the taste acquires a neural interpretation of adequate food (Bernays & Weiss, 1996). This type of process has been demonstrated for *Locusta migratoria* (Linnaeus, 1758) (Orthoptera, Acrididae) (Simpson & White, 1990) and *Exorista mella* (Walker, 1859) (Diptera, Tachinidae) (Stireman, 2002). Several lines of evidence suggest that learning, as well as other aspects of behavior, has evolved in response to specific problems of a species in its particular niche in such a way that it cannot be defined as a phenotypic trait of different populations of the same species occupying different niches (Healy, 1992).

The fruit fly *Anastrepha obliqua* (Macquart, 1835) is a polyphagous species showing wide behavioral flexibility, which needs to feed on a protein source during adulthood in order to permit egg maturation (Braga & Zucoloto, 1981; Aluja, 1994). Due to the importance of protein for reproduction of this fruit fly, the precise determination as to where and how much protein is acquired remains an interesting target for the study of these insects. Various aspects of protein acquisition and utilization by female *A. obliqua* can be investigated. The aim of the present study was to determine whether *A. obliqua*, due to its dependence on protein for reproduction, is able to associate a component that does not constitute part of its habitual diet with a food rich in protein. Since sucrose is known to be a powerful phagostimulant for most insects, including *A. obliqua*, the flies were submitted to tests using diets without sucrose in order to determine whether phagostimulation influenced food selection in the tests in which the diets had an adequate nutritional value.

MATERIAL AND METHODS

Wild adult *A. obliqua* females were obtained from infested "cajá-mirim" (*Spondias lutea*, Anacardiaceae) fruits, Universidade de São Paulo, Ribeirão Preto *campus*, Brazil (21°17'75"S, 47°81'2"W). The infested fruits were collected

and placed in plastic boxes (60 x 40 x 10 cm) containing a 5 cm layer of sand. After complete drying of their pulp, the fruits were removed and the puparia were separated from the sand with a sieve, washed under running water and placed in acrylic boxes (11 x 11 x 3 cm) containing sterile sand.

The boxes containing the puparia and the experimental boxes were maintained in a wooden incubator covered with a glass lid at a mean temperature of $28 \pm 1^{\circ}C$ and relative humidity of 70 to 80% under a 10/14 h light/dark photoperiod using a 400-lux fluorescent lamp.

Shortly after emerging from the puparia, the females were randomly put into acrylic boxes (11 x 11 x 3 cm) with 10 individuals each (1 box = 1 replicate) containing four lateral orifices (1 cm in diameter) through which food and water were offered. Water was offered in small test tubes stoppered with cotton. Food was offered in pieces (400 mg each) attached to a pin stuck into a cork. The food pieces were weighed on an analytical scale. Both water and diet were changed daily.

The secondary compound used in the present study was quinine sulphate, which was chosen for not being used on A. obliqua natural diet.

Six types of diet were used: 1) control diet consisting of 11.0 g sucrose, 6.5 g brewer yeast, 3.0 g agar, 1.5 ml Nipagin (fungicide; alcoholic solution 20%), and 100 ml distilled water; 2) control diet + quinine sulphate; 3) agar diet of the same composition as the control diet except it lacked sucrose and brewer yeast; 4) agar diet + quinine sulphate; 5) brewer yeast diet of the same composition as the control diet except it lacked sucrose, and 6) brewer yeast diet + quinine sulphate. The quinine sulphate quantity is defined in experiment 1. Diets were prepared and stored in a refrigerator.

Daily ingestion was measured using the following formula (Cangussu & Zucoloto, 1995): I = (TDM-RDM)/N, where I = intake (mg/female/day), TDM = total dry matter (total weight of the diet placed in the box without flies for the control of evaporation), RDM = remaining dry matter (total weight of the diet placed in the experimental box), and N = number of live flies in the box at the day measure. The food removed from the boxes after 24 hours was left to dry in an incubator for additional 24 hours at a temperature of $80 \pm 1^{\circ}C$ and then weighed for the calculation of food intake.

The results were analyzed by the Wilcoxon test (α=0.05) using the SigmaStat for Windows program (Jandel, 1997).

Experiment 1. The aim of this experiment was to determine whether quinine sulphate is attractive to A. obliqua. For this purpose, 15 mg and 25 mg quinine sulphate were added to the diet type 1. The experiment consisted of 10 replicates for each quinine sulphate quantity, in which the flies could choose between the agar diet and the agar + quinine sulphate diet. Food ingestion was measured in the first day after emergence of the females.

Experiment 2 (15 mg quinine sulphate). Four groups with 10 replicates each were set up. Groups 1 and 2 were fed on control diet + 15 mg quinine sulphate for 5 days, and groups 3 and 4 were fed on control diet for 5 days. After this period, groups 1 and 3 were allowed to choose between the control diet vs the control diet + 15 mg quinine sulphate, meanwhile groups 2 and 4 were allowed to choose between the agar diet vs the agar diet + 15 mg quinine sulphate.

Food ingestion was measured in the first day after the experience period.

Experiment 3 (25 mg quinine sulphate). The same procedure as described for experiment 2 was adopted for this experiment using 25 mg quinine sulphate.

Experiment 4. In this experiment, females received the brewer yeast + 25 mg quinine sulphate diet for 2 days before the test since flies do not survive longer than 5 days without sucrose (Fontellas & Zucoloto, 1999). The flies were then submitted to the same procedure as described for experiment 2 (groups 1 and 2) using the brewer yeast + 25 mg quinine sulphate diet.

RESULTS

Experiment 1. The results showed that quinine sulphate is not attractive to A. obliqua. Ingestion of the agar diet was significantly higher (0.08 ± 0.02 mg/female/day) than that of the diet containing 25 mg quinine sulphate (0.03 ± 0.02 mg/female/day) (p=0.0038). Ingestion of the agar diet was not significantly different between agar diet (0.03 ± 0.02 mg/female/day) and that of the agar diet containing 15 mg quinine sulphate (0.02 ± 0.01 mg/female/day) (p=0.5843). Than, these amounts of quinine sulphate are not attractive or phagostimulant in females. Agar diets containing 25 mg quinine sulphate may have been repellent to the flies. Both amount of quinine sulphate were used in the experiments.

Experiment 2. Flies previously fed on control diet + 15 mg QS did not differentiate between control vs control + 15 mg QS diets, but they did when tested with agar vs agar + 15 mg QS (Tab. I). No significant difference in the ingestion rates was observed for groups that fed on control diet in the experience period and after being allowed to choose between control diet vs control diet + QS (Tab. II). But these groups preferred agar diet when the choose was between agar diet vs agar diet + QS (Tab. II).

Experiment 3. Flies previously fed on control diet + 25 mg QS preferred control diet when the choose was between control vs control + 25 mg QS diets, but they preferred agar diet + QS when tested with agar vs agar + 15 mg QS (Tab. I). No significant difference in the ingestion rates was observed for groups that fed on control diet in the experience period (Tab. II).

Experiment 4. The findings were the same as those obtained for experiment 2 (Tab. III), supporting the presence of associative learning in cases when the diet offered does not have an adequate nutritional value (agar). Food ingestion was low, probably due to the absence of sucrose-mediated phagostimulation.

DISCUSSION

In all experiments, when the diet offered did not have any nutritional value (diets based on agar), the flies that previously consumed nutritive diets containing quinine sulphate preferred diets with this compound. When the diet of choice had a high nutritional value for this species, the results did not clearly show the occurrence of learning. Since learning is based on positive post-ingestion nutritional feedback, consumption of the control diet with or without quinine sulphate results in a metabolic benefit, as the concentration of the secondary

Table I. Selection of diets with or without quinine sulphate (QS) by females of *Anastrepha obliqua*, which have previously tasted diets containing different contents of quinine sulphate. Means followed by different letters on the same row are significantly different (Wilcoxon test, $p < 0.05$).

Experiment	Previous diet	Group	Ingestion (mg/female/dia)		p
2		1	Control diet + 15 mg QS	Control diet	
	Control diet		$0.81 \pm 0.42a$	$1.07 \pm 0.25a$	0.2324
	+				
	15 mg QS	2	agar + QS	agar	
			$0.05 \pm 0.03a$	$0.02 \pm 0.02b$	0.0391
3		1	Control diet + 25 mg QS	Control diet	
	Control diet		$0.41 \pm 0.23a$	$1.70 \pm 0.49b$	0.0039
	+				
	25 mg QS	2	agar + QS	agar	
			$0.04 \pm 0.03a$	$0.01 \pm 0.03b$	0.0234

Table II. Selection of diets with or without quinine sulphate (QS) by females of *Anastrepha obliqua*, which have previously tasted diets with no quinine sulphate (control diet). Means followed by different letters on the same row are significantly different (Wilcoxon test, $p < 0.05$).

Experiment	Group	Ingestion (mg/female/dia)		p
2	3	Control diet + 15 mg QS	Control diet	
		$0.96 \pm 0.39a$	$1.27 \pm 0.48a$	0.1934
	4	agar + QS	agar	
		$0.02 \pm 0.03a$	$0.04 \pm 0.02b$	0.0039
3	3	Control diet + 25 mg QS	Control diet	
		$0.88 \pm 0.60a$	$1.26 \pm 0.47a$	0.1309
	4	agar + QS	agar	
		$0.02 \pm 0.04a$	$0.03 \pm 0.02a$	0.3828

Table III. Selection of diets with or without quinine sulphate (QS) by females of *Anastrepha obliqua*, which have previously tasted brewer yeast diets with quinine sulphate. Means followed by different letters on the same row are significantly different (Wilcoxon test, $p < 0.05$).

Ingestion (mg/female/day)		p
Brewer yeast + 25 mg QS	Brewer yeast	
$0.22 \pm 0.08a$	$0.24 \pm 0.19a$	1.000
agar + 25 mg QS	agar	
$0.07 \pm 0.10a$	$0.01 \pm 0.02b$	0.0036

compound was not toxic to *A. obliqua*. Although females displayed typical behavior of generalist species by consuming nutritious diets with or without QS, experience with QS modified feeding behavior toward acceptance of non-nutritive diets that contained QS.

According to PAPAJ & PROKOPY (1989), the effect of experience on behavior is considered to be learning when it fulfills the three following rules: 1) the individual behavior changes repeatedly as a consequence of the experience, 2) behavior changes gradually with continuous experience, and 3) the behavioral change that accompanies the experiences is reduced in the absence of continuous experience of the same type or as a consequence of a new or unpleasant experience.

The capacity of generalists to obtain an adequate balance of nutrients as a result of a mixture of these nutrients selected from different diets permits the dilution of secondary compounds present in diets which could be potentially poisonous (BERNAYS & MINKENBERG, 1997). In the present study, the groups receiving brewer yeast diets might have been "confused" by the nutritional value of the food items involved in those experiments. Feeding on the two diet blocks (with and without quinine sulphate) might have been possible due to the dilution of any positive or negative effect resulting from the presence of quinine sulphate. It is interesting to note that protein was the unconditioned stimulus and not sucrose present in the four experiments. Since protein is of vital importance to the reproduction of *A. obliqua*, especially during the first days after emergence, and since it is not always present in abundant amounts in the natural diet of these insects, it seems to be more advantageous that females spend energy to develop mechanisms to detect diets with a high protein content. The same should not apply to carbohydrates which represent an abundant nutrient in the natural diet of these flies (BATEMAN, 1960).

The results obtained in experiments using control diet should not be taken as evidence that *A. obliqua* is not capable of associating quinine sulphate to the presence of brewer yeast on the diet. It is not possible to evaluate experimentally how the taste of the diet is changed by the quinine sulphate and how it affects the insect's perception.

Simpson & White (1990), in a study on the desert grasshopper *Schistocerca gregaria* (Forskal, 1775) (Orthoptera, Acrididae), showed that the insects were trained to respond to specific odors associated with essential macronutrients. The studies showed a significant response to odors associated with protein-rich food but not to odors associated with carbohydrate-rich food. Recently emerged *Periplaneta americana* (Linnaeus, 1758) (Blattodea, Blattidae) adults learn to associate protein-rich food to odors that have no nutritive meaning, whereas the same was not observed for carbohydrates (Gadd & Raubenheimer, 2000).

The direct involvement of positive learned associations in nutritional homeostasis depends on two factors: the nutritional status of the insect must determine when the behavior is expressed, as shown by Simpson & White (1990), and the nutritional status needs to be involved, although not necessarily, in the formation of memory. In the latter case, a positive association between a signaling substance and a nutrient is more readily established when the animal lacks this nutrient. Foresteel *et al.* (2001) observed that rats acquire associations between odors and a food source and discriminate between them; however, the expression of this discrimination of odors depends on the presence or absence of food. On the other hand, latent learning, whose extent (duration/intensity) is relatively independent of the nutritional status, has been observed (Simpson & Raubenheimer, 1993).

Since a dietary deficit or the toxicity of a substance is often only perceived after ingestion, the results of the present study become clear when analyzing the ingestion rates obtained for each day during experiment 2. Behavior is probably altered as a consequence of post-ingestion feedback. It seems quite unlikely that the *A. obliqua* females no longer preferred the diet containing quinine sulphate due to a toxic effect, since the groups receiving nutritious diets containing quinine sulphate continued to ingest these diets. Probably, the lack of a nutritional value was the determinant factor for the change in behavior.

Learning by fruit flies has been demonstrated mainly in the laboratory. Prokopy *et al.* (1989) have shown that some *Rhagoletis* (Loew, 1862) (Diptera, Tephritidae) species are able to learn the colors, forms and odors of different host fruits on which they lay eggs so that they can also learn signs associated with food sources. Learning in terms of acceptance of fruits has been demonstrated for female *Ceratitis capitata* (Wiedemann, 1824) (Diptera, Tephritidae) which oviposited on a certain fruit and subsequently tended to reject other types of equally acceptable fruits (Cooley *et al.*, 1986). The size of the fruit is the main characteristic learned and is used for finding a familiar fruit, whereas color and odor seem to be of little importance (Prokopy *et al.*, 1989).

The aspects of the feeding behavior of wild *A. obliqua* females observed in the present study indicate that these insects are highly flexible, with a marked capacity to adapt to new conditions and new food items, a fact that renders them the target of many behavioral studies.

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