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[SHORT COMMUNICATION]

Honeybee Queens Lay Fertilized Eggs When No Comb Cells for Oviposition Are Available

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ABSTRACT—To study the causal mechanisms underlying the control of egg sex by honeybee queens (*Apis mellifera*), the queens were allowed to lay eggs in experimental cages without comb cells. The sex of the eggs laid were then determined by counting the number of chromosomes, and by observation of female and male pronuclei in the eggs and sperm cells on the surface of the eggs. It was found that queens laid normally fertilized diploid eggs under the experimental conditions. These results suggested that honeybee queens lay fertilized eggs when no information of comb cell size is available, thus the idea that queens would be stimulated to release sperm by small worker cells fitting queen's abdomen is not supported.

INTRODUCTION

Haplodiploidy in hymenopteran insects should be manipulated by a maternal physiological mechanism to determine the sex of eggs to be laid; unfertilized eggs develop into males and fertilized eggs develop into females. Mothers can potentially control the sex of offspring at the oviposition in response to external cues in order to enhance their fitness (Charnov *et al.*, 1981), although its mechanism remains unknown.

Honeybee queens (*Apis mellifera*) have been shown to control the sex of eggs on the basis of different types of comb cells available for oviposition; males in larger cells and females in smaller ones (Ratnieks and Keller, 1998). Two different processes to respond to the cell size have been suggested; (1) fitness of the abdomen inserted into the cell (Petrunkewitsch, 1901; Michener, 1974), and (2) inspection of the cell by forelegs or antennae (Flanders, 1950; Koeniger, 1970). Koeniger (1970) insisted the importance of the queen's forelegs based upon the experiments using queens with their forelegs amputated or with flags on intact forelegs to prevent them from inspecting cells.

By the idea of Petrunkewitsch (1901) and Michener (1974) that queens would be stimulated to release sperm by small worker cells fitting queen's abdomen, the queens should lay unfertilized eggs under the experimental condition without information of cell size. In this paper, we present evidence suggesting that queens should fertilize eggs when sensory inputs from the cells are lacking, thus their idea is not supported.

MATERIALS AND METHODS

Before the ovipositional experiments, honeybee queens in colonies with 8000–10000 workers were observed to have produced male and female brood (larvae and pre-pupae) in male and female comb cells, respectively. Prior to the experiment queens were also observed during oviposition to determine in which comb cells they actually laid eggs. Queens were then transferred into small cages (8×3.5×1.5 cm) and kept at 32°C with 10 workers who were fed sugar dissolved in honey, wherein queens were allowed to lay eggs. No external cues originating from comb cells were thus available to the queens. A Petri dish was placed under the cage to collect the eggs dropping from queen's ovipositional pore. The collected eggs were then incubated at 32°C in an incubator for 18–20 hr for Giemsa staining and for 5 min–5.5 hr for Feulgen staining. The ovipositional experiments were carried out from April to July, 1997 and from April to August, 1998.

The sex of eggs was determined by (1) counting the number of chromosomes, and by (2) observation of female and male pronuclei in the eggs and sperm cells on the surface of the eggs. The former could detect ploidy of the embryo developing but not egg fertilization, since automictic parthenogenesis occasionally appears in several races of honeybee (Tucker, 1958). The latter could determine whether the eggs laid were fertilized and whether sperm were released to the eggs.

The egg's chromosomes were stained by modified Giemsa staining (Imai *et al.*, 1977). For this the collected eggs were immersed in 1% sodium citrate solution with 0.01% colchicine for 10 min and prefixed in EA solution (ethanol : acetic acid=1:1). The chorion of the egg was removed with a sharp needle for pre-fixation. The cells of embryo were then fixed in fresh EA solution for 2 min, washed in acetic acid solution and dried at 60°C overnight. The preparations were then stained in Giemsa's solution (Merck, Germany) for 10 min, washed in water for 2 sec and dried at 60°C overnight. The number of stained chromosomes were counted in at least 10 cells per preparation to assess whether the egg was haploid ($n=16$) or diploid ($2n=32$) (Sanderson and Hall, 1948).

To observe female and male pronuclei in the eggs laid and sperm

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cells on the surface of the eggs, the eggs were submitted to Feulgen staining. Eggs were first fixed in an FAA solution (95% ethanol : 50% acetic acid : formalin=2 : 0.5 : 0.2) at 4°C for 3 hr, cut at the midline under a dissecting microscope, fixed again in fresh FAA solution at 4°C overnight, and washed in 70% ethanol for 30 min. The eggs were then washed in distilled water three times, hydrolyzed in 1 N HCl for 10 min at room temperature, then in 1 N HCl for 15 min at 60°C, and washed in ice-cold distilled water. They were stained with Schiff's solution (Wako, Japan) for 1 hr at room temperature, washed briefly in 0.5% $K_2S_2O_8$ solution containing 0.05 N HCl and dehydrated in ethanol series. Dehydrated eggs were cleared in xylene and mounted with Bioleat (Oken, Japan).

RESULTS AND DISCUSSION

Under the present experimental conditions, queens were observed not to lay eggs when they were not in contact with workers. They started to oviposit only when touched on the abdomen by the worker's antennae. Once the queens laid their first egg, however, they laid eggs continuously thereafter even in the absence of worker antennal contact. During oviposition, queens moved their abdomen along the longitudinal axis without bending ventrally and then raised the sting to push out an egg from the ovipositional pore.

All the queens laid diploid eggs (Table 1). Seventy-nine of 84 eggs were diploid and one was haploid. The sex of four eggs could not be determined because of the small number of cells that had discernible chromosomes. All the queens, except queen C, had laid eggs in worker cells before being transferred into the experimental cages. Queen C who had laid eggs previously in male cells, laid exclusively diploid eggs in the experimental cage.

Sixty-five eggs laid were stained by Feulgen staining. All eggs developed in the same manner as normally fertilized eggs, as described previously by several authors (Nachtsheim, 1913; Counce and Waddington, 1973). There were no eggs developing automictically. On the surface of the eggs laid, several nuclei supposed to be those of sperm cells were stained (Fig. 1a). No nuclei, however, were observed on the surface of unfertilized eggs ($n=15$), taken out from queen's lateral oviducts, which were stained by the same procedure. The stained nuclei formed slender shapes (3–4 μ m in length) which seemed similar to those of sperm cells taken from the spermathecae of queens (Fig. 1b) and they were distinguishable from the nuclei of normal somatic cells which formed round

shapes. The nuclei of sperm cells were observed mainly on the outer surface of the posterior end of the egg (Fig. 1a). The number of sperm cells observed in that position varied from egg to egg (range: 6–122; mean \pm s.e. = 28.6 ± 6.1 , $n=25$). On the anterior end, on the other hand, significantly less sperm cells (range: 0–15; mean \pm s.e. = 3.2 ± 0.8 , $n=25$) were observed ($p < 0.001$, $z = -5.56$, Mann-Whitney U test). No sperm cells were observed on the intermediate zone of the egg ($n=25$).

In this study, queens were allowed to lay eggs in an open place where no comb cells for oviposition were available. This experimental set-up was abnormal for the queens in respect to oviposition in three ways; (1) absence of cell inspection, (2) no ventral bending of abdomen and (3) no insertion of abdomen into cells. The results of chromosome examination of the deposited eggs demonstrate that queens laid diploid eggs under our experimental conditions. It has been reported that unmated queens occasionally lay automictic diploid eggs during accelerated oviposition following an artificially induced cessation of oviposition (Mackensen, 1943; Tucker, 1958). The automictic diploid eggs were derived from the fusion of two haploid egg nuclei formed by complete meiosis (Tucker, 1958). In our experiments, however, it is unlikely that the queens have laid automictic diploid eggs, since the diploid eggs must have resulted from normal egg fertilization with female and male pronuclei fused. The findings of sperm cells on the surface of eggs show that the sperm release have occurred in the vagina during egg laying under our experimental conditions. This means that fertilization of eggs by queens is independent of the presence of cells. It also suggests that the ventral bending of the abdomen and insertion of the abdomen into cells, that proceed oviposition, are not necessarily required for the release of sperm.

Our results do not support the idea of Petrunkevitch (1901) nor of Michener (1974), but are consistent with the results obtained by Koeniger (1970). Koeniger (1970) reported that the queens did not distinguish between male and worker cells and laid female eggs in male cells, if their forelegs were amputated or if they had intact forelegs with flags to prevent cell inspection. He concluded that egg fertilization is prevented by specific stimuli originating from the male cell, and that oviposition of fertilized eggs in worker cells only took place when these stimuli were lacking. In our experiment, the queens in the cages would extend their forelegs more than normal

Table 1. Karyotypes of eggs laid in the experimental cages without comb cells for oviposition.

Queens	Comb cells used for previous oviposition	No. of eggs examined	No. of haploid eggs	No. of diploid eggs	No. of eggs in which the karyotype could not be determined
A	worker	10	0	10	0
B	worker	14	0	12	2
C	male	8	0	8	0
D	worker	12	0	11	1
E	worker	11	0	10	1
F	worker	19	0	19	0
G	worker	10	1	9	0
Total		84	1	79	4

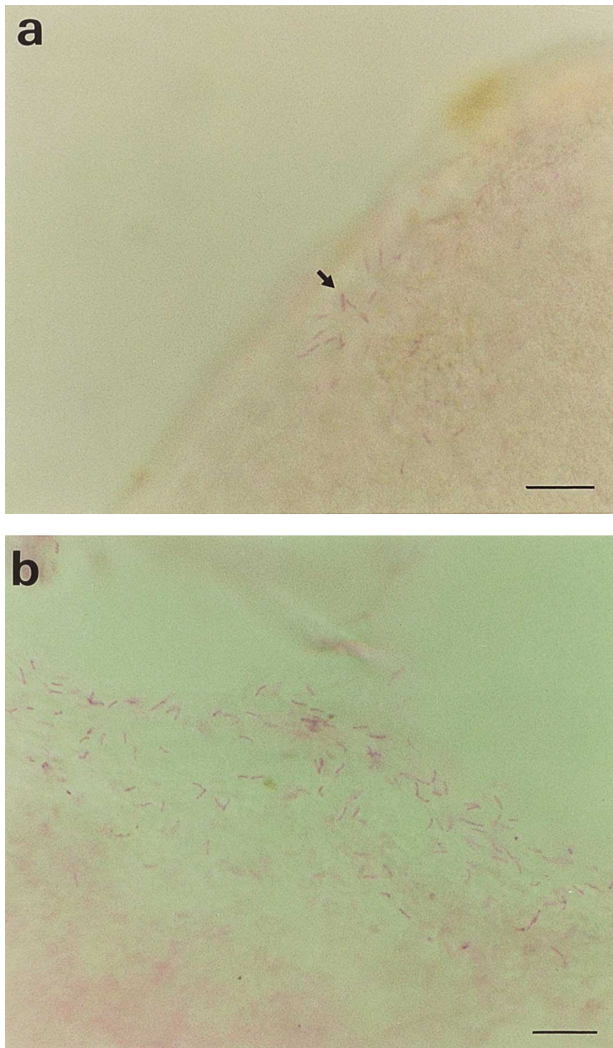


Fig. 1. Nuclei of sperm cells stained by Feulgen staining on the posterior end of the laid egg (a) and those in the sperm cells taken out of the spermathecae of queen bees (b). Arrow indicates the sperm nuclei stained red with slender shapes. Bars: 5 μ m

queens when inspecting into worker cells, looking more like queens inspecting into male cells. However, the queens were not stimulated to prevent egg fertilization by foreleg extension and laid fertilized eggs. How do queens recognize male cells for controlling egg fertilization? Queens might be stimulated to prevent egg fertilization by the exact size of male cells or other unknown cues including chemical substances in male cells.

Honeybee queens are able to control egg fertilization accurately depending on comb cell types (Koeniger, 1970; Sasaki *et al.*, 1996; Ratnieks and Keller, 1998). However, the mechanism by which unused sperm left in the vagina is cleared off so as not to fertilize subsequent eggs mistakenly has been unclear (Michener, 1974). The observation that the stained sperm cells appeared on the posterior end of eggs but not on the intermediate zone suggests that eggs may push off the remaining semen left unused in previous fertilizations with their posterior end when they pass through the vagina with the

posterior end ahead. The mean number of observed sperm nuclei per egg was 28.6 on the posterior end, which coincides with the estimated sperm number released at each fertilization (20–30 sperm cells per egg) (Harbo, 1979). The results indicate that the sperm cells left in the vagina should be trapped on the posterior end of eggs and pushed out. Since the micropyle of an egg is located at the anterior end (Retnakaran and Percy, 1985), an egg should be exposed to the fresh semen on the micropyle to be fertilized, or to the vaginal liquid that contain no sperm to be not. It is, therefore, likely that the posterior end first manner of egg passage through the vagina constitutes the mechanism of cleansing the vagina of unused semen, thereby ensuring accurate control over sex determination of the eggs to be laid by the queen.

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