Insectes soc. 49 (2002) 234–240
0020-1812/02/030234-07

Insectes Sociaux

Research article

Egg activation and timing of sperm acceptance by an egg in honeybees (*Apis mellifera* L.)

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Received 26 November 2001; revised 25 February and 25 March 2002; accepted 2 April 2002.

Summary. Mechanical stresses by a narrow glass capillary were applied to unfertilized eggs of honeybees to determine whether the removal of meiotic blocks of the eggs could be caused by simple mechanical stimuli. The treated eggs developed into the anaphase of the first meiotic division at 15 min after treatment, whereas the untreated eggs remained arrested at the metaphase of the first meiotic division. The results of histological examination of the common oviduct showed that its inner widths were sufficiently narrow to cause the distortion of eggs passing through it. The distorted eggs could be fertilized and develop into diploid embryos if they were exposed to the semen immediately (within 30 sec) after egg distortion. However, this would not happen if the distorted eggs were exposed to semen later (30 min). The eggs exposed to the semen but not given mechanical stimuli could initiate the embryonic development with diploid chromosomes. The interval between mechanical distortion and sperm acceptance by eggs in vitro is compatible with that of natural oviposition of fertilized eggs by honeybee queens. These results suggest that egg activation by mechanical stresses in the common oviduct is valid for the natural oviposition in honeybees.

Key words: Eggs, sperm, fertilization, development, *Apis mellifera*.

Introduction

In many animals with sexual reproduction, egg development is triggered by sperm invasion. The mechanisms underlying this process have received considerable attention in echinoderms, amphibians and mammals (Monroy and Tyler, 1967; de Petrocellis et al., 1974; Epel, 1978; Smith, 1981). Such mechanisms in these animals, however, cannot operate in insects that have an ability to reproduce parthenogenetically.

In many Hymenopteran insects, males develop from unfertilized eggs with a haploid set of chromosomes. Haploid eggs do not develop unless they receive specific stimuli including mechanical pressure, different osmotic pressure and cold shock (Went, 1982; Vinson and Jang, 1987; Sawa and Oishi, 1989a). These stimuli have been shown to initiate the early development of an egg. It remains controversial, however, what kinds of cues cause meiosis of oocyte during or after natural oviposition. In Parasitica including Ichneumonoidea, the development of haploid eggs may be initiated by mechanical stimuli (King and Rafai, 1973; Went and Krause, 1973; Vinson and Jang, 1987) applied to the eggs during their passage through the female's ovipositor (Went and Krause, 1973, 1982).

It is generally accepted that Aculeata including honeybees and ants have diverged from Parasitica (Dowton and Austin, 1994), in most of which divergence has been accompanied by a change in ovipositor function from egg laying to stinging. The eggs in Aculeata are thus laid through the ovipositional pore without being mechanically distorted by the ovipositor. It is shown that the development of eggs of honeybees may be initiated by similar artificial mechanical stresses as those in Parasitica eggs (Sasaki et al., 1997). Sasaki et al. additionally suggested that the unfertilized eggs in Aculeata might receive the mechanical stimuli to commence the development by the queen's reproductive tract, instead of the stimuli by the ovipositor in Parasitica. However, several questions, including the timing of egg fertilization and egg activation of the fertilized egg, the time course of sperm acceptance of eggs and other possible sites in the reproductive tract responsible for the activation, remain unsolved.

This study aims to determine: (1) whether the meiotic blocks in the oocyte can be released by artificial mechanical stresses and (2) whether the common oviduct of a queen is responsible for causing the mechanical stress to eggs. If so, the oviducts may cause a trigger stimulus for the egg activa-

tion before sperm invasion. Therefore, we examined (3) whether the eggs can be fertilized in vitro after being mechanically stimulated. We also discuss the probable mechanisms underlying egg activation occurring in the queen's reproductive tracts.

Material and methods

Production of new queens

The standard procedures for producing new queens were employed. Bees in colonies of mixed Italian strain were used for the queen production. The procedure for rearing queens is as follows: A frame holding two bars with each 10 commercial plastic queen-rearing cups is introduced into a queenless colony for 2 days. During this time the worker bees cleaned and polished the cups, and placed additional wax on the cups. After 2 days, the frame was removed from the queenless colony and then each hatched larva taken from a queenright colony was transferred into each queen-rearing cup on the frame. The frame with queen cups was returned to the queenless colony and allowed the queenless workers to rear new queens. When the queen cells were capped, the cells containing queen larvae were removed from the frame and incubated at 32 °C. After emergence of adult new queens, each queen was introduced into a small queenless colony containing 4000–5000 workers and kept for a few weeks so that the virgin queens could mate naturally. A month later, the mated queens were observed to confirm their egg laying and used for the experiments.

Observation of meiosis in the mechanically distorted oocyte

To observe whether the meiotic blocks in the oocyte can be released by simple mechanical stress, unfertilized mature eggs were taken out of the lateral oviducts of queens and then passed through narrow capillaries of 0.2 mm inner diameter. These capillaries were designed to apply sufficient mechanical pressure to eggs with a majority of the eggs surviving (Sasaki et al., 1997). The eggs were then incubated at 32 °C for 15 min in saline solution (128.33 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$, pH 6.7) (Bailey, 1952). As a control, eggs without mechanical stress were incubated under the same conditions. After the incubation, the eggs were submitted to Feulgen staining which could selectively stain DNA of chromosomes. Eggs were first fixed in an FAA solution (95% ethanol : 50% acetic acid : formalin = 2 : 0.5 : 0.2) at 4 °C for 3 h, cut at the midline under a dissecting microscope (SZH, Olympus), 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 1 N HCl at 60 °C for 15 min, and washed in ice-cold distilled water. They were stained with Schiff's solution (Wako, Japan) at room temperature for 1 h, washed briefly in 0.5% $K_2S_2O_5$ solution containing 0.05 N HCl and dehydrated in ethanol series. Dehydrated eggs were cleared in xylene and mounted with Bioleit (Oken, Japan). Total 16 unfertilized mature eggs taken from two queens were examined.

Histology of the reproductive tract

Serial sections for light microscopy were made to determine the inner structure of the reproductive tract, particularly the common oviduct and the attachment of semi-circular muscles. The reproductive tracts dissected from 23 queens were fixed for 1 h in Bouin's solution and washed in distilled water for 5 min. The fixed tissues were then dehydrated by a graded ethanol-xylene-paraffin series and embedded into paraffin wax. A microtome was used to prepare 6-µm-thick sections which were mounted on gelatin-coated microscope slides. The sections were dewaxed and hydrated by a xylene-ethanol series before staining with Ehrlich's hematoxylin and eosin.

Specimens for scanning electron microscope (SEM) observation were made to determine the location of the micropyle of an egg in the reproductive tract. Mature eggs taken from the lateral oviducts were fixed in 2% glutaraldehyde, buffered at pH 7.3 with 100 mM sodium cacodylate for 2 h at 4 °C. The eggs were then cut off at the posterior end region for SEM observation of the anterior end or at the anterior end for the observation of the posterior end to facilitate the fixation and also to mark the direction of eggs. The eggs were postfixed in 2% osmium tetroxide in the same buffer for 2 h at 4 °C, dehydrated in a graded acetone series, dried using a critical point dryer (JCPD-3, JEOL), coated with a 40 nm gold layer and examined with an SEM (JSM-T330A, JEOL). In total 14 eggs taken from 3 queens were observed.

To measure the inner diameters of common oviducts, the queen reproductive tracts were dissected from 15 queen abdomens under the dissecting microscope, fixed for 1 h in Bouin's solution and washed in distilled water for 5 min. The tissues were then transferred into 10% NaOH solution at 60 °C for 15 min. After the incubation, the transparentized tissues were observed under the dissecting microscope and their inner diameters measured by a micrometer.

In vitro egg fertilization

To analyze sperm acceptance by an egg, artificial egg fertilizations in vitro were carried out. Spermatozoa were taken out from the spermatheca of queens by using a microdispenser (S-200, Drummond) and diluted by Hyes's solution (NaCl, KCl, $CaCl_2$, $NaHCO_4$, pH 8.5). The semen was dropped on the anterior surface of an unfertilized egg so that the spermatozoa could enter the egg through its micropyle. After 30 min, the eggs were transferred into the saline solution and incubated therein at 32 °C for 18 h. As a control, unfertilized eggs not exposed to the semen without mechanical egg deformation were incubated in saline solution at 32 °C for 18 h. Several eggs were first passed through a narrow glass capillary (0.2 mm in diameter) and then, within 30 sec or after 30 min, were placed on the semen at room temperature for 30 min and incubated in the saline solution at 32 °C for 18 h. After the incubation, the eggs were observed for embryonic development. The developed embryos were then stained by Giemsa staining (Sasaki and Obara, 1999), and their karyotypes were observed to judge whether the eggs had been fertilized. The survivorship of eggs was judged on the basis of two different criteria of egg morphology. One is the yolk coming out from the membrane of oocytes or from the chorion of eggs, which may be caused by the severe injuries of membrane of oocytes and indicates apparently dead eggs. The other is the change of yolk transparency from normal white opaque states without any sign of development for 18h. Although these transparent eggs might initiate the development abnormally and stop the embryogenesis, we failed to observe any stained cell nuclei in these eggs by Giemsa staining, and therefore we did not count the transparent eggs as surviving eggs. A total of 156 mature unfertilized eggs taken from 22 queens were examined.

Results

First meiotic division in oocyte

The eggs were subjected to mechanical stresses and were distorted by passing through a capillary (Fig. 1 A). The ratio of whole length to minimum width of an egg was 4.253 ± 0.238 (mean ± s. d., n = 6) in undistorted mature eggs, whereas that in the mechanically distorted eggs was 7.846 ± 0.587 (n = 4). The nuclei of the oocytes stained clearly by Feulgen staining were located near the egg surface at the ventral side of the anterior pole. The untreated control eggs did not show any sign of meiosis and remained arrested at the metaphase of the

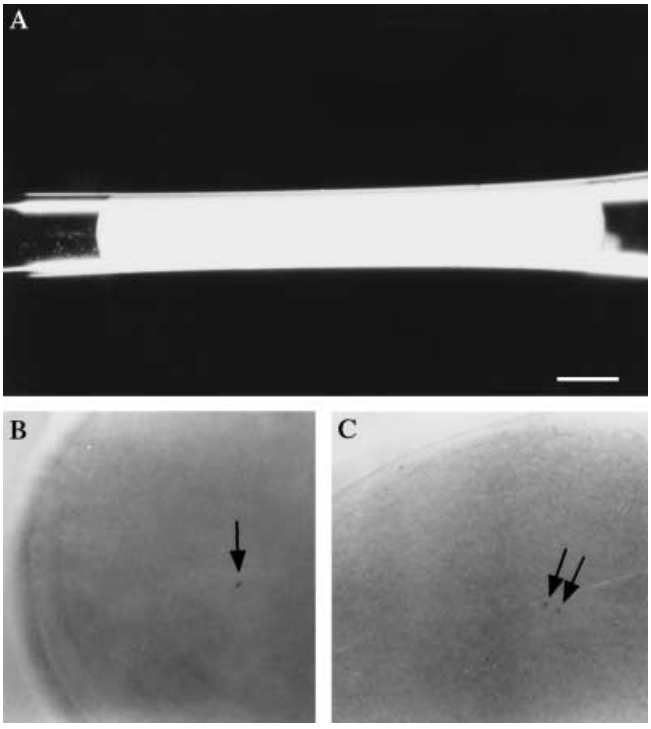

Figure 1. Mechanically distorted egg in the capillary (A). The nuclei of an unfertilized egg without (B) and with mechanical stresses (C). The nuclei are indicated by the arrows. In (B), the egg remained arrested at the metaphase of the first meiotic division at 15 min after the control treatment. In (C) the egg developed into the anaphase of the first meiotic division at 15 min after the stimulation. Scale bar in (A): 0.2 mm

first meiotic division at 15 min after treatment (n = 8, Fig. 1B). The nuclei of the mechanically distorted eggs, on the other hand, developed into the anaphase of the first meiotic division at 15 min after stimulation (n = 8, Fig. 1C). Neither male pronuclei nor nuclei of sperm cells were observed in both eggs with and without the mechanical stress.

Inner structure of reproductive tract

The reproductive tract of a queen consists of four major parts: a pair of ovaries, a pair of lateral oviducts (Fig. 2A), a common oviduct (Fig. 2B) and a vagina (Fig. 2C). Mature eggs produced from a pair of ovaries are stored in the lateral oviducts until the queen begins an oviposition. The micropyle was observed on the anterior pole of the egg (Fig. 2D). The micropylar area of an egg consisted of a dense network of canals of various shapes and sizes. The center canals are perpendicular to the margin of the micropylar area, whereas the outer canals generally slant toward the center. The surface of the chorion at the posterior end of an egg was smooth without any radial structure or canals (Fig. 2E).

Muscles of the lateral oviducts were attached on the outer surface of the connective tissue, but could not make a strong force to distort eggs because of their wide expandable tracts in the lateral oviducts (Fig. 2A, 3A). In contrast to the thin layer of muscles surrounding the lateral oviducts, muscles of the common oviduct are well developed in a relatively thicker layer (Fig. 2B). The muscle fibres in the common oviduct are attached to the connective tissue with the inner cuticle of the tract from the dorsal to lateral region, but not to the connective tissue of the ventral floor of the tract, and terminate on the median antecosta of sternum VII like the other muscles of the tract (Laidlaw, 1944; Ruttner, 1956, 1961; Camargo and Mello, 1970). This structure can move the inner cuticle of the tract inside and squeeze the egg with a strong force of the semi-circular muscle when the muscles contract with an egg within. Thus, the inner diameter of the tract of the common oviduct is kept small by the semi-circular muscles. There were small numbers of muscle fibres attached to the ventral outer layer of the valve-fold in the vagina which originate from the median antecosta of sternum VII (Fig. 2). The valve-fold would be raised normally and fixed posteriorly during copulation (Ruttner, 1956), which may be caused by the action of the muscle attached to the valve-fold in the vagina.

The common oviduct is elliptic in shape with a lateral inner diameter of 0.283 ± 0.079 mm (n = 10) and a dorsoventral inner diameter of 0.343 ± 0.068 mm (n = 8). The width of matured eggs sampled from the lateral oviducts ranged from about 0.4–0.45 mm, being much larger than the inner diameter of the common oviduct (Fig. 3A). Some of the queens, which were sampled just when they were in oviposition, were found to have an egg staying in the common oviduct. The egg was actually distorted in the thin canal of the common oviduct surrounded by the muscle (Fig. 3B). In the common oviduct, the ratio of the distorted egg length to its minimum width was 7.5, which is a similar value to that of the egg mechanically distorted in the capillary.

Timing of sperm acceptance by an egg

The eggs tended to survive longer when they were not subjected to mechanical stresses than when artificial distortion was applied by the glass capillary (Table 1), but the trends were not statistically significant ($p = 0.119$, $\chi^2 = 2.431$, $df = 1$, χ^2-test).

Artificial egg distortion caused embryonic development as reported in previous experiments (Sasaki et al., 1997). The mechanical distorted eggs developed into early blastoderm stages at 18h in the same manner as normally embryonic development (Counce, 1973; Fleig and Sander, 1985). Egg fertilization in vitro also caused egg development. The eggs immersed in the semen showed initiation of embryonic development with diploid chromosomes (Table 1). All eggs but one that survived in the control group did not show any sign of development.

Sperm acceptance for egg fertilization depended on when the egg was mechanically stimulated. The eggs mechanically distorted and fertilized in vitro within 30 sec after the distortions developed into diploid blastoderm, whereas the eggs treated likewise but fertilized 30 min later developed into haploid embryos (Table 1).

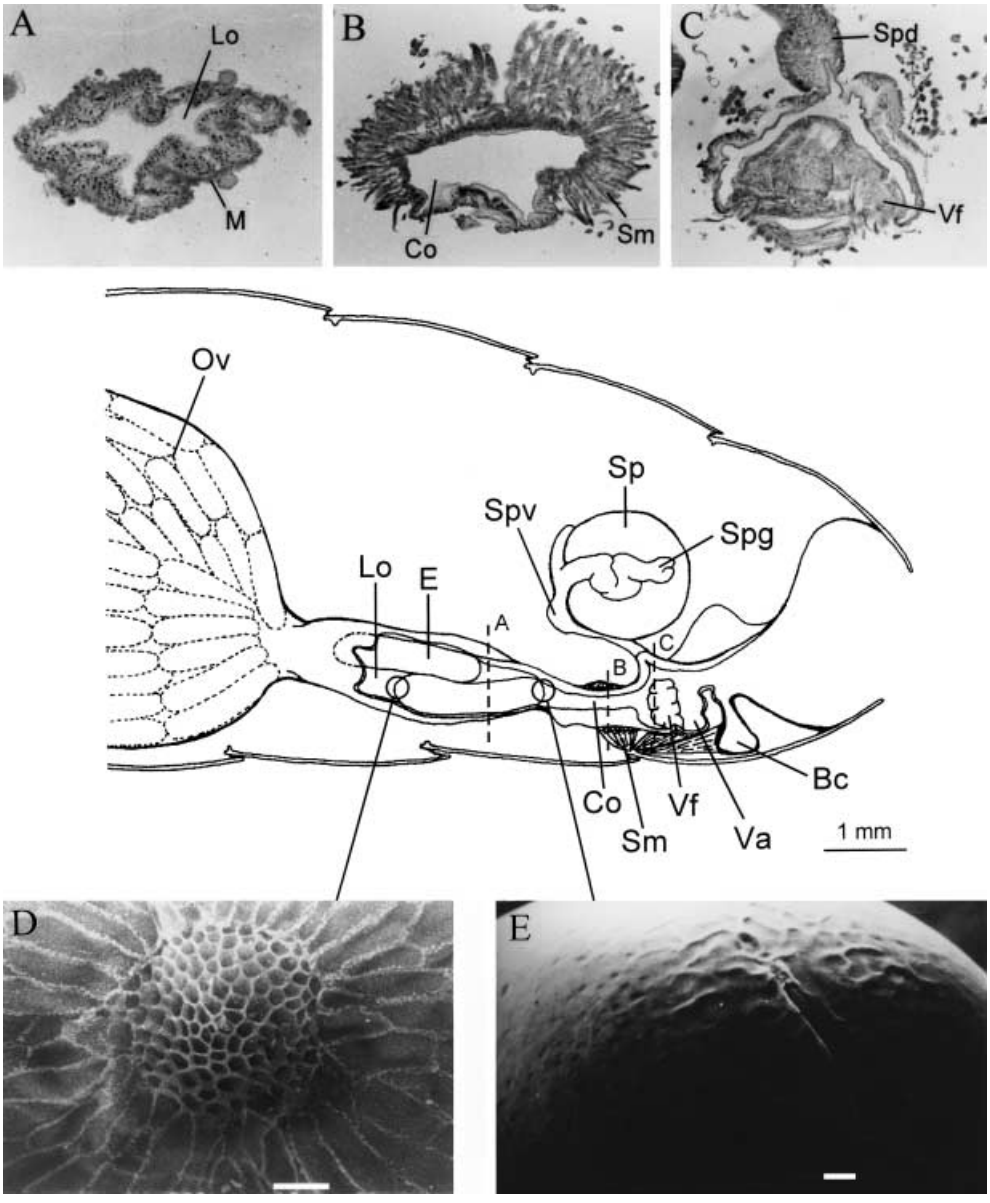

Figure 2. Reproductive tract of a honeybee queen. Serial cross sections for light microscopy were made from each portion of tracts indicated by dashed lines (A, B, C). Photos on top correspond to the cross section at each dashed line A, B and C. SEM images of the anterior surface of an egg (D) and the posterior surface of an egg (E). Muscles originating from the median antecosta of sternum VII are illustrated. Bc: bursa copulatrix, Co: common oviduct, E: egg, Lo: lateral oviduct, M: muscle fibres Ov: ovary, Sm: semi-circular muscle, Sp: spermatheca, Spd: spermathecal duct, Spg: spermathecal gland, Spv: spermathecal valve, Va: vagina, Vf: valve-fold. Scale bars in (D) and (E): 10 μm

Table 1. Effect of mechanical distortions and artificial fertilizations on egg development

Treatment	No. of eggs tested	No. of eggs surviving (%)	No. of eggs developed	Karyotype
M.D.	52	34 (65.4)	34	n
A.F.	28	22 (78.6)	22	2n
M.D. + A.F. (within 30 sec)	26	17 (65.4)	17	2n
M.D. + A.F. (after 30 min)	25	14 (56.0)	14	n
No stimulation	25	18 (72.0)	1	– *

M.D.: Mechanical distortion, A.F.: Artificial fertilization. *: Karyotype was not determined as most of the eggs did not develop.

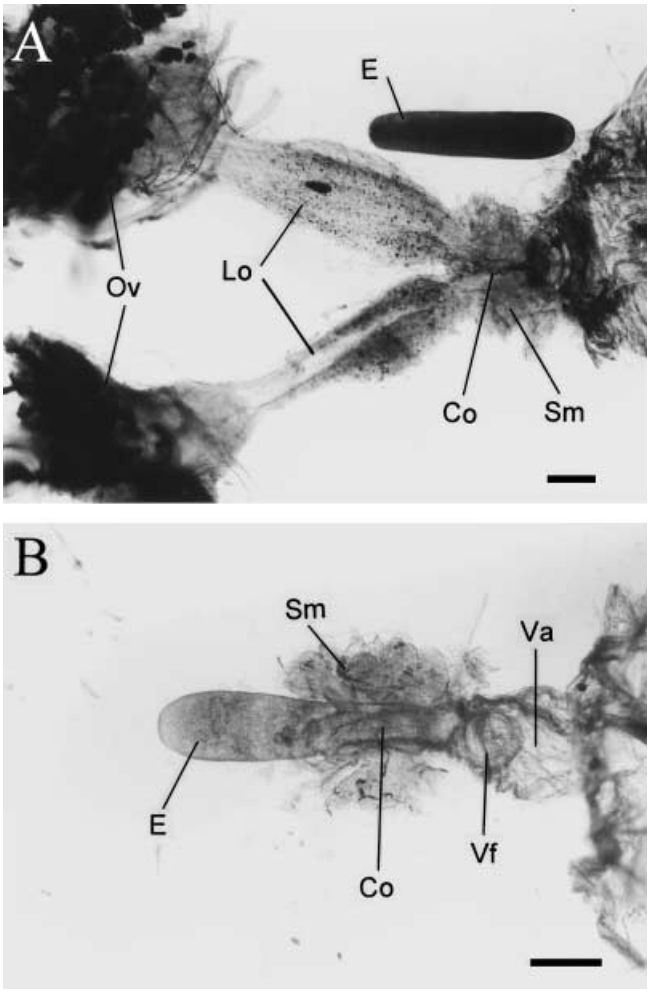

Figure 3. Queen's reproductive tracts transparentized by NaOH. Inner structures of cuticle in the tracts can be observed (A). The common oviduct with an egg distorted (B). The spermatheca located at dorsal reproductive tracts was removed. Lettering as in the legend to Fig. 2. Scale bars in (A) and (B): 0.4 mm

Discussion

Activation of the unfertilized egg is the first physiological step to parthenogenesis in Hymenoptera. Our study provides evidence suggesting that mechanical stress in the common oviduct is responsible for the initiation of development of eggs in honeybees.

The observation that experimental distortion of the eggs caused meiosis suggests that the distortion removes the meiotic block at the metaphase of the first meiotic division. It has been reported in several species of Hymenoptera that egg development is triggered by mechanical stresses or pricking (King and Rafai, 1973; Went and Krause, 1973; Vinson and Jang, 1987; Sawa and Oishi, 1989a). This is also true in other invertebrates (Went, 1982) and vertebrates (Uehara and Yanagimachi, 1977). The mechanism shared by these treatments seems to be that they cause damage or stimulate the membrane of the oocyte. This may be the case for Hymenopteran eggs which are developmentally activated by different osmotic pressure and cold shock (Vinson and Jang, 1987; Sawa and Oishi, 1989a). Sperm invasion into the egg might also cause a similar effect on the membrane of the oocyte. One possible mechanism underlying the phenomena may be that the injury of the membrane in oocytes causes changes in membrane potential. It is suggested that the change of membrane potential is caused by intracellular ion concentration changes which in turn trigger egg development (Steinhardt et al., 1974; Nuccitelli, 1988).

The matured unfertilized eggs are stored in the lateral oviducts and they remain arrested at the metaphase of the first meiotic division until they receive the stimulus for activating the egg development (Nachtsheim, 1913; Counce and Waddington, 1973; Yu and Omholt, 1999). Our results shows that the micropyle of an egg is located on the anterior end of an egg which moves down with the posterior end head and cannot be exposed to the semen in the lateral and common oviducts. Thus, the lateral oviducts clearly have no function in causing mechanical distortion in eggs and consequently are not responsible for initiating the development. The functions of lateral oviducts may be the storage of matured unfertilized eggs and the transport of eggs to the common oviduct, but we do not know the mechanisms how one single egg can be selected from the pool of eggs by the action of the lateral oviducts to be transported into the common oviduct. The light microscopic observation of the common oviduct showed that the common oviduct is surrounded by well-developed semi-circular muscles with smaller inner diameter than that of eggs. The semi-circular muscles surrounding the common oviduct and their nerve innervation have been previously described by several authors (Laidlaw, 1944; Ruttner, 1956, 1961; Camargo and Mello, 1970). The semi-circular muscles are thought to have multi-functions for the egg transport (Laidlaw, 1944) and for holding an egg with the valve-fold into the right position at the orifice of the spermathecal duct to ensure the egg fertilization (Camargo and Mello, 1970). We suggest a new hypothesis that the semi-circular muscles have another function for mechanical egg distortions to activate the egg development when the egg passes through the common oviduct. The function for mechanical egg activation in the common oviduct is partly demonstrated by the present experiments of the in vitro artificial egg activation and the observations of the reproductive system of queens. There have been several reports about the functions of valve-fold in the vagina (Bresslau, 1905; Ruttner, 1956; Fyg, 1966; Camargo and Mello, 1970). The functions of the valve-fold are thought to support the migration of spermatozoa into the spermatheca after copulation (Ruttner, 1956) and to lodge the spermatozoa to the micropyle of egg by the valve-fold indentations and prevent the dispersion of spermatozoa in the vagina during egg fertilization (Camargo and Mello, 1970). Our results revealing the anterior micropyle of eggs indicate that the valve-fold would be vent posteriorly when the egg passes though the vagina and could not serve to lodge the spermatozoa to the micropyle by its indentations. The valve-fold may have a function for lifting up the anterior end of eggs during sperm release so that the micropyle can fit the opening of spermathecal duct.

In the case of diploid egg oviposition, it is supposed that the post-activated eggs can additionally accept sperm invasion in the vagina. This speculation was supported by the results of in vitro egg fertilization. The post-activated eggs could be fertilized if they encountered semen immediately (30 sec) after egg distortions. However, this was not the case if they encountered semen much later (30 min). It is observed that honeybee queens oviposit a fertilized egg within an average of 10–28 sec (Fyg, 1943; Koeniger, 1970; Dietz, 1969; Michener, 1974; Yu and Omholt, 1999). This indicates that the egg is exposed to semen in the vagina within 10–28 sec after egg distortion in the common oviduct. Thus the mechanisms of egg activation by mechanical stress in the common oviduct do not interfere with the fertilization by sperm. A similar temporal relation between egg activation and egg fertilization has been reported in the sawfly *Athalia rosae* (Sawa and Oishi, 1989b; Oishi et al., 1993). In this species, in vitro fertilization is possible when sperm are injected into the egg 20 min post-activation. More than 20% of the eggs develop as fertilized diploid females. No fertilized eggs were observed, on the other hand, when sperm were injected into the egg 60 min post-activation. This property of eggs in primitive species of Hymenoptera might be inherited in their descendants as honeybees.

There have been several studies on the mechanisms of egg activation in Hymenoptera. In the sawfly, which is one of the primitive species in Hymenoptera, the eggs are potentially activated for development by several modes of stimulation (Sawa and Oishi, 1989a). In other species of Hymenoptera, the eggs might also have a similar potential property for egg development. No experimental studies to test the possibility, however, have been done. In the ichneumoid wasp *Nemeritis canescens* (Salt, 1965), *Pimpla turionellae* (Went and Krause, 1974), the branconid wasps *Habrobracon* (von Borstel, 1960) and *Dinotomus mactator* (Sasaki, unpubl.), the eggs collected at the base of the ovipositor did not develop, suggesting that the reproductive tract other than the ovipositor was not sufficient for egg activation. In honeybees, we cannot exclude the possibility that the development of egg is activated by other unknown stimuli in the queen's reproductive tract. Honeybee eggs, however, are unlikely to be exposed to fluid with different osmotic pressure in the queen's reproductive tract, because secretion into the reproductive tract comes only from spermathecal glands for releasing semen and should not occur in unfertilized egg oviposition (Bresslau, 1905; Flanders, 1950; Gerber and Klostermeyer, 1970). Furthermore, the vaginal fluid would be cleared out by an egg itself in each egg laying (Sasaki and Obara, 1999). It seems, therefore, that mechanical distortion plays a critical role in initiating egg development in honeybees. In several species of stingless bees, the workers lay trophic eggs which cannot initiate the development and are consequently consumed by the queen (Sakagami et al., 1977; Engels and Imperatriz-Fonseca, 1990). Since the trophic eggs are discharged from immature reproductive tracts of workers, the phenomena that the trophic eggs cannot initiate the development might be responsible for the insufficient stimuli for egg activation in their reproductive tracts. However, this possibility cannot explain the trophic eggs showing various signs of deficiency. It has been reported that the trophic eggs lack a nucleus, the reticulate chorion pattern and the micropyle (Akahira et al., 1970; Koedam et al., 1996). Thus, the phenomena of the trophic eggs without initiating their development may be involved in the irregular egg formations.

In Aculeata, the divergence from Parasitica has been accompanied by a change in ovipositor function from that of ovipositing to that of stinging. The role of relevant mechanical stimulation to eggs is supposed to be taken over by the common oviduct in Aculeata from the ovipositor in Parasitica, in parallel with a change in the ovipositor role. It is unlikely that the conversional evolution of ovipositor function occurred before acquisition of the common oviduct's function for egg distortion, and so there are three possibilities for the evolution of egg activation mechanisms in Aculeata. The first is that the common oviduct acquired a function of egg distortion in the course of conversion of the ovipositor into the sting. The second is that both the common oviduct and ovipositor have the function to cause mechanical stress in the primitive species of Aculeata. The third is that another unknown stimulus contributes to egg activation before completion of the function of egg distortion in the common oviduct in the primitive species of Aculeata. To determine which of these possibilities is the case, the structure of the reproductive tract in primitive species of Aculeata needs to be studied.

Acknowledgement

We thank two anonymous referees for giving useful information about the queen's reproductive system in honeybees and critical comments of the manuscript. This work was supported by a Grant in Aid for JSPS Fellows. K.S. was supported by a JSPS Research Fellowship.

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