Interspecific Instrumental Insemination Using Mixed Semen of Japanese and European Honeybees (Hymenoptera: Apidae)¹

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The queen honeybee copulates with several drones during her mating flight in the drone congregation area (DCA) which may be several kilometers from the hive (RUTTNER, 1985; KOENIGER, 1986; KOENIGER and KOENIGER, 1991). Interspecific reproductive isolation has evolved in regions where two or more honeybee species are distributed sympatrically. In Sri Lanka, three species, *Apis florea*, *A. cerana*, and *A. dorsata*, take mating flights at different times of the day (KOENIGER and WIJAYAGUNASEKERA, 1976). However, in regions where non-indigenous honeybee species have been artificially introduced, interspecific copulation might occur because of overlapping mating flight times and possible shared sex attractant. Examples have been observed in Germany (RUTTNER and MAUL, 1983). Environmental conditions required for the DCA in the indigenous Japanese honeybee, *A. cerana japonica* (Acj) and the introduced European honeybee, *A. mellifera* (Am) are different (YOSHIDA, 1994). However, some Am drones are sometimes trapped in the Acj DCA. Furthermore, Am drones can actually copulate with Acj queens fixed 8 m above the ground in the Am DCA (YOSHIDA and YAMAZAKI, 1993). In this research, I simulated an interspecific cross between these two species of honeybees using instrumental insemination to investigate what might happen if queens and drones actually mated.

MATERIALS AND METHODS

Am queens were produced from 2 to 27 May 1993 by routine grafting at the apiary of Honeybee

Science Research Center, Tamagawa University. Am and Acj drones trying to fly for mating were collected in traps fixed to the entrance of colonies.

Counting semen and spermatozoa. The semen volumes and number of spermatozoa were examined according to RUTTNER et al. (1973).

Spermatozoa in spermatheca. The number of spermatozoa in the queen spermatheca was counted using the method of WOYKE (1973, 1975) 45 days after the start of egg-laying.

Collecting semen for insemination. Semen was collected into calibrated syringes by squeezing the drone thorax (WOYKE, 1973). The collected volume of semen was 4 mm³ (WOYKE, 1973). To get mixed semen, 1 or 2 mm³ of semen was collected alternately from Am and Acj drones, making 3 syringes of each combination. The mixed semen was used to confirm a blend of spermatozoa in spermatheca, and well-attained insemination.

Instrumental insemination. Three Am queens, 7-to-11 days old were inseminated using the SCHLEY apparatus with dorsal-pierced hook (SCHLEY, 1988). After insemination, wings were cut and the queens were introduced into nucleus hives with a queen excluder at the entrance. One day after insemination, the queens were treated with CO₂ to accelerate oviposition (MACKENSEN, 1947). Eggs (200–280) were marked randomly at the 3rd or 4th day after insemination, and the cells were examined again after 4 and 15 days. Adults emerging from these cells were also observed in an incubator.

RESULTS AND DISCUSSION

The volume of semen per Am drone was calculated as 0.51 mm³, which is similar to the result of HARBO (1985). Acj drones have 0.14 mm³ of semen slightly less than *A. cerana indica*, which has 0.16 mm³ (WOYKE, 1973), or 0.2 mm³ (RUTTNER et al., 1973) (Table 1). The number of spermatozoa per 1 mm³ was 3.74 million for Am and 2.56 million for Acj, or an approximate ratio of 3:2 (Table 1). These results agree with those of RUTTNER et al. (1973). For artificial insemination, about two Am drones and 8 to 9 Acj drones were required to collect 1 mm³ of semen.

The average hatchability (normal cells in Table 2) for naturally-mated Am queens was 91.6%, but was 84.5% for artificially-inseminated queens. When Am queens were inseminated with 4 mm³ of

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Table 1. Amount of semen and spermatozoa of *A. mellifera* and *A. cerana japonica* drones

	<i>A. mellifera</i>			<i>A. cerana japonica</i>		
	No. drones	Range	Mean±S.D.	No. drones	Range	Mean±S.D.
Volume of semen collected per drone (mm ³) ^a	123	0.50–0.57	0.51±0.04	320	0.13–0.18	0.14±0.01
Number of spermatozoa (10 ⁶ /mm ³)	25	2.2–6.4	3.74±1.48	30	1.9–2.9	2.56±0.34

^a Only those drones from which semen was successfully collected in the syringe are included.

Acj semen, 99.2% of the 720 cells laid were abnormal: 676 (93.9%) cells remained with unhatched eggs (or eggs were removed then replaced with new eggs), and 38 (5.3%) were either empty or contained only bee milk (Table 2). Only 6 eggs developed into larvae (0.8% hatchability) but it was not determined whether they were workers or drones because the larvae died immediately after hatching. The queens laid eggs in 32 drone cells in worker bee comb. Larvae were found in 18 cells, and all emerged as drones. The egg-laying behavior of queens seemed normal. Normal development was not observed in previously-reported interspecific instrumental insemination (RUTTNER and MAUL, 1983). Once the semen is in the spermatheca it is thoroughly mixed (LAIDLAW and PAGE, 1984; MORITZ, 1986). The hatchability in insemination with 3 mm³ of Am semen and 1 mm³ of Acj semen (approximate spermatozoa concentration ratio of 9:2), 2 mm³ of Am semen and 2 mm³ of Acj semen (6:4), and 1 mm³ Am and 3 mm³ Acj (3:6) was 79.5%, 53.6%, and 26.5%, respectively, and produced only Am workers (Table 2). Interspecific fertilization resulted in non-viable larvae. RUTTNER and MAUL (1983) reported embryonic development was blocked during the later stages for interspecific insemination between *A. cerana cerana* and Am. In the present study, I confirmed the earlier embryonic development and the following blockage in the artificially inseminated egg between Acj (different subsp.) and Am through microscopic observations. The results show that Am workers are produced in direct relationship to the ratio of Am spermatozoa in the mixed semen. The number of spermatozoa in the

spermatheca of each queen was 1.39 to 1.85 million; all spermatozoa seemed to be alive and their activity in the spermatheca was normal similar to the results of WOYKE (1973) and RUTTNER and MAUL (1983). If interspecific copulation occurred, interspecific-fertilized eggs do not hatch, thereby having a deleterious effect on normal colonies.

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REFERENCES

- HARBO, J. R. (1985) *Am. Bee J.* **125**: 282–287.
- KOENIGER, G. (1986) *Bee Genetics and Breeding* (T. E. RINDERER, ed.). Academic Press, Orlando, pp. 255–280.
- KOENIGER, N. and H. N. P. WIJAYAGUNASEKERA (1976) *J. Apic. Res.* **15**: 67–71.
- KOENIGER, N. and G. KOENIGER (1991) *Apidologie* **22**: 581–590.
- LAIDLAW, H. H. and R. E. PAGE (1984) *Genetics* **108**: 985–997.
- MACKENSEN, O. (1947) *J. Econ. Entomol.* **40**: 344–349.
- MORITZ, R. F. A. (1986) *Experientia* **42**: 445–448.
- RUTTNER, F. (1985) *Experimental Behavioral Ecology and Sociobiology* (B. HÖLLDOBLER and M. LINDAUER, eds.). Gustav Fischer Verlag, Stuttgart, pp. 225–236.
- RUTTNER, F. and V. MAUL (1983) *Apidologie* **14**: 309–327.
- RUTTNER, F., J. WOYKE and N. KOENIGER (1973) *J. Apic. Res.* **12**: 21–34.

Table 2. Fate of eggs laid by *A. mellifera* queen instrumentally inseminated with *A. mellifera* and *A. cerana japonica* semen

Composition of injected semen (mm ³)		Fate of eggs ^a				No. cells observed	Abnormal cells with		Normal cells with larvae (%)	No. pupae ^b (%)	Spermatozoa number in spermatheca ^c (10 ⁶ /mm ³)
<i>A. mellifera</i>	<i>A. cerana japonica</i>	Eggs	Empty	Bee milk only	Total (%)						
4	0	39	0	0	39 (19.4)	201	0	0	162 (80.6)	146 (90.1)	1.75
4	0	28	0	0	28 (12.7)	220	0	0	192 (87.3)	177 (92.2)	1.45
4	0	33	0	0	33 (14.5)	228	0	0	195 (85.5)	174 (89.2)	1.71
	Average (Av.)	33.3	0	0	33.3(15.5)	216.3	0	0	183.0(84.5)	165.7(90.5)	1.64
3	1	66	2	2	70 (26.9)	260	2	2	190 (73.1)	159 (83.7)	1.85
3	1	35	14	0	49 (17.4)	281	14	0	232 (82.6)	209 (90.1)	1.44
3	1	39	2	0	41 (17.3)	237	2	0	196 (82.7)	170 (86.7)	1.80
	Av.	46.7	6.0	0.6	53.3(20.5)	259.3	6.0	0.6	206.0(79.5)	179.3(86.8)	1.70
2	2	100	3	3	106 (45.3)	234	3	3	128 (54.7)	118 (92.2)	1.49
2	2	81	4	1	86 (40.8)	211	4	1	125 (59.2)	110 (88.0)	1.39
2	2	119	10	0	129 (53.1)	243	10	0	114 (46.9)	85 (74.6)	1.59
	Av.	100.0	5.7	1.3	107.0(46.4)	229.3	5.7	1.3	122.3(53.6)	104.3(84.9)	1.49
1	3	144	8	0	152 (66.1)	230	8	0	78 (33.9)	65 (83.3)	1.59
1	3	166	6	3	175 (85.8)	204	6	3	29 (14.2)	26 (89.7)	1.40
1	3	164	9	7	180 (68.7)	262	9	7	82 (31.3)	79 (96.3)	1.56
	Av.	158.0	7.7	3.3	169.0(73.5)	232.0	7.7	3.3	63.0(26.5)	56.7(89.8)	1.52
0	4	211	19	7	237 (98.3)	241	19	7	4 (1.7)	0 (0)	1.36
0	4	220	3	1	224 (100)	224	3	1	0 (0)	0 (0)	1.39
0	4	245	5	3	253 (99.2)	255	5	3	2 (0.8)	0 (0)	1.68
	Av.	225.3	9.0	3.7	238.0(99.2)	240.0	9.0	3.7	2.0(0.8)	0 (0)	1.48
Naturally mated		19	0	0	19 (8.2)	233	0	0	214 (91.8)	196 (91.5)	4.88
		23	0	0	23 (9.6)	240	0	0	217 (90.4)	202 (93.1)	5.98
		15	0	0	15 (7.4)	202	0	0	187 (92.6)	167 (89.3)	2.77
	Av.	19.0	0	0	19.0(8.4)	225.0	0	0	206.0(91.6)	188.3(91.3)	4.54

^a Cell contents investigated on 4th day after eggs laid during first 4 days after insemination.^b Observations made on day 15 (%) is the number of normal cells. All pupae were female.^c Examined after end of experiment.

- SCHLEY, P. (1988) *Am. Bee J.* **128**: 282-284.
WOYKE, J. (1973) *J. Apic. Res.* **12**: 151-158.
WOYKE, J. (1975) *J. Apic. Res.* **14**: 153-159.
YOSHIDA, T. (1994) *Jpn. J. Appl. Entomol. Zool.* **38**: 85-90. (in Japanese with English summary).
YOSHIDA, T. and M. YAMAZAKI (1993) *Asian Apiculture* (L. J. CONNER, T. RINDERER, H. A. SYLVESTER and S. WONGSIRI, eds.). Wicwas Press, Connecticut, pp. 99-103.
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