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Diapause and Non-Delayed Eusociality in a Univoltine
and Basically Solitary Bee, *Ceratina japonica*
(Hymenoptera, Anthophoridae)

I. Diapause Termination by Cooling and Application of
Juvenile Hormone Analog

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Abstract *Ceratina japonica* is a univoltine and basically solitary bee, but it can occasionally form semisocial or delayed eusocial colonies. For this species the artificial termination of reproductive diapause within the active season seems necessary to induce the non-delayed eusociality, the life mode common to all temperate social insects. In this study the possibility of diapause termination was tested by two standard methods, cooling pre-hibernating adult females (also for an allied species, *C. flavipes*) and topical application of juvenile hormone analog (JHA) to prepupae and pupae. The rate of termination rises over 50% by cooling at 5–8°C for 3–4 months. However, this method is too time-consuming to match with the univoltine life cycle of *C. japonica* and JHA application is the only known possibility to induce non-delayed eusociality in this species, in spite of its low rate of diapause termination, only 35% by our procedure.

Key words: *Ceratina* bees, non-delayed eusociality, diapause termination, cooling, juvenile hormone analog.

Introduction

Ceratina (*Ceratinidia*) *japonica* COCKERELL is a Japanese xylocopine bee distributed from Hokkaido to Kyushu and univoltine everywhere. It is basically a solitary species, but we were able to induce multiple female (mostly 2 ♀♀) colonies with a rudimentary caste system, and found also some such colonies in natural populations (SAKAGAMI & MAETA, 1985). These colonies are mostly formed among females that overwintered in the natal nests and are usually semisocial (associations among

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sisters), but some are delayed eusocial (associations between the mother and her old, post-hibernating daughters). No colonies were non-delayed eusocial (associations between the mother and her pre-hibernating daughters that emerged in early summer), which is the life mode common to most, if not all, temperate social insects. This is understandable because the univoltine life cycle of *C. japonica* does not permit the insertion of a non-delayed eusocial period.

However, the foundress of this species can start oviposition again, when deprived of the early brood immatures. We were able to produce 5 stepmother/stepdaughter colonies and 5 colonies of mothers and their own daughters. The growth of female immatures was accelerated by warming at 32°C and returning them into the natal nests when the mothers started second oviposition periods. The experiments for induction of non-delayed eusociality by this procedure were futile, because the daughters did not forage for larval food as do the daughter workers of all real eusocial bees. Mothers performed again both foraging and laying activities. Daughters, instead, mechanically disturbed the mothers' work by their presence in nests (SAKAGAMI & MAETA, 1989).

This failure was possibly because the daughters were under the reproductive diapause until the next spring. To test this inference we repeated the same experiments with a consubgener, *C. (C.) okinawana* MATSUMURA et UCHIDA. This species is also basically solitary but produces occasional multifemale colonies, and the mothers can rear a second brood, often even in the presence of the juvenile adults of the first brood. Furthermore, this species is multivoltine (OKAZAKI, 1987, up to 3 generations per year) and females of the first and second generations can start brood rearing within the active season, *i. e.*, without intervention of reproductive diapause. Combining the mother and her daughters, we obtained 5 successful non-delayed eusocial colonies (later 6 additional ones), and also confirmed the compatibility of solitary nests and non-delayed eusocial colonies in a natural population, for the first time in the bees (SAKAGAMI & MAETA, 1989).

Encouraged by these results, which favor our inference mentioned above, we attempted to induce non-delayed eusocial colonies in *C. japonica* by artificial termination of diapause. This first report describes the results of diapause termination by the two standard methods, cooling and JHA application. Prior to this work we had also tried to terminate the diapause in *C. flavipes* SMITH, a consubgener with similar life cycle (SAKAGAMI & MAETA, 1977) but almost completely solitary (SAKAGAMI & MAETA, 1987). These results are incorporated in this paper.

Methods

All Japanese bees for which diapause had so far been artificially terminated overwintered as prepupae or adults in the natal cells (Andrenidae, Megachilidae and Anthophorinae), unlike *Ceratina* which overwintered as adults that at least once had departed from nests for self-feeding. Their diapause was terminated after about 3



Fig. 1. Rearing apparatus of *Ceratina* bees.

months cooling (MAETA, unpub.). This experience became a guide for our work. Below, the cooling means the maintenance of diapausing adult females at temperatures lower than the minimum temperature necessary to start the flight activity by the 2 *Ceratina* species ($\pm 17^{\circ}\text{C}$).

1. *Diapause termination in C. flavipes by cooling under constant temperatures*

In both species only adults who took sufficient food can resist cooling. Hence, the treatment must be made from late October onward, which corresponds to the pre-winter dispersal period (P_5) in the life cycle (SAKAGAMI & MAETA, 1985).

Experiments were made in 1986 and 1987 with bees collected on Mt. Sanbe near Matsue. Cooling was started on 14 November, 1986 and 23–26 October, 1987. Juvenile post-feeding adults were exposed to $-1, 2, 5, 8, 11, 14$ and 17°C during 1–4 months in the darkness, then warmed at 24°C for 30 days under either long day (16L: 8D) or short day (8L: 16D) regimes. At cooling about 10 females were put in old nest tubes excavated in pithy stems of *Rubus palmatus* var. *coptophyllus*. The entrances were closed with cotton plugs. At warming these bees were transferred to a petri-dish (9 cm, height 2 cm) provided with a corrugated cardboard as shelter, and a glass tube filled with diluted honey ($\pm 35\%$) and some pollen loads of honeybees as food (Fig. 1). About 20 adult females (10–30) were used for each trial. After the above cooling/warming treatments, bees were dissected and the ovaries were fixed with CARNOY's fluid and stained with carbol-thionine (GÔUKON *et al.*, 1987).

In this and the next 2 experiments, the termination of diapause was determined

by examining the stages of the oldest (=basalmost) oocyte in each of 8 ovarioles (KURIHARA *et al.*, 1980). Bees which have only oocytes of previtellogenic stage (=I) or those degenerated from I (=I') were considered to be still in the diapausing state. Bees with vitellogenic or mature oocytes (=II, III) or those degenerated from II and III (=II', III') were regarded as in the diapause terminated state.

2. *Diapause termination in C. japonica by cooling with daily warming*

The seasonal sequence of the life cycle in *C. japonica* is similar to that of *C. flavipes* (SAKAGAMI & MAETA, 1977). To realize earlier termination of diapause for our purpose, the formation of non-delayed eusociality, we inserted warming hours daily through the cooling period.

In 1989, the bees collected in Sendai, northern Japan, where this species is commoner than in southern Japan, were reared in Matsue since the previous year. On 11 May and 3–4 June, 1990, immatures were removed from nests and reared at 28°C in order to accelerate their development. The females which emerged were fed with diluted honey and pollen under the 28°C and 16L: 8D regime for 5 days. Then, they were cooled for 2, 3 or 4 months under the 10°C and 16L (0600–2200): 8D (2200–0600) regime, but warming at 28°C for 4 hours (0900–1300) was inserted during the long-day period. The bees were allowed to take food freely at 28°C. Finally, the bees were reared for 10 days at 28°C before examining the ovarian states.

3. *Diapause termination in C. japonica by JHA application*

Prepupae and young pupae before attaining fully-pigmented eyes were taken on 7–30 May, 1989 from the nests kept in a greenhouse. The juvenile hormone analog (SUMITOMO Chemical Co. Ltd., S-31183) was diluted with acetone and smeared topically on the upper surfaces of head and thorax of the prepupae and pupae. The doses tested are shown in Fig. 2. For each dose 5–20 individuals ($\bar{x}=12$) were used and for each individual 1 μ l was smeared with a microcap glass tube. The treated individuals were put in a small vial (inner diameter 7 mm, height 3.5 mm) placed in a plastic case, and kept at 28°C, 75% R. H. (adjusted by super-saturated NaCl solution) and 16L: 8D photoperiod. Adults which emerged were reared as were post-cooling bees, fed with diluted honey and pollen for 14 days, until the examination of ovarian states.

Results

Table 1 shows the results of cooling experiments on *C. flavipes*. The photoperiodic effect on the diapause termination is unknown, because the cooling in all trials was under constant darkness. However, it is certain that the thermal conditions played a major role in diapause termination. The post-diapause adults with mature basal oocytes were obtained only by coolings at temperatures below 17°C. The sudden decrease of the termination rate at -1°C suggests that the

Table 1. Rate (%) of diapause termination in *Ceratina flavipes* by cooling at different temperatures.

Cooling temperature (°C)	Year	Post-cooling photoperiod regime	Rate of diapause termination (mortality during and after cooling) by cooling for different months (%)			
			1	2	3	4
24 (no cooling)	1986	16L: 8D	0 (0, 0)			
	1986	8L: 16D	0 (0, 0)			
17	1987	16L: 8D	0 (0, 0)	38.9 (5.0, 5.3)	60.0 (70.0, 16.7)	— (100, —)
14	1987	16L: 8D	0 (5.0, 0)	36.8 (0, 5.0)	38.9 (10.0, 0)	37.5 (55.0, 11.1)
11	1986	16L: 8D	0 (0, 0)	80.0 (0, 33.3)	96.4 (9.4, 3.5)	100 (22.6, 0)
	1986	8L: 16D	33.3 (0, 0)	86.7 (0, 0)	100 (4.8, 5.0)	100 (26.1, 5.9)
	1987	16L: 8D	0 (0, 0)	10.0 (0, 0)	94.1 (15.0, 0)	
8	1986	16L: 8D	5.3 (0, 0)	100 (0, 0)	100 (3.4, 0)	78.9 (13.8, 8.0)
	1986	8L: 16D	10.0 (0, 0)	100 (0, 0)	100 (4.3, 0)	100 (13.0, 0)
	1987	16L: 8D	0 (0, 0)	15.0 (0, 0)	63.2 (0, 5.0)	
5	1986	16L: 8D	10.0 (0, 0)	100 (3.3, 0)	100 (9.4, 0)	95.0 (18.8, 0)
	1986	8L: 16D	20.0 (0, 0)	100 (0, 6.7)	89.5 (5.0, 0)	100 (19.0, 0)
	1987	16L: 8D	0 (0, 0)	21.1 (0, 5.0)	72.2 (0, 10.0)	
2	1986	16L: 8D	16.7 (3.6, 0)	85.7 (3.3, 0)	93.3 (9.1, 0)	100 (13.3, 3.9)
	1986	8L: 16D	25.0 (0, 0)	78.6 (0, 6.7)	85.0 (9.1, 0)	100 (13.0, 0)
-1	1987	16L: 8D	0 (0, 10.0)	5.6 (5.0, 5.3)	6.3 (10.0, 11.1)	— (100, —)

lower limit of the effective thermal range would lie around about 0°C. Higher termination rates were obtained by coolings between 2 and 11°C, suggesting this thermal range to be needed for termination of diapause.

In the same year, the termination rate by shorter cooling periods tended to be higher at 5 and 8°C than at the other temperatures. This tendency was pronounced in 1986. Full termination was obtained by the cooling for only 2 months. Probably the thermal optimum for diapause termination may lie between 5 and 10°C. There was, however, a year-to-year difference in the sequence of the termination process; full (100%) termination was delayed more in 1987 than in 1986 under the same cooling temperatures (see 5, 8 and 11°C, Table 1). Probably, the delay of cooling until mid November in 1986 is responsible for this difference. The juvenile bees used in 1986 might have experienced low temperatures for a longer time before being put in cooling cabinets than those in 1987. Meteorological records showed, in fact, that the cumulative time until the start of the experiment, during which the field temperature went below 11°C, was 370 hours (15.4 days) in 1986, in contrast to 52 hours (2.2 days) in 1987. This longer exposure to the low temperature before the cooling experiment must have accelerated diapause termination in 1986.

The result in 1986 may permit an estimate of the period required for diapause termination under favorable cooling temperatures as 2.5 months (15.4 days in the field and 2 months in the cooling cabinet). In 1987, however, 3 months of cooling at the effective temperatures was insufficient for full termination of diapause. This discrepancy suggests the presence of unknown factors. In any event, it is difficult to terminate the reproductive diapause in a period shorter than 3 months by thermal control alone.

The seasonal sequence and life cycle are quite similar in *C. flavipes* and *C. japonica*. Therefore, the diapause of *C. japonica* may be terminated with the same procedure as in *C. flavipes*. Table 2 shows the results obtained with *C. japonica* for the ovarian states. The rate of diapause termination was about 50% for 3 months and about 100% for 4 months at low temperatures. As about 20 cumulative days (4 hrs × 4 months) within 4 months were spent for warming at 28°C, the actual duration of cooling for termination in *C. japonica* is estimated at about 3 months

Table 2. Mean number of oocytes of various developmental stages in *Ceratina japonica* after cooling (10°C, 20 hrs) with temporary warming (28°C, 4 hrs) for different months.

Duration of cooling (months)	Percent ratio of diapause termination	Mortality	Mean number of oocytes of stages I-III'*							Number of bees examined
			I	I'	II	II'	III	III'		
2	0	0	8.00						11	
3	45.5	0	6.68	0.14	0.73	0.36	0.05	0.05	22	
4	96.2	27.8	5.77	0.04	1.15	0.65	0.19	0.19	36	

* Explained in text.

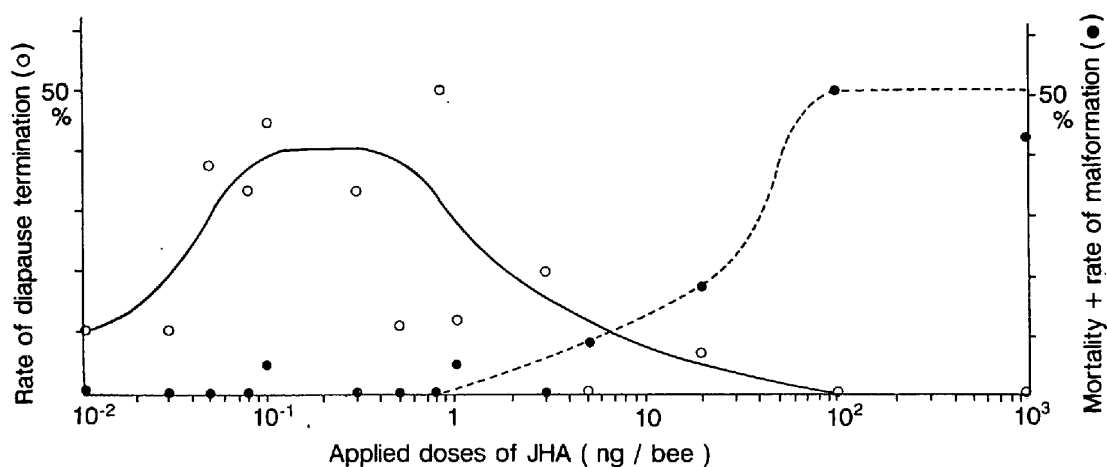


Fig. 2. Percent ratio of diapause termination, mortality and percent ratio of malformation by topical application of JHA at various doses to the prepupae and pupae of *Ceratina japonica*. Lines are drawn not statistically by combining the results for prepupae and pupae.

by subtracting the 20 days.

By artificial warming of juvenile adults and allowance of food intake during the warm spell in each cooling day, the time necessary for diapause termination (4 months) was shortened to about 3 months, yet post-diapause juveniles can be obtained only in October. Thus, this procedure was also inappropriate for our purpose, because the time limit at which mothers can lay eggs is the end of August.

Figure 2 shows the combined results of topical application of JHA to the prepupae and pupae of *C. japonica* at different JHA doses. The rate of diapause termination was only 11–50% ($34.9 \pm 12.2\%$) even at the most effective dose range (0.05–0.8 ng/bee). The termination rate seemed slightly higher for pupae than prepupae but without significant differences. The ratio of ovipositing females after JHA application was 37.5% (3 out of 8 bees) in 1989 and 15% (3/20) in 1990. Using this method, JHA treated juvenile females are available since early July, *i. e.*, sufficiently before the cessation of ovipositions by mothers at the end of August to induce mother-daughter colonies of adults artificially.

The mortality and the rate of malformation (shrunk-antennae, -legs, and -wings) increased distinctly at higher JHA doses. In 1990 JHA was smeared twice on each female at the prepupal and pupal stages, but the termination rate was lower than that of 1989. It is possible that the low termination rate may be resulted from the developmental stages used rather than the JHA doses and the number of topical smearings.

Discussion

Most true temperate bees (*i. e.*, excluding honeybees and subtropical allodapine bees) overwinter in one of the 3 stages: A – prepupae, B – adults without leaving natal brood cells, and C – adults after pre-hibernating extranidal self-feeding (see HOSHIKAWA *et al.*, 1992). Overwintering as eggs and feeding larvae is impossible in both brood rearing and cleptoparasitic bees. It is noteworthy that pupal overwintering is very rare in Apoidea. The only reported case is *Chilicola albida* (Colletidae) (STEPHEN *et al.*, 1969). Most social or socially disposed temperate bees (halictine bees, *Ceratina*, bumblebees) adopt type C, although *Rhopalomesoma esakii*, a nomiid bee with a low level sociality, belongs to type A (MAETA & SAKAGAMI, unpub.).

Detailed studies on the diapause of bees have not been made, although something is known for *Megachile rotundata* (type A, BITNER, 1976; MAETA, 1983), *Osmia cornifrons*, *O. taurus*, *O. pedicornis*, *O. imaii*, *O. coerulea*, *Chalicodoma sculpturalis*, *C. spissula*, *M. pseudomonticola* (all type B, MAETA, 1978; MAETA, unpub.), *Anthophora pilipes villosula* (type B, MAETA, unpub.) and *Andrena prostomias* (type A, MAETA, unpub.). In all these species, diapause is terminated by cooling for about 3 months. The present study revealed that cooling for the same period was required also in the type C bees: The reproductive diapause of young females is terminated by cooling for 3 months or more under a low temperature range with the optimum around 5 to 8°C.

Our main aim is to induce non-delayed eusociality in *Ceratina japonica*, which is basically solitary but occasionally forms semisocial and delayed eusocial colonies (SAKAGAMI & MAETA, 1989). For this purpose, we must match the mother who lays her second brood (=the prospective queen) and the daughter who forages for larval food (=the prospective worker) appropriately. The difficulty is due to the following facts: The mother's second laying can not be extended after late August when the day length diminishes beyond the critical point for oviposition activity at 13 hours (MAETA, 1993). Besides, the diapause termination of juvenile females requires the effective cooling at least for 3 months even though a warm spell has been given for food intake every day. It is difficult to induce the non-delayed eusociality within such limited duration by control of thermal conditions alone. Consequently, JHA-application is at the present the only available method for our purpose, even though further improvement of the procedure is necessary.

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