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Artificial Induction of Prepupal Diapause in a Partially Bivoltine Bee, *Megachile rotundata* (Fabricius) (Hymenoptera, Megachilidae)

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Abstract *Megachile rotundata* is a partially bivoltine bee, in which genetically univoltine and potentially bivoltine strains are compatible within the same population. The emergence rate of the first generation in those original populations introduced from the USA and Canada was very high every year (36.7−93.7%) in Morioka, northern Japan (N 39°70'), showing that *M. rotundata* was a bivoltine species. This became an obstacle to maintain large bee populations needed for alfalfa pollination. To solve the problem, artificial induction of prepupal diapause and selection for a univoltine strain were tried. The results were as follows. 1) No prepupal diapause was induced by rearing only the stages of egg, larva and prepupa under low temperatures (a little higher than the developmental threshold temperatures were used, 20℃ for eggs and 18℃ for other stages) and subsequent later stages (prepupae and pupae) under 30℃. 2) High rate of prepupal diapause (up to 92.0%) was induced by rearing the larvae and subsequent later stages at 18℃. 3) It was also proven in rearing the 5th instar larvae which had commenced defecation and subsequent later stages at 18℃ (91.8%). 4) High rate of induction of prepupal diapause (69.2−100%) also occurred by rearing the larvae and subsequent later stages at the combination of 2 different temperatures (22℃/14℃ and 26℃/10℃), with a mean became 18℃. Thus, the critical time perceived by bees seemed to be larval stage (also perceivable at the 5th instar larvae even after they commenced defecation). Prepupal diapause can be induced by rearing them and subsequent later stages at 18℃. Day length did not affect the induction of prepupal diapause. In the bivoltine strain, those prepupae in which diapause was naturally and artificially induced, it was not necessary to break their diapause by subjecting them to low temperatures. In contrast, the Spanish univoltine strain needs to be subjected to 8−20℃ for 2 months to break diapause. Selection of a univoltine strain was made possible by rearing the first introduced parental population consecutively for more than 4 years.

Keywords: Bees, management for alfalfa pollination, bi-voltinism, immature development, induction and breaking of prepupal diapause, *Megachile rotundata*

Introduction

The univoltine bee group, Megachilidae, includes some partially bivoltine species, *e.g., Osmia coerulescens* (Linnaeus), *Megachile ainu* Hirashima et Maeta, *M. kobensis* Cockerell, *M. rotundata* (Fabricius), *M. subalbuta* Yasumatsu and *M. willughbiella sumizome* Hirashima et Maeta (Tasei, 1972; Westrich, 1989; Maeta, 2016; Maeta and Miyanaga, 1997; Maeta and Minagi, 1999; Parker *et al*., 1976; Present study; Maeta, 1999; Maeta *et al*., 1996).

Megachile rotundata was first introduced into Japan from the United States in 1971 (Maeta *et al*., 1973), and then the introduction has been continued to use bees for breeding and seed production of alfalfa in Japan (Maeta and Kitamura, 2005; Maeta, 2016). The emergence rate of the first generation was remarkably high every year in Japan. The first generation (G_1) means that is offspring of the overwintered generation (G_0), but those prepupae entered obligatory diapause hibernate as the overwintered generation. The high rate of emergence of the first generation became an obstacle to maintain bee populations, because the nesting activity of the first generation was inhibited by decreasing temperatures and the shortage of flowers when the first generation emerged in late summer to early autumn. Moreover, some or majority of pupae could not develop into adults, depending on years. To solve this problem, the selection of a univoltine strain emerging in summer only, and the mechanisms to induce prepupal diapause in *M. rotundata* were studied.

Up to now, the cues and mechanism(s) that induce diapause or bivoltinism are unknown, as in other above-mentioned partially bivoltine species. Suspected factors that elicit summer emergence include maternal and/or larval responses to long photoperiods, excessive heat, and poor larval nutrition, as well as maternal inheritance, were reported by many authors (Theresa *et al*., 2011).

Material and Methods

The study was conducted at the campus of Tohoku National Agricultural Experiment Station (TNAES) (current name: Tohoku Agricultural Research Center), Morioka, Iwate Pref. (N 39° 70') for 7 years (1975－1981) and Hokkaido National Agricultural Experiment Station (HNAES) (Hokkaido Agricultural Research Center), Sapporo, Hokkaido (N 43° 04') for 2 years (1971 and 1972). Cocoons of *M. rotundata* were introduced from Alberta, Canada (in 1975, 1978, 1979 and 1980), Oregon, USA (in 1977, 1979 , 1980 and 1981), Idaho, USA (in 1972 and 1976) and Utah, USA (in 1971). Years in parentheses show that bees were introduced and reared. Canadian bees originated from the populations introduced from the Pacific Northwest region of the United States in 1962 (Hobbs and Richards, 1976).

Univoltine strains of *M. rotundata* were introduced 6 times from Spain on May 19, 1980, on January 1, 1981, on February 17, 1982, on February 2, 1983, on February 14, 1986 and on February 10, 1987. Bees were reared in the greenhouse and field at TNAES for 2 years (1980－1981) and at the campus of Shimane University, Matsue, Shimane Pref. (N 35° 29') for 3 years (1986－1988). These foreign bees were introduced with a permit. Only cocoons that contained healthy adults were liberated, using equipment designed to control and exclude chalcid parasites (Johansen *et al*., 1973). When liberating the bees, 5 female adults in cocoons were placed in each new reed tube ("replaced releasing method" also used for *Osmia* bees, Maeta, 1978).

1. Rate of emergence of the first generation in two districts, Sapporo and Morioka

Bees were liberated for 6 years at the campus of TNAES and 2 years at the campus of HNAES. To confirm the emergence of the first generation in the Spanish univoltine strain, populations reared for 4 years at the campus of Shimane University were examined. Emergence rate of the first generation was examined in the winter period, and obtained by A/A + B (A: Adults + Pupae, irrespective their living or not; B: Living prepupae). Calculation of the emergence rate of the first generation was adopted in the following experiments of 2 – 4 and 6.

2. Rate of emergence of the first generation under different constant temperatures

Rearing of immatures in the following experiments was conducted under the following method. Those provisioned cells including immatures, which were obtained by cutting the paper tube nests, were placed vertically on the floor of the petri-dishes by cementing their bottoms with glue, and then these petri-dishes were placed into a container box in which was filled with saturated solution of salt (NaCl) (controllable 73 – 78% relative humidity). Provisioned cells contained only eggs and young larvae (1st and 2nd instars) were subjected as studied stages in 1977, and incubated under 6 constant temperatures (18℃, 20℃, 22℃, 26℃, 30℃ and 34℃). However, in 1978 – 1980 those provisioned cells contained the stages of eggs to 5th instar larvae, and were incubated under 18℃ and 30℃, and all these stages were included as objective materials.

3. Rate of emergence of the first generation in relation to the nesting sequence

To examine the emergence rate in relating to the nesting period, bees produced in Oregon were reared at the campus of the TNAES in 1976. Liberation of bees was conducted on July 23. Dates of the completion were described on the nest tubes when they were completed every times. Nest contents were examined in the winter.

4. Selection of the univoltine strain

Bee cocoons introduced from Idaho in 1976 and Oregon in 1977 were reared at the campus of TNAES. Those dormant cocoons, which had not emerged as the first generation, were kept as the next source populations and consecutively reared them in the field. The Idaho population was reared for 2 years (1976 – 1977) and the Oregon population for 5 years (1977 – 1981). The dormant cocoons were examined every winter.

5. Development of immatures in relation to the temperatures

To compare the development of immatures derived from 3 different localities, USA (Oregon), Canada (Alberta) and Spain (Valladolid) were used. Reed tubes with nests were taken from the field nesting sites. Provisioned cells with eggs were treated following the same method mentioned in 2. Eggs were incubated under 4 constant temperatures (22℃, 26℃, 30℃ and 34℃). Dates on which incubation was commenced were June 13, 1979 for Oregonian eggs, June 15, 1978 for Canadian eggs and on June 17 – 25, 1981 for Spanish eggs. Developmental stages were recorded once a day at a fixed time.

Post development of free-diapaused prepupae of the Spanish univoltine strain was examined, and incubation of these prepupae was commenced on July 21, 1981 under 20℃, 24℃, 28℃ and 32℃.

6. Artificial induction of prepupal diapause

The number of larval instars of *M. rotunda* is five (Trostle and Torchio, 1994), as in other bee species that belong to various taxa (reviewed in Maeta, 2000). Defecation occurred at the 5th instar larval stage (within a few days after becoming this instar) and cocoon spinning commenced as soon as they consumed pollen loaves.

Temperature a little higher than the developmental threshold temperatures (20℃ for egg and 18℃ for larvae and subsequent prepupae) seemed to function to induce prepupal diapause, then those immatures were reared under both temperatures. The following 12 temperature regimes (I－XII) were planned. Provisioned cells including eggs were treated following the same method as mentioned in 2. For regime I, young eggs were selected from nests. To determine the critical time perceived by bees, the subjected immature stages were divided into egg, 1st to 5th instar larvae and prepupa. Temperatures and day lengths used for rearing of the immatures are indicated in parentheses. These regimes are illustrated in Fig. 1. All experiments were conducted in 1978 except VII in 1980.

I Eggs (20℃ for 1－7 days, dark, 7 sub-regimes I－1 to I－7)→ later stages (30℃, dark)

II Eggs (20℃, dark)→ 1st to 3rd instar larvae (18℃, dark)→ later stages under (30℃, dark)

III Eggs (20℃, dark)→ 1st to 4th instar larvae (18℃, 12hr and 16 hr, 2 sub-regimes III－1 and III－2)→ later stages (30℃, dark)

IV Eggs (20℃, dark)→ 1st to 5th instar larvae (18℃, 12 hr and 16 hr, 2 sub-regimes IV－1 and IV－2)→ later stages (30℃, dark)

V Eggs to 4th instar larvae (30℃, dark)→ 5th instar larvae were exposed temporally to 10℃ for 5－10, 14 days, dark, 7 sub-regimes V－1 to V－7)→ later stages under (30℃, dark)

VI Eggs (30℃, dark)→ later stages (18℃, dark)

VII Eggs to 5th instar larvae and subsequent later stages (combined 2 different temperatures, 22℃/14℃ and 26℃/10℃ of which mean became 18℃ (dark), exposed alternatively to each temperature for 12 hr, dark, 2 sub-regimes VII－1 and VII－2).

VIII Eggs to 2nd instar larvae (30℃, dark)→ later stages (18℃, dark)

IX Eggs to 3rd instar larvae (30℃, dark)→ later stages (18℃, dark).

X Eggs to 4th instar larvae (30℃, dark) → later stages (18℃, dark, 12 hr and 16 hr, 3 sub-regimes X－1, X－2 and X－3)

XI Eggs to early period of 5th instar larvae before defecation (30℃, dark)→ later stages (18℃, dark)

XII Eggs to 5th instar larvae (30℃, dark) → later stages (18℃, 12hr and 16 hr, 2 sub-regimes XII－1 to XII－2)

7. Breaking prepupal diapause

Treatment of the dormant stages under low temperatures are essential to break their diapause in temperate solitary bees (Maeta *et al.*, 2009). "Low temperatures" mean temperatures that are lower than at the usual time of commencement of nesting activity of adult bees. *Megachile rotundata* begins nesting activity when air temperature stays above 24℃, on sunny days (Maeta and Adachi, 2005).

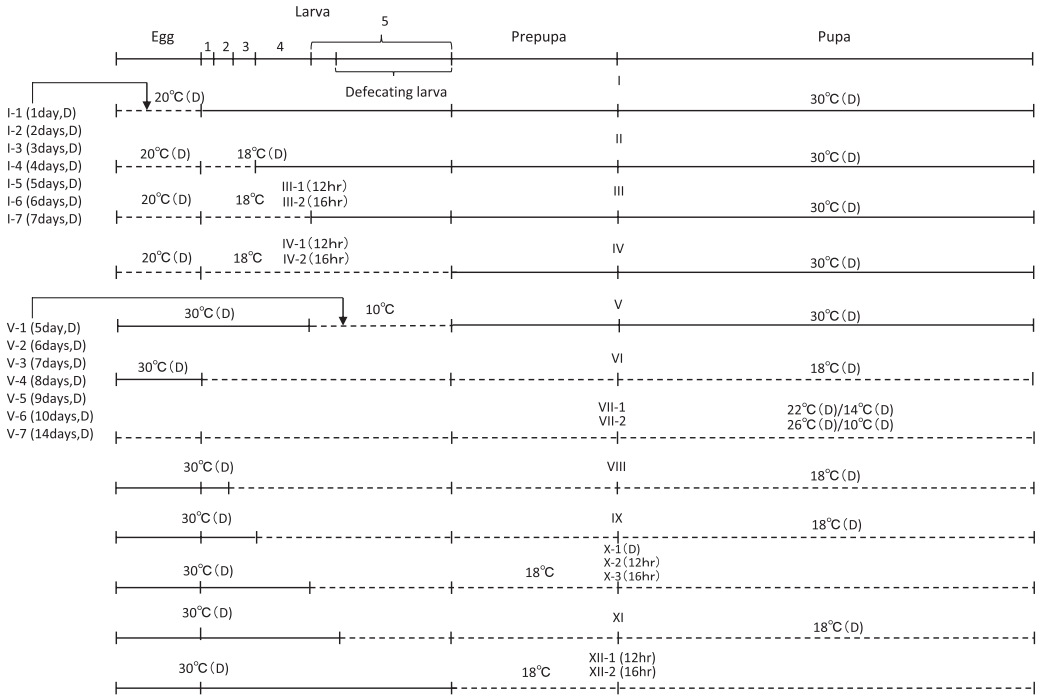

Fig. 1. Schema showing that twelve experimental regimes (I–XII) in which immatures are exposed sequentially under different temperatures (10℃, 18℃, 20℃ and 30℃) and day lengths (dark (D), 12 hr and 16 hr). Prepupae who entered diapause were kept with further developed pupae at 18℃. Larval stages, 1–5 in the top of the figure show the first to 5th instars.

Effective low temperatures to break diapause in prepupae were studied. Diapausing prepupae were grouped into 3: 1) Offspring of the first generation naturally and obligatorily entered diapause; 2) Artificially induced diapause by rearing immatures under various regimes (see **6**); and 3) Diapausing prepupae of Spanish univoltine strain. 1) Prepupae were subjected to 7 constant low temperatures, 0℃, 5℃, 10℃, 12℃, 15℃, 18℃, and 20℃ for one to 4 months on November 29, 1978. 2) Prepupae were subjected to 4 constant low temperatures, 9℃, 12℃, 15℃ and 18℃ for 15 to 90 days in July soon after they had entered diapause in 1979. 3) Prepupae were obtained by yearly rearing bees introduced from Spain. Dormant prepupae were subjected to 7 constant low temperatures, −1℃, 5℃, 8℃, 11℃, 14℃, 17℃, and 20℃ for one to 4 months on September 24, 1987. Final checking of the dead and living individuals was done on April 20, 1988 (208 days after the commencement of treatments). A total 322 prepupae remained in dormant state in 28 treatments. These prepupae were moved to 14℃ so as to break diapause on April 30.

Incubation after low temperature treatments was conducted at 30℃ in all 3 groups. Emergence rate (%) of diapause-broken individuals was calculated by total number of dead pupae + emerged adults/total number of normal cocoons used in 3 groups. When the rate of diapause-broken prepupae exceeded 94%, it was regarded that diapause was broken completely, because dead prepupae during cold treatments occurred 0–6%.

8. Suitable storing temperature for cocoons with artificially induced diapause

Materials used for the experiment were obtained by using Canadian cocoons reared under 18℃ in 1980. To determine the suitable temperature to store artificially induced dormant cocoons, they were kept at 5℃ and 18℃ for 6 months. Mortality of prepupae during storing under 2 low temperatures and incubating under 30℃ was examined. To discriminate the mortality between the 2 periods of storing and incubating, the anterior part of the cocoons was cut off with the razor blade.

Results and Discussion

1. Rate of emergence of the first generation in two districts, Sapporo and Morioka

Foraging activity of *M. rotundata* was observed from mid July to early September in the campus of TNAES (Maeta, 2018). Alfalfa, *Lespedeza bicolor* var. *japonica*, *Rhus javanica* var. *ruxburghii* and *Aralia elata* were used as major floral resources. In the campus of HNAES alfalfa was supplied as floral resources in the greenhouses.

As shown in Table 1, the emergence rates of the first generation were (12.6－24.7%) in Sapporo, and 36.7－93.7% in Morioka. The rates were relatively lower in the northern locality, and higher in Morioka every year, irrespective of difference of the source of cocoons introduced. It is reported as ca. 30% in Utah (Parker *et al*., 1976), and almost perfect univoltine (the emergence rate of the first generation is less than 1%) in the cooler habitat, Canada (Hobbs and Richards, 1976). Thus, voltinism seems to be determined by thermal conditions, depending on the localities related to the latitude where bees are inhabiting (Krunic, 1972).

In the Spanish univoltine strain reared at the campus of TNAES and Shimane University, none of the first generation had emerged from populations reared by liberating the bees at the usual early summer nesting period in the field (1986－1988, Table 1). On the other hand, when the population released on April 5, 1981 in the greenhouse, the nesting activity commenced on April 26 and continued until August 15 (111 days), and 15.5% of the total cocoons emerged as the first generation (Table 1). The partial first generation also occurs in French univoltine strain (Tasei, 1977; Tasei and Masure, 1978).

2. Rate of emergence of the first generation under different constant temperatures

The rate of the emergence of the first generation was becoming higher, according to the rise of temperatures used for rearing of the immatures (Table 2). We reared stages including eggs to the 5th instar larvae. It was very low in both sources of cocoons (Oregon and Canada) reared at 18℃, which was a little higher than the developmental threshold temperature. However, in those individuals reared under 18℃ from egg stage, no individuals completed development and emerged during period of observation (Kemp and Bosch, 2000). This may be related to the the threshold temperature is higher in eggs than in larvae. On the other hand, a majority of cocoons reared by us emerged as the first generation when reared above 20℃. Comparing the emergence rate between 2 source populations, no clear difference was recognized between them. This may suggest that both sources are basically multivoltine. Parker and Bosch (2001) described that elevated post-cocooning temperatures were associated with a higher prevalence of nondiapausing individuals.

In our present laboratory experiments, those individuals did not emerge as the first generation when reared above 20℃ and seem to be univoltine strain, although their ratios against bivoltine

Table 1. Emergence rate of the first generation in populations liberated in the field and greenhouse.[1]

Locality	Year	Emergence rate (%)[2]	Date of liberation of adults	Source of cocoons[3]
Sapporo	1971*	12.6 (22/174)	July 9	Utah
	1972*	24.7 (128/519)	July 13	Idaho
Morioka	1975	84.3 (156/185)	July 3	Canada
	1976	87.4 (1780/2037)	July 23	Idaho
	1977	84.9 (923/1087)	July 14	Oregon
	1978	93.7 (5949/6349)	July 11	Canada
	1979	65.6 (347/529)	July 14	Canada
	1979	64.1 (436/680)	July 20	Oregon
	1979*	0 (0/151)	August 27	Oregon
	1980	36.7 (72/196)	July 9	Canada
	1981	78.0 (4192/5374)	July 16	Oregon
	1981*	15.5 (249/1603)	April 5	Spain
	1981	0 (0/161)	July 9	Spain
Matsue	1986*	0 (0/899)	June 30	Spain
	1987*	0 (0/1997)	June 10	Spain
	1988	0 (0/67)	June 11	Spain

[1] Those populations reared in the greenhouses were indicated with an asterisk.
[2] Obtained by A/A+B (A: Adults+Pupae, irrespective their living or not; B: Living prepupae). In 1976 many dead pupae were found in nests, due to low ambient temperature in autumn.
[3] The country where cocoons were introduced.

Table 2. Emergence rate of the first generation of eggs and larvae reared under constant different temperatures.

Temperature (%)	Year[1]	Source of cocoons	Emergence rate (%)[2]	Mortality (%)[3]	Date of incubation commenced[4]	No. of individuals incubated
18	1977	Oregon	33.1 (40/121)	27.1	Aug. 4−6	166
	1978	Canada	23.4 (97/414)	16.5	May 19	496
	1979	Oregon	8.1 (17/211)	9.8	June 28	234
	1980	Oregon	12.5 (3/24)	4.0	Aug. 7−9	25
	1980	Canada	0 (0/142)	6.0	Aug. 12−14	151
20	1977	Oregon	88.4 (137/155)	4.3	Aug. 3−5	162
22	1977	Oregon	95.2 (59/62)	20.0	July 9−Aug. 3	75
26	1977	Oregon	98.4 (63/64)	9.9	July 9−Aug. 3	71
30	1977	Oregon	100 (99/99)	14.9	July 9−Aug. 3	114
	1978	Canada	100 (50/50)	10.0	June 14−15	50
	1980	Oregon	100 (125/125)	1.6	Aug. 7−9	127
	1980	Canada	89.7 (70/78)	3.7	Aug. 10	81
34	1977	Oregon	100 (108/108)	7.8	July 9−Aug. 3	116

[1] Those provisioned cells that contained eggs and young larvae (1st and 2nd instars) were incubated in 1977, while eggs to the 5th instar larvae were done in 1978−1980.
[2] Obtained by A/A+B (A: Adults+Pupae, irrespective of their living or not; B: Living prepupae).
[3] Excluded individuals which were dead by parasitic associates. Only dead immatures, possibly died during incubation, were counted.
[4] Provisioned cells were taken out from the nests soon after their completion.

were quite low.

3. Rate of emergence of the first generation in relation to the nesting sequence

The nesting activity in the population reared in the campus of TNAES in 1976 was begun from July 24 and continued until September 15 (53 days). The nests were dissected between December 24, 1976 and January 7, 1977. As shown in Table 3, the number of nests, which produced the first generation, was reduced in accordance with elapse of the nesting period, showing the synchronization with the decrease in the emergence of the first generation. The same tendency was recognized in other populations reared in the same campus (1975－1979). It was surprising there were nests as much as 21.3% (39/183) in which emerged adults and diapaused prepupae existed together (Table 3), showing the occurrence of univoltine and multivoltine strains within the same nests. Such occurrence seems to be individual variation, not genetical difference, as suggested in *Osmia coerulescens* (Maeta, 2016).

Similar traits were reported in nests of *M. rotundata* constructed during the second half of the nesting season, which traditionally exhibit a high proportion of diapausing bees compared with individuals from nests constructed earlier in the nesting season (Krunic, 1972; Richards, 1984; Kemp and Bosch, 2001).

4. Selection of the univoltine strain

Table 4 shows the emergence rate (%) of the first generation in 2 trials of selection of the univoltine strain (parental sources were Idaho and Oregon, respectively). It was gradually decreased, according to the elapse of the years. In the first trial the second year population (1977) was heavily infested by *Melittobia acasta* Walker, and obliged to cease continuous selection. On the other hand, in the second trial the rate of emergence of the first generation became 0% on the 4th year population in 1980. The climate of this year was cool in summer and reproduction of bees was very poor, thus only 56 normal cocoons containing dormant prepupae were obtained. These cocoons were released again in 1981, the emergence rate of the first generation became 1.3% in the 5th year population.

Comparing the emergence rates of the first generation between populations which were

Table 3. Rate of nests in which prepupae entered diapause in relation to the nesting periods.[1)]

Period of nests completed	Number of nests				Emergence rate of the first generation (%)[4)]
	completed	all did not emerge (%)	partially emerged (%)[2)]	all emerged (%)[3)]	
Late July	9	0 (0.0)	1 (11.1)	8 (88.9)	90.4 (49/52)
Early August	64	0 (0.0)	10 (15.6)	54 (84.4)	94.8 (328/346)
Middle August	67	7 (10.4)	17 (25.4)	43 (64.2)	80.7 (218/270)
Late August	43	18 (41.9)	11 (25.6)	14 (32.6)	59.8 (76/127)
Total	183	25 (13.7)	39 (21.3)	119 (65.1)	84.2 (669/795)

[1)] Cocoons introduced from Idaho in 1976 were liberated in the field. Nests were dissected in winter periods (December 24 to January 7).
[2)] Some adults in the same nests emerged as the first generation.
[2)] and [3)] Dead pupae were regarded as adults of the first generation. Most of them did not develop into adults, due to low ambient temperature in late summer and early autumn.
[4)] The rate was obtained by A/A + B (A: Adults + Pupae, irrespective of their living or not; B: Living prepupae).

Table 4. Process of the selection of a univoltine strain in *Megachile rotundata* introduced from USA.

Year	Time	Emergence rate of first generation (%)[1]	No. of cocoons examined[2]
	Cocoons from Idaho		
1976	1st	87.4	2037
1977	2nd	76.4	242
	Cocoons from Oregon		
1977	1st	84.9	1087
1978	2nd	60.3	1302
1979	3rd	48.6	315
1980	4th	0	56
1981	5th	1.3	594

[1] Obtained by A/A + B (A: Adults + Pupae, irrespective of their living or not; B: Living prepupae).
[2] Total numbers of A + B.

introduced every year from the same localities and liberated at the campus of TNAES (Tables 1 and 4) with those which were selected univoltine strain, it was conspicuous the decrease of the emergence rates in the selected populations. The effect of selection of the univoltine strain was prominent and seems to be promising, because *M. rotundata* was not established so far in Japan, then no crossing between previously reared populations occurs. Gradual yearly decrease of the emergence rate of the first generation in the process of selecting a univoltine strain implies that univoltine and multivoltine strains are compatible in the same *M. rotundata* populations. Hobbs and Richards (1976) mentioned a strain of the alfalfa leafcutting bee that has produced less than 1% second-generation adults for 3 consecutive years was produced by selection against bivoltinism.

5. Development of immature stages in relation to the temperatures

Immatures were divided into the following 5 stages. Stage 0: Eggs; Stage I: Larvae, from hatching to commencement of defecation; Stage II: Larvae from commencement of defecation to commencement of cocoon spinning; Stage III: Larvae from commencement of cocoon spinning to completion of cocoon; Stage IV: Prepupae from completion of cocoon to pupation; and Stage V: Pupae from pupation to eclosion of adult. Stage 0 was examined to select those eggs whose duration was the longest among incubated eggs. Therefore, the duration in some eggs was not always accurate.

Duration in days of stages 0－V in 3 source populations, *i.e.*, introduced from Oregon, Canada and Spanish, which were reared under 3 or 4 constant temperatures, is shown in Tables 5－8. Figure 2 (A－F) shows the developmental rate of stages 0 (egg), I + II (feeding larva) and V (pupa). Developmental rates of immatures was expressed by 100/developmental durations (in days).

The duration of prepupal stages of the first generation in non-diapausing prepupae (multivoltine strain) were very short, only 3－12 days under 30℃－22℃, while that in the Spanish univoltine strain was extremely long. Post development of free-diapaused prepupae was shown in Table 8. Durations of prepupae and pupae incubated under 20℃ were remarkably longer than those under 24℃, 28℃ and 32℃.

The developmental rates of stages of eggs, feeding larvae and pupae were slightly faster in

Table 5. Developmental duration of the immature stages in Oregonian *Megachile rotundata* incubated under three different constant temperatures in 1979.[1)]

Temperature (℃)	Sex	Duration in days of six stages[2)]							
		0	I	II	I+II	III	IV	V	I−V
22	Female	5.0±0.0	7.7±0.2	5.6±0.3	13.3±0.3	1.4±0.4	12.5±1.0	26.0±0.4	53.5±1.7
	N	2	7	7	7	7	7	7	7
	Male	5.0±0.0	7.8±0.2	4.4±0.2	12.2±0.2	1.4±0.1	10.1±0.2	23.6±0.1	47.3±0.3
	N	4	39	39	39	39	39	39	39
26	Female	3.0±0.0	4.7±0.2	1.9±0.2	6.7±0.2	1.2±0.1	7.1±0.4	15.0±0.1	29.9±0.5
	N	5	15	15	15	15	15	15	15
	Male	3.0±0.0	4.6±0.1	2.2±0.1	6.7±0.1	0.8±0.1	5.4±0.1	13.1±0.1	25.9±0.2
	N	16	37	37	37	37	37	37	37
30	Female	2.0±0.0	3.8±0.1	1.9±0.2	5.7±0.2	0.7±0.1	4.5±0.3	9.9±0.3	20.8±0.3
	N	2	11	11	11	11	11	11	11
	Male	2.0±0.0	3.4±0.1	2.0±0.1	5.4±0.1	0.7±0.1	2.8±0.2	9.2±0.3	18.1±0.2
	N	17	36	36	36	36	36	36	36
Developmental threshold temp.(℃)	Female	16.8	13.9	16.5	15.4	14.9	17.7	17.3	16.8
	Male	16.8	15.7	14.4	15.2	13.3	19.4	16.9	16.9

[1)] Incubation was started on June 13, 1979. Values are given as mean±SD.

[2)] Stage 0: Egg; Stage I: From hatching to commencement of defecation; Stage II: From commencement of defecation to commencement of cocoon spinning; Stage III: From commencement of cocoon spinning to completion of cocoon; Stage IV: From completion of cocoon to pupation (prepupa); Stage V: From pupation to eclosion (pupa).

Table 6. Developmental duration of the immature stages in Canadian *Megachile rotundata* incubated under three different constant temperatures in 1978.[1)]

Temperature (℃)	Sex	Duration in days of six stages[2)]							
		0	I	II	I+II	III	IV	V	I−V
22	Female	5	7.2±0.3	4.3±0.2	11.5±0.3	1.3±0.2	9.3±0.4	23.5±0.3	45.2±0.6
	N	1	13	13	13	13	13	13	13
	Male	5.3±0.0	7.8±0.2	4.0±0.2	11.7±0.2	1.1±0.1	8.0±0.2	21.0±0.1	41.9±0.3
	N	4	35	35	35	35	35	35	35
26	Female	3	5.1±0.1	2.8±0.2	7.9±0.2	0.8±0.1	5.3±0.2	13.9±0.1	28.0±0.4
	N	1	12	12	12	12	12	12	12
	Male	3.0±0.0	4.8±0.1	2.6±0.1	7.4±0.1	0.8±0.1	4.8±0.1	12.7±0.1	25.8±0.2
	N	7	34	34	34	34	34	34	34
30	Female	2	3.3±0.2	2.1±0.2	5.4±0.1	1.0±0.1	3.6±0.2	9.8±0.1	19.8±0.3
	N	1	12	12	12	12	12	12	12
	Male	2.0±0.0	3.4±0.1	1.8±0.3	5.2±0.1	0.8±0.1	3.2±0.1	8.6±0.1	17.8±0.1
	N	17	33	33	33	33	33	33	33
Developmental threshold temp.(℃)	Female	16.8	15.6	14.3	15.1	−	17.0	16.4	16.0
	Male	17.3	15.9	15.6	15.2	−	16.8	16.5	16.4

[1)] Incubation was started on June 15, 1978. Values are given as mean±SD.

[2)] Stage 0: Egg; Stage I: From hatching to commencement of defecation; Stage II: From commencement of defecation to commencement of cocoon spinning; Stage III: From commencement of cocoon spinning to completion of cocoon; Stage IV: From completion of cocoon to pupation (prepupa); Stage V: From pupation to eclosion (pupa).

Table 7. Developmental duration of the immature stages in Spanish univoltine *Megachile rotundata* incubated under four different constant temperatures in 1981.[1)]

Temperature (℃)	Sex	Duration in days of four stages[2)]					
		0	I	II	I+II	III	I − III
22	Female + Male	4.3±0.5	8.9±0.9	5.5±1.1	14.2±0.8	1.5±0.7	15.8±0.8
	N	3	19	19	19	19	19
26	Female + Male	3.0±0.0	6.2±0.5	3.5±0.6	9.7±0.8	1.5±0.6	11.2±0.9
	N	3	19	19	19	19	19
30	Female + Male	−	4.5±0.6	2.8±0.7	7.3±0.9	1.2±0.5	8.4±0.8
	N		18	18	18	18	18
34	Female + Male	2.0±0.0	3.6±0.7	2.4±0.6	5.9±0.7	1.1±0.4	7.0±0.5
	N	8	20	20	20	20	20
Developmental threshold temp.(℃)	Female + Male	11.2	14.1	12.0	13.5	6.4	12.6

[1)] Incubation was started on June 17 − 25, 1981. Values are given as mean±SD. All prepupae entered diapause in the Spanish univoltine strain.
[2)] Stage 0: Egg; Stage I: From hatching to commencement of defecation; Stage II: From commencement of defecation to commencement of cocoon spinning; Stage III: From commencement of cocoon spinning to completion of cocoon.

Table 8. Duration in days from pupation to adult in free-diapaused prepupae in Spanish univoltine *Megachile rotundata* under four different temperatures (1982).[1)]

Temperature incubated (℃)	Sex	Duration in days of			
		Prepupa[2)]	Pupa	Total	*N*
20	Female	46.6±3.4	49.0±1.1	95.5±3.7	15
	Male	43.8±1.6	40.7±0.7	84.5±1.3	12
24	Female	28.1±2.9	15.4±0.8	43.5±3.3	14
	Male	20.6±2.3	14.0±1.0	34.6±2.9	10
28	Female	20.5±1.6	11.4±1.4	31.9±1.3	10
	Male	22.0±0.0	9.5±0.5	31.5±0.5	2
32	Female	20.5±2.0	10.0±1.9	30.5±2.0	6
	Male	18.0±0.9	6.6±0.9	24.6±1.1	8
Developmental threshold temp.(℃)	Female	13.7	15.7	13.0	
	Male	11.6	17.6	13.3	

[1)] Cocoons were kept in uncontrolled room temperature until March 5, 1982, then moved to the temperature cabinet controlled at 10℃. Incubation was commenced on July 21, 1982. Values are given as mean±SD.
[2)] Duration implies that for later development phase of free-diapaused prepupae, *i.e.*, from the day on which incubation started to the day on which pupation occurred.

males than in females, in all 3 source materials. The developmental threshold temperatures of the eggs, feeding larvae and pupae were around 17℃, around 15℃ and around 17℃, respectively in the multivoltine Oregonian and Canadian populations, while those were remarkably lower: 11.2℃ (included both sexes), 13.5℃ (ditto) and 15.7℃ for females and 17.6℃ for males in the Spanish univoltine strain (Tables 7 and 8). As shown in Fig. 2, the patterns of the regression lines of the developmental rates in stages of egg, feeding larva and pupa were quite similar between Oregonian and Canadian populations. However, those in the Spanish univoltine strain differed from the above 2 multivoltine strains. The developmental rates of these stages were lower under 24℃, 28℃ and 32℃ in the Spanish univoltine strain, showing that the development of immatures was progressed gradually in accordance with the increase of temperatures.

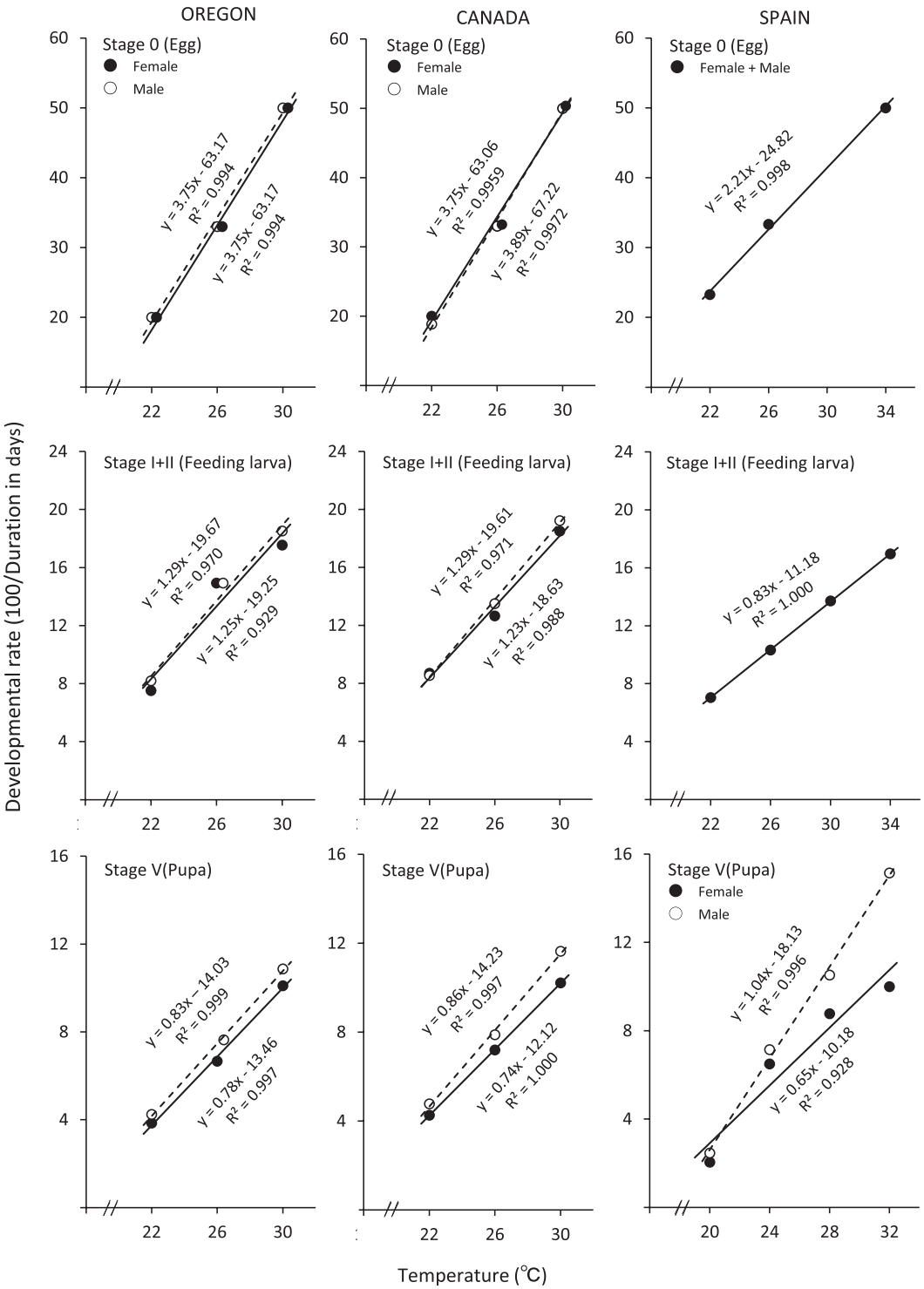

Fig. 2. Relationship between three or four different constant temperatures and developmental rates of the stages of egg, feeding larva and pupa in three different parental cocoons of *Megachile rotundata* introduced from USA, Canada and Spain. These figures are drawn based on Tables 5 – 8.

The occurrence of developmental inhibition by high temperatures was recognized only at the prepupal stage in the Spanish univoltine strain under 32℃ (Table 8) and the Oregonian bivoltine strain under 34℃. The latter case was examined in 1977, but not tabulated in the this paper. Tasei and Masure (1978) mentioned that for the French strain to develop from egg to prepupa, it needed 18－19 days at 22℃ and 10－11 days at 29℃. These durations coincided well with ours for the Spanish univoltine strain (Table 7). They described that the temperature of 15℃ killed all eggs and young larvae.

Kemp and Bosch (2000) compared duration in days of development periods for non-diapausing and diapausing forms reared under differing temperature regimes. Bees were obtained from nesting populations released in pasture in Utah. Prepupae of diapausing form were transferred to 4℃ for 203 days and then incubated. Comparing the development between nondiapausing and diapausing forms, there was no clear difference in the development of 3 comparable stages, egg, larva to commencement of spinning and pupa between the 2 forms. However, total duration from egg to adult of the non-diapausing form examined by our study (Tables 5 and 6) was generally shorter than that examined by Kemp and Bosch (2000). Those populations obtained by Kemp and Bosch in Utah and by ours in Oregon and Canada seem to belong to genetically different biotypes.

6. Artificial induction of prepupal diapause

From those experiments on the emergence rate of the first generation reared under 6 different constant temperatures (18－34℃), it became obvious that low temperature (18℃) was effective to induce prepupal diapause (Table 2). Respective immature stages from egg to prepupa were begun to rarer at 18℃ and kept rearing them under the same temperature. Results are shown in Table 9. High rate of prepupal diapause (above 75%) was induced in those regimes/sub-regimes, VI, VII－1 and VII－2, VIII, IX, X－1 and XI. On the other hand, few dormant prepupae (less than 10%) were induced in those regimes/sub-regimes, I－1 to I－7, II, III, IV, XII－1 and XII－2.

Apparently high rates of prepupal diapause were induced by keeping immatures from 1st instar (VI), 2nd and 3rd instars (VIII), 4th instar (IX), 5th instar, (X) and defecating 5th instar (XI) at 18℃, and then subsequent prepupae reared at the same temperature. Similar high rates were also obtained by subjecting immatures, from egg to 5th instar and subsequent later stages at 2 different combined temperatures (22℃/14℃ and 26℃/10℃ (mean 18℃), alternately for 12 hr (VII－1 and VII－2). A higher rate of dormant prepupae was induced with a smaller difference of 2 combined temperatures. Day length did not affect the induction of prepupal diapause at all. In a partial bivoltine *Osmia coerulecens* (Linnaeus), the day length also did not affect the induction of adult diapause (Maeta, 2016), In this experiment of *M.rotundata*, 18℃ was used for the larval stages (stages I+II), which was higher than the developmental threshold temperature (15－16℃, Table 5 and 6). If closer to 15－16℃ was used, a higher rate of prepupal diapause seems to be inducted. Tepedino and Parker (1966) mentioned that an extremely high mortality occurred when immatures from the egg or early larval stages to the adult stage were reared at 16℃. However, all living prepupae entered diapause. Similarly high rates of diapaused prepupae with lesser larval mortality were obtained in our regimes in which more advanced larval stages were exposed.

A very high rate of prepupal diapause was also induced by temporarily subjecting the 5th instar larvae to 10℃ for 5 to 14 days (V－1 to V－7). However, the mortality was extremely high over 5

Table 9. Rate of prepupae of which diapause was artificially induced under various regimes.[1)]

Regime/sub-regime[1)]	Reate(%) of diapaused prepupae[2)]	Mortality (%)[3)]	No. of reared individuals
I－1	0	7.7	13
I－2	2.9	10.3	78
I－3	2.3	8.5	47
I－4	4.3	4.1	49
I－5	0	8.9	56
I－6	6.1	10.9	55
I－7	1.7	7.8	64
II	0	0.0	33
III－1	2.2	4.3	92
III－2	10.3	4.5	88
IV－1	0	5.6	108
IV－2	0	10.0	80
V－1	100	14.3	14
V－2	100	47.4	38
V－3	97.1	51.4	70
V－4	100	94.7	19
V－5	92.8	85.7	98
V－6	100	76.5	17
V－7	100	95.3	85
VI	76.7	34.8	112
VII－1	88.5*/100**	10.3*/11.2**	29*/109**
VII－2	69.2*/98.2**	2.5*/6.8**	40*/59**
VIII	75.6	38.3	120
IX	92.0	11.6	250
X－1	75.3	7.1	98
X－2	51.0	18.0	111
X－3	43.3	16.8	107
XI	91.8	8.3	205
XII－1	2.6	0.9	116
XII－2	1.6	6.2	129

[1)] Explanation of regimes see the text (p. 184 and Fig. 1). All experiments were conducted in 1978, except VII－1 and VII－2 in 1980. In VII－1 and VII－2 experiments, the source of cocoons from Oregon (with*) and Canada (**) were used.
[2)] Expressed by number of diapaused prepupae/(number of reared individuals－number of dead individuals).
[3)] Excluded those individuals infested by parasitic associates.

days exposure. Apparently the low temperature of 10℃ was harmful for their further development. Additional experiments are needed to subject the 5th instar larvae for less than 5 days in order to confirm the above procedures.

In *M. rotundata,* the critical time perceived by immatures, which induce the prepupal diapause, seems to be larval stages from 1st to 5th instars and possibly even after 5th instar larvae who had commenced defecation, because few dormant prepupae occurred when only the prepupal stage was reared under 18℃ (XII－1 and XII－2).

Bitner (1976) mentioned that prepupal diapause could be induced in ca. 90% of the offspring by

subjecting mature pupae for consecutively 8 days at 10℃ for 3hr/day while incubating them at 30℃. However, those prepupae of the overwintering (2nd) generation were known to enter diapause obligatorily, due to decreasing ambient temperature, as in a partially bivoltine *O. coerulescens* (Maeta, 2016).

7. Breaking prepupal diapause

7.1. Prepupae naturally diapaused

The offspring of the first generation obligatorily entered diapause. The studied material was obtained by rearing of the first generation at the campus of TNAES in 1978. The first generation emerged on August 18, and their nesting activity commenced around August 26 and continued until around October 3 (ca 38 days), due to the warm autumn in this year. These naturally diapaused prepupae were subjected to 7 different low constant temperatures for one to 4 months to break prepupal diapause. When those dormant prepupae, which were incubated at 30℃ on November 26 before the experiments commenced, all adults emerged from cocoons, spending 33.6±4.8 ($N=37$) days for females and 30.3±3.4 ($N=7$) days for males (mortality, including both sexes, was 12.0%). In those prepupae which entered obligatory diapause without emerging as the first generation in the population released on July 20 at the field of TNAES in 1979 (Oregonian cocoons), and kept at uncontrolled room temperature until incubation at 30℃ on November 30. The adults emerged from dormant cocoons, spending 41.6±2.9 ($N=14$) for females and 35.8±2.8 ($N=28$) for males (mortality, including both sexes, was 16.7%). These cases show no necessity for low temperature treatments to break prepupal diapause, as well as the following case of prepupae artificially induced diapause. These facts indicate that dormancy of prepupae was already broken before overwintering (overwintering diapause), and the later development of prepupae gradually progressed during hibernation. It quite differed from the other temperate solitary bee species, in which diapause was broken by subjecting them to lower temperatures for several months (Maeta *et al*., 2009). Stephen and Osgood (1965) mentioned that the development of overwintering prepupae of *M. rotundata* occurred between 19℃ and 38℃ with the optimum of a constant 32℃. Their description seems to suggest no necessity of cold treatments. In the experiments of the length of cold storage and percentage emergence in a Canadian strain, 61% of dormant prepupae had emerged without cold storage, by incubation at 30℃ (Richards *et al*., 1987). The Canadian population seems to be composed of multivoltine and univoltine strains, with no necessity for chilling to break prepupal diapause in the former strain.

In those prepupae naturally entered diapause, which were stored under 7 low temperatures for one to 4 months, the duration in days from the commencement of incubation at 30℃ to the emergence of adults from cocoons (henceforth, emergence duration) was the longest under all 7 treatments for one month storage, but was not conspicuously shortened under 0℃, 5℃, 10℃, 12℃ and 15℃ in accordance with the increase of storing durations (2 to 4 months). Mortality was generally very low under these temperatures, irrespective of storing durations (Table 10). On the other hand, the emergence durations were clearly shortened in accordance with the increase of storing durations at 18℃ and 20℃, because both temperatures functioned to incubate cocoons during storing. The temperatures of 18℃ and 20℃ are unfavorable to match the timing of releasing bees with the blooming period of alfalfa. The mean emergence durations in those naturally induced

Table 10. Duration in days from prepupa to adult emergence from cocoons in naturally diapaused prepupae subjected to seven different temperatures (1978).[1]

Temperatures subjected	Sex and mortality	Days from incubation of prepupae to adult emergence and mortality (%)[2]			
		Prepupae subjected to different temperatures for			
		one month	two months	three months	four months
0℃	Female	32.4±2.0 (34)	28.2±2.1 (18)	27.6±2.0 (20)	26.1±2.1 (32)
	Male	29.1±2.8 (14)	24.9±1.7 (30)	23.7±1.9 (28)	21.6±1.1 (16)
	Mortality	2.0	4.0	4.0	4.0
5℃	Female	30.7±2.7 (41)	26.4±2.0 (37)	25.8±1.6 (31)	25.5±1.5 (44)
	Male	26.9±1.5 (7)	25.3±1.4 (7)	22.8±1.4 (14)	20.0±0.8 (3)
	Mortality	4.0	12.0	10.0	6.0
10℃	Female	31.7±2.5 (37)		Not available	
	Male	28.7±3.2 (6)			
	Mortality	4.0			
12℃	Female	31.3±2.9 (18)	26.4±1.8 (25)	26.5±1.9 (34)	–
	Male	29.7±3.1 (29)	24.6±2.0 (24)	23.2±1.8 (13)	–
	Mortality	2.0	2.0	6.0	–
15℃	Female	28.0±1.9 (27)	23.9±1.5 (34)	24.7±1.3 (38)	23.3±1.4 (44)
	Male	25.7±2.5 (20)	20.7±0.7 (9)	21.0±0.5 (6)	20.3±0.8 (3)
	Mortality	4.0	10.0	10.0	6.0
18℃	Female	25.5±2.7 (31)	19.1±1.2 (31)	17.7±1.6 (32)	14.0±3.9 (45)
	Male	21.6±1.4 (10)	16.5±0.8 (12)	14.1±3.4 (8)	7.7±1.2 (3)
	Mortality	14.0	14.0	18.0	5.9
20℃	Female	24.4±2.2 (25)	15.1±1.6 (38)	12.4±2.6 (36)	4.5±1.6 (13)
	Male	20.0±1.2 (15)	13.5±1.0 (6)	7.0±0.0 (8)	–
	Mortality	10.0	4.0	12.0	54.0

[1] Values are given mean±SD. Naturally diapaused prepupae reared in the field were used. Subjection to 7 low temperatures commenced on November 29.

[2] Combined female and male individuals (prepupae and pupae). Included both periods of subjecting under respective low temperatures and incubating at 30℃.

diapaused prepupae stored at 5℃ and incubated at 30℃ (Table 10) were similar to those (22.5－26.8 days for females and 20.1－24.8 days for males) of cocoons obtained at different latitudes in Canada and incubated at ca. 29℃ after storing at 4℃ for 6 months (Pankiw *et al*., 1980).

7.2. Prepupae with artificially induced diapause

Prepupae with artificially induced diapause were subjected to 4 low constant temperatures for 15 days to 90 days in 1979. Four treatments were commenced soon after confirmation that prepupae had entered into diapause. Those prepupae which had not continued further development, were regarded as dormant prepupae. In diapausing prepupae of the parent generation which were incubated at 30℃ on April 20, 1979, the emergence duration (values are given as mean±SD) was 21.1±0.4 days (N=16) for females and 19.2±1.3 days (N=36) for males (mortality including both sexes was 1.9%). Those prepupae shown in Table 11 were apparently being diapausing. No tendency was found of the strengthening of prepupal diapause by storing at low temperatures for 15－90 days.

In Table 12 the emergence durations of prepupae with artificially induced diapause by incubating those cells including eggs to 5th instar larvae under the following 3 different regimes in 1980, are shown. 1) reared under constant 18℃, 2) reared under 2 different combined temperatures exposed immatures alternatively for 12 hours every 22/14℃ and 3) 26/10℃. The emergence duration of dormant prepupae reared under 2) and 3) were apparently longer than that of 1). Combined 2 different temperatures, (fluctuated to became 18℃ mean), seems to have functioned to induce deeper diapause.

Some differences in diapausing situation were recognized between naturally and artificially induced prepupae as follows. 1) The emergence duration was clearly shorter in the latter than that in the former, when compared with those of the same treatments, subjecting temperatures (10℃, 12℃ and 15℃) and duration (one month). 2) The emergence durations did not differ among 4 treatments in the latter, irrespective of storing durations. However, mortality was remarkably increased in those cocoons stored for 60 days and 90 days under 15℃ and 18℃. Temperatures above 12℃ are apparently unfavorable to store the cocoons.

The cause of the above-mentioned differences is difficult to explain. It might be derived from the thermally situational differences when they had been encountered during the development of larvae between naturally and artificially induced diapause. The former was obligatorily diapaused under daily fluctuated temperatures in the field, while the latter were artificially induced by rearing immatures under constant 18℃. The fluctuated temperatures effect to enhance deeper diapause is proved, as mentioned above. In both populations, naturally and artificially induced prepupae, included some individuals of which emergence durations were extremely longer than those of the mean durations. These prepupae seem to be a genetically univoltine strain.

7.3. Prepupae of Spanish univoltine strain

Diapaused prepupae in the univoltine strain of *M. rotundata* need to be exposed to a period of low temperature to resume development next year, and the length of time required for adult emergence is intensively related to the duration of the chill period was reported by many authors (Krunic and Hinks, 1972; Johansen and Eves, 1973; Tasei and Masure, 1978; Richards *et al*., 1987; Kemp

Table 11. Duration in days from prepupa to adult emergence from cocoons in artificially induced diapausing prepupae subjected to four different temperatures (1979).

Temperature subjected	Sex and mortality	Days from incubation of prepupae to adult emergence (*N*)[1] and mortality (%)[2]			
		Prepupae subjected to different temperature for			
		15 days	30 days	60 days	90 days
9℃	Female	21.7±4.7 (77)	20.3±2.4 (61)	19.2±1.6 (18)	−
	Male	18.2±5.0 (124)	18.1±2.9 (70)	17.0±1.5 (45)	−
	Mortality	5.0	8.4	13.7	−
12℃	Female	21.8±5.6 (79)	19.6±1.3 (67)	21.2±1.3 (49)	20.0±0.8 (9)
	Male	18.5±3.8 (93)	18.2±2.3 (138)	19.7±2.7 (38)	18.3±1.3 (12)
	Mortality	3.9	1.4	7.4	0.0
15℃	Female	22.7±5.5 (90)	21.6±2.6 (74)	23.1±2.3 (47)	24.4±2.4 (23)
	Male	18.7±2.9 (88)	19.4±2.7 (107)	21.0±2.2 (92)	21.4±2.9 (11)
	Mortality	1.7	4.3	8.6	15.0
18℃	Female	22.0±4.9 (68)	23.2±3.2 (83)	22.7±2.5 (59)	21.5±4.0 (15)
	Male	18.5±4.5 (130)	19.8±2.7 (52)	20.0±3.2 (47)	18.3±3.2 (32)
	Mortality	0.5	2.2	23.2	44.7

[1] Incubation of prepupae at 30℃ after subjecting to low temperatures. Values are given as mean±SD.
[2] Combined female and male individuals (prepupae and pupae). Included both periods of subjecting under respective low temperatures and incubating at 30℃.

Table 12. Comparison of the emergence duration from cocoons in artificially induced dormant prepupae, which were subjected to different treatments (1980).

Temperatures (℃)[1]	Source of cocoons	Sex	Emergence duration of adults[2] (*N*)	Mortality (%)[3]
18	Oregon	Female	22.8±1.3 (18)[a]	4.8 (1/21)
		Male	21.5±0.5 (2)	
	Canada	Female	27.9±2.6 (14)[a]	9.1 (4/44)
		Male	25.0±3.2 (26)[a]	
22/14	Oregon	Female	37.5±8.1 (15)[b]	0.0 (0/23)
		Male	32.1±4.0 (8)[b]	
	Canada	Female	38.0±4.5 (39)[b]	3.2 (3/94)
		Male	30.7±3.4 (52)[b]	
26/10	Oregon	Female	36.7±1.5 (9)[b]	0.0 (0/26)
		Male	33.7±4.5 (17)[b]	
	Canada	Female	39.2±3.7 (17)[b]	3.8 (2/53)
		Male	31.8±4.4 (34)[b]	

[1] Subjected temperatures used for immatures to induce artificial prepupal diapause. Two different temperatures were used alternatively every 12 hours.
[2] Duration in days from commencement of incubation at 30℃ to emergence of adults from cocoons. Incubation was commenced on November 20, 1980. Value are given as mean±SD. Significant difference is indicated by different letters (a and b) among each sex within the same cocoon sources treated under different temperature regimes. Bonforroni's multiple comparison ($p < 0.05$) was used, except for males of Oregon which was used Student t-test ($p < 0.01$), but males of Oregon at 18℃ was skipped, due to small sample size.
[3] Combined females and males.

and Bosch, 2001). As mentioned above, in prepupae of the multivoltine strains, subjection to low temperature was not necessary to break their diapause. On the other hand, in the univoltine Spanish strain, it was essential to subject dormant prepupae to low temperatures. In the parent population used for the study, the nesting period in the greenhouse was between June 16 and August 2, 1987

(47 days). The subjection to low temperature of those individuals which developed into prepupae commenced on September 24, 1987, therefore, some individuals used for the experiment were possibly encountered low temperature for a short time before subjection to the selected 7 low temperatures. Prepupae were incubating at 30℃ prior to subject them low temperatures, 15.2% of 46 individuals were developed into adults, spending 50 days ($N=1$) in females and 66.3±21.0 days ($N=6$) in males (values are given as mean±SD). Other 39 prepupae were moved to 14℃ on April 30, 1988 so as to break diapause of these living prepupae. As mentioned above, 15.5% of the offspring emerged as the first generation from the population liberated earlier than usual period in the greenhouse (Table 1). These facts show that exposure to the low temperatures is essential in the univoltine strain, but also suggests that no occurrence of the genuinely genetical univoltine strain in *M. rotundata*. Krunik and Hinks (1972) described that over 50% prepupae stored at 28℃ developed without prior treatment at a lower temperature but were less synchronized. They also proved that preincubation temperatures of 5℃, 10℃ and 15℃ were equally effective for diapause development and for the synchronization of adult emergence.

In the Spanish univoltine bees, the emergence durations in those prepupae subjected to effective temperatures were longer than those in naturally and artificially diapaused prepupae in multivoltine strains (Fig. 3, Tables 10 and 11). This may suggest that prepupal diapause in the Spanish univoltine strain was deeper than in the other partially bivoltine strains.

Effective low temperatures to break diapause of dormant prepupae were 8℃－20℃ and to subject them for 2 months was enough (Fig. 3). As compared with other temperate megachilid bees, such as *Megachile* and *Osmia,* which require treatments for 3 to 4 months under effective low temperatures (Maeta *et al*., 2009), the duration subject to low temperature was extremely short in the Spanish univoltine strain. The emergence duration of prepupae which had been subjected to 8℃－20℃ ranged ca. 30 to 90 days, depending on the exposed low temperatures and their durations (Fig. 3). Mortality of prepupae was very low, mostly none and the highest 9.0%, irrespective of low temperatures (－1℃－20℃) and their subjected durations (one to 4 months). The emergence duration was the shortest at 20℃, because prepupae apparently were had been incubated during storage. Possibly, a little higher temperature than 20℃ may function to break diapause, but 30℃ was not functional to break diapause completely as mentioned above.

Rank and Rank (1989) compared the relative diapause intensity of prepupae between French univoltine and Saskatchewan commercial strains undergoing diapause development at 10℃ for different time periods. Diapause intensity was estimated by scoring days to emergence after incubation (at 30℃ and 60% RH) at different times (fall incubation, winter incubation and spring incubation). In all comparisons made, the univoltine strain had a significantly increased diapause intensity compared with the commercial strain. They concluded that diapause intensity was heritable.

Diapausing prepupae exhibit a median emergence time of 210－241 days when stored at 31.1℃ versus 28－31 days when chilled at 1.7℃ for 5 months (Johansen and Eves, 1973). Richards *et al.* (1987) chilled dormant prepupae at －5℃, 0.5℃, 5℃ and 10℃ for 0－22 months, and incubated them at 30℃. Similar results as those in Johansen and Eves (1973) were reported after 4 months of chilling diapausing prepupae at 5－10℃. High prepupal survival and a narrow period of adult emergence during incubation were observed when cocoons were stored at 5℃ for 7 to 10 months.

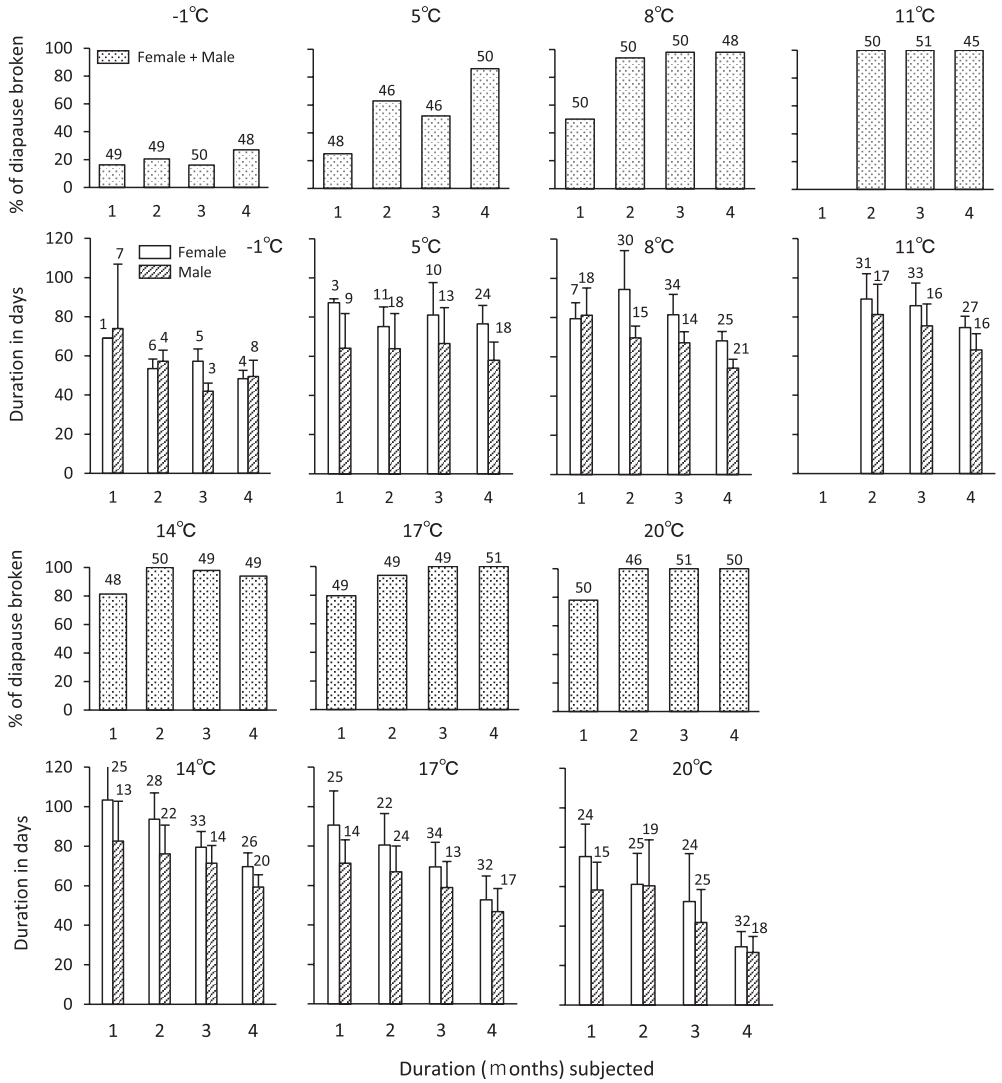

Fig. 3. Percentage of adults in which diapause was broken (upper) and mean duration (days) from commencement of incubation at 30℃ to emergence from their cocoons (lower) in the Spanish univoltine strain. Dormant adults were subjected to seven different low temperatures (−1℃, 5℃, 8℃, 11℃, 14℃, 17℃ and 20℃) for one to four months. The values of duration are given as mean±SD. Numerals in the figure show the number of dormant prepupae used (upper) and those that emerged as adults (lower).

Kemp and Bosch (2001) released a population in pasture in Utah. They mentioned that diapausing females and males, of which prepupae were subjected to 4℃ for 213 days before commencement of incubation at 29℃, required 27−30 days and 29−32 days, respectively, to develop from prepupae to adult emergence. The temperature 4℃ used by them seems to be not optimum to break prepupal diapause, so far as our diapause experiments in the Spanish univoltine bees (Fig. 3). However, their the above-mentioned very short emergence durations seem to be arisen by exposing long time at 4℃ extended for 213 days. *Megachile rotundata* should terminate diapause development by December

to early January in most years in Utah (Kemp *et al*., 2004). A similar case was reported in summer univoltine bee, *M. spissula* Cockerell examined in Morioka, Japan (Maeta *et al*., 2009).

8. Suitable storing temperature for cocoons in artificially induced diapause

Artificially induced diapausing prepupae were divided into 2 groups by different temperatures used for storing, one was kept at 18℃ and the other removed to 5℃. Mortality of dormant prepupae during storing for 6 months and during incubating was apparently lower under 5℃ than 18℃ as shown in Table 13. The emergence duration (values are given as mean±SD) examined prior to the moving to 5℃ on November 20, 1980 was 27.9±2.6 days for females (N=14) and 25.0±3.2 days for males (N=26) (Canadian strain in Table 12). No clear decrease of the emergence duration after storing under 5℃ for 6 months occurred. The temperature of 18℃ was unfavorable to keep dormant cocoons, because of the increase of mortality and shortening of the emergence duration. A similar case was recognized in naturally diapaused prepupae (Table 10). If the anterior part of the cocoons were not cut off, less mortality might have occurred.

In *M. rotundata* management, overwintered cells are kept under refrigeration at 2℃ to 4℃, and subsequently incubated at 29℃ to 30℃ on a schedule to synchronize their emergence with peak alfalfa bloom (Stephen and Osgood, 1965). Richards *et al*. (1987) recommend that cells containing prepupae were kept at 0−10℃ for 7 to 10 months to reduce losses by parasites and predators and to arrest prepupal development until the spring. Theresa *et al*. (2011) found that prepupae that spend the winter in cold storage (at 4−5℃ for 7−10 months), and then incubate at constant 30℃ and 50−60% relative humidity provide for safe, synchronous, and timely adult emergence. Prepupal weights, prepupal lipid and water contents, adult emergence and adult female longevity were affected by the timing of the onset of winter storage and incubation were reported by Theresa *et al*. (2009).

9. Additional remarks on the voltinism and prepupal diapause

Megachile rotundata accidentally invaded the USA from the Eurasian continent about the 1930s, first found in eastern USA (Bohart, 1971; Stephen, 2003). Now it is used as an important pollinator of alfalfa, which is also of Eurasian origin, worldwide (reviewed in Maeta *et al*., 1973). American and Canadian strains are partially bivoltine, although it is genetically almost univoltine in Eurasia

Table 13. Comparison of mortality of artificially induced dormant prepupae kept under two low temperatures (5℃ and 18 ℃) for six months (1980).[1)]

Stored temp. (℃)[2)]	Sex	Emergence duration of adults (N)[3)]	Mortality (%)[4)] during			No. of individuals examined
			storing	incubating	Total	
5	Female	23.9 (19)	0	8.0	8.0	50
	Male	21.6 (27)				
18	Female	19.4 (19)	8.0	10.9	18.9	50
	Male	16.8 (22)				

[1)] Canadian cocoons, which were reared under a constant temperature of 18℃, were used. Values are given as mean.
[2)] Subjected on November 20, 1980 and incubated at 30℃ after storing.
[3)] Duration in days from commencement of incubation at 30℃ to emergence of adults from cocoons.
[4)] Combined females and males.

(Parker, 1979). As mentioned above, there were remarkable differences in the patterns of immature development and prepupal diapause between partially bivoltine and Spanish univoltine strains.

Parker (1979) crossed these populations of North America and Spain to determine if these populations were races or a distinct species. He concluded that these populations are biotypes of a widespread polymorphic Eurasian species. In the partially bivoltine population, univoltine and multivoltine strains are compatible within the same population and habitat. Similar compatibility is known in *Megachile rixator sakishimana* Yasumatsu et Hirashima, *Nomia chalybeata* Smith and *N. incerta* Gribodo (Kitamura *et al*., 2001; Hannan *et al*., 2013; Masuda, 1943). In the latter 2 species univoltine and potentially multivoltine strains occurred within the same nests. *Megachile rixator sakishimana* and *N. chalybeata* may have a potentiality to inhabit widely throughout different climatic zones. *Megachile rotundata* seems to have a similar potentiality.

Artificial induction of prepupal diapause in the basically bivoltine strain was possible by continuous rearing of the larvae and subsequent prepupae at 18℃, which is a little higher than the developmental threshold temperature (15－16℃, Tables 5 and 6). The critical time perceived by bees seems to be larval stages (perceivable in the 5th instar larvae even after they commenced defecation).

The emergence rate of the first generation depended on the difference of latitude, which was closely related to the thermal sequence in the nesting period where bees were inhabiting. *Megachile rotundata* is a typical summer bee and commences their foraging activity when the temperature attained 24℃ on fine days. The Canadian strain is reported as almost perfectly univoltine (Hobbs and Richards, 1976; Parker, 1979). The emergence rate was usually very high (36.7－93.7%) when reared in Morioka (Table 1). Canadian populations might be partially bivoltine. Univoltine seems to have resulted from low temperatures, relating to the cool climate in Canada where the mean temperature during the 3 months' nesting period of July, August and September, at Lethbridge, Alberta, Canada is 18℃, 17.7℃ and 12.6℃ which induce prepupal diapause at a very high rate. On the other hand, the mean temperature is much higher in Morioka (21.8℃, 23.4℃, 18.7℃ and 12.1℃ in the 4 months'nesting period of July, August, September and October), where the emergence rate of the first generation was very high every year (Table 1). Actually, none of adults emerged and all 151 prepupae obligatorily entered diapause in the population (Oregonian cocoons) which was reared in the greenhouse of TNAES by releasing the overwintering generation delayedly on August 27, 1979 (Table 1).

In *M. rotudnata*, the rate of dormant prepupae (not emerged as the first generation) produced by the overwintered generation increased toward late August (Table 3), according to the decrease of ambient temperatures. On the other hand, in a partial bivoltine *Osmia coerulescens*, the rate of dormant adults (remained in their cocoons, not emerged as the first generation) increased toward middle July, according to the rise of ambient temperatures (Maeta, 2016). Both cases of *M. rotundata* and *O. coerulescens* are contrastive, but the rate of their dormant individuals seems to be determined by sequential changes of the temperatures.

In naturally diapaused prepupae, which were offspring of the first generation of the bivoltine strain and artificially induced diapausing prepupae, the subjection of them to low temperatures to break their diapause was not necessary. Their diapause was broken by just incubating at high temperatures (above 18℃). On the other hand, the Spanish univoltine prepupae needed to be

cooled for only 2 months to low temperatures (8℃－20℃) to break their diapause, but their emergence durations were longer as compared with those of other temperate megachilid bee species (Maeta *et al*., 2009). The cues and mechanisms that induce or enhance prepupal diapause, and occurrence of bivoltinism of *M. rotundata* were reported or suspected by many authors.

Both Bitner (1976) and Parker and Tepedino (1982) suggested the possible influence of day length on adult females and the subsequent frequency of nondiapusing individuals in their progeny, although in neither study was this hypothesis explicitly tested (Kemp and Bosch, 2001). Our present studies denied the effect of day length during larval development to induce prepupal diapause. Long-term post harvest storage of diapausing prepupae at constant chilling temperatures (5－15℃) results in pronounced diapause development and a decreased emergence time (Stephen and Osgood, 1965; Krunic and Hinks, 1972; Johansen and Eves, 1973; Tasei and Masure, 1978). Prior to storage at 10℃, removing of nests from the field as soon as they filled and subsequently incubating at constant 30℃ for 2－3 weeks would have the effect of minimizing loss to the next generation by repressing diapause development in the Canadian partially bivoltine strain (Rank and Rank (1990). Tepedino and Parker (1966) reared from the egg or early larval stages to the adult stage at one of four temperature regimes (16, 24, 29, 29/16℃ [12h:12h]). They mentioned that rearing *M. rotundata* immatures at temperatures of ≤ 24℃ is ineffective in increasing the number of diapausing bees and has the additional detrimental effects of increasing pre-overwintering mortality. As mentioned above, 18℃ is functional to induce a high rate of prepupal diapause. The 16℃ is below or nearly threshold temperature for the eggs and feeding larvae, and the emergence rate of the first generation was very high when immatures were reared above 20℃ (Table 2).

Parker (1979) crossed Spanish univoltine and North American partially bivoltine strains, and found that incidence of diapause was influenced by the origin of bees. Females from Spain crossed with either Spanish or American males produced mostly univoltine progeny. Progeny of the reversed crosses produced the first generation emergent adults ranging between 32 and 67%. Afterward, Parker and Tepedino (1982) confirmed that parental crosses with Spanish univoltine females yielded a significantly larger proportion of diapausing offspring than did crosses with American multivoltine females, irrespective of origin of male parent. They suggested that the sex-linked diapause trait is completely dominant and is under polygenic control.

It is very interesting to know how Eurasian univoltine bees have changed to partially bivoltine bees since invading to North America. Probably, the Eurasian strain is not genetically perfectly univoltine.

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部分的2化性種，アルファルファハキリバチにおける前蛹態休眠の人為的誘起

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北米からアルファルファの育種と採種用に導入したアルファルファハキリバチは，部分的2化性で前蛹態で休眠越冬する．1化性系統と潜在的多化性系統が同一個体群内に併存している．アメリカ合衆国とカナダから導入した個体群における2化期（第1世代）の出現率は，導入した盛岡市（北緯39°70′）では毎年非常に高かった（36.7～93.7%）．これはアルファルファハキリバチが2化性であることを示している．2化期の出現は個体群の維持にあたり障害となった．この問題を解決するために，前蛹態休眠の人為的誘引と1化性系統の選抜を試みた．結果は以下のとおりであった．1）卵，幼虫，前蛹だけを低温（発育限界温度よりもやや高い温度，卵には20℃，幼虫には18℃を用いた）で飼育し，その後のステージ（前蛹と蛹）を30℃で飼育した場合は前蛹態休眠は誘起されなかった．2）高い休眠率（最大92.0%）は幼虫とそれ以降のステージを18℃での継続飼育でもたらされた．3）同じように高率誘起は，5齢幼虫（脱糞開始後でも）それ以降のステージの18℃での継続飼育でも認められた（91.8%）．4）高率での休眠誘起（69.2～100%）は，2つの異なる温度を組み合わせた（22℃/14℃と26℃/10℃，2温度の平均は18℃，各温度で12時間交互に加温）飼育でも誘起された．両温度差が小さい組わせの方が高い休眠率が得られた．以上の結果から，低温を感知したハチにおいて休眠が誘起される臨界ステージは幼虫（脱糞後の5齢幼虫でも可能）で，前蛹態休眠はこれらのステージとその後のステージを低温（18℃）で継続飼育することで誘起されると推定された．日長は休眠誘起にまったく関与しなかった．多化性系統の個体群では，自然休眠したあるいは人為的休眠させた前蛹では休眠覚醒には低温処理は不要であった．一方，1化性系統のスペイン産のアルファルファハキリバチでは，前蛹態休眠の覚醒には8～20℃での2ヵ月間の低温処理が必要であった．1化性の選抜も4年以上の経年飼養で可能であった．

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