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▶ **To cite this version:**

Jm Flores, Ja Ruiz, Jm Ruz, F Puerta, M Bustos, et al.. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. Apidologie, 1996, 27 (4), pp.185-192. hal-00891337

HAL Id: hal-00891337
https://hal.science/hal-00891337

Submitted on 11 May 2020

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Apidologie (1996) 27, 185-192
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Original article

Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions

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(Received 4 April 1996; accepted 6 June 1996)

Summary — This paper reports improvements on a previous technique of ours for producing chalkbrood disease in *Apis mellifera* under controlled conditions. Mummification reached almost 95% (the previous technique reached 70%) when fifth instar larvae were chilled at 18 °C 24 h before sealing and kept at 25 °C for 6 days after operculation. When the larvae were chilled, but the temperature after operculation was 30 or 35 °C, mummification reached 43.65 and 29%, respectively. Percentages of mummification were lower when chilling prior to sealing cells was not applied: 77.62% (at 25 °C after sealing), 15.31% (at 30 °C) and 2.22% (at 35 °C). The effect of a high relative humidity (rh) (87%) combined with slight chilling (30 °C) induced a higher percentage of mummification (7.75%) then compared to the same temperature but lower rh (only 0.95% of larvae were mummified at 68% rh).

***Apis mellifera* / chalkbrood / *Ascosphaera apis* / brood chilling / humidity**

INTRODUCTION

Development of chalkbrood in *Apis mellifera* requires predisposing conditions; the presence of spores of *Ascosphaera apis* is not enough to induce the disease (Heath, 1982; Puerta et al, 1994). Chilling of brood and high humidity have been indicated as two of the main predisposing factors by many authors and are reviewed by Heath (1982).

Cooling of brood was considered as the predisposing condition by Bailey (1967), Lunder (1971) and Cooper (1980). However, Mehr et al (1976), Gilliam et al (1978) and Gilliam (1990) suggested that chilling was not a predisposing condition in Wyoming (cold climate), although excessive heat in a hot climate (Arizona) appeared to be a predisposing condition. Thus chilling may not be the only factor inciting chalk-

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brood. Puerta et al (1994) suggested that the average temperature of a climate does not guarantee the maintenance of uniform conditions throughout all brood developmental stages. Influence of temperature on the development of the disease could depend on the duration and severity of cooling and on the stage of brood development at which this cooling is present.

Bailey (1967) linked development of chalkbrood with chilling of the brood at about the time when larvae are sealed. Puerta et al (1994) demonstrated that chilling (22 ± 2 °C) applied 24 h before or after operculation induces about 60% mummification and the same chilling applied to older brood produces a much lower incidence.

There are few data concerning the influence of humidity on chalkbrood development, most authors assuming prevalence of the disease in damp regions (Dreher, 1938; Roussy, 1962; Lunder, 1971; Dallman, 1974) or during periods of high humidity inside the colony (Tabarly and Monteira, 1961; Albisetti and Brizard, 1979). However, none of these opinions has been supported by scientific study.

In this paper, additional studies were made of the effect of the intensity and duration of cooling on chalkbrood expression, improving the technique described before (Puerta et al, 1994). Moreover, a preliminary study of the influence of humidity on chalkbrood under controlled conditions was made.

MATERIALS AND METHODS

To study the influence of temperature during the sealing period on chalkbrood in larvae cooled just before operculation, tests were performed on unsealed fifth instar larvae (Rembold et al, 1980) taken from 12 Langstroth hives. Each larva was fed with a homogeneous suspension of 5 x 10^5 spores mixed with honey (Puerta et al, 1994), cooled (18 ± 2 °C) over 24 h and reintroduced afterwards into the hive until their cells were sealed. All brood used was sealed over a period of 16 h (larvae not sealed during this period were eliminated) and maintained after sealing in an incubator at three different temperatures (25, 30 and 35 °C) and a relative humidity (rh) of 68%. Six days after sealing, the cells were opened and the percentage of larvae mummified (PM) was checked. This first experiment (*Exp 1*) was repeat seven times (eight tests).

To study the influence of temperature of sealed brood on chalkbrood, the procedure was the same as before, but the larvae did not receive the cooling stress before operculation. This second experiment (*Exp 2*) was also repeated seven times.

To study the influence of humidity as a predisposing condition in chalkbrood, the procedure differed from *Exp 1* in only two details: fifth instar larvae were kept at 30 °C throughout the test (24 h before sealing and the period of 6 days after sealing) and two levels of rh (68 and 87%) were applied in the incubator. This third experiment (*Exp 3*) was repeated six times (seven tests)

Larvae used in each replicate were taken from only one colony. The number of larvae used per test are presented in tables I and II. In *Exp 1* and *Exp 2*, all live larvae of the 25 °C group not mummified after 6 days were kept at 35 °C to check viability and pupation.

Data were analysed using a general linear models procedure (SAS Institute, 1982). Data from a group were considered to differ significantly from the others within a 95% fiducial limit. A two-way ANOVA (stress before sealing x temperature after sealing) and single ANOVA was made, with multiple range analysis.

RESULTS

Data concerning temperature (*Exp 1* and *Exp 2*) are presented in figure 1 and table I. In both tests, all live larvae kept at 25 °C and not mummified were still prepupae when the cells were opened 6 days after sealing. After being maintained at 35 °C afterwards, all prepupae turned into pupae and none of them showed chalkbrood symptoms.

When the influence of cooling before sealing was checked (*Exp 1*), there were

significant differences in PM between 25 and 30 °C but not between 30 and 35 °C. There was also a progressive increase in percentage mummified (PM) when temperature of development of sealed brood decreased: 95% at 25 °C, 43.65% at 30 °C and 29% at 35 °C.

Differences in PM at three temperatures also appeared when cooling was not applied (*Exp 2*): 77.62%, 15.31% and 2.22% at 25, 30 and 35 °C, respectively. At the same temperature, PM in *Exp 1* and *Exp 2* differed significantly only at 25 °C. No interaction between cooling and tem-

Table I. Effect of three temperatures (25, 30 and 35 °C) of sealed brood on chalkbrood. (**A**) with cooling stress on L5 (18 °C 24 h before operculation) and (**B**): without cooling stress on L5.

		Test								*Total*
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
A										
25 °C	Larvae	10	7	15	12	0	20	24	38	126
	Mummies	9	6	14	12	0	19	23	38	121
	Healthy	0	0	0	0	0	1	1	0	2
	Dead	1	1	1	0	0	0	0	0	3
30 °C	Larvae	31	13	16	16	9	7	30	3	125
	Mummies	13	7	8	12	2	3	9	1	55
	Healthy	18	5	8	4	7	3	21	2	68
	Dead	0	1	0	0	0	1	0	0	2
35 °C	Larvae	33	16	0	20	10	15	20	25	139
	Mummies	25	1	0	0	1	3	4	0	34
	Healthy	7	15	0	19	8	12	16	25	102
	Dead	1	0	0	1	1	0	0	0	3
B										
25 °C	Larvae	37	52	21	57	45	39	31	87	369
	Mummies	34	39	13	49	17	38	22	87	299
	Healthy	3	13	8	8	27	1	9	0	69
	Dead	0	0	0	0	1	0	0	0	1
30 °C	Larvae	63	70	58	71	33	35	32	64	426
	Mummies	8	5	5	7	2	6	10	19	62
	Healthy	55	65	53	63	31	29	21	45	362
	Dead	0	0	0	1	0	0	1	0	2
35 °C	Larvae	27	62	44	93	35	33	10	23	327
	Mummies	1	0	1	3	3	0	0	0	8
	Healthy	26	61	42	89	32	29	10	23	312
	Dead	0	1	1	1	0	4	0	0	7

Number of larvae used in each test (larvae) are composed of mummies produced (mummies), larvae alive (healthy) and dead not mummified larvae (dead).

Table II. Effect of two levels of humidity in chalkbrood disease.

		Test							*Total*	*Larvae mummified* $\bar{x} \pm sd$ (%)
		1	*2*	*3*	*4*	*5*	*6*	*7*		
68% rh	Larvae	17	32	19	40	41	24	21	194	
	Mummies	0	0	0	1	0	1	0	2	
	Healthy	17	31	19	39	41	22	20	189	0.95 ± 1.70
	Dead	0	1	0	0	0	1	1	3	
87% rh	Larvae	25	30	10	26	49	8	39	187	
	Mummies	0	2	2	1	3	1	2	11	
	Healthy	23	26	8	24	46	7	36	170	7.75 ± 6.57
	Dead	2	2	0	1	0	0	1	6	

perature after sealing was detected with respect PM when the two-way ANOVA was applied.

Data concerning humidity (*Exp 3*) are presented in table II. Although there were significant differences between PM at the two levels of humidity, a few mummies were detected (0.95 and 7.75% at 68 and 87% rh, respectively).

Significant differences were also found in PM between tests of *Exp 3* and *Exp 2* at 30 °C; PM was higher in the latter.

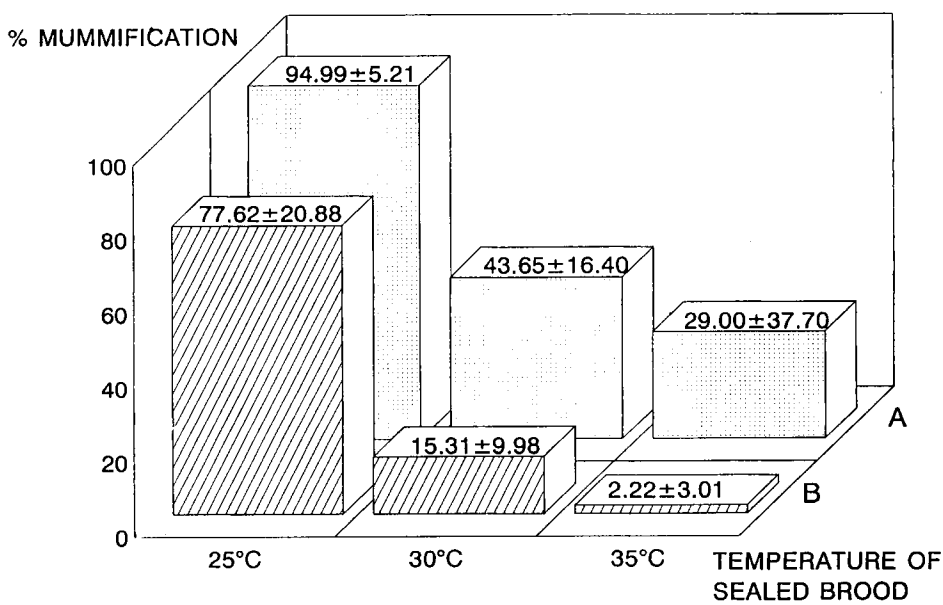


Fig 1. Percentage of mummification (mean + sd) at three temperatures: (**A**) with a cooling stress on L5 (18 °C 24 h before sealing) and (**B**) without the cooling stress. Data from table I.

At the highest humidity, a permanent condensation of water was detected inside the incubator, and fungal growth from the pieces of comb containing larvae was usual, but this growth was not identified as *A apis* when examined microscopically.

DISCUSSION

These results confirm again that *A apis* is an opportunistic pathogen that kills larvae only when they are subjected to a predisposing condition (Bailey, 1967; Heath, 1984; Puerta et al, 1994). Although temperature in the brood area can fluctuate between 35.5 and 18 °C (Cooper, 1980), the optimal temperature range for brood rearing is 30–35 °C (Winston, 1987). Several studies have linked high rh in the hive with chalkbrood (Tabarly and Monteira, 1961; Albisetti and Brizard, 1979). At appropiate conditions for brood development (30–35 °C and moderate humidity), mummification reaches low rates: 2.22% in *Exp 2* (35 °C, 67% rh), and 0.95% in *Exp 3* (30 °C, 67% rh) in spite of the large amount of spores (5×10^5) used.

One of the main obstacles in investigating of chalkbrood in *A mellifera* is the difficulty in inducing this disease in a repeatable way. The usual technique has been to supply spores to the hive inside pollen cake patties or in a sugar suspension (Gilliam et al, 1978; Milne, 1982; Gilliam et al, 1983; Gilliam, 1986; Gilliam et al, 1988, Befus-Nogel et al, 1992; Spivak and Gilliam, 1993). This method shows some problems like the appearance of unforeseeable chalkbrood levels in the control colonies or the variability in chalkbrood levels in inoculated colonies. Supplying a high number of spores to the hive has been considered a predisposing condition itself (Koenig et al, 1987). As the concentration of spores rises in the colony, the probability of an outbreak of chalkbrood also increases. This can occur because the simultaneous presence of spores inside the gut of susceptible brood and a predisposing condition is then a more likely event. However the distribution and amount of spores inside the brood population and the real influence of predisposing conditions on susceptible brood are not easily controlled in hives with the cited techniques. These factors may account for the variability in their results.

A technique we reported previously (Puerta et al, 1994) reduced these problems, but PM only reached 70%. The new technique described in this paper gives a higher PM (near 95%) when brood is cooled before operculation at 18 °C and maintained afterwards at 25 °C for several days. Besides, it is possible to reach different chalkbrood levels within a wide range (from 2.22 to 94.99% of PM).

Bailey (1967) pointed out that chilling applied immediately after the brood is sealed is unlikely to occur frequently in the hive. From *Exp 2* it is clear that an intense and short cooling after the sealing of cells is not the only situation which incites the disease. A slighter but longer period of cooling during the prepupal stage can also induce chalkbrood (77.62% of brood mummified when 25 °C is maintained after sealing).

Some of the techniques used for inciting the disease, such as reducing the number of adult bees (Gilliam et al, 1978; Koenig et al, 1987) or changing the situation of combs (Befus-Nogel et al, 1992) could result in cooling. Furthermore, as suggested by Heath (1982), peaks of chalkbrood induced in observation hives and mating nuclei can be produced by low temperatures in brood development. A short chilling followed by a moderate cooling could be a common situation after division of colonies.

Data from *Exp 3* show differences in PM when the highest humidity is applied. A level of humidity of 87% could be reached in a colony in the nectar flow, but the PM at this rh is not very high (7.75%). This is lower than the 15.31% reached in *Exp 2* at the

same temperature, but a lower humidity. Besides, there is also a difference in the mortalities between *Exp 2* at 30 °C (15.31 ± 9.98%) and *Exp 3* at 68% rh (0.95 ± 1.70). There is thus an uncontrolled factor which induces a wide variability in some of our results at 30 °C. Perhaps 30 °C is a critical temperature for measuring chalkbrood incidence, representing the mid-point between a cooling which incites symptoms in most of the brood (25 °C) and an optimal temperature for brood development (35 °C) which incites a low PM.

The technique used in *Exp 1* allowed us to reach almost 95% mummification in a controlled way. This high percentage will enable us to test treatments, predisposing conditions, epidemiology and other aspects of the disease which may help to control it.

ACKNOWLEDGMENTS

Funds were provided by a grant from INIA 'Chemotherapeutic control of chalkbrood and population dynamics of *Varroa jacobsoni* in *Apis mellifera iberica*' (SC93-029-C44) and by the Consejerìa de Agricultura, Pesca y Alimentación (Junta de Andalucía, Spain), which also gave financial support. This study was developed in the Centro Andaluz de Apicultura Ecológica (Universidad de Córdoba-UAGA). Thanks are also given to C Moralobo and RMoreno.

Résumé — Effet de la température et du taux d'humidité du couvain operculé sur le couvain plâtré en conditions contrôlées. Cette expérimentation a non seulement amélioré la technique de Puerta et al (1994), par laquelle on peut induire de façon contrôlée le couvain plâtré (ou ascosphérose, causée par *Ascosphaera* apis), en obtenant un taux de momification des nymphes de 95 %, mais a permis aussi d'étudier l'influence de la température sur le développement des larves operculées et l'influence du taux d'humidité sur le développement de l'ascosphérose. Trois types d'expériences (Exp) ont été réalisées. À chaque fois des larves de stade 3 ont été infestées artificiellement avec des spores d'*A apis* par voie orale. Dans les deux premières expériences (Exp 1 et Exp 2) les larves ont été maintenues après l'operculation aux températures suivantes : 25, 30 et 35 °C. Elles ont subi un refroidissement à 18 °C durant les 24 heures précédant l'operculation dans Exp 1, mais pas dans Exp 2. Le taux de momification (PM) a atteint 94,99, 43,7 et 29,0 % dans Exp 1 et 77 %, 15,3 et 2,2 % dans Exp 2, respectivement pour les trois températures étudiées (fig 1 ; tableau I). La discussion a porté sur les conditions dans lesquelles ces trois situations thermiques peuvent se rencontrer dans la colonie (refroidissement fort mais court, suivi d'un refroidissement plus long mais moins fort). Dans Exp 3, deux taux d'humidité relative (HR) ont été étudiés : 68 et 87 %. Comme décrit plus haut, les larves ont reçu en nourrissement des spores d'*A apis* et ont été maintenues à 30 °C, température limite pour le développement de l'ascosphérose. Dans les deux cas le PM a été très bas : 1,03 % pour le taux d'HR le plus bas et 5,88 % pour le plus élevé (tableau II). Bien qu'il existe une différence significative du PM entre les deux taux d'HR, en faveur du plus élevé, il ne semble pas que l'humidité ambiante soit un facteur déclenchant pour la maladie.

***Apis mellifera* / couvain plâtré / *Ascosphaera apis* / refroidissement / humidité**

Zusammenfassung — Einfluß von Temperatur und Feuchtigkeit der verdeckelten Brut auf die Entwicklung der Kalkbrut unter kontrollierten Bedingungen. Durch die Verbesserung der Technik von Puerta et al (1994), die eine kontrollierte Entwicklung der Kalkbrut (Ascosphaeriosis) induziert, wurde eine Mumifizierung von 95% erreicht. Dazu wurden die Larven

während der 24 Stunden vor der Verdeckelung auf 18 °C abgekühlt und in den 6 Tagen nach der Verdeckelung eine Temperatur von 30 °C eingestellt. Dieser Versuch hat nicht nur die Technik verbessert, mit der man kontrolliert die Ascosphaeriosis hervorrufen kann, es wurde mit dieser Methode auch der Einfluß der Temperatur auf die Entwicklung der verdeckelten Larven und der Einfluß des Feuchtigkeitsgrades auf die Entwicklung von Ascosphaeriosis untersucht. Es wurden drei Verschiedene Versuche (EXP) durchgeführt, bei denen in allen Fällen Larven im dritten Stadium oral mit Sporen von *Ascosphaera apis* künstlich infiziert wurden. In den ersten beiden Versuchen (EXP1 und EXP2) wurden die Larven nach der Verdeckelung konstant auf drei verschiedenen Temperaturen gehalten: 25 °C, 30 °C und 35 °C. Beim ersten Versuch (EXP1) wurden die Larven in den ersten 24 Stunden vor der Verdeckelung auf 18 °C gekühlt gehalten, im zweiten Versuch (EXP2) jedoch nicht. Die Prozentsätze der Mumifizierung (PM) des ersten Versuches (EXP1) betrugen 94,99 %, 43,7 % und 29,0 % für die entsprechenden Temperaturen nach der Verdeckelung. Im zweiten Versuch (EXP2) waren 77,6 %, 15,3 % und 2,2 % der Puppen bei den entsprechenden Temperaturen mumifiziert (Abb 1; Tab I). Es wurden die Bedingungen diskutiert, unter denen diese drei thermischen Situationen in den Bienenvölkern entstehen können (eine starke, jedoch relativ kurze Abkühlung, gefolgt von einer weiteren jedoch weniger starken Abkühlung). Bezüglich der Rolle des Feuchtigkeitsgrades wurden zwei verschiedene Werte untersucht: 68 % und 87 % relative Luftfeuchtigkeit. Wie oben beschrieben' wurden Larven mit Sporen von *A apis* gefüttert und bei 30 °C, der Grenztemperatur für eine Entwicklung von Ascosphaeriosis, gehalten. Der PM ist in beiden Fällen sehr niedrig: 1,03 % beim niedrigen und 5,8 % beim höheren relativen Feuchtigkeitsgrad (Tab II). Obwohl ein deutliche höherer PM bei den höheren Feuchtigkeitsgraden besteht, scheint der Ausbruch der Krankheit nicht durch die Luftfeuchtigkeit hervorgerufen zu werden.

***Apis mellifera* / Kalkbrut / *Ascosphaera apis* / Abkühlung / Feuchtigkeitsgrad**

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