Apidologie 40 (2009) 524–534
© INRA/DIB-AGIB/ EDP Sciences, 2009
DOI: 10.1051/apido/2009029

Available online at:
www.apidologie.org

Original article

Differential susceptibility across honey bee colonies in larval chalkbrood resistance*

Annette Bruun JENSEN[1], Bo Vest PEDERSEN[2], Jørgen EILENBERG[1]

[1] Department of Ecology and Agriculture, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
[2] Centre for Social Evolution, Department of Biology, University of Copenhagen, Universitetsparken 15, 2100 Copenhagen, Denmark

Received 25 June 2008 – Revised 18 November 2008 – Accepted 9 December 2008

Abstract – Chalkbrood susceptibility of in vitro reared honey bee larvae was investigated. Larvae were grafted from 3–4 colonies headed by pure mated queens of *Apis mellifera carnica*, *A. m. ligustica* and *A. m. mellifera*, respectively. Three day old larvae were fed with different dosages of *Ascophaera apis* spores and a clear dose-response relationship was shown. Over the whole experiment LD_{50} estimates ranged from 55 to 905 spores. The response differed significantly (up to a factor ten) between colonies of the same subspecies. The mean time to death decreased with increased dose, with more larvae dying faster after eating more fungal spores. The *A. m. ligustica* larvae used in this study were less susceptible to *A. apis* than *A. m. mellifera* and *A. m. carnica* larvae. However due to the limited number of colonies included and the high variation shown we cannot predict that any *A. m. ligustica* colony is better adapted to cope with *A. apis* than colonies of *A. m. carnica* and *A. m. mellifera*.

Ascosphaera apis **/ bioassay / brood disease / insect pathogen / in-vitro rearing**

1. INTRODUCTION

Ascosphaera apis (Maassen ex Claussen) Spiltoir and Olive (1955) is an important fungal pathogen of the honey bee (*Apis mellifera* L.) and it is the causal agent of the chalkbrood disease (Gilliam and Vandenberg, 1997). *A. apis* only produces sexual spores and is heterothallic, thus spores are only produced when mycelia of the two opposite mating types come together and fruiting bodies are formed (Aronstein et al., 2007). The honey bee larvae primarily get infected upon ingesting these spores with their food. In the lumen of the gut the spores germinate due to the presence of a high CO_2 content (Heath and Gaze, 1987). The hyphae then penetrate the peritrophic membrane, epithelia cells and basal membrane before entering the haemocoel for further growth. Mycelia growth has also been observed in the fat bodies and other larval tissue (Chorbiński, 2004). After colonizing the larva cavity, the hyphae penetrate the cuticle from the inside and finally the entire larvae body surface will be covered with white mycelium and /or eventually dark fruiting bodies. However, *A. apis* does not produce chitinase like many other insect pathogenic fungi (St. Leger et al., 1993), but N-acetyl-β-glycosaminidase and protease, enzymes that destroy protective insect body barriers (Alonso et al., 1993; Theantana and Chantawannakul, 2008).

To combat a fungal disease like chalkbrood the honey bee has developed individual as well as social immune systems. The larval resistance can be divided into two main

Corresponding author: A.B. Jensen, abj@life.ku.dk
* Manuscript editor: David Tarpy

categories of defence reactions: cell-mediated responses such as phagocytosis or encapsulation performed by haemocytes and cell free defence mechanisms such as antimicrobial peptides, small proteins and lysosymes all which mainly are produced in cells by the fat body (Glinsky and Buczek, 2003; Schmid-Hempel, 2005). Honey bees, however, carry fewer known immune system genes than the fruit fly *Drosophila melanogaster* Meigen or the malaria mosquito *Anopheles gambiae* Giles as revealed by genomic analyses. This finding was unexpected since social life with crowded quarters and close relatedness between the individuals comes with additional risks for diseases to spread (Honeybee Genome Sequencing Consortium, 2006) and indicates that powerful compensatory mechanism to secure diseases resistance are probably in place.

The honey bees have developed several types of behaviour in order to avoid, control or eliminate an intruding pathogen (Cremer et al., 2007). The age dependent division of labour and nest mate recognition will reduce the uptake and intake of spores from the environment as well as it will decrease the spread between colonies (Naug and Camazine, 2002). More profound is the hygienic behaviour where fungal killed larvae and pupae, also called mummies, are pulled out of the cells by young worker bees and dumped outside the colony; an example of a sophisticated social behaviour (Gilliam et al., 1988). Slightly chilling of newly capped brood increases the incidence of *A. apis* significantly. This can easily occur in colonies that temporarily have an insufficient numbers of adult bees to incubate their brood adequately around 34–35 °C (Bailey, 1967; Puerta et al., 1994; Flores et al., 1996). Such chilling is more likely to occur in colder climates, however colonies exposed to *A. apis* can increase nest temperature, that supposedly inhibit the development of the chalkbrood disease (Starks et al., 2000). In addition, honey bees collect antimicrobial substances from the environment and use them prophylactically e.g. propolis or antagonistic microorganisms collected from pollen that inhibit *A. apis* growth (Sahinler and Kurt, 2004; Gilliam et al., 1988).

Recent molecular analyses suggest that *A. mellifera* originated in Africa and expanded into Europe twice, resulting in a C lineage South East of the Alps and a M lineage North West of the Alps, that are geographically close but genetically distant (Whitfield et al., 2006). Within the two lineages several locally adapted races and subspecies then developed, which can be distinguished based on morphology and ecological traits (Ruttner, 1988). The subspecies *A. m. mellifera* belongs to the M lineages and is supposed to be better adapted to survive in the colder North European climate as opposed to the Mediterranean subspecies *A. m. ligustica* and *A. m. carnica* which belong to the C lineage and *A. m. mellifera* may therefore also be better adapted to cope with chalkbrood. Many honey bee breeders around the world today are breeding bees derived from these three subspecies either in pure stocks or as hybrids. Assessing chalkbrood resistance in different breeding stocks might be valuable in future breeding programs.

The aim of the current study was to develop a method for investigation of the susceptibility of honey bee larvae of *A. mellifera* to *A. apis.* We aimed to compare colonies of *A. m. carnica*, *A. m. ligustica* and *A. m. mellifera*. This was done by testing the individual immunity of larvae exposed to different controlled dosages of *A. apis.*

2. MATERIALS AND METHODS

2.1. Honey bee

Three groups of colonies headed by queens of three different European honey bee subspecies: *A. m. carnica*, *A. m. ligustica* and *A. m. mellifera* were established each in three different apiaries. All queens were naturally mated with drones of their respective subspecies in to ensure progeny of pure races. The four established *A. m. carnica* queens originated from three different breeding lines, three of the queens were bought from Danish queen breeders that utilise island mating (two of the queens were sisters) and one queen was imported from Slovenia. The three established Italian queens all originated from different breeding lines, two of those were bought from Danish queen breeders that

utilise island mating and the third queen was imported from Italy. The four *A. m. mellifera* queens originated from Læsø (an isolated island hosting the main population of *A. m. mellifera* in Denmark (Jensen et al., 2005)), but they were all mated on another isolated island in Denmark.

To verify the subspecies of the *A. m. carnica* and *A. m. ligustica* queens the mtDNA region including the tRNALeu gene, the COI-COII intergenic region and the 5' end of the COII gene was amplified from one bee per colony using a protocol described by Garnery et al. (1993). The PCR product was run on 1.5% agarose gel for size determination and sequenced from both ends.

2.2. In vitro rearing of worker larvae

Larvae were reared in vitro following the protocol of Aupinel et al. (2005) with a few modifications. Larvae were reared individually directly in 48-well tissue culture plates with a diet consisting of 50% of Chinese fresh frozen royal jelly (v/v) (Sonnentracht Imkerei GmbH, Bremen, Germany), 6% D-glucose (w/v), 6% D-fructose (w/v) and sterile deionised water. The diet was mixed and frozen in smaller aliquots and was pre-heated to 34 °C before being used for feeding. Larvae younger than 24 hours were taken from the combs using a Swiss grafting tool (Swinty, Sønderborg, Denmark) and carefully transferred to a droplet of 10 μL diets. Larval age was estimated by size, since mean weights of different age groups differs significantly (Brødsgaard et al., 1998). The subsequent days the larvae were feed once a day with 10 μL (day 2), 20 μL (day 3), 30 μL (day 4), 40 μL (day 5) and 50 μL (day 6) summing up to 160 μL diet in all. By this feeding regime no additional transfer of the larvae with the risk of injury was necessary. The larvae were placed within a humid chamber to ensure a high relative humidity and thereby avoiding larval dehydration. The humid chambers were placed in an incubator at 34 °C except day 6 where they were incubated at 28 °C for 24 hours. The incubation at a low temperature was included, since chilling of the brood just upon capping has a significant effect on the development of the chalkbrood disease (Puerta et al., 1994; Flores et al., 1996). When the larvae started to defecate the wells were gently cleaned with small cotton sticks.

2.3. Preparation of *A. apis* spore suspensions and spore viability test

A stock of 50 black mummies originating from a single honey bee colony naturally infected by *A. apis* was grinded dry and stored at 4 °C. Spore suspensions were made by transferring approximately 0.01 gram dry stock to a sterile glass tissue homogenizer. 50 μL sterile deionised water was added and thereafter grinded using a glass pistil for two minutes to break apart spore cysts and spore balls. The released spores were mixed with 1 mL sterile deionised water. Large particles in the suspension were allowed to settle for 20 min, and a sample of approximately 0.5 mL was taken from the middle of the suspension using a pipette. A haemocytometer was used to determine the spore concentrations which, were usually around 1.0×10^8 spores per ml. Fresh spore solutions were made the day of each infection experiment.

Spore viability was tested following the protocol of James and Buckner (2004) with a few modifications. 100 μL spore suspension of a concentration of 2.0×10^7 spores per ml was mixed with 500 μL GLEN, a liquid medium suitable for germination and in vitro growth of fastidious insect pathogenic fungi (Beauvais and Latgé, 1988; Jensen et al., 2001). Ten μL was placed onto each of the six mm spot on sterile Teflon coated slides, which were placed in sterile Petri dishes lined with wet filter paper. Each Petri dish was subsequently placed in an airtight container. The container was flushed with CO_2 several times and incubated 24 hours at 34 °C. A cover slip was added and the germination percentage was determined using differential interference contrast microscopy at 400× magnification. 100 spores were evaluated for enlargement or germ tube formation in three different fields of view.

2.4. Fungus treatment procedure

A. apis spores were thoroughly mixed with the larval diet by vortexing. Five different concentrations were prepared: 2.0×10^5, 1.0×10^5, 2.0×10^4, 1.0×10^4 and 2.0×10^3 spores per mL. According to Bailey (1967) larvae are most susceptible to chalkbrood disease when they are three-four days old thus on the second day after grafting the larvae were feed five μL infectious diet and approximately six hours later 15 μL normal diet. The larvae were fed five μL infectious diet first to increase the changes of a quick ingestion of all the spores. Control larvae

were treated similarly; they were first fed five μL uninfectious normal diet and later on 15 μL diet.

We grafted 24–48 larvae (a half or one plate) per spore concentration and control, giving a total of 144–192 larvae per colony per experiment. Larvae dying during the first two days after grafting were excluded from the experiment, since they died before the fungus treatment. All colonies of the same apiary were grafted on the same day and all experiments were repeated on different dates.

Control and treated larvae were observed every day under a dissection microscope. Dead larvae were classified as dead by ceased respiration, loss of body elasticity or a change to gray or brown colours. Larval mortality and *A. apis* infection in laboratory bioassays was recorded daily. Dead larvae were classified as dead of *A. apis* when hyphae were growing out of the body. All experiments were terminated nine days after the treatment since we in a preliminary experiment showed that only a very few individuals that were kept until the emergence of the adults, died of *A. apis* after nine days. In total 5364 larvae were exposed to different dosages of *A. apis* spores.

2.5. Statistical analysis

Data on mortality due to *A. apis* nine days post treatment was subjected to probit analysis to generate dose–mortality regression lines using the Polo Plus ver 2.0 in the LeOra software package (Robertson and Preisler, 1992). Dose–mortality regression lines were generated for colony separately, and the slopes and LD_{50}'$_{ies}$ and LD_{90}'$_{ies}$ were compared as described by Robertson and Preisler (1992). LD's of the colonies were considered significantly different if the LD ratios of the 95% confidence limit (CL) did not include 1.0, since this is a more powerful data interpretation than just using the 95% confidence intervals (Payton et al., 2003).

Mean time to death defined as the number of days needed to achieve an accumulated 50% mortality was estimated by use of the Kaplan–Meier product-limit estimator survivorship analysis (PROC LIFETEST, SAS ver. 9.1) since it can handle censored as well as uncensored data. Censored data are those that arise when the dependent variable represents the time to a terminal event, in our case nine days. We only included those larvae that died due to the fungal infection and survivors. Pairwise comparisons were performed using a log-rank chi-squared test.

3. RESULTS

3.1. Haplotype designation

Sequences of the mtDNA region including the tRNALeu gene, the COI-COII intergenic region and the 5' end of the COII gene of the *A.m. ligustica* colonies were in agreement with the subspecies designation given by the breeders. They all had the C1 haplotype which is typical *A. m. ligustica* (Frank et al., 2001). In the four *A. m. carnica* colonies we found two different haplotypes. Colony 1, 2 and 3 had the C11 haplotype which is found in Turkey (Solorzano et al., 2009) and is common in US (Szalanski A.L., pers. comm.) and colony 4 had the C2C haplotype which is typical for *A. m. carnica* in Slovenia (Sušnik et al., 2004).

3.2. Spore germination and control mortality

The germination was determined based on the enlargement after 24 hours. Mean (±SE) spore germination across all bioassays was 80.3 (±2.3) %, which was regarded fully satisfactory. Control mortality was below 20%.

3.3. Dose mortality relationship

The dose–mortality regression lines for each colony nine days post treatment within each of the three groups were significantly different from each other (χ^2 = 68.40; df = 6; $P < 0.05$) *A. m. carnica*; 68.40, (χ^2 = 19.22; df = 4; $P < 0.05$) *A. m. ligustica* and (χ^2 = 68.39; df = 6; $P < 0.05$) *A. m. mellifera.*

The LD_{50} over all the colonies span from 55 to 905 spores and the LD_{90} from 1.27×10^3 to 4.18×10^4 spores, thus the lethal dose ratio between of the LD_{50} of the most (*A. m. carnica*) and least (*A. m. ligustica*) susceptible colony was 16.4, and the lethal dose ratio between the LD_{90} of the most (*A. m. mellifera*) and least (*A. m. ligustica*) susceptible colony was 33.4 (Tab. I).

There were significant differences of the LD_{50}'$_{ies}$ and LD_{90}'$_{ies}$ between some of the colonies of the same group, except for the

Table I. Lethal doses LD_{50} and LD_{90} of *Ascosphaera apis* spores feed to third instars honey bee (*Apis mellifera)* larvae originating from three different subspecies, *A. m. carnica*, *A. m. ligustica* and *A. m. mellifera.*

Subspecies	n^a	Slope±SE	H-index[b]	LD^C_{50} (95% CL)	All[d]	LD^c_{90} (95% CL)	All[d]
A. m. carnica							
Colony 1	347	0.74 ± 0.09	1.78	113.3 (44.7–219.1)	a	6.20×10^3 (2.37–37.01)	de
Colony 2	408	0.99 ± 0.09	1.51	530.4 (341.7–831.2)	c	1.02×10^4 (0.49–3.35)	e
Colony 3	388	0.88 ± 0.09	1.88	113.6 (55.3–199.3)	a	3.29×10^3 (1.49–12.56)	cd
Colony 4	293	0.93 ± 0.16	1.14	55.1 (11.1–123.0)	a	1.33×10^3 (0.63–5.47)	abc
A. m. ligustica							
Colony 1	715	0.67 ± 0.06	1.82	534.6 (329.8–918.5)	c	4.18×10^4 (1.43–24.56)	f
Colony 2	593	0.68 ± 0.07	1.88	446.4 (234.7–854.8)	bc	3.51×10^4 (1.11–25.91)	f
Colony 3	626	0.88 ± 0.80	2.19	905.0 (570.2–1567)	d	2.55×10^4 (1.02–11.54)	ef
A. m. mellifera							
Colony 1	422	0.69 ± 0.09	1.60	78.0 (28.8–149.5)	a	5.61×10^3 (2.15–34.55)	cde
Colony 2	578	0.88 ± 0.10	1.69	259.9 (124.3–444.2)	b	7.41×10^3 (3.52–26.57)	cde
Colony 3	487	1.26 ± 0.11	1.90	291.9 (198.1–421.9)	b	3.07×10^3 (1.78–7.03)	bcd
Colony 4	507	1.18 ± 0.09	1.65	104.2 (68.6–145.0)	a	1.27×10^3 (0.79–2.44)	a

[a] Total number of larvae tested from each colony. [b] Goodness of fit χ^2 divided by the degree of freedom. [c] Total number of spores ingested. [d] LD's within each column followed by the same letter are not significant different.

LD_{90} between the three *A. m. ligustica* (Tab. I). Among the three groups the highest variation was found within *A. m. carnica*, here lethal dose ratios of a factor 9.6 for the LD_{50} and a factor 7.6 for the LD_{90} between the most and the least susceptible colony were shown.

Due to the different dose mortality responses between the colonies from the same group we could not merge all data per group to test for the overall group response. However in the pair wise comparison of LD_{50} and LD_{90}, all three *A. m. ligustica* colonies were significant less susceptible than several of the *A. m. carnica* and *A. m. mellifera* colonies (Tab. I).

3.4. Time mortality relationships

Already two days after the larvae were fed with *A. apis* spores a few *A. m. mellifera* larvae died of fungal infections and hyphae penetration were observed. The first *A. m. carnica* and *A. m. ligustica* larvae that died of fungal infections were recorded three days post inoculation.

The mean time to death decreased with increased spore concentration. Within each colony there was significant difference of the mean time to death when the larvae were feed with 1000 and 10 000 spores respectively (Tab. II) and as seen in Figure 1 the cumulative mortality of the infected larvae was throughout dependent on the spore concentration. The higher concentration the higher a percentages of the exposed larvae died earlier of fungal infection.

When the larvae were fed the highest dosage, 10 000 spores, there was no significant differences in the mean time to death between colonies of the same group ($\chi^2 = 3.38$; df = 3; $P = 0.34$) *A. m. carnica*; ($\chi^2 = 2.372$; df = 2; $P = 0.31$) *A. m. ligustica* and ($\chi^2 = 1.29.$; df = 3; $P = 0.73$) *A. m. mellifera*. At a dosage of 1000 spores there was significant difference in the mean time to death between the four *A. m. carnica* colonies ($\chi^2 = 11.68.$; df = 3; $P = 0.009$) and between the four *A. m. mellifera* colonies ($\chi^2 = 9.91.$; df = 3; $P = 0.019$) respectively, however no significant difference was observed between the three *A. m. ligustica* colonies ($\chi^2 = 0.26.$; df = 2; $P = 0.88$).

The larvae from *A. m. carnica* and *A. m. mellifera* died significant quicker than *A. m. ligustica* larvae ($\chi^2 = 34.16$; df = 1; $P > 0.0001$) and ($\chi^2 = 27.51$; df = 1; $P > 0.0001$) when fed 10000 spores, however there were no significant differences between the mean time

Table II. Mean time to death (LT_{50}) of third instar honey bee (*Apis mellifera*) larvae fed with two different doses of *Ascosphaera apis* spores. The larvae originated from three different honey bee subspecies, *A. m. carnica*, *A. m. ligustica* and *A. m. mellifera.*

Subspecies	Dose of 1000 spores		Dose of 10 000 spores	
	n[a]	LT_{50} ± SE (days p.i.)	n[a]	LT_{50} ± SE (days p.i.)
A. m. carnica				
Colony 1	104	6.57 ± 0.21	71	4.96 ± .025
Colony 2	127	6.65 ± 0.21	63	5.24 ± 0.28
Colony 3	120	6.43 ± 0.17	62	4.61 ± .020
Colony 4	72	6.06 ± 0.2	51	5.20 ± 0.2
All				5.00 ± 0.12
A. m. ligustica				
Colony 1	197	6.79 ± 0.17	109	5.83 ± 0.23
Colony 2	115	6.97 ± 0.21	79	5.82 ± 0.24
Colony 3	203	6.89 ± 0.16	116	6.24 ± 0.22
All				5.98 ± 0.13
A. m. mellifera				
Colony 1	90	6.58 ± 0.21	45	4.96 ± 0.25
Colony 2	154	6.65 ± 0.21	80	5.24 ± 0.28
Colony 3	119	6.43 ± 0.17	83	4.61 ± 0.20
Colony 4	142	6.06 ± 0.20	75	5.20 ± 0.23
All				5.26 ± 0.11

[a] Total number of larvae tested from each colony.

to death of *A. m. carnica* and *A. m. mellifera* (χ^2 = 1.92.; df = 1; P = 0.17). A pair wise comparison on the merged dataset of each group was not performed at the 1000 dose due to the significant within group variation; however the three *A. m. ligustica* colonies had the highest mean time to death (Tab. II).

4. DISCUSSION

We have shown that in vitro rearing is a practicable routine method to study susceptibility of individual honey bee larvae to *A. apis* under controlled conditions excluding the social immunity. Previous in vitro rearing of larvae have been used successfully to study bacterial brood pathogens; American or European foulbrood (Brødsgaard et al., 1998; McKee et al., 2004; Genersch et al., 2005). All the eleven honey bee families included in the current study were found to be susceptible to *A. apis* spores harvested from mummies collected in a single naturally infected colony.

Three day old larvae were fed with different dosages of *A. apis* spores and a clear dose-response relationship were shown. Over the whole experiment LD_{50} estimates ranged from 55 to 905 spores. Glinski (1981) found LD_{50} values between 580 and 4700 spores using 3–4 days old larvae from a single *A. mellifera* colony thus it is in the upper range of what we found. These differences might be due to the experimental settings; Glinski (1981) used in vitro spores from different fungal isolates grown on agar plates whereas we used spores harvested directly from infected mummies and also the temperature regimes varied. In Glinski (1981) the larvae were held at constantly at 25 °C, whereas our larvae were held at 34 °C, the normal brood rearing temperature, and only chilled to 28 °C 24 hours when they reached the prepupal stage.

The progeny of the different pure breed queens was found to differ in susceptibility to the tested spore concentration within each of the three subspecies tested *A. m. carnica*, *A. m. ligustica* and *A. m. mellifera*. The *A. m. carnica* colonies had the most variable dose-mortality response with up to a factor 10 difference in the LD_{50} estimates between larvae

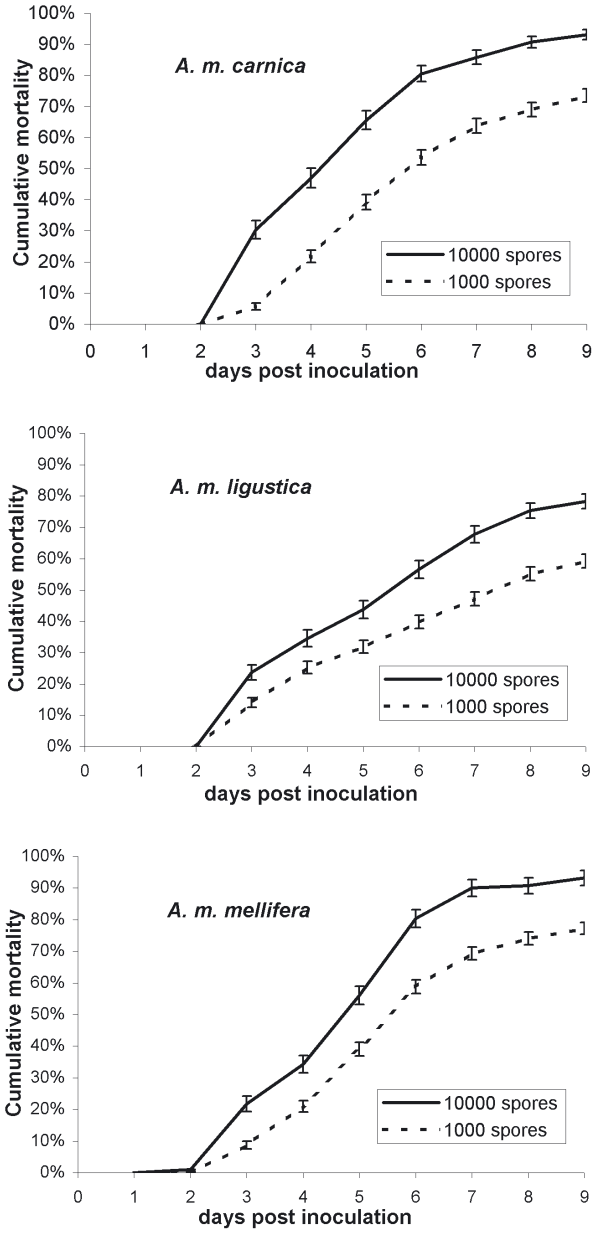

Figure 1. Dose-mortality effect. Three day old larvae from three different honey bee subspecies were fed with different doses of *Ascosphaera apis* spores. The time course of chalkbrood infection at two doses (1000 or 10000 spores) are shown as the average cumulative mortality (± SE indicated by error bars) of larvae that died due to fungal infection.

from the different queens. The within subspecies variation in susceptibility was not so pronounced when considering the mean time to death of larvae exposed to *A. apis* spores. Thus at the highest dose 10000 spores there were no significant difference in the mean time to death of colonies, whereas significant differences were found in between larvae of the four *A. m. carnica* and the four *A. m. mellifera* colonies when exposed to 1000 *A. apis* spores. This indicates that the larval immune system inevitably succumbs to infections that are over a certain threshold dose. A similar result has been found when infecting leafcutter ants with *Metarhizium* (Hughes and Boomsma, 2004) and indicates that pilot experiments are always necessary to find the appropriate dose that can detect differences in susceptibility at the colony or (sub) species level.

Honey bee larvae infected with *A. apis* have an increased expression of certain antimicrobial peptides and genes implicated in the cellular immunity (Evans, 2006). Differences in the expression level of larvae from the different colonies (Evans and Pettis, 2005), might account for the observed variance in susceptibility to chalkbrood. Such colony variation in the response might not only be restricted to *A. apis*. Considerable colony variation in the abaecin expression of larvae exposed to American Foulbrood has been observed (Evans and Pettis, 2005; Decanini et al., 2007). A recent study showed that even drone larvae from sister queens differed in susceptible to American Foulbrood (Behrens et al., 2007), which genetically are much more homogenous compared to the worker larvae we used.

Hygienic behaviour is supposed to be the prime chalkbrood tolerance actor and a negative correlation was found between colonies that quickly removed freeze killed brood and development of the chalkbrood disease (Gilliam et al., 1988). Some colonies however deviate from this pattern which might be explained by the variable larval response, thus if a highly hygienic colony have highly *A. apis* susceptible larvae symptoms of chalkbrood might been seen eventhough. The genetic variability of the individual colony by means of mating frequency of the queen might be another exploratory factor; since multiple-drone-inseminated queens did not suffer of severe natural chalkbrood infections as did single-drone-inseminated queens (Tarpy and Seeley, 2006). Multiple mating of honeybee queens adds an additional component of susceptibility variation within each colony; differential patriline susceptibility. This has so far only been shown for American foulbrood (Palmer and Oldroyd, 2003). However, the chalkbrood prevalence varied significantly more in single-drone-insemated compared to multiple-drone-inseminated colonies exposed to chalkbrood spores (Tarpy, 2003). This might be explained by fact that the variable paternal inheritance are equalled out in multiple mated colonies.

Requening colonies which suffer of chalkbrood, utilising queens breed for higher chalkbrood tolerance, has long been the recommended measure (Lunder, 1972). The requeening success regarding chalkbrood resistance will thus depend both on the hygienic capacity, mating frequency and the larvae susceptibility.

Little work has been done to investigate differences in susceptibility to honey bee diseases between subspecies. Malone and Stefanovic (1999) were not able to detect any significant different responses of *Nosema apis* infection. Larvae from the *A. m. ligustica* queens used in this study were found to be less susceptible to chalkbrood, as shown both in the dose-response experiments and time-mortality analysis, than larvae from *A. m. carnica* and *A. m. mellifera* queens. Considering the low number of colonies used we can, however, not predict that any *A. m. ligustica* colony is more resistant to chalkbrood compared to *A. m. carnica* and *A. m. mellifera* colonies. Our data do not support any major differences in the response at larval level to *A. apis* between honey bees derived of the two European *A. mellifera* lineages. This could be due to the low number of colonies included or because the *A. m. mellifera* queens originate from a single population where conservation and genetic purity of the population is in focus. Still other adaptations might have evolved including social defences like hygienic behaviour or better control over brood rearing temperature e.g. by adjusting the spring build up according to climate.

In conclusion, our study proved that the individual larvae response toward *A. apis* vary substantial among honey bee colonies and that variation can be found within and between different subspecies. The variation might be higher than found in the eleven colonies examined; however, even with the modest number included, it is clear that there is breeding potential toward higher larval chalkbrood tolerance in honey bees.

ACKNOWLEDGEMENTS

The authors wish to thank Christina Wolsted and Camilla Falk for technical support. Per Kryger and Ejner Olsen for apiary facilities, Koos Boomsma for encouragements and comments, and the Danish National Research Foundation and The Danish Council for Strategic Research for funding.

Sensibilité au couvain plâtré (*Ascosphaera apis*) des diverses sous-espèces d'abeilles : *Apis mellifera carnica*, *A. m. ligustica et A. m. mellifera*.

maladie du couvain / pathogène / champignon / test biologique / élevage in vitro

Zusammenfassung – Unterschiedliche Empfänglichkeit verschiedener Honigbienenvölker für Kalkbrut-Resistenz. Larven aus verschiedenen Honigbienenvölkern wurden mit Kalkbrut-Sporen (*Ascosphaera apis*) infiziert. Die Larven stammten von Königinnen, die sich an isolierten Paarungsplätzen (z.B. Inseln) natürlich gepaart hatten. Wir verwendeten Königinnen von *Apis mellifera carnica*, *A. m. ligustica* und *A. m. mellifera*. Dadurch konnten wir sowohl zwischen den drei Gruppen als auch innerhalb der Gruppen die Unterschiede in der Anfälligkeit gegen eine Kalkbrutinfektion testen. Dreitägige Larven wurden dazu mit unterschiedlichen Dosen von *A. apis*-Sporen gefüttert, während die Kontrollgruppe nicht kontaminiertes Futter erhielt. Alle Larven wurden täglich unter dem Mikroskop beobachtet und nach den Kriterien „lebend", „tot" oder „durch Pilzbefall getötet" (mit Pilzhyphen am Körper) eingeteilt. Es gab einen klaren Zusammenhang zwischen der gefütterten Sporenmenge und der Anzahl infizierter Larven. Es waren zwischen minimal 55 und maximal 905 Sporen notwendig, um 50 % der behandelten Larven zu töten (Tab. I). Die Unterschiede in der Anfälligkeit betragen also mehr als den Faktor 10 und erwartungsgemäß nahm die durchschnittliche Überlebensdauer mit zunehmender Sporendosis ab (Abb. 1). Bei der höchsten verabreichten Sporendosis von 10000 Sporen gab es keine Unterschiede in der Überlebensdauer zwischen bzw. innerhalb der Gruppen. Wurden die Larven aber nur mit 1.000 Sporen gefüttert, traten zwischen den Gruppen signifikante Unterschiede auf. Insgesamt weisen die LD_{50}–Werte und die durchschnittliche Überlebensdauer darauf hin, dass die in dieser Untersuchung verwendeten *A. m. ligustica*-Larven weniger anfällig gegen *A. apis*-Infektionen waren als Larven von *A. m. mellifera* und *A. m. carnica*. Allerdings werden aufgrund der begrenzten Anzahl an Testvölkern und der hohen Variation innerhalb der Unterarten weitere Daten benötigt, um zuverlässige Vorhersagen zur spezifischen Anfälligkeit bzw. Toleranz verschiedener *Apis*-Unterarten gegenüber einer Kalkbrutinfektion zu machen. Die großen Unterschiede in der Kalkbrut-Anfälligkeit zwischen den verschiedenen Völkern zeigen aber, dass es durchaus ein Potential für die Zucht auf Kalkbrut-Resistenz gibt.

***Ascosphaera apis* / Biotest / Brutkrankheit / Insektenpathogene / in-vitro Aufzucht**

REFERENCES

Alonso J.M., Rey J., Puerta F., Hermoso de Mendosa J., Hermoso de Mendosa M., Flores J.M. (1993) Enzymatic equipment of *Ascosphaera apis* and the development of infection by this fungus in *Apis mellifera*, Apidologie 24, 383–390.

Aronstein K.A., Murray K.D., De Leon J.H., Qin X., Weinstock G.M. (2007) High mobility group (HMG-box) genes in the honey bee fungal pathogen *Ascosphaera apis*, Mycologia 99, 553–561.

Aupinel P., Fortini D., Dufouri H., Taséi J.-N., Michaud B., Odoux F., Pham-Delègue M.-H. (2005) Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae, Bull. Insectol. 58, 107–111.

Bailey L. (1967) The effect of temperature on the pathogenecity of the fungus *Ascosphaera apis* for the larvae of the honeybee, *Apis mellifera*, in: Van der Laan PA (Ed.), Insect Pathology and Microbial Control, North Holland Publishing Co, Amsterdam, The Netherlands, pp. 162–167.

Beauvais A., Latgè J.P. (1988) A simple medium for growing entomophthoralean protoplasts, J. Invertebr. Pathol. 51, 175–178.

Behrens D., Forsgren E., Fries I., Moritz R.F.A. (2007) Infection of drone larvae (*Apis mellifera*) with American foulbrood, Apidologie 38, 281–288.

Brødsgaard C.F., Ritter W., Hansen H. (1998) Response of in vitro reared honey bee larvae to various doses of *Paenibacillus larvae larvae* spores, Apidologie 29, 569–578.

Chorbiński P. (2004) The development of the infection of *Apis mellifera* larvae by *Ascosphaera apis*, Electronic J. Polish Agric. Univ., Vet. Med., 7, #03 [online]. http://www.ejpau.media.pl/volume7/issue2/veterinary/art-03.html.

Cremer S., Armitage S., Schmid-Hempel P. (2007) Social Immunity, Curr. Biol. 17, 693–702.

Decanini L.I., Collins A.M., Evans J.D. (2007) Variation and heritability in immune gene expression by diseased honeybees, J. Hered. 98, 195–201.

Evans J.D. (2006) Beepath: An ordered quantitative-PCR array for exploring honey bee immunity and diseases, J. Invertebr. Pathol. 93, 135–139.

Evans J.D., Pettis J.S. (2005) Colony-level impacts of immune responsiveness in honey bees, *Apis mellifera*, Evolution 59, 2270–2274.

Flores J.M., Ruiz J.A., Ruz J.M., Puerta F., Bustos M., Padilla F., Campano F. (1996) Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions, Apidologie 27, 185–192.

Franck P., Garnery L., Loiseau A., Oldroyd B.P., Hepburn H.R., Solignac M., Cornuet J.-M. (2001) Genetic diversity of the honeybee in Africa: microsatellite and mitochondrial data, Heredity 86, 420–430.

Garnery L., Solignac M., Celebrano G., Cornuet J.-M. (1993) A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L., Experientia 49, 1016–1021.

Gilliam M., Vandenberg J. (1997) Fungi, in: Morse R.A., Flottum K. (Eds), Honey Bee Pests, Predators and Diseases, 3rd ed., The A.I. Root Company,Ohio), pp. 79–112.

Gilliam M., Taber S., Lorenz B.J., Prest D.B. (1988) Factors affecting development of chalkbrood disease in colonies of honey bees, *Apis mellifera*, fed pollen contaminated with *Ascosphaera apis*, J. lnvertebr. Pathol. 52, 314–325.

Genersch E., Ashiralieva A., Fries I. (2005) Strain and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honeybees, Appl. Environ. Microbiol. 71, 7551–7555.

Glinski Z. (1981) Studies on the effect of the fungus *Ascosphaera apis* on the larvae of the honey bee, *Apis mellifera* L., Pol. Archiw. Wet. 23, 9–15.

Glinski Z., Buczek K. (2003) Response of Apoidea to fungal infections, Apiacta 38, 183–189.

Heath L.A.F., Gaze B.M. (1987) Carbon dioxide activation of spores of the chalkbrood fungus *Ascosphaera apis*, J. Apic. Res. 26, 243–246.

Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*, Nature 443, 931–949.

Hughes W.O.H., Boomsma J.J. (2004) Genetic diversity and disease resistance in leaf-cutting ant societies, Evolution 58, 1251–1260.

James R.R., Buckner J.S. (2004) Lipids stimulate spore germination in the entomopathogenic ascomycete *Ascosphaera aggregata*, Mycopathology 158, 293–302.

Jensen A.B., Thomsen L., Eilenberg J. (2001) Intraspecific variation and host specificity of *Entomophthora muscae s.str.* isolates revealed by random amplified polymorphic DNA, universal primed PCR, PCR-restriction fragment length polymorphism, and conidial morphology, J. Invertebr. Pathol. 78, 251–259.

Jensen A.B., Palmer K.A., Boomsma J.J., Pedersen B.V. (2005) Varying degrees of *Apis mellifera ligustica* introgression in protected populations of the black honeybee, *Apis mellifera mellifera*, in northwest Europe, Mol. Ecol. 14, 93–106.

Lunder R. (1972) Undersøkelse av kalkyngel i 1971 [Investigation on chalkbrood in 1971], Birøkteren 88, 55–60.

Malone L.A., Stefanovic S. (1999) Comparison of the responses of two races of honeybees to infection with *Nosema apis* Zander, Apidologie 30, 375–382.

McKee B., Goodman R.D., Hornitzky M.A. (2004) The transmission of European foulbrood (*Melissococcus plutonius*) to artificially reared honey bee larvae (*Apis mellifera*), J. Apic. Res. 43, 93–100.

Naug D., Camazine S. (2002) The role of colony organization on pathogen transmission in social insects, J. Theor. Biol. 215, 427–439.

Palmer A.K., Oldroyd B.P (2003) Evidence for intra-colonial genetic variance in resistance to American foulbrood of honey bees (*Apis mellifera*): further support for the parasite/pathogen hypothesis for the evolution of polyandry, Naturwissenschaften 90, 265–268.

Payton M.E., Greenstone M.H., Schenker N. (2003) Overlapping confidence intervals or standard error intervals: What do they mean in terms of statistical significance? J. Insect Sci. 3, 34.

Puerta F., Flores J.M., Bustos M., Padilla F., Campano F. (1994) Chalkbrood development in honey brood under controlled conditions, Apidologie 25, 1–7.

Robertson J.L., Preisler H.K. (1992) Pesticide Bioassays with Arthropods, CRC Press, Boca Raton.

Ruttner F. (1988) Biogeography and taxonomy of honey bees, Springer Verlag, Berlin.

Sahinler N., Kurt S. (2004) A study on antifungal activity of formic acid and propolis extract against Chalkbrood disease pathogen *Ascosphera apis*, J. Anim. Vet. Adv. 3, 554–556.

Schmid-Hempel P. (2005) Evolutionary ecology of insect immune defenses, Annu. Rev. Entomol. 50, 529–551.

Solorzano C.D., Szalanski A.L., Kence M., McKern J.A., Austin J.W., Kence A. (2009) Phylogeography and population genetics of honey bees (*Apis mellifera*) from Turkey based on COI-COII sequence data, Sociobiology 53, 1–10.

Spiltoir C.F., Olive L.S. (1955) A reclassification of the genus *Pericystis* Betts, Mycologia 47, 238–244.

Starks P.T., Blackie C.A., Seeley T.D. (2000) Fever in honeybee colonies, Naturwissenschaften 87, 229–231.

St. Leger R.J., Staples R.C., Roberts D.W. (1993) Entomopathogenic isolates of *Metarhizium anisopliae*, *Beauveria bassiana*, and *Aspergillus flavus* produce multiple extracellular chitinase isozymes, J. Invertebr. Pathol. 61, 81–84.

Sušnik S., Kozmus P., Poklukar J., Meglič V. (2004) Molecular characterisation of indigenous *Apis mellifera carnica* in Slovenia, Apidologie 35, 623–636.

Tarpy D.R. (2003) Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth, Proc. R. Soc. Lond. B Biol. Sci. 270, 99–103.

Tarpy D.R., Seeley T.D. (2006) Lower disease infections in honeybee (*Apis mellifera*) colonies headed by polyandrous vs monandrous queens, Naturwissenschaften 93, 195–199.

Theantana T., Chantawannakul P. (2008) Protease and beta-N-acetylglucosaminidase of honey bee chalkbrood pathogen *Ascosphaera apis*, J. Apic. Res. 47, 68–76.

Whitfield C.W., Behura S.K., Berlocher S.H., Clark A.G., Johnston J.S., Sheppard W.S., Smith D.R., Suarez A.V., Weaver D., Tsutsui N.D. (2006) Thrice out of Africa: Ancient and recent expansions of the honey bee, *Apis mellifera*, Science 314, 642–645.