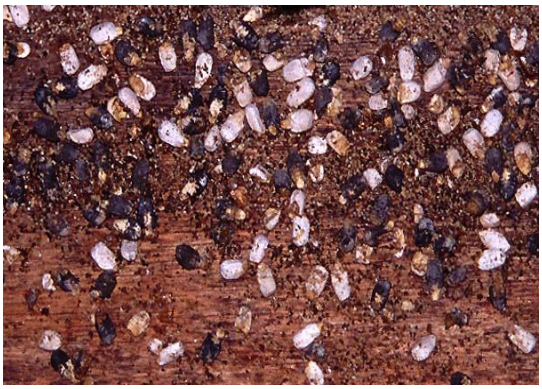
Action FA0803

COLOSS Workshop

WG 2

The future of brood disease research – guidelines, methods and developments



Welcome

Dear colleagues

It is our pleasure to welcome you to Copenhagen and the international COLOSS workshop on the future of honey bee brood diseases.
We are grateful to all the contributing participants and we look forward to stimulating and fruitful discussions concerning fungal and bacterial diseases in honey bee brood with the overall goal of increased knowledge and future collaboration.

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The organizing committee
Annette Bruun Jensen, Eva Forsgren and Elke Genersch

Conference Venue
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Transcriptional responses in Honey Bee larvae infected with chalkbrood fungus, *Ascosphaera apis.*

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Diseases and other stress factors working synergistically weaken honey bee health and may play a major role in the losses of bee populations in recent years. Among a large number of bee diseases, chalkbrood has been on the rise. We present here the experimental identification of honey bee genes that are differentially expressed in response to infection of honey bee larvae with the chalkbrood fungus, *Ascosphaera apis*. Using a combination of cDNA-AFLP and qRT-PCR analyses, we were able to determine several key transcriptional events that constitute to the overall effort in the honey bee larvae to fight natural fungal infection. Honey bee transcripts identified in this study are involved in critical functions related to transcriptional regulation, apoptotic degradation of ubiquitinated proteins, nutritional regulation, and RNA processing. One of the most interesting differentially-regulated transcripts is for a chitinase-like enzyme that may be linked to anti-fungal activities in the honey bee larvae, similarly to gut and fat-body specific chitinases found in mosquitoes and the red flour beetle. We have also found that immune regulation of the anti-fungal responses in honey bee involves highly coordinated activation of both NF-κB signaling pathways, leading to production of anti-microbial peptides. Significantly, activation of immune responses in the infected bee larvae was associated with down-regulation of major storage proteins, leading to depletion of nutritional resources.

Recent advances in our understanding of European Foulbrood in England and Wales

Giles E. Budge*[1], Ben Jones[1], Michelle Powell[1], Liz Anderson[1], Lynn Laurenson[1], Stéphane Pietravalle[1], Gay Marris[1], Ed Haynes[12], Richard Thwaites[1], Jan Bew[1], Selwyn Wilkins[1] and Mike Brown[1]

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Information will be presented on the recent history of European foulbrood disease in England and Wales, including geographical spread. Data on disease prevalence from the prioritised inspection service will be compared with unbiased data from a large 'random' apiary study still in progress. Case studies of disease spread will be presented with information on the predictive power of diagnostics as a marker for disease. Finally plans for future work will be shared with the aim of promoting collaborative working.

Virulence of different *Melissococcus plutonius* strains on larvae tested by an in vitro larval test.

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In Switzerland, European foulbrood (EFB) has become a worrying sanitary problem and cases have been reported with increased frequency in the last decade. Between 1970 and 1998, approximately 20 to 50 diseased apiaries per year were sanitized by the veterinary authorities. However, since 1999 there has been a significant increase of reported cases, with more than 994 apiaries affected in 2010 alone. This represents a prevalence of 5.4 %.
At the moment, we have no explanation for the dramatic expansion of this infectious brood disease. In most of Europe EFB reports are anecdotal and present with low morbidity. An exception being some regions in UK and Norway where the prevalence of EFB is also rather high.
One hypothesis to explain the resurgence of EFB in Switzerland and the other isolated regions where EFB is problematic could be the emergence of more virulent *Melissococcus plutonius* strains. To test this hypothesis, we used the larval in vitro rearing method developed by Aupinel et al. (Pest. Manag. Sci., 2007) with minor modifications. We will present results on the comparison of the virulence of different Swiss and foreign EFB strains. We will also show results about the effect of the secondary invader *Paenibacillus alvei* on the morbidity of *M. plutonius*.

European foulbrood in Norway: How to deal with a major outbreak after 30 years absence

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In Norway, EFB is a rare disease with the last verified case in 1980. Beekeepers should notice the Norwegian Food Safety Authority (NFSA) which deals with animal diseases when they suspect a notifiable disease in their honey bees. August 18. EFB was confirmed by PCR in the first sample in Aust-Agder County. Two days after EFB was verified in the first sample, a standstill notice was declared for the whole County of Aust-Agder (9.158 km^2). Brood samples from colonies in contact apiaries. In total 154 samples from 72 beekeepers were analysed and 45 of the beekeeping operations tested positive for EFB. In all but one case outside Aust-Agder County, the presence of *M plutonius* could be tracked back to infected apiaries in Aust-Agder.

The NFSA implemented a strategy with the aim to eradicate *M. plutonius* from Norwegian apiaries. This strategy means that beekeepers that have tested positive for the presence of *M. plutonius* in one or more of their colonies have to destroy all their colonies and all the equipment have to be disinfected. In 2010 about 3000 colonies were destroyed but the beekeepers will be compensated for their losses.

If sampling in 2011 reveals that the bacterium is common and widespread there will be a change in the strategy to control the disease. We will apply the Norwegian Department of Agriculture for funding of a four year research project on EFB. In this project we will focus on several topics, among them, virulence of different *M. plutonius* strains, disease transfer within and among apiaries and efficacy of different alternative strategies such as shook swarm treatment at different time of the year

Temperature and climate in chalkbrood disease

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Chalkbrood in honey bee is a fungal disease affecting brood after sealing. Chalkbrood is caused by *Ascosphaera* sp, but is necessary a predisposing condition for disease development. Temperature is the main predisposing condition. Chilled colonies with contaminated larvae around capping time show disease symptoms, but disease appear so much in cold climate as temperate climate; spring, autumn or summer time. How are affected larvae by different temperatures? What are the cooled brood causes?

On the other hand, other predisposing conditions had been considered. Damp environment is one of them. Can humidity induce chalkbrood on contaminated brood? When humidity increase into beehive? Are there any relationships between temperature and humidity? How environmental conditions, like big flowering, can generate predisposing conditions?

To answer above questions is necessary to understand chalkbrood disease and design tools for disease control.

Omics of *Paenibacillus larvae*

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"Omics" are a broad field of biology that imply analyses of complete datasets consisting of similar single elements. For example *genomic*s involves the exploration of the total of all genetic information of any organism, and *proteomic*s approaches refer to all proteins of an organism. To gain a better understanding of *Paenibacillus larvae* (*P. larvae*), the causative agent of American Foulbrood and one of the most important honey bee pathogens, both such analyses, *genomics* and *proteomics*, were carried out within this species. Four different genotypes of *P. larvae* (ERIC I – ERIC IV) are described, which are phenotypically distinct, e.g. in colony and spore morphology, in metabolism and in virulence. For comparative genomic of *P. larvae* we analyzed all four genotypes via Suppression Subtractive Hybridization. Comparative proteomics of the genotypes ERIC I and ERIC II were performed via two-dimensional SDS-PA gel electrophoresis. Combining the results of both comparative -*omics*-approaches we obtained comprehensive genetic as well as protein information which allowed us to identify several factors putatively responsible for phenotypic variation within the species *P. larvae*.

Are histological methods relevant in detecting the pathogenic and un-pathogenic effects on cellular and tissue level in honeybee brood

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A number of techniques were employed to assess cellular changes induced in honeybee larvae midgut after *per os* inoculation of bacterium *Paenibacillus larvae*, the causative agent of American foulbrood disease. Our data indicate that *Paenibacillus* induces necrosis only as opposed to other external influences (acaricide Amitraz) which are also triggers for programmed cell death in the midgut epithelial cells of honeybee larvae. The effect on cellular level could be quantified *in situ* using the TUNEL techniques. In *Paenibacillus* infected larvae all midgut epithelial cells died by necrotic means as indicated by morphological changes, too. Approximately 5 to 10 % programmed cell death was found in the uninfected tissue.
The immunohistochemical localisation of the heat shock proteins (Hsp70 and Hsp90) and the histone protein in healthy and *Paenibacillus larvae* infected honeybee larvae were studied. Hsp70 was found in the nuclei and the cytoplasm of infected midgut and salivary gland cells, but not in uninfected larvae. Exposed histone proteins were localised in the nuclei of dying uninfected cells undergoing programmed cell death. After applying histone protein antibodies to *P. larvae* infected honeybee larvae, the DAB based reaction product was located in the nuclei or in immediate surroundings of all larval cells.
The data show that immunohistochemical methods are useful for studying *in situ* tissue pathology and indicate possibilities for monitoring the effects of infective and additionally chemical environmental stressors on cell death in honeybee larvae tissue.

Virulence of brood diseases in South African honey bees (*Apis mellifera scutellata*): a comparison between strains of different origins

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Both honey bee sub-species of South Africa, *Apis mellifera capensis* and *A.m. scutellata,* seems to be very resistant/ tolerant to pests and diseases. Despite the presence of all major threats for honeybees no extensive countrywide colony collapses occurred in South Africa. The punctual occurrence of large scale colony losses (up to 70% of colonies per beekeeper) reported in the last season, especially in the *Scutellata* region, seems to see also an increase in the occurrence of brood diseases e.g. European foulbrood and chalkbrood and the recent outbreak and rapid spread of American foulbrood through the country is a cause of major concern. Up until now our bees has been considered more resistant to brood diseases especially since the outbreak of AFB has not resulted in major colony losses yet. The effect of brood diseases on our bees is tested under controlled conditions to investigate the statement that they have a higher resistance. Our study aims to investigate firstly the virulence of the three major brood diseases, EFB, AFB and Chalkbrood in *A. m. scutellata* honey bees and secondly we want to compare the virulence of these diseases through larval rearing experiments with strains of different origin (Europe and South Africa).

Basic and applied approaches for exploiting the cell surface of *Paenibacillus alvei* with a focus on the glycosylation aspect

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Paenibacillus alvei is a mesophilic, Gram-positive organism isolated from bee larvae infected with European foulbrood. The outermost cell surface of *P. alvei* is a 2D glycoprotein layer (S-layer). In our research focuses *P. alvei* serves as a model organism (i) for unraveling molecular details of bacterial glycosylation and (ii) for establishing it as a nanometer-controlled bacterial cell surface display system of functional epitopes. The basis for this endeavor is a gene knockout system developed in our laboratory that is using bacterial mobile group II intron-mediated gene disruption [1].

The S-layer *O*-glycans are polymers of [→3)-βDGal-(1[αDGlc-(1→6)]→4)-βDManNAc-(1→] repeating units that are linked via the adaptor -[GroA-2→OPO_2→4-βDManNAc-(1→4)]→3)-αLRha-(1→3)-αLRha-(1→3)-αLRha-(1→3)-βDGal-(1→ to specific tyrosine residues of the S-layer protein SpaA [2]. The genetic information is encoded in an *slg* gene locus governing the step-wise assembly of the glycan on a lipid-carrier, its export across the cytoplasmic membrane, and is transfer onto the S-layer [3]. Based on this knowledge, a strategy for nanopatterned *in vivo* cell surface co-display of peptide and glycan epitopes enabled by S-layer glycoprotein self-assembly was recently established [4].

The described strategy is the starting point for the future *in vivo* cell surface presentation of different peptides and proteins combined with bioactive glycans which may have great value in the fields of receptor mimics, vaccine development, and drug delivery. Considering the importance of a bacterium's cell surface as a contact zone with the environment, in the same way, the life-style of the bacterium may be influenced, affecting, for instance, bacterial virulence.

[1] Ristl R, K Steiner, K Zarschler, P Messner, C Schäffer (2011) *Int J Microbiology*, article ID 127870, 16 pages. [2] Zarschler K, B Janesch, S Zayni, C Schäffer, P Messner (2009) *Appl Environ Microbiol* 75:3077-3085. [3] Zarschler K, B Janesch, M Pabst, F Altmann, P Messner, C Schäffer (2010) *Glycobiology* 20:787-798. [4] Zarschler K, B Janesch, B Kainz, R Ristl, P Messner, C Schäffer (2010). *Carbohydr Res* 345:1422-1431

Tools to investigate bacterial *Bacillus* and *Enterococccus* virulence and pathogenesis in insects

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The presentation will focus on aspects and tools developed to investigate on virulence properties and pathogenesis of entomopathogenic *Bacillus thuringiensis*/*B. cereus* and the human opportunistic pathogen *Enterococcus faecalis* in the infection model *Galleria mellonella* (Greater Wax Moth) larvae. This insect is used to study infections and virulence genes of several insect and human pathogens particularly by injection into the hemocoel. Attempts to make parallels to the agents of American foulbrood disease (*Peanibacillus larvae*) and European Foulbrood (*Melissococcus plutonius* will be searched when possible, although this is actually not our domain of research.
Approaches based on gene comparison, to search for homology of known virulence factors (pore-forming toxin, proteases, lipases etc.) will be shown. Molecular tools, for targeted and non-targeted gene deletions used to uncover of virulence factors will be mentioned. Results from application of *in vivo* gene expression systems like RIVET (Recombinase *in vivo* expression technology) used to identify genes expressed during infection will be shown. Transcriptional promoter fusions to marker molecules like GFP (green fluorescent protein) or LacZ (ß-Galactosidase) used to determine and localize gene expression will also be highlighted. These techniques along with classical histopathological observations permit to uncover some steps of the infection process and to identify genes, which are important for bacterial development in the host. Bacterial infection capacity also depends to a large extent on the host. Indeed, opportunistic pathogens are only capable to infect immuno-compromised host, thus stress situations like starvation and temperature variations can contribute to increased host susceptibility resulting in lethal infection.

Apis mellifera-Ascosphaera apis: Study of hygienic behavior

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Chalkbrood is a nice model for testing hygienic behavior. Spore-cysts obtained from pure strains preserved in integral rice were used to inoculate fifth instar larvae. Activities performed by honeybees of different ages on cells containing brood affected by *Ascosphaera apis* in hygienic (H) and non-hygienic (NH) colonies was studied by filming labeled bees and analyzed using Observer Video-Pro. The average age of honeybees performing inspection was 15 and 16 days for uncapping and removing and no difference was detected between treatments. It was registered honeybees performing hygienic behavior in both groups of colonies but this percentage was higher in H colonies. In NH colonies the bees that perform this behavior were more persistent (more time on the cells, more visits /bee, more time dedicated to these activities) because they were in a lower proportion in the colony. H colonies were more efficient in mummies removal. Filming allowed describing the sequence of hygienic behavior. Activities performed by H and NH honeybees in presence of brood affected by *Ascosphaera apis* (MO) or pin-killed brood (PB) was compared. H colonies were more efficient in the start of uncapping, with a fast response to the stimulus of dead brood independent of the method used to kill it. Once uncapping begun, NH colonies reacted in a similar way to MO treatment than H colonies when uncapping ending, removal starting and ending were considered. This would indicate a different stimulus level in both treatments. H colonies removed a greater percentage of dead brood than NH colonies.

Manipulation of *Paenibacillus larvae*

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Genotypic analyses of *Paenibacillus larvae* genotypes (ERICI-IV) clearly revealed differences in virulence during honey bee larvae infection. In order to understand genotype specific pathogeneses, molecular tools for genetic manipulation are strongly required. The expression of recombinant proteins in *P. larvae* is a new tool to specifically investigate vegetative bacterial cells and significant virulence factors during infection. Therefore we successfully transformed the GFP expression Gram-positive – *E. coli* shuttle plasmid pAD43-25 (BGSC) in genotypes ERIC I and ERIC II. The expression of recombinant GFP in *P. larvae* is a new important tool for genetic manipulation of these bacteria and helps to understand the infection cycle. Following the electroporation protocol under different experimental conditions bacterial cells are manipulated by the uptake of foreign plasmid DNA. The *Bacillus cereus* UW85 *upp* promotor upstream of a mutant GFP facilitates high-level constitutive expression of a Green Fluorescent Protein variant in vegetative cells. Recombinant clones were grown and detected during different stages of growth in liquid media. Expression of recombinant GFP allows visualisation of bacterial cells with an optimal excitation wavelength of 498 nm. The expression of GFP is an important molecular marker for visualization and quantification of vegetative cells, as well as for analyses of specific virulence factors that are expressed and accumulated in and outside of bacterial cells during larvae infection

Incidence and prevalence of AFB in Europe

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The data, which were based on an email-survey requesting the incidence of AFB in Europe, are presented by AFB cases per year for several European countries. Furthermore the methods to treat against this disease and the respective laws which apply to these methods are compared. For Germany, the epidemiology of AFB is presented in more detail for the past 15 years. German data were collected from the official sources of the German Epizootic Diseases News Service and the estimated honey bee colony numbers of the German Beekeepers Association. However, since data from all European countries is not always easily available and sources of data often differ in quality, the comparison between European countries has been inevitably limited. Nevertheless we should encourage discussions to commence for the development of standard methods for the diagnosis and treatment of AFB in Europe

Do antimicrobial compounds in host plant pollen protect solitary bees from developing chalkbrood?

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Solitary bees, along with their social relatives, the honey bees, suffer from the disease chalkbrood. Chalkbrood, caused by several species of the fungus *Ascosphaera*, is often seen the solitary bees *Osmia rufa* and *Megachile rotundata* but it has not been found in *Chelostoma florisomne*, a wild solitary bee which nests gregariously in the reeds of thatched roofs. *Chelostoma florisomne* is an oligolectic bee, provisioning its eggs almost exclusively with pollen collected from *Ranunculus* species (buttercups). *Ranunculus* contains a compound that exhibits both antifungal and antibacterial activity. We found that a diet of *Ranunculus* pollen inhibits the development of chalkbrood in *C. florisomne* and *O. rufa* larvae, and that ethanolic extracts of *Ranunculus* pollen significantly inhibit *in vitro* spore germination of pathogenic Ascosphaereae. The evolution of host plant choice has been linked to nutrition, to avoidance of competition for pollen, as well as to recognition of floral morphology. But pathogens such as *Ascosphaera*, which likely have a long evolutionary history with bees, may also play a role in the evolution of host plant choice. The selective advantage of a narrow diet may be prophylactic.

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