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Genetic diversity and effects of the bacterial pathogen Melissococcus plutonius in Swiss honey bee populations (Apis mellifera)

Grossar Daniela

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Genetic diversity and effects of the bacterial pathogen *Melissococcus plutonius* in Swiss honey bee populations (*Apis mellifera*)

Thèse de doctorat ès sciences de la vie (PhD)

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Faculté de biologie et de médecine
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par

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Genetic diversity and effects of the bacterial pathogen *Melissococcus plutonius* in Swiss honey bee populations (*Apis mellifera*)

Lausanne, le 26 mai 2023

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Jan van der Meer

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Abstract

European Foulbrood (EFB) is a bacterial brood disease of honey bees (*Apis mellifera*), caused by *Melissococcus plutonius*. In recent years, a significant increase in EFB cases has been observed in Switzerland and also other countries, and this situation threatens honeybee health, causes damages to apiculture, and requires extensive measures to control the spread of the disease. In this thesis, I study the virulence of 16 *M. plutonius* isolates, on the basis of *in vitro* infection assays (chapter I). The virulence levels of individual isolates varied greatly and were correlated with the growth dynamics of an isolate. Three of the tested *M. plutonius* isolates contained the gene *mtxA*, which codes for the putative toxin melissotoxin A. Mortality rates in honey bee larvae after experimental infection with one of these three isolates were significantly higher than after infection with isolates lacking this gene, suggesting *mtxA* to increase virulence of *M. plutonius*. In chapter II, I investigate the genetic diversity and spatial distribution of Swiss *M. plutonius*, by applying multi locus sequence typing (MLST) to 160 isolates sampled in two periods (2006-07 and 2013). I also analyze the presence of *mtxA*. Five novel sequence types (ST) have been only identified in Swiss *M. plutonius* samples, and *mtxA* was detected more often in second sampling period isolates. In search of new solutions to control EFB in chapter III, I collect honey bee queens from apiaries with acute EFB outbreaks: queens from colonies being severely affected by EFB (EFB^+), and queens from colonies showing no symptoms of the disease (EFB^-). I infect the larvae of these queens *in vitro* with *M. plutonius* strain CH 49.3 or with strain CH 40.2. In parallel, I test the colonies established by these queens for hygienic behavior, by freeze-killed brood bioassays. Larvae of EFB^- queens die off faster after infection with the lower virulence *M. plutonius* strain CH 40.2, than larvae of EFB^+ queens. No such difference was observed after infection with high virulence isolate CH 49.3. Colonies headed by EFB^- queens showed higher levels of hygienic behavior than colonies founded by EFB^+ queens. EFB^- colonies could therefore have developed a strategy to keep *M. plutonius* levels low within their colonies, consisting of earlier death of infected larvae, combined with elevated hygienic behavior. Overall, this thesis expands our knowledge of EFB, by providing new insights into the virulence and genetic diversity of the pathogen *M. plutonius*, as well as on the disease resistance mechanism of honey bees.

Résumé

La loque européenne (EFB) est une maladie bactérienne du couvain des abeilles mellifères (*Apis mellifera*), causée par *Melissococcus plutonius*. Une augmentation significative des cas d'EFB a été observée en Suisse et dans d'autres pays ces dernières années. Cette situation menace la santé des abeilles, provoque des pertes dans l'apiculture et nécessite des mesures couteuses pour contrôler la propagation de la maladie. Dans cette thèse, j'étudie la virulence de 16 isolats de *M. plutonius*, sur la base d'essais d'infection *in vitro* (chapitre I). Les degrés de virulence des différents isolats sont très variables et en corrélation avec leur dynamique de croissance. Trois isolats de *M. plutonius* contenaient le gène *mtxA*, qui code pour la toxine putative melissotoxin A et est situé sur un plasmide qui peut être échangé entre les bactéries. Les taux de mortalité des larves d'abeilles après une infection par l'un de ces trois isolats étaient significativement plus élevés qu'après une infection par des isolats dépourvus de ce gène. Dans le chapitre II, j'étudie la diversité génétique et la distribution spatiale de *M. plutonius* Suisse en appliquant le typage séquentiel multilocus (MLST) à 160 isolats prélevés au cours de deux périodes (2006-07 et 2013). De plus, j'analyse la présence de mtxA. Les isolats analysés ont été classés en 12 types de séquences (ST), dont cinq n'ont été identifiés que dans les échantillons suisses de *M. plutonius*. Le *mtxA* a été détecté plus souvent dans les isolats de la période récente d'échantillonnage. Dans le chapitre III, je collecte des reines d'abeilles mellifères provenant de ruchers présentant des foyers aigus d'EFB. Certaines reines viennent de colonies fortement touchées par l'EFB (EFB^+), et d'autres de colonies ne présentant aucun symptôme de la maladie (EFB^-). J'infecte les larves de ces reines *in vitro* avec la souche CH 49.3 ou CH 40.2 de *M. plutonius*. Parallèlement, je teste le comportement hygiénique des colonies nouvellement fondées par ces reines à l'aide de tests biologiques sur le couvain congelé. Les larves des reines EFB^- meurent plus rapidement après une infection par la souche CH 40.2 de *M. plutonius*, qui est moins virulente, que les larves des reines EFB^+. Les colonies fondées par des reines EFB^- présentaient des niveaux d'hygiène plus élevés que les colonies fondées par des reines EFB^+. Les colonies EFB^- pourraient donc avoir développé une stratégie pour maintenir les niveaux de *M. plutonius* bas au sein de leurs colonies, consistant en une mort plus précoce des larves infectées, combinée à un comportement hygiénique plus performant. En résumé, cette thèse élargit nos connaissances sur

l'EFB, en apportant de nouveaux éléments sur la virulence et la diversité génétique du pathogène *M. plutonius* et sur le mécanisme de résistance des abeilles mellifères à une maladie infectieuse.

List of publications

This thesis is based on the following articles, each presented in a separate chapter:

Chapter I:

Grossar, D., Kilchenmann, V., Forsgren, E., Charrière, J. D., Gauthier, L., Chapuisat, M., & Dietemann, V. (2020). Putative determinants of virulence in *Melissococcus plutonius*, the bacterial agent causing European foulbrood in honey bees. *Virulence*, 11(1), 554-567.

Chapter II:

Grossar, D., Haynes, E., Budge, G. E., Parejo, M., Gauthier, L., Charrière, J. D., Chapuisat, M. & Dietemann, V. (2023). Population genetic diversity and dynamics of the honey bee brood pathogen *Melissococcus plutonius* in a region with high prevalence. *Journal of Invertebrate Pathology*, 196, 107867.

Annex:

Dainat, B., **Grossar, D.**, Ecoffey, B., & Haldemann, C. (2018). Triplex real-time PCR method for the qualitative detection of European and American foulbrood in honeybee. *Journal of Microbiological methods*, 146, 61-63.

Related publication

A co-authored publication with a topic linked to this thesis was published in:

Djukic, M., Erler, S., Leimbach, A., **Grossar, D.**, Charrière, J. D., Gauthier, L., Hartken, D., Dietrich, S., Nacke, H., Daniel, R. & Poehlein, A. (2018). Comparative genomics and description of putative virulence factors of *Melissococcus plutonius*, the causative agent of European foulbrood disease in honey bees. *Genes*, 9(8), 419.

General Introduction

Threats to honey bees

The Western honey bee (*Apis mellifera* L.) is an insect of major ecological and economic importance, and the most commonly managed insect in the world. In addition to producing economically and medically relevant products, such as honey, beeswax, pollen, propolis, royal jelly and apitoxin, honey bees provide an essential ecosystem service by pollinating many wild plants and agricultural crops (Kleijn, Winfree et al. 2015), contributing to plant biodiversity and food security. Recently reported large-scale losses of managed honey bee colonies in many regions of the world, however, potentially threaten the services and products honey bees provide (Neumann and Carreck 2010; Potts, Biesmeijer et al. 2010; Liu, Chen et al. 2016; Kulhanek, Steinhauer et al. 2017; Steinhauer, Kulhanek et al. 2018; Decourtye, Alaux et al. 2019; Gray, Adjlane et al. 2022). Multiple factors such as parasites and pathogens, habitat loss, climatic changes, agricultural intensification including the use of chemicals and monocultures, and combinations of all these factors are suspected to cause colony losses (Neumann and Carreck 2010; VanEngelsdorp and Meixner 2010; Vanbergen 2013; Nath, Singh et al. 2023). Researchers, policy-makers, beekeepers and the public are therefore interested in identifying the main drivers of honey bee declines, and disease has become a major concern for these stakeholders (Moritz, De Miranda et al. 2010; Evans and Schwarz 2011; Smith, Loh et al. 2013). Honey bee health and welfare is affected by pests and pathogens including viruses (Highfield, El Nagar et al. 2009; Berthoud, Imdorf et al. 2010; Genersch 2010), protozoa (Morimoto, Kojima et al. 2013; Ravoet, Maharramov et al. 2013), fungi (Higes, Martín-Hernández et al. 2008; Paxton 2010), parasitic mites (McMullan and Brown 2009; Dahle 2010; Guzmán-Novoa, Eccles et al. 2010; Dainat, Evans et al. 2012; Dietemann,

Pflugfelder et al. 2012; Kielmanowicz, Inberg et al. 2015) and bacteria (Wilkins, Brown et al. 2007; Roetschi, Berthoud et al. 2008; Genersch 2010; Evans and Schwarz 2011; Cornman, Tarpy et al. 2012). One of the most detrimental bacterial diseases affecting honey bees is European foulbrood (EFB).

European foulbrood disease

European foulbrood (EFB) is a bacterial disease of honey bee brood caused by the gram-positive bacterium *Melissococcus plutonius* (Fig. 1; ex. White (1912); Lactobacillales, Enterococcaceae). Although, EFB is known since more than a century (Cheshire and Cheyne 1885; Burri 1906; Maassen 1907), comparatively little is known on the underlying etiology of the disease. White (1912) identified *M. plutonius* to be the causative agent of EFB, but this is only decades later Bailey (1957) managed to successfully cultivate the pathogen.

M. plutonius enters the intestinal tract of immature honey bees presumably within food, which consists of gland secretions of adult nurse bees, mixed with pollen and honey (Brodschneider and Crailsheim 2010). Once ingested, *M. plutonius* rapidly multiplies in the mid-gut lumen (Tarr 1938; Takamatsu, Sato et al. 2016). This brood food is distributed between the larvae of a colony by nurse bees, so that pathogens are transmitted easily via this route. However, the infection routes of the disease are complex and not fully understood. Some honey bee larvae are able to survive an infection with *M. plutonius*, pupate and emerge as adults, but an infection with *M. plutonius* frequently results in death of honey bee larvae, and in consequence in a decreased adult honey bee population, leading to reduced colony strength and productivity, and eventually honey bee colony collapses (Forsgren 2010). Mainly young, i. e. unsealed honey bee larvae are susceptible to an infection. Infected larvae typically change their color from white to yellowish and brown (Fig. 1), become flaccid and often die a few days after

infection (Bailey and Ball 1991; Forsgren 2010). In contrast to brood nests of healthy honey bee colonies, the brood nests of colonies suffering from EFB show irregular capping patterns of bee brood. This visual symptom of diseased colonies is also known as pepper pot brood, where diseased, but alive and dead larvae that form a scale on the bottom of the brood cell, are scattered irregularly over the brood frame (Forsgren 2010; Forsgren, Budge et al. 2013). Additionally, EFB infected honey bee larvae often emit a foul and sour odor. The initial infection with the primary pathogen *M. plutonius* also promotes secondary infections with Enterococci or saprophytic bacteria as *Paenibacillus alvei* or *Brevibacillus laterosporus*, which intensify disease symptoms and accelerate the death of larvae (Bailey 1957; McKee, Goodman et al. 2004; Giersch, Barchia et al. 2010). *P. alvei* is the most common secondary invader isolated from EFB infected larvae (Giersch, Barchia et al. 2010) and was mistaken to cause EFB (Cheshire and Cheyne 1885).

Adult bees are carriers *of M. plutonius*, but typically do not show any disease symptoms themselves (Belloy, Imdorf et al. 2007; Roetschi, Berthoud et al. 2008). Within the honey bee colony adult bees are in charge of the nest hygiene, and forager bees easily get in contact with pathogens outside of the colony, and can import them into their hives. It is therefore supposed that adult bees play a role in the intra- and intercolonial transmission of the bacteria (McKee, Goodman et al. 2004).

M. plutonius is also known to be able to persist for several years within beeswax, pollen and honey, without causing disease symptoms in honey bee colonies (Forsgren, Lundhagen et al. 2005; Belloy, Imdorf et al. 2007; The National Bee Unit 2017). EFB symptoms in bee colonies often become apparent when they suffer environmental or nutritional stress. For example, in regions with seasonal

colder climate, honey bee colonies often do not have enough young nurse bees or food resources to adequately tend their growing larval population, after a reproduction pause during winter. Brood care is often impaired and increases larval susceptibility to EFB (Forsgren 2010). Generally, the nurse bee population and also the food supply increases with the progression of the season, and colonies may spontaneously recover from EFB (Bailey and Locher 1968; Forsgren 2010; The National Bee Unit 2017).

EFB is usually diagnosed initially via visual inspection of brood combs directly in the field, and the suspicion for EFB disease is verified only in a second step e.g. by antibody-based field test kits (Tomkies, Flint et al. 2009), or laboratory diagnostics which can include bacterial culture, PCR, and DNA sequencing (Bailey 1957; Pinnock and Featherstone 1984; Alippi 1991; Allen and Ball 1993; Govan, Brözel et al. 1998; Roetschi, Berthoud et al. 2008; Forsgren 2010; Arai, Miyoshi-Akiyama et al. 2014; Grangier, Belloy et al. 2015; Dainat, Grossar et al. 2018; Nesvorna, Sopko et al. 2021).

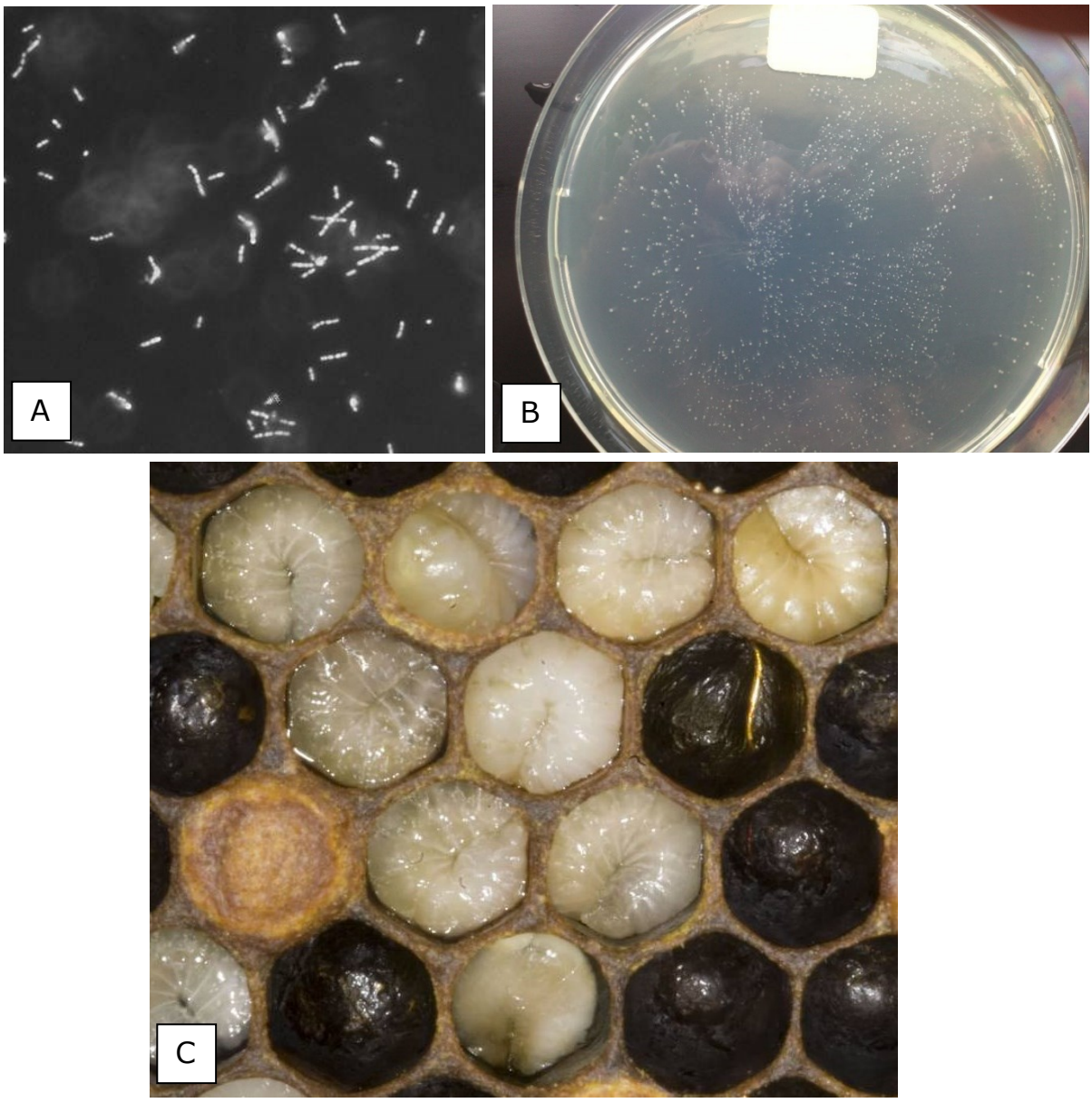

Fig. 1: *M. plutonius* under the light microscope (A) and on an agar plate (B). Honey bee larvae with EFB symptoms in brood comb (C; photo by K. Ruoff).

Prevalence and measures to control EFB worldwide and in Switzerland

Despite the descriptor "European" in its name, the disease is distributed worldwide (Ellis and Munn 2005; Boncristiani, Ellis et al. 2020). About 80 countries worldwide have classified EFB as notifiable disease (OIE) 2020) and in about a quarter of these countries the destruction of EFB-symptomatic colonies is required. Numerous countries have reported severe outbreaks in the last years (Wilkins, Brown et al. 2007; Roetschi, Berthoud et al. 2008; Takamatsu, Morinishi et al. 2014; De León-Door, Romo-Chacón et al. 2018; Grossar, Kilchenmann et al. 2020; Nesvorna, Sopko et al. 2021; Pietropaoli, Carpana et al. 2022; Thebeau, Liebe et al. 2022; Grossar, Haynes et al. 2023; Peña-Chora, Toledo-Hernández et al. 2023). EFB has emerged as infectious disease in the United Kingdom since the mid-eighties (Wilkins, Brown et al. 2007), since the year 2000 in Switzerland (Roetschi, Berthoud et al. 2008; Von Büren, Oehen et al. 2019; Grossar, Haynes et al. 2023) and since 2010 in Norway (Dahle, Sørum et al. 2011). A considerable increase in EFB outbreaks have been also reported from Finland, France, Greece, the Netherlands, Czech Republic, Italy and Canada (Dahle, Wilkins et al. 2014; Hendrikx, Saussac et al. 2015; Erban, Ledvinka et al. 2017; Thebeau, Liebe et al. 2022).

In Switzerland, the prevalence of reported EFB cases varies regionally. The disease is detected more often in the central midland and eastern parts, than in the western or southern parts of the country. Most of registered EFB cases have been reported in the canton of Bern (41 %), which holds about 23 % of the Swiss active apiaries, followed by the cantons of Zurich (9 %) and of Luzern (8 %) (InfoSM, Federal Food Safety and Veterinary Office FSVO; N= 8596 cases 1992-2021; (Von Büren, Oehen et al. 2019)). Many abiotic and biotic factors, as

geographical and climatic differences, colony and apiary density, and also anthropological influences (e.g. beekeeping practices, or exchange of materials within language groups) have been suspected to cause elevated prevalence of EFB in certain Swiss regions. However, so far no single key factor influencing prevalence has been identified, and interactions of multiples factors are suspected.

The number of reported EFB cases rose from 1999 to peak in 2010, with 992 annually reported cases (Fig. 1.2; InfoSM, Federal Food Safety and Veterinary Office FSVO), which corresponds to approximately 5 % of the Swiss active apiaries at that time (Charrière, Frese et al. 2018). Since then, strict and intensified control measures were implemented (TSV/OFE 916.401, Art. 273, 1995;FSVO (2015)), and the number of reported EFB cases in Switzerland is decreasing (Fig. 2). In 2022, 91 cases of EFB were reported.

An EFB outbreak in a Swiss apiary entails the application of costly sanitation actions to prevent further spread. Symptomatic honey bee colonies have to be immediately sacrificed and all bee material has to be if possible disinfected or disposed. If more than half of the honey bee colonies of an apiary are symptomatic for EFB, all colonies of the apiary have to be destructed. A containment zone with a radius of one kilometer around the affected apiary is established and no bees, bee colonies or beekeeping material is permitted to exit this containment zone. The affected apiary itself and all neighboring apiaries within the containment zone are visited several times and monitored closely by trained bee inspectors upon next spring, for the occurrence of EFB symptomatic colonies (FSVO 2015).

In some countries, broad-spectrum antibiotics, such as oxytetracycline, are authorized to treat EFB-affected colonies. However, antibiotic resistance development and a potential accumulation of residues in honey are undesirable side effects of this treatment (Miyagi, Peng et al. 2000; Waite, Jackson et al. 2003;

Mosca, Giannetti et al. 2022), and relapses after antibiotic treatments are reported (Waite, Jackson et al. 2003; Budge, Barrett et al. 2010; The National Bee Unit 2017). The Shook Swarm method, in which all the honeybees are shaken off infected combs and transferred onto new comb foundations into a new clean hive, offers an alternative method to control EFB without sacrificing the honey bee colony (Waite, Brown et al. 2003; Budge, Barrett et al. 2010; Fried 2011; Mosca, Bubnic et al. 2023). However, this method can involve significant labor and expense (The National Bee Unit 2017).

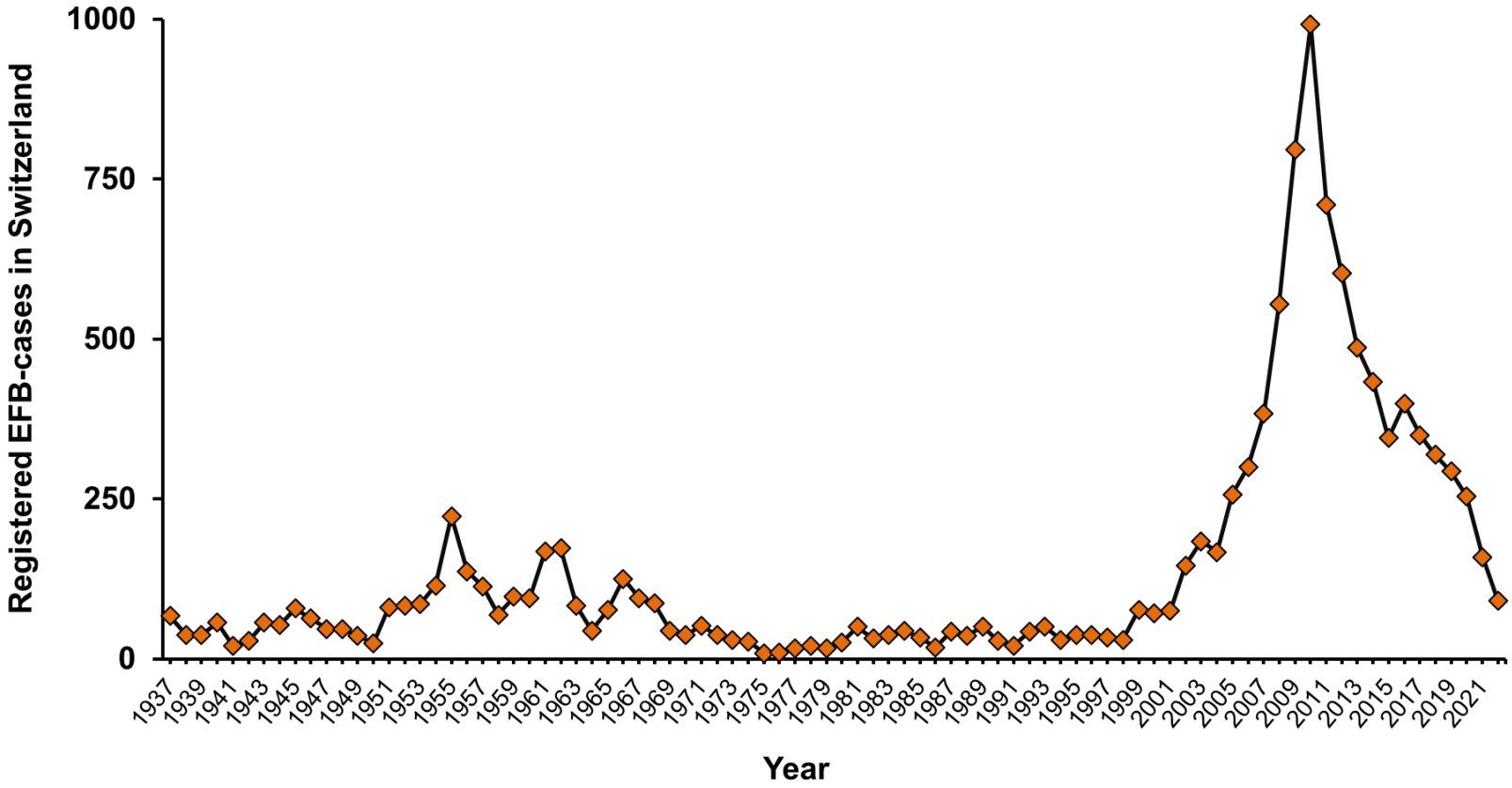

Fig. 2: Registered EFB cases in Switzerland from 1937 to 2022. Data from Info-SM, FSVO and Agroscope.

Different isolates of the pathogen *M. plutonius*

M. plutonius strains, even though isolated from different sources, regions and *Apis* species (*A. florea* (Saraithong, Li et al. 2015); *A. cerana* (Singh Rana, Mohan Rao et al. 2012); *A. mellifera* and *A. dorsata* (Allen and Ball 1993)), were thought to be remarkably homogenous (Allen and Ball 1993; Djordjevic, Smith et al. 1999). This opinion was reconsidered, as newer genetic studies identified notable differences among *M. plutonius* strains (Okumura, Arai et al. 2011; Arai, Tominaga et al. 2012; Okumura, Arai et al. 2012; Haynes, Helgason et al. 2013; Arai, Miyoshi-Akiyama et al. 2014; Budge, Shirley et al. 2014; Takamatsu, Morinishi et al. 2014; De León-Door, Romo-Chacón et al. 2018; Djukic, Erler et al. 2018; Okumura, Takamatsu et al. 2018; Grossar, Kilchenmann et al. 2020; Nakamura, Okumura et al. 2020; Grossar, Haynes et al. 2023).

A relatively new method, multi-locus sequence typing (MLST), enables the detection of relationships between bacterial isolates and to group them accordingly, by analyzing only small pieces of the genome and using online platforms such as the Bacterial Isolate Genome Sequence Database (BIGSdb) (Maiden, van Rensburg et al. 2013). For *M. plutonius*, a MLST-scheme, based on four loci, has been developed recently (Haynes, Helgason et al. 2013), and was used to successfully classify *M. plutonius* strains from all over the world into 47 sequence types (ST) (Haynes, Helgason et al. 2013; Budge, Shirley et al. 2014; Takamatsu, Morinishi et al. 2014; De León-Door, Romo-Chacón et al. 2018; Thebeau, Liebe et al. 2022; Grossar, Haynes et al. 2023).

Whole genome sequencing allows an in-depth analysis of bacterial genomes and phylogenomic lineages within a bacterial species (Okumura, Arai et al. 2011; Okumura, Arai et al. 2012; Djukic, Erler et al. 2018; Okumura, Takamatsu et al. 2018). Whole genome sequenced *M. plutonius* isolates, reference strain

ATCC35311 from United Kingdom (Bailey and Collins 1982; Okumura, Arai et al. 2011), 2011) and strain DAT561 isolated in 2009 in Japan (Arai, Tominaga et al. 2012; Okumura, Arai et al. 2012; Okumura, Takamatsu et al. 2018) both carry a circular 1.8 Mbp chromosome plus an accessory mobile genetic element, a plasmid pMP1. Djukic, Erler et al. (2018) whole-genome sequenced 12 *M. plutonius* isolates from Norway and Switzerland and found that the genomes are very similar in gene content. While all analyzed isolates also had plasmid pMP1, four Swiss isolates harbored an additional 19.4 kbp plasmid pMP19. Interestingly, plasmid pMP19 was also detected in a re-analysis of *M. plutonius* isolate DAT561 (Okumura, Takamatsu et al. 2018).

Mobile genetic elements, including plasmids, bacteriophages and pathogenicity islands are vectors for gene exchange between bacteria and play an important role in bacterial evolution (Ochman, Lawrence et al. 2000; Frost, Leplae et al. 2005; Wiedenbeck and Cohan 2011). The main source for innovations in the bacterial genome is horizontal gene transfer, which refers to the acquisition of DNA material from another, but non-parental cell (Ochman, Lawrence et al. 2000; Frost, Leplae et al. 2005). Their high genome plasticity helps bacteria to quickly adapt to changing environmental conditions and to establish in new niches. Plasmids are extrachromosomal DNA molecules which can replicate autonomously within a bacterial cell and frequently contain genes which encode traits that bring a selection advantage for the cell. Genes commonly found on plasmids are for antimicrobial resistance, for catabolism of unique nutrient sources, bacteriocin synthesis, growth inhibition of competitors, for heavy metal tolerance or virulence (Rankin, Rocha et al. 2011; Carroll and Wong 2018). In microbial pathogens, factors involved in infection are often encoded on plasmids, e.g. for the development of fimbriae that facilitate the attachment to host cell surfaces, or for

exotoxins (Pallen and Wren 2007). The possession of a plasmid containing a gene involved in infection can significantly increase the virulence of a bacterial strain, as repeatedly confirmed in animal infection models (Takai, Sekizaki et al. 1991; Chow, Thal et al. 1993; Ben-Dov, Boussiba et al. 1995; Bakshi, Singh et al. 2003; Cusumano, Hung et al. 2010). These plasmids are therefore often called virulence plasmids.

Genotypic differences can have a significant influence on the phenotype of a bacterial strain, as already demonstrated for Japanese *M. plutonius* isolates (Takamatsu, Arai et al. 2013; Arai, Miyoshi-Akiyama et al. 2014; Nakamura, Yamazaki et al. 2016; Takamatsu, Osawa et al. 2017; Takamatsu, Okumura et al. 2020; Nakamura, Okumura et al. 2021). Pulsed field gel electrophoresis showed that Japanese strains are clustered into two distinct genetic groups, typical and atypical *M. plutonius* (Arai, Tominaga et al. 2012). Atypical strains, including the whole genome sequenced *M. plutonius* strain DAT561 (Okumura, Arai et al. 2011; Okumura, Arai et al. 2012; Arai, Miyoshi-Akiyama et al. 2014), are genetically distinct from typical *M. plutonius* strains, and differ also in their cultural and biochemical traits (Arai, Tominaga et al. 2012; Takamatsu, Arai et al. 2013). Notably, atypical strains cause significantly higher mortality rates in laboratory based infection assays (Arai, Tominaga et al. 2012; Nakamura, Yamazaki et al. 2016; Nakamura, Okumura et al. 2020; Nakamura, Okumura et al. 2021). Differences in virulence also occur between typical *M. plutonius* strains, depending on experimental conditions (Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019; Grossar, Kilchenmann et al. 2020; Nakamura, Okumura et al. 2020). This suggests that virulence difference in *M. plutonius* strains have a genetic basis.

Virulence

Virulence is defined as the relative capacity of a pathogen to cause damage in a host in comparison to other species or strains of the same pathogen (Casadevall and Pirofski 2003; Ebert and Bull 2008; Pirofski and Casadevall 2012). A very general definition of virulence is the increase in host mortality, or at least reduction of the host's fitness, due to infection (Schmid-Hempel 2014). Although virulence is an attribute of pathogens, it is the outcome of a host-pathogen interaction. Hence virulence is also dependent on host reaction, and is modulated by environmental conditions (Schmid-Hempel 2014). From a microorganism-centered viewpoint virulence is determined by the presence or absence of microbial gene products (e.g. capsular polysaccharides or toxins), that distinguishes a pathogenic from a non-pathogenic microorganism. A purely host-centered view, in contrast, emphasizes the failure of host defense mechanism (e.g. immune reactions) in this interaction. Each host-pathogen interaction is unique and neither immune function, nor microbial fitness are constant factors (Casadevall and Pirofski 2003; Pirofski and Casadevall 2012).

Several factors are likely to influence the virulence of pathogens. One is the impact of secondary agents (Blaser and Cohn 1986). In the case of EFB, saprophytic species are assumed to cause secondary bacterial infections that increase damage to larvae (Forsgren 2010; Erler, Lewkowski et al. 2018). However, their influence in EFB is not fully understood. Infection of healthy honey bee larvae in the laboratory with smears of EFB diseased larvae, which is equivalent to an infection with the primary pathogen *M. plutonius* in combination with a set of bacterial secondary invaders involved in EFB, that are also commonly found in natural infection within the honey bee colony, results in increase larval mortality (McKee, Goodman et al. 2004). In contrast, better standardizable co-

infections of honey bee larvae in the laboratory with *M. plutonius* and only the most commonly secondary invader, *Paenibacillus alvei*, did not increase mortality of honey bee larvae beyond to the mortality induced by single infections with *M. plutonius* alone (Giersch, Barchia et al. 2010; Lewkowski and Erler 2019; Grossar, Kilchenmann et al. 2020). These experiments were performed *in vitro* in the laboratory, and the virulence shown by pathogens *in vitro* compare to *in vivo*, within the complex environment of honey bee colonies might be inverse, as discussed and shown for the other bacterial honey bee brood pathogen *Paenibacillus larvae* (Genersch, Ashiralieva et al. 2005; Rauch, Ashiralieva et al. 2009). However, *in vivo* infections of whole honey bee colonies in the field often carry a high sanitary risk, especially when dealing with a fast and highly spreading pathogen as *M. plutonius*. Therefore, simulating natural infections in the colony by standardized infection assays *in vitro*, in which individual pathogen isolates are tested on single hosts, is a suitable method to evaluate the virulence of *M. plutonius*, while decoupling individual larval response from social defense mechanisms.

In vitro larval rearing tests

Besides the risk of disease transmission in the field, artificial infection assays of honey bee larvae *in vitro* avoid the complex social environment and transmission dynamics of pathogens within the honey bee hive. The first reports on rearing honey bee larvae in the laboratory appeared as early as 1927 (Bertholf 1927; Velich 1930; Von Rhein 1933), even though the aim of these early studies was a better understanding of physiological and developmental processes in larvae (Crailsheim, Brodschneider et al. 2013). These early protocols were improved over time, with optimization of the diet consisting today mainly of diluted royal jelly mixed with fructose and glucose, which led to two principal *in vitro* rearing

methods. In the first method, one larva is reared per cup and the exact amount of diet a larva consumes daily is administered (Rembold and Lackner 1981; Vandenberg and Shimanuki 1987; Aupinel, Fortini et al. 2005). In the second method, multiple larvae are transferred first into a single culture plate with food in excess, and then are progressively isolated into new dishes with increasing age (Peng, Mussen et al. 1992; Genersch, Ashiralieva et al. 2005; Genersch, Forsgren et al. 2006; Behrens, Forsgren et al. 2010). Both methods are similar and enable the standardized infection of bee brood with larval pathogens (Brødsgaard, Ritter et al. 1998; Brødsgaard, Ritter et al. 2000; McKee, Goodman et al. 2004; Genersch, Ashiralieva et al. 2005; Genersch, Forsgren et al. 2006; Jensen, Pedersen et al. 2009; Behrens, Forsgren et al. 2010). In the past, such infection assays were considered unfeasible with cultivated *M. plutonius*, as the pathogen showed a rapid decrease in infectivity and virulence during bacterial sub-cultivation in artificial media (Bailey 1957; Bailey 1963; Bailey and Locher 1968; McKee, Goodman et al. 2004). More recently, several studies have successfully used *in vitro* larval rearing to infect honey bee larvae with previously cultivated *M. plutonius* isolates and induced pathogen related mortality in infected larvae (Giersch, Barchia et al. 2010; Arai, Tominaga et al. 2012; Vásquez, Forsgren et al. 2012; Wu, Sugimura et al. 2014; Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019; Grossar, Kilchenmann et al. 2020; Nakamura, Okumura et al. 2020).

The *in vitro* larval rearing methods offer not only the ability to test the virulence of the pathogen *M. plutonius* and secondary bacterial invaders in EFB disease, but also proved useful to investigate the susceptibility of individual larvae to the disease e.g. after transgenerational immune priming (Ory, Duchemin et al. 2022), or based on the patriline of the larvae (Ameline, Beaurepaire et al. 2023).

Varying mortality rates among larvae after infection with *M. plutonius* (Ameline, Beaurepaire et al. 2023) indicate a genetic origin of disease resistance in honey bees, and supports the theory that polyandry, which increases genetic diversity within the honey bee colony, contributes to disease resistance (Wilson-Rich, Spivak et al. 2008).

Polyandry in honey bee colonies

In honey bee colonies the queen is the main reproductive female, who mates multiple times during nuptial flights and conserves a sperm blend of 5-20 drones in her spermatheca (Estoup, Solignac et al. 1997), creating a high level of intra-colony genetic diversity. All other female nestmates develop from eggs fertilized with this stored sperm (Brodschneider, Arnold et al. 2012) and are half-sisters, sharing the same mother, or full-sisters, sharing also the same father, and are therefore members of the same patriline.

There is empirical evidence that individual patrilines differ in resistance to pathogens and parasites (Palmer and Oldroyd 2003; Tarpy and Seeley 2006; Invernizzi, Peñagaricano et al. 2009; Bourgeois, Rinderer et al. 2012). The organization of the honey bee society, featuring polyandry, increases the intracolonial genetic diversity, and thereby influences the susceptibility of honey bee diseases. A higher host genetic diversity is generally thought to reduce the risk of spread for infectious diseases in host populations as predicted in mathematical models (Lively 2010), and therefore might be beneficial for disease resistance. This relationship between polyandry and disease resistance is also known as the "genetic diversity for disease resistance hypothesis", and the anticipated elevated disease resistance in polyandrous broods following this hypothesis was repeatedly found in eusocial hymenopterans (Soper, Ekroth et al. 2021). Indeed, several studies demonstrated that polyandry increases disease and

parasite resistance in honey bees (Tarpy 2003; Seeley and Tarpy 2006; Tarpy and Seeley 2006; Evison, Fazio et al. 2013; Lee, McGee et al. 2013; Delaplane, Pietravalle et al. 2015; Desai and Currie 2015; Simone-Finstrom, Walz et al. 2016). This might explain why polyandry has evolved and persisted in honey bees, despite the costs associated with multiple mating and the lack of direct benefits from such behavior (Palmer and Oldroyd 2003).

Polyandry also affects other traits in honey bees, as hygienic behavior (Pérez-Sato, Châline et al. 2009), which indirectly affects disease resistance at the colony level.

Hygienic behavior

In the honey bee colony, several behavioral defense mechanisms have evolved, including necrophoric behavior, behavioral fever, grooming behavior and hygienic behavior (Evans and Spivak 2010). Hygienic behavior means the detection and effective removal of unhealthy and dead brood from the honey bee colony by adult worker bees to prevent the intra-colonial transmission of brood diseases, and is one of the key factors of colony level immunity (Cremer, Armitage et al. 2007; Wilson-Rich, Spivak et al. 2008; Pérez-Sato, Châline et al. 2009; Evans and Spivak 2010; Spivak and Danka 2021). As adult bees act as carriers of *M. plutonius*, they potentially play a key role in the transmission of the bacteria within and between colonies. However, nurse bees can also perform hygienic brood removal after detecting sick and dead brood, and transport the contagious remains out of the colony (Wilson-Rich, Spivak et al. 2008; Evans and Spivak 2010). This behavior can significantly repress disease outbreak, even if *M. plutonius* can persist for several years in the colony (Pinnock and Featherstone 1984; Forsgren, Lundhagen et al. 2005; Belloy, Imdorf et al. 2007).

Essentially, all bioassays used to quantify the hygienic behavior honey bee colonies consist of two main steps: In the first step, a defined portion of bee brood is sacrificed, inoculated with pathogen, or infested with a parasite; In a second step, the removal rate, corresponding to the number of cleaned out cells, is evaluated after a certain period of time (Leclercq, Francis et al. 2018). Several bioassays have been developed to quantify the occurrence of hygienic behavior, and all of them have inherent limitations and advantages, which should be considered when choosing an appropriated assay for the research question of an experiment (Spivak and Gilliam 1998; Leclercq, Francis et al. 2018). Among the available assays, the freeze killed brood removal assay is the most appropriate for testing hygienic behavior of bees against EFB, as commonly used for American foulbrood (AFB) (Spivak and Reuter 1998). There is no inoculation of brood with a pathogen involved, which eliminates the risk of spreading the disease, and unlike to the pin-killed brood removal assay there is no leaking of haemolymph of needle pinned brood, which could bias the result of the assay, as leaking haemolymph could enhance the stimulus to adult workers for removing dead brood (Leclercq, Francis et al. 2018). Indeed, there is evidence, that olfactory signals from inside the brood cell could trigger hygienic removal of brood wounded by a needle (Wagoner, Spivak et al. 2018). The application of liquid nitrogen directly onto a section of a honey bee brood comb to sacrifice bee brood is a more recent method (Spivak and Gilliam 1998). Compared to the freezer method, in which a section of a comb with already sealed brood in their later larval stages or pupal stage, is frozen in a conventional freezer, liquid nitrogen is more convenient and the freezing process is shorter (Leclercq, Francis et al. 2018). In both methods, the removal rate of sacrificed brood off the treated comb is determined after 24 h and 48 h in the bee colony (Spivak and Gilliam 1998; Leclercq, Francis et al. 2018).

However, for screening honey bee colonies for hygienic behavior as defense against EFB, utilizing frozen, unsealed brood (cf. Al Toufailia, Evison et al. (2018)), would be a better approach, as most larvae with EFB die early in unsealed cells, so that uncapping might not be necessary for hygienic behavior against EFB (Spivak and Gilliam 1998). Such assays to assess the capacity for hygienic behavior of honey bee colonies have been widely used in research and implemented into breeding programs of honey bee livestock resistant to various diseases and parasite infestations (Leclercq, Pannebakker et al. 2017).

Resistance to disease in honey bees

There are two distinct ways by which hosts can deal with infections and protect themselves: The first, resistance, means reduction of pathogen load and to limit the pathogen burden. The second, tolerance, stands for the limitation of harm caused by a pathogen. These two mechanisms complement each other and increase the capacity of the hosts to endure and survive infection (Ayres and Schneider 2008; Råberg, Graham et al. 2008; Schmid-Hempel 2014). Host health and ultimately fitness hence depends on the one hand on the ability of a host to limit parasite burdens (resistance), and on the other hand on the ability to limit the damage caused by a given parasite burden (tolerance) (Råberg, Graham et al. 2008).

Resistance and tolerance to a certain pathogen or parasite, whether acquired via natural selection or selective breeding efforts, span a wide range of behavioral adaptations or are based on individual immunity. In the particular case of honey bee colonies counteracting EFB disease, social and individual immune defenses, e.g. hygienic behavior for reducing the pathogen load, hence clearing the infection at the colony level, might play an important role, and rather represent

resistance mechanisms against EFB. Whereas, tolerance rather confers to the ability of infected brood or honey bee lines to withstand and survive an infection with the pathogen *M. plutonius* (c.f. Kurze, Routtu et al. (2016) described individual and colony level defenses, which could be attributed to tolerance and resistance of honey bees, against the parasites *Varroa destructor* and *Nosema* sp.). Resistance and tolerance mechanisms can act concurrently, to reach resilience of an organism towards a parasite or disease (Blacquière and Panziera 2018; Knap and Doeschl-Wilson 2020).

Aims of this PhD

In this thesis, I explore interactions between the Western honey bees (*A. mellifera*) host and its bacterial pathogen *M. plutonius*, causing European foulbrood disease. In the first section of this thesis, I deal with the pathogen *M. plutonius*, particularly with its virulence towards honeybee larvae (chapter I) and its genetic diversity and spatial population structure (chapter II). In chapter I, I investigate the virulence of sixteen *M. plutonius* isolates, using a standardized assay to infect honey bee larvae with the pathogen *in vitro.* The presence of melissotoxin A (*mtxA*), a recently discovered gene, that codes for a putative toxin is discussed as key virulence factor of *M. plutonius*. I further analyze the differences in the growth dynamics of Swiss *M. plutonius* isolates in culture medium, and link them to the ability of the pathogen *M. plutonius* to cause mortality in infected larvae. Since secondary bacteria in EFB disease are suspected to intensify disease symptoms, I investigate the effect of co-infection of honey bee larvae with *M. plutonius* and the common secondary invader *P. alvei*.

In chapter II of this thesis, I apply multi-locus sequence typing (MLST) to 160 *M. plutonius* isolates to study their genetic diversity and geographical

distribution in Switzerland. I also screen the isolates for melissotoxin A (*mtxA*), the gene that putatively increases virulence of *M. plutonius* following the results of chapter I, and notice an increase in frequency of *mtxA* in the Swiss *M. plutonius* population.

In the second part of this thesis, I combine field and laboratory based assays to investigate how the host, *A. mellifera*, can protect itself effectively from EFB infections. In chapter III, I evaluate differences in the resilience against EFB of two groups of newly established honey bee colonies. One group of colonies is headed by honey bee queens collected from colonies showing clear EFB symptoms during acute EFB outbreaks in their former apiary. While the other group of newly formed colonies consists of the offspring of queens originating from colonies without EFB symptoms during an EFB outbreak in their apiary of provenance. I applied freeze killed brood bioassays, to test for differences in hygienic behavior between these two groups of colonies, and subjected the larvae of these two groups of queens to infections with two *M. plutonius* isolates in the laboratory. Based on the results of these experiments, I discuss a potential strategy of honey bee colonies to effectively combat EFB disease.

In Annex I, I present a publication on the development of a PCR method for the diagnosis of European and American foulbrood diseases, via parallel detection of the two main bacterial honey bee brood pathogens *M. plutonius* and *P. larvae*. This method is a useful tool to simplify the early detection of these two honey bee diseases and to effectively hinder their spread via control measures, in Switzerland and worldwide.

Overall, the first part of this thesis contributes to a better understanding of the pathogen *M. plutonius*, by identifying characteristics which influence its virulence at the level of individual honey bee larvae, and by shedding light on its

genetic diversity and population structure in Switzerland. The second part of this thesis examines the collective resistance mechanism of honey bee colonies to EFB, and increases knowledge of the mechanisms underlying the resistance of these social insects to infectious diseases.

Chapter I: Putative determinants of virulence in *Melissococcus plutonius*, the bacterial agent causing European foulbrood in honey bees

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Abstract

Melissococcus plutonius is a bacterial pathogen that causes epidemic outbreaks of European foulbrood (EFB) in honey bee populations. The pathogenicity of a bacterium depends on its virulence, and understanding the mechanisms influencing virulence may allow for improved disease control and containment. Using a standardised *in vitro* assay, we demonstrate that virulence varies greatly among sixteen *M. plutonius* isolates from five European countries. Additionally, we explore the causes of this variation. In this study, virulence was independent of the multilocus sequence type of the tested pathogen, and was not affected by experimental co-infection with *Paenibacillus alvei*, a bacterium often associated with EFB outbreaks. Virulence *in vitro* was correlated with the growth dynamics of *M. plutonius* isolates in artificial medium, and with the presence of a plasmid carrying a gene coding for the putative toxin Melissotoxin A. Our results suggest that some *M. plutonius* strains showed an increased virulence due to the acquisition of a toxin-carrying mobile genetic element. We discuss whether strains with increased virulence play a role in recent EFB outbreaks.

Introduction

The Western honey bee (*Apis mellifera* L.) is an insect of major worldwide ecological and economic importance. Honey bees produce honey and wax and pollinate many economically important crops (Klein, Vaissière et al. 2007). The recent loss of managed honey bee colonies in several regions of the world threatens the ecological services provided by this pollinator (Ellis, Evans et al. 2010; Neumann and Carreck 2010; Potts, Biesmeijer et al. 2010; Gray, Brodschneider et al. 2019). Consequently, honey bee health has become a major concern not only for scientists, but also for the public and policy-makers (Moritz, De Miranda et al. 2010; Evans and Schwarz 2011; Smith, Loh et al. 2013). Current research indicates that pathogens are a major cause of colony losses. Honey bee pathogens include viruses (Genersch 2010), protozoa (Morimoto, Kojima et al. 2013), fungi (Higes, Martín-Hernández et al. 2008), parasitic mites (Dietemann, Pflugfelder et al. 2012) and bacteria (Wilkins, Brown et al. 2007; Roetschi, Berthoud et al. 2008; Genersch 2010; Evans and Schwarz 2011). One of the most detrimental bacterial diseases affecting honey bees is European foulbrood (EFB). EFB is reported worldwide (Ellis and Munn 2005) and has emerged as an infectious disease since the mid-eighties in the United Kingdom, since the year 2000 in Switzerland and since 2010 in Norway (Wilkins, Brown et al. 2007; Roetschi, Berthoud et al. 2008; Dahle, Sørum et al. 2011; Von Büren, Oehen et al. 2019). High numbers of clinical cases have also been reported from Finland, France, Greece, Holland, Czechia and Italy (Dahle, Wilkins et al. 2014; Hendrikx, Saussac et al. 2015; Erban, Ledvinka et al. 2017), making EFB an economically important veterinary disease (Dahle, Wilkins et al. 2014; Grangier, Belloy et al. 2015).

The pathogenic agent of EFB, *Melissococcus plutonius* (*Lactobacillales*, *Enterococcaceae*) (White 1912), enters the intestinal tract of honey bee larvae

through contaminated food provided by adult bees (Forsgren 2010). Once ingested, *M. plutonius* rapidly multiplies in the mid-gut lumen, possibly depriving the host of nutrients (Bailey 1983). Diseased larvae typically change from white to a yellowish colour, become flaccid and die 4-5 days after infection (Bailey and Ball 1991; Forsgren 2010). The massive loss of brood resulting from severe infection weakens the colony and can lead to its collapse (Bailey and Ball 1991).

To date, only broad-spectrum antibiotics such as oxytetracycline are available to treat EFB-affected colonies. Due to the risk of antibiotic resistance development (Miyagi, Peng et al. 2000; Waite, Jackson et al. 2003) and an accumulation of residue in honey, the use of antibiotics is not a sustainable method to control EFB and is banned in some countries. In the absence of efficient treatment and given the severity of EFB outbreaks, 79 countries worldwide have classified EFB as a notifiable disease (World Animal Health Information Database, OIE (World Organisation for Animal Health (OIE)) (OIE) 2020)). In 22 of these countries, veterinary authorities destroy symptomatic colonies and monitor neighbouring apiaries to avoid the further spread of the pathogen, which is costly and time-consuming. This situation calls for new control strategies, but their development is constrained by the limited knowledge of the pathogenesis of EFB (Forsgren 2010).

Virulence is central to pathogenesis. A better knowledge of the mechanisms that determine this trait could contribute to the design of improved control methods. Indeed, virulence factors are promising targets for specific drugs or management measures (Vale, McNally et al. 2016). Such measures could consist in curing honey bee colonies hosting less virulent pathogens and restricting the use of destructive control methods to treat infections caused by more virulent pathogens (Abadi and Kusters 2016). An assessment of the extent and causes of

virulence variation in strains of *M. plutonius* may thus prove useful in controlling EFB more efficiently and more sustainably to improve honey bee health.

Early reports (Bailey and Gibbs 1962; Djordjevic, Smith et al. 1999) suggested that *M. plutonius* isolates were genotypically and phenotypically homogeneous. As a result, variation in virulence was not expected. The discovery of genetic differences between isolates (Okumura, Arai et al. 2011; Arai, Tominaga et al. 2012; Okumura, Arai et al. 2012; Haynes, Helgason et al. 2013; Budge, Shirley et al. 2014; Takamatsu, Morinishi et al. 2014; Djukic, Erler et al. 2018; Okumura, Takamatsu et al. 2018) has challenged this view. Differences in virulence have indeed been documented within *M. plutonius*, with strains defined as atypical by Arai, Tominaga et al. (2012), killing a higher proportion of hosts in a shorter time period than typical strains. Differences in virulence also occur between typical strains, depending on experimental conditions (Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019). However, no virulence factor has yet been clearly identified.

Several factors are likely to influence the virulence of pathogens. One is the impact of secondary agents (Blaser and Cohn 1986). In the case of EFB, saprophytic species such as *Paenibacillus alvei*, *Enterococcus faecalis*, *Brevibacillus laterosporus* or *Achromobacter eurydice* cause secondary bacterial infections that might increase damage to larvae (Forsgren 2010; Erler, Lewkowski et al. 2018). However, their influence in EFB is debated (Bailey 1957; McKee, Goodman et al. 2004; Giersch, Barchia et al. 2010; Lewkowski and Erler 2019). Another potential cause of variation in virulence is the growth dynamics of the bacterial pathogen. Bacterial strains that multiply rapidly and reach high densities can cause more damage to the host (North and Izzo 1993; Zhang, Gong et al. 1998). Other major factors influencing virulence include the production of

biologically active compounds, such as adhesins improving attachment to host cells, enzymes degrading host tissues, or toxins disturbing the physiological processes of the host (Finlay and Falkow 1997; Wu, Wang et al. 2008; Antúnez, Anido et al. 2011; Fünfhaus, Poppinga et al. 2013; Garcia-Gonzalez, Poppinga et al. 2014; Krska, Ravulapalli et al. 2015).

Experiments aimed at quantifying the virulence of *M. plutonius* and identifying factors that influence virulence have been hampered by the complex social environment of the honey bee colony that affects the spread and growth of the bacteria (Bailey 1963; Evans and Spivak 2010), and by the legal obligation to destroy colonies showing disease symptoms (Roetschi, Berthoud et al. 2008). These constraints can be overcome by experimentally infecting honey bee larvae reared *in vitro*. In the past, such assays were deemed impossible due to *M. plutonius* loss of infectivity during bacterial sub-cultivation (Bailey 1957; Bailey 1963; McKee, Goodman et al. 2004). More recently, several studies using the *in vitro* larval rearing method (Aupinel, Fortini et al. 2005) have circumvented this problem (Giersch, Barchia et al. 2010; Arai, Tominaga et al. 2012; Vásquez, Forsgren et al. 2012; Wu, Sugimura et al. 2014; Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019; Nakamura, Okumura et al. 2020), making standardized quantification of virulence possible without any sanitary risk to the colonies in the field.

In this study, we reared honey bee larvae *in vitro* and infected them with *M. plutonius* to investigate the causes and extent of variation in virulence. We screened 17 *M. plutonius* isolates collected from five European countries for genetic differences and measured their virulence over the entire developmental period of worker brood. We examined whether virulence was associated with *M. plutonius* multilocus sequence type (MLST), co-infection with *P. alvei*,

differences in growth dynamics in a culture medium and presence of a putative toxin-coding gene (Djukic, Erler et al. 2018). We discuss whether *M. plutonius* strains with high virulence play a role in recent EFB outbreaks and whether they could be targets for more sustainable control measures of the disease.

Results

Virulence of *M. plutonius* strains

The *M. plutonius* isolates collected in various regions of Europe belonged to six multilocus sequence types and two clonal complexes (CC 3 and CC 13, Tab. 1.1) of the typical form. After excluding cases with excessive control mortality, we obtained enough replicates to quantify the virulence of 16 out of the 17 isolates tested. The virulence of these isolates in terms of honey bee brood mortality *in vitro* varied greatly (Fig. 1.1). Three Swiss isolates (CH 21.1, CH 49.3 and CH 60) were highly virulent, causing over 80 % mortality in infected brood up to the imago stage (Tab. 1.1, Tab. S2, Fig. S3). These isolates caused significant mortality compared to the controls (Fig. 1.1; pairwise log-rank tests, Bonferroni-Holm corrected, $p < 0.001$, Tab. S1, Fig. S3). Eight isolates (FR 27.1, CH 119, CH 54.1, CH 46.1, CH 45.1, CH 40.2, NO 764-5B, NO 765-6B) had low to intermediate virulence, causing a brood mortality of 15 to 55 %, which was significantly higher than that of the controls (Fig. 1.1; pairwise log-rank tests, Bonferroni-Holm corrected, $p < 0.001$, Tab. S1 and S2, Fig. S3). The mortality of the brood infected with each of the remaining five isolates (UK 36.1, UK 31.1, CH MeplS1, CH 82, IT 1.3) was not significantly higher than that of the non-infected control brood. These isolates were therefore categorized as avirulent (Fig. 1.1; pairwise log-rank tests, Bonferroni-Holm corrected, n.s., Tab. S1 and S2, Fig. S3).

Isolate code	Collection location (city, region and/or country)	Year	GenBank accession number	Sequence type (clonal complex)	Generation time ± SD [h]	Presence of melissotoxin A gene	Mortality	Virulence degree
UK 36.1	Somerset, England	2006	NA	ST 3 (3)	3.46 ± 0.98	NEGATIVE	1.24 %	Avirulent
UK 31.1	Norfolk, England	2006	NA	ST 13 (13)	3.83 ± 0.16	NEGATIVE	4.67 %	Avirulent
CH MeplS1[1]	Graubünden, Switzerland	2007	GCA_000747585.1	ST 3 (3)	3.49 ± 0.62	NEGATIVE	5.88 %	Avirulent
CH 82	Bern, Switzerland	2007	GCA_001047595.1	ST 32 (13)	5.40 ± 3.99	NEGATIVE	6.80 %	Avirulent
IT 1.3	Turin, Italy	2006	NA	ST 3 (3)	5.07 ± 1.42	NEGATIVE	7.43 %	avirulent
FR 27.1	Gard, France	2006	NA	ST 20 (13)	4.28 ± 0.95	NEGATIVE	16.46 %	low-intermediate
CH 90	Fribourg, Switzerland	2006	GCA_001047445.1	ST13 (13)	5.53± 2.77	NEGATIVE	22.55 %	low-intermediate
CH 119	Bern, Switzerland	2007	GCA_001047515.1	ST 20 (13)	4.70 ± 1.25	NEGATIVE	28.28 %	low-intermediate
CH 54.1	St. Gallen, Switzerland	2007	NA	ST 35 (13)	5.22 ± 1.42	NEGATIVE	32.82 %	low-intermediate
CH 46.1	Zürich, Switzerland	2006	NA	ST 7 (3)	4.19 ± 2.30	NEGATIVE	39.44 %	low-intermediate
CH 45.1	St. Gallen, Switzerland	2007	NA	ST 3 (3)	3.63 ± 1.02	NEGATIVE	42.93 %	low-intermediate
CH 40.2	Zürich, Switzerland	2007	NA	ST 35 (13)	2.82 ± 0.70	NEGATIVE	43.97 %	low-intermediate
NO 764-5B	Norway	2011	GCA_001047465.1	ST 3 (3)	4.52 ± 1.21	NEGATIVE	48.74 %	low-intermediate
NO 765-6B	Norway	2011	GCA_001047435.1	ST 3 (3)	4.79 ± 1.26	NEGATIVE	53.50 %	low-intermediate
CH 49.3	Graubünden, Switzerland	2007	GCA_001047395.1	ST 3 (3)	5.05 ± 3.98	POSITIVE	79.98 %	high
CH 21.1	Bern, Switzerland	2006	GCA_001047455.1	ST 7 (3)	4.55 ± 0.80	POSITIVE	84.85 %	high
CH 60	Bern, Switzerland	2007	GCA_001047545.1	ST 7 (3)	4.39 ± 1.49	POSITIVE	91.88 %	high

[1] CH MeplS1 derives from re-cultivation of *M. plutonius* isolate CH 49.3 in the laboratory, and lost plasmid pMP19 including *melissotoxin A* (Djukic, Erler et al. 2018).

Table 1.1. *Melissococcus plutonius* isolates with their collection location, year of isolation, multilocus sequence type and clonal complex. The presence of the melissotoxin A gene was assessed with a PCR screening test (Djordjevic, Smith et al. 1999). The post-infection mortality rate is expressed as a Henderson-Tilton corrected percentage. Avirulent isolates did not cause significant mortality compared to non-infected controls (Tab. S1) and Henderson-Tilton corrected mortality rates are inferior to 10 %. Low to intermediate virulence degrees correspond to mortality rates in the range of 15-55 %. High virulent isolates cause mortality rates above 80 % (see Fig. S3)

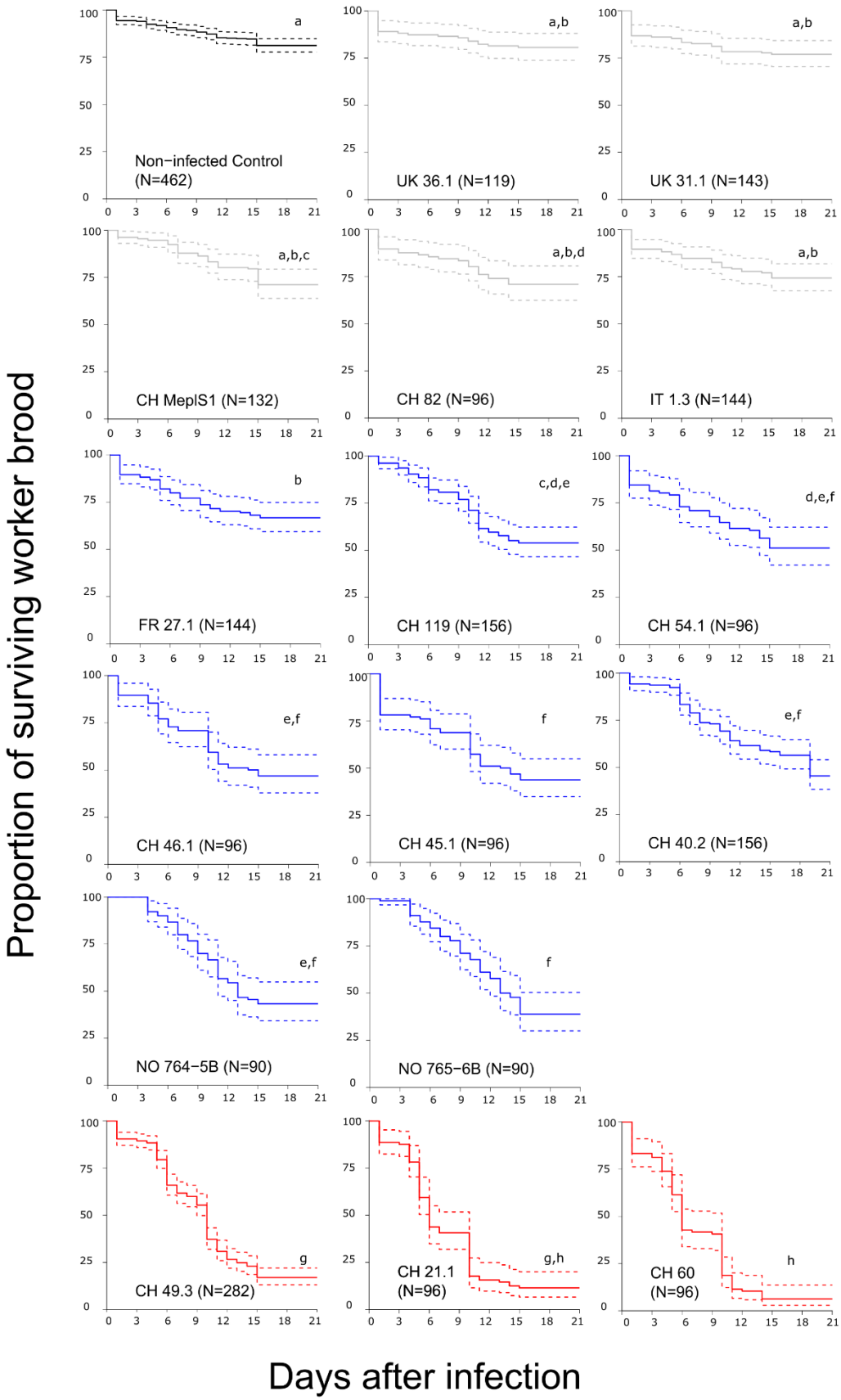
Proportion of surviving worker brood
Days after infection
Non−infected Control (N=462)
a
UK 36.1 (N=119)
a,b
UK 31.1 (N=143)
a,b
CH MeplS1 (N=132)
a,b,c
CH 82 (N=96)
a,b,d
IT 1.3 (N=144)
a,b
FR 27.1 (N=144)
b
CH 119 (N=156)
c,d,e
CH 54.1 (N=96)
d,e,f
CH 46.1 (N=96)
e,f
CH 45.1 (N=96)
f
CH 40.2 (N=156)
e,f
NO 764−5B (N=90)
e,f
NO 765−6B (N=90)
f
CH 49.3 (N=282)
g
CH 21.1 (N=96)
g,h
CH 60 (N=96)
h

Fig. 1.1. Survival of *in vitro* reared honey bee brood infected with 16 *M. plutonius* isolates (isolate code indicated in each panel). Grey, blue and red curves correspond to avirulent, low to intermediate and high virulence isolates, respectively. The black curve indicates survival of non-infected controls. N = number of larvae tested. Dashed lines represent 95 % confidence intervals. Significant differences in the survival of brood due to treatments (uncorrected for control mortality) are indicated by different letters (pairwise log-rank tests, Bonferroni-Holm corrected, $p < 0.001$

Mortality caused by *M. plutonius*, *P. alvei* and co-infection

The survival of the honey bee brood after a single infection with *M. plutonius* CH 90 (Mean Henderson-Tilton corrected mortality 22.6 %, Tab. S2) was significantly lower than the survival of the non-infected control brood (Fig. 1.2, Tab. S4; pairwise log-rank tests, Bonferroni-Holm corrected p= 0.006). Single infection with *P. alvei* DSM29 (Mean Henderson-Tilton corrected mortality 8.82 %, Tab. S2) also caused significant mortality within the brood (Fig. 1.2, Tab. S4; pairwise log-rank tests, Bonferroni-Holm corrected p= 0.007), as did co-infection with *M. plutonius* CH 90 and *P. alvei* DSM29 (Fig. 1.2, Tab. S2 and S4; pairwise log-rank tests, Bonferroni-Holm corrected p= 0.004; Mean Henderson-Tilton corrected mortality 23.08 %). However, no significant increase in mortality was observed after co-infection with *M. plutonius* and *P. alvei* compared to single infections with one bacterial species alone (Fig. 1.2 Tab. S4, pairwise log-rank tests, Bonferroni-Holm corrected p> 0.01).

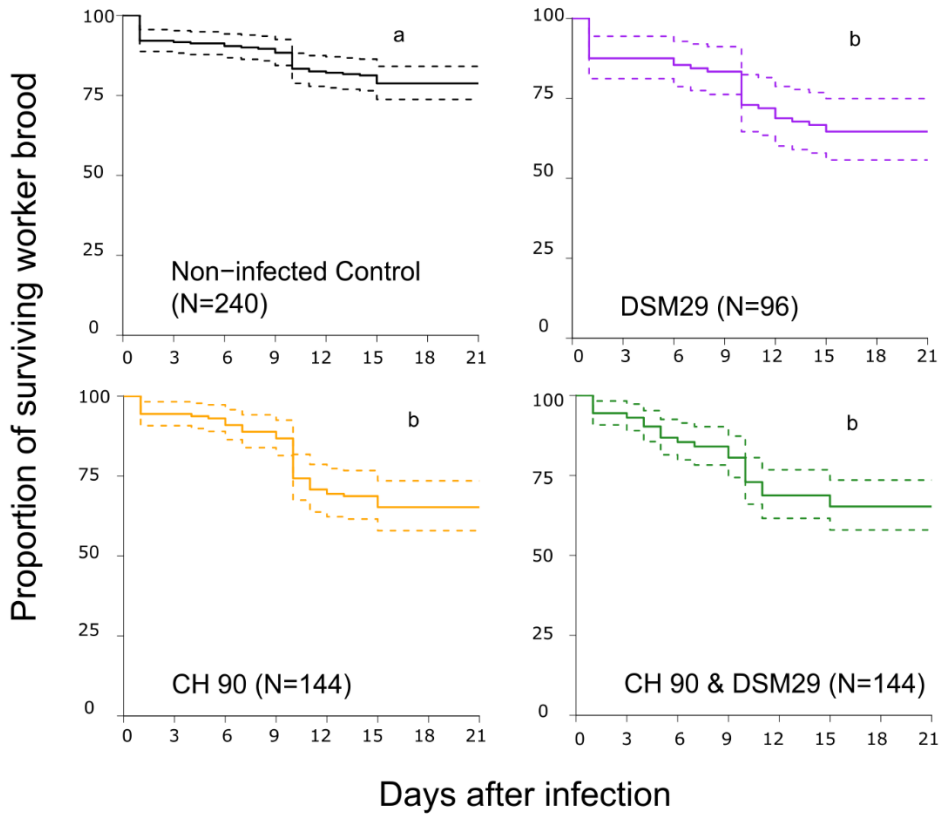

Fig. 1.2. Survival of *in vitro* reared honey bees exposed to *M. plutonius* isolate CH 90, orange curve) or to *P. alvei* reference strain DSM29 (purple curve) only, or after co-infection with *M. plutonius* CH 90 and *P. alvei* DSM29 (green curve). The black curve indicates the survival of non-infected controls. Dashed lines represent 95 % confidence intervals and N designates the number of larvae tested. Significant differences in the survival of brood due to treatments are indicated by different letters (pairwise log-rank tests, Bonferroni-Holm corrected, $p < 0.01$).

Growth dynamics and virulence of *M. plutonius*

The *M. plutonius* isolates varied in their *in vitro* growth dynamics (Fig. 1.3). Some bacterial isolates multiplied rapidly to high cell densities, while others multiplied slowly and reached low final cell densities in liquid basal medium at the end of the observation period. Other strains showed mixed features, multiplying rapidly to low final cell densities and inversely. Four isolates of low to intermediate virulence (FR 27.1, CH 45.1, NO 764-5B and NO 765-6B) and one avirulent isolate (IT 1.3) showed no significantly different growth pattern as isolates of high virulence (CH 49.3, CH 60 and CH 21.1; Fig. 1.3, Tab. S5 and S6). OD_{600} of most isolates decreased abruptly after 84h, indicating bacterial mortality. We thus considered this time point as indicative of final density reached. Four isolates of low to intermediate virulence (FR 27.1, CH 45.1, NO 764-5B and NO 765-6B) reached final densities as high as those measured in high virulence isolates (CH 49.3, CH 21.1 and CH 60; Fig. 1.3, Tab. S5). *M. plutonius* isolates causing over 40 % mortality (corrected through a Henderson-Tilton calculation) in the *in vitro* larval infection assay, also reached high final densities in basal medium with OD_{600} values at 0.7 or above, and five out of the seven most virulent isolates reached OD_{600} values above 0.8 (Tab. S5).

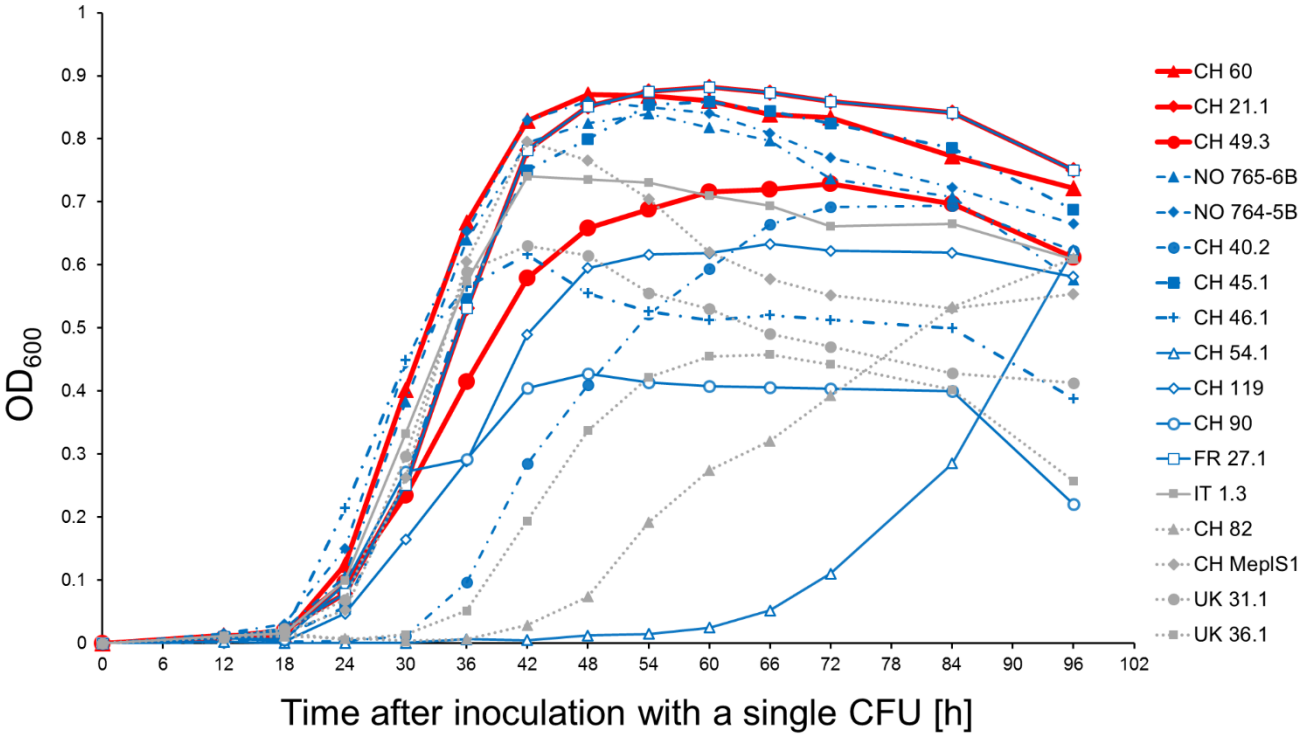

Fig. 1.3. Growth curves of 17 *M. plutonius* isolates in artificial medium. Red, blue and grey curves correspond to high, low-intermediate virulence and avirulent isolates, respectively. See Tab. S5 for mean OD600-values and SD and table S6 for statistical differences in growth dynamics between isolates.

Presence of melissotoxin A gene

The three highly virulent *M. plutonius* isolates CH 21.1, CH 49.3 and CH 60 were positive for the gene encoding Melissotoxin A in PCR screening, while all other isolates were negative (Tab. 1.1, S8).

Relative contribution of sequence type, clonal complex, growth rate, final cell density and Melissotoxin A to the virulence of *M. plutonius*

The multiple regression model, including sequence type, clonal complex, generation time, final cell densities and the presence of Melissotoxin A gene, explained a significant part of brood mortality (R^2= 0.806, $F_{5,15}$= 7.483, p< 0.005; Tab S7). While the presence of the plasmid detected through the amplification of a 1.36 kbp-fragment of a putative toxin gene significantly predicted brood mortality (B= 45.802, β= 0.617, p< 0.007), neither sequence type (B= 0.757, β= 0.270, p= 0.422), nor clonal complex (B= -2.419, β= -0.384, p= 0.277), nor generation time (B= -2.952, β= -0.068, p= 0.671), nor cell density reached at 84 h (B= 74.226, β= 0.342, p= 0.068) was significantly associated with brood mortality. The model did not show a correlation with the mortality rate induced in the honey bee brood (N= 15, Kendall's τ= 0.175, p= 0.266; Tab. S7) and the generation time during the exponential growth phase in artificial medium, which ranged from 3 to 6 h (Tab. 1.1). In contrast, final cell density (at 84 h) was positively correlated with mortality (N= 15, Kendall's τ= 0.641, p< 0.005, Tab. S7).

Discussion

Virulence varies among *M. plutonius* isolates

Mortality of the honey bee brood caused by the *M. plutonius* isolates tested varied greatly. The variation in virulence among the 16 isolates was independent of their multi-locus sequence type and clonal complex affiliation. Variation in virulence among strains of the so called 'atypical' *M. plutonius* belonging to different clonal complexes has recently been documented (Nakamura et al., 2016). Our results indicate that variation in virulence also occurs in clonal complexes belonging to the "typical" strains of *M. plutonius* as observed in (Lewkowski and Erler 2019). Mortality rates also varied among replicates of *in vitro* infection tests using the same *M. plutonius* isolate, but larvae of different colonies. This is in line with the results from two recent studies (Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019) and may be due to variation in host susceptibility. Although all but one isolate (CH Mepl S1) tested in this study were derived from honey bee colonies with explicit symptoms of an acute EFB infection, four isolates (UK 36.1, UK 31.1, CH 82 and IT 1.3) did not cause elevated larval mortality *in vitro*, and were therefore ranked as avirulent. It is possible that this discrepancy is a methodological artefact due to the loss of the plasmid carrying the putative virulence factor during cultivation on artificial media. Although the tested isolates were subjected to the same laboratory procedures, random loss of the plasmid cannot be excluded. Alternatively, negative effects on the development or physiology of the larvae not detected in our assay could have triggered increased removal by adult hygienic workers, generating the typical spotty brood pattern in EFB-diseased colonies. It is also possible that bacterial isolates less virulent at individual level are highly virulent at colony level, and vice versa, as was hypothesized for *Paenibacillus larvae* (Genersch, Ashiralieva et al. 2005). Larvae

infected with low virulence isolates might be removed slower than larvae affected by high virulence isolates. A low removal rate could facilitate the production and spread of the pathogenic bacteria within the colony. It is highly likely that the *in vivo* virulence of *M. plutonius* at colony level is modulated by the effect of the social immunity of the colony (Cremer, Pull et al. 2018), as proposed for *P larvae* (Spivak and Reuter 2001; Genersch, Ashiralieva et al. 2005) be different from virulence quantified *in vitro*.

No evidence that co-infection with *P. alvei* increases mortality

Our results indicate that the presence of the secondary agent *P. alvei* is not required to induce brood mortality. We observed high larval mortality after single infection with some of the *M. plutonius* isolates, in line with former studies (Arai, Tominaga et al. 2012; Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019; Nakamura, Okumura et al. 2020). To our knowledge, this is the first report on single infections with *P. alvei* in an *in vitro* infection assay. In this study, single infection with *P. alvei* strain DSM29 caused significant mortality to the honey bee brood. In contrast, co-infection with *M. plutonius* did not increase brood mortality beyond the effect of single infections with the tested *M. plutonius* isolate (CH 90). These results are similar to those of Lewkowski and Erler (2019), but differ from those of Giersch, Barchia et al. (2010), probably because of methodological differences (e.g., different infection time, higher concentration of *P. alvei* used in their study). To better understand the role of secondary agents, other bacteria associated with EFB should be tested singly and in co-infections with a larger set of *M. plutonius* isolates *in vitro* (Lewkowski and Erler 2019) as well as *in vivo* in honey bee colonies.

Relationship between *M. plutonius* growth dynamics and virulence

The growth dynamics of *M. plutonius* isolates in the culture medium were highly variable. The bacterial generation time in the artificial medium did not predict mortality in the honey bee brood. The final bacterial density may play a more important role in determining pathogenicity (*sensu* Casadevall and Pirofski (1999)) than the generation time. All isolates defined as being of intermediate or high virulence reached high final densities in the culture medium, and isolates with lower final densities caused low mortality. This pattern obtained *in vitro* suggests that strains must reach a damage threshold to induce high mortality. Although the growth dynamics of *M. plutonius* isolates in culture medium as observed in this study may be different from that in honey bee larvae, the hypothesis that a high bacterial load is a prerequisite for high virulence is supported by the occurrence of a positive correlation between bacterial numbers and virulence *in vivo* (Nakamura, Yamazaki et al. 2016; Nakamura, Okumura et al. 2020). However, some low virulence isolates reached high final densities in the culture medium at 84 h, suggesting that factors other than the number of *M. plutonius* bacteria are involved in causing high mortality in honey bee brood.

Role of Melissotoxin A in virulence

The Melissotoxin A gene was restricted to three highly virulent Swiss isolates (CH 21.1, CH 49.3 and CH 60) and the presence of this gene was a significant predictor of honey bee brood mortality (Tab. S5). In our experiment, the bacterial isolate CH MeplS1 was avirulent. This isolate originated from a bacterial culture of the highly virulent isolate CH 49.3 and lost plasmid pMP19 (19.4 kbp; GenBank: JSBA01000009.1) after repeated sub-cultivation (Djukic, Erler et al. 2018). Plasmid pMP19 encodes melissotoxin A (GenBank: KMT29105) and another

putative virulence factor, the extracellular matrix-binding protein (Djukic, Erler et al. 2018). While the additional loss of genome-encoded virulence factors in CH MeplS1 cannot be excluded, the simultaneous loss of pMP19 and virulence suggests a causal link.

A further piece of evidence for the possible implication of the melissotoxin A gene in virulence is that it is expressed by *M. plutonius* during infection *in vivo* (Djukic, Erler et al. 2018; Nakamura, Okumura et al. 2020). This gene shares a high sequence similarity with the epsilon toxins of the ETX/MTX2 family (pfam03318 of *Clostridium perfringens*, 33 % amino acid sequence identity (Djukic, Erler et al. 2018) and of *Bacillus pumilus* with 48 % sequence identity, 90 % coverage and an Expect (E)-value of $7e^{-73}$, NCBI query; https://www.ncbi.nlm.nih.gov/). Epsilon toxins change the cell permeability for ions by forming large membrane pores, causing cell death and oedema in animal models (Petit, Maier et al. 2001). It was recently suggested that a protein of the ETX/MT2 family is an important virulence factor in two subtypes of *P. larvae*, the causative pathogen of American foulbrood (Erban, Zitek et al. 2019). It is thus plausible that Melissotoxin A increases the virulence of *M. plutonius* by corrupting the cells of the larva's digestive tract.

The melissotoxin A gene is situated on plasmid pMP19 (Djukic, Erler et al. 2018). Plasmids can easily be exchanged between individual bacterial cells within and between species (Frost, Leplae et al. 2005). The plasmid pMP19 found in certain *M. plutonius* strains, including atypical isolates from Japan (Okumura, Takamatsu et al. 2018; Nakamura, Okumura et al. 2020), may originate from other bacterial invaders co-existing with *M. plutonius* in the intestinal tract of honey bee larvae. The fact that the toxin gene is located on a mobile genetic

element can explain the absence of association between brood mortality and the sequence type or clonal complex of the isolates used in this study.

The conventional explanation for the negative effect of *M. plutonius* is a competition for nutrients in the gut (Bailey and Ball 1991). Although not mutually exclusive, our finding that a toxin-carrying mobile genetic element could confer high pathogenicity to certain *M. plutonius* isolates suggests a direct detrimental effect on gut cells. This mode of action is in line with the findings of McKee, Goodman et al. (2004) and with evidence that *M. plutonius* still caused high larval mortality in *in vitro* experiments where food was given in excess (McKee, Goodman et al. 2004; Giersch, Barchia et al. 2010; Arai, Tominaga et al. 2012; Vásquez, Forsgren et al. 2012; Wu, Sugimura et al. 2014; Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019; Nakamura, Okumura et al. 2020).

Further experiments will be needed to demonstrate the direct role of melissotoxin A in the pathogenicity of *M. plutonius*. Such experiments include producing genetically modified strains of *M. plutonius*, for example by removing plasmid pMP19 from highly virulent strains, knocking-out melissotoxin A gene on pMP19 (pMP19Δmelissotoxin A gene), transforming wildtype pMP19 and pMP19Δmelissotoxin A gene into low virulent isolates of *M. plutonius*, as well as producing the putative toxin in the laboratory. The impact of mutant strains or the synthesized toxin on honey bee larvae could then be directly tested *in vitro*. Curing pMP19 from Japanese *M. plutonius* isolates belonging to CC 3 resulted in a loss of virulence (Nakamura, Okumura et al. 2020), supporting our hypothesis for the role of the melissotoxin A gene. However, transformation experiments aimed at introducing the plasmid back in the cured isolates did not result in recuperation of virulence (Nakamura, Okumura et al. 2020). A reduced growth of these isolates due to the transformation protocol was provided as an explanation (Nakamura,

Okumura et al. 2020), in line with our suggestion that rapid multiplication is also required to produce highly virulent phenotypes. The role of the putative virulence gene on pMP19 could not be confirmed in a Japanese isolate of CC 13 (Nakamura, Okumura et al. 2020). A reduced growth of this isolate could also explain its low virulence despite the gene's presence.

Is the recent emergence of EFB due to highly virulent strains?

The question then arises as to whether the recent emergence of EFB is linked to the occurrence of bacterial strains of particularly high virulence at the colony level. Virulence trade-off models predict that the virulence of a pathogen is inversely correlated to its transmission, because excessive virulence causes early host death, hindering the spread of the pathogen amongst the population (Anderson and May 1982; Ewald 1983; Ebert and Bull 2008; Alizon, Hurford et al. 2009). However, human-induced changes in the rate of horizontal transmission may have altered the adaptive compromise between virulence and transmission in *M. plutonius* (Govan, Brözel et al. 1998; Fries and Camazine 2001; Von Büren, Oehen et al. 2019). Increased contact between honey bee colonies due to human management may allow for the spread of highly virulent strains unable to spread to new hosts under more natural settings.

More extensive field screening of the prevalence of different *M. plutonius* sequence types and of the plasmid carrying the melissotoxin A gene will reveal if highly virulent strains of *M. plutonius* arose by acquiring this toxin-carrying mobile genetic element. This will also contribute to a better understanding of the origin of recent epidemic outbreaks of EFB. If a link between virulence and disease outbreaks in the field can be established, strain-specific, virulence-based control methods can be promising avenues to better control the spread of EFB (Abadi and

Kusters 2016; Vale, McNally et al. 2016). The melissotoxin A gene could provide a putative marker for such specific control methods.

Conclusion

Infection with *M. plutonius* alone caused European foulbrood symptoms in honey bee brood reared *in vitro*. Bee mortality varied substantially among 16 investigated *M. plutonius* isolates, independently of their sequence type. Mortality did not increase when larvae were infected with the potential secondary agent *P. alvei* in combination with a *M. plutonius* isolate of low to intermediate virulence. Variation in the virulence of *M. plutonius* isolates was not significantly linked to growth dynamics. A high final density in the culture medium was reached by highly virulent isolates, but was not sufficient to explain the high mortality caused by these *M. plutonius* isolates. High virulence was associated with the presence of a plasmid carrying the putative toxin melissotoxin A, suggesting that this mobile genetic element is a major factor in the virulence of *M. plutonius*. These new insights into the pathogenicity of a poorly understood, but important honey bee disease may serve as a basis for the development of more sustainable control methods.

Experimental procedures

Origin and culture of *M. plutonius* isolates

Diseased larvae were collected from European foulbrood (EFB) outbreaks in several European regions (Tab. 1.1) and sent to our laboratory for the purpose of *M. plutonius* isolation. The only exception was the Norwegian samples, which were processed elsewhere. *Melissococcus plutonius* was isolated from smears of diseased larvae on basal medium. The medium contained 1 % yeast extract, 1 % glucose, 1 % saccharose, 0.04 % L-cysteine and 0.1 M KH_2PO_4 in distilled water, with a pH adjusted to 6.7 with 5 M KOH. The medium was solidified with 18 g agar/liter and autoclaved at 121 °C for 18 min (Bailey 1957; Forsgren, Budge et al. 2013). After incubation for four days at 36 °C under anaerobic conditions (GENbox anaer, bioMérieux), individual bacterial colonies identified as *M. plutonius* based on colony morphology were picked from the Petri dishes and inoculated in a liquid basal medium or on plates. The plates were incubated anaerobically for another four days at 36 °C. Isolate stock solutions were supplemented with 15 % glycerol and stored at -80 °C until further use.

PCR identification of *M. plutonius* and detection of the gene encoding melissotoxin A

Isolates were confirmed as *M. plutonius* by PCR as described by Govan, Brözel et al. (1998). The strains were further identified as either "typical" or "atypical" following the protocol described in Arai, Tominaga et al. (2012). The multi-locus sequence type and clonal complex of each isolate were assessed as described in Haynes, Helgason et al. (2013). We screened for the presence of melissotoxin A gene (GenBank: KMT29105) (Djukic, Erler et al. 2018) using specific primers (tox_MEPL_for: 5'-GCTCAAGCAGCAACTTTTACG-3' and tox_MEPL_rev: 5'-TTCCCCTGGTATTACTTGTAGATG-3'; fragment size approx.

1.36 kbp) in a conventional PCR reaction using KAPA2G Fast DNA Polymerase (KAPA Biosystems). DNA was extracted with the NucleoSpin Tissue kit from Macherey-Nagel according to the manufacturer's instructions, the DNA-concentration was measured with NanoDrop® ND-1000 spectrophotometer (NanoDrop, Thermo Fischer Scientific, USA) and each DNA-extract diluted to 5 ng DNA per µl. Each reaction in the PCR consisted of 2 µl template DNA-extract, 13 µl KAPA2G fast ready mix (2x), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), and 8 µl water added to a total reaction volume of 25 µl. The PCR started with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s and DNA extension at 72 °C for 20 s, and a final extension step at 72 °C for 2 min (TProfessional Basic Thermocycler, Biometra). PCR products were visualised under UV light after staining the 1.5 % agarose gel with GelRed™ (Biotium).

Infection assays of honey bee larvae reared *in vitro*

Honey bee brood originating from healthy, queenright colonies was used in the infection assays. Same aged larvae were obtained by confining queens to empty combs for the purpose of egg-laying, using excluder cages. After 36 hours, the queens were removed from the cages for three days, until first instar larvae hatched. First instar larvae were grafted individually in plastic queen starter cells (Nicoplast™) that had been sterilised in 70 % ethanol for 30 min. The plastic cells were placed in the cells of 48-well tissue culture plates, on top of a piece of wet dental roll imbibed with 15.5 % glycerol in 0.4 % methyl benzethonium chloride to prevent unwanted microorganism growth. The larvae were reared *in vitro*, according to standard methods (Aupinel, Fortini et al. 2005). In brief, the culture plates were placed in a hermetic desiccator containing a dish filled with saturated

K_2SO_4 solution, which ensured a relative humidity of 95 % needed to prevent dehydration of larvae. The desiccator containing the larvae was placed into an incubator at 34.5 °C for the first six days of larval development. At day 7, when the larvae defecate and start to pupate, the plates were moved into a desiccator containing a dish filled with saturated NaCl solution to ensure an optimal relative humidity of 75 %. The larvae were fed daily with pre-warmed diet (34.5 °C), according to the following feeding program: on the day of grafting, larvae were provided with 10 µl of diet A (1.2 g glucose, 1.2 g fructose, 0.2 g yeast extract and 8.4 g pure water, filter sterilized (0.2 µm) and mixed with 10 g pure royal jelly). On day 3, larvae were fed 20 µl of diet B (1.5 g glucose, 1.5 g fructose, 0.3 g yeast extract and 8 g pure water, filter sterilized (0.2 µm) and mixed with 10 g pure royal jelly). On days 4, 5 and 6, larvae were fed with 30, 40 and 50 µl of diet C, respectively. Diet C consisted of 1.8 g glucose, 1.8 g fructose, 0.4 g yeast extract and 7.45 g pure water, filter sterilized (0.2 µm) and mixed with 10 g pure royal jelly (Aupinel, Fortini et al. 2005; Crailsheim, Brodschneider et al. 2013). The royal jelly was obtained from healthy colonies and stored at -20 °C.

For *in vitro* infection experiments, *M. plutonius* isolates were cultivated in a liquid basal medium at 36 °C under anaerobic conditions for four days. In order to standardize the number of bacteria administered in the infection assays, we determined the concentration of viable bacterial cells using colony forming units (CFUs) counting. The CFUs from a serial dilution spread on basal medium agar plates were counted after four days of incubation at 36 °C under anaerobic conditions (Forsgren, Budge et al. 2013). During this time and before administration, bacterial cultures were stored at 4 °C.

Paenibacillus alvei type strain DSM29 was obtained from the German Culture Collection (DSMZ, Braunschweig, Germany) and cultivated on casein-

peptone agar plates under aerobic conditions at 30 °C for three weeks, until spores formed. Spores were harvested from agar plates by scraping them off the plate and suspending them in saline (0.9 % NaCl in distilled water). The spores were washed once with saline, diluted in saline and heat treated for 5 minutes at 90 °C. The total *P. alvei* spore concentration was determined via 10-fold dilutions plated on casein-peptone agar plates and CFU counting (Human, Brodschneider et al. 2013). Spore suspensions were stored at 4 °C.

Infection with *M. plutonius* was induced by administering a droplet of 10 µl diet A spiked with 10^7 CFU ml^{-1} of the respective *M. plutonius* isolate (1:9 *M. plutonius* inoculum-diet mix) on day 1, within two hours of grafting. Hence, each larva was fed 10^5 CFU of *M. plutonius*. Larvae were infected with *P. alvei* on day 1 by receiving 10 µl of diet A spiked with 10^5 spores of *P. alvei* per ml of saline (*i.e.* 10^3 *P. alvei* spores per larva). Co-infections were performed by feeding 10 µl diet A spiked with a mix of 10^5 *P. alvei* DSM29 spores ml^{-1} and 10^7 CFU ml^{-1} of *M. plutonius* isolate CH 90. This isolate was selected for co-infection assays for its relatively low virulence, which facilitates the detection of a putative increased mortality due to *P. alvei*. Non-infected control larvae received 10 µl of diet A mixed with sterile saline. We administered a lower number of *P. alvei* spores per larva (10^3) than in previous studies (6 x 10^4; (Giersch, Barchia et al. 2010; Lewkowski and Erler 2019)). This lower dose is closer to the one eliciting American foulbrood symptoms in infections by the congeneric bacteria *P. larvae* (20 spores/larva; (Hernández López, Schuehly et al. 2014)), and comparable to the number of *P. alvei* spores larvae were exposed to in a recent study (8.3 x 10^3; (Lewkowski and Erler 2019)).

Honey bee larvae were subjected to single infections with *M. plutonius*, single infections with *P. alvei* and co-infections with *M. plutonius* and *P. alvei*. We

monitored brood survival and compared brood mortality caused by the isolates, alone or in combination. The status of each larva (dead or alive) was recorded every 24 h by observing it under a microscope. Larvae or pupae without signs of respiration or reaction to mechanical stimulus were recorded as dead and removed from the plates (Crailsheim, Brodschneider et al. 2013). In contrast to previous studies (McKee, Goodman et al. 2004; Giersch, Barchia et al. 2010; Arai, Tominaga et al. 2012; Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019), we monitored brood survival until completion of development (*i.e.* until imaginal stage) to take into account the whole brood developmental period to assess its mortality. For each strain or combination of strains, 2-6 replicates were performed, each with 24-84 larvae produced by 2-8 queens (Tab. S2).

Growth dynamics of *M. plutonius* isolates

For each *M. plutonius* isolate used in the infection assays (17 isolates), ten replicates (two runs with five replicates each) of 20 ml liquid basal medium were inoculated with a single *M. plutonius* colony pre-cultured on an agar plate for three days and incubated under anaerobic conditions at 36 °C. Bacterial growth was monitored by measuring optical density at 600 nm (OD600) with a spectrophotometer (DR/2000, HACH), every 6 h until 96 h past inoculation (Hall et al., 2013). OD_{600} was averaged over the ten replicates. Based on this data, we calculated the generation time and compared the growth dynamics of the strains (see below).

Statistics

Survival differences in honey bee brood after experimental infection with *M. plutonius* or *P. alvei*, or with a combination of *M. plutonius* and *P. alvei*, were

illustrated with Kaplan-Meier survival curves with dashed 95 % confidence intervals (Kaplan and Meier, 1958). Differences in survival between hosts infected by various isolates, and between the latter and uninfected controls were tested using pairwise log-rank tests (Mantel-Haenszel test (Therneau 2015)) and adopting a significance level α of 0.05, corrected by a Bonferroni-Holm procedure for multiple comparisons (Holm 1979). We calculated the Henderson-Tilton-corrected mortality rate of individuals dead until day 21 of development, when the imago stage is reached, as follows: mortality ratio= 1- ((number of live test bees after treatment * number of live control bees after treatment^{-1}) * (number of live control bees before treatment * number of live bees before treatment^{-1})) (Medrzycki, Giffard et al. 2013), and state in Tab. 1.1.

We used the R package growthrates (Petzoldt 2016) to compute the growth constant k for each isolate, by fitting segments of linear models to the log-transformed OD_{600} values during the exponential growth phase (Hall, Acar et al. 2013). The generation time (g) of each isolate was then calculated (Madigan, Martinko et al. 1997) as g = ln (2) / k. As recommended by Hall, Acar et al. (2013), we verified the fit to the exponential phase generated by the algorithm implemented in Petzoldt (2016). For isolate CH 54.1, the algorithm did not identify the exponential phase correctly, due to a long and irregular lag phase. We obtained a corrected dataset by excluding the first six points for this isolate well ahead of the exponential phase, which led to correct fitting. The overall correlation between growth rate and mortality, as well as the results of the regression model, were similar between the corrected dataset, the original dataset, and after excluding this isolate from the dataset (Tab. S7). We thus present the results based on the scenario where isolate CH 54.1 was excluded from the regression model analysis.

Generation time only considers the exponential phase of the bacterial growth cycle. To analyse the growth dynamics over the entire experiment (96 h), we used permutation tests with 100,000 iterations to conduct pairwise comparisons between growth curves (Elso, Roberts et al. 2004; Baldwin, Sakthianandeswaren et al. 2007). These statistical analyses were done in R (R Foundation for Statistical Computing, Vienna, Austria) with the statmod package (Smyth, Hu et al. 2015).

A multiple regression analysis (McClelland, Bernhardt et al. 2006) was conducted to examine the relationship between the Henderson-Tilton corrected mortality and the potential virulence factors multi-locus sequence type, clonal complex, generation time, final density (density reached at 84 h) and presence of melissotoxin A gene. For this model, we used SPSS Statistics, version 21 (IBM Corp.).

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Supplementary material

	UK 36.1	UK 31.1	CH MeplS1	CH 82	IT 1.3	FR 27.1	CH 119	CH 54.1	CH 46.1	CH 45.1	CH 40.2	NO 764-5B	NO 765-6B	CH 49.3	CH 21.1	CH 60
C	0.81944	0.19992	0.02017	0.01780	0.06229	**0.00011**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**
UK 36.1		0.45221	0.13169	0.10928	0.24421	0.01355	**0.00002**	**0.00001**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**
UK 31.1			0.44487	0.35222	0.66856	0.06782	**0.00023**	**0.00007**	**0.00001**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**	**0.00000**
CH MeplS1				0.77814	0.73391	0.23749	0.00149	**0.00050**	**0.00004**	**0.00000**	**0.00003**	**0.00001**	**0.00000**	**0.00000**	**0.00000**	**0.00000**
CH 82					0.57259	0.44857	0.01447	0.00569	**0.00098**	**0.00016**	**0.00060**	**0.00055**	**0.00009**	**0.00000**	**0.00000**	**0.00000**
IT 1.3						0.14614	**0.00070**	**0.00024**	**0.00002**	**0.00000**	**0.00001**	**0.00001**	**0.00000**	**0.00000**	**0.00000**	**0.00000**
FR 27.1							0.07506	0.02285	0.00550	**0.00087**	0.00364	0.00397	**0.00061**	**0.00000**	**0.00000**	**0.00000**
CH 119								0.48027	0.17461	0.05892	0.30672	0.16880	0.07024	**0.00000**	**0.00000**	**0.00000**
CH 54.1									0.62092	0.31620	0.87371	0.55212	0.30228	**0.00000**	**0.00000**	**0.00000**
CH 46.1										0.62450	0.60145	0.98617	0.69490	**0.00000**	**0.00000**	**0.00000**
CH 45.1											0.30298	0.66099	0.96055	**0.00002**	**0.00000**	**0.00000**
CH 40.2												0.54112	0.24030	**0.00000**	**0.00000**	**0.00000**
NO 764-5B													0.75317	**0.00000**	**0.00000**	**0.00000**
NO 765-6B														**0.00001**	**0.00000**	**0.00000**
CH 49.3															0.00164	**0.00002**
CH 21.1																0.51168

Table S1. Results matrix of Kaplan-Meier survival curves comparisons after experimental infection of honey bee larvae with *M. plutonius* isolates. Survival of non-infected control larvae (C) and larvae infected with 16 *M. plutonius* isolates was compared using pairwise log rank tests (Mantel-Haenszel-test), of which the p-values are reported in the table. Significant differences after Bonferroni-Holm correction method are shown in bold.

	Treatment				Mean Henderson Tilton corrected mortality	SD	Min	Max	Runs	N	Queens
	M. plutonius		*P. alvei*								
	Isolate	CFU/ larva	Isolate	CFU/ larva							
Single Infections	UK 36.1	10^5			1.24%	4.61%	-5.26%	4.88%	3	119	4
	UK 31.1	10^5			4.67%	6.55%	-2.13%	13.51%	3	143	4
	CH MeplS1	10^5			5.88%	7.15%	-0.84%	15.79%	3	132	3
	CH 82	10^5			6.80%	1.09%	5.71%	7.89%	2	96	4
	IT 1.3	10^5			7.43%	9.15%	-2.63%	19.51%	3	144	4
	FR 27.1	10^5			16.46%	19.89%	-2.63%	43.90%	3	144	4
	CH 119	10^5			28.28%	23.03%	-4.17%	60.00%	4	156	5
	CH 54.1	10^5			32.82%	1.39%	31.43%	34.21%	2	96	4
	CH 46.1	10^5			39.44%	26.28%	13.16%	65.71%	2	96	3
	CH 45.1	10^5			42.93%	11.35%	31.58%	54.29%	2	96	3
	CH 40.2	10^5			43.97%	27.98%	18.18%	82.86%	3	156	5
	NO 764-5B	10^5			48.74%	24.36%	23.08%	81.48%	3	90	3
	NO 765-6B	10^5			53.50%	23.45%	29.17%	85.19%	3	90	3
	CH 49.3	10^5			79.98%	15.38%	59.09%	100.00%	6	282	8
	CH 21.1	10^5			84.85%	1.99%	82.86%	86.84%	2	96	4
	CH 60	10^5			91.88%	2.41%	89.47%	94.29%	2	96	4
	Mean ± SD mortality in non-infected control: 17.98% ± 5.45%										
Co-Infections	CH 90	10^5			22.55%	12.71%	4.88%	34.21%	3	144	5
	CH 90	10^5	DSM29	10^5	23.08%	22.66%	-2.44%	52.63%	3	144	6
			DSM29	10^5	8.82%	5.88%	2.94%	14.71%	2	96	3
	Mean ± SD mortality in non-infected control: 21.25% ± 7.02%										

Table S2. Henderson-Tilton corrected mortality rates in percent with standard deviation (SD), minimal (Min) and maximal (Max) value, the number of runs (Runs), number of larvae (N) and number of mother honey bee queens (Queens) used per treatment. Control mortality in percent (Mean ± SD) is given for single (N= 462, N_{Queens}= 10, N_{Runs}= 10) and co-infection assays (N= 240, N_{Queens}= 8, N_{Runs}= 5).

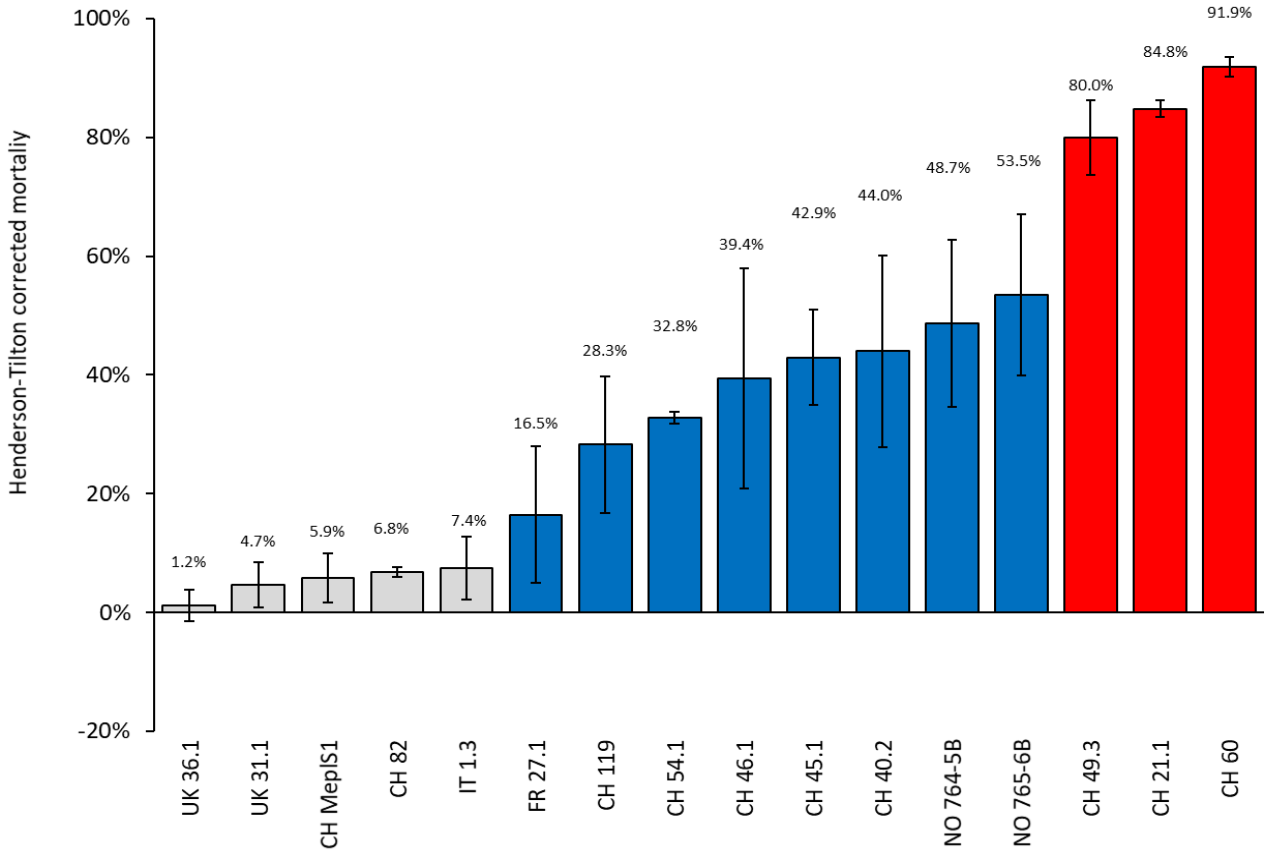


Fig. S3. Henderson-Tilton corrected honey bee brood mortality rates (mean±SE) after infection with 16 *M. plutonius* isolates. Isolates are ordered by increasing virulence. Blue and red bars correspond to low to intermediate and high virulence isolates, respectively. Grey bars represent avirulent *M. plutonius* isolates. Three categories were determined based on statistical comparisons of virulence with controls (mortality of brood infected with avirulent strains was not significantly different from that of non-infected brood) and on large differences in virulence range (i.e., between NO 765-6B and CH 49.3).

	DSM29	CH 90	CH 90 & DSM29
C	**0.00701**	**0.00569**	**0.00395**
DSM29		0.82214	0.92963
CH 90			0.84250

Table S4. Results matrix of Kaplan-Meier survival curves comparisons after experimental co-infection of honey bee larvae with *M. plutonius* and *P. alvei*. The Survival of non-infected control larvae (C), of larvae infected with *P. alvei* isolate DSM29, of larvae infected with *M. plutonius* strain CH 90, and of larvae co-infected with *M. plutonius* CH 90 and *P. alvei* DSM29, was compared using pairwise log rank tests (Mantel-Haenszel-test) and the p-values for these tests are reported in the table. Significant differences after Holm-Bonferroni correction method are bold.

	12 h	18 h	24 h	30 h	36 h	42 h	48 h	54 h	60 h	66 h	72 h	84 h	96 h
UK 36.1	**0.009** ± 0.002	**0.017** ± 0.004	**0.006** ± 0.006	**0.014** ± 0.010	**0.052** ± 0.049	**0.194** ± 0.116	**0.337** ± 0.135	**0.422** ± 0.100	**0.455** ± 0.063	**0.458** ± 0.091	**0.442** ± 0.117	**0.402** ± 0.124	**0.258** ± 0.038
UK 31.1	**0.009** ± 0.002	**0.023** ± 0.005	**0.070** ± 0.032	**0.297** ± 0.108	**0.589** ± 0.030	**0.630** ± 0.020	**0.615** ± 0.021	**0.555** ± 0.013	**0.531** ± 0.010	**0.491** ± 0.016	**0.471** ± 0.032	**0.428** ± 0.015	**0.413** ± 0.015
CH MepIS1	**0.010** ± 0.002	**0.018** ± 0.005	**0.053** ± 0.038	**0.263** ± 0.192	**0.606** ± 0.232	**0.795** ± 0.061	**0.766** ± 0.043	**0.704** ± 0.078	**0.621** ± 0.075	**0.577** ± 0.060	**0.552** ± 0.038	**0.532** ± 0.027	**0.554** ± 0.008
CH 82	**0.010** ± 0.001	**0.012** ± 0.008	**0.008** ± 0.011	**0.004** ± 0.005	**0.006** ± 0.009	**0.029** ± 0.036	**0.074** ± 0.100	**0.192** ± 0.263	**0.275** ± 0.341	**0.321** ± 0.368	**0.393** ± 0.345	**0.534** ± 0.339	**0.611** ± 0.180
IT 1.3	**0.012** ± 0.001	**0.016** ± 0.002	**0.100** ± 0.066	**0.333** ± 0.214	**0.574** ± 0.071	**0.741** ± 0.087	**0.735** ± 0.088	**0.730** ± 0.099	**0.710** ± 0.103	**0.694** ± 0.106	**0.661** ± 0.127	**0.666** ± 0.110	**0.609** ± 0.106
FR 27.1	**0.011** ± 0.003	**0.020** ± 0.009	**0.079** ± 0.062	**0.252** ± 0.189	**0.531** ± 0.200	**0.782** ± 0.148	**0.852** ± 0.108	**0.875** ± 0.094	**0.883** ± 0.082	**0.874** ± 0.081	**0.859** ± 0.090	**0.841** ± 0.084	**0.750** ± 0.046
CH 90	**0.010** ± 0.001	**0.006** ± 0.004	**0.094** ± 0.103	**0.272** ± 0.272	**0.292** ± 0.209	**0.405** ± 0.220	**0.428** ± 0.213	**0.414** ± 0.217	**0.408** ± 0.214	**0.406** ± 0.218	**0.404** ± 0.219	**0.400** ± 0.212	**0.220** ± 0.107
CH 119	**0.009** ± 0.002	**0.003** ± 0.004	**0.047** ± 0.037	**0.165** ± 0.131	**0.289** ± 0.159	**0.490** ± 0.191	**0.595** ± 0.082	**0.616** ± 0.044	**0.618** ± 0.031	**0.633** ± 0.032	**0.623** ± 0.032	**0.620** ± 0.029	**0.582** ± 0.022
CH 54.1	**0.002** ± 0.002	**0.000** ± 0.000	**0.000** ± 0.001	**0.000** ± 0.001	**0.006** ± 0.004	**0.005** ± 0.003	**0.013** ± 0.008	**0.015** ± 0.005	**0.025** ± 0.013	**0.052** ± 0.037	**0.110** ± 0.094	**0.285** ± 0.275	**0.624** ± 0.080
CH 46.1	**0.003** ± 0.001	**0.019** ± 0.011	**0.214** ± 0.199	**0.450** ± 0.256	**0.565** ± 0.109	**0.616** ± 0.153	**0.555** ± 0.160	**0.527** ± 0.154	**0.513** ± 0.149	**0.521** ± 0.133	**0.513** ± 0.120	**0.500** ± 0.116	**0.388** ± 0.031
CH 45.1	**0.002** ± 0.002	**0.012** ± 0.005	**0.058** ± 0.051	**0.260** ± 0.176	**0.548** ± 0.123	**0.750** ± 0.132	**0.799** ± 0.097	**0.854** ± 0.082	**0.859** ± 0.071	**0.844** ± 0.072	**0.824** ± 0.081	**0.786** ± 0.088	**0.688** ± 0.064
CH 40.2	**0.002** ± 0.001	**0.003** ± 0.002	**0.004** ± 0.004	**0.011** ± 0.011	**0.097** ± 0.075	**0.285** ± 0.170	**0.409** ± 0.146	**0.522** ± 0.110	**0.594** ± 0.049	**0.665** ± 0.059	**0.692** ± 0.082	**0.694** ± 0.081	**0.623** ± 0.030
NO 764-5B	**0.015** ± 0.002	**0.030** ± 0.010	**0.150** ± 0.090	**0.444** ± 0.185	**0.653** ± 0.078	**0.830** ± 0.056	**0.859** ± 0.049	**0.850** ± 0.054	**0.841** ± 0.049	**0.809** ± 0.046	**0.770** ± 0.065	**0.722** ± 0.044	**0.665** ± 0.046
NO 765-6B	**0.015** ± 0.002	**0.022** ± 0.006	**0.105** ± 0.068	**0.383** ± 0.190	**0.640** ± 0.071	**0.794** ± 0.120	**0.824** ± 0.109	**0.840** ± 0.108	**0.818** ± 0.098	**0.797** ± 0.095	**0.736** ± 0.123	**0.708** ± 0.106	**0.576** ± 0.029
CH 49.3	**0.008** ± 0.004	**0.020** ± 0.009	**0.096** ± 0.143	**0.235** ± 0.259	**0.415** ± 0.280	**0.579** ± 0.313	**0.658** ± 0.292	**0.688** ± 0.251	**0.716** ± 0.213	**0.720** ± 0.117	**0.729** ± 0.110	**0.697** ± 0.128	**0.612** ± 0.051
CH 21.1	**0.011** ± 0.003	**0.020** ± 0.009	**0.079** ± 0.062	**0.252** ± 0.189	**0.531** ± 0.200	**0.782** ± 0.148	**0.852** ± 0.108	**0.875** ± 0.094	**0.883** ± 0.082	**0.874** ± 0.081	**0.859** ± 0.090	**0.841** ± 0.084	**0.750** ± 0.046
CH 60	**0.013** ± 0.002	**0.012** ± 0.004	**0.125** ± 0.106	**0.402** ± 0.253	**0.667** ± 0.136	**0.828** ± 0.091	**0.870** ± 0.058	**0.868** ± 0.048	**0.860** ± 0.043	**0.839** ± 0.044	**0.834** ± 0.061	**0.773** ± 0.049	**0.722** ± 0.019

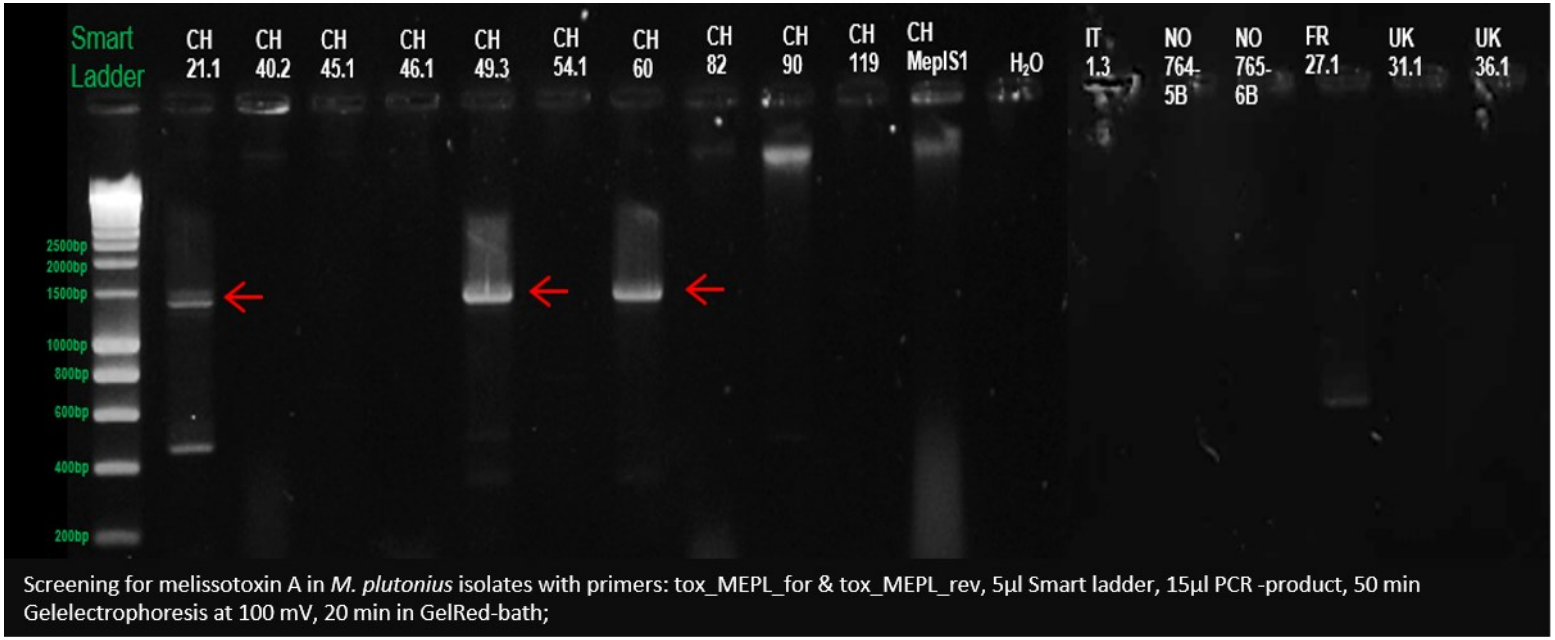
Table S5. Mean OD_{600}-values ± SD as measure for growth of 17 *M. plutonius* isolates in liquid basal medium over a time period of 96 h. Final cell densities are defined as an OD_{600}-value above 0.7 after 84 h.

	UK 31.1	CH MeplS1	CH 82	IT 1.3	FR 27.1	CH 90	CH 119	CH 54.1	CH 46.1	CH 45.1	CH 40.2	NO 764-5B	NO 765-6B	CH 49.3	CH 21.1	CH 60
UK 36.1	**0.00001**	**0.00000**	0.01035	**0.00000**	0.00223	0.42267	**0.00001**	**0.00002**	**0.00001**	**0.00000**	**0.00032**	**0.00000**	**0.00001**	**0.00011**	**0.00003**	**0.00002**
UK 31.1		**0.00003**	**0.00003**	0.01863	0.94752	0.10996	**0.00067**	**0.00000**	0.80021	**0.00006**	**0.00001**	**0.00002**	0.00085	0.06510	**0.00000**	**0.00001**
CH MeplS1			**0.00002**	0.15437	0.90064	0.00247	0.09775	**0.00002**	0.15223	**0.00004**	**0.00001**	**0.00003**	**0.00022**	0.19493	**0.00000**	**0.00005**
CH 82				**0.00001**	0.00172	0.01952	**0.00000**	0.00243	**0.00001**	**0.00000**	0.00094	**0.00002**	**0.00003**	**0.00021**	**0.00001**	**0.00000**
IT 1.3					0.45977	0.00101	0.00136	**0.00000**	0.04555	0.09402	**0.00000**	0.00418	0.06644	0.78434	0.01799	0.00551
FR 27.1						0.03713	0.15581	**0.00002**	0.74525	0.25970	0.01872	0.03038	0.08224	0.94779	0.13733	0.09960
CH 90							0.09213	**0.00007**	0.05852	**0.00001**	0.58644	**0.00001**	**0.00002**	0.01314	**0.00001**	**0.00000**
CH 119								**0.00000**	0.85927	**0.00001**	0.02366	**0.00002**	**0.00000**	0.04493	**0.00002**	**0.00001**
CH 54.1									**0.00001**	**0.00001**	**0.00002**	**0.00001**	**0.00001**	**0.00001**	**0.00002**	**0.00001**
CH 46.1										**0.00017**	0.01395	**0.00001**	0.00177	0.16161	**0.00000**	**0.00000**
CH 45.1											**0.00001**	0.30851	0.71297	0.11232	0.37932	0.30521
CH 40.2												**0.00002**	**0.00001**	0.00669	**0.00004**	**0.00000**
NO 764-5B													0.36196	0.02193	0.80796	0.62003
NO 765-6B														0.11177	0.49919	0.38737
CH 49.3															0.03602	0.02573
CH 21.1																0.91577

Table S6. Comparisons between growth dynamics of 17 *M. plutonius* isolates. Optical density of culture medium at 600 nm every six hours was compared pairwise with a permutation test with 100,000 iterations. P-values are reported in the table, and differences that are significant after Holm-Bonferroni correction method are shown in bold.

Scenario		Correlation between mortality and generation time		Correlation between mortality and final density		Multiple regression model																	
						model			ST			CC			Generation time			Final density			*Melissotoxin A*		
	N	τ	p	τ	p	R^2	F	p	B	β	p	B	β	p	B	β	p	B	β	p	B	β	p
Original dataset	16	0.153	0.285	0.553	**0.013**	0.660	6.818	**0.005**	1.278	0.524	0.149	-3.220	-0.542	0.146	-0.171	-0.004	0.980	50.614	0.280	0.136	46.815	0.635	**0.007**
First six data points of CH 54.1 excluded	16	0.050	0.427	0.553	**0.013**	0.667	7.728	**0.004**	1.072	0.440	0.186	-2.970	-0.500	0.172	2.691	0.133	0.486	61.355	0.340	0.097	44.071	0.598	**0.008**
Data of CH 54.1 excluded	15	0.175	0.266	0.641	**0.005**	0.806	7.483	**0.005**	0.757	0.270	0.422	-2.419	-0.384	0.277	-2.952	-0.068	0.671	74.226	0.342	0.068	45.802	0.617	**0.007**

Table S7. Results of the statistical analyses of growth constant k based on original dataset, corrected dataset (first six data points of the lag phase of isolate CH 54.1 excluded due to due to a long and irregular lag phase observed in this isolate, see data analysis section of methods) and original dataset without isolate CH 54.1.



Screening for melissotoxin A in *M. plutonius* isolates with primers: tox_MEPL_for & tox_MEPL_rev, 5µl Smart ladder, 15µl PCR -product, 50 min Gelelectrophoresis at 100 mV, 20 min in GelRed-bath;

S8. Picture of the agarose gel showing the results of the PCR screening for melissotoxin A gene in *M. plutonius* isolates used in this study.

Chapter II: Population genetic diversity and dynamics of the honey bee brood pathogen *Melissococcus plutonius* in a region with high prevalence

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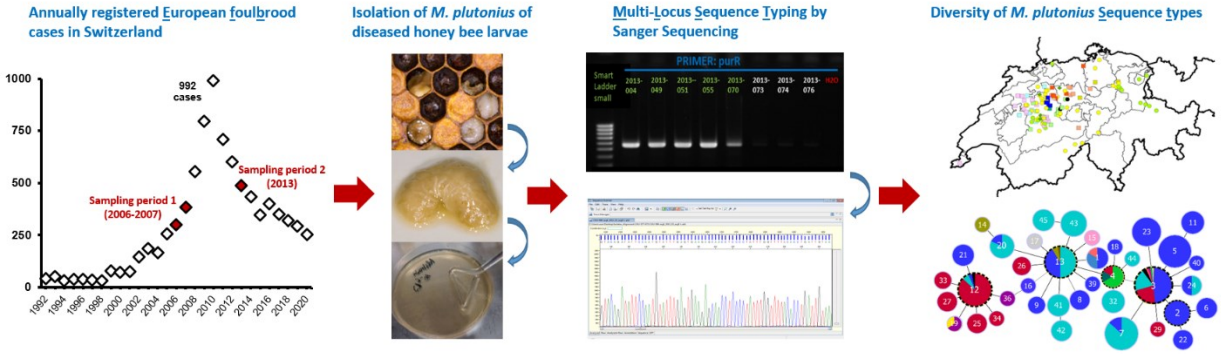
Graphical Abstract



Abstract

European foulbrood (EFB) is a honey bee brood disease caused by the bacterium *Melissococcus plutonius*. Large-scale EFB outbreaks have been reported in several countries in recent decades, which entail costly sanitation measures of affected apiaries to restrict the spread of this contagious pathogen. To mitigate its impact, a better understanding of the population dynamics of the etiological agent is required. We here used multi-locus sequence typing (MLST) to infer the genetic diversity and geographical distribution of 160 *M. plutonius* isolates collected from EFB symptomatic honey bee colonies seven years apart. Isolates belonged to three clonal complexes (CC) known worldwide and to 12 sequence types (STs), of which five were novel. Phylogenetic and clustering analyses showed that some of these novel sequence types have likely evolved locally during a period of outbreak, but most disappeared again. We further screened the isolates for melissotoxin A (*mtxA*), a putative virulence gene. The prevalence of STs in which *mtxA* was frequent increased over time, suggesting that this gene promotes spread. Despite the increased frequency of this gene in the population, the total number of cases decreased, which could be due to stricter control measures implemented before the second sampling period. Our results provide a better understanding of *M. plutonius* population dynamics and help identify knowledge gaps that limit efficient control of this emerging disease.

Introduction

The Western honey bee (*Apis mellifera* L.) is exploited for their natural products since at least the Neolithic era (Roffet-Salque, Regert et al. 2015), and more recently, to provide pollination service to many wild plants and economically important crops (Kleijn, Winfree et al. 2015). Consequently, *A. mellifera* is one of the most commonly managed insects in the world. The large-scale losses of managed colonies reported in recent times in the Northern Hemisphere potentially threaten the services honey bees provide (Neumann and Carreck 2010; Potts, Biesmeijer et al. 2010; Liu, Chen et al. 2016; Kulhanek, Steinhauer et al. 2017; Steinhauer, Kulhanek et al. 2018; Decourtye, Alaux et al. 2019; Gray, Adjlane et al. 2022). Honey bee health has thus become a major concern for scientists, policy-makers, beekeepers and the public (Moritz, De Miranda et al. 2010; Evans and Schwarz 2011; Smith, Loh et al. 2013). Honey bee colony losses are primarily attributed to pests and pathogens such as viruses (Highfield, El Nagar et al. 2009; Berthoud, Imdorf et al. 2010; Genersch 2010), protozoa (Morimoto, Kojima et al. 2013; Ravoet, Maharramov et al. 2013), fungi (Higes, Martín-Hernández et al. 2008; Paxton 2010), parasitic mites (McMullan and Brown 2009; Dahle 2010; Guzmán-Novoa, Eccles et al. 2010; Dainat, Evans et al. 2012; Kielmanowicz, Inberg et al. 2015) and bacteria (Wilkins, Brown et al. 2007; Roetschi, Berthoud et al. 2008; Genersch 2010; Evans and Schwarz 2011; Cornman, Tarpy et al. 2012).

One of the major bacterial diseases affecting the honey bee is European foulbrood (EFB). EFB is present worldwide (Ellis and Munn 2005; Boncristiani, Ellis et al. 2020) and has become a problem in numerous countries that have reported severe outbreaks (Wilkins, Brown et al. 2007; Roetschi, Berthoud et al. 2008; De León-Door, Romo-Chacón et al. 2018; Grossar, Kilchenmann et al. 2020;

Nesvorna, Sopko et al. 2021; Pietropaoli, Carpana et al. 2022; Thebeau, Liebe et al. 2022). Adult honey bees act as carriers of the bacterial pathogen *M. plutonius*, but typically show no symptoms, while immature honey bees (brood) often die upon infection (Belloy, Imdorf et al. 2007; Forsgren 2010). The massive loss of brood due to the disease weakens the colony and can lead to its collapse (Forsgren, Budge et al. 2013). To prevent the further spread of the bacteria, in case of outbreaks, the authorities of many countries require the destruction of affected colonies and occasionally of whole apiaries, and the implementation of costly sanitation measures, including the establishment of control zones around affected apiaries and recurrent inspections of neighboring apiaries (Forsgren 2010).

Despite the application of strict control measures since 2010, immediately after a rise in outbreaks frequency, the situation in Switzerland has improved, but the situation is still not under control. This is in part due to a lack of effective preventive or control methods. The development of such methods is limited by our poor knowledge of the epidemiological processes that underlie the expression and spread of this disease. A better understanding of transmission routes, and of biotic and abiotic risk factors for disease outbreaks can be achieved by determining the structure, genetic diversity and dynamics of *M. plutonius* populations, as well as the phenotypic traits associated with the virulence and ability to spread of genetic variants. Knowing the genetic landscape of *M. plutonius* variants and their effects on honey bees could also help monitoring the emergence of new problematic variants, and possibly prevent their negative impacts on honey bee stocks.

In Switzerland, the frequency of EFB symptomatic apiaries rose from 1999 to peak in 2010, with 992 reported cases (InfoSM, Federal Food Safety and Veterinary Office FSVO), which corresponds to approximately 5 % of the Swiss active apiaries at this time (Charrière, Frese et al. 2018). The prevalence of EFB

shows striking spatial differences in the country. The disease is more frequently detected in the central midland and eastern parts than in the western or southern parts of the country. Strikingly, the canton of Bern, which holds 23 % of the Swiss active apiaries, reported as many as 41 % of all registered EFB cases in Switzerland (N= 8596 cases 1992-2021; InfoSM, Federal Food Safety and Veterinary Office FSVO; Von Büren, Oehen et al. (2019)). This pattern is unexplained and might be due to multifactorial interactions between topographical and climatic differences; to anthropological influences (e.g., apiary density, Von Büren, Oehen et al. (2019), beekeeping practices or exchange of materials within language groups); and/or to regionally specific strains of *M. plutonius*. Also, newly emerging variants of *M. plutonius* could contribute to higher local prevalence and impact of the pathogen.

In the present study, we aimed to obtain a better understanding of the factors influencing the regional distribution of *M. plutonius* outbreak by investigating the genetic diversity, population structure and dynamics of this pathogen. For this, we applied a multi-locus sequence typing (MLST) scheme to 160 isolates collected from EFB-diseased colonies across Switzerland, sampled over two periods seven years apart. MLST is a robust method to distinguish between genetic subtypes of clonal bacterial species (Killgore, Thompson et al. 2008), and has been used to unravel the epidemiology of numerous human and animal pathogens (Dingle, Colles et al. 2002; Ruiz-Garbajosa, Bonten et al. 2006; Freitas, Novais et al. 2009; Lowder, Guinane et al. 2009). For *M. plutonius*, a MLST-scheme incorporating four genomic loci has been developed (Haynes, Helgason et al. 2013) to classify *M. plutonius* strains into sequence types, themselves grouped into clonal complexes (CCs), of which three have been described to date (CC 3, CC 12 and CC 13).

Although genotyping is important to identify cases or outbreaks and to track the source and spread of infections, it is currently unclear whether the markers used reflect phenotypic variation relevant to disease progression, such as virulence (Freitas, Novais et al. 2009; Lowder, Guinane et al. 2009). This is also the case for *M. plutonius*. The study of correlations between sequencing types and virulence factors could hence help to better predict EFB outbreaks and spread of the disease in the field. For instance, such correlation was shown for *M. plutonius* strains from CC 3, which exhibited stronger resistance to the antimicrobial activity of royal jelly, and could explain the higher virulence of this CC (Takamatsu, Osawa et al. 2017). In contrast, isolate virulence at the individual level was not related to its sequence type, but was correlated to the occurrence of a mobile-genetic element, plasmid pMP19, which carries the mtxA gene encoding the protein melissotoxin A (Djukic, Erler et al. 2018; Grossar, Kilchenmann et al. 2020; Nakamura, Okumura et al. 2020). Because of its putative role in virulence, this gene could affect the ability of an isolate harboring it to cause an outbreak. We therefore screened the collected isolates for the presence of *mtxA*. Our results increase knowledge of *M. plutonius* genetic diversity, population dynamics, and distribution in a country with high disease prevalence. Such data on *M. plutonius* help explain differences in the spread and impact of EFB between regions, and could lead to the development of refined mitigation measures.

Methods

Sampling and isolation of *M.* plutonius

Routine inspections of Swiss apiaries as well as controls following the announcement of suspect cases by beekeepers are conducted by trained bee inspectors, as part of a national control program (Massnahmen im Seuchenfall von Sauerbrut (Europäische Faulbrut) bei Bienen, 10.08.2015 based on Art. 273a TSV 27. 06. 1995; SR 916.401; (FSVO) (2015)). Such inspections entail the sampling of brood combs from colonies suspected to show EFB symptoms. These samples are sent to one of three accredited laboratories (Laboratoire vétérinaire Institut Galli-Valerio, Lausanne; IDEXX Diavet AG, Bäch; Zentrum für Labormedizin, St. Gallen) for diagnostic disease confirmation via microscopical analysis and/or PCR (Roetschi, Berthoud et al. 2008). Brood combs that contained larvae diagnosed positive for *M. plutonius* were obtained from these laboratories for MLST typing. To isolate *M. plutonius*, we collected 1-3 symptomatic larvae (yellowish-brown color, unpleasant smell and signs of decay) from one brood comb per infected apiary and ground them in sterile saline solution (0.9 % NaCl w/V). A droplet of 25 µl of each larval homogenate was then streaked on basal medium plates, containing 1 % yeast extract, 1 % glucose, 1 % saccharose, 0.04 % L-cysteine and 0.1 M KH_2PO_4 in distilled water, pH adjusted to 6.7, and 18 g agar/l, and incubated at 36 ± 1 °C for 4 to 5 d under anaerobic conditions (GENbox anaer, bioMérieux, France) (Forsgren, Budge et al. 2013). Individual bacterial colonies suspected to be *M. plutonius* were picked from the plates with sterile plastic needles and dipped into the *M. plutonius*-specific PCR mix, to confirm the bacterial species. This mix contained the primers Mp-SodA-F (5'-ACTGAAACAATGCATTTGCACC-3') and Mp-SodA-R (5'-AGTGGTGAATCTTGGTTGGCT-3') that target the sodA gene (Manganese

dependent superoxide dismutase) of *M. plutonius* (Roetschi, Berthoud et al. 2008). Primers Mp-SodA-F and Mp-SodA-R were designed for this study using the tool Primer BLAST at NCBI (https://www.ncbi.nlm.nih.gov/)(Ye, Coulouris et al. 2012). The protocol was adapted for conventional PCR, instead of RT-PCR, and the PCR amplification size was 401 bp. PCR positive subcultures were then again inoculated into liquid basal medium (1 % yeast extract, 1 % glucose, 1 % saccharose, 0.04 % L-cysteine and 0.1 M KH_2PO_4 in distilled water, pH adjusted to 6.7 with 5 M KOH) (Forsgren, Budge et al. 2013) and incubated anaerobically at 36 ± 1 °C for 4 to 5 d, supplemented with 15 % Glycerol and stored at - 80 °C until further use.

Our dataset comprised 160 *M. plutonius* isolates from EFB symptomatic colonies. We sequence-typed 91 field isolates originating from EFB outbreaks that occurred between 2006 and 2007, and 63 originating from EFB outbreaks in 2013. The single *M. plutonius* isolate from each infected apiary was used for further analysis, except for four EFB outbreaks in 2006-2007 from which two isolates were available and analyzed. To complete the dataset, published MLST-data of four Swiss isolates collected in 2006 (http://pubmlst.org/mplutonius) were included in the analyses. The total number of EFB cases analyzed corresponded to approximately 14 % and 13 % of all reported EFB cases in 2006-2007 and 2013, respectively. In Switzerland, EFB case numbers reached a peak in 2010 (InfoSM, Federal Food Safety and Veterinary Office FSVO). Therefore, the first sampling (2006-2007) occurred during a period of increasing EFB case numbers, while the second sampling (2013) occurred during a period of decrease in numbers of notified EFB cases.

Genetic typing and phylogenetic analysis of *M. plutonius* isolates

DNA was extracted from single step *M. plutonius* cultures grown from isolate stock solutions in liquid basal medium, using the NucleoSpin® Tissue DNA extraction kit (Macherey-Nagel, Germany). The DNA samples were stored at - 20 °C until further use. For multi-locus sequence typing (MLST) of *M. plutonius*, we PCR-amplified and sequenced the following four loci: argE (Acetylornithine deacetylase, 503bp; argE-for: 5'-GGTGGGACATTTAGACGTAG-3' and argE-rev: 5'-AAATTAAGACCCAACCCTTC-3'), galK (Galactokinase, 471bp; galK-for: 5'-TTTCCAGCAGCAATTACAA-3' and galK-rev: 5'-GGGTAGGGATTTTTGAAGAG-3'), gbpB (Putative secreted antigene, 314-557bp; gbpB-for: 5'-AGCAGCTAAACAGAATGAGC-3' and gbpB-rev: 5'-GCCAACGTCTAACAGATACC-3') and purR (Purine operon repressor, 382bp; purR-for: 5'-GCCAACGTCTAACAGATACC-3' and purR-rev: 5'-CGATTTTGTTCTGATAACCTG-3') (Haynes et al., 2013). For each locus, we used the KAPA2G fast Polymerase (Kapa Biosystems, USA) and a PCR mix consisting of 13 µl Kapa (2x) mix, 1 µl forward primer at 10 µM, 1 µl reverse primer at 10 µM, 8 µl water and 2 µl template DNA (approx. 10 ng DNA) in elution buffer (5 mM Tris/HCl, pH 8.5; total reaction volume 25 µl). Each reaction was run for 15 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s and DNA extension at 72 °C for 20 s, and a final extension step at 72 °C for 2 min in a TProfessional Basic Thermocycler (Biometra, Germany). PCR products were loaded on agarose gels (1.5 % agarose in 0.5 % TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8)), run for 40 min at 100 mV in an electrophoresis tank and stained for 20 min in a GelRed™ bath (Biotium, USA). Bands visible under UV-light were sized according to a DNA ladder (SmartLadder SF, 100 to 1,000 bp molecular weight marker; Eurogentec, Belgium). PCR products of the expected size were purified with the

NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). The DNA content of purified extracts dissolved in elution buffer (5 mM Tris/HCl, pH 8.5) was measured using NanoDrop™ (Thermoscientific, USA). Extracts were diluted, supplemented with the corresponding primer, and sent for Sanger sequencing (Eurofins Scientific, Germany or Fasteris SA, Switzerland). Sequencing chromatograms were manually checked for their quality using Sequence Scanner v1.0 (Applied Biosystems, 2005) and aligned to reference sequences from the PubMLST database (http://pubmlst.org/mplutonius, as per June 2, 2022; Bacterial Isolate Genome Sequence database (BIGSdb), Jolley and Maiden (2010)).

Based on the sequences of the four loci, the isolates were assigned to sequence types. Sequence types newly identified in this study were then attributed to clonal complexes using the clustering approach implemented in the program goeBURST v. 1.2.1 (http://goeburst.phyloviz.net, Francisco et al. (2009)). Sequence types belong to the same clonal complex when they share at least three identical alleles (out of four). To place our 160 Swiss *M. plutonius* isolates in the global phylogenetic context, we used the PHYLOViZ 2.0 program (Francisco, Vaz et al. 2012; Nascimento, Sousa et al. 2016) to construct a goeBURST tree including our samples and the 385 isolates available from the PubMLST database (http://pubmlst.org/mplutonius, as per June 2, 2022).

Further analyses to confirm the phylogenetic relationship were conducted on concatenated sequences of each sequence type (1904 bp). The allele sequences aligned using MAFFT (Kuraku, Zmasek et al. 2013) were downloaded from the PubMLST database in fasta format. The phylogenetic history was inferred with the maximum likelihood method (500 replications) and the Tamura-Nei model (Tamura and Nei 1993) as implemented in MEGA11 (Tamura, Peterson et al. 2011).

mtxA screening

To identify the presence of the putative virulence factor Melissotoxin A coded by *mtxA* (Djukic, Erler et al. 2018; Grossar, Kilchenmann et al. 2020), we screened all 160 isolates using PCR. As *mtxA* is located on pMP19, a plasmid that is prone to be lost by repeated sub-cultivation *in vitro* (Djukic, Erler et al. 2018; Grossar, Kilchenmann et al. 2020; Nakamura, Okumura et al. 2020), we used DNA extracts of single step *M. plutonius* cultures of every isolate. Each isolate was screened for the corresponding gene with specific primers (mtxA, 897 bp; tox_MEPL_for: 5'-GCTCAAGCAGCAACTTTTACG-3' and tox_MEPL_rev: 5'-TTCCCCTGGTATTACTTGTAGATG-3'). These primers were run in a conventional PCR reaction (initially 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 15 s, 72 °C for 20 s, and final extension at 72 °C for 2 min), using KAPA2G Fast DNA Polymerase (KAPA Biosystems, USA). The total reaction volume was 25 µl consisting of 13 µl Kapa (2x) mix, 1 µl tox_MEPL_for at 10 µM, 1 µl tox_MEPL_rev at 10 µM, 8 µl water and 2 µl template DNA extract (10 ng DNA) in elution buffer (5 mM Tris/HCl, pH 8.5). The PCR-products were loaded on agarose gels, as described above for MLST.

Isolate mapping

Each *M. plutonius* sample (isolate) was mapped based on the zip code of the collection location using Quantum GIS (QGIS, version 1.8.0-Lisboa; http://www2.qgis.org/en/site, GNU General Public License; background maps and geographical information were obtained from www.toposhop.admin.ch).

Statistical analysis

To test whether the presence of *mtxA* was associated with the sequence type, clonal complex, or period of sampling, we calculated χ^2 for maximum likelihood contingency tables (SPSS v. 26 (IBM, USA)). To determine whether the presence of *mtxA* promoted the spread of sequence types that have many isolates carrying this gene, we performed a Spearman rank correlation analysis between the frequency of each sequence type among all *M. plutonius* samples and the frequency of isolates harboring *mtxA* within each sequence type, among the samples of 2006-2007, of 2013 and for both sampling periods.

Results

Sequence types of *M. plutonius* from Switzerland

The 160 Swiss isolates of *M. plutonius* belonged to 12 sequence types (ST 3, ST 7, ST 12, ST 13, ST 20, ST 24, ST 32, ST 41, ST 42, ST 43, ST 44, and ST 45; Fig. 2.1 and Fig. 2.2) nested within three known clonal complexes (Fig. 2.3). The predominant clonal complex was CC 3 (52 % of the isolates analyzed), followed by CC 13 (46 %) and CC 12 (2 %; Fig. 2.1 and Fig. 2.2). ST 7 (34 % of the isolates analyzed) and ST 3 (16 %), both within CC 3, were the most frequently detected sequence types (Fig. 2.1 and Fig. 2.2). In four cases, two *M. plutonius* isolates were analyzed per sample, and both isolates belonged to the same sequence type. Of the 12 sequence types detected, five (ST 41 to ST 45) had never been identified previously in any of the 385 isolates listed in the PubMLST database. One previously unreported allele at locus galK distinguished three new sequence types (ST 43, ST 44 and ST 45). This new allele (galK 12) differs from the known allele galK 1 by a single nucleotide polymorphism (C > A) at position 257. The two other newly identified sequence types (ST 41 and ST 42) were based on new MLST profiles (new allele combinations), without novel sequence variation. Six sequence types were detected in both sampling periods (ST 3, ST 7, ST 13, ST 20, ST 32 and ST 43), comprising all the five sequence types that had been reported before. The rare sequence types ST 12 and ST 24 were not detected in isolates from 2006-2007, but occurred in two and four isolates from 2013, respectively (Fig. 2.2). Four of the five novel identified sequence types (ST 41, ST 42, ST 44 and ST 45) occurred only in the samples of the first period (2006-2007), but not in samples of 2013. ST 43 was the sole novel sequence type found in both sets of samples (Fig. 2.1 and Fig. 2.2).

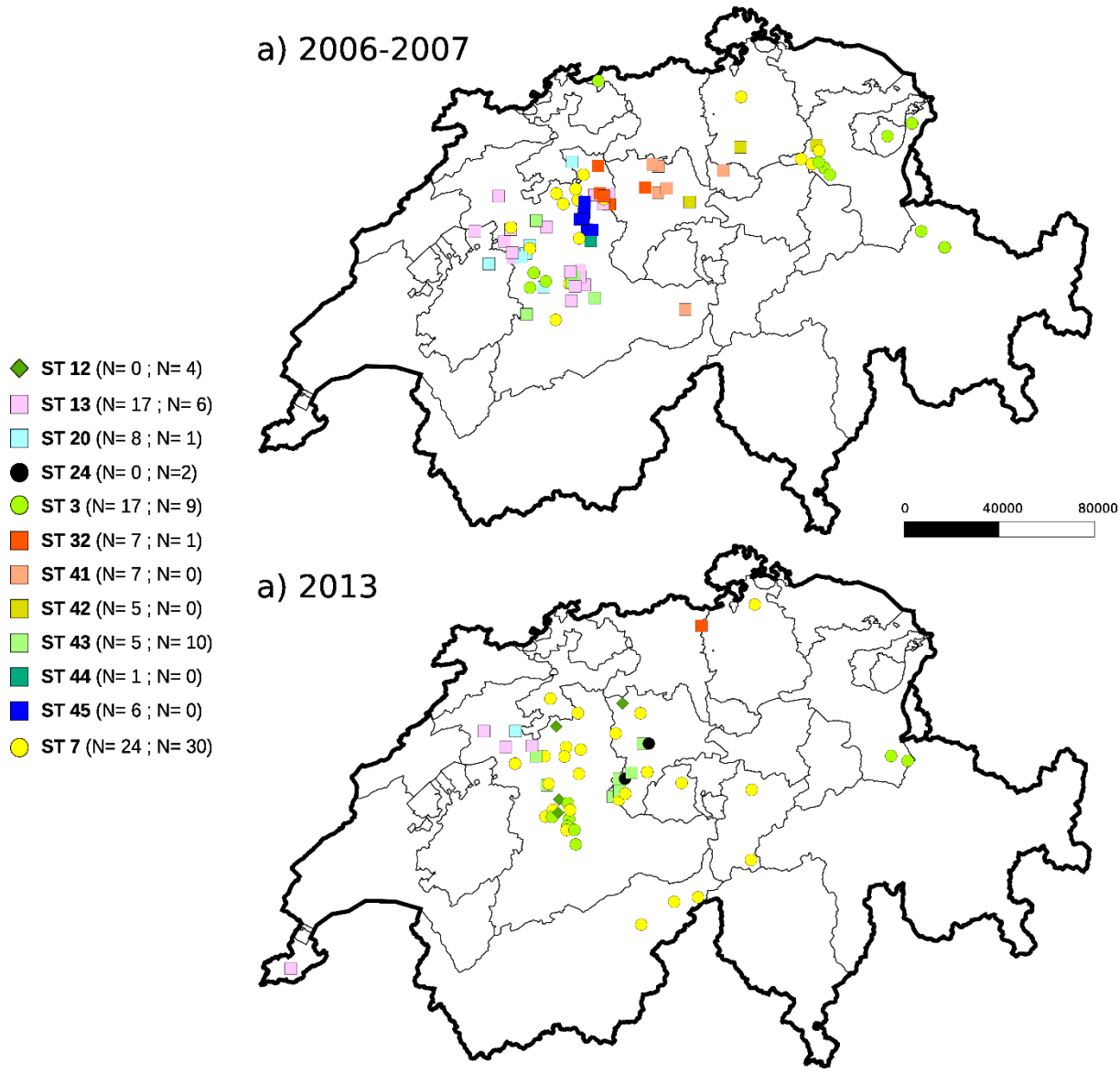


Fig. 2.1: Distribution of sequence types of the 160 *M. plutonius* isolates collected in Switzerland over two sampling periods: a) 2006-2007 (N= 97) and b) 2013 (N= 63). The isolates belonged to 12 sequence types (in parentheses: number of isolates of each sequence type detected in the first and second sampling, respectively), which grouped into three clonal complexes (circles = CC 3, diamonds = CC 12, and squares = CC 13).

Phylogenetic analysis of *M. plutonius* sequence types

As for the *M. plutonius* sequence typed in other countries, Swiss isolates clustered in three clonal complexes and their frequency distribution confirmed that sequence types occur at variable frequencies across countries and continents (Fig. 2.3). ST 3 was the most abundant sequence type identified worldwide, whereas it was the second most abundant sequence type in Swiss samples (Fig. 2.1 and Fig. 2.2). The maximum likelihood phylogenetic tree based on concatenated allele sequences of all 43 *M. plutonius* sequence types (Fig. 2.2) was highly congruent with the grouping inferred with goeBURST (Fig. 2.3). The goeBURST program recovered the three clonal complexes, the central position of the presumed founder sequence type in each clonal complex, and the phylogenetic relationships among most sequence types. The only major difference between the two analyses was the position of ST 39. This sequence type was grouped with CC 13 in the goeBURST clustering analysis (Fig. 2.3), because ST 39 is a double locus variant of ST 17 (differing at loci argE and gbpB). In contrast, ST 39 was assigned to CC 3 in the phylogenetic tree (Fig. 2.2), because ST 39 shares a single locus variant (galK) with many sequence types of CC 3 (ST 3, ST 5, ST 7, ST 23, and ST 24; De León-Door, Romo-Chacón et al. (2018), Budge, Shirley et al. (2014)).

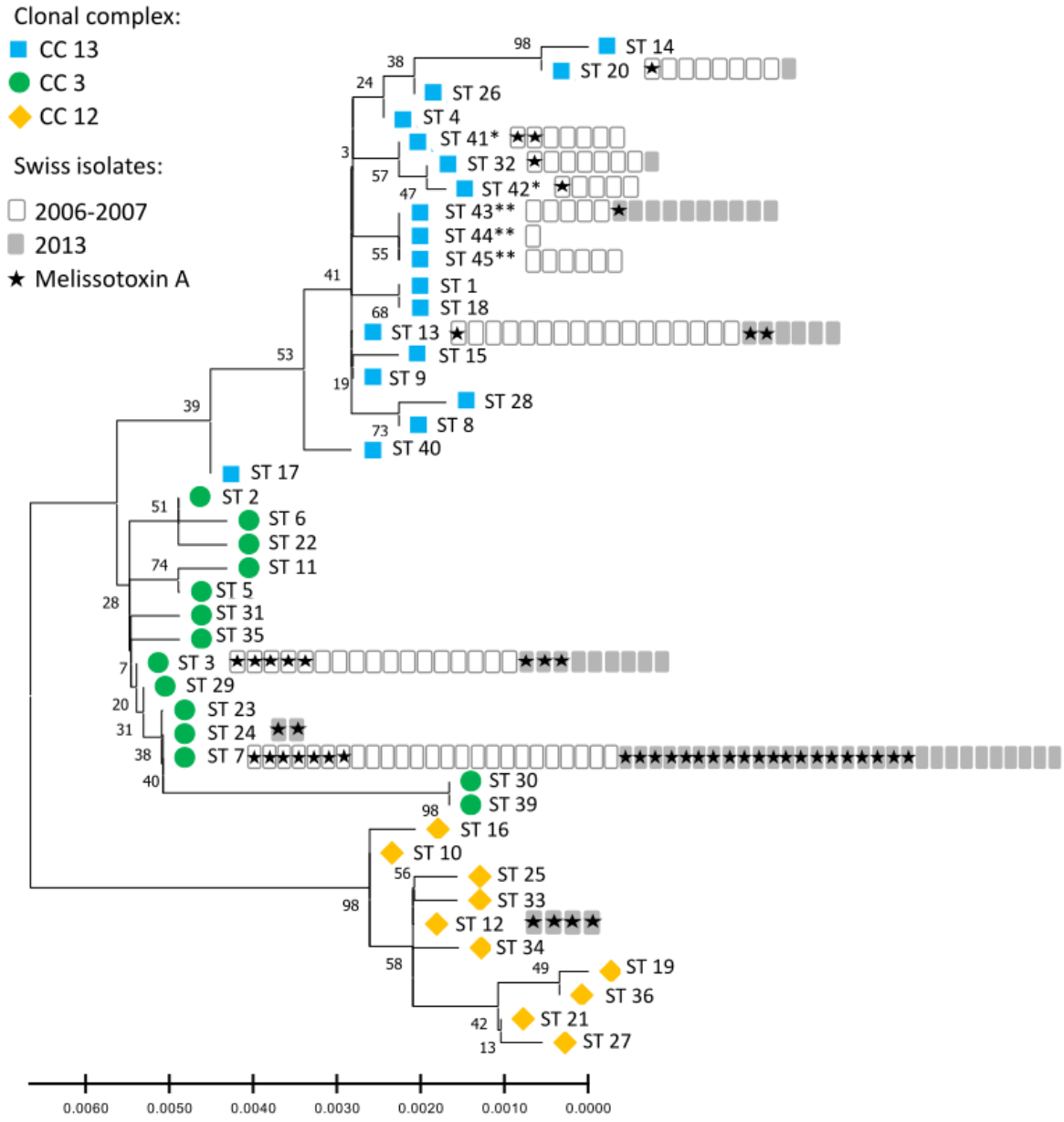


Fig. 2.2: Phylogenetic tree of all known *M. plutonius* sequence types (ST) and clonal complex (CC), inferred with the maximum likelihood method and Tamura-Nei model, using the concatenated sequences of each sequence type. The tree with the highest log likelihood (-2949) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. * New sequence types identified in this study. ** New sequence types identified in this study with a new allele, galk12. *mtxA* was only screened in Swiss isolates collected in our study.

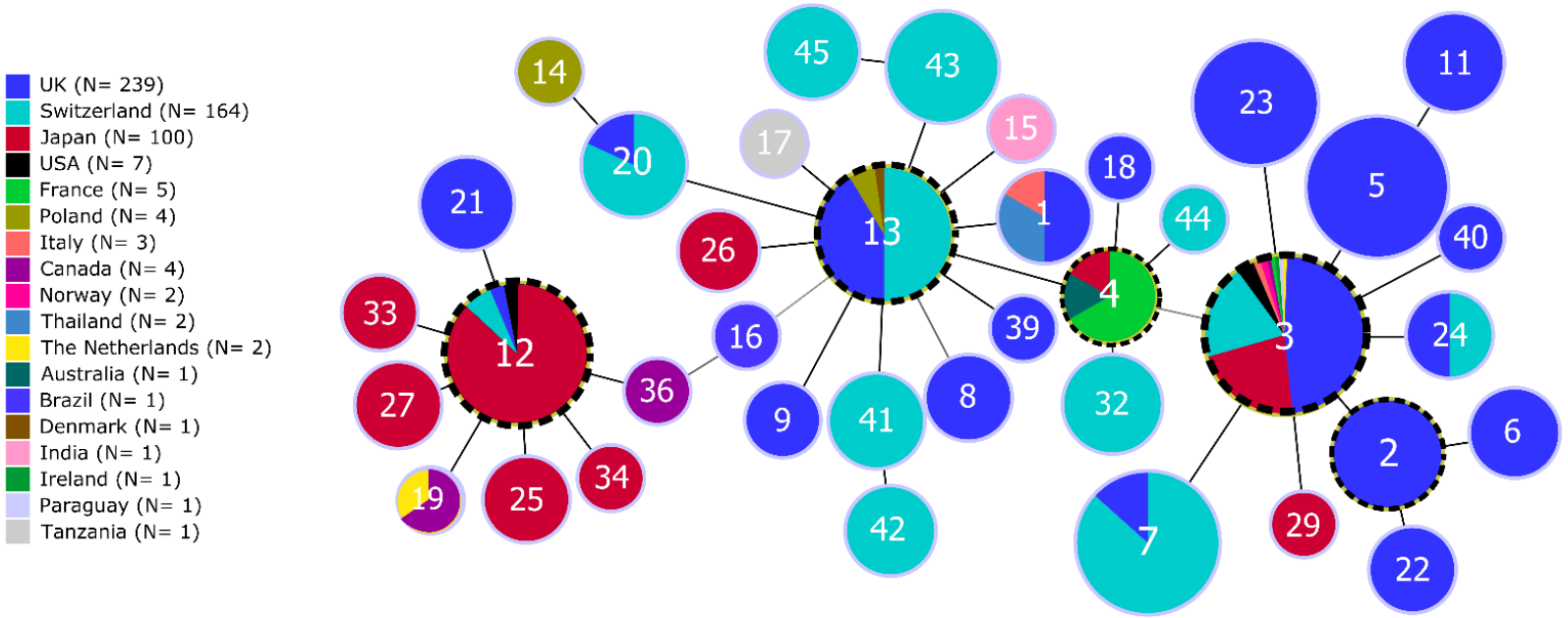


Fig. 2.3: Clustering of *M. plutonius* sequence types based on sequence similarity, as per goeBURST analysis. The graph is based on MLST data from the current study and from the publicly available PubMLST database. Each circle represents a sequence type with its number of isolates in the center. Circle diameter correlates with sequence type frequency. Lines between circles link sequence types to their closest relatives. Black lines indicate single allelic changes between sequence types, whereas grey lines indicate differences at two loci. Circles with a dashed ring highlight putative founder sequence types. Colors indicate countries where the isolates of each sequence type were found (18 countries, including Switzerland). The three known clonal complexes are recognizable around the presumed founders ST 12, ST 13 and ST3

Distribution of *mtxA*

Fifty out of 160 tested *M. plutonius* isolates (31 %) harbored *mtxA* (Fig. 2.2). The proportion of isolates carrying the *mtxA* gene increased from 19 % to 51 % between the 2006-2007 and the 2013 samples (Fig. 2.2). The proportion of isolates harboring *mtxA* within a sequence type was positively correlated with the frequency of this sequence type among all samples (samples of 2006-2007: Spearman's-rho= 0.734, p= 0.007; samples of 2013: Spearman's-rho= 0.923, p< 0.001; all samples: Spearman's-rho= 0.684, p= 0.014). The sampling period was a significant factor explaining the occurrence of this putative virulence factor (maximum likelihood contingency table, χ^2= 5.40, df= 1, p= 0.02). All four Swiss *M. plutonius* isolates belonging to CC 12 harbored *mtxA*, whereas 46 % of the isolates of CC 3 (N= 83) and only 11 % of the isolates of CC 13 (N= 74) harbored the gene. This frequency pattern leads to a significant association between *mtxA* occurrence and clonal complex (maximum likelihood contingency table, χ2= 30.95, df= 2, p< 0.001, N= 160). The same was true for sequence type (maximum likelihood contingency table, χ^2= 38.90, df= 11, p< 0.001, N= 160; Fig. 2.2). In CC 3, *mtxA* was found in the two ST 24 isolates, in 51 % of the ST 7 isolates (N= 55) and in 31 % of the ST 3 isolates (N= 26). In CC 13 sequence types, *mtxA* frequency ranged from 0 % for ST 45 (N= 6) to 29 % for ST 41 (N= 7; Fig. 2.2). All four Swiss *M. plutonius* isolates belonging to ST 12 of CC 12 harbored *mtxA*.

Discussion

European foulbrood is a honey bee brood disease with a worldwide distribution. Although the disease has been known for a long time, we still lack information on the epidemiology of its etiological agent, *M. plutonius*. Here, we studied the genetic diversity, distribution, and population dynamics of *M. plutonius* in Switzerland. The Swiss honey bee populations serve as an excellent small-scale model with high apiary densities and therefore a high probability of disease transmission, which is attested by a high number of EFB cases. Using a multi-locus sequence typing (MLST) scheme, we identified 12 sequence types (of which five are novel) within 160 *M. plutonius* isolates sampled across Switzerland. The geographical distribution of the sequence types detected remained largely unchanged between the two sampling periods, but their prevalence, as well as that of *mtxA*, a gene coding for a putative virulence factor varied over time. The proportion of *mtxA* carrying isolates increased over time, in spite of the fact that the second sampling period occurring when the total number of cases reported in the country was decreasing.

The 12 sequence types detected among the 160 isolates belonged to the three known clonal complexes (CC; Budge, Shirley et al. (2014)). About half of the isolates were assigned to CC 3, the other half to CC 13, whereas CC 12 was rare and comprised only four 2013 samples. Sequence types ST 7 and ST 3, both belonging to clonal complex CC 3, were the most abundant sequence types in Switzerland, and are also very common worldwide (Haynes, Helgason et al. 2013; Budge, Shirley et al. 2014; Takamatsu, Morinishi et al. 2014). In England and Wales, ST 3 dominates, while ST 7 is less prevalent (Haynes, Helgason et al. 2013; Budge, Shirley et al. 2014). ST 13, which is the third most abundant *M. plutonius* type in Switzerland, has been detected in one sample from Denmark,

three samples from Poland (Haynes, Helgason et al. 2013) and 19 samples from England and Wales (Budge, Shirley et al. 2014). The ubiquity of these sequence types suggests a large natural distribution range. In contrast, ST 12, the most abundant *M. plutonius* variant in Japan (Takamatsu, Morinishi et al. 2014), is only occasionally detected in other countries (De León-Door, Romo-Chacón et al. 2018) and was absent from the 2006-2007 samples, but detected in four Swiss samples from 2013. ST 12 may thus have been recently introduced to Switzerland from one of the locations in which it was previously reported. Similarly, in England and Wales, one out of over 200 analyzed samples from 2011-2012 belonged to ST 12, consistent with a recent introduction (Budge, Shirley et al. 2014). However, large-scale screenings of *M. plutonius* genetic diversity are rare, which limits our knowledge of the natural distribution range of sequence types and the role of honey bee related trade in explaining the observed biogeographic patterns (Mutinelli 2011). Similarly, within country, human-mediated migration can contribute to spread sequence types. This latter effect is likely restricted in Switzerland, as only approximately 7 % of the beekeepers migrate their colonies to track nectar flows (Charrière, Frese et al. 2018).

Among the 12 sequence types detected in Switzerland, we identified five novel types (ST 41 to ST 45), to date unique to Swiss isolates. Three of these novel sequence types (ST 43, ST 44 and ST 45) included a yet unreported allele at locus galK (galK 12). The novel sequence types could either have been imported via trade of honey bees or of contaminated beekeeping material (Mutinelli 2011)), or have evolved in Switzerland. Evidence for a local evolution can be detected in the phylogenetic tree and goeBURST analysis (Fig. 2.2, 2.3). Indeed, ST 42 and ST 41 are closely related, and the first could have derived from the second. The same pattern was observed for ST 45 and ST 43. ST 44 is a single locus variant

of ST 43 and ST 45. All three sequence types share the novel galK allele 12, which suggests that they are related by descent and thus of local origin. The goeBURST analysis also linked ST 44 to ST 4, because ST 44 is a single locus variant of ST 4, differing only by a single nucleotide polymorphism at the galK locus. ST 4 has not yet been detected in Switzerland, but it might have been introduced from France, Ireland or Japan, and remained undetected. Similarly, ST 32 is another sequence type found in Switzerland and closely related to ST 4. Finally, ST 4 is closely related to ST 3 and ST 13, both present in the country (Fig. 2.3). Overall, the phylogenetic pattern is largely consistent with local evolution of most novel sequence types, possibly following a transient introduction, *e.g.* of ST 4.

As there were numerous disease outbreaks in Swiss apiaries and the disease incidence was increasing rapidly at the time the 2006-2007 samples were collected, a local evolution of at least some of these novel types is in line with theoretical population genetic models. These models state that more mutations arise under expansion (i.e*.* increase in EFB cases), but either quickly die out or 'surf', i.e. become dominant (Miller 2010). This scenario also helps account for why in 2013, when the incidence rates of EFB were already declining, only ST 43 appeared to have increased in prevalence, while all other novel sequence types (ST 41, ST 42, ST 44 and ST 45) were not detected again and likely had disappeared.

Other sequence types (ST 13, ST 20, ST 3 and ST 32), which had already been detected before our study, also decreased in prevalence in the second sampling period, despite a similar sampling effort. These results reveal high temporal dynamics in the genetic composition of the Swiss *M. plutonius* population. In contrast, the spatial dynamics appeared stable, as the same sequence types occurred in the two sampling periods (Fig. 2.1). This spatial

stability indicates that the pathogen had not spread over long distances in Switzerland, over seven years.

To investigate a potential link between *M. plutonius* virulence at the individual level (Lewkowski and Erler 2019; Grossar, Kilchenmann et al. 2020) and spread (Alizon, Hurford et al. 2009), we screened the *M. plutonius* variants for the presence of the recently discovered plasmid-encoded melissotoxin A toxin gene (*mtxA*) (Djukic, Erler et al. 2018). The toxin gene was found in all but two novel sequence types (ST 44 and ST 45) and was significantly associated with sequence type and clonal complex. Most interestingly, the gene was less frequently detected in isolates from 2006-2007, compared to isolates from 2013, which suggests that this gene could be an emerging virulence factor in the Swiss *M. plutonius* population.

In previous infection assays of honey bee larvae in the laboratory, we had detected significantly higher mortality when the larvae were infected with *M. plutonius* isolates harboring *mtxA*, compared to isolates lacking the toxin gene (Grossar, Kilchenmann et al. 2020). Because *mtxA* is carried by a plasmid, a mobile genetic element (Djukic, Erler et al. 2018), one can expect that *mtxA* is not present in all isolates of a given sequence type. Indeed, *mtxA* was found in varying proportions of the isolates of the detected sequence types (Fig. 2.2), suggesting this element can be acquired or lost by the pathogen. The conditions under which this plasmid is acquired or lost, and the dynamics of this phenomenon, are currently not understood.

In general, highly virulent pathogens are less likely to spread and persist in a population (Alizon, Hurford et al. 2009), so that an inverse relationship between the percentage of *mtxA* carrying isolates and their prevalence in the field was expected. However, we observed the opposite trend, with the percentage of

isolates carrying *mtxA* increasing between the first and the second sampling. This suggest that *mtxA* increased disease spread in Switzerland over a period of seven years, despite increased virulence. This pattern could be explained by an inverse relationship between individual and colony level virulence, due to earlier or more efficient hygienic behavior of adult honey bees directed against brood infected by highly virulent and damaging pathogens (Cremer, Armitage et al. 2007; Rauch, Ashiralieva et al. 2009; Page, Lin et al. 2016). The relationship between individual and colony level virulence needs to be better understood to determine whether virulence-dependent control strategies can be applied (Grossar et al. 2020). In addition, *mtxA* is only one of several putative virulence factors (Djukic, Erler et al. 2018) and their respective roles in determining the spread of their carriers requires investigation.

To further complicate the relationship between virulence and spread of this honey bee pathogen, an interaction between the genome of each isolate and the presence of virulence-amplifying plasmids is likely as the presence of *mtxA* was significantly associated with sequence type and clonal complex. This is in line with results from genetic manipulation of Japanese *M. plutonius* isolates and infections of honey bee larvae *in vitro*. Nakamura, Okumura et al. (2020) demonstrated that the plasmid containing *mtxA*, pMP19 (Djukic, Erler et al. 2018) increased the virulence of CC 3 isolates, but isolates of CC 13 were avirulent, irrespective of the presence of pMP19, and CC 12 isolates always caused high mortality rates, even in absence of pMP19. A further factor likely affecting the spread of isolates in the host population is the infections of colonies by multiple sequence types. Although in the four cases in which we typed two isolates per sample their sequence type was identical, infections by multiple sequence types were previously suspected (Takamatsu, Morinishi et al. 2014; De León-Door, Romo-Chacón et al. 2018;

Djukic, Erler et al. 2018). The interactions between sequence types via their effect on brood and colony health is likely influencing the ability of each sequence type to spread.

Despite a putative effect of *mtxA* gene on the spread of its carrier *M. plutonius* and an increase in the frequency of *mtxA* between the sampling periods, the total number of EFB cases in Switzerland did not further increase beyond 2010. The decrease in the number of cases was likely due to the stricter control measures implemented by veterinary authorities since 2010 (FSVO 2015) to counter the increased frequency of outbreaks experienced in the previous decade. From the mandatory destruction of symptomatic colonies and a control of the apiary at the end of the season before this date, later measures included the mandatory destruction of all colonies in an apiary if more than half of the colonies were symptomatic, and the monitoring of neighboring apiaries within a one kilometer radius after the report as well as at the next spring.

The data presented here for Swiss *M. plutonius* isolates, and comparison with data from other regions where EFB is currently becoming a sanitary problem, advance the knowledge of the epidemiology of EFB and offer a better understanding of the spread of genetic subtypes of *M. plutonius*. We found evidence that a high individual level virulence vectored by a mobile genetic element could favor the spread of *M. plutonius* isolates, but that increased control measures may have countered their propagation within the country. An improved understanding of the virulence factors within the genetically diverse *M. plutonius* is required to better mitigate future outbreaks. Combining large-scale monitoring of *M. plutonius* genotypes in data deficient regions with more detailed characterization of how *M. plutonius* impacts honey bees at the individual and

colony level would be highly desirable, as this could provide key information to control this pathogen more efficiently and halt its spread worldwide.

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Chapter III: Investigating the resistance of honey bee colonies to European foulbrood

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Abstract

In apiaries with acute European foulbrood (EFB) outbreaks, honey bee colonies heavily affected by EFB (EFB^+) frequently co-occur with colonies without any disease symptoms (EFB^-). Understanding the mechanisms that promote resistance to the disease in EFB^- colonies could help to establish higher levels of EFB resistance in honey bee populations. Queens from each type of colonies were therefore collected and transferred into host colonies. Their brood was subjected to freeze-kill bioassays to test for hygienic behavior by adult offspring, and subjected to standardized *in vitro* infection assays with two strains of *M. plutonius*, the bacterial pathogen causing EFB. Levels of hygienic behavior were higher in newly established EFB^- colonies than in EFB^+ colonies, whereas larvae of EFB^- colonies died more quickly after infection with *M. plutonius*. The more resilient honey bee colonies (EFB^-) could therefore have developed a two-tier strategy to better control infection with *M. plutonius*, consisting of early death of infected larvae, coupled with elevated levels of hygienic behavior. In combination, the two mechanisms might facilitate early suppression of *M. plutonius*, helping to keep the colony free from EFB.

Introduction

In *A. mellifera* colonies, the queen is the main reproductive female. After mating with multiple drones in nuptial flights, a sperm blend of about 5-20 drones is stored in the queen's spermatheca (Estoup, Solignac et al. 1997). All other females of the colony develop from eggs fertilized with this preserved sperm, which is used randomly for fertilization (Franck, Coussy et al. 1999; Brodschneider, Arnold et al. 2012). Hence, honey bee colonies consist of many patrilines, corresponding to distinct genetic lineages. Most of the female worker bees in a colony are therefore half-sisters, sharing only the same mother, or full-sisters, sharing the same father and mother, and belonging to the same patriline. These individual patrilines differ in resistance to pathogens and parasites (Palmer and Oldroyd 2003; Tarpy and Seeley 2006; Invernizzi, Peñagaricano et al. 2009; Bourgeois, Rinderer et al. 2012; Ameline, Beaurepaire et al. 2023). The polyandrous mating system in honey bees increases intracolonial genetic diversity, and is positively correlated to the ability of a honey bee colony to withstand to diseases and parasitism (Palmer and Oldroyd 2003; Tarpy 2003; Seeley and Tarpy 2006; Tarpy and Seeley 2006; Invernizzi, Peñagaricano et al. 2009; Bourgeois, Rinderer et al. 2012; Evison, Fazio et al. 2013; Lee, McGee et al. 2013; Desai and Currie 2015).

A honey bee colony consists of several thousands of individual bees, living together in densely populated nests, that provide optimal conditions for parasite growth and horizontal pathogen and parasite transmission (Kurze, Routtu et al. 2016). Honey bees have therefore developed not only individual defense mechanisms to control the impact of pathogens and parasites, but also a wide range of mechanisms improving social immunity, including necrophoric behavior, behavioral fever, grooming and hygienic behavior (Wilson-Rich, Spivak et al.

2008; Evans and Spivak 2010). The removal of unhealthy and dead brood from their brood cells can improve colony health by limiting horizontal transmission of pathogens and population growth of parasites (Spivak and Danka 2021).

The resilience of an organism towards parasites and diseases can be achieved through resistance or tolerance, and the two mechanisms can act concurrently (Schmid-Hempel 2014; Blacquière and Panziera 2018). On the one hand, resistance means the limitation of pathogen burden or reduction of pathogen load. In honey bees, this includes social and individual immune defenses. On the other hand, tolerance implies the limitation of the harm caused by a pathogen, which improves the ability of infected colonies, genetic bee lines or individual bees to survive an infection. Honey bee resistance due to hygienic behavior, can be tested in bioassays. Honey bee tolerance is usually tested by infection of honey bee brood with a pathogen in the laboratory. This method was mainly used to evaluate differences in the virulence of pathogen strains (Arai, Tominaga et al. 2012; Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019; Grossar, Kilchenmann et al. 2020; Nakamura, Okumura et al. 2020), but it is also suitable to explore the hosts capacity to tolerate an infection with the pathogen, and there is some previous evidence that the survival of larvae depends on their genetic background (Lewkowski and Erler 2019; Ameline, Beaurepaire et al. 2023). Resistance to EFB in honey bees might therefore be of genetic origin.

Honey bee colonies of the same apiary are frequently located close to each other and usually experience identical management practices by the beekeeper (Sperandio, Simonetto et al. 2019). High colony density generally facilitates the transmission of contagious diseases between colonies (Brosi, Delaplane et al. 2017), e.g. due to high drifting rates of workers, or the use of contaminated tools and material by the beekeeper (The National Bee Unit 2017). During an EFB

outbreak in an apiary, not all colonies appear to be sick. Some of the colonies lack disease symptoms and appear to be unaffected by EFB, although located in direct vicinity to sick colonies and therefore are under high risk of *M. plutonius* infection. A temporal component may also play a role in infection variation among colonies, as some colonies do not show EFB symptoms at the time of apiary inspection, but may do so later. Overall, it is still unclear why some honey bee colonies can resist exposure to EFB and better endure infection than other colonies.

In this study, we test whether the offspring of honey bee queens, collected from apiaries affected by EFB vary in their resistance to EFB disease. Queens from colonies with and without EFB disease symptoms, all originating from apiaries during acute EFB outbreaks, were collected. To exclude spread of EFB, solely the collected queens, without any other beekeeping material or potentially infected worker bees, were introduced into new, queenless colonies at another location. After the queens' newly produced offspring had replaced the worker bees in the host colony, the colonies were tested for hygienic behavior performance by examining the removal of freeze killed brood. Larvae of the collected queens were tested for survival after artificial infections with two *M. plutonius* isolates *in vitro*. To cover a range of pathogen virulence, a highly virulent *M. plutonius* isolate and an intermediate virulence isolate were used to infect honey bee larvae. The results show, that colonies headed by queens from colonies without EFB symptoms have a faster hygienic response. First, the larvae of these queens died more rapidly after *in vitro* infection with the intermediate virulent *M. plutonius* isolate, than the larvae of queens from colonies showing clear EFB disease symptoms. Second, adult bees performed more hygienic behavior. The implications of this pattern for resistance to EFB disease in honey bees at the individual larval level and at the colony level are discussed.

Material and Methods

Sampling of *A. mellifera* queens

Queens from "EFB^{+}" colonies, displaying clinical EFB symptoms, or "EFB^{-}" colonies, displaying no disease symptoms - were collected from apiaries with EFB outbreaks. The queens were introduced into smaller queenless host colonies, kept in Mini plus sized hives. In total, 54 queens from 15 apiaries with an acute EFB outbreak in the canton of Bern were collected in 2013 and 2014. The offspring of queens that could successfully establish in their host colonies was then subjected to hygienic behavior assays and *in vitro* infections in 2014.

Hygienic behavior assay

All experimental colonies were established at least eight weeks before the test, to ensure that the offspring of the former queen was completely replaced by the offspring of the introduced queen, and the worker bees old enough to carry out hygienic behavior. The performance in cleaning out freeze killed brood of eight colonies headed by queens of former EFB symptomatic colonies, was compared to the performance of eleven colonies headed by queens collected from colonies non-symptomatic for EFB. For the hygienic behavior assay, a limited area of brood on a frame was sacrificed with liquid nitrogen (area ~50 cm^2, delimited by a steel ring). After the frozen area thawed, the frames were returned into their colonies of origin, and removal of dead brood by workers was monitored by taking photos (Fig. 3.1) after 24 h and 48 h (Spivak and Gilliam 1998; Leclercq, Francis et al. 2018). This test has been performed on capped brood, i.e. larvae and pupae at a late developmental stage (brood cells sealed with wax cap; from fifth instar until hatching), as commonly done in hygiene tests (Büchler, Andonov et al. 2013; Spivak and Danka 2021). Additionally, the test was performed on uncapped brood,

i.e. on younger larval stages (brood cells not sealed with wax caps yet; from first to fifth larval instar), which renders the test biologically more relevant, because during an EFB outbreak affected larvae are mostly dying before the capping of their brood cell takes place (Spivak and Gilliam 1998). As in-hive worker bees are suspected to remove uncapped brood much quicker than capped brood, observation intervals in this experiment were scheduled 4, 8, 24 and 48 hours after the brood was freeze-killed.

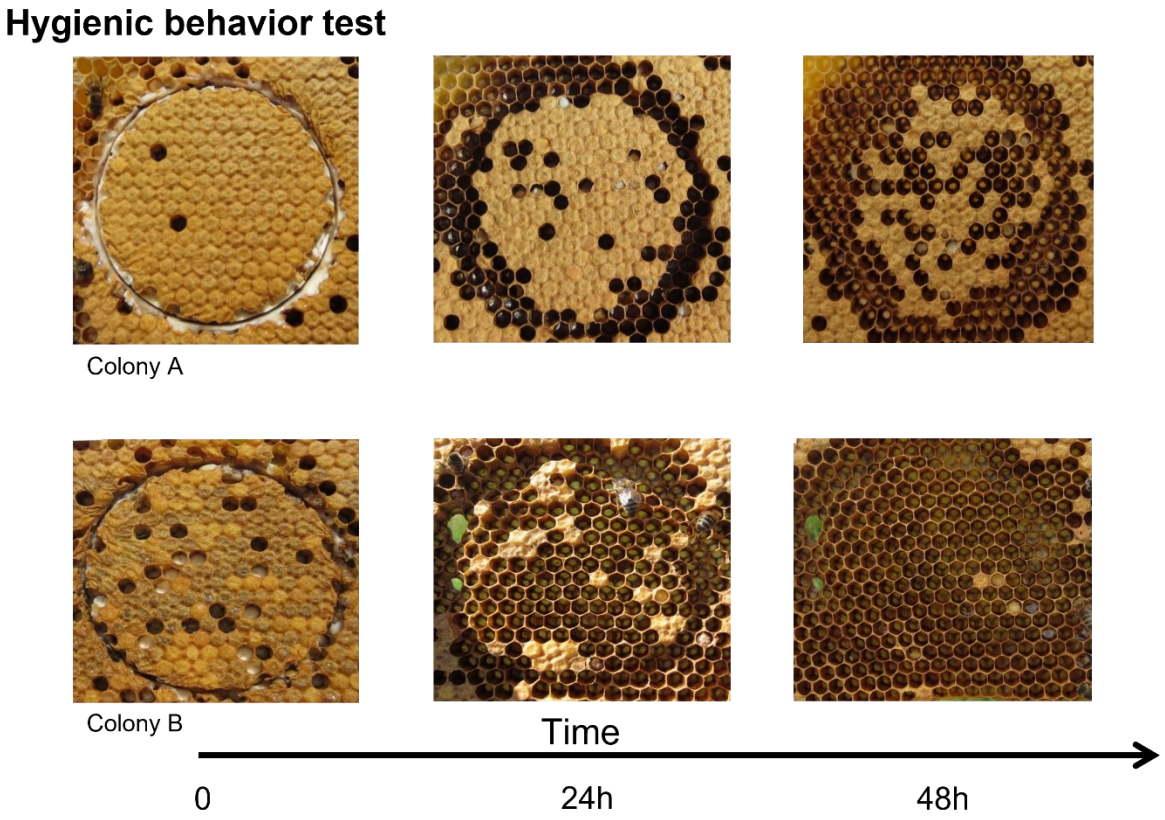


Fig. 3.1: Hygienic behavior test set up. The larvae and pupae of a circular part of a brood comb were sacrificed using liquid nitrogen, and the removal of dead bee brood was monitored 24 h and 48 h after freeze kill of brood. Here, combs with closed brood cells, i.e. holding older bee brood, are depicted. In the upper row, colony A shows slow hygienic behavior with incomplete removal of dead brood after 48 h. Colony B (lower row) shows faster and more complete removal of dead brood.

In vitro infection assays on honey bee larvae

Honey bee brood originating from the queens collected from EFB outbreaks, was reared *in vitro* following standard methods (Aupinel, Fortini et al. 2005; Crailsheim, Brodschneider et al. 2013), and artificially infected with Swiss *M. plutonius* isolate CH 40.2 or isolate CH 49.3 as described before (Grossar, Kilchenmann et al. 2020). In total 24-48 larvae of four to five queens of each group were tested in five independent replicates from June to August 2014. The test queens were therefore confined for 24 h for laying eggs onto a brood comb. After 72 h, the synchronized eggs developed into 1st instar larvae and were individually grafted with a fine paint brush into 10 µl of larval diet within pre-sterilized plastic queen starter cells (Nicoplast™; sterilized for 30 min in 70 % ethanol and then dried). The plastic cells were then placed into the cells of 48-well tissue culture plates (Fig. 3.2), equipped with a piece of wet dental roll wetted with 0.4 % methyl benzethonium chloride and 15.5 % glycerol in water, to avoid desiccation and microbial contamination. The 48 well tissue culture plates with the larvae in queen starter cells were then placed in a hermetic plastic desiccator, provided with a dish filled with saturated K_2SO_4 to maintain 95 % relative humidity necessary for the development of larvae, and the desiccator positioned into an incubator at a constant temperature of 34 °C for the first six day of larval development. At day 7, the plates containing the bee brood were transferred into a desiccator containing a dish filled with saturated NaCl solution to ensure an optimal relative humidity of 75 % for pupal development (Fig. 3.2), placed within an incubator at 34 °C.

The larvae were fed daily with a droplet of pre-warmed diet (34 °C), following a feeding program as follows (Aupinel, Fortini et al. 2005): On day one, the day of grafting, 10 µl of diet A, consisting of 50 % royal jelly and 50 % of an

aqueous solution containing 2 % yeast extract, 12 % glucose and 12 % fructose, was pipetted into the queen starter cups for facilitating grafting of 1st instar larvae. Each larva received another 10 µl of diet A within two hours of grafting. Test larvae artificially infected with *M. plutonius* received 10 µl of diet A spiked with 10^7 CFU ml^{-1} vegetative cells of isolate CH 40.2 or CH 49.3 in saline (9 parts food + 1 part 10^8 CFU ml^{-1} *M. plutonius*, each larva was infected with 10^5 CFU of *M. plutonius* of the respective isolate; for cultivation conditions see below), whereas non-infected control larvae received a mix of nine parts diet A with one part sterile saline (0.9 % NaCl). On day 3 after grafting, 20 µl diet B, which consisted of 50 % fresh royal jelly and 50 % of an aqueous solution containing 3 % yeast extract, 15 % glucose and 15 % fructose, was fed to larvae. On days 4, 5 and 6 after grafting, 30 µl, 40 µl and 50 µl of diet C (mix of 50 % royal jelly and 50 % of 4 % yeast extract, 18 % glucose and 18 % fructose dissolved in water) was fed to the larvae, respectively. The royal jelly used in these experiments, was obtained from healthy colonies located in Liebefeld, Bern and stored in aliquots at – 20 °C until usage.

M. plutonius field strains CH 49.3 and CH 40.2 (Tab. 1.1 in chapter I) were cultivated anaerobically (GENbox, bioMérieux, France) at 36 °C for four days in basal medium (Forsgren et al., 2013). Bacterial concentrations were determined by calculating the mean CFU, which grew on three independent basal medium agar plates (Forsgren et al., 2013). The *M. plutonius* suspensions were adjusted to receive a 10^8 CFU ml^{-1} stock solution in sterile EFB broth before spiking diet A.

The survival of brood was assessed on a daily basis until completion of development (*i.e.* until successful emergence of the imago). Larvae which showed no respiration, had either lost body elasticity, did not react to a mechanical stimulus, developed edema or underwent color change, were recorded as dead

(Crailsheim, Brodschneider et al. 2013). The time of death of honey bee pupae was estimated and recorded based on morphological features when their development had stopped (Rembold, Kremer et al. 1980), as pupae mostly lack respiration movements and reactions to mechanical stimuli. Dead individuals were removed from cells for sanitary reasons.

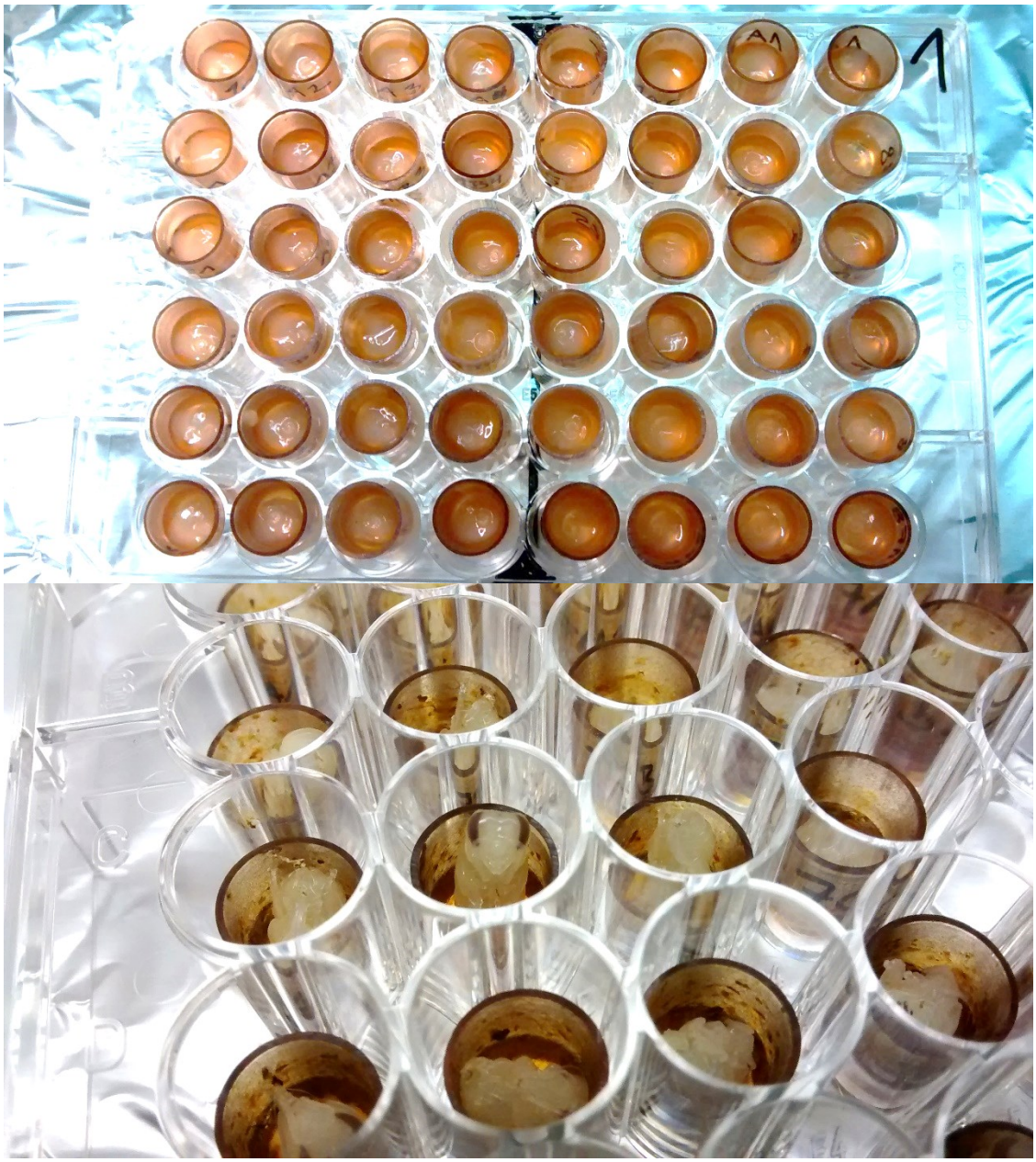


Fig. 3.2: *In vitro* larval rearing set up. In the upper picture 3rd instar larvae in queen starter cups floating in larval diet are visible. In the lower part of the figure artificially reared *A. mellifera* pupae are visible.

Statistics

Hygienic behavior performance was tested by comparing the removal rates of freeze killed capped brood in colonies of the EFB^+ and EFB^- group, after 24 h and 48 h, using a non-parametric, Kruskal-Wallis Test. Further, the overall success at freeze killed brood removal was estimated by comparing the proportion of colonies in the EFB^+ and EFB^- group, reaching a removal rate superior to 90 % after 4 h, 8 h, 24 h and 48 h, with Pearson's Chi square tests. The removal threshold of 90 % was set based on the results of former studies on the removal of freeze killed brood among unselected honey bee colonies (Spivak and Reuter 1998; Waite, Brown et al. 2003; Pérez-Sato, Châline et al. 2009; Bigio, Al Toufailia et al. 2014).

Brood survival after experimental infection with *M. plutonius* were illustrated with Kaplan-Meier survival curves (Kaplan and Meier, 1958). Differences in survival between honey bee larvae infected by *M. plutonius* isolates CH 40.2 and CH 49.3 were tested using pairwise log-rank tests (IBM SPSS Statistics, version 26). A significance level of $p < 0.05$ was adopted for all statistical tests.

Results

Differences in hygienic behavior

Colonies headed by queens collected from colonies without clinical EFB symptoms, EFB^- group, removed on average 50 % of the freeze killed capped brood (N= 8 colonies, mean removal rate (SD) after 24 h= 49.6 % (19.5 %), whereas colonies headed by queens collected from colonies with EFB symptoms, EFB^+ group, removed only 43 % of the freeze killed brood on average (N= 7

colonies, mean removal rate (SD) after 24 h= 42.7 % (14.9 %); Fig. 3.3). After 48 h, the mean removal rates (SD) in the two groups of colonies are almost identical with a removal rate $_{EFB–,\ 48\ h}$= 70.4 % (17.1 %) and a removal rate (SD) $_{EFB+,\ 48\ h}$= 71.43 % (19.8 %), respectively. Differences in the freeze killed capped brood removal between the EFB^- and EFB^+ group of colonies are statistically not significant (Kruskal-Wallis tests, freeze killed brood removal after 24 h: p= 0.345 and freeze killed brood removal after 48 h: p= 0.836).

EFB^- and EFB^+ did not differ significantly in the removal of ≥ 90 % of uncapped freeze killed brood after four hours (EFB^+, N= 7 colonies, mean removal rate (SD) after 4 h= 61.4 % (24.3 %); EFB^-, N= 6 colonies, mean removal rate (SD) after 4 h= 81.8 % (17.0 %); X^2 Pearson= 1.935, df= 1, p= 0.164; Fig. 3.3). After eight hours in the colonies, the freeze killed brood was removed mostly completely in the colonies of the EFB^- group (N= 6, mean removal rate (SD) = 99.5 % (0.9 %)), whereas only about 85 % of freeze killed brood was effectively removed in colonies of the EFB^+ group (N= 7, mean removal rate (SD)= 84.4 % (15.4 %). A higher proportion of colonies reached a removal rate threshold of ≥ 90 % of freeze killed brood after eight hours in the group of EFB^- colonies (6 out of 6 colonies) compared to colonies in the EFB^+ group (3 out of 7 colonies), and this difference is statistically significant (X^2 Pearson = 4.952, df= 1, p= 0.026). The removal of freeze killed uncapped brood was completed at the checks after 24 h and after 48 h in both groups of colonies, hence no statistical difference in the removal rate was detected between these two groups of colonies (≥ 90 % removal rate after 24 h: X^2 Pearson= 0.016, df= 1, p= 0.898; ≥ 90 % removal rate after 48 h: X^2 Pearson= 0.109, df= 1, p= 0.741)

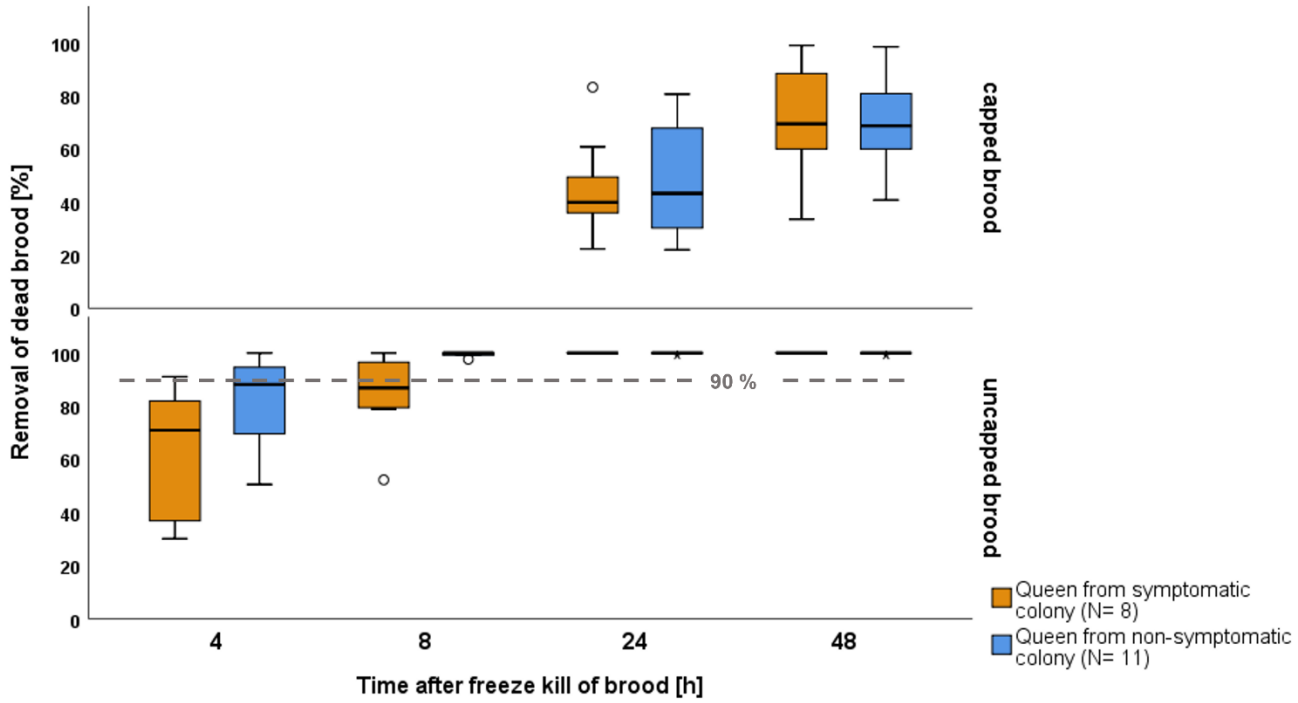
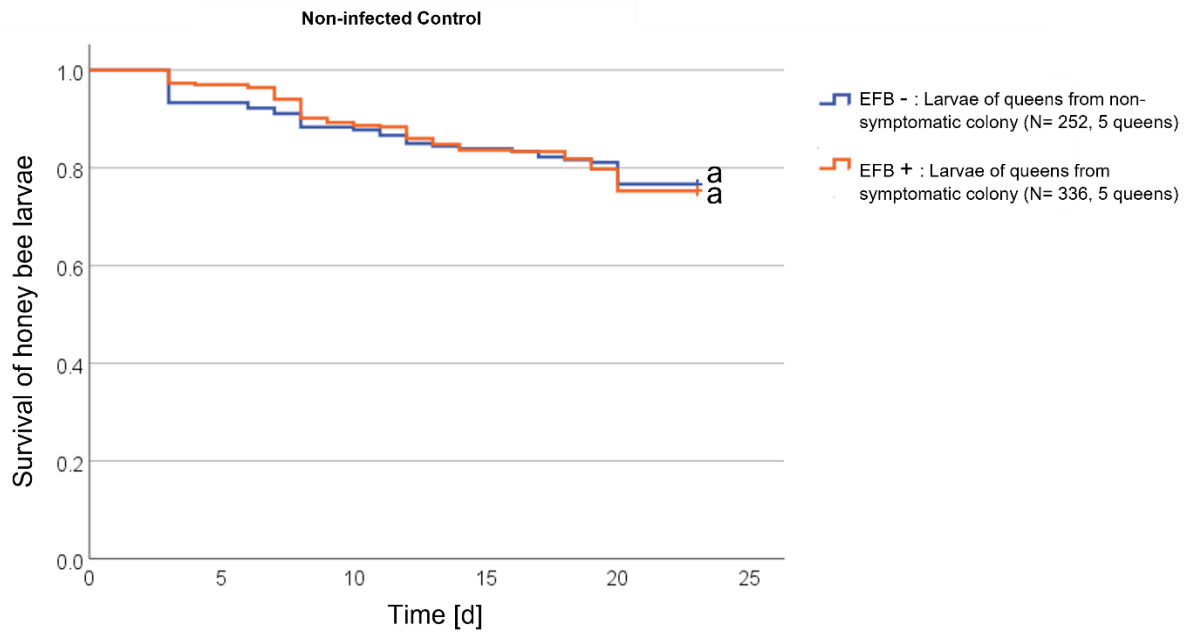


Fig. 3.3: Removal rates of freeze killed, capped brood in honey bee colonies after 24 h and 48 h (upper part), and removal rates of freeze killed, uncapped brood after 4 h, 8 h, 24 h and 48 h (lower part of graph). Orange boxplots represent colonies headed by queens collected from colonies with EFB symptoms (EFB^+); blue boxplots represent colonies headed by queens collected from colonies without EFB symptoms (EFB^-).

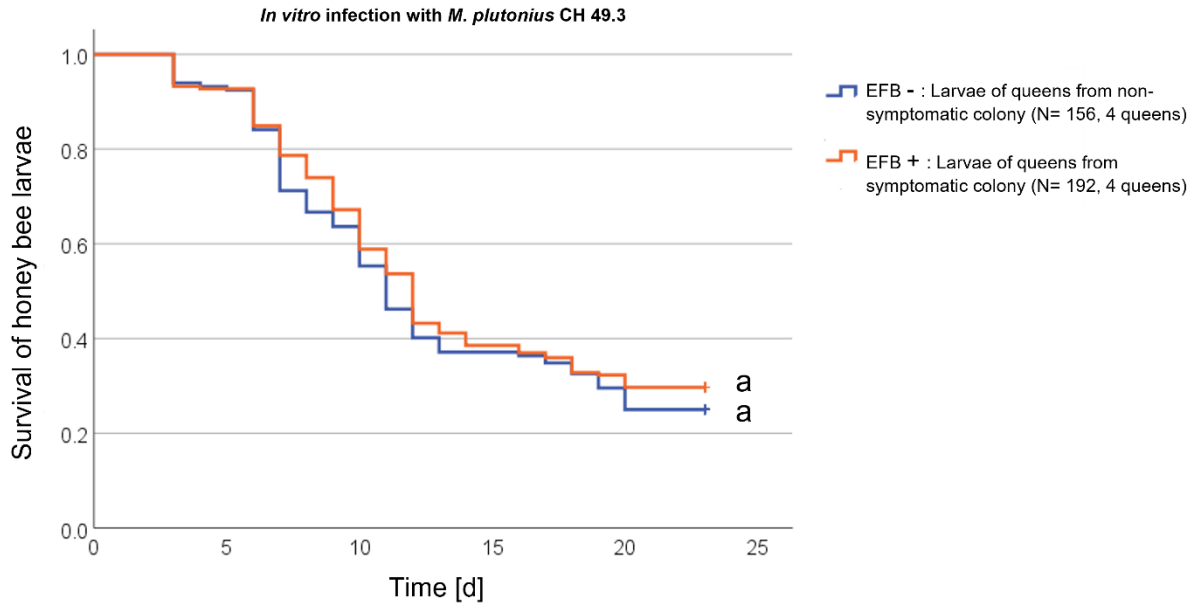
Differences in the survival of individual larvae

The survival of non-infected control larvae of the two groups of colonies, headed by queens of former colonies with EFB symptoms (EFB^+) or by queens from symptomless colonies (EFB^-), does not significantly differ from each other (Log rank Mantel-Cox test, X^2= 0.062, d*f*= 1, p= 0.804, Fig 3.4 A). Also, the survival of larvae from queens originating from EFB^+ or EFB^- colonies, after *in vitro* infection with the highly virulent *M. plutonius* CH 49.3 does not significantly differ from each other (Log rank Mantel-Cox test, X^2= 0.919, d*f*= 1, p= 0.338, Fig 3.4 B). Larvae of EFB^- queens survive on average for 13.3 days [12.2-14.3 days] after infection with *M. plutonius* CH 49.3, and larvae of EFB^+ queens for 13.8 days [12.9-14.3 days]. The average survival time of honey bee larvae

after infection with the less virulent *M. plutonius* isolate CH 40.2, originating from EFB⁻ queens is with 17.4 days [16.5-18.3 days] slightly shorter, than the 18.4 days [17.7-19.4 days] survival time of larvae of the EFB⁺ queens after infection, a difference that was statistically significant (Log rank Mantel-Cox test, X^2= 5.088, d*f*= 1, p= 0.024, Fig. 3.4 C).



A



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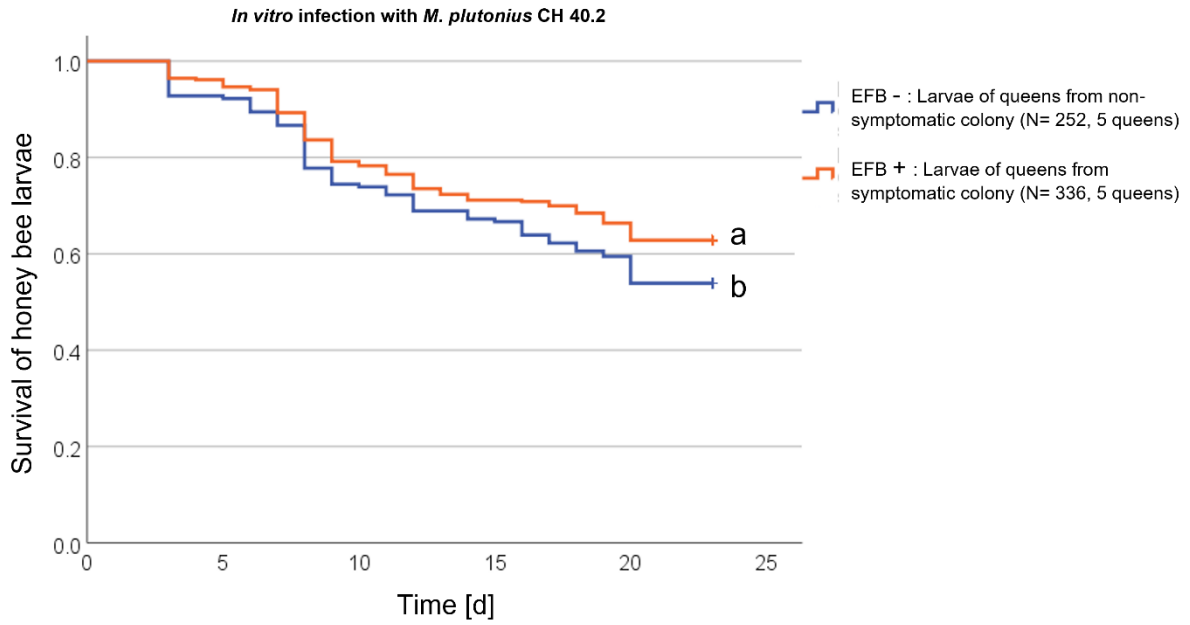

C

Fig. 3.4: Survival of non-infected control honey bee larvae (A), or survival of larvae after infection with *M. plutonius* strain CH 49.3 (B), or CH 40.2 (C). Non-capitalized letters indicate significantly differences in survival (pairwise log-rank Mantel-Cox test, $p < 0.05$).

Discussion

Hygienic behavior assays are often used to select highly hygienic bee lines, which are either challenged with parasites or pathogens or monitored for prevalence of disease (Leclercq, Pannebakker et al. 2017; Leclercq, Francis et al. 2018; Spivak and Danka 2021). In contrast, this study followed a converse approach, as hygienic behavior assays were applied to examine colonies which had been naturally exposed to *M. plutonius*. Both types of colonies in this study, EFB^+ and EFB^- colonies, showed high levels of hygienic behavior. This is consistent with the results of Bigio, Al Toufailia et al. (2014), who also found high levels of hygienic behavior in colonies from naturally mated and unselected queens. showed. of No difference in the removal rates of freeze killed brood from sealed cells was detected between EFB^+ and EFB^- colonies. However, when adapting the

freeze killed brood hygienic behavior bioassay to the characteristics of EFB disease, by sacrificing unsealed, younger brood (Spivak and Gilliam 1998), significantly more of the EFB^- colonies than of the EFB^+ were able to remove a large part (≥ 90 %) of the freeze killed brood within 8 hours. In a former study on the removal of freeze killed unsealed brood (Al Toufailia, Evison et al. 2018), no difference between hygienic and less hygienic honey bee lines was detected, all colonies had removed almost all of the freeze killed unsealed brood within 48 hours. Likely, this result is an artefact, though, as in the other study the removal of freeze killed unsealed brood in the test colonies was controlled only after 24 hours and after 48 hours, as suggested in the standard protocol for the hygienic behavior bioassay on sealed brood (Leclercq, Francis et al. 2018). A time span of 24 hours or 48 hours, for the monitoring of the removal of unsealed brood (Al Toufailia, Evison et al. 2018), thus is presumably too long, because in contrast to the removal of capped brood the detection of dead brood in their already capped cells and the removal of the cell capping, is not required if the test is performed on unsealed brood. In this study, virtually all test colonies had completed removal of freeze-killed, unsealed brood within 24 hours, with several colonies having removed ≥ 90 % of the brood after only 8 hours.

The results show that the progeny of queens collected from colonies free of EFB symptoms (EFB^-) is more susceptible to infection with an intermediate virulence strain of *M. plutonius*, but not to infection with the high virulence strain, than the progeny of queens from formerly diseased colonies (EFB^+). This finding is surprising, because larvae from queens of disease-free colonies were predicted to be more resistant to infection than larvae from queens of colonies with visible EFB symptoms. Although this finding is unexpected, it is supported by theoretical models where higher mortality rates of diseased individuals are correlated to a

better winter survival of the whole honey bee colony (Betti, Wahl et al. 2014). The early death of infected larvae can therefore ensure the survival of the whole colony. Overall, the results suggest that larvae from EFB$^-$ colonies are not less susceptible to *M. plutonius* infection, but their earlier death facilitates their hygienic removal by adult bees and suppression of disease within the colony. Indeed, a connection between increased worker brood susceptibility and colony-level resistance to *Varroa* mite infestation was documented recently and referred to as "social apoptosis" (Page, Lin et al. 2016). The early death and removal of infected individuals minimize infection costs for the whole colony, by hindering the spread of infection (Page, Lin et al. 2016). Conroy and Holman (2022) experimentally challenged adult honey bees leaving the hive with bacterial lipopolysaccharides and found that these bees altruistically banished themselves from the colony. This form of social immunity could therefore be a widely spread, fundamental defense mechanism in social insect colonies, irrespective of the pathogen or parasite (Page, Lin et al. 2016; Aanen 2018). Likewise, social apoptosis could also be expressed by the honey bee larvae infected with *M. plutonius*.

Early mortality coupled with elevated hygienic behavior could be an effective mechanism to prevent disease outbreak in honey bee colonies. First, the early death of infected larvae, before defecation, limits pathogen transmission to other susceptible larvae within the colony, and keeps *M. plutonius* confined to the intestinal tract (Tarr 1938; Takamatsu, Sato et al. 2016). Second, the quicker removal of contagious material from the hive lowers the pathogen load within the whole colony and limits intra-colonial EFB transmission.

Since many honey bee queens of both experimental groups were lost during the experiment, e.g. due to replacement by the host colonies, the number of

colonies that could be tested in this study was limited. In view of the interesting first results of this study, it will be of interest to therefore be necessary repeat the experiments and collect more EFB^{+} and EFB^{-} queens from apiaries with acute EFB outbreaks. To confirm a genetic component in EFB resilience, a next experiment would be to establish two distinct populations via breeding of a F1-generation of honey bee colonies based on daughter queens and drones reared from the collected EFB^{+} and EFB^{-} queens. These two populations of honey bee colonies could then be tested for their performance in hygienic behavior and brood survival after *in vitro* infection with *M. plutonius*. If the observed tolerance mechanisms against EFB indeed have a genetic component, one would expect differences in the survival of these two groups of F1-larvae after infection and efficiency of freeze killed brood removal.

Honey bees have been naturally selected for resistance against parasitic mite infestations (van Alphen and Fernhout 2020; Guichard, Dainat et al. 2023). Under the assumption that resistance to EFB is heritable and is subjected to natural selection, the collected queens from EFB outbreaks could be a starting point for a breeding program. To this end, queens from EFB outbreaks which do not show disease symptoms and have presumably undergone natural selection, or alternatively their F1-generation, could serve as breeding material for an EFB resistant honey bee line.

General Discussion

European Foulbrood (EFB) is an important disease of the Western honey bee (*A. mellifera*) caused by the bacterial pathogen *M. plutonius*. This disease was first described more than a century ago (Cheshire and Cheyne 1885; Burri 1906; Maassen 1907; White 1912), yet knowledge on EFB is still limited. EFB has become more prevalent in recent years in several countries, including Switzerland, which stimulated numerous research projects. This thesis has focused on the host-pathogen interaction between honey bees (*A. mellifera*) and *M. plutonius*, which causes EFB disease. I assessed the virulence of the pathogen *M. plutonius* at the level of individual honey bee larvae, and factors that drive it. In addition, I studied the distribution and occurrence of individual genetic subtypes of the pathogen in Switzerland. Finally, I also considered the hosts side in the interaction with the pathogen, and described a strategy of honey bees to withstand EFB disease at the colony level.

In chapter I, I found evidence that the presence of *mtxA*, a gene that is situated on plasmid pMP19 and codes for the putative toxin Melissotoxin A, increases the virulence of *M. plutonius* isolates. Three of the *M. plutonius* isolates used to infect honey bee larvae *in vitro,* contained *mtxA*, and caused mortality rates above 80 %, whereas infection with an isolate lacking *mtxA* killed below 55 % of larvae. *M. plutonius* isolate CH MeplS1, an isolate that was repeatedly sub-cultivated from the highly virulent isolate CH 49.3 and had lost pMP19 which contained *mtxA* (Djukic, Erler et al. 2018), was practically avirulent in the applied honey bee larvae infection model. This is in line with former studies, in which standardized infection assays with *M. plutonius* were not feasible, as the pathogen quickly lost infectivity during sub-cultivation in artificial media (Bailey 1957; Bailey 1963; Bailey and Locher 1968; McKee, Goodman et al. 2004; Giersch, Barchia et

al. 2010). Moreover, this result substantiates the potential of plasmid pMP19 containing *mtxA* as virulence factor of *M. plutonius*, although experiments that re-inserted pMP19 into cured isolates, did not result in recuperation of virulence (Nakamura, Okumura et al. 2020). However, this might be explained by reduced bacterial growth of the modified isolates (Nakamura, Okumura et al. 2020). A positive correlation between the ability to grow in artificial medium and the virulence of an isolate was also detected in chapter I, suggesting that growth abilities are an important factor of *M. plutonius* virulence. In contrast, co-infection with *M. plutonius* and common secondary invader *P. alvei*, neither increased EFB symptoms, nor did co-infection cause higher mortality rates in infected honey bee larvae.

In chapter II, I expanded the sample size, from only a few isolates, to 160 *M. plutonius* samples isolated from Swiss EFB outbreaks in 2006-07 and in 2013, to assess their genetic diversity. I combined multi locus sequence typing (MLST) (Haynes, Helgason et al. 2013), with PCR screening for *mtxA* (chapter I), phylogenetic and spatial analyses to explore the distribution and population structure of these 160 Swiss *M. plutonius* isolates (chapter II). I discovered twelve sequence types in Switzerland, of which five were novel. These novel sequence types probably have evolved locally, four of the five novel sequence types have been only found in samples from the first sampling period, but not in samples of the second sampling period. The *mtxA* gene, was detected more frequently in isolates from the second sampling period, indicating an increased prevalence of this potential virulence factor (chapter I) in Swiss *M. plutonius* isolates over time. However, EFB disease was detected less frequently in the Swiss honey bee population during the second sampling period, thus the number of registered EFB cases in Switzerland decreased from the first sampling period to the second (Fig.

2) possibly due to the stricter control measures implemented in 2010 (FSVO 2015). As an alternative explanation, the elevated virulence of *mtxA* containing *M. plutonius* isolates at the larval level *in vitro* (chapter I), might be converse to the virulence of such isolates at the colony level *in vivo*. Bacterial isolates that are less virulent at individual level, could be highly virulent at colony level, and vice versa, as was already hypothesized for *P. larvae* (Genersch, Ashiralieva et al. 2005).

The virulence trade-off model (Anderson and May 1982; Ewald 1983; Ebert and Bull 2008; Alizon, Hurford et al. 2009) predicts that pathogens have to adapt virulence in order to maximize their own transmission. Pathogen strains of low virulence, which are easily outcompeted by more aggressive strains, and strains of excessive virulence, which kill the host as a side effect before their own transmission are both prone to become extinct. In fact, pathogens of medium virulence should be favored by evolution, as their lifetime transmission success is supposed to be the highest (Ebert and Bull 2008). This simplified model might apply to the here studied host-pathogen relationship of *A. mellifera* and *M. plutonius*: Honey bee larvae often survive an infection with *M. plutonius*, but generally defecate and pupate later than uninfected control larvae (Bailey and Ball 1991). A delay in pupation might be beneficial for *M. plutonius*, as the pathogen gains time for multiplication in the gut of infected larvae. Consequently, more viable bacterial cells can exit the larval gut within feces, which is expelled and smeared on the cell walls by the larvae during pupation, and infect other larvae (Bailey and Ball 1991). A too high virulence, expressed by fast killing of honey bee larvae, might hinder the transmission of *M. plutonius* within the colony (Fries and Camazine 2001), and on a larger scale also in the host population. Thus, highly virulent pathogen strains, as *M. plutonius* CH 49.3, are estimated to represent

only a small proportion in the population and their virulence evaluated on the individual larva level is supposed to be contrary to virulence measured on the colony level. In fact, all *M. plutonius* isolates tested and analyzed (chapter I and chapter II), were derived from honey bee colonies with explicit symptoms of EFB disease, and therefore are estimated to be pathogenic at the colony level. A quarter of the tested isolates, however, did not cause larval mortality above the baseline mortality rates observed in uninfected control larvae (chapter I) and were therefore ranked as avirulent, which provides further evidence that virulence of *M. plutonius* at the colony level might be negatively correlated to the virulence at individual larva level.

The honey bee host could also have different susceptibilities to the pathogen. It is known, that individual patrilines differ in their resistance to pathogens and parasites (Palmer and Oldroyd 2003; Tarpy and Seeley 2006; Invernizzi, Peñagaricano et al. 2009; Bourgeois, Rinderer et al. 2012), and it was only recently shown that the genetic origin of honey bee larvae has an effect on their susceptibility to *M. plutonius* in *in vitro* infection assays (Nakamura, Yamazaki et al. 2016; Lewkowski and Erler 2019; Ameline, Beaurepaire et al. 2023). In chapter III, I tested this variation in host susceptibility based on honey bee larvae from mother queens, that I collected from colonies situated in apiaries with EFB outbreaks. One group of honey bee queens was sampled from colonies showing clear EFB symptoms (EFB^+), while the other group of queens originated from colonies without EFB symptoms (EFB^-). EFB^+ and EFB^- queens were fostered into queenless colonies to found new colonies. I subjected the larvae of EFB^+ and EFB^- queens to *in vitro* infection with *M. plutonius* isolate CH 49.3, or isolate CH 40.2, which I both had tested before (chapter I). The results show that larvae of EFB^- queens, which were derived from EFB symptomless colonies, are more

sensitive to an infection by medium virulence *M. plutonius* isolate CH 40.2 (chapter III). The colonies formed by EFB^+ and EFB^- queens, were also tested for their hygienic behavior performance, via freeze killed brood assays. Colonies descending from EFB^- queens appeared to be faster in removal of dead brood than colonies of EFB^+ queens. Together these results suggest EFB^- colonies suppress EFB disease symptoms by more efficient and quicker removal of infected brood, which lowers the pathogen load within their colonies. the first, early death of infected larvae hinders the growth and spread of infective *M. plutonius* cells, because if an infected larva dies before defecation, *M. plutonius* has less chance to be transmitted in the honey bee colony, as the bacteria stay confined in the intestinal tract (Tarr 1938; Takamatsu, Sato et al. 2016). Second, fast hygienic removal of infected larvae from the colony prevents the spread of *M. plutonius* to other susceptible larvae and keeps the colony free of EFB symptoms.

In conclusion, the results presented in this thesis advance the knowledge of the epidemiology of EFB and offer a better understanding of the prevalence and distribution of genetic subtypes and virulence of *M. plutonius* in Switzerland. Field screenings of genetic subtypes of *M. plutonius* in regions where EFB is an emerging disease, in combination with tests on the virulence at the individual larva and colony levels, are needed to clarify if novel, highly virulent strains of *M. plutonius* are responsible for recent epidemic outbreaks of EFB worldwide (Boncristiani, Ellis et al. 2020). The genetic variant and the presence of *mtxA* or plasmid pMP19 (Arai, Tominaga et al. 2012; Nakamura, Yamazaki et al. 2016; Djukic, Erler et al. 2018; Grossar, Kilchenmann et al. 2020; Nakamura, Okumura et al. 2020; Nakamura, Okumura et al. 2021) could provide a predictive marker for virulence at the individual larva level. If a link between virulence of *M. plutonius* and disease outbreak is found, or if responses to control measures or treatment

differ among genetic variants of the pathogen, then differential, virulence-based control options could be initiated, dependent upon the infecting *M. plutonius* variant, (Abadi and Kusters 2016; Vale, McNally et al. 2016), to better control the spread of EFB.

Experiments at the individual bee level are not ideal to provide realistic estimates of effects on entire honey bee colonies, which consist of several thousand individuals showing complex social interactions. In the second part of the thesis, the focus was therefore shifted from the individual honey bee to the whole honey bee colony. Identifying behavioral strategies by which this social living insects combat EFB, and eventually breeding resistant honey bee lines from naturally selected bee stocks, could improve effective control of EFB and ultimately honey bee health. Studies at both the individual and colony level are thus important to fill the present knowledge gaps and provide the required information to take all necessary measures to improve honey bee health in the future.

Annex I: Triplex real-time PCR method for the qualitative detection of European and American foulbrood in honeybee

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Abstract

The bacteria *Melissococcus plutonius* and *Paenibacillus larvae*, causative agents of respectively European and American foulbrood, damage honeybee health worldwide. Here, we present a specific and sensitive qualitative triplex real-time PCR method to detect simultaneously those microbial agents and a honeybee gene, validated through a study involving 7 laboratories through Europe.

Short Note:

American foulbrood (AFB) caused by *Paenibacillus larvae* and European foulbrood (EFB) induced by *Melissococcus plutonius* are both major honeybee (*Apis mellifera*) brood disease globally widespread (Ellis and Munn 2005) and contribute to colony declines (Forsgren 2010; Genersch 2010). Both pathogens develop in the gut when ingested by the larvae at any stage for EFB (Bailey and Ball 1991) or only within the first 72 hours for AFB (Bamrick and Rothenbuhler 1961; Brødsgaard, Ritter et al. 1998). Four AFB types, so called ERIC types have been described (Genersch, Forsgren et al. 2006). Only ERIC I and II are usually detected in the field (Genersch 2010). AFB and EFB are notifiable diseases in many countries where the authorities usually destroy the colonies with clinical symptoms. Hence, both foulbrood can lead to serious economic loss for beekeepers and considerable efforts from veterinaries authorities.

In Switzerland EFB prevalence dramatically increased the last decade where a first real-time PCR system has been published (Roetschi, Berthoud et al. 2008). Therefore, early diagnostic is from utmost importance, using accurate, rapid, cheap and easy to handle detection tools, to prevent its spread. Here we present a qualitative real-time triplex PCR method for the detection of EFB, AFB (including ERIC type I to IV) and a specific gene of the honeybee host as PCR amplification control verified by a validation study involving 7 laboratories European Union wide. To our knowledge this is the first DNA-based method to detect all three listed target organisms simultaneously.

The DNA was extracted with the NucleoSpin Tissue kit from Macherey-Nagel according to the user manual with the following modifications: During the pre-lyse step, prior to the proteinase K, a lysozyme digestion (20 mg lysozyme/ml at 37 ˚C for 1 h) and after the Proteinase K digestion a RNAse A Incubation (20 mg/ml

at room temperature for 5 minutes) was added. DNA concentration in extracts was measured with NanoDrop® ND-1000 spectrophotometer (NanoDrop, USA). For the primer-probe design, performed with the Beacon Designer 8.20, the following gene sequences were used: for *Melissococcus plutonius*: napA pseudogene, Na+/H+ antiporter, strain: ATCC 35311, Accession #: AB778538.1 (Arai, Miyoshi-Akiyama et al. 2014), for *Paenibacillus larvae* the sequences of the amplicon of primer pair ERC0390F/ERC03906R, including gene tnp60 (Djukic, Brzuszkiewicz et al. 2014). This region belongs to *P. larvae* DSM 25430, Accession #: CP003355.1, and for *Apis mellifera* mRNA for actin, partial cds, accession number AB023025,1 (Roetschi, Berthoud et al. 2008). All primers and probes were examinated for similarity using the NCBI BLAST tool. The results confirmed the theoretical specifity of the target regions.

Information on primer and probe sequences, the labelling fluorophores and with its quenchers and the amplicons are listed in in Table A1. The PCR was set up in a 20 µl volume, with 2x Mastermix (SensiFast Probe No-ROX Kit from Bioline) containing 400 nM of each primer, 100 nM of each probe and 5 µl DNA-extract (1 ng/µl)

Target organism	Primer Probe	Oligonucleotide sequence (5'-3') and probe labelling	Amplicon length
M. plutonius	MP-F	GAC CTG TTT AGC TAT TAT CAC TA	92 bp
	MP-R	CAC CTA CAA TGA ATG ATT CAT TC	
	MP-Probe	FAM – TCC GCC TAA GCT ACC ACC TAA GAA C - BHQ1	
P. larvae	PL-F	TAC GCT TTT CGA TTC TCT G	87 bp
	PL-R	GTC TGT ACT GAA CCA AGT C	
	PL-Probe	Yakima Yellow – ATC TGC TTC CAC TTG TTC ACT CAC CA - BHQ1	
A. mellifera	AM-F	TCC AGA TGG TCA AGT AAT TAC	87 bp
	AM-R	GCT TCC ATT CCT AAG AAG G	
	AM-Probe	ROX – TCC GTT GTC CCG AGG CTC TTT - BHQ2	

Table A1: Primer and probe information

70.0% AFB + 20.0% *A. mellifera* + 10.0% EFB
75.0% AFB + 20.0% *A. mellifera* + 5.0% EFB
80.0% AFB + 17.5% *A. mellifera* + 2.5% EFB
70.0% EFB + 20.0% *A. mellifera* + 10.0% AFB
75.0% EFB + 20.0% *A. mellifera* + 5.0% AFB
80.0% EFB + 17.5% *A. mellifera* + 2.5% AFB
70.0% *A. mellifera* + 15.0% EFB + 15.0% AFB
90.0% *A. mellifera* + 5.0% EFB + 5.0% AFB
95.0% *A. mellifera* + 2.5% EFB + 2.5% AFB

Table A2: Mixtures of EFB *M. plutonius*, AFB *P. larvae* and *Apis mellifera* with different concentrations.

The amplifications were performed with the Rotor-Gene 6000, Qiagen (formerly Corbett Life Science). According to the Bioline recommendations the following cycling program was applied: 5 min at 95 °C and 45 cycles of 10 s at 95 °C and 30 s at 60 °C. The Rotor-Gene software version 2.3.2.49 was used to analyze the raw data.

Prior to a validation study the triplex real-time PCR was tested with DNA extracted from the following target organism: *Melissococcus plutonius*, *Paenibacillus larvae* (ERIC I, II, III and IV) and *Apis mellifera*. All possible combinations in practice-oriented concentrations were tested (Table A2).

The limit of detection (LOD) for EFB and AFB was set at a quantification cycle Cq-value of 30 (corresponding to 0.001 ng/PCR or 456 copies number resp. 0.0001 ng/PCR or 20 copies) and for *Apis mellifera* at a Cq-value of 32 (corresponding to 0.1 ng/PCR or 380 copies). From the view of the practical relevance these LODs are considered as absolutely satisfactory. For each target organism and each concentration, the Cq-value was always significantly above the LOD, meaning that even in a mixture of the two pathogens low contamination levels can be detected.

To test the fitness of the method an international validation study was organized. Thirteen blind samples (Table A3a), each in a tube labelled with a randomized number, containing approximately 100 µl extracted DNA (10 ng/µl) together with 2 tubes containing the lyophilized primers and probes and a detailed working instruction was shipped to 7 laboratories.

The fitness of this method was assessed by computation of the accuracy (AC), sensitivity (SE) and specifity (SP) for each single laboratory and target organism and calculated as follows:

Accuracy (AC) is the fraction of correct positive and negative results; it is calculated by the following equation:

AC is: $\frac{PA+NA}{PA+ND+PD+NA}$

Sensitivity (SE) is the ability of classifying positive results as positive, it is calculated as follows:

SE is: $\frac{PA}{PA+ND}$

Specificity (SP) is the ability of classifying negative results as negative, it is calculated as follows:

SP is: $\frac{NA}{PD+NA}$

With:

PA: positive agreement (i.e. number of times detection was done when expected)

NA: negative agreement (i.e. number of times there was no detection when expected)

PD: positive deviation (i.e. number of times detection was done even though detection was not expected)

ND: negative deviation (i.e. number of times there was no detection even though detection was expected)

The results of the statistic parameters AC, SE and SP reported from each laboratory with all target organisms are listed in Table A3b. All 7 participating laboratories could successfully detect EFB and AFB in the 13 samples (Table A3a).

Sample	DNA extract	expected qPCR result EFB	expected qPCR result AFB	expected qPCR result bee
1	*E. coli* DH5α	negative	Positive[1)]	negative
2	*M. plutonius* ATCC 35311 (Reference)	positive	negative	negative
2	*M. plutonius* 49.3 (CH)	positive	negative	negative
4	*M. plutonius* 27.1 (FR)	positive	negative	negative
5	*P. larvae* DSM 7030 (ERIC I, Reference)	negative	positive	negative
6	*P. larvae* BK 199-13 (ERIC II, GER)	negative	positive	negative
7	*P. larvae* CCUG 48972 (ERIC II, SE)	negative	positive	negative
8	10% *M. plutonius* ATCC 35311 & 90% *P. larvae* DSM 7030	positive	positive	negative
9	90% *M. plutonius* ATCC 35311 & 10% *P. larvae* DSM 7030	positive	positive	negative
10	90% *A. mellifera* ATCC 35311 & 10 % *P. larvae DSM 7030*	negative	positive	positive
11	90% *A. mellifera* & 10 % *M. plutonius ATCC 35311*	positive	negative	positive
12	*A. mellifera*	negative	negative	positive
13	80% *A. mellifera* & 10 % *P. larvae DSM 7030* & 10 % *M. plutonius ATCC 35311*	positive	positive	positive

Table A3a

[1)] contaminated with *P. larvae*

	Melissococcus plutonius			***Paenibacillus larvae***			***Apis mellifera***		
Lab	**AC**	**SE**	**SP**	**AC**	**SE**	**SP**	**AC**	**SE**	**SP**
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3	1.00	1.00	1.00	1.00	1.00	1.00	0.77	0.25	1.00
4	1.00	1.00	1.00	0.92	0.88	1.00	1.00	1.00	1.00
5	1.00	1.00	1.00	0.92	1.00	0.80	1.00	1.00	1.00
6	1.00	1.00	1.00	1.00	1.00	1.00	N/A	N/A	N/A
7	1.00	1.00	1.00	1.00	1.00	1.00	0.46	0.50	0.44
9	0.85	0.71	1.00	1.00	1.00	1.00	N/A	N/A	N/A

Table A3: a) List of the samples with its expected results used for the validation study b) Validation study results of the accuracy, sensitivity and specificity. N/A: non measured.

For *M. plutonius* only one laboratory reported 2 false negative results of a total of 49 samples. For *P. larvae* 5 laboratories reported results with no errors. One laboratory reported 1 false negative of a total of 56 positive samples and another 1 false positive result of a total of 35 negative samples. Due to the fact that the probe for the *A. mellifera* PCR-system was labelled with ROX, 2 laboratories were not able to work with this probe: one laboratory had to use a mastermix containing ROX as fluorophore for the instrument calibration, the second had a PCR machine with two channels only.

From the remaining 5 laboratories 3 reported results with no errors for *A. mellifera*, one with false positive and one with false negative and false positive results (Table A3b). Taking into account that the two laboratories reporting false results did not use a mastermix optimized for triplex real-time PCR (compared to the 3 laboratories submitting no errors for *A. mellifera*) these results are absolutely plausible.

Based on these facts this method can be considered as fit for purpose.

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References

Aanen D.K. (2018) Social immunity: The disposable individual. Current Biology **28**(7): R322-R324.

Abadi A.T.B., J.G. Kusters. (2016) Management of *Helicobacter pylori* infections. BMC Gastroenterology **16**(1): 94.

Al Toufailia H., S.E.F. Evison, W.O.H. Hughes, F.L.W. Ratnieks. (2018) Both hygienic and non-hygienic honeybee, *Apis mellifera*, colonies remove dead and diseased larvae from open brood cells. Philosophical Transactions of the Royal Society B: Biological Sciences **373**(1751): 20170201.

Alippi A.M. (1991) A comparison of laboratory techniques for the detection of significant bacteria of the honey bee, *Apis mellifera*, in Argentina. Journal of Apicultural Research **30**(2): 75-80.

Alizon S., A. Hurford, N. Mideo, M. van Baalen. (2009) Virulence evolution and the trade-off hypothesis: History, current state of affairs and the future. Journal of Evolutionary Biology **22**(2): 245-259.

Allen M.F., B.V. Ball. (1993) The cultural characteristics and serological relationships of isolates of *Melissococcus pluton*. Journal of Apicultural Research **32**(2): 80-88.

Ameline C., A. Beaurepaire, F. Ory, M. de La Harpe, B. Dainat, et al. (2023) Differential resistance across paternal genotypes of honey bee brood to the pathogenic bacterium *Melissococcus plutonius*. Journal of Applied Entomology **147**(1): 85-93.

Anderson R.M., R.M. May. (1982) Coevolution of hosts and parasites. Parasitology **85**(2): 411-426.

Antúnez K., M. Anido, D. Arredondo, J.D. Evans, P. Zunino. (2011) *Paenibacillus larvae* enolase as a virulence factor in honeybee larvae infection. Veterinary Microbiology **147**(1): 83-89.

Arai R., T. Miyoshi-Akiyama, K. Okumura, Y. Morinaga, M. Wu, et al. (2014) Development of duplex PCR assay for detection and differentiation of typical and atypical *Melissococcus plutonius* strains. Journal of Veterinary Medical Science **76**(4): 491-498.

Arai R., K. Tominaga, M. Wu, M. Okura, K. Ito, et al. (2012) Diversity of *Melissococcus plutonius* from honeybee larvae in Japan and experimental

reproduction of European foulbrood with cultured atypical Isolates. PloS one **7**(3): e33708.

Aupinel P., D. Fortini, H. Dufour, J.N. Tasei, B. Michaud, et al. (2005) Improvement of artificial feeding in a standard *in vitro* method for rearing *Apis mellifera* larvae. Bulletin of Insectology **58**(2): 107-111.

Ayres J.S., D.S. Schneider. (2008) A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections. PLoS Biology **6**(12): e305.

Bailey L. (1957) European foul brood: A disease of the larval honeybee (*Apis mellifera* L.) caused by a combination of *Streptococcus pluton* (*Bacillus pluton* White) and *Bacterium eurydice* White. Nature **180**: 1214-1215.

Bailey L. (1957) The isolation and cultural characteristics of *Streptococcus pluton* and further observations on *Bacterium eurydice*. Microbiology **17**(1): 39-48.

Bailey L. (1963) The pathogenicity for honey-bee larvae of microorganisms associated with European foulbrood. Journal of Insect Pathology **5**(2): 198-205.

Bailey L. (1983) *Melissococcus pluton*, the cause of European foulbrood of honey bees (*Apis* spp.). Journal of Applied Bacteriology **55**(1): 65-69.

Bailey L., B.V. Ball. (1991) Honey bee pathology. Harcourt Brace Jovanovich, Sidcup, Kent.

Bailey L., M.D. Collins. (1982) Reclassification of '*Streptococcus pluton*'(White) in a new genus *Melissococcus*, as *Melissococcus pluton* nom. rev.; comb. nov. Journal of Applied Bacteriology **53**(2): 215-217.

Bailey L., A.J. Gibbs. (1962) Cultural characters of *Streptococcus pluton* and its differentiation from associated Enterococci. Microbiology **28**(3): 385-391.

Bailey L., N. Locher. (1968) Experiments on the etiology of European foul brood of the honeybee. Journal of Apicultural Research **7**(2): 103-107.

Bakshi C.S., V.P. Singh, M. Malik, R.K. Singh, B. Sharma. (2003) 55 kb plasmid and virulence-associated genes are positively correlated with *Salmonella enteritidis* pathogenicity in mice and chickens. Veterinary Research Communications **27**(6): 425-432.

Baldwin T., A. Sakthianandeswaren, J.M. Curtis, B. Kumar, G.K. Smyth, et al. (2007) Wound healing response is a major contributor to the severity of cutaneous Leishmaniasis in the ear model of infection. Parasite Immunology **29**(10): 501-513.

Bamrick J.F., W.C. Rothenbuhler. (1961) Resistance to American foulbrood in honey bees. 4. Relationship between larval age and inoculation and mortality in a resistant and in a susceptible line. Journal of Insect Pathology **3**(4): 381-&.

Behrens D., E. Forsgren, I. Fries, R.F.A. Moritz. (2010) Lethal infection thresholds of *Paenibacillus larvae* for honeybee drone and worker larvae (*Apis mellifera*). Environmental Microbiology **12**(10): 2838-2845.

Belloy L., A. Imdorf, I. Fries, E. Forsgren, H. Berthoud, et al. (2007) Spatial distribution of *Melissococcus plutonius* in adult honey bees collected from apiaries and colonies with and without symptoms of European foulbrood. Apidologie **38**(2): 136-140.

Ben-Dov E., S. Boussiba, A. Zaritsky. (1995) Mosquito larvicidal activity of *Escherichia coli* with combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. Journal of Bacteriology **177**(10): 2851-2857.

Bertholf L.M. (1927) The utilization of carbohydrates as food by honeybee larvae. Journal of Agricultural Research **35**: 429-452.

Berthoud H., A. Imdorf, M. Haueter, S. Radloff, P. Neumann. (2010) Virus infections and winter losses of honey bee colonies (*Apis mellifera*). Journal of Apicultural Research **49**(1): 60-65.

Betti M.I., L.M. Wahl, M. Zamir. (2014) Effects of infection on honey bee population dynamics: A model. PloS one **9**(10): e110237.

Bigio G., H. Al Toufailia, W.O.H. Hughes, F.L.W. Ratnieks. (2014) The effect of one generation of controlled mating on the expression of hygienic behaviour in honey bees. Journal of Apicultural Research **53**(5): 563-568.

Blacquière T., D. Panziera. (2018) A plea for use of honey bees' natural resilience in bekeeping. Bee World **95**(2): 34-38.

Blaser M.J., D.L. Cohn. (1986) Opportunistic infections in patients with AIDS: Clues to the epidemiology of AIDS and the relative virulence of pathogens. Reviews of Infectious Diseases **8**(1): 21-30.

Boncristiani H., J.D. Ellis, T. Bustamante, J. Graham, C. Jack, et al. (2020) World honey bee health: The global distribution of Western honey bee (*Apis mellifera* L.) pests and pathogens. Bee World **98**(1): 2-6.

Bourgeois A.L., T.E. Rinderer, H.A. Sylvester, B. Holloway, B.P. Oldroyd. (2012) Patterns of *Apis mellifera* infestation by *Nosema ceranae* support the parasite hypothesis for the evolution of extreme polyandry in eusocial insects. Apidologie **43**(5): 539-548.

Brodschneider R., G. Arnold, N. Hrassnigg, K. Crailsheim. (2012) Does patriline composition change over a honey bee queen's lifetime? Insects **3**(3): 857-869.

Brodschneider R., K. Crailsheim. (2010) Nutrition and health in honey bees. Apidologie **41**(3): 278-294.

Brødsgaard C.J., W. Ritter, H. Hansen. (1998) Response of *in vitro* reared honey bee larvae to various doses of *Paenibacillus larvae larvae* spores. Apidologie **29**(6): 569-578.

Brødsgaard C.J., W. Ritter, H. Hansen, H.F. Brødsgaard. (2000) Interactions among *Varroa jacobsoni* mites, acute paralysis virus, and *Paenibacillus larvae* larvae and their influence on mortality of larval honeybees *in vitro*. Apidologie **31**(4): 543-554.

Brosi B.J., K.S. Delaplane, M. Boots, J.C. de Roode. (2017) Ecological and evolutionary approaches to managing honeybee disease. Nature Ecology & Evolution **1**(9): 1250-1262.

Büchler R., S. Andonov, K. Bienefeld, C. Costa, F. Hatjina, et al. (2013) Standard methods for rearing and selection of *Apis mellifera* queens. Journal of Apicultural Research **52**(1): 1-30.

Budge G.E., B. Barrett, B. Jones, S. Pietravalle, G. Marris, et al. (2010) The occurrence of *Melissococcus plutonius* in healthy colonies of *Apis mellifera* and the efficacy of European foulbrood control measures. Journal of Invertebrate Pathology **105**(2): 164-170.

Budge G.E., M.D. Shirley, B. Jones, E. Quill, V. Tomkies, et al. (2014) Molecular epidemiology and population structure of the honey bee brood pathogen M*elissococcus plutonius*. The ISME journal **8**(8): 1588-1597.

Burri R. (1906) Bakteriologische Untersuchungen über die Faulbrut und Sauerbrut der Bienen.

Carroll A.C., A. Wong. (2018) Plasmid persistence: Costs, benefits, and the plasmid paradox. Canadian Journal of Microbiology **64**(5): 293-304.

Casadevall A., L.-a. Pirofski. (1999) Host-pathogen interactions: Redefining the basic concepts of virulence and pathogenicity. Infection and Immunity **67**(8): 3703-3713.

Casadevall A., L.-a. Pirofski. (2003) The damage-response framework of microbial pathogenesis. Nature Reviews Microbiology **1**(1): 17-24.

Charrière J.-D., S. Frese, P. Herren. (2018) Bienenhaltung in der Schweiz. Agroscope Transfer **250**: 1-24.

Cheshire F.R., W.W. Cheyne. (1885) The pathogenic history and history under cultivation of a new *Bacillus* (*B. alvei*), the cause of a disease of the hive bee hitherto known as foul brood. Journal of the Royal Microscopical Society **5**(4): 581-601.

Chow J.W., L.A. Thal, M.B. Perri, J.A. Vazquez, S.M. Donabedian, et al. (1993) Plasmid-associated hemolysin and aggregation substance production contribute to virulence in experimental enterococcal endocarditis. Antimicrobial Agents and Chemotherapy **37**(11): 2474-2477.

Conroy T.E., L. Holman. (2022) Social immunity in the honey bee: Do immune-challenged workers enter enforced or self-imposed exile? Behavioral Ecology and Sociobiology **76**(2): 32.

Cornman R.S., D.R. Tarpy, Y. Chen, L. Jeffreys, D. Lopez, et al. (2012) Pathogen webs in collapsing honey bee colonies. PloS one **7**(8): e43562.

Crailsheim K., R. Brodschneider, P. Aupinel, D. Behrens, E. Genersch, et al. (2013) Standard methods for artificial rearing of *Apis mellifera* larvae. Journal of Apicultural Research **52**(1): 1-16.

Cremer S., S.A.O. Armitage, P. Schmid-Hempel. (2007) Social immunity. Current biology **17**(16): R693-R702.

Cremer S., C.D. Pull, M.A. Fürst. (2018) Social immunity: Emergence and evolution of colony-level disease protection. Annual Review of Entomology **63**(1): 105-123.

Cusumano C.K., C.S. Hung, S.L. Chen, S.J. Hultgren. (2010) Virulence plasmid harbored by uropathogenic *Escherichia coli* functions in acute stages of pathogenesis. Infection and Immunity **78**(4): 1457-1467.

Dahle B. (2010) The role of *Varroa destructor* for honey bee colony losses in Norway. Journal of Apicultural Research **49**(1): 124-125.

Dahle B., H. Sørum, J. Weidemann. (2011) European foulbrood in Norway: How to deal with a major outbreak after 30 years absence, COLOSS Workshop: The Future of Brood Disease Research–Guidelines, Methods and Development, Copenhagen, Denmark, pp. 8.

Dahle B., S. Wilkins, J.-D. Charrière. (2014) European foulbrood (EFB) and American foulbrood (AFB) - distribution, status and control and management strategies throughout Europe, EURBEE, Murcia, Spain.

Dainat B., J.D. Evans, Y.P. Chen, L. Gauthier, P. Neumann. (2012) Predictive markers of honey bee colony collapse. PLoS one **7**(2): e32151.

Dainat B., D. Grossar, B. Ecoffey, C. Haldemann. (2018) Triplex real-time PCR method for the qualitative detection of European and American foulbrood in honeybee. Journal of Microbiological Methods **146**: 61-63.

De León-Door A.P., A. Romo-Chacón, C. Rios-Velasco, P.B. Zamudio-Flores, J. de Jesús Ornelas-Paz, et al. (2018) Prevalence, typing and phylogenetic analysis of *Melissococcus plutonius* strains from bee colonies of the State of Chihuahua, Mexico. Journal of Invertebrate pathology **159**: 71-77.

Decourtye A., C. Alaux, Y. Le Conte, M. Henry. (2019) Toward the protection of bees and pollination under global change: Present and future perspectives in a challenging applied science. Current opinion in insect science **35**: 123-131.

Delaplane K.S., S. Pietravalle, M.A. Brown, G.E. Budge. (2015) Honey bee colonies headed by hyperpolyandrous queens have improved brood rearing efficiency and lower infestation rates of parasitic *Varroa* mites. PloS one **10**(12): e0142985.

Desai S.D., R.W. Currie. (2015) Genetic diversity within honey bee colonies affects pathogen load and relative virus levels in honey bees, *Apis mellifera* L. Behavioral Ecology and Sociobiology **69**(9): 1527-1541.

Dietemann V., J. Pflugfelder, D. Anderson, J.-D. Charrière, N. Chejanovsky, et al. (2012) *Varroa destructor*: Research avenues towards sustainable control. Journal of Apicultural Research **51**(1): 125-132.

Dingle K.E., F.M. Colles, R. Ure, J.A. Wagenaar, B. Duim, et al. (2002) Molecular characterization of *Campylobacter jejuni* clones: A basis for epidemiologic investigation. Emerging infectious diseases **8**(9): 949.

Djordjevic S.P., L.A. Smith, W.A. Forbes, M.A. Hornitzky. (1999) Geographically diverse Australian isolates of *Melissococcus pluton* exhibit minimal genotypic diversity by restriction endonuclease analysis. FEMS Microbiology Letters **173**(2): 311-318.

Djukic M., E. Brzuszkiewicz, A. Fünfhaus, J. Voss, K. Gollnow, et al. (2014) How to kill the honey bee larva: Genomic potential and virulence mechanisms of *Paenibacillus larvae*. PLoS one **9**(3): e90914.

Djukic M., S. Erler, A. Leimbach, D. Grossar, J.-D. Charrière, et al. (2018) Comparative genomics and description of putative virulence factors of *Melissococcus plutonius*, the causative agent of European foulbrood disease in honey bees. Genes **9**(8): 419.

Ebert D., J.J. Bull. (2008) The evolution and expression of virulence, in: Stearns S.C. and Koella J.C. (Eds.), Evolution in Health and Disease, Oxford University Press.

Ellis J.D., J.D. Evans, J. Pettis. (2010) Colony losses, managed colony population decline, and Colony Collapse Disorder in the United States. Journal of Apicultural Research **49**(1): 134-136.

Ellis J.D., P.A. Munn. (2005) The worldwide health status of honey bees. Bee world **86**(4): 88-101.

Elso C.M., L.J. Roberts, G.K. Smyth, R.J. Thomson, T.M. Baldwin, et al. (2004) Leishmaniasis host response loci (lmr1–3) modify disease severity through a Th1/Th2-independent pathway. Genes & Immunity **5**(2): 93-100.

Erban T., O. Ledvinka, M. Kamler, B. Hortova, M. Nesvorna, et al. (2017) Bacterial community associated with worker honeybees (*Apis mellifera*) affected by European foulbrood. PeerJ **5**: e3816.

Erban T., J. Zitek, M. Bodrinova, P. Talacko, M. Bartos, et al. (2019) Comprehensive proteomic analysis of exoproteins expressed by ERIC I, II, III and IV *Paenibacillus larvae* genotypes reveals a wide range of virulence factors. Virulence **10**(1): 363-375.

Erler S., O. Lewkowski, A. Poehlein, E. Forsgren. (2018) The curious case of *Achromobacter eurydice*, a gram-variable pleomorphic bacterium associated with European foulbrood disease in honeybees. Microbial Ecology **75**(1): 1-6.

Estoup A., M. Solignac, J.-M. Cornuet. (1997) Precise assessment of the number of patrilines and of genetic relatedness in honeybee colonies. Proceedings of the Royal Society of London. Series B: Biological Sciences **258**(1351): 1-7.

Evans J.D., R.S. Schwarz. (2011) Bees brought to their knees: Microbes affecting honey bee health. Trends in Microbiology **19**(12): 614-620.

Evans J.D., M. Spivak. (2010) Socialized medicine: Individual and communal disease barriers in honey bees. Journal of Invertebrate Pathology **103**: S62-S72.

Evison S.E.F., G. Fazio, P. Chappell, K. Foley, A.B. Jensen, et al. (2013) Host–parasite genotypic interactions in the honey bee: the dynamics of diversity. Ecology and Evolution **3**(7): 2214-2222.

Ewald P.W. (1983) Host-parasite relations, vectors, and the evolution of disease severity. Annual Review of Ecology and Systematics **14**(1): 465-485.

Finlay B.B., S. Falkow. (1997) Common themes in microbial pathogenicity revisited. Microbiology and Molecular Biology Reviews **61**(2): 136-169.

Forsgren E. (2010) European foulbrood in honey bees. Journal of Invertebrate Pathology **103**: S5-S9.

Forsgren E., G.E. Budge, J.-D. Charrière, M.A. Hornitzky. (2013) Standard methods for European foulbrood research. Journal of Apicultural Research **52**(1): 1-14.

Forsgren E., A.C. Lundhagen, A. Imdorf, I. Fries. (2005) Distribution of *Melissococcus plutonius* in honeybee colonies with and without symptoms of European foulbrood. Microbial Ecology **50**: 369-374.

Francisco A.P., C. Vaz, P.T. Monteiro, J. Melo-Cristino, M. Ramirez, et al. (2012) PHYLOViZ: Phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics **13**: 1-10.

Franck P., H. Coussy, Y. Le Conte, M. Solignac, L. Garnery, et al. (1999) Microsatellite analysis of sperm admixture in honeybee. Insect Molecular Biology **8**(3): 419-421.

Freitas A.R., C. Novais, P. Ruiz-Garbajosa, T.M. Coque, L. Peixe. (2009) Dispersion of multidrug-resistant *Enterococcus faecium* isolates belonging to major clonal complexes in different Portuguese settings. Applied and Environmental Microbiology **75**(14): 4904-4908.

Fried B. (2011) Sauerbrut-Projekt Werdenberg – ein voller Erfolg. Schweizerische Bienen-Zeitung(6): 13-15.

Fries I., S. Camazine. (2001) Implications of horizontal and vertical pathogen transmission for honey bee epidemiology. Apidologie **32**(3): 199-214.

Frost L.S., R. Leplae, A.O. Summers, A. Toussaint. (2005) Mobile genetic elements: The agents of open source evolution. Nature Reviews Microbiology **3**(9): 722-732.

FSVO. (2015) Technische Weisungen über die Massnahmen im Seuchenfall von Sauerbrut (Europäische Faulbrut) bei Bienen, in: office F.F.S.a.V. (Ed.).

Fünfhaus A., L. Poppinga, E. Genersch. (2013) Identification and characterization of two novel toxins expressed by the lethal honey bee pathogen *Paenibacillus larvae*, the causative agent of American foulbrood. Environmental Microbiology **15**(11): 2951-2965.

Garcia-Gonzalez E., L. Poppinga, A. Fünfhaus, G. Hertlein, K. Hedtke, et al. (2014) *Paenibacillus larvae* chitin-degrading protein PlCBP49 is a key virulence factor in American foulbrood of honey bees. PLoS Pathogens **10**(7): e1004284.

Genersch E. (2010) American foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. Journal of Invertebrate Pathology **103**: S10-S19.

Genersch E. (2010) Honey bee pathology: Current threats to honey bees and beekeeping. Applied Microbiology and Biotechnology **87**(1): 87-97.

Genersch E., A. Ashiralieva, I. Fries. (2005) Strain- and genotype-specific differences in virulence of *Paenibacillus larvae* subsp. *larvae*, a bacterial pathogen causing American foulbrood disease in honeybees. Applied and Environmental Microbiology **71**(11): 7551-7555.

Genersch E., E. Forsgren, J. Pentikäinen, A. Ashiralieva, S. Rauch, et al. (2006) Reclassification of *Paenibacillus larvae* subsp. *pulvifaciens* and *Paenibacillus larvae* subsp. *larvae* as *Paenibacillus larvae* without subspecies differentiation. International Journal of Systematic and Evolutionary Microbiology **56**(3): 501-511.

Giersch T., I. Barchia, M. Hornitzky. (2010) Can fatty acids and oxytetracycline protect artificially raised larvae from developing European foulbrood? Apidologie **41**(2): 151-159.

Govan V.A., V. Brözel, M.H. Allsopp, S. Davison. (1998) A PCR detection method for rapid identification of *Melissococcus pluton* in honeybee larvae. Applied and Environmental Microbiology **64**(5): 1983-1985.

Grangier V., L. Belloy, J.-D. Charrière, M.G. Doherr, A. Fritsche, et al. (2015) Real-time PCR as a decision aid in the control of European foulbrood. Journal of Apicultural Research **54**(4): 366-372.

Gray A., N. Adjlane, A. Arab, A. Ballis, V. Brusbardis, et al. (2022) Honey bee colony loss rates in 37 countries using the COLOSS survey for winter 2019–2020: The combined effects of operation size, migration and queen replacement. Journal of Apicultural Research: 1-7.

Gray A., R. Brodschneider, N. Adjlane, A. Ballis, V. Brusbardis, et al. (2019) Loss rates of honey bee colonies during winter 2017/18 in 36 countries participating in the COLOSS survey, including effects of forage sources. Journal of Apicultural Research **58**(4): 479-485.

Grossar D., E. Haynes, G.E. Budge, M. Parejo, L. Gauthier, et al. (2023) Population genetic diversity and dynamics of the honey bee brood pathogen *Melissococcus plutonius* in a region with high prevalence. Journal of Invertebrate Pathology **196**: 107867.

Grossar D., V. Kilchenmann, E. Forsgren, J.-D. Charrière, L. Gauthier, et al. (2020) Putative determinants of virulence in *Melissococcus plutonius*, the bacterial agent causing European foulbrood in honey bees. Virulence **11**(1): 554-567.

Guichard M., B. Dainat, V. Dietemann. (2023) Prospects, challenges and perspectives in harnessing natural selection to solve the 'varroa problem' of honey bees. Evolutionary Applications **16**(3): 593-608.

Guzmán-Novoa E., L. Eccles, Y. Calvete, J. Mcgowan, P.G. Kelly, et al. (2010) *Varroa destructor* is the main culprit for the death and reduced populations of overwintered honey bee (*Apis mellifera*) colonies in Ontario, Canada. Apidologie **41**(4): 443-450.

Hall B.G., H. Acar, A. Nandipati, M. Barlow. (2013) Growth rates made easy. Molecular Biology and Evolution **31**(1): 232-238.

Haynes E., T. Helgason, J.P.W. Young, R. Thwaites, G.E. Budge. (2013) A typing scheme for the honeybee pathogen *Melissococcus plutonius* allows detection of disease transmission events and a study of the distribution of variants. Environmental Microbiology Reports **5**(4): 525-529.

Hendrikx P., M. Saussac, F. Meziani, S. Wendling, S. Franco, et al. (2015) Résabeilles : Résultats de deux campagnes de surveillance programmée de la mortalité des abeilles en France. Bulletin épidémiologique, santé animale et alimentation: 19-23.

Hernández López J., W. Schuehly, K. Crailsheim, U. Riessberger-Gallé. (2014) Trans-generational immune priming in honeybees. Proceedings of the Royal Society B: Biological Sciences **281**(1785): 20140454.

Higes M., R. Martín-Hernández, C. Botías, E. Garrido Bailón, A.V. González-Porto, et al. (2008) How natural infection by *Nosema ceranae* causes honeybee colony collapse. Environmental Microbiology **10**(10): 2659-2669.

Highfield A.C., A. El Nagar, L.C.M. Mackinder, M.-L.J.N. Laure, M.J. Hall, et al. (2009) Deformed wing virus implicated in overwintering honeybee colony losses. Applied and Environmental Microbiology **75**(22): 7212-7220.

Holm S. (1979) A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics **6**(2): 65-70.

Human H., R. Brodschneider, V. Dietemann, G. Dively, J.D. Ellis, et al. (2013) Miscellaneous standard methods for *Apis mellifera* research. Journal of Apicultural Research **52**(4): 1-53.

Invernizzi C., F. Peñagaricano, I.H. Tomasco. (2009) Intracolonial genetic variability in honeybee larval resistance to the chalkbrood and American foulbrood parasites. Insectes Sociaux **56**(3): 233-240.

Jensen A.B., B.V. Pedersen, J. Eilenberg. (2009) Differential susceptibility across honey bee colonies in larval chalkbrood resistance. Apidologie **40**: 524-534.

Jolley K.A., M.C.J. Maiden. (2010) BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics **11**: 1-11.

Kielmanowicz M.G., A. Inberg, I.M. Lerner, Y. Golani, N. Brown, et al. (2015) Prospective large-scale field study generates predictive model identifying major contributors to colony losses. PLoS Pathogens **11**(4): e1004816.

Killgore G., A. Thompson, S. Johnson, J. Brazier, E. Kuijper, et al. (2008) Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: Restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. Journal of Clinical Microbiology **46**(2): 431-437.

Kleijn D., R. Winfree, I. Bartomeus, L.G. Carvalheiro, M. Henry, et al. (2015) Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. Nature Communications **6**(1): 7414.

Klein A.-M., B.E. Vaissière, J.H. Cane, I. Steffan-Dewenter, S.A. Cunningham, et al. (2007) Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences **274**(1608): 303-313.

Knap P.W., A. Doeschl-Wilson. (2020) Why breed disease-resilient livestock, and how? Genetics Selection Evolution **52**(1): 60.

Krska D., R. Ravulapalli, R.J. Fieldhouse, M.R. Lugo, A.R. Merrill. (2015) C3 larvin toxin, an ADP-ribosyltransferase from *Paenibacillus larvae*. Journal of Biological Chemistry **290**(3): 1639-1653.

Kulhanek K., N. Steinhauer, K. Rennich, D.M. Caron, R.R. Sagili, et al. (2017) A national survey of managed honey bee 2015–2016 annual colony losses in the USA. Journal of Apicultural Research **56**(4): 328-340.

Kuraku S., C.M. Zmasek, O. Nishimura, K. Katoh. (2013) aLeaves facilitates on-demand exploration of metazoan gene family trees on MAFFT sequence alignment server with enhanced interactivity. Nucleic Acids Research **41**(W1): W22-W28.

Kurze C., J. Routtu, R.F.A. Moritz. (2016) Parasite resistance and tolerance in honeybees at the individual and social level. Zoology **119**(4): 290-297.

Leclercq G., F. Francis, N. Gengler, T. Blacquière. (2018) Bioassays to quantify hygienic behavior in Honey Bee (*Apis mellifera* L.) colonies: A review. Journal of Apicultural Research **57**(5): 663-673.

Leclercq G., B. Pannebakker, N. Gengler, B.K. Nguyen, F. Francis. (2017) Drawbacks and benefits of hygienic behavior in honey bees (*Apis mellifera* L.): A review. Journal of Apicultural Research **56**(4): 366-375.

Lee G.M., P.A. McGee, B.P. Oldroyd. (2013) Variable virulence among isolates of *Ascosphaera apis*: Testing the parasite–pathogen hypothesis for the evolution of polyandry in social insects. Naturwissenschaften **100**(3): 229-234.

Lewkowski O., S. Erler. (2019) Virulence of *Melissococcus plutonius* and secondary invaders associated with European foulbrood disease of the honey bee. MicrobiologyOpen **8**(3): e00649.

Liu Z., C. Chen, Q. Niu, W. Qi, C. Yuan, et al. (2016) Survey results of honey bee (*Apis mellifera*) colony losses in China (2010–2013). Journal of Apicultural Research **55**(1): 29-37.

Lively Curtis M. (2010) The effect of host genetic diversity on disease spread. The American Naturalist **175**(6): E149-E152.

Lowder B.V., C.M. Guinane, N.L. Ben Zakour, L.A. Weinert, A. Conway-Morris, et al. (2009) Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus*. Proceedings of the National Academy of Sciences **106**(46): 19545-19550.

Maassen A. (1907) Ueber die sogenannte Faulbrut der Honigbienen. Mitteilungen aus der Kaiserlichen biologischen Anstalt für Land- und Forstwirtschaft **4**: 51-53.

Madigan M.T., J.M. Martinko, J. Parker. (1997) Brock biology of microorganisms. Prentice hall, Upper Saddle River, NJ.

Maiden M.C.J., M.J.J. van Rensburg, J.E. Bray, S.G. Earle, S.A. Ford, et al. (2013) MLST revisited: The gene-by-gene approach to bacterial genomics. Nature Reviews Microbiology **11**(10): 728-736.

McClelland E.E., P. Bernhardt, A. Casadevall. (2006) Estimating the relative contributions of virulence factors for pathogenic microbes. Infection and Immunity **74**(3): 1500-1504.

McKee B.A., D.R. Goodman, A.M. Hornitzky. (2004) The transmission of European foulbrood (*Melissococcus plutonius*) to artificially reared honey bee larvae (*Apis mellifera*). Journal of Apicultural Research **43**(3): 93-100.

McMullan J.B., M.J.F. Brown. (2009) A qualitative model of mortality in honey bee (*Apis mellifera*) colonies infested with tracheal mites (*Acarapis woodi*). Experimental and Applied Acarology **47**: 225-234.

Medrzycki P., H. Giffard, P. Aupinel, L.P. Belzunces, M.-P. Chauzat, et al. (2013) Standard methods for toxicology research in *Apis mellifera*. Journal of Apicultural Research **52**(4): 1-60.

Miller J.R. (2010) Survival of mutations arising during invasions. Evolutionary Applications **3**(2): 109-121.

Miyagi T., C.Y.S. Peng, R.Y. Chuang, E.C. Mussen, M.S. Spivak, et al. (2000) Verification of oxytetracycline-resistant American foulbrood pathogen *Paenibacillus larvae* in the United States. Journal of Invertebrate Pathology **75**(1): 95-96.

Morimoto T., Y. Kojima, M. Yoshiyama, K. Kimura, B. Yang, et al. (2013) Molecular detection of protozoan parasites infecting *Apis mellifera* colonies in Japan. Environmental Microbiology Reports **5**(1): 74-77.

Moritz R.F.A., J. De Miranda, I. Fries, Y. Le Conte, P. Neumann, et al. (2010) Research strategies to improve honeybee health in Europe. Apidologie **41**(3): 227-242.

Mosca M., J. Bubnic, L. Giannetti, L. Fortugno, M. Pietropaoli, et al. (2023) Adoption of partial shook swarm in the integrated control of American and European foulbrood of honey bee (*Apis mellifera* L.). Agriculture **13**(2): 363.

Mosca M., L. Giannetti, A. Franco, M. Iurescia, M. Milito, et al. (2022) Impact of oxytetracycline on colonies: Preliminary results on residues and antibiotic resistance. Journal of Apicultural Science **66**(2): 159-170.

Mutinelli F. (2011) The spread of pathogens through trade in honey bees and their products (including queen bees and semen): Overview and recent developments. Revue Scientifique et Technique **30**(1): 257-271.

Nakamura K., K. Okumura, M. Harada, M. Okamoto, M. Okura, et al. (2020) Different impacts of pMP19 on the virulence of *Melissococcus plutonius* strains with different genetic backgrounds. Environmental Microbiology **22**(7): 2756-2770.

Nakamura K., K. Okumura, M. Harada, M. Okamoto, M. Okura, et al. (2021) Peritrophic matrix-degrading proteins are dispensable virulence factors in a virulent *Melissococcus plutonius* strain. Scientific Reports **11**(1): 8798.

Nakamura K., Y. Yamazaki, A. Shiraishi, S. Kobayashi, M. Harada, et al. (2016) Virulence differences among *Melissococcus plutonius* strains with different genetic backgrounds in *Apis mellifera* larvae under an improved experimental condition. Scientific Reports **6**(1): 33329.

Nascimento M., A. Sousa, M. Ramirez, A.P. Francisco, J.A. Carriço, et al. (2016) PHYLOViZ 2.0: Providing scalable data integration and visualization for multiple phylogenetic inference methods. Bioinformatics **33**(1): 128-129.

Nath R., H. Singh, S. Mukherjee. (2023) Insect pollinators decline: An emerging concern of Anthropocene epoch. Journal of Apicultural Research **62**(1): 23-38.

Nesvorna M., B. Sopko, M. Bodrinova, J. Zitek, M. Markovic, et al. (2021) A scientific note on the comparison of PCR based quantification methods of *Melissococcus plutonius* in honey bees. Journal of Apicultural Research **60**(2): 255-259.

Neumann P., N.L. Carreck. (2010) Honey bee colony losses. Journal of Apicultural Research **49**(1): 1-6.

North R.J., A.A. Izzo. (1993) Mycobacterial virulence. Virulent strains of *Mycobacteria tuberculosis* have faster *in vivo* doubling times and are better equipped to resist growth-inhibiting functions of macrophages in the presence and absence of specific immunity. Journal of Experimental Medicine **177**(6): 1723-1733.

Ochman H., J.G. Lawrence, E.A. Groisman. (2000) Lateral gene transfer and the nature of bacterial innovation. Nature **405**(6784): 299–304.

OIE) W.f. (2020) World Animal Health information database (WAHIS interface) - version 1, in: Health W.O.f.A. (Ed.), https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home

Okumura K., R. Arai, M. Okura, T. Kirikae, D. Takamatsu, et al. (2011) Complete genome sequence of *Melissococcus plutonius* ATCC 35311. Journal of Bacteriology **193**(15): 4029-4030.

Okumura K., R. Arai, M. Okura, T. Kirikae, D. Takamatsu, et al. (2012) Complete genome sequence of *Melissococcus plutonius* DAT561, a strain that shows an unusual growth profile and is representative of an endemic cluster in Japan. Journal of Bacteriology **194**(11): 3014-3014.

Okumura K., D. Takamatsu, M. Okura. (2018) Complete genome sequence of *Melissococcus plutonius* DAT561, a strain that shows an unusual growth profile, obtained by PacBio sequencing. Genome Announcements **6**(23): e00431-00418.

Ory F., V. Duchemin, V. Kilchenmann, J.-D. Charrière, B. Dainat, et al. (2022) Lack of evidence for trans-generational immune priming against the honey bee pathogen *Melissococcus plutonius*. PLoS one **17**(5): e0268142.

Page P., Z. Lin, N. Buawangpong, H. Zheng, F. Hu, et al. (2016) Social apoptosis in honey bee superorganisms. Scientific Reports **6**(1): 27210.

Pallen M.J., B.W. Wren. (2007) Bacterial pathogenomics. Nature **449**(7164): 835-842.

Palmer K.A., B.P. Oldroyd. (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**(6): 265-268.

Paxton R.J. (2010) Does infection by *Nosema ceranae* cause "Colony Collapse Disorder" in honey bees (*Apis mellifera*)? Journal of Apicultural Research **49**(1): 80-84.

Peña-Chora G., E. Toledo-Hernández, C. Sotelo-Leyva, P. Damian-Blanco, A.G. Villanueva-Flores, et al. (2023) Presence and distribution of pests and diseases of *Apis mellifera* (Hymenoptera: *Apidae*) in Mexico: A review. The European Zoological Journal **90**(1): 224-236.

Peng Y.-S.C., E. Mussen, A. Fong, M.A. Montague, T. Tyler. (1992) Effects of chlortetracycline of honey bee worker larvae reared *in vitro*. Journal of Invertebrate Pathology **60**(2): 127-133.

Pérez-Sato J.A., N. Châline, S.J. Martin, W.O.H. Hughes, F.L.W. Ratnieks. (2009) Multi-level selection for hygienic behaviour in honeybees. Heredity **102**(6): 609-615.

Petit L., E. Maier, M. Gibert, M.R. Popoff, R. Benz. (2001) *Clostridium perfringens* Epsilon toxin induces a rapid change of cell membrane permeability to ions and forms channels in artificial lipid bilayers. Journal of Biological Chemistry **276**(19): 15736-15740.

Petzoldt T. (2016) Growthrates: Estimate growth rates from experimental data

Pietropaoli M., E. Carpana, M. Milito, M. Palazzetti, M. Guarducci, et al. (2022) Use of *Lactobacillus plantarum* in preventing clinical cases of American and European foulbrood in central Italy. Applied Sciences **12**(3): 1388.

Pinnock D.E., N.E. Featherstone. (1984) Detection and quantification of *Melissococcus pluton* infection in honeybee colonies by means of enzyme-linked immunosorbent assay. Journal of Apicultural Research **23**(3): 168-170.

Pirofski L.-a., A. Casadevall. (2012) Q&A: What is a pathogen? A question that begs the point. BMC biology **10**(1): 6.

Potts S.G., J.C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, et al. (2010) Global pollinator declines: Trends, impacts and drivers. Trends in Ecology & Evolution **25**(6): 345-353.

Råberg L., A.L. Graham, A.F. Read. (2008) Decomposing health: Tolerance and resistance to parasites in animals. Philosophical Transactions of the Royal Society B: Biological Sciences **364**(1513): 37-49.

Rankin D.J., E.P.C. Rocha, S.P. Brown. (2011) What traits are carried on mobile genetic elements, and why? Heredity **106**(1): 1-10.

Rauch S., A. Ashiralieva, K. Hedtke, E. Genersch. (2009) Negative correlation between individual-insect-level virulence and colony-level virulence of *Paenibacillus larvae*, the etiological agent of American foulbrood of honeybees. Applied and Environmental Microbiology **75**(10): 3344-3347.

Ravoet J., J. Maharramov, I. Meeus, L. De Smet, T. Wenseleers, et al. (2013) Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. PLoS one **8**(8): e72443.

Rembold H., J.-P. Kremer, G.M. Ulrich. (1980) Characterization of postembryonic developmental stages of the female castes of the honey bee, *Apis mellifera* L. Apidologie **11**(1): 29-38.

Rembold H., B. Lackner. (1981) Rearing of honeybee larvae *in vitro*: Effect of yeast extract on queen differentiation. Journal of Apicultural Research **20**(3): 165-171.

Roetschi A., H. Berthoud, R. Kuhn, A. Imdorf. (2008) Infection rate based on quantitative real-time PCR of *Melissococcus plutonius*, the causal agent of European foulbrood, in honeybee colonies before and after apiary sanitation. Apidologie **39**(3): 362-371.

Roffet-Salque M., M. Regert, R.P. Evershed, A.K. Outram, L.J.E. Cramp, et al. (2015) Widespread exploitation of the honeybee by early Neolithic farmers. Nature **527**(7577): 226-230.

Ruiz-Garbajosa P., M.J.M. Bonten, D.A. Robinson, J. Top, S.R. Nallapareddy, et al. (2006) Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. Journal of Clinical Microbiology **44**(6): 2220-2228.

Saraithong P., Y. Li, K. Saenphet, Z. Chen, P. Chantawannakul. (2015) Bacterial community structure in *Apis florea* larvae analyzed by denaturing gradient gel electrophoresis and 16S rRNA gene sequencing. Insect Science **22**(5): 606-618.

Schmid-Hempel P. (2014) Evolutionary parasitology: The integrated study of infections, immunology, ecology, and genetics. Oxford University Press, New York.

Seeley T.D., D.R. Tarpy. (2006) Queen promiscuity lowers disease within honeybee colonies. Proceedings of the Royal Society B: Biological Sciences **274**(1606): 67-72.

Simone-Finstrom M., M. Walz, D.R. Tarpy. (2016) Genetic diversity confers colony-level benefits due to individual immunity. Biology Letters **12**(3): 20151007.

Singh Rana B., K. Mohan Rao, S.K. Chakravarty, S. Katna. (2012) Characterization of *Melissococcus plutonius* causing European foulbrood disease in *Apis cerana* F. Journal of Apicultural Research **51**(4): 306-311.

Smith K.M., E.H. Loh, M.K. Rostal, C.M. Zambrana-Torrelio, L. Mendiola, et al. (2013) Pathogens, pests, and economics: Drivers of honey bee colony declines and losses. EcoHealth **10**(4): 434-445.

Smyth G., Y. Hu, P. Dunn, B. Phipson, Y. Chen. (2015) Statmod: Statistical modeling.

Soper D.M., A.K.E. Ekroth, M.J.F. Martins. (2021) Direct evidence for increased disease resistance in polyandrous broods exists only in eusocial Hymenoptera. BMC Ecology and Evolution **21**(1): 189.

Sperandio G., A. Simonetto, E. Carnesecchi, C. Costa, F. Hatjina, et al. (2019) Beekeeping and honey bee colony health: A review and conceptualization of beekeeping management practices implemented in Europe. Science of the Total Environment **696**: 133795.

Spivak M., R.G. Danka. (2021) Perspectives on hygienic behavior in *Apis mellifera* and other social insects. Apidologie **52**(1): 1-16.

Spivak M., M. Gilliam. (1998) Hygienic behaviour of honey bees and its application for control of brood diseases and varroa. Bee World **79**(4): 169-186.

Spivak M., G. Reuter, S. (1998) Performance of hygienic honey bee colonies in a commercial apiary. Apidologie **29**(3): 291-302.

Spivak M., G.S. Reuter. (2001) Resistance to American foulbrood disease by honey bee colonies *Apis mellifera* bred for hygienic behavior. Apidologie **32**(6): 555-565.

Steinhauer N., K. Kulhanek, K. Antúnez, H. Human, P. Chantawannakul, et al. (2018) Drivers of colony losses. Current Opinion in Insect Science **26**: 142-148.

Takai S., T. Sekizaki, T. Ozawa, T. Sugawara, Y. Watanabe, et al. (1991) Association between a large plasmid and 15- to 17-kilodalton antigens in virulent *Rhodococcus equi*. Infection and Immunity **59**(11): 4056-4060.

Takamatsu D., R. Arai, T. Miyoshi-Akiyama, K. Okumura, M. Okura, et al. (2013) Identification of mutations involved in the requirement of potassium for growth of typical *Melissococcus plutonius* strains. Applied and Environmental Microbiology **79**(12): 3882-3886.

Takamatsu D., K. Morinishi, R. Arai, A. Sakamoto, M. Okura, et al. (2014) Typing of *Melissococcus plutonius* isolated from European and Japanese honeybees suggests spread of sequence types across borders and between different *Apis* species. Veterinary Microbiology **171**(1): 221-226.

Takamatsu D., K. Okumura, A. Tabata, M. Okamoto, M. Okura. (2020) Transcriptional regulator SpxA1a controls the resistance of the honey bee pathogen *Melissococcus plutonius* to the antimicrobial activity of royal jelly. Environmental Microbiology **22**(7): 2736-2755.

Takamatsu D., A. Osawa, K. Nakamura, M. Yoshiyama, M. Okura. (2017) High-level resistance of *Melissococcus plutonius* clonal complex 3 strains to antimicrobial activity of royal jelly. Environmental Microbiology Reports **9**(5): 562-570.

Takamatsu D., M. Sato, M. Yoshiyama. (2016) Infection of *Melissococcus plutonius* clonal complex 12 strain in European honeybee larvae is essentially confined to the digestive tract. Journal of Veterinary Medical Science **78**(1): 29-34.

Tamura K., M. Nei. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution **10**(3): 512-526.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution **28**(10): 2731-2739.

Tarpy D.R. (2003) Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proceedings of the Royal Society of London. Series B: Biological Sciences **270**(1510): 99-103.

Tarpy D.R., T.D. Seeley. (2006) Lower disease infections in honeybee (*Apis mellifera*) colonies headed by polyandrous vs monandrous queens. Naturwissenschaften **93**(4): 195-199.

Tarr H.L.A. (1938) Studies on european foul brood of bees: IV. On the attempted cultivation of *Bacillus pluton*, the susceptibility of individual larvae to inoculation with this organism and its localization within its host. Annals of Applied Biology **25**(4): 815-821.

The National Bee Unit. (2017) Foulbrood disease of honey bees and other common brood disorders, The Animal and Plant Health Agency, Sand Hutton, York, United Kingdom.

Thebeau J.M., D. Liebe, F. Masood, I.V. Kozii, C.D. Klein, et al. (2022) Investigation of *Melissococcus plutonius* isolates from 3 outbreaks of European foulbrood disease in commercial beekeeping operations in western Canada. Canadian Veterinary Journal **63**(9): 935-942.

Therneau T. (2015) A package for survival analysis in S.

Tomkies V., J. Flint, G. Johnson, R. Waite, S. Wilkins, et al. (2009) Development and validation of a novel field test kit for European foulbrood. Apidologie **40**(1): 63-72.

Vale P., L. McNally, A. Doeschl-Wilson, K. King, R. Popat, et al. (2016) Beyond killing: Can we find new ways to manage infections? Evolution, Medicine, and Public Health(1): 148-157.

van Alphen J.J.M., B.J. Fernhout. (2020) Natural selection, selective breeding, and the evolution of resistance of honeybees (*Apis mellifera*) against *Varroa*. Zoological Letters **6**(1): 6.

Vanbergen A.J. (2013) Threats to an ecosystem service: Pressures on pollinators. Frontiers in Ecology and the Environment **11**(5): 251-259.

Vandenberg J.D., H. Shimanuki. (1987) Technique for rearing worker honeybees in the laboratory. Journal of Apicultural Research **26**(2): 90-97.

VanEngelsdorp D., M.D. Meixner. (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology **103**: S80-S95.

Vásquez A., E. Forsgren, I. Fries, R.J. Paxton, E. Flaberg, et al. (2012) Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. PLoS one **7**(3): e33188.

Velich A.V. (1930) Entwicklungsmechanische Studien an Bienenlarven. Zeitschrift für wissenschaftliche Zoologie **136**: 210-222.

Von Büren R.S., B. Oehen, N.J. Kuhn, S. Erler. (2019) High-resolution maps of Swiss apiaries and their applicability to study spatial distribution of bacterial honey bee brood diseases. PeerJ **7**: e6393.

Von Rhein W. (1933) Über die Entstehung des weiblichen Dimorphismus im Bienenstaate. Wilhelm Roux' Archiv für Entwicklungsmechanik der Organismen **129**(4): 601-665.

Wagoner K.M., M. Spivak, O. Rueppell. (2018) Brood affects hygienic behavior in the honey bee (Hymenoptera: Apidae). Journal of Economic Entomology **111**(6): 2520-2530.

Waite R., M. Brown, H. Thompson. (2003) Hygienic behaviour in honey bees in the UK: A preliminary study. Bee World **84**(1): 19-26.

Waite R., S. Jackson, H. Thompson. (2003) Preliminary investigations into possible resistance to oxytetracycline in *Melissococcus plutonius*, a pathogen of honeybee larvae. Letters in Applied Microbiology **36**(1): 20-24.

Waite R.J., M.A. Brown, H.M. Thompson, M.H. Bew. (2003) Controlling European foulbrood with the shook swarm method and oxytetracycline in the UK. Apidologie **34**(6): 569-575.

White G.F. (1912) The cause of European foulbrood. US Department of Agriculture Bureau of Entomology, Circular **no. 157**.

Wiedenbeck J., F.M. Cohan. (2011) Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. FEMS Microbiology Reviews **35**(5): 957-976.

Wilkins S., M.A. Brown, A.G.S. Cuthbertson. (2007) The incidence of honey bee pests and diseases in England and Wales. Pest Management Science **63**(11): 1062-1068.

Wilson-Rich N., M. Spivak, N.H. Fefferman, P.T. Starks. (2008) Genetic, individual, and group facilitation of disease resistance in insect societies. Annual Review of Entomology **54**(1): 405-423.

Wu H.-J., A.H.J. Wang, M.P. Jennings. (2008) Discovery of virulence factors of pathogenic bacteria. Current Opinion in Chemical Biology **12**(1): 93-101.

Wu M., Y. Sugimura, K. Iwata, N. Takaya, D. Takamatsu, et al. (2014) Inhibitory effect of gut bacteria from the Japanese honey bee, *Apis cerana japonica*, against *Melissococcus plutonius*, the causal agent of European foulbrood disease. Journal of Insect Science **14**.

Ye J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, et al. (2012) Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics **13**(1): 134.

Zhang M., J. Gong, Y. Lin, P.F. Barnes. (1998) Growth of virulent and avirulent *Mycobacterium tuberculosis* strains in human macrophages. Infection and Immunity **66**(2): 794-799.