Received: 12 May 2022 | Revised: 25 August 2022 | Accepted: 25 September 2022

DOI: 10.1111/jen.13087

ORIGINAL CONTRIBUTION

JOURNAL OF APPLIED ENTOMOLOGY WILEY

Differential resistance across paternal genotypes of honey bee brood to the pathogenic bacterium *Melissococcus plutonius*

Camille Ameline[1] | Alexis Beaurepaire[2] | Florine Ory[1] | Marylaure de La Harpe[1] | Benjamin Dainat[1] | Vincent Dietemann[1,3]

[1]Swiss Bee Research Center, Agroscope, Bern, Switzerland

[2]Institute of Bee Health, University of Bern, Bern, Switzerland

[3]Department of Ecology and Evolution, Biophore, UNIL-Sorge, University of Lausanne, Lausanne, Switzerland

Correspondence
Camille Ameline, Swiss Bee Research Center, Agroscope, Schwarzenburgstrasse 161, 3003 Bern, Switzerland.
Email: cameline8@gmail.com

Present address
Camille Ameline, Evolutionary Biology group, Instituto Gulbenkian de Ciência, Oeiras, Portugal

Marylaure de La Harpe, Remote Sensing Laboratories - Spatial Genetics, Department of Geography, University of Zurich, Zurich, Switzerland

Abstract

Melissococcus plutonius is a pathogenic bacterium affecting immature stages of the western honey bee (*Apis mellifera*) and leads to European foulbrood (EFB) disease. Despite EFB outbreaks increasing in frequency in several countries in recent decades, there is little knowledge on the epidemiology of *M. plutonius* or on the defence mechanisms of honey bees against this pathogen. Mating of honey bee queens with multiple males (polyandry) can be such a mechanism, as it has been shown to be beneficial to colony health and fitness. It is hypothesized that a high level of polyandry was selected for in response to pathogen pressure to maximize the probability that at least some patrilines among nestmates in a colony possess a high degree of resistance to specific pathogens, ultimately protecting colonies against infections. We show that *M. plutonius* infection provokes differential mortality among patrilines of immature honey bee workers. Such differences indicate a genetic origin of resistance against this pathogen—supporting the polyandry hypothesis—and open up avenues to improve control of EFB disease via selective breeding.

KEYWORDS
Apis mellifera, brood survival, drone genotype, genetic diversity, *Melissococcus plutonius*, microsatellite analysis, patriline

1 | INTRODUCTION

The bacterium *Melissococcus plutonius* is the causative agent of European foulbrood (EFB), a disease affecting immature stages (i.e., brood) of honey bees (Forsgren, 2010). This gram-positive bacterium is transmitted by symptomless adult honey bees to young larvae, which become infected after ingesting the pathogen through contaminated food. The bacterium then multiplies in the larvae's gut, which can lead to their death. As a result, within-colony population dynamics are disturbed, to the point that colonies can collapse (Forsgren, 2010). Transmission of EFB between colonies and apiaries is high, leading to local and recurrent outbreaks of increasing frequency in several countries over the last decades (Belloy et al., 2007; Budge et al., 2014; Grossar et al., 2020). Thus, EFB poses a major threat to honey bee health and contributes, together with other biotic and abiotic factors, to recent non-sustainable increases in colony losses in many regions of the Northern hemisphere (Goulson et al., 2015; Neumann & Carreck, 2010).

To mitigate the impact of EFB disease, one option for beekeepers is to apply antibiotics, e.g. oxytetracycline hydrochloride. However, antibiotic treatment is not sustainable, as it may (i) remove the symptoms but not the causative agent of the disease (Thompson &

Benjamin Dainat and Vincent Dietemann contributed equally to the work.

Brown, 2001); (ii) lead to the accumulation of residues in hive products, such as honey and propolis (Thompson et al., 2006); (iii) alter honey bee microbiota (Raymann et al., 2017); and (iv) increase the likelihood that pathogen resistance evolves (Tian et al., 2012; Waite, Jackson, & Thompson, 2003). Furthermore, antibiotic treatment may not prevent the spread of the pathogen (Roetschi et al., 2008). Due to the aforementioned limitations, several countries prohibit the use of antibiotics to mitigate EFB (Grossar et al., 2020). A laborious alternative to antibiotics is the shook swarm method, consisting of transferring adult honey bees from symptomatic colonies to new wax combs and destroying the infected brood (Waite, Brown, et al., 2003; Wilkins et al., 2007). Given the absence of an effective, easy-to-implement and sustainable approach to combat EFB and the severity of its outbreaks, EFB is classified as a notifiable disease in many countries. Affected apiaries are submitted to strict sanitation measures, with the destruction of symptomatic colonies and monitoring of surrounding apiaries (Grossar et al., 2020). These costly measures highlight the need for the development of improved preventive or control measures against this disease. Exploiting natural mechanisms of honey bee immunity may offer a sustainable solution to combat EFB (Simone-Finstrom, 2017).

Honey bees possess natural resistance mechanisms against several pathogens, such as *Varroa destructor* (Locke, 2016), *Paenibacillus larvae* (Laidlaw & Page, 1984; Spivak & Reuter, 2001) and *Ascospharea apis* (Invernizzi et al., 2011), leading to varroosis, American foulbrood and chalkbrood diseases, respectively. Attempts to exploit these mechanisms by selective breeding have been undertaken to protect managed stock against these pathogens (Guarna et al., 2017; Guichard et al., 2020; Spivak & Reuter, 2001). Resistance of honey bee colonies against specific pathogens has been linked to the presence of patrilines possessing beneficial traits against these pathogens (Castelli et al., 2021; Invernizzi et al., 2009; Palmer & Oldroyd, 2003). The mating of queens with a dozen males on average leads to the presence of multiple patrilines in *Apis mellifera* (*A. mellifera*) colonies (Estoup et al., 1994; Hernández-García et al., 2009; Tarpy et al., 2004). Such a level of polyandry has been shown to drive diversity in various traits, such as foraging behaviours (Robinson & Page, 1989) and resistance to pathogens (van Baalen & Beekman, 2006). One of the conditions for polyandry to be beneficial is that pathogens are of high genetic diversity (van Baalen & Beekman, 2006). In this situation, pathogens are less likely to spread within genetically heterogenous colonies and damage them (Sherman et al., 1988). As *M. plutonius* is a genetically highly diverse pathogen (Grossar et al., 2020; Lewkowski & Erler, 2019), we can expect variation in host resistance to infection. Previous research reported that honey bee larvae survived after the inoculation of a high dosage of *M. plutonius* in colonies (McKee et al., 2015) and that offspring of distinct queens survived differentially (Lewkowski & Erler, 2019). There is, however, no strong evidence that genetic factors play a role in resistance to EFB (Forsgren, 2010; Lewkowski & Erler, 2019).

The aim of this study was to determine whether patrilines present in honey bee colonies varied in resistance to *M. plutonius* infection. We conducted in vitro brood rearing assays, inoculated honey bee worker larvae under controlled laboratory conditions and used microsatellite markers to identify their patrilines post hoc (Brodschneider et al., 2012; Estoup et al., 1994). We then assessed whether larvae of different patrilines differed in their ability to survive infection, an endpoint required to show an effective difference in resistance (van Baalen & Beekman, 2006). Unlike previous studies that showed differences in resistance to other pathogens among patrilines (Invernizzi et al., 2009), we used in vitro brood rearing. This method allowed for more precise control of the inoculum dose delivered to each individual and thus reduced the probability that the fate of honey bee larvae and pupae was due to infections of varying intensities (Invernizzi et al., 2009). Our results revealed differential survival across infected patrilines, indicating a genetic basis for resistance, which opens avenues for the breeding of honey bee lineages resistant to *M. plutonius*.

2 | METHODS

2.1 | Honey bee and bacterial material

We used larvae produced by two unrelated, naturally mated honey bee queens (Q12 and Q79), both slightly older than 1 year. We reared the larvae in vitro using standard methods (Aupinel et al., 2005; Grossar et al., 2020) and inoculated them with the highly virulent strain of *M. plutonius*, CH 49.3. This strain had been isolated from diseased colonies found in 2007 in Graubünden, Switzerland and induced about 80% brood mortality in its native host population (Grossar et al., 2020). We chose a highly virulent strain to apply strong pathogenic pressure to the brood and thus enable the detection of differences in survival.

To minimize the probability of genetic changes due to recultivation (Grossar et al., 2020), we collected an aliquot of the bacterium from the original 15% glycerol stock solution stored at −80°C and performed a single cultivation step before inoculation. We cultivated the bacterium on five plates with a solid basal medium composed of 20 g/L of agar, 10 g/L of yeast extract, 10 g/L of glucose, 10 g/L of starch, 0.25 g/L of L-cysteine and 1 M of KH_2PO_4 in distilled water, adjusted to pH 6.7 using 2.5 M of KOH and autoclaved at 115°C for 15 min (Forsgren et al., 2013). After incubation for 4 days at 36°C under anaerobic conditions consisting of a hermetic box with an anaerobic generator sachet and anaerobic indicator (GENbox anaer: bioMérieux), we confirmed culture purity by visual investigation of the colonies, as shown in Figure 4 of Forsgren et al. (2013). We also confirmed *M. plutonius* identity with MALDI-TOF mass spectrometry (data not shown). We then suspended the bacterial colonies from each of the five culture plates in 3 ml of liquid basal medium. This medium consisted of the same components as described above but without agar and with 10 g/L of saccharose instead of starch. To determine the number of viable bacteria in the inoculum administered to the larvae, we determined the bacterial concentration in the liquid medium by making 10-fold serial dilutions (10^{-4}–10^{-7}) and by

counting colony-forming units (CFUs) grown on Petri dishes under the cultivation conditions described above. We then adjusted the bacterial inocula to the desired bacterial concentration (see section: *Infection experiment*) using sterile saline suspension buffer (0.9% NaCl). We prepared the inocula immediately before feeding the larvae.

2.2 | Infection experiment

Figure 1 summarizes the experimental design. Additional images of the experimental steps and larval development stages are shown in Figure S1. We conducted eight biological replicates of inoculation of 12 larvae from each queen (96 per queen), resulting in a total sample size of 192 larvae. We performed inoculations between July and September 2019 at seven-day intervals to take the effect of seasonal variation in external conditions on brood survival into account. For this, we treated replicates as a random factor in the models (see section: *Survival analysis*). In parallel, and following the same experimental design except for bacteria inoculation, we used the same number of offspring ($n = 192$) from the two queens as a negative control to verify inoculation success.

For larval rearing until the emergence of the imago, we followed the methods of (Grossar et al., 2020). Briefly, to produce a brood to be reared in vitro, we obtained first instar larvae by placing the queens on empty wax combs in queen excluder cages for 24 h (cages placed on Day −3, removed on Day −2, Figure 1). Caging restricted the queens' egg-laying activity to empty combs, and the workers were able to move freely to care for the brood produced. Three days after uncaging the comb (Day 1), we transferred (i.e., grafted) newly hatched first instar larvae from worker cells on the comb to Nicotplast (Maisod, France) plastic cups placed in an incubator at 34.5°C and relative humidity of 75% or 95%, according to the larval developmental stage (Grossar et al., 2020). The stability of temperature and humidity conditions was verified at each feeding event using a data logger.

We fed larvae within 2 h of transfer to the plastic cups with 10 µl of a diet containing the 2×10^5 *M. plutonius* CFU inoculum (Ory et al., 2022). We fed control individuals with 10 µl of the same diet, including saline suspension bacteria-free buffer. Changes in diet composition during the larval development have been described elsewhere (Ory et al., 2022). We prepared this diet with royal jelly from 10 healthy *A. mellifera* colonies, harvested under sterile conditions and stored at −25°C before use (Ory et al., 2022). As a royal jelly and sugar diet can reduce the bacterial load due to their antibacterial properties (de La Harpe et al., 2022; Vezeteu et al., 2017), we plated 50 µl of this diet at three dilutions (10^{-2}, 10^{-3} and 10^{-4}) on solid medium within 2 h after larval feeding. This enabled quantification of the minimal bacterial load the larvae were exposed to. This load was measured after 4 days

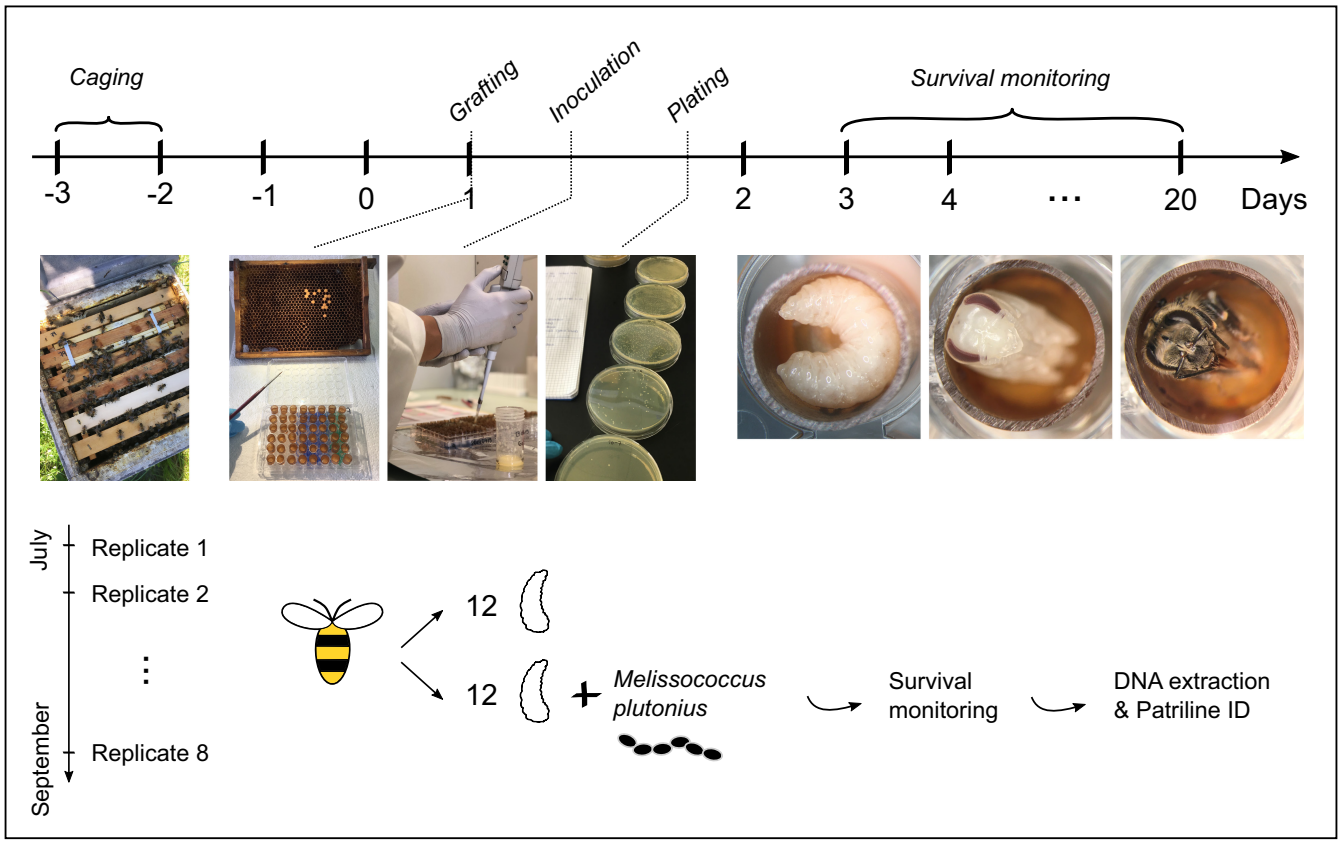

FIGURE 1 Experimental setup for the infection of honey bee larvae with *Melissococcus plutonius*. Brood stages of fifth larval instar, dark brown eyes pupa stage and pre-emergence adult worker are presented. [Colour figure can be viewed at wileyonlinelibrary.com]

of anaerobic incubation at 36°C. We checked for the absence of *M. plutonius* colonies in the control diet by plating 50 µl of this diet at 10^{-2} dilution.

We monitored brood survival from the larval stage on Day 3 until the imaginal stage on Day 20 (Figure 1, Figure S1). During this period, we observed all individuals daily under a binocular microscope and considered them dead when we did not detect respiration movement or reaction to a mechanical stimulus applied with a sterile rod. After metamorphosis, pupae are mostly immobile, and respiration or reactions to stimuli are difficult to observe. Hence, from Day 13 onwards, we recorded the time of death based on the developmental stage of the worker pupae, following (Rembold et al., 1980). We considered individuals dead on a particular day when their developmental stage was less advanced than that of uninfected controls on this day and when they did not develop further in the following days. We removed larvae and pupae once death was ascertained (i.e., at least 2 days after the day of considered death). After removal, we stored the inoculated dead individuals at −80°C for later genotyping. On Day 20, we stored all remaining live inoculated individuals until genotyping. As recommended in standard procedures (Crailsheim et al., 2013), we included a replicate in the analyses only if control mortality was less than 25%. We did not collect control individuals because they were not challenged with the pathogen.

2.3 | Microsatellite analysis

To identify patrilines of the inoculated larvae, we chose a set of six linked polymorphic microsatellite markers: SV240 (Moritz et al., 2008), At012 (Solignac et al., 2003), HB002, HB003, HB010 and HB012 (Lattorff et al., 2007) (see Information on markers: Table S1).

We extracted honey bee DNA using a Nucleospin 96 Tissue kit (Macherey-Nagel), following manufacturer instructions. We amplified microsatellite markers using fluorescently labelled primers in a PCR reaction using QIAGEN Multiplex PCR master mix (Qiagen) in an Applied BioSystems 2720 thermocycler. We provide primer and PCR information in Tables S1 and S2, respectively. We diluted the PCR products to one-third in water and added 1 µl of this diluted solution to 9.8 µl of Hi-Di Formamide and 0.2 µl of GeneScan 500 LIZ Size Standard (Applied BioSystems). We then determined amplicon sizes in an ABI GA3730 sequencer and analysed the data using PeakScanner v. 1.0 (Applied BioSystems).

2.4 | Patriline identification

From the offspring genotype data, we identified patrilines and matrilines (maternal haplotypes), i.e., for each marker, we determined which of the two alleles came from the father (drone) and the mother (queen). We defined a patriline as a specific combination of paternal genotypes at all markers. To reconstruct the patrilines, we utilized several elements of the general Mendelian rules of inheritance. We describe these methods in detail in Appendix S1.

To validate the identification of patrilines in the brood samples, we calculated the non-detection error (NDE) (Human et al., 2013). The NDE assesses the resolution of the markers used, as the probability of obtaining two identical genotypes in two individuals by chance. We then calculated the non-sampling error (NSE) to estimate the number of undetected patrilines due to insufficient sampling (Human et al., 2013).

2.5 | Survival analysis

We performed mixed effect Cox proportional-hazards regression modelling of brood survival data after inoculation with *M. plutonius*. Two models tested the effect of the fixed explanatory variables on brood survival: (i) inoculation of larvae with *M. plutonius* versus the non-inoculated controls and (ii) the patriline of the inoculated individuals. To obtain a sufficient sample size to test for the effect of the patriline on survival, we excluded patrilines represented by less than six offspring from our dataset (Figure S2). The models included two crossed random effects: queen identity and inoculation replicate. We implemented the full models as follows: survival ~ inoculation or patriline + (1|queen identity) + (1|replicate). In both models, we tested the effect of each random factor on survival using an Analysis of Variance (ANOVA) and removed non-significant factors from the final models. Mean values are presented with standard error (SE).

We used R v. 4.0.5 and RStudio v. 1.4.1717 (R Core Team, 2021) to perform all statistical analyses and draw all graphs. We subsequently edited the graphs in Inkscape v. 1.1 (https://inkscape.org/). We used the following R packages: package installation: devtools v. 2.4.1 (Wickham et al., 2019), data manipulation: tidyverse v. 1.3.1 (Wickham, 2017) (containing the ggplot2 graphics package), data.table v. 1.14.0 (Dowle & Srinivasan, 2021), naniar v. 0.6.0 (Tierney et al., 2020) and xlsx v. 0.6.5 (Dragulescu & Arendt, 2018). For graphs, we used randomcoloR v. 1.1.0.1 (Ammar, 2019), RColorBrewer v. 1.1–2 (Neuwirth, 2014), plotrix v. 3.8–1 (Lemon, 2006) and cowplot v. 1.1.1 (Wilke, 2019). For survival plots and linear models, we used survival v. 3.2–10 (Therneau, 2021; Therneau & Grambsch, 2000), survminer v. 0.4.9 (Kassambara et al., 2021) and coxme v. 2.2–16 (Therneau, 2020).

3 | RESULTS

3.1 | *Melissococcus plutonius* inoculum

From the initial dose of 2×10^5 *M. plutonius* CFU, we recovered, 2 h after feeding, mean (±SE) bacterial concentrations of $3.00\times10^4\pm1.55\times10^4$ CFU (range: 2.8×10^3 to 4.97×10^4) across the eight diets prepared.

3.2 | Effect of *M. plutonius* infection on brood survival

All the larval rearing assay replicates were included in the analysis, as none of the negative control groups experienced more than 25% brood mortality (mean ± SE: 13.5% ± 7.3% of the 96 offspring from each queen).

Queen identity had no significant effect on brood survival (ANOVA, $X^2 = 3.3$, df = 1, $p = 0.07$; Figures 2 and 3). However, the factor 'replicate' had a significant effect on brood survival (ANOVA, $X^2 = 14.0$, df = 1, $p = 0.0002$; Figure 3). Taking into account variations among the replicates, inoculation with *M. plutonius* had a significant negative effect on brood survival (mixed effects Cox model; $X^2 = 29.6$, df = 13, $p = 0.005$; Figure 3). For model details, see Table S3.

3.3 | Effect of patriline on brood survival

Non-detection error values ($NDE_{Q12} = 0.004$ and $NDE_{Q79} = 0.012$) indicated that the level of variation of the genetic markers was high enough to accurately differentiate patrilines. Non-sampling error values ($NSE_{Q12} = 0.76$ and $NSE_{Q79} = 0.08$, Figure S3) indicated a high probability that we had sampled all patrilines in the test colonies. There were 26 and 17 unique patrilines among the offspring from queen Q12 and queen Q79, respectively (Figure S2 and Table S4). No patrilines common to both queens were found among the offspring, and patrilines were homogeneously distributed across replicates (Figure S2). We retained for the subsequent analysis eight and six patrilines present in more than six individuals each, totalling $n = 70$ and $n = 69$ individuals for queen Q12 and Q79, respectively (Figure S2).

We found a significant effect of patriline on brood survival (mixed effect Cox model; $X^2 = 29.6$, df = 1, $p = 0.005$; Figure 4). In this model, brood survival varied significantly across replicates. Queen identity was excluded because it had no significant effect (ANOVAs, replicate: $X^2 = 5.5$, df = 1, $p = 0.02$; queen identity: $X^2 = 0.004$, df = 1, $p = 0.9$). Details on the model are given in Table S3. The survival probability of each patriline on Day 20 according to the model and the genotypes of all patrilines are presented in Table S4.

4 | DISCUSSION

Inoculation of honey bee larvae with the strain CH49.3 of *M. plutonius* led to similarly high levels of mortality in both colonies. However, the patrilines in both colonies differed significantly in the rate of survival after inoculation with a standardized number of bacteria.

The number of patrilines ($n_{Q12} = 26$ and $n_{Q72} = 17$) found among the queens' offspring was not biased by non-detection (NDE) nor by non-sampling errors (NSE) and corresponded to expectations based on previous studies (Estoup et al., 1994; Hernández-García et al., 2009; Tarpy et al., 2004). All patrilines were detected among the offspring throughout the experimental period (Figure S2), in line with the random mixing of sperm in the queen's spermatheca (Brodschneider et al., 2012; Estoup et al., 1994), and indicating a balanced experimental design. Thus, patriline imbalance could not explain the significant effect of the factor 'replicate' in our models.

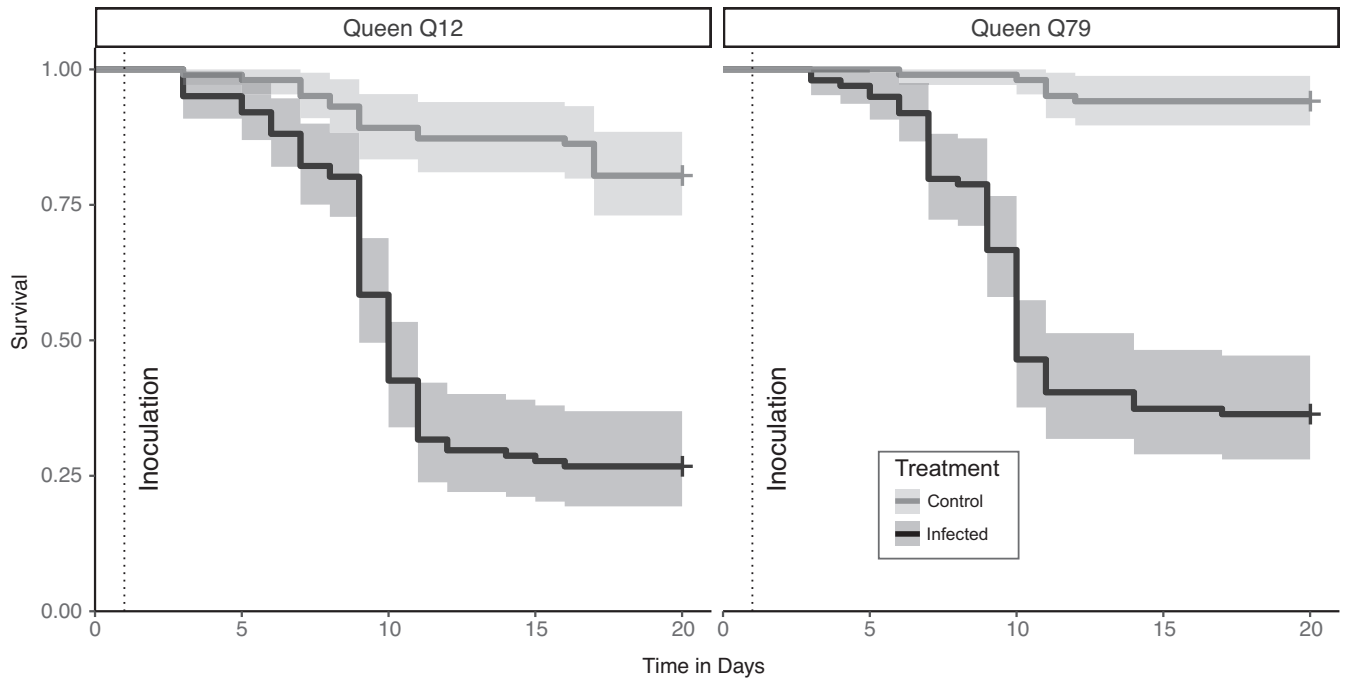

FIGURE 2 Survival of larvae infected with *Melissococcus plutonius* and control larvae. The grey area around the curve represents the 95% confidence interval.

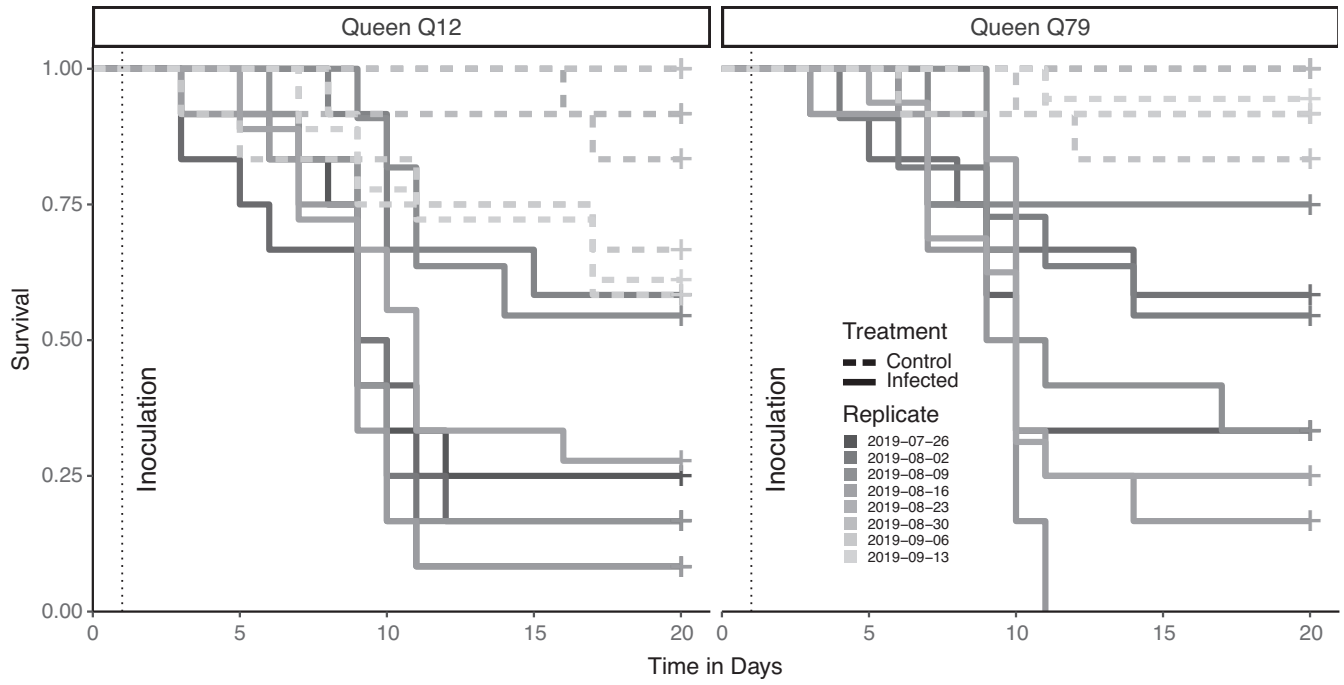

FIGURE 3 Survival of larvae infected with *Melissococcus plutonius* and control larvae for each replicate.

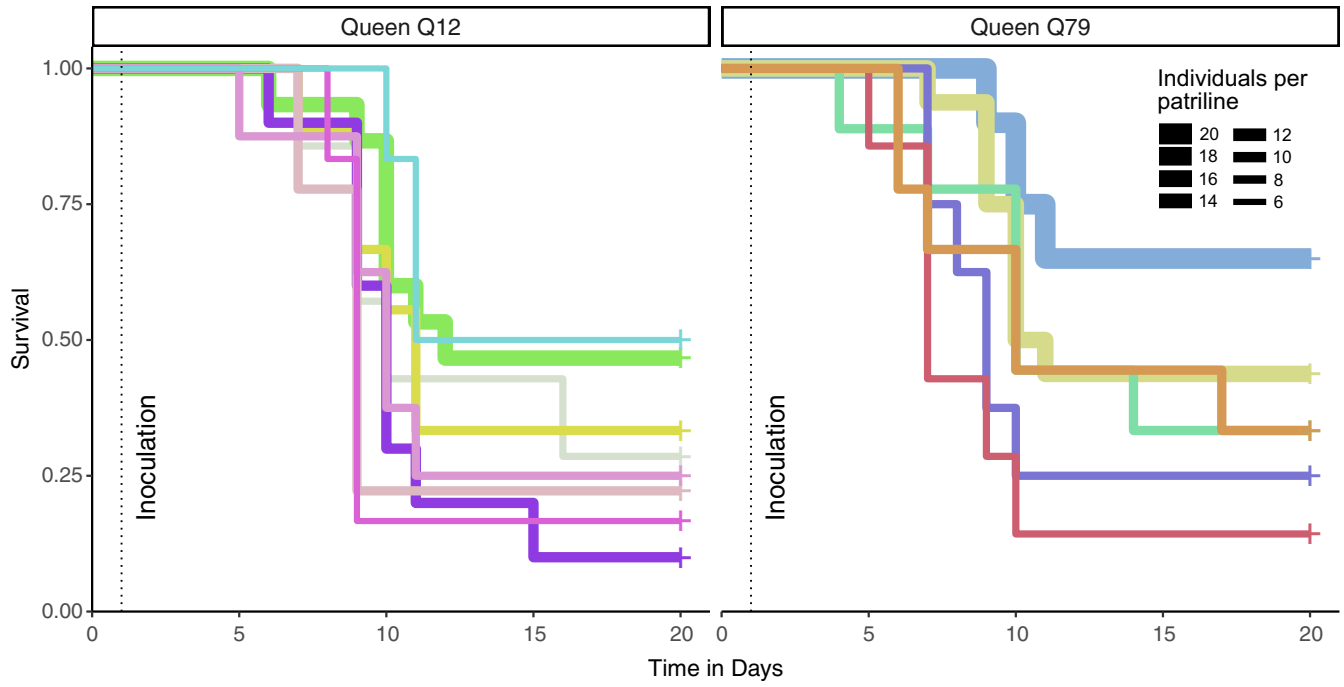

FIGURE 4 Survival of larvae infected with *Melissococcus plutonius* according to their patriline. Each colour represents a unique patriline. No patrilines were shared between the two colonies. Patrilines distribution and genotypes are presented in Figure S2 and Table S4. [Colour figure can be viewed at wileyonlinelibrary.com]

This effect might be due to variations in environmental conditions affecting brood viability and survival over the honey bee season; to which the larvae used in vitro rearing system had been exposed for up to 24 h before collection (Blaschon et al., 1999; Schmickl & Crailsheim, 2002). The lack of significance of queen identity effect on brood survival in the model using only inoculated individuals may be due to the smaller sample size in this analysis, as this factor marginally influenced brood survival in the model that included the complete dataset (i.e., inoculated and non-inoculated individuals; Table S3). With a larger sample size, queen identity might have been found to influence the survival of the *M. plutonius* infected brood, as it was found in a previous study using two colonies, in

which intracolony genetic variation was not tested (Lewkowski & Erler, 2019). Such a queen effect would suggest genetic resistance to the pathogen through the dam (maternal) pathway.

In our study, genetic variability in resistance to *M. plutonius* via the sire (paternal) pathway was clearly indicated, with observed differences in survival after infection with this pathogen among six and nine patrilines with at least six individuals. The bactericidal effect of the nutritive jelly—which the larvae ingest and contaminate themselves—makes it challenging to precisely standardize the number of bacteria across replicates. Despite this effect, our inoculates 2 h post-feeding were in a narrow range of doses ($3.00\times10^4\pm1.55\times10^4$ CFUs per larva). Variations in the inoculum dose could thus be excluded as the cause of the differences in survival between patrilines. The role of cell cleaning, hygienic or corpse removal behaviours by adult workers in the differences of survival among patrilines (Invernizzi et al., 2011; Spivak & Reuter, 2001) could also be excluded because our in vitro rearing assays were performed in the absence of adult workers.

The fact that the larvae from the observed patrilines differed in their ability to resist infections by *M. plutonius* suggests a paternally transmissible mechanism, which raises the prospect of breeding honey bee lineages resistant to this pathogen. Population genetics simulations and modelling studies have shown that controlled breeding of honey bees using high-quality drones is beneficial for colonies because it increases breeding success (Du et al., 2021; Plate et al., 2019). Such a medication-free approach is highly desirable, as there is currently no sustainable method in the beekeeping industry for combating EFB infection.

Using a powerful and standardized assay, we showed that infection with *M. plutonius* led to differential survival of larvae of distinct patrilines co-occurring in a honey bee colony. Differences among patrilines in their resistance to *M. plutonius* provide the opportunity to design selective breeding programs aimed at increasing the level of resistance of managed honey bee stock against this pathogen, which has a major negative impact on beekeeping in several countries. Moreover, the occurrence of differential resistance to *M. plutonius* across worker patrilines of the same colony indicates that honey bee pathogens may have contributed to selection for intracolonial genetic diversity and supports the pathogen hypothesis for the evolution of multiple mating in honey bees (Delaplane et al., 2015; Sherman et al., 1988; Simone-Finstrom et al., 2016; van Baalen & Beekman, 2006).

AUTHOR CONTRIBUTIONS

VD and BD designed the experiment. FO performed the infection experiment. MdLH performed DNA extraction. CA, AB and MdLH performed the microsatellite analysis. CA performed the data analysis, designed the figures and wrote the manuscript. VD, BD, MdLH and AB contributed to the data analysis. All authors reviewed the manuscript.

ACKNOWLEDGEMENT

We thank Matthieu Guichard for his help during the experiment, Cord Drögenmüller for constructive discussions and Nathalie Besuchet Schmutz for laboratory assistance and running the PCR reactions at the Institute of Genetics, University of Bern. CA and MdLH were supported by the BNF National Qualification Program of the University of Bern. We thank two anonymous reviewers for their constructive comments on the manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data underlying this article and the scripts of calculations and statistical analyses are available in the Figshare Repository at https://doi.org/10.6084/m9.figshare.19352411.v1.

ORCID

Camille Ameline https://orcid.org/0000-0001-6892-2819
Alexis Beaurepaire https://orcid.org/0000-0002-9832-5609
Florine Ory https://orcid.org/0000-0002-7460-7943
Marylaure de La Harpe https://orcid.org/0000-0002-8353-714X
Benjamin Dainat https://orcid.org/0000-0002-1740-7136
Vincent Dietemann https://orcid.org/0000-0002-0791-9939

REFERENCES

Ammar R. 2019. *randomcoloR: Generate attractive random colors*.
Aupinel, P., Fortini, D., Dufour, H., Tasei, J.-N., Michaud, B., Odoux, J.-F., & Pham-Delègue, M.-H. (2005). Improvement of artificial feeding in a standard in vitro method for rearing *Apis mellifera* larvae. *Bulletin of Insectology*, *58*, 107–111.
Belloy, L., Imdorf, A., Fries, I., Forsgren, E., Berthoud, H., Kuhn, R., & Charrière, J.-D. (2007). Spatial distribution of *Melissococcus plutonius* in adult honey bees collected from apiaries and colonies with and without symptoms of European foulbrood. *Apidologie*, *38*, 136–140.
Blaschon, B., Guttenberger, H., Hrassnigg, N., & Crailsheim, K. (1999). Impact of bad weather on the development of the broodnest and pollen stores in a honeybee colony (Hymenoptera: Apidae). *Entomologia Generalis*, *24*, 49–60.
Brodschneider, R., Arnold, G., Hrassnigg, N., & Crailsheim, K. (2012). Does patriline composition change over a honey bee queen's lifetime? *Insects*, *3*, 857–869.
Budge, G. E., Shirley, M. D. F., Jones, B., Quill, E., Tomkies, V., Feil, E. J., Brown, M. A., & Haynes, E. G. (2014). Molecular epidemiology and population structure of the honey bee brood pathogen *Melissococcus plutonius*. *The ISME Journal*, *8*, 1588–1597.
Castelli, L., Genchi García, M. L., Dalmon, A., Arredondo, D., Antúnez, K., Invernizzi, C., Reynaldi, F. J., Le Conte, Y., & Beaurepaire, A. (2021). Intra-colonial viral infections in western honey bees (*Apis mellifera*). *Microorganisms*, *9*(5), 1087.
Crailsheim, K., Brodschneider, R., Aupinel, P., Behrens, D., Genersch, E., Vollmann, J., & Riessberger-Gallé, U. (2013). Standard methods for artificial rearing of *Apis mellifera* larvae. In V. Dietemann, J. D. Ellis, & P. Neumann (Eds.), *The COLOSS BEEBOOK, volume I: Standard methods for Apis Mellifera research* (pp. 1–16). COLOSS. Journal of Apicultural Research, 52(1).
de La Harpe, M., Gütlin, A., Chiang, C., Dietemann, V., & Dainat, B. (2022). Influence of honey bee nutritive jelly type and dilution on its bactericidal effect on *Melissococcus plutonius*, the etiological agent of European foulbrood. *Microbial Ecology*, *9*, 1–7.
Delaplane, K. S., Pietravalle, S., Brown, M. A., & Budge, G. E. (2015). Honey bee colonies headed by hyperpolyandrous queens have

improved brood rearing efficiency and lower infestation rates of parasitic *varroa* mites. *PLoS One*, *10*, e0142985.

Dowle M, Srinivasan A. 2021. *data.table: Extension of 'data.frame'*.

Dragulescu AA, Arendt C. 2018. *xlsx: Read, write, format Excel 2007 and Excel 97/2000/XP/2003 files*.

Du, M., Bernstein, R., Hoppe, A., & Bienefeld, K. (2021). A theoretical derivation of response to selection with and without controlled mating in honeybees. *Genetics, Selection, Evolution*, *53*, 17.

Estoup, A., Solignac, M., & Cornuet, J.-M. (1994). 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*, 1–7.

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

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

Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*, 1255957.

Grossar, D., Kilchenmann, V., Forsgren, E., Charriere, 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*, 554–567.

Guarna, M. M., Hoover, S. E., Huxter, E., Higo, H., Moon, K.-M., Domanski, D., Bixby, M. E. F., Melathopoulos, A. P., Ibrahim, A., Peirson, M., Desai, S., Micholson, D., White, R., Borchers, C. H., Currie, R. W., Pernal, S. F., & Foster, L. J. (2017). Peptide biomarkers used for the selective breeding of a complex polygenic trait in honey bees. *Scientific Reports*, *7*, 8381.

Guichard, M., Dietemann, V., Neuditschko, M., & Dainat, B. (2020). Advances and perspectives in selecting resistance traits against the parasitic mite *varroa destructor* in honey bees. *Genetics, Selection, Evolution*, *52*, 71.

Hernández-García, R., De la Rúa, P., & Serrano, J. (2009). Mating frequency in *Apis mellifera iberiensis* queens. *Journal of Apicultural Research*, *48*, 121–125.

Human, H., Brodschneider, R., Dietemann, V., Dively, G., Ellis, J. D., Forsgren, E., Fries, I., Hatjina, F., Hu, F.-L., Jaffé, R., Jensen, A. B., Köhler, A., Magyar, J. P., Özkýrým, A., Pirk, C. W. W., Rose, R., Strauss, U., Tanner, G., Tarpy, D. R., … Zheng, H.-Q. (2013). Miscellaneous standard methods for *Apis mellifera* research. In V. Dietemann, J. D. Ellis, & P. Neumann (Eds.), *The COLOSS BEEBOOK, volume I: Standard methods for Apis mellifera research* (pp. 1–53). COLOSS. Journal of Apicultural Research, 52(4).

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

Invernizzi, C., Rivas, F., & Bettucci, L. (2011). Resistance to chalkbrood disease in *Apis mellifera* L. (Hymenoptera: Apidae) colonies with different hygienic behaviour. *Neotropical Entomology*, *40*, 28–34.

Kassambara A, Kosinski M, Biecek P. 2021. *survminer: Drawing survival curves using 'ggplot2'*.

Laidlaw, H. H., & Page, R. E. (1984). Polyandry in honey bees (*Apis mellifera* L.): Sperm utilization and intracolony genetic relationships. *Genetics*, *108*, 985–997.

Lattorff, H. M., Moritz, R. F., Crewe, R. M., & Solignac, M. (2007). Control of reproductive dominance by the *thelytoky* gene in honeybees. *Biology Letters*, *3*, 292–295.

Lemon, J. (2006). Plotrix: A package in the red light district of R. *R-News*, *6*, 8–12.

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

Locke, B. (2016). Natural *varroa* mite-surviving *Apis mellifera* honeybee populations. *Apidologie*, *47*, 467–482.

McKee, B. A., David Goodman, R., & Alan Hornitzky, M. (2015). The transmission of European foulbrood (*Melissococcus plutonius*) to artificially reared honey bee larvae (*Apis mellifera*). *Journal of Apicultural Research*, *43*, 93–100.

Moritz, R. F. A., Dietemann, V., & Crewe, R. (2008). Determining colony densities in wild honeybee populations (*Apis mellifera*) with linked microsatellite DNA markers. *Journal of Insect Conservation*, *12*, 455–459.

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

Neuwirth E. 2014. *RColorBrewer: ColorBrewer palettes*.

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

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

Plate, M., Bernstein, R., Hoppe, A., & Bienefeld, K. (2019). The importance of controlled mating in honeybee breeding. *Genetics, Selection, Evolution*, *51*, 74.

R Core Team. (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Raymann, K., Shaffer, Z., & Moran, N. A. (2017). Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLoS Biology*, *15*, e2001861.

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

Robinson, G. E., & Page, R. E. (1989). Genetic determination of nectar foraging, pollen foraging, and nest-site scouting in honey bee colonies. *Behavioral Ecology and Sociobiology*, *24*, 317–323.

Roetschi, A., Berthoud, H., Kuhn, R., & Imdorf, A. (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*, 362–371.

Schmickl, T., & Crailsheim, K. (2002). How honeybees (*Apis mellifera* L.) change their broodcare behaviour in response to non-foraging conditions and poor pollen conditions. *Behavioral Ecology and Sociobiology*, *51*, 415–425.

Sherman, P. W., Seeley, T. D., & Reeve, H. K. (1988). Parasites, pathogens, and polyandry in social hymenoptera. *The American Naturalist*, *131*, 602–610.

Simone-Finstrom, M. (2017). Social immunity and the superorganism: Behavioral defenses protecting honey bee colonies from pathogens and parasites. *Bee World*, *94*, 21–29.

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

Solignac, M., Vautrin, D., Loiseau, A., Mougel, F., Baudry, E., Estoup, A., Garnery, L., Haberl, M., & Cornuet, J.-M. (2003). Five hundred and fifty microsatellite markers for the study of the honeybee (*Apis mellifera* L.) genome. *Molecular Ecology Notes*, *3*, 307–311.

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

Tarpy, D. R., Nielsen, R., & Nielsen, D. I. (2004). A scientific note on the revised estimates of effective paternity frequency in *Apis*. *Insectes Sociaux*, *51*, 203–204.

Therneau T. 2020. *coxme: Mixed effects Cox models*.

Therneau T. 2021. *A package for survival analysis in R*.

Therneau, T., & Grambsch, P. (2000). *Modeling survival data: Extending the cox model*. Springer.

Thompson, H. M., & Brown, M. A. (2001). Is contact colony treatment with antibiotics an effective control for European foulbrood? *Bee World*, *82*, 130–138.

Thompson, H. M., Waite, R. J., Wilkins, S., Brown, M. A., Bigwood, T., Shaw, M., Ridgway, C., & Sharman, M. (2006). Effects of shook swarm and supplementary feeding on oxytetracycline levels in honey extracted from treated colonies. *Apidologie*, *37*, 51–57.

Tian, B., Fadhil, N. H., Powell, J. E., Kwong, W. K., & Moran, N. A. (2012). Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honeybees. *MBio*, *3*(6), e00377-12.

Tierney N, Cook D, McBain M, Fay C. 2020. *naniar: Data structures, summaries, and visualisations for missing data*.

van Baalen, M., & Beekman, M. (2006). The costs and benefits of genetic heterogeneity in resistance against parasites in social insects. *The American Naturalist*, *167*, 568–577.

Vezeteu, T. V., Bobiş, O., Moritz, R. F. A., & Buttstedt, A. (2017). Food to some, poison to others - Honeybee royal jelly and its growth inhibiting effect on European foulbrood bacteria. *MicrobiologyOpen*, *6*, e00397.

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

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

Wickham H. 2017. *tidyverse: Easily install and load the 'Tidyverse'*.

Wickham H, Hester J, Chang W. 2019. *devtools: Tools to make developing R packages easier*.

Wilke CO. 2019. *cowplot: Streamlined plot theme and plot annotations for 'ggplot2'*.

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

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ameline, C., Beaurepaire, A., Ory, F., de La Harpe, M., Dainat, B., & Dietemann, V. (2023). Differential resistance across paternal genotypes of honey bee brood to the pathogenic bacterium *Melissococcus plutonius*. *Journal of Applied Entomology*, *147*, 85–93. https://doi.org/10.1111/jen.13087