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Original Article

Polyandry and paternity affect disease resistance in eusocial wasps

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Polyandry (multiple mating by females) is a central challenge for understanding the evolution of eusociality. Several hypotheses have been proposed to explain its observed benefits in eusocial Hymenoptera, one of which, the parasite–pathogen hypothesis (PPH), posits that high genotypic variance among workers for disease resistance prevents catastrophic colony collapse. We tested the PPH in the polyandrous wasp *Vespula shidai*. We infected isolated workers with the entomopathogenic fungus *Beauveria bassiana* and quantified their survival in the laboratory. Additionally, we conducted a paternity analysis of the workers using nine microsatellite loci to investigate the relationship between survival and the matriline and patriline membership of the workers. As predicted by the PPH, nestmate workers of different patrilines showed differential resistance to *B. bassiana*. We also demonstrated variation in virulence among strains of *B. bassiana*. Our results are the first to directly support the PPH in eusocial wasps and suggest that similar evolutionary pressures drove the convergent origin and maintenance of polyandry in ants, bees, and wasps.

Key words: **disease resistance, patrilines, polyandry, social wasp, *Vespula*.**

INTRODUCTION

Explaining the adaptive significance of polyandry (multiple mating by females) in eusocial insects has been a central challenge in sociobiology for the past three decades (Crozier and Pamilo 1996). However, because polyandry causes dilution of kin relationships among nestmates, the phenomenon seems contradictory to kin selection theory because high intracolony relatedness would appear necessary for the evolution of eusociality (Crozier and Pamilo 1996; Queller and Strassmann 1998). Despite there being costs to female multiple mating (e.g., time and energy costs; Thornhill and Alcock 1983; predation risk; Arnqvist 1989; pathogen or virus transmission risk; Sherman et al. 1988; Amiri et al. 2016), polyandry and polygyny (the presence of multiple queens in a nest) have repeatedly evolved from ancestral monandry in eusocial insects, suggesting selective forces favoring genetic diversity among nestmates (Hughes et al. 2008). Large-scale phylogenetic studies have demonstrated that increased colony size is associated with greater paternity frequency and reduced paternity skew, both of which increase intracolony genetic diversity across species of eusocial Hymenoptera (Jaffé et al. 2012; Loope et al. 2014).

Several plausible hypotheses have been proposed to explain the evolution of multiple mating given these costs (e.g., parasite–pathogen hypothesis [PPH], facilitating division of labor hypothesis, and gaining sperm hypothesis; reviewed in Crozier and Fjerdingstad 2001). The PPH invokes the benefits of high intracolony genetic diversity to explain the evolution of polyandry in social insects (Sherman et al. 1988; Shykoff and Schmid-Hempel 1991). The PPH assumes that different matrilines or patrilines of workers within the colony have different susceptibility or resistance to a single pathogen genotype and also that different pathogen genotypes have different virulence to individuals of matrilines or patrilines (Sherman et al. 1988, 1998; Kraus and Page 1998). The PPH proposes that the increased intracolony genetic diversity arising from polyandry could prevent catastrophic colony collapse

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following challenge by parasites and pathogens, particularly if resistance to them among workers is variable depending on genotype (Sherman et al. 1988).

Social insects that live in groups of genetically homogeneous individuals are likely to be more vulnerable to parasites and pathogens in their nests than solitary species, given the high potential for transmission among frequently interacting nestmates. Negative fitness effects of diseases by fungi and viruses have been observed in various eusocial Hymenopterans (Schmid-Hempel 1998), including *Vespula* wasps (Dobelmann et al. 2017; Gruber et al. 2019). The PPH has been supported only in Apidae and Formicidae, where polyandry has originated independently (Baer and Schmid-Hempel 2003; Palmer and Oldroyd 2003; Hughes and Boomsma 2004; Seeley and Tarpy 2007). The workers of different patrilines within a colony and those of different colonies showed differing levels of disease resistance in *Apis mellifera* and the leaf-cutting ant *Acromyrmex echinator* (Palmer and Oldroyd 2003; Hughes and Boomsma 2004). In addition, genetic diversity within a colony has been positively associated with disease resistance in *A. mellifera* (Tarpy and Seeley 2006; Seeley and Tarpy 2007) and in the leaf-cutting ants (Hughes and Boomsma 2004).

Although both positive and negative effects of polyandry on populations have been discussed, the empirical data are insufficient (Holman and Kokko 2013). Significant positive correlations between the number of times a queen mates and the number of offspring produced have been reported in eusocial insects (Wiernasz et al. 2004; Goodisman et al. 2007; Mattila and Seeley 2007), but such findings do not reveal the mechanisms by which these benefits occur. In addition to providing pathogen resistance benefits, the high genetic diversity within the nest due to polyandry could improve the division of labor among workers or make colonies more capable of flexibly responding to changes in the environment, such as shifts in climate or prey or pathogen type, which could allow for the production of more offspring. In *Apis cerana*, the high frequency of multiple mating by the queen is thought to mitigate the loss of genetic diversity, known as the founder effect, during a recent introduction to Australia (Ding et al. 2017). Some other polyandrous eusocial insects are notorious as invasive species (e.g., *Vespa velutina*; Kishi and Goka 2017; *Vespula pensylvanica*; Hanna et al. 2014), possibly because the queen's multiple mating contributes to a stable expansion of the population following a genetic bottleneck.

Here, we conducted two experiments to test the PPH in a eusocial wasp in Japan. The first aimed to evaluate fungal virulence differences among colonies, and the second aimed to evaluate fungal virulence differences among patrilines, worker emergence date, and their interaction in a more detailed fashion. We challenged multiple patrilines and matrilines of *Vespula shidai* with multiple strains of the entomopathogenic filamentous fungus *Beauveria bassiana*. *Beauveria bassiana* infects a broad range of insect hosts, and one of its identifying features is the formation of its characteristic conidiospore balls (Hajek and Leger 1994; Schmid-Hempel 1998; Schmidt et al. 2011).

The nesting biology of *V. shidai* is similar to that of other *Vespula* (Matsuura 1995; Saga et al. 2017). A new colony is initiated by a single foundress queen that emerges from hibernation in spring. After hibernation, the queen founds the colony and produces workers and the colony grows through the summer, switching to the production of reproductives (males and new queens) from the beginning of fall to the beginning of winter. Mating occurs in the fall and, after the beginning of winter (early December in central Japan), only new queens hibernate and all workers and males die (Matsuura and Yamane 1990).

In addition, we investigated whether differences in emergence date, which could be associated with differences in the environment experienced as larvae, affected resistance to disease and whether workers that emerged on the same day showed any bias toward specific patrilines. Simultaneous emergence of workers from various patrilines would also increase the genetic diversity within the nest. In many organisms, the effects of epigenetics and phenotypic plasticity mean that even individuals of the same genotype exhibit variability in disease resistance due to differences in environmental factors (Pigliucci 2005). However, given the homeostasis common within advanced eusocial societies (Oldroyd and Fewell 2007), it seems likely that workers emerging contemporaneously would have experienced very similar environments as larvae (e.g., exposure to stresses from temperature, nutrition, or disease).

The PPH has not yet been directly tested in *Vespula*, a genus phylogenetically independent of Apidae and Formicidae. In this study, our aims were to first test the prediction that matrilines and patrilines differ in their resistance to particular strains of pathogen and, then, to determine if pathogen strains vary in their virulence to hosts of the same genotype, which would suggest the potential for classic coevolutionary host–pathogen dynamics (Schmid-Hempel and Ebert 2003). We also hoped to extend the sparse literature available (Barribeau et al. 2014) on variation in pathogen strain virulence to the same eusocial host genotype.

MATERIAL AND METHODS

Pathogens

We followed the experimental protocol by Okuno et al. (2012) and conducted a fungal inoculation experiment with some modifications. We used five fungal strains (A–E) of the entomopathogenic filamentous fungus *B. bassiana* for experiment 1 and, then, selected the two most lethal strains (A and C) for experiment 2. The fungal strains were isolated from five mummy queen larvae from different colonies of *V. shidai* collected in Nakatsugawa City, Gifu Prefecture, Japan, November 3, 2013. The strains were stored on Sabouraud agar medium (0.01 g/mL yeast extract, 0.01 g/mL bacto peptone, 0.02 g/mL L-glucose, 0.02 g/mL agar, and distilled water) in a refrigerator. The fungus was cultured on Sabouraud medium three times prior to the experiments. Conidiospore suspensions were prepared from sporulating culture plates (cultured at 25 °C, 8–14 days, 24-h light) in a 0.03% Tween 80 solution. The fungal spore suspension (30 mL) was quantified using a hemocytometer (Sigma, St. Louis, MO) and diluted to the required dosage for both experiments: low (10^6 spores/mL) and high (10^8 spores/mL).

Wasps and experiment setup

Experiment 1: interaction of matriline and pathogen strain

We collected about 400 workers from each of five colonies, on November 9, 2014 at Akechi Wasp Festival in Toyota City, Aichi prefecture, Japan (regarding wasp festivals, refer to Nonaka 2010). On the day of collection, the workers were confined within wire mesh cages, one cage per colony, and placed into a room at constant temperature (25 °C with a 15:9 h light:dark photoperiod). A 30% honey solution was provided daily for 1 week from the collection date, and the workers remaining alive were used in experiment 1. The workers were placed in a freezer at −20 °C for 20 s

to anesthetize them just before conidiospore infection. Individual workers were then completely submerged for 1 s either into the high dose fungal spore solution (10^8 spores/mL, 30 mL) or into sterile water (30 mL; control). Excess fluid on the workers' body surface was absorbed using filter paper. Each worker was then housed singly in a plastic box (3 × 8 × 10 cm) lined with filter paper and provided with 500 µL water daily. Their survival was checked every 24 h for 7 days. If a worker died, we moisturized the filter paper in the box with water to maintain humidity at 90% for two more weeks. We attributed death to *B. bassiana* where whitish hyphae, a typical feature of the fungus, were seen growing from the corpse.

Experiment 2: comparison of strain virulence between different worker patrilines and emergence dates

We collected three colonies in Nakatsugawa City, Gifu prefecture, Japan, in the middle of July 2015 using traditional methods for wasp hunting and keeping (Saga 2019) and labeled them TK, TG, and KM. We placed each colony in a wooden box (30 × 30 × 60 cm) and provided the colonies with meat (purchased chicken heart) and a 30% honey solution, permitting free outdoor foraging within the original collection areas until October 11, 2015. On this day, we collected combs filled with larvae and pupae from each colony, put the combs in a plastic box with a mesh roof, and set the boxes in a room at constant temperature (25 °C with a 15:9 h light:dark photoperiod). Every 12 h, we transferred newly emerged workers to individual plastic boxes (3 × 8 × 10 cm) lined with filter paper. We conducted experiment 2 using the workers 12–24 h postemergence, administering the low concentration of fungal spore suspension (10^6 spores/mL; 30 mL) of either strain A or strain C, and using the same infection protocol as in experiment 1, including controls. Strains A and C were used because their apparent virulence differed most (Table 1). After inoculation, we placed the workers into individual boxes and fed them with 30% honey solution (2 mL) every 48 h, checking for death every 12 h for 15 days. Following death, we collected the worker's antennae and middle legs for genotyping. As in the preliminary experiment, high humidity was maintained in the box to confirm that the cause of death was *B. bassiana*.

Genetic analysis

We extracted DNA from the workers killed by *B. bassiana* but we did not extract and analyze DNA for the surviving workers. Template DNA was extracted from antenna or leg of the individuals by placing them in 50 µL of 5% Chelex solution (Chelex 100,100–200 mesh, Bio Rad) and 0.5 µL of proteinase K (20 mg/mL, Takara Bio Inc.), then incubating for 24 h at 56 °C and 5 min at 95 °C. We froze 5 µL of the supernatant in 45 µL of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, Takara Bio Inc.) before analysis by polymerase chain reaction (PCR). We genotyped workers at nine polymorphic microsatellite loci: List2001, List2003, List2004, List2019, List2020, Rufa5, Rufa19, VMA3, and VMA6 (Thorén et al. 1995; Daly et al. 2002; Hasegawa and Takahashi 2002) using multiplex PCR. We used dye-labeled primers (Applied Biosystems) in combination with a three-primer labeling method (Schuelke 2000) to perform multiplex PCR with nine primers. Each 10 µL PCR reaction included 1 uL extracted DNA, 5 µL Qiagen master mix (Qiagen Type-It Microsatellite Kit, Qiagen Inc.), 0.2 µL of each reverse primer, 0.2 µL (dye-labeled) or 0.1 µL (three-primer labeled) of each forward primer, 0.15 µL FAM-labeled three-primer tag for each three-primer-labeled primer pair, and water to total 10 µL. PCR reaction conditions were 95 °C for 15 min, 35 cycles at 95 °C for 30 s, 50 °C for 90 s, and 72 °C for 60 s, followed by 60 °C for 30 min. Fragment analysis was performed on an ABI-3730 × l sequencer using 0.5 µL PCR product combined with 15 µL HiDi Formamide and 0.15 µL LIZ 500 internal size standard (Applied Biosystems). Allele sizes were called using GeneMarker (SoftGenetics LLC) and checked twice by eye. We used 10 samples to check for linkage, departures from Hardy–Weinberg equilibrium, and null alleles using Genepop 4.4 (Rousset 2008; Supplementary Material and Supplementary Table S1).

Estimating maternity and paternity

We used nine microsatellite markers, which were suitable for estimating maternity and paternity (see Supplementary Material). To determine patriline membership, we analyzed microsatellite genotypes using the software Colony v2.0.6.5 (Wang 2004). First, genotypes at locus List2001 were set to unknown for individuals from colony TK because genotypes suggested a maternal null allele. We, then, ran Colony using a maternal sibship constraint for each colony (*V. shidai* is an obligatory monogynous species). We then reexamined genotypes that were identified as possibly erroneous by Colony and also rescored genotypes for individuals whose putative fathers' genotypes differed from other fathers at only a single locus as these are likely the result of genotyping errors. Colony was then rerun using the corrected genotypes to produce the final sibship assignments. Settings for the Colony

Table 1

Results of statistical test for experiment 1. Cox survival analysis investigating the relationship between the survival and the strain used for infection, the colony, and the interaction of the both

Dependent variable	Predictor	Coefficient	Hazard ratio	Standard error	*z*	*P*
Survival	Strain A vs. B	−0.163	0.85	0.215	−0.759	0.448
	Strain A vs. C	−0.885	0.413	0.225	−0.393	<0.001**
	Strain A vs. D	−0.374	0.688	0.208	−1.800	0.072
	Strain A vs. E	−0.608	0.544	0.214	−2.846	0.004**
	Colony 3 vs. 1	−1.541	0.214	0.209	−7.381	<0.001**
	Colony 3 vs. 2	−0.715	0.489	0.206	−3.469	<0.001**
	Colony 3 vs. 4	−0.702	0.495	0.200	−3.519	<0.001**
	Colony 3 vs. 5	−0.840	0.432	0.238	−3.526	<0.001**

Akaike information criterion (AIC) calculated in the full model was 1368.1. AIC calculated in the final model was 1351.3. Any interactions were excluded as a variable by the stepwise model selection. The strain or colony with lowest survival was used as the reference strain or colony in Cox regressions (strain A and colony 3).
Significance level: $^{**}P = 0.01$.

runs were: updating allele frequencies, inbreeding absent, polygamy for females, monogamy for males, no scaling of full sibship, no sibship size prior, unknown allele frequencies, a long full likelihood run, and 0.01 dropout and other error rates for all loci.

Sibship assignments using Colony identified three or four major patrilines per colony. One worker in colony TK and two in colony KM were not assigned to one of the common patrilines and likely represented minor patrilines, foreign workers, or genotyping errors. These individuals were removed from further analyses.

Statistical analysis

For experiment 1, we compared each of the Kaplan–Meier survival curves between the infection and control treatment by log-rank test. We also tested the influence of each colony and each strain and interaction between both on survival by Cox regression survival analysis with variable selection by stepwise method (stepAIC function, MASS package, R). For Cox regression survival analysis, we used the colony and strain with the lowest survival rate in experiment 1 as a reference for each comparison. All statistical analyses were conducted in R 3.5.1 (R 2018).

For experiment 2, we analyzed the relationship between worker emergence date and patrilines using an Anova. We compared each of the Kaplan–Meier survival curves in a pairwise fashion between the infection and control treatment by log-rank test. We also tested the influence of each patriline, each strain, each emergence date and their interactions on survival by Cox regression survival analysis with variable selection using the stepwise method (stepAIC function, MASS package, R). We analyzed each colony separately because patrilines are nested within colonies. For survival analyses we used the patriline that had the lowest survival rate in experiment 2 as a reference for comparison within each colony. All statistical analyses were conducted in R 3.5.1 (R 2018).

RESULTS

Experiment 1: interaction of matriline and pathogen strain

We evaluated fungal virulence differences between colonies. The survival of controls was significantly greater than that of the workers infected with two strains of *B. bassiana* in two out of the five colonies (Supplementary Table S2). Control individuals were more likely to survive until the end of the experiment (26%, $n = 50$) and have greater survival time (106.56 ± 0.95 h, mean ± standard error) than treated individuals (0% survival until the end of the experiment, 83.44 ± 0.08 h average survival time, $n = 430$). Of the treated individuals, those from the colony 3 × strain B combination had the lowest average survival time (55.20 ± 1.30 h, $n = 20$), whereas the colony 1 × strain D combination had the greatest (112.36 ± 0.62 h, $n = 20$; see Supplementary Table S2 for details). Cox regression models indicated that both colony membership (i.e., matriline) and fungal strain had a significant influence on survival in experiment 1; in particular, strain A and colony 3 had negative effects on survival (Table 1; Figure 1).

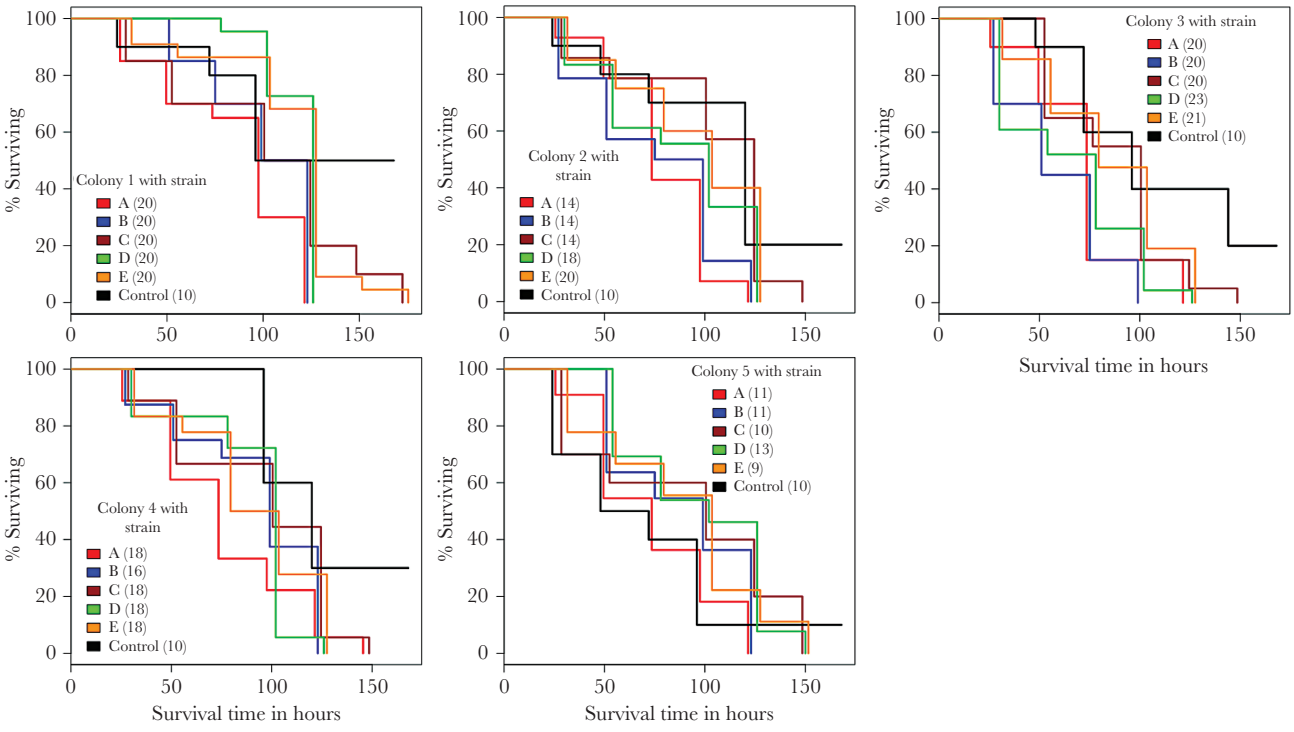

Figure 1
Survival curves for controls and infected workers in each colony in experiment 1. Legends show strains of *B. bassiana*. Values in parentheses are number of samples. Results of statistical analysis are shown in Table 1. Survival time measurements were terminated at 168 h. Individuals that survived for 168 h were treated as alive (censored) in the survival analysis. We have slightly adjusted these figures to reveal the overlapping lines of the survival curves (added survival times in the order A to E so that they were 1.5 h apart from each other.).

Experiment 2: comparison of strain virulence between different worker patrilines and emergence dates

The second aim of this study was to examine the effects of patrilines, worker emergence dates, strain differences, and their interactions on worker survival. The PPH hypothesis predicts that patrilines within a colony vary in their susceptibility to a given strain in a pathogen challenge. To test for such a pattern, we first needed to establish that our genotyping methods would accurately establish parentage in our study colonies. We did not detect any deviation from Hardy–Weinberg equilibrium, departure from linkage disequilibrium, or null alleles in this population (Supplementary Table S1), indicating that our chosen nine microsatellite markers were appropriate for matriline and patriline detection. The observed paternity number (the number of fathers; k) was four in colony TK and KM and three in colony TG. The effective paternity number (k_{e3}) averaged across the three nests examined in this study was 3.23 ± 0.32 using the equation of Nielsen et al. (2003). There was no significant relationship between worker emergence date and patriline in any of the three colonies in experiment 2 (Anova: TK, n = 197, F = 0.690, P = 0.407; TG, n = 155, F = 0.005, P = 0.945; KM, n = 215, F = 3.023, P = 0.083).

Given that our genotyping allowed us to accurately assign paternity, we then examined the effects of paternity and strain on individual survival. As in experiment 1, Control individuals were more likely to survive until the end of the experiment (60.6% survival until 360 h, n =284) and had greater mean survival time (312.13 ± 0.24 h, n = 284) than treated individuals (25.6% survival until 360 h, n = 567; mean survival time = 222.22 ± 0.17 h; log-rank test on survival curves, $P < 0.001$). To test for effects of patriline and strain, we performed Cox survival analysis and stepwise model selection using the results of experiment 2 (dependent variable: survival; predictor variables: each patriline, each strain, each emerged date, and their interaction; Table 2). As predicted by the PPH, we detected significant effects of patriline, as well as emergence date, and the interaction of both on survival of *V. shidai* workers in the TK colony (Table 2; Figure 2). Similarly, we detected significant effects of patriline and strain on survival in the TG colony (Table 2; Figure 2). We detected significant effects of patriline and emergence date on survival in the KM colony (Table 2; Figure 2). The patriline F11 in colony TG × strain A combination had the lowest survival (survival for 360 h: 6.2%; survival time: 170.25 ± 4.44 h, n = 16), and the patriline F4 in colony TK × strain A combination had the greatest survival (survival for 360 h: 57.1%; survival time: 279.42 ± 14.94 h, n = 7; see Supplementary Table S3 for details).

DISCUSSION

This study supports the PPH, the hypothesis that genetic variation between individuals within a colony leads to resistance to diverse pathogens at the colony level, by demonstrating that resistance to pathogens differs among individuals from different matrilines and patrilines. In experiment 1, we investigated the effect of matrilines on survival and the difference of pathogenicity of different fungus strains. Survival of the workers infected with the fungus varied significantly both between colonies (= matrilines) and strains of *B. bassiana* (Tables 1 and 2). Because *V. shidai* is an obligatory monogynous species, the observed differences in virulence between colonies suggest that matriline differences and/or the interaction of matriline and strain likely play a crucial role in colony resistance to parasites such as *B. bassiana*. In experiment 2, we demonstrated that the survival of the workers infected with the fungus varied among patrilines, showing, for the first time in eusocial wasps, that resistance to pathogens varies depending on patriline, a key prediction of the PPH for the evolution of polyandry in social insects.

Such patriline differences have been shown in some bees and ants (Baer and Schmid-Hempel 2003; Palmer and Oldroyd 2003;

Table 2

Results of statistical test for experiment 2. Cox survival analysis investigating the relationship between the survival and the strain, the patrilines, the emergence date, and those interactions

Dependent variable	Matriline	Predictor	Coefficient	Hazard Ratio	Standard error	z	P
Survival	TK, n = 197,AIC = 956.4	F3 vs. F1	−1.062	0.346	0.498	−2.133	0.033 *
		F3 vs. F2	0.212	1.236	0.559	0.379	0.704
		F3 vs. F4	−2.402	0.090	0.990	−2.427	0.015 *
		Strain (A vs. C)	1.171	3.227	0.662	1.771	0.077
		Emergence date	0.002	1.002	0.009	0.03	0.486
		F3 × emergence date vs. F1 × emergence date	0.105	1.111	0.081	1.295	0.195
		F3 × emergence date vs. F2 × emergence date	−0.091	0.913	0.090	−1.009	0.313
		F3 × emergence date vs. F4 × emergence date	0.277	1.319	0.137	2.019	0.043 *
		Strain × emergence date	−0.152	0.859	0.093	−1.630	0.103
	TG, n = 155,AIC = 756.1	F10 vs. F9	0.274	1.315	0.221	1.237	0.216
		F10 vs. F11	0.508	1.662	0.252	2.018	0.044*
		Strain (A vs. C)	−0.608	0.544	0.193	−3.150	0.002**
	KM, n = 215,AIC = 1044.8	F6 vs. F12	0.629	1.876	0.237	2.650	0.008**
		F6 vs. F13	0.153	1.166	0.207	0.740	0.359
		F6 vs. F15	0.351	1.420	0.275	1.274	0.202
		Emergence date	−0.084	0.919	0.030	−2.828	0.005**

Akaike information criterion (AIC) calculated in the full model of TK, TG, and KM colony were 959.7, 767.2, and 1055.0, respectively. The result after performing stepwise model selection by AIC are presented. The patriline with lowest survival for each colony was used as the reference patriline in Cox regressions (patriline F3 in colony TK, patriline F10 in colony TG, and patriline F6 in colony KM).
Significance levels: *P = 0.05, **P = 0.01.

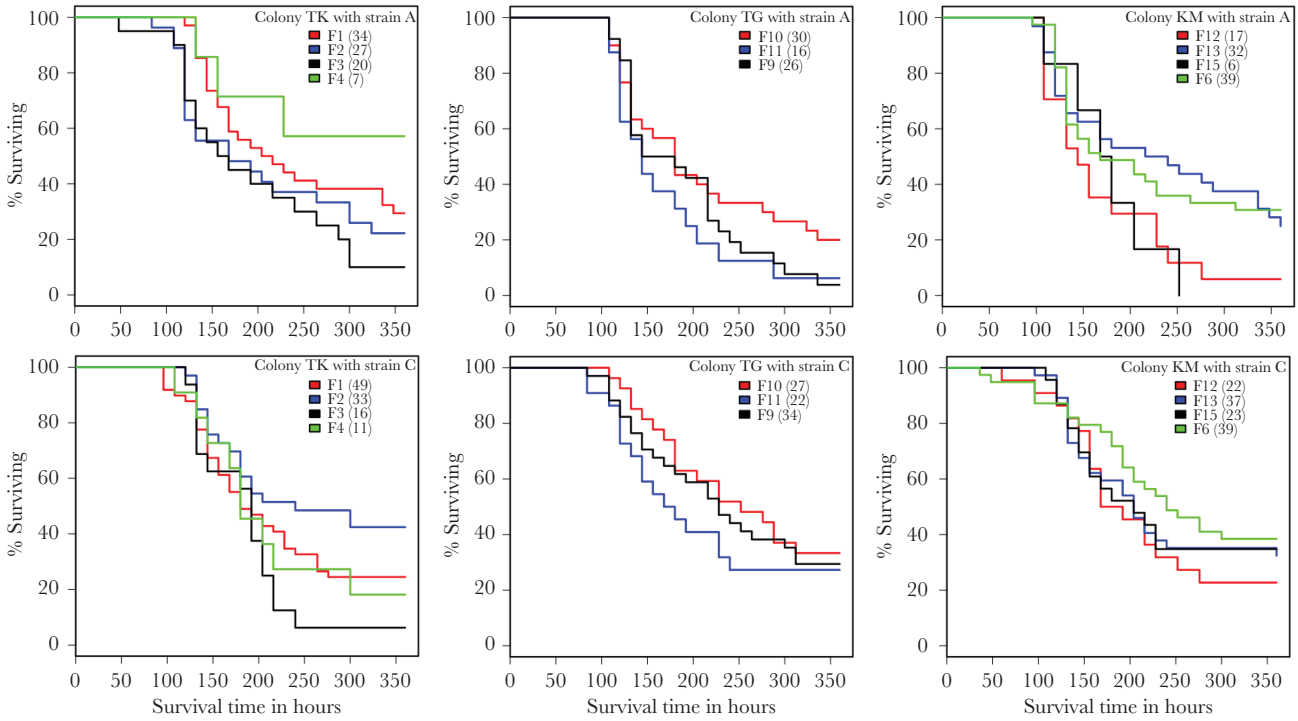

Figure 2

Survival curves for patrilines in each colony with fungal strains A and C in experiment 2. Legends show patrilines. Values in parentheses are number of samples. Results of statistical analysis are shown in Table 2. Survival time measurements were terminated at 360 h. Individuals that survived for 360 h were treated as alive (censored) in the survival analysis.

Hughes and Boomsma 2004) and suggest that worker matrilines and/or patrilines probably have different levels of resistance to a single pathogen genotype. Such variation in resistance among matrilines and patrilines would reduce the chances of colony failure as a whole due to reduced disease transmission among nestmates, if individual susceptibility depends on an interaction between host and pathogen genotypes (Sherman et al. 1988). There have been few reports that host survivorship depends on pathogen genotype (but see Barribeau et al. 2014). In this study, we assumed that the different strains of *B. bassiana* possessed different genotypes because they were isolated from different sources, and we demonstrated that the different strains varied in virulence at least for some patrilines and matrilines. On this basis, our results clearly support the PPH.

Our results suggest that the PPH likely explains why high colony genetic diversity is associated with high reproductive success in eusocial wasps as found by Goodisman et al. (2007) and Dobelmann et al. (2017). We also confirmed polyandry of *V. shidai* with three or four major patrilines per colony, similar to other *Vespula* species (Goodisman et al. 2007; Bonckaert et al. 2008; Loope et al. 2014). As observed in other *Vespula* species (Ross 1986; Goodisman et al. 2007), we found that sperm was mixed in the queens' spermatheca because patrilines were evenly distributed across emergence dates, resulting in increased genetic diversity within colonies.

What determines the number of patrilines in a *Vespula* wasp colony? Reproductive-conflict-based benefits likely only select for relatively low levels of polyandry. Queens in colonies with at least two patrilines may avoid conflict between workers and queens over the production of male offspring because, in this case, workers are more related to the queen's sons than to other workers' sons (Bourke 1994). Loope (2015) found that, in the social wasp *Dolichovespula arenaria*, queens in colonies with effective paternity less than 2.0 were more likely to be killed by their workers, probably because of competition over male production. This could select for multiple mating by queens to avoid matricide but does not explain why queens would mate with more than two or three males. Although intracolonial genetic diversity sharply increases with paternity frequency up to roughly three patrilines, the diversity plateaus when the number of patrilines is six or more (Kraus and Moritz 2010). Thus, benefits from colony genetic diversity, including enhanced disease resistance (i.e., the PPH) and division of labor (Waibel et al. 2006), are perhaps unlikely to explain paternity frequencies higher than this. In contrast, the cost to the queen from mating to acquire additional patrilines for her colony (e.g., time and energy costs, increased predation risk, and sexually transmitted disease risk) increases linearly with each mating. Given the diminishing diversity benefits of more than three to six patrilines, the costs to the queen of additional mating beyond this number may be what has maintained the moderate polyandry observed in most *Vespula* species (Loope et al. 2014). Extreme polyandry, where queens mate with more than 10 times, is known to occur in ants and honeybees (reviewed in Kraus and Moritz 2010) but not in yellowjacket wasps and hornets (Loope et al. 2014). The reason for this difference may be that *Vespula* queens have a 1-year lifespan (Matsuura and Yamane 1990), but the queen of ants and honey bees survive for multiple years (more than 10 years for some species; Keller and Genoud 1997). Such long queen survivals could favor mating with more males to ensure sufficient sperm supply and genetic diversity for the duration of the colony's life.

In experiment 2, we reared the adult wasps under the same experimental conditions, but we could not control the environment of larval and pupal workers before emergence. In the KM colony,

the survival time of infected workers was dependent on emergence day, suggesting that resistance to pathogens may also be affected by environmental factors during the larval stage. However, even if some variation in survival is attributable to variation in the larval environment, this is almost certainly independent of the observed association with patriline.

Understanding the evolutionary processes that maintain polyandry in social insects has been one of the central challenges of social insect evolutionary biology in the past three decades (Mattila and Seeley 2007). Here, for the first time, we have demonstrated that the PPH is one of the evolutionary factors contributing to polyandry in a eusocial wasp. Colonies we tested were shown to contain individuals of some patrilines that did not die after infection with *B. bassiana*. Patrilines conferring increased resistance may help a colony to maintain a minimum number of individuals to ensure colony survival and reproduction, and this may be particularly important in the early stages of colony development. *V. shidai* queens are known to usurp young nests of the same or other species (Saga et al. 2017). This intraspecific and interspecific usurpation by *V. shidai* leads to an increase in genetic diversity of nestmates in the early stages and is likely to contribute to an increase in disease resistance. We suggest that our methods should be applied to other species of wasp because few have been studied. One might expect to find similar results in other obligately polyandrous Vespidae species, although the selective forces that favor facultative polyandry may be quite different (Loope 2015; Loope et al. 2017). The alternative hypotheses explaining polyandry (e.g., sperm limitation; Kraus et al. 2004; enhanced division of labor; Waibel et al. 2006; conflict reduction; Mattila et al. 2012, Loope 2015), which are not mutually exclusive with the PPH, also deserve future research.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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