Polyandry and Paternity influence disease resistance in a eusocial wasp – support for the pathogen parasite hypothesis

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Short running title: Polyandry and paternity influence disease resistance in a eusocial wasp

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Abstract

Polyandry (multiple mating by females) is a central challenge for understanding the evolution of eusociality. Several hypotheses have been proposed to explain this observed benefit of polyandry in eusocial Hymenoptera, and the parasite-pathogen hypothesis (PPH: Sherman et al. 1988, 1998) proposes that high genotypic variance of workers for resistance to disease among workers prevents catastrophic colony collapse. We test the PPH in the polyandrous wasp *Vespula shidai*. We individually placed each infected worker with an experimentally administered entomopathogenic fungus (*Beauveria bassiana*) in a plastic box and quantified their survivorship in the laboratory. We also conducted paternity analysis of the workers using 9 microsatellite loci. Then we investigated the relationship between survivorship and the matriline/patriline 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 similar evolutionary pressures drove the convergent origin and maintenance of polyandry in ants, bees and wasps.

Introduction

Explaining the adaptive significance of polyandry (multiple mating by females) in the eusocial insects has been a central challenge in sociobiology for the past three decades (Crozier and Pamilo 1996). However, Polyandry causes dilution of kin relationships among nestmates, and this phenomenon seems contradictory to kin selection theory, because the high intra-colonial relatedness appears necessary for the evolution of eusociality (Crozier and Pamilo 1996; Queller and Strassmann 1998). Although female multiple mating is also likely to have several costs (e.g. time and energetic 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 (presence of multiple queens in a nest) have evolved from ancestral monandry independently in eusocial insects, suggesting selective forces favoring genetic diversity among nest-mates (Hughes et al. 2008). Large-scale phylogenetic studies demonstrated that colony size is associated with both greater paternity frequency and reduced paternity skew which increase intra-colony genetic diversity across species of eusocial Hymenoptera (Jaffe et al. 2012; Loope et al. 2014).

Several hypotheses have been proposed to explain the evolution of multiple mating given these costs (e.g. parasite-pathogen hypothesis, facilitating division of labor hypothesis and gaining sperm hypothesis, reviewed in Crozier and Fjerdingstad 2001). The parasite-pathogen hypothesis (PPH) is one of the plausible ideas which is besed on the benefits of high intra-colony genetic diversity to explain the evolution of polyandry in social insects (Sherman et al. 1988; Shykoff and Schmid-Hempel 1991). The PPH assumed that different matrilines or patrilines of workers within the colony have different susceptibility or resistance to a single pathogen genotype, and also, different pathogen genotypes have different virulence to individuals of matrilines or patrilines (Sherman et al. 1988; Kraus and Page 1998; Sherman et al. 1998). The PPH proposed that increasing intra-colony genetic diversity via polyandry could prevent catastrophic colony collapse by parasites and pathogens, if resistance to parasites and pathogens among workers is variable depending on genotype (Sherman et al. 1988). The PPH has been supported in only Apidae and Formicidae, two independent origins of polyandry (Baer and Schmid-Hempel 2003, Palmer and Oldroyd 2003, Hughes and Boomsma 2004, Seeley and Tarpy 2007).

Here, we conducted two types of experiments to test the PPH in a eusocial wasp. The first aimed to evaluate the virulence differences among colonies, and the second aimed to evaluate the virulence differences among patrilines, emergence date of workers, and their interactions, in a more detailed fashion. We performed pathogen challenge among multiple patrilines and matrilines of *Vespula shidai* versus multiple strains of an entomophagous filamentous fungi, *Beauveria bassiana*. *B. bassiana* is a broad range of insect hosts, and can be identified by characteristic conidiospore balls (Hajek & Leger 1994; Schmid-Hempel 1998; Schmidt et al. 2012).

Nesting biology of *V. shidai* is similar as the other *Vespula* (Matsuura 1995, Saga et al. 2016). A new colony is initiated by a single foundress queen that emerges from hibernation in spring. The queen founds the colony and produces workers after hibernation 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 gynes hibernate, and all workers and males die (Matsuura & Yamane 1990).

In addition, we investigate whether differences in growth environment affect resistance to disease in the social wasp. We investigate whether workers emerge on the same day without being biased to specific patrilines. Because assuming that the benefits of high intra-colony genetic

diversity are the evolutionary cause of the polyandry, the simultaneous emergence of workers from various patrilines increase the genetic diversity within the nest. Considering the epigenetics and phenotypic plasticity, even individuals of the same genotype have variability in disease resistance due to differences in environmental factors in many organisms (Pigliucci 2005). In addition, Given the homeostasis common within advanced eusocial societies (Oldroyd and Fewell 2007), it seems likely that workers of similar emergence day experienced very similar environments as larvae (e.g. kind of food and exposed diseases).

In this study, we investigate PPH in *Vespula* wasps, phylogenetic-independently of Apidae and Formicidae, in which the PPH has not been directly tested. Firstly, we test the prediction that matrilines/patrilines differ in their resistance to particular strains of pathogen. Second, we determine if pathogen strains vary in their virulence when exposed to the same host genotype, which would suggest the potential for classic co-evolutionary host-pathogen dynamics (Schmid-Hempel and Ebert 2003). There are few studies showing variation in virulence among strains of pathogens that exposed to the same host eusocial wasp genotype (but see Barribeau et al. 2014).

Material and Methods

Pathogen

We used five fungal strains (A-E) of the entomophagous filamentous fungi *Beauveria bassiana* for experiment 1, and then we selected two highest lethality strains (A and C) for experiment 2. These fungal strains were separated and isolated from five mummy queen larvae from different colonies of *V. shidai* collected in Nakatsugawa City, Gifu Prefecture, Japan, 3 November 2013. We stored these strains on Sabouraud 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. For these experiments, conidiaspore 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 (30mL) was quantified using a hemocytometer (Sigma, St. Louis, MO, USA) and diluted to the required dosages for both experiments: low (10^6 spores/mL) and high (10^8 spores/mL).

Wasps and Experiment setting

Experiment 1: Interaction of matriline and pathogen strain

We collected about 400 workers from each of five colonies, on 9 November 2014 at Akechi Wasp Festival in Toyota City, Aichi prefecture, Japan (regarding wasp festivals, refer to Nonaka 2011). We confined the collected workers within one wire mesh cage for each colony and kept them in a constant temperature room (25 ºC with a 15L: 9D photoperiod on that day. We provided the workers with 30% honey solution every day for one week from the collection date. We conducted experiment 1 using the live workers remaining after one week. We put all the workers in the freezer (-20 ºC) for 20 seconds to anesthetize just before conidiospore infection. We completely submerged individual workers on a fungal spore dosage in sterilized water (high; 10^8 spores/mL, 30mL) to exposure, or only sporeless control of the water (30mL) for 1 second. We absorbed excess fluid on the workers' body surfaces using filter paper. After that, we housed one treated worker within a plastic box (3 × 8 ×10 cm) lined with filter paper. Then, we reared them, checked for their live or death every 24 hours for seven days, and recorded their survival time in days. We provided 500 µL water every 24 hours during the rearing period. If we confirmed worker death, we moisturized the filter paper with water to keep more than 90% of humidity in the box for two weeks following worker death to identify the cause of the death. We attributed the workers' death to *B. bassiana* when we observed whitish hyphae growing from the corpse, which is a typical feature of the fungus.

Experiment 2: Comparison of strain virulence between patriline and emergence date of worker differences

We collected three colonies in Nakatsugawa City, Gifu prefecture, Japan in middle of July 2015 by using traditional methods for wasp hunting and keeping (Saga 2019). We fixed each colony in a woody box (30 × 30 × 60 cm) and provided the colonies with purchased meat of chicken heart and 30% honey solution, permitting free outdoor foraging within original collection areas until 11th October 2015. We then collected combs filled with larvae and pupae from each colony, put the combs in a plastic box with mesh roof, and set the boxes in a constant room temperature (25 ºC with a 15L: 9D) on the 11th October 2015. Every 12 hours, we transferred newly emerged workers from the combs to individual plastic boxes (3 × 8 ×10 cm) lined with filter paper. We conducted experiment 2 using the workers 12 – 24h post emergence, administering a low concentration of fungal spore suspension (10^6 spores/mL; 30mL) of either strain A or strain C, with the same infection

protocol as the preliminary experiment including controls. The reason for using strain A and C is that we found most apparent virulence differences as in Table 1. After that, we individually placed each worker in the box, feeding with 30% honey solution (2mL) every 48 hours and checking for worker death every 12 hours for 15 days. Following worker death, we collected the antenna and middle legs of the corpses of workers for genotyping. We maintained high humidity in the box using the similar procedure as in the preliminary experiment.

Genetic analysis

We extracted DNA from the workers killed by *B. bassiana*. 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 (20mg/mL, Takara Bio inc.), then incubating for 24h at 56°C and 5 minutes 95°C. We froze five μL of the supernatant in 45 μL of Tris-EDTA buffer (10mM Tris, 1mM EDTA, Takara Bio inc.) before PCR. We genotyped workers at nine polymorphic microsatellite loci List2001, List2003, List2004, List2020, Rufa5, Rufa13, Rufa15, VMA-3 and VMA-6 (Thorén et al. 1995, Daly et al. 2002, Hasegawa & Takahashi 2002) using multiplex PCR. We used dye-labeled primers (Applied Biosystems) in combination with a 3-primer labeling method (Schuelke 2002) to perform multiplex PCR with nine primers. Each 10 μL PCR reaction included 1uL 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 (3-primer labeled) of each forward primer, 0.15 μL FAM-labeled 3-primer tag for each 3-primer-labeled primer pair, and water to total 10 μL. PCR reaction conditions were 95°C for 15-minutes, 35 cycles of 95°C for 30 seconds, 50°C for 90 seconds, 72°C for 60 seconds, followed by 60°C for 30 minutes. 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, depatures from Hardy-Weinberg equilibrium, and null alleles by using Genepop 4.4 (Rousset 2008, see in supplemental materials and Table S2).

Estimating maternity and paternity

We used nine microsatellite markers which were suitable for estimating maternity and paternity (see supplemental material). To determine patriline membership, we analyzed microsatellite

genotypes using the Colony program v2.0.6.5 (Wang 2004). First, genotypes at locus L2001 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 (*Vespula shidai* is an obligatory monogynous species). We then re-examined genotypes that were identified as possibly erroneous by Colony, and also re-scored 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 re-run using the corrected genotypes to produce the final sibship assignments. Settings for the Colony 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 3 – 4 major patrilines per colony. One worker in colony TK and two individuals 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 the experiment 1, we compared each of 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 of the both on the lifespan checked in every 24 hours by Cox regression survival analysis with variable selection by stepwise method (stepAIC function, MASS package, R). For Cox regression survival analysis, we used colony and strain, which had the lowest survival rate in Experiment 1, as a reference for each comparison. All statistics analysis was conducted in R 3.2.4 (R 2018).

For the experiment 2, we analyzed the relationship between worker emergence date and patrilines using ANOVA. We compared each of Kaplan-Meier survival curves with a pair-wised fashion between the infection and control treatment by log-rank test. We also tested the influence of each patriline, each strain, each emerged date and those interaction on the lifespan by Cox regression survival analysis with variable selection by stepwise method in each colony, since patrilines are nested within colonies (stepAIC function, MASS package, R). For cox regression survival analysis, we used the patriline that had the lowest survival rate in Experiment 2 as a reference for comparison within each colony. All statistics analysis was conducted in R 3.2.4 (R 2018).

Results

The survival time of control was significantly longer than the workers infected with each strain of *Beauveria bassiana* (Table S1). Akaike Information Criterion (AIC) calculated in the full model of the result of experiment 1(dependent variables: survival time and rate; predictor variable: each colony and each strain, the interaction of the both) was 1368.1. AIC calculated in the final model (dependent variables: survival time and rate; predictor variable: each colony and each strain) after performing stepwise model selection was 1351.3. Any interactions were excluded as a variable by the stepwise model selection. We found that differences of colonies and strains significantly influenced on the lifespan in the experiment 1 (Table 1).We also showed the results of the experiment 1 in Figure 1.

Table 1. Cox survival analysis investigating the relationship between the lifespan and the strain used for infection, the colony, and the interaction of the both.

Dependent variable	Predictor	Coef	Hazard ratio	SE	z	P	
Lifespan	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	**

significance level, **; P = 0.01

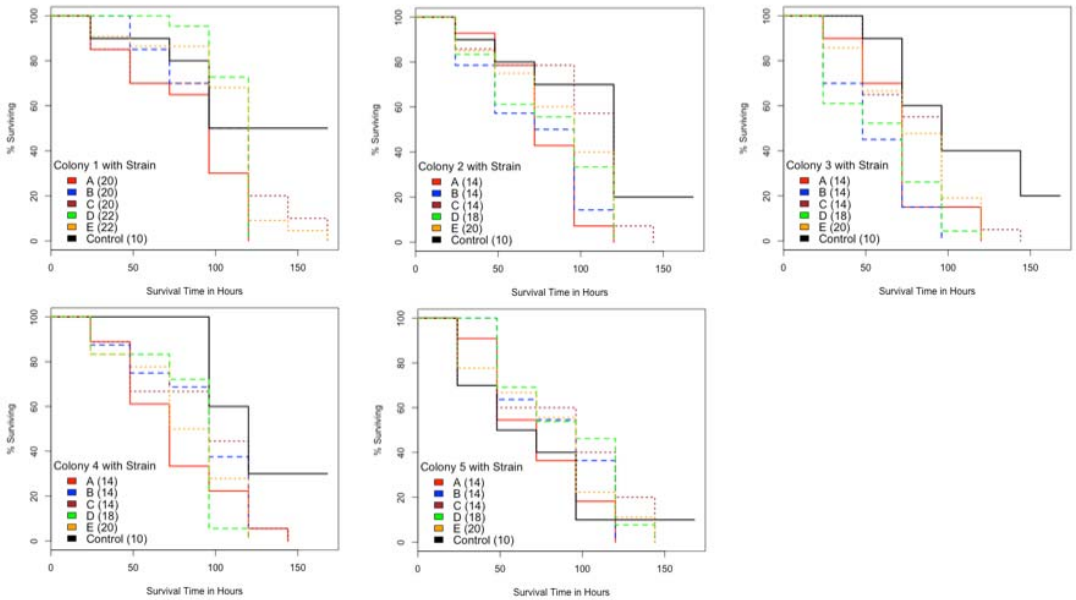

Figure 1. The survival curves of the infected workers with strains and control in each colony. Legends show strains of *Beauveria bassiana*. Parentheses are number of samples. We showed results of statically analysis in Table 1.

We did not detect any deviation from Hardy-Weinberg equilibrium, departure from linkage disequilibrium, and null alleles in this population (Table S2). Therefore, the nine microsatellite markers were useful for our matriline and patriline detection. Effective number of paternity (k_{e3}) was 3.23 ± 0.32 SE using the equation of Nielsen et al. (2003).

There were not significant relationships between the worker emergence date and patrilines in all three colonies in the 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). The survival time of the control was significantly longer than those of the workers infected with the two strains in all three colonies (log-rank test, $P < 0.001$). Akaike Information Criterion (AIC) calculated in the full model of the result of experiment 2 (dependent variables: survival time and rate; predictor variable: each patriline, each strain, each emerged date and those interaction) in TK, TG and KM colony were 959.7, 767.2, 1055.0. The result after performing stepwise model selection by AIC are presented on Table 2. We detected significant effects of patriline differences, emergence date, and the interaction of the both on lifespan of *Vespula shidai* workers in TK colony (Table 2). Similarly, we detected significant effects of patriline differences and strain differences on the lifespan in TG colony (Table 2). We detected

significant effects of patriline differences and emergence date on the lifespan in KM colony (Table 2). We also showed the results of the experiment 2 in Figure 2.

Table 2. Cox survival analysis investigating the relationship between the lifespan and the strain, the patrilines, the emergence date, and those interactions.

Dependent variable	Matriline	Predictor	Coef	Hazard ratio	SE	z	P	
Lifespan	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	0.09	0.99	-2.427	0.015	*
		strain	1.171	3.227	0.662	1.771	0.077	
		emergence date	0.002	1.002	0.009	0.03	0.486	
		F3 × emegence date vs F1 × emergence date	0.105	1.111	0.081	1.295	0.195	
		F3 × emegence date vs F2 × emergence date	-0.091	0.913	0.090	-1.009	0.313	
		F3 × emegence 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	-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	**

significance level *; P = 0.05, **; P = 0.01

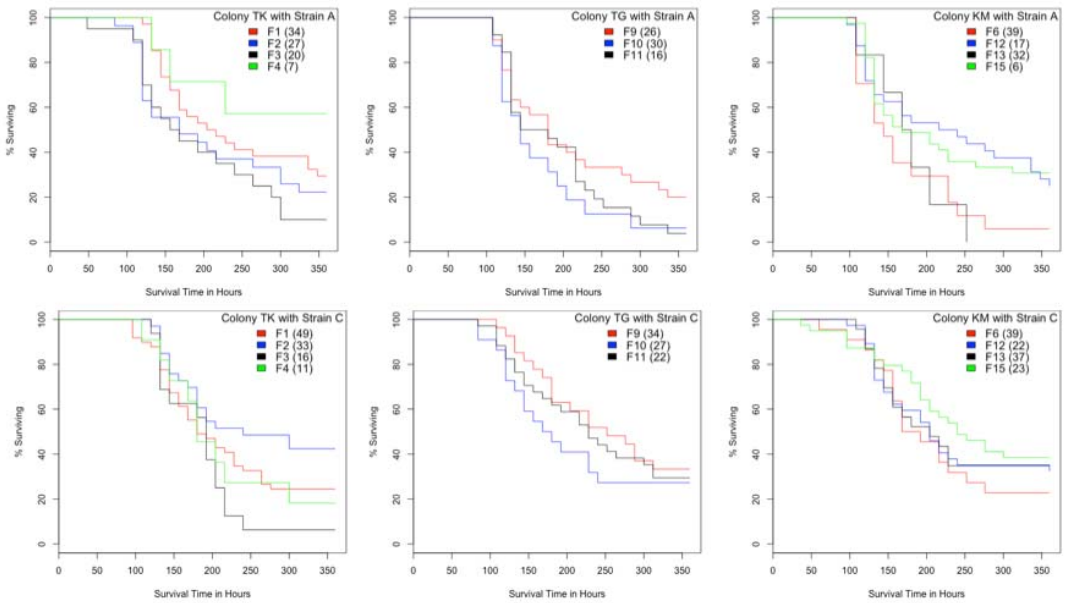

Figure 2. The survival curves of each patriline of each colony on the strain A and C in experiment 2. Legends show patrilines. Parentheses are number of samples. We showed results of statically analysis in Table 2.

Discussion

Our results showed that the five strains of *Beauveria bassiana* have virulence to *Vespula shidai* because the survival time of the workers infected with the strains was significantly shorter than the control in two of five colonies (Table S1). We also found that *V. shidai* is polyandrous, with 3 – 4 major patrilines per colony, similar to other *Vespula* species (Goodisman et al. 2007; Bonckaert et al. 2008; Loope et al. 2014). We demonstrated that lifespan varied significantly both among colonies (= matrilines) and strains of *B. bassiana* (Table 1, 2). Because *V. shidai* is a obligatory monogynous species, such virulance differences among colonies suggests that matriline differences and/or the interaction of matriline and the strain differences likely play a crucial role in colony resistance against parasite like *B. bassiana*.

We also demonstrated that the lifespan of the workers infected with the fungus varied among patrilines, showing that resistant ability against this fungus varied depending on patriline differences in eusocial wasps for the first time. Such patriline differences have been shown in some bees and ants (Palmer and Oldroyd 2003, Baer and Schmid-Hempel 2003, Hughes and Boomsma 2004), as predicted by PPH; worker matrilines and/or patrilines probably have different resistance ability to a

single pathogen genotype. Pathogen variants has different virulence to individual matrilines or patrilines. Such variation in resistance among matrilines and patrilines would prevent colony failure as a whole due to reduced disease transmission among nestmates under genotype – genotype interaction of host – parasite relationship (Sherman et al. 1988). There have been few reports that host survivorship depends on pathogen genotype (but see Barribeau et al. 2014). It is considered that different strains of *B. bassiana* which show different genotypes in this study since they were isolated from different sources at the beginning of experiment 1. We demonstrated that the different strains of *B. bassiana* have different virulence, a least for some patrilines and matrilines. Our results are clearly supporting the PPH.

Although Dobelmann et al. (2017) compared natural viral infection rates and immune responses among patrilines within colonies of polyandrous *Vespula vulgaris*, they found that no effects of patriline differences, perhaps due to lower sample sizes than those employed here. Our results suggest that the PPH likely explains why high colony genetic diversity is associated with high reproductive success in eusocial wasps found by Goodisman et al. (2007) and Dobelmann et al. (2017). As observed in other *Vespula* species (Ross 1986, Goodisman et al. 2007), we found that sperm was mixed in queens' spermatheca, because patrilines were evenly distributed across emergence dates. This has the effect of increasing genetic diversity within colonies.

We managed to rear the adult wasps after emergence for experiment 2 in the same environment, but we did not control the environment of larval and pupal workers before emergence. The survival time of infected workers was dependent on emergence day in KM colony; that suggested that the resistivity to pathogens is also affected by the environmental factor during the larval stage. However, even if some variation in survival is attributable to variation in larval environment, this is almost certainly independent of the observed association with patriline.

Understanding the evolutionary processes that maintain multiple mating by female social insects has been one of the central challenges of social insect evolutionary biology in the past three decades (Mattila and Seeley 2007). Here we demonstrated PPH is one of the evolutionary factors of polyandry in eusocial wasps for the first time. According to experiment 2, there were individuals of some patrilines who did not die after infection with *B. bassiana*. Colonies with patrilines conferring increased resistance may maintain a minimum number of individuals to continue colony survival and reproduction, and this may be particularly important in the early stages of colony development. *V. shidai* queens usurp young nests of same species or other species (Saga et al. 2017). This intra- and

interspecific usurpation of *V. shidai* leads to an increase in genetic diversity of nestmates in the early stages, is likely to contribute an increase in disease resistance. It is thought likely that increasing resistance to disease was a driving and maintenance force of evolution of polyandry in eusocial insects. We suggest that our methods should be applied to other wasps, since few have been studied. One might expect to find similar results in other highly, obligately, polyandrous Vespidae species, although the selective forces that favor facultative polyandry may be quite different (Loope 2015, Loope et al. 2017). Several other hypotheses (e.g. sperm limitation, Kraus et al. 2004; enhanced division of labor, Waibel et al. 2006; conflict reduction, Mattila et al. 2012, Loope 2015) has also proposed to explain the evolution of polyandry. In future we have to test these hypotheses that are not mutually exclusive with the PPH.

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