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ORIGINAL PAPER

Mating triggers an up-regulation of *vitellogenin* and *defensin* in ant queens

Sarah Chérasse[1] · **Pauline Dacquin**[1] · **Serge Aron**[1]

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Abstract

Mating induces a range of physiological changes in female insects. In species that mate during several reproductive bouts throughout their life, mating causes an increase in oviposition, affects immune function, and decreases female lifespan and receptivity to further mating. Social Hymenoptera (ants, social bees, and wasps) are unique, since queens mate during a single reproductive effort at the beginning of their life. Their reproductive strategy is thus fundamentally different from that of other insects and one might expect the effects of mating on social Hymenoptera queens to be altered. We tested the effect of mating and multiple mating on the expression of six genes likely to be involved in post-mating changes, in queens of the ant *Lasius niger* L. We show that mating induces oviposition, and is followed by an up-regulation of *vitellogenin* and *defensin* expression. The expression of *juvenile hormone esterase, insulin receptor 2*, *Cu−Zn superoxide dismutase 1,* and *prophenoloxidase* is not significantly affected by mating. Queen-mating frequency did not affect the expression of the tested genes. Altogether, our results indicate that certain effects of mating on female insect physiology are generalized across species independent of their mating strategies, while others seem species specific.

Keywords Ant queen · Defensin · Mating · Oviposition · Vitellogenin

Introduction

Sex is the prevailing reproductive strategy in the animal kingdom. The main hypotheses for the maintenance of sexual reproduction propose that it provides an advantage in the coevolutionary arms race against parasites and reduces the accumulation of deleterious mutations (West et al. 1999). On a more proximate scale, mating also has a range of direct effects on female behavior and physiology. Insects are no exception, and in females, mating has been found to stimulate rates of oviposition (Venkatesh et al. 1988; Wolfner 2002), affect immune function (Lawniczak et al. 2007), decrease lifespan (Wolfner 2002; Fedorka et al. 2004), and lower receptivity to further matings (Sugawara 1979; Wolfner 2002).

Social Hymenoptera have a particular reproductive strategy making them appealing models for studying the effect of mating on female physiology (Baer 2011). As opposed to females of other insect orders that can mate on several occasions throughout their life, queens of ants and social bees and wasps mate only during a single reproductive event. After this unique mating bout, males die and queens store the sperm that they acquired during copulation and use it to fertilize eggs for the rest of their life. Social Hymenoptera queens are extremely long-lived compared to other insect females, several decades in some species (Keller and Genoud 1997), and queens will, therefore, need to use stored sperm parsimoniously (den Boer et al. 2009), as they can never mate again to renew their stock. Previous studies have shown that mating increases queen longevity in the ant *Cardiocondyla obscurior* (Schrempf et al. 2005), a result that contrasts with those reported in *Drosophila* (Wolfner 2002) and in crickets (Fedorka et al. 2004), where mating decreases female lifespan. This suggests that the fundamental differences between the mating strategies of social

Sarah Chérasse and Pauline Dacquin are co-first authors.

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✉ Serge Aron
saron@ulb.ac.be

[1] Evolutionary Biology and Ecology, Université Libre de Bruxelles, Avenue Franklin Roosevelt 50, 1050 Brussels, Belgium

Hymenoptera and other insects translate into different effects on the physiology of reproductive females. Despite this, little is known about the impact of mating on the physiology of social Hymenoptera queens.

We measured the effect of mating in ant queens on the expression of six focal genes likely to be involved in post-mating changes in female physiology. The genes were *vitellogenin*, *juvenile hormone esterase*, *insulin receptor 2* (involved in the Insulin–IGF-1 pathway), *Cu−Zn superoxide dismutase 1*, *prophenoloxidase*, and *defensin*. Vitellogenin is the major yolk protein precursor and is thus directly linked to egg production (Engelmann 1979). Vitellogenesis is stimulated by juvenile hormone (Hartfelder 2000; Flatt et al. 2005). Male insects might be able to coerce females into increasing the rate of oviposition by either directly transferring juvenile hormone to females or by stimulating its synthesis using accessory gland proteins (Shirk et al. 1980, 1983; Borovsky et al. 1994; Moshitzky et al. 1996; Fan et al. 1999, 2000). Juvenile hormone is degraded by juvenile hormone esterase (Gilbert et al. 2000) and female insects might thus be able to counteract male control over oviposition by producing high levels of juvenile hormone esterase after mating. Measuring *juvenile hormone esterase* expression could thus help suggest whether or not a conflict between the sexes is present. In honeybees, insulin–IGF-1 signaling (IIS) could be important for regulating longevity (Corona et al. 2007). In line with this, the down-regulation of IIS pathway increases longevity in *Drosophila* (Finch and Ruvkun 2001). Superoxide dismutase is one of the prominent defenses against reactive oxygen species (Felton and Summers 1995). Honeybee queens have higher superoxide dismutase activity in their muscle tissue compared to virgins (Weirich et al. 2002), suggesting that superoxide dismutase could play a role in managing mating-associated stress responses (Kocher et al. 2008, 2010). Prophenoloxidase is an essential enzyme regulating insect melanization response during which melanin is deposited around wounds or pathogens (Nakhleh et al. 2017). Mating induces a decrease in melanization-related immune capacity in various insects (Rolff and Siva-Jothy 2002; Fedorka et al. 2004; Baer et al. 2006; Castella et al. 2009; Dávila et al. 2015). The insect immune system also relies on antimicrobial peptides (Bulet and Stocklin 2005), among which defensin is a major and highly conserved representative (Hoffmann and Hetru 1992). In *Drosophila*, mating triggers an up-regulation of several antimicrobial peptides (Lawniczak and Begun 2004; McGraw et al. 2004), indicating that they could play an important part in the insect immune response to mating.

We used queens of the black garden ant *Lasius niger* L. as a model organism. *L. niger* queens are facultatively polyandrous (Fjerdingstad et al. 2002, 2003), meaning that they can mate with either a single male (monandry) or multiple males (polyandry) during a unique mating flight. To test the effect of mating and of multiple mating on the expression of the six focal genes, we quantified expression fold changes in virgin, monandrous, and polyandrous queens. As the effects of mating might be delayed in time, we measured gene expression 1 and 5 days after mating.

Materials and methods

Queen collection and rearing

Virgin and mated *Lasius niger* queens were collected during a mating flight on 4 July 2017 in Brussels, Belgium. Virgin queens were gathered at the entrance of their natal colonies right before they took off to mate. A couple of hours later on the same day, mated queens were collected, while they scurried on the ground to find a nesting site to found their colony. A first batch of virgin queens was flash frozen in liquid N_2 immediately after collection to serve as a calibrator sample for gene expression analysis (see "RNA extraction and reverse transcription"). The remaining virgin and mated queens were placed into artificial nest tubes and reared in the dark at room temperature (ca. 20 °C). After 24 h, half of the virgin and mated queens were flash frozen (one day group). The other half was flash frozen 5 days after the mating flight (five days group). Frozen queens were kept at – 80 °C until further analysis. When present, eggs laid by founding queens were counted to estimate their fecundity.

Mating frequency

The number of males that each queen had mated with was determined by genotyping the sperm stored in their spermatheca at four previously identified microsatellite loci (Ln10-53, Ln10-282, Ln10-174, Ln1-5; Fjerdingstad et al. 2003). To avoid errors in the inferred mating frequency of queens due to non-detection of certain father genotypes (Chapuisat 1998), we genotyped queen eggs (when they were present). To avoid errors in the inferred mating frequency of queens due to contamination of the sperm with queen DNA, we also genotyped a leg from each queen.

For each frozen queen, the tip of the abdomen containing the spermatheca was removed by rapidly sectioning the membrane between the third and fourth tergite. One leg was also removed for each queen. The queen, minus the tip of her abdomen and one leg, was immediately placed back at – 80 °C. To extract the sperm from the spermatheca, the spermathecal membrane was removed and the sperm was allowed to flow out in a droplet of sterile water. DNA was extracted from the total amount of sperm contained in each queen spermatheca using 5% Chelex 100 (Walsh et al. 1991). This method was also used to extract DNA from queen legs and each individual egg. DNA samples were amplified at

the four microsatellite loci by PCR (PTC-100, Bio-Rad). The reaction mix consisted of 1 µl of sample DNA, 5 µl of 2×Type-it multiplex PCR mastermix (Qiagen), 1 µl of primer mix (each primer at 2 µM, all forward primers had a fluorescent marker), and 3 µl dH_2O. The amplification cycling was as follows: 5 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C; and 10 min at 72 °C. The PCR products were sequenced using a 3730 DNA Analyzer (Thermo Fisher Scientific). Each reaction consisted of 1.5 µl of amplified DNA, 0.3 µl of MapMarker (Eurogentec), and 8.7 µl of Hi-Di formamide (Thermo Fisher Scientific). Results were analyzed using Peak Scanner software version 1.0.

RNA extraction and reverse transcription

To determine the effect of mating on the expression of the six focal genes, we randomly selected virgin ($n=8$), monandrous ($n=8$), and polyandrous ($n=8$) queens in the 1 day group and virgin ($n=8$), monandrous ($n=8$) and polyandrous ($n=8$) queens in the 5 day group. Total RNA was extracted from their entire body, minus the tip of the abdomen containing the spermatheca and one leg. For virgin queens that were not genotyped, as they had not mated, we removed the tip of the abdomen and a leg so that the tissue extracted was equivalent to that of mated queens. To compare gene expression between samples, we used a calibrator sample that consisted of a pool of 10 virgin queens collected before the mating flight. For this sample, RNA was extracted from the pool of virgin queens to have enough material to include the calibrator in each quantitative PCR run.

Queens were removed from storage at −80 °C and were homogenized in TRI Reagent solution (Thermo Fisher Scientific) in a Mixer Mill MM301 (Retsch GmbH) with 2.8 mm Precellys® zirconium oxide beads (Bertin Corp). The rest of the extraction procedure followed the TRI Reagent® solution RNA isolation protocol (TRI Reagent® solution, RNA/DNA/protein isolation reagent, RNA isolation procedure, 2010, Thermo Fisher Scientific). The total amount and quality of the extracted RNA were measured with a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific). RNA integrity was assessed by band inspection after electrophoresis on a 2% agarose gel. To remove remaining genomic DNA, each queen RNA sample was then treated with DNase I, amplification grade (Thermo Fisher Scientific). 230 ng of each DNase I treated total RNA queen sample was used to reverse transcribe mRNA into cDNA. Reverse transcription was carried out using SuperScript III reverse transcriptase (Thermo Fisher Scientific) and oligo$(dT)_{20}$ primer (Thermo Fisher Scientific) following the manufacturer's protocol (SuperScript™ III reverse transcriptase, first-strand cDNA synthesis, 2004, Thermo Fisher Scientific). To make sure that no genomic DNA contamination was present despite DNase I digestion, we used a control sample with dH_2O instead of SuperScript III reverse transcriptase (no RT control) for each queen sample. After reverse transcription, cDNA samples were diluted five times in buffer AE (Qiagen) and stored at − 20 °C until quantitative PCR.

Primer design

When selecting the genes analyzed in this study, we had to make a compromise between genes whose expression we believed would be affected by mating and genes for which sequences were known in *Lasius niger* or other, preferably social, insects. Gene sequences for *defensin* (EU401743.1), *vitellogenin* (DY543805.1), and *Cu−Zn superoxide dismutase 1* (AY309973.1) had previously been identified in *L. niger*. For *prophenoloxidase*, *juvenile hormone esterase,* and *insulin receptor 2*, homologous gene sequences were identified by BLAST® (tBLASTn and BLASTn, Altschul et al. 1990) of sequences known in other insect species (*prophenoloxidase*: *Drosophila melanogaster,* NP_476812.1, NP_610443.1, NP_524760.1; *Apis mellifera,* AAO72539.2; *Atta laevigata,* JI332441.1; *Bombus terrestris,* ADK91829.1—*juvenile hormone esterase*: *A. mellifera,* AAU81605.1; *D. melanogaster,* NP_523758.3, NP_001163166.1, NP_001286476.1—*insulin receptor 2*: *A. mellifera,* DAA34971.1; *Solenopsis invicta,* ADZ56366.1) against a *L. niger* transcriptome (courtesy of E. R. Lucas and L. Keller). We had initially planned to study the expression of several genes involved in the Insulin–IGF-1 pathway (i.e., *insulin receptor 1*, *insulin receptor 2,* and *insulin-like peptides*). However, except for *insulin receptor 2*, we were unable to find homologous sequences in the transcriptome, so we abandoned the analysis of *insulin receptor 1* and *insulin-like peptides*. We selected *elongation factor 1α* as a housekeeping gene (sequence known in *L. niger*: EU43083.1). *Elongation factor 1α* had previously been shown to have stable expression in *L. niger* queens (Chérasse and Aron 2017). In the present study, we confirmed the stable expression of *elongation factor 1α* between virgin, monandrous, and polyandrous queens (Schmittgen and Zakrajsek 2000) (1 day group: one-way ANOVA: $F_{2,21}=1.96$, $P=0.17$; 5 day group: Kruskal−Wallis test: $\chi^2=0.45$, $df=2$, $P=0.8$). Primer pairs were designed with Primer3Plus (Untergasser et al. 2012). Primer sequences, melting temperatures (T_m), and amplicon sizes are given in Table S1. Primer specificity was checked with PCR followed by electrophoresis on a 2% agarose gel. All primers produced single bands of the expected amplicon size. Specificity was subsequently confirmed by melt curve analysis after quantitative PCR.

Gene expression

Quantitative PCR were carried out on a Rotor-Gene Q cycler (Qiagen). Reaction volumes were as follows: 10 µl SYBR Premix Ex taq (Takara), 0.4 µl of forward primer, 0.4 µl of reverse primer, 7.2 µl of dH_2O, and 2 µl of sample cDNA. Samples were run in triplicate and no RT controls were included for each sample. The calibrator sample was included in each run for all genes. Quantitative PCR cycles were the same for all genes: 95 °C for 1 min 45 s; 40 cycles consisting of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min. Melt curves were run after the last cycle. To determine the efficiency of primer pairs, we ran standard curves for each gene. This efficiency was used to determine relative fold changes in gene expression between queen samples and the calibrator sample following the method proposed by Pfaffl (2001).

Statistical analyses

To determine if mating had an effect on the expression of the six focal genes, we used one-way ANOVA with mating status as a factor with three levels: virgin, monandrous, and polyandrous queens. The analysis was done separately for each gene and for each time group (1 day and 5 days). Gene expression fold changes were $\log_{10}$ transformed to meet assumptions of normality and homoscedasticity. When the transformation did not suffice, we used a Kruskal–Wallis test (non-normal data) or a Welsh ANOVA (heteroscedastic data) instead.

The only queens that had laid eggs were the mated queens in the 5 day group. We used a *t* test to determine if the number of eggs laid varied between monandrous and polyandrous queens. All statistical analyses were carried out in R, version 3.4.3 (R Core Team 2015).

Results

Mating frequency

We genotyped a total of 24 mated queens in the 1 day group and of 27 mated queens in the 5 day group. In the 1 day group, we found 14 monandrous queens and 10 polyandrous queens. In the 5 day group, we found 14 monandrous queens and 13 polyandrous queens. Polyandrous queens had all mated twice except for one queen in the 5 day group that had mated three times. This queen was not used for the gene expression analysis. The number of fathers detected in the spermatheca and the eggs, when present, was the same for all samples. None of the queens in the 1 day group had started laying eggs, whereas all queens in the 5 day group had laid eggs. The raw genotyping data can be found in Table S3.

Gene expression

One day after mating, *vitellogenin* expression did not vary between virgin, monandrous, and polyandrous queens ($F_{2,21}=1.63$, $P=0.22$) (Fig. 1). However, 5 days after mating, monandrous and polyandrous queens, but not virgins, had started to lay eggs and *vitellogenin* expression was significantly higher in mated queens than in virgin queens (Welsh ANOVA: $F_{2,9.66}=18.87$, $P<0.001$; Games–Howell post hoc test: monandrous > virgin, $P=0.02$; polyandrous > virgin, $P=0.002$) (Fig. 1). There was no difference in *vitellogenin* expression between monandrous and polyandrous queens (Games–Howell post hoc test: $P=0.25$), and neither did they lay different numbers of eggs (*t* test: $t=-1.12$, $df=14$, $P=0.28$).

Mating significantly affected the expression of *defensin* (one-way ANOVA: $F_{2,21}=6.29$, $P=0.007$) (Fig. 1). One day after mating, both monandrous (Tukey HSD post hoc test: $P=0.02$) and polyandrous (Tukey HSD post hoc test: $P=0.01$) queens indeed had a higher expression than virgin queens. *Defensin* expression did not vary significantly between monandrous and polyandrous queens (Tukey HSD post hoc test: $P=0.95$). Five days after mating, *defensin* expression was not affected by queen-mating status anymore (one-way ANOVA: $F_{2,21}=2.54$, $P=0.10$) (Fig. 1).

Queen-mating status did not significantly affect the expression of *prophenoloxidase* (one-way ANOVA—1 day: $F_{2,21}=0.63$, $P=0.54$; one-way ANOVA—5 days: $F_{2,21}=2.83$, $P=0.08$), *juvenile hormone esterase* (one-way ANOVA—1 day: $F_{2,21}=2.2$, $P=0.13$; Welsh ANOVA—5 days: $F_{2,9.57}=3.53$, $P=0.07$), *insulin receptor 2* (one-way ANOVA—1 day: $F_{2,21}=1.2$, $P=0.32$; Kruskal–Wallis test—5 days: $\chi^2=1$, $df=2$, $P=0.6$) and *Cu–Zn superoxide dismutase* (one-way ANOVA—1 day: $F_{2,21}=2.96$, $P=0.07$; Kruskal–Wallis test—5 days: $\chi^2=0.73$, $df=2$, $P=0.69$) (Fig. S1).

Fold changes in gene expression for each sample are given in Table S2.

Discussion

In insects, mating has been shown to impact female physiology in a variety of ways. In species that mate repeatedly throughout life, mating stimulates oviposition (*Trichoplusia ni*, Venkatesh et al. 1988—*Drosophila melanogaster*, Wolfner 2002), influences immune function (*Matrona basilaris japonica*, Siva-Jothy et al. 1998—*Tenebrio molitor*, Rolff and Siva-Jothy 2002—*Allenomobius socius*, Fedorka

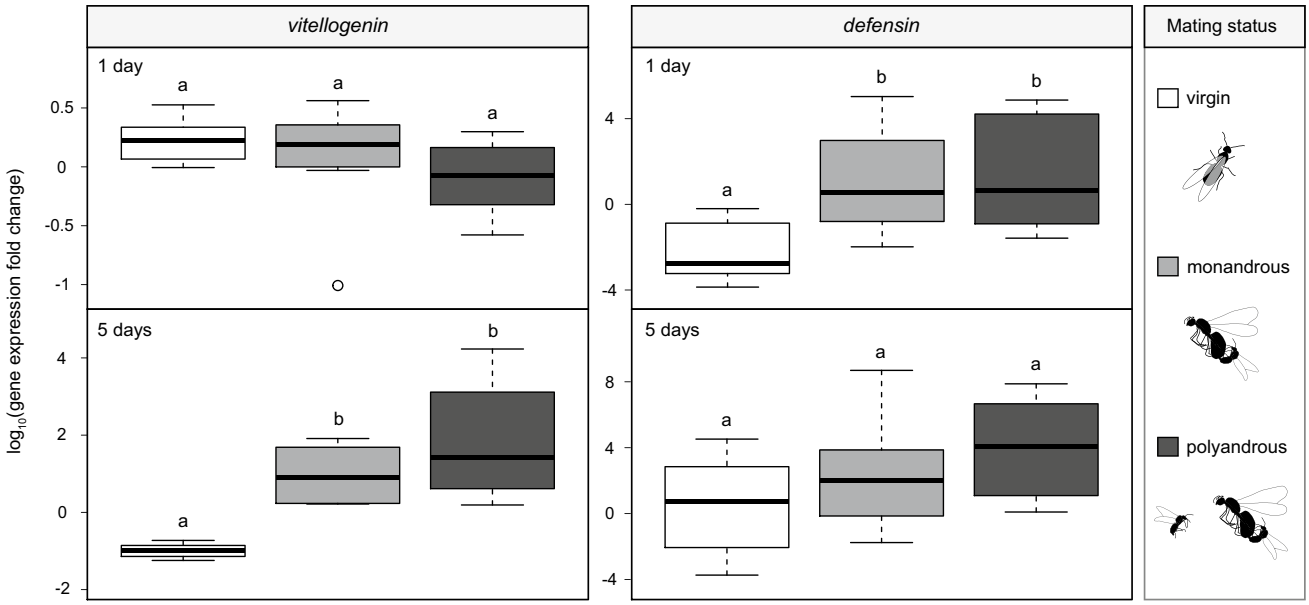

Fig. 1 Effect of mating on *vitellogenin* and *defensin* expression in *Lasius niger* ant queens. The box and whiskers plot shows $\log_{10}$ transformed relative fold changes in *vitellogenin* or *defensin* gene expression for virgin (no mating), monandrous (mating with a single male), and polyandrous (mating with two male) *Lasius niger* ant queens. Gene expression was measured 1 and 5 days after mating. Each box corresponds to gene expression measured in eight queens. The midline of each box is the median; the lower and upper edges of the boxes are the first and third quartiles, respectively. The whiskers extend to the most extreme data points that are less than 1.5 times the distance between the first and third quartile away from the lower or upper edges of the box. Above this distance, values are given as outliers (open circles). Letters above the upper whiskers indicate statistically significant differences (or not, when letters are the same) between virgin, monandrous, and polyandrous queens

et al. 2004—*D. melanogaster*, Lawniczak and Begun 2004; McGraw et al. 2004) and decreases female lifespan (*D. melanogaster*, Wolfner 2002—*A. socius*, Fedorka et al. 2004). Queens of ants as well as social bees and wasps mate on a single occasion early in life and will never engage in additional matings after this initial event. Our study on the expression of six genes likely to be involved in post-mating responses in females shows that mating triggers oviposition, and is followed by an up-regulation of *vitellogenin* and *defensin* gene expression. In contrast, we found no effect of mating on the expression of *prophenoloxidase, juvenile hormone esterase, insulin receptor 2,* and *Cu−Zn superoxide dismutase 1*. Monandrous and polyandrous queens always had similar levels of gene expression, indicating, to our knowledge for the first time, that ant queen-mating frequency does not influence post-mating regulation of the tested genes. Hereafter, we discuss our results in comparison with the previous studies in other social Hymenoptera and in insects, where remating is common.

One day after mating, there was no effect of mating status on *vitellogenin* expression. At this time, none of the queens had started laying eggs. Five days after mating, both monandrous and polyandrous queens had laid eggs and *vitellogenin* expression was significantly higher in mated queens compared to virgin queens that never oviposited. *Vitellogenin* up-regulation and initiation of oviposition were thus synchronous, indicating that the synthesis of vitellogenin, the major yolk protein precursor, is activated to start producing eggs. A similar result was found in honeybee queens, where queens 2 days after mating had similar levels of *vitellogenin* expression as virgin queens (Kocher et al. 2010), whereas egg-laying queens 5 days after mating had higher *vitellogenin* expression than virgin queens (Kocher et al. 2008). Likewise, queens of the ant *Solenopsis invicta* up-regulated *vitellogenin* after mating (Tian et al. 2004). Our results, therefore, confirm that, after mating, *vitellogenin* is up-regulated and oviposition is initiated. In *Drosophila*, mating also induces increased rates of oviposition, but these are prompted by male transferred proteins from the accessory glands (Wolfner 2002). If the male-induced increase in egg-laying rate is above the optimal level for females, the interests of both sexes become conflictual (Chapman et al. 2003). Male control over egg laying should be selected in species, where females seek additional mates after an initial reproductive bout. This is because, by stimulating female oviposition, males increase their reproductive success, as their mates will lay more eggs fertilized with their own sperm. The reproductive strategy of social Hymenoptera precludes such a mechanism. Males die after copulating and queens are restricted to using the sperm that they stored during mating,

since they never remate later in life. The reproductive success of both sexes thus depends entirely on the survival of the queen and her precious sperm stock, meaning that males would not benefit from coercing their mates into ovipositing especially if this has negative effects on queen physiology and/or survival. It thus seems unlikely that our finding of up-regulation of *vitellogenin* expression and oviposition in mated *L. niger* queens is under male control.

We found that *defensin* expression was significantly higher in mated queens than in virgin queens 1 day after mating but not 5 days after mating. This result is consistent with Dávila et al. (2018) who showed that *L. niger* queen antibacterial activity peaked 1 day after mating before decreasing drastically within the next few days. In addition, mating up-regulates the expression of antimicrobial peptides in *S. invicta* ant queens (Tian et al. 2004) and *Drosophila* females (Lawniczak and Begun 2004; McGraw et al. 2004). In honeybee queens, immune genes were also up-regulated after mating or instrumental insemination (Kocher et al. 2008, 2010; Niño et al. 2013; Manfredini et al. 2015); however, among these genes, the expression pattern of *defensin* differed across studies. These results suggest that, independently of the reproductive strategy, mating triggers the up-regulation of immune defense genes and that this resistance is likely to depend on the action of antimicrobial peptides. Whether such an immune response to mating is caused by an actual bacterial infection during copulation or acts as a prophylactic measure requires further study.

Prophenoloxidase, an important regulator of the melanization immune response, was not affected by queen-mating status. This contrasts with other studies in ants (Baer et al. 2006; Castella et al. 2009; Dávila et al. 2015), crickets (Fedorka et al. 2004), damselflies (Siva-Jothy et al. 1998), and mealworms (Rolff and Siva-Jothy 2002), reporting that melanization-related immune capacity decreases after mating. However, these studies all used either direct measures of enzymatic activity or of melanization capacity—not gene expression analysis. Hence, the absence of effect in our experiment could stem from a lack of correlation between *prophenoloxidase* gene expression intensity and levels of enzymatic activity.

It has been suggested that male insects achieve control over oviposition by either directly transferring juvenile hormone to females or by stimulating its synthesis using accessory gland proteins (Shirk et al. 1980, 1983; Borovsky et al. 1994; Moshitzky et al. 1996; Fan et al. 1999, 2000). Females, on the other hand, might be able to reduce male control over oviposition by increasing the rate of juvenile hormone degradation. Such a mechanism could rely on stimulating the synthesis of juvenile hormone esterase, the enzyme responsible for juvenile hormone hydrolysis (Gilbert et al. 2000). This idea remains very speculative as the only indication that such a process might take place comes from females of non-social moths, where levels of juvenile hormone esterase are higher after mating (Cusson and Delisle 1996). In social Hymenoptera, male-induced stimulation of oviposition, through, e.g., increasing juvenile hormone levels, is unlikely to exist (see above). In line with this, we found no effect of mating on the expression of juvenile hormone esterase in *L. niger* queens, suggesting that males do not manipulate egg-laying. This result does, however, not provide formal proof of the absence of male–female conflict in *L. niger* ants, as the regulation of juvenile hormone degradation probably depends on many different factors. To fully study male manipulation of oviposition through juvenile hormone, the whole synthesis pathway should be studied in detail as levels of juvenile hormone might be regulated by both its synthesis and degradation.

Mating status did not influence the expression of *insulin receptor 2*, a gene involved in the IIS pathway. In *Drosophila*, down-regulation of the IIS pathway increases lifespan (Tatar et al. 2001; Broughton et al. 2005) and in honeybees, old queens (long-lived) have lower expression of IIS genes (insulin-like peptide, *AmILP-1,* and its putative receptors, *AmInR-1* and *AmInR-2*) than old workers (short-lived) (Corona et al. 2007). Furthermore, Schrempf et al. (2005) showed that in the ant *Cardiocondyla obscurior*, mating increased queen lifespan. To gain a complete understanding of how mating influences the IIS pathway and might affect lifespan in ant queens, one would need to test all the genes involved in IIS. Especially, further studies are needed to determine if the regulation of the effect of mating on ant queen longevity depends on other pathways than IIS or on other IIS genes such as the *insulin-like peptide* and *insulin receptor 1,* whose expression levels were not examined in the present work.

In contrast with the previous results in honeybee queens (Weirich et al. 2002), *Cu–Zn superoxide dismutase 1* expression did not vary between virgin and mated *L. niger* queens, suggesting that this antioxidant does not play a role in regulating mating-associated oxidative stress in this species. Superoxide dismutase is not the only antioxidant found in insects (Felton and Summers 1995) and *L. niger* queens could thus rely on other antioxidant enzymes such as catalases and peroxidases. Interestingly, vitellogenin has an antioxidant effect in honeybee workers (Seehuus et al. 2006). The post-mating up-regulation of *vitellogenin* discussed above might, therefore, also help protect queens from oxidative stress after mating.

None of the tested genes were influenced by the number of males queens had mated with (single or double mating). In *Atta colombica*, a strictly polyandrous ant, immune capacity was negatively correlated with the number of males that contributed to the sperm stored by queens (between two and five) (Baer et al. 2006). *L. niger* queens are facultatively polyandrous, and in our experiment, all

polyandrous queens had mated only twice. The impact of polyandry on ant queen physiology might thus only be detected when queens are obligatorily polyandrous and/or mate with a substantial number of males.

The present study was designed as an initial test of the effect of mating on social Hymenoptera queen physiology. Given that the subject is still scarcely studied, we included a range of different factors (i.e., genes involved in various pathways, time after mating, and mating frequency) to identify the most fruitful path for further experiments. Such an exploratory design meant that we did not focus on studying one particular factor in detail, as we did not have clear-cut predictions.

Overall, our work confirms the previous results in both social Hymenoptera and insects with remating promiscuity, showing that mating is followed by oviposition and an up-regulation of *vitellogenin* and antimicrobial peptide expression. Conversely, the expression of *prophenoloxidase*, *juvenile hormone esterase*, *insulin receptor 2,* and *Cu−Zn superoxide dismutase* is not affected by queen-mating status which differs from findings either in other species of social Hymenoptera or in female insects that mate repeatedly throughout life. More generally, our results indicate that certain impacts of mating on female insect physiology are generalized across species independent of their mating strategies, while others seem to be species specific. Furthermore, queen-mating frequency does not affect gene regulation providing a first result relative to the effect of multiple mating on queen physiology. We hope that these results will help to guide researchers interested in studying post-mating effects in social Hymenoptera queens in their selection of the genes to study, the time to study them and whether or not to control for mating frequency. Future studies could concentrate on investigating, for example, tissue-specific gene expression and/or quantifying final protein levels of defensin and vitellogenin in mated *L. niger* queens to improve our understanding of the mechanisms underlying their up-regulation after mating.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The species *Lasius niger* is not listed on the IUCN Red List of Threatened Species. The ants were handled humanely in accordance with current ethical standards. Special ethical approval is not required to carry out this study.

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