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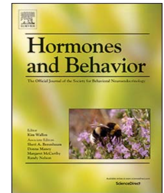

Measuring *inotocin receptor* gene expression in chronological order in ant queens

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ABSTRACT

In vertebrates and invertebrates, oxytocin/vasopressin-like peptides modulate a variety of behaviors. The recent discovery of the gene and receptor sequences of inotocin, the insect ortholog of oxytocin/vasopressin, opens new opportunities for understanding the role of this peptide family in regulating behaviors in the most populated class of living animals. Ants live in highly organized colonies. Once a year, they produce future queens that soon leave the nest to mate and found new colonies. During the first months of their lives, ant queens display a sequence of behaviors ranging from copulation and social interactions to violent fighting. In order to investigate the potential roles of inotocin in shaping queen behavior, we measured gene expression of the *inotocin receptor* in the heads of *Lasius niger* ant queens at different points in time. The highest levels of expression occurred early in queen life when they experience crowded conditions in their mother nests and soon thereafter set out to mate. Inotocin could thus be involved in regulating social and reproductive behaviors as reported in other animals. While oxytocin and vasopressin are also involved in aggression in mammals, we found no direct link between these behaviors and *inotocin receptor* expression in *L. niger*. Our study provides a first glimpse into the roles the inotocin receptor might play in regulating important processes in ant physiology and behavior. Further studies are needed to understand the molecular function of this complex signaling system in more detail.

1. Introduction

The existence of oxytocin/vasopressin-like peptide signaling has been documented in both vertebrates and invertebrates, and is believed to have evolved from an ancestral peptide before the split between Proto- and Deuterostomia, 640–760 million years ago (Acher and Chauvet, 1995; Douzery et al., 2004). Vertebrates have two peptide homologs, oxytocin and vasopressin, whereas invertebrates usually have only one. In either case, receptors belong to the G protein-coupled receptor family (Beets et al., 2013; Donaldson and Young, 2008; Gimpl and Fahrenholz, 2001). Oxytocin/vasopressin-like peptides regulate a wide range of peripheral physiological processes. In particular, oxytocin plays a major role in stimulating uterus contractions during parturition in mammals (Gimpl and Fahrenholz, 2001), and in annelids and possibly also mollusks, oxytocin/vasopressin-like peptides are involved in egg-laying behaviors (Fujino et al., 1999; Oumi et al., 1996; Van Kesteren et al., 1995; Wagenaar et al., 2010). The peptide family also has a number of important central effects in the invertebrate and vertebrate brain (Beets et al., 2013; Feldman et al., 2016; Gimpl and Fahrenholz, 2001; Gruber, 2014). For instance, both vertebrate oxytocin (Borrow and Cameron, 2012; Goodson, 2013) and invertebrate oxytocin/vasopressin-like peptides (Gruber, 2014) have been linked to reproductive behaviors in several species. In rodents (Dore et al., 2013; Young and Wang, 2004), birds (Goodson et al., 2009; Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012) and even humans (Carter et al., 2009; Hurlemann et al., 2010; Kosfeld et al., 2005; Rilling et al., 2012), the peptide appears to be involved in mediating a range of social interactions such as pair bonding and cooperation. In contrast, vasopressin and oxytocin also seem to modulate certain aggressive behaviors in both rodents and humans (Pagani et al., 2013).

Oxytocin/vasopressin signaling and function have been primarily studied in vertebrates. Surprisingly, the role of these peptides in shaping behavior and physiology remains largely unknown in insects, which represent 58% to 80% of all animal species (Scudder, 2009). The oxytocin/vasopressin-like peptide in insects was first discovered in the locust *Locusta migratoria* (Orthoptera) (Proux et al., 1987). Later, the genes coding for this same oxytocin/vasopressin-like peptide and its G protein-coupled receptor were identified in the genome of the red flour beetle *Tribolium castaneum* (Coleoptera) (Aikins et al., 2008; Stafflinger et al., 2008). Stafflinger et al. (2008) named the peptide inotocin, for insect oxytocin/vasopressin-like peptide, and showed that inotocin strongly activated its receptor in vitro. The gene expression of the

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inotocin preprohormone matched that of the receptor in different body parts and developmental stages in *T. castaneum* (Stafflinger et al., 2008). To date, the gene sequences of inotocin and its receptor have been identified in the genomes of over 100 insect species from at least 20 representative orders (Gruber and Muttenthaler, 2012; Liutkeviciute et al., 2016; Stafflinger et al., 2008). However, inotocin peptides and receptors are absent from the genome sequences of certain orders, namely Diptera and Lepidoptera, suggesting that inotocin signaling is restricted to basal insect lineages (Liutkeviciute et al., 2016; Stafflinger et al., 2008).

Ants are excellent biological models for studying the functional role of inotocin, as they display diverse and remarkable behaviors. They live in highly organized societies characterized by reproductive division of labor, whereby one or a few individuals (the queens and the males) specialize in reproduction, whereas the others (the workers) forego their own reproduction to participate in cooperative tasks such as building the nest, collecting food, rearing the young or defending the colony (Hölldobler and Wilson, 1990). Typically, once a year mature colonies produce new queens and males that soon leave the safety of their mother colony to mate during large nuptial flights. After mating, males die, while newly mated queens land on the ground and start searching for an adequate nesting site to establish their future colony. Once queens have settled in their new nest, they start to lay eggs and will continue doing so for the rest of their decade long lives while never mating again (Boomsma, 2013, 2009, 2007). Young mated ant queens can either found their colony alone (haplometrosis) or in cooperation with one or more unrelated co-founding queens (pleometrosis) (Bernasconi and Strassmann, 1999; Hölldobler and Wilson, 1977). Pleometrotic colony foundations are usually more competitive and survive better compared to haplometrotic foundations. However, pleometrosis is a risky endeavor since in most species queen cooperation breaks down at the time of emergence of the first workers, as queens engage in violent fights where only the winner survives and continues to head the colony alone.

We measured, to our knowledge for the first time, the gene expression levels of the *inotocin receptor* during insect reproductive, cooperative and aggressive behaviors, using *Lasius niger* ant queens as a biological model. In this species, queens can found their colony either by haplometrosis or pleometrosis, allowing us to investigate both scenarios. We compared the expression levels of the *inotocin receptor* at specific points in time, from sexual maturity to one year after mating, i.e. when queens sequentially engage in mating, social interactions, and fighting. Relative mRNA levels were measured by quantitative PCR in virgin queens, newly mated queens, haplo- and pleometrotic founding queens and one year old queens. Expression levels were quantified in the queen head, to exclude any potential background noise that could arise from involvement of inotocin in physiological processes in other parts of the body.

2. Materials and methods

2.1. Queen collection and rearing

Virgin and freshly mated queens were collected in Brussels, Belgium, during June and July 2015 and 2016. We first collected virgin queens by excavating three colonies during the course of one day, approximately two weeks before the nuptial flight (referred to as *young virgin queens* hereafter). At the onset of the nuptial flight, we collected virgin queens preparing to take off from the entrances of three different colonies (referred to as *flight ready virgin queens*) (Fig. 1). Young virgin queens ($n = 6$) and flight ready virgin queens ($n = 6$) were snap-frozen in liquid nitrogen immediately after collection. During the nuptial flight, when queens start landing after mating, we collected a large batch of freshly mated queens on the ground. A first, randomly selected, group of these ($n = 6$) were killed by snap-freezing within two hours (time needed to complete the sampling and bring queens back to the laboratory) after collection (referred to as *freshly mated queens*) (Fig. 1). The rest were installed into artificial nests (glass tubes divided into three parts: water, a cotton plug and a dry area for the actual nest space) either by themselves (haplometrosis) ($n = 30$) or two by two (pleometrosis) ($n = 36$). Pleometrotic queen pairs were assigned at random. After the emergence of the first workers (ca. 30 days after the mating flight) colonies were given ad libitum sugar water and mealworms.

The nesting haplometrotic and pleometrotic founding queens were randomly assigned to separate groups that were snap-frozen either, one, 20, 40 or 80 days after mating (n = 6 queens in each group) (Fig. 1). For haplometrotic queen nests, we added an extra group consisting of queens that were killed one year after mating ($n = 6$). For pleometrotic queen nests, we added two extra groups in which queens were killed either during or one day after their fight for colony rule (each, $n = 6$). Because we were interested in measuring gene expression according to queen behavior, and not by a comparison between winners and losers during fights, we sampled only one of the two foundresses from pleometrotic foundations. Before the fights (i.e., one, 20 and 40 days after mating) and during the fights, the queens used for gene expression measurements were selected at random, as it was not straightforward to predict which queen would win the fight (Aron et al., 2009; Holman et al., 2010; Sommer and Hölldobler, 1995). After the fight (i.e., one day after the fight and 80 days after mating), the queen used was invariably the winner, as the loser always died during or soon after the fights, precluding their use for gene expression quantification.

After snap-freezing, all queens were stored at − 80 °C until further use.

2.2. RNA extraction and reverse transcription

We chose to use whole heads and not only brains as the latter are small and timely to dissect which might jeopardize RNA integrity due to degradation during dissection. Queens were removed from storage at − 80 °C and their heads were immediately homogenized in TRI Reagent® Solution (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a Mixer Mill MM301 (Retsch GmbH, Haan, Germany) using 2.8 mm Precellys® zirconium oxide beads (Bertin Corp, Rockville, Maryland, USA). Total RNA was then extracted following the TRI Reagent® Solution RNA isolation protocol (TRI Reagent® Solution, RNA/DNA/Protein Isolation Reagent, RNA Isolation Procedure, 2010, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The amount of extracted RNA was measured with a Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and RNA quality and integrity were verified using Nanodrop™ absorption ratios and band inspection after electrophoresis on a 2% agarose gel.

Each individual queen head RNA sample was then treated with DNase I, Amplification Grade (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to remove traces of genomic DNA (Deoxyribonuclease I, Amplification Grade, Protocols, 2002, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Reverse transcription of the mRNA was carried out on 160 ng of DNase I treated total RNA using Oligo(dT)$_{20}$ Primer (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and SuperScript™ III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's protocol (SuperScript™ III Reverse Transcriptase, First-Strand cDNA Synthesis, 2004, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The reverse transcription program consisted of 60 min at 50 °C, followed by 15 min at 70 °C. To control for genomic DNA contamination in the following quantitative PCR step, we included a no reverse transcription (no RT) control containing water instead of reverse transcriptase for each sample. cDNA samples and no RT controls were diluted 10 times in Buffer AE (Qiagen, Venlo, Netherlands) and stored at − 20 °C until gene expression analysis.

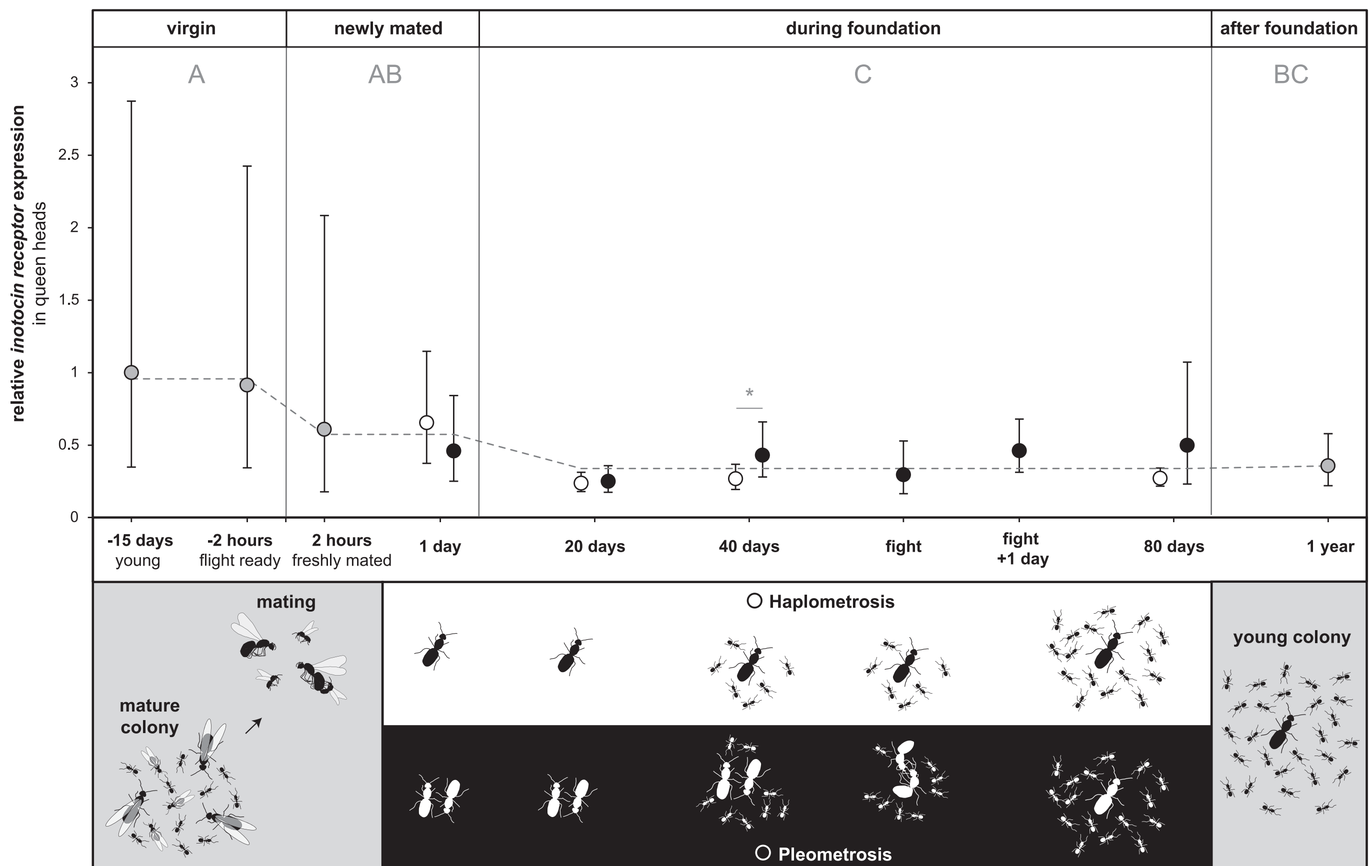

Fig. 1. Relative *inotocin receptor* expression in *Lasius niger* ant queens.
Mean transformed (Willems et al., 2008) relative *inotocin receptor* gene expression (± 95% CI) in the heads of *Lasius niger* queens is represented in the following groups: virgin (young virgin queens, ca. 15 days before mating; flight ready queens, ca. 2 h before mating), freshly mated (ca. 2 h after mating), haplometrotic foundation (one, 20, 40 and 80 days after mating; white dots) and pleometrotic foundation (one, 20, 40 and 80 days after mating, during and one day after queen fights; black dots) and one year after mating. Each mean comprises six queen replicates. *Inotocin receptor* expression was calibrated relative to young virgin queens (mean = 1). Schematic ants under the graph illustrate the changing environment experienced by queens and the associated behaviors. Significant statistical differences between virgin, newly mated, founding and one year old queens are indicated by capital letters in the top center of each panel. Significant statistical difference between haplometrotic and pleometrotic founding queens 40 days after mating is indicated with an asterisk.

Table 1
Primer sequences and melting temperatures.

Gene	Forward primer	Reverse primer	Tm (°C)
inotocin receptor	ACGTCCAAATATTATGTCACCATCATC	CTTGCGAAGTACCAGTGATCATG	61.5
elongation factor 1α	ACCTAACCATGAGGAGCATTTCG	TTGTCTAAATCGGTGACTGGATACG	61.9
ribosomal protein L8	AGCCCTTAATCTCCAAAGCAATG	AATGGGAAACTGGTGGCAATATAC	62.8

2.3. Gene expression

The putative sequence of the *inotocin receptor* in *L. niger* was identified by BLAST® (Altschul et al., 1990) searching (tBLASTn and BLASTn) a transcriptome (courtesy of L. Keller) for sequences homologous to those previously identified in *Nasonia vitripennis* (GenBank accession no. NM_001172274.1) and *Tribolium castaneum* (GenBank accession no. NM_001085361.1) (Stafflinger et al., 2008). During the course of this study, Di Giglio et al. (2017) identified, cloned and characterized the inotocin signaling system in *L. niger* (GenBank accession no. AOC59508.1). As demonstrated previously in *T. castaneum* (Stafflinger et al., 2008), they showed that the inotocin peptide strongly activated the *L. niger* receptor in vitro. The partial gene sequence they identified as coding for the *inotocin receptor* was identical to the one we had selected. We chose two stably expressed (verified with geNorm; Vandesompele et al., 2002) housekeeping genes: *elongation factor 1α* (GenBank accession no. EU143083.1) and *ribosomal protein L8* (GenBank accession no. EE049695.1), whose mRNA sequences had previously been identified in *L. niger*. Primer pairs were designed with Primer3Plus (Untergasser et al., 2012) to amplify sequences between 80 and 100 bp depending on the gene in question. Their specificity was tested by PCR followed by electrophoresis on a 2% agarose gel which produced single bands of the desired length for each gene (*inotocin receptor*, 85 bp; *elongation factor 1α*, 82 bp; *ribosomal protein L8*, 97 bp). Finally, after subsequent quantitative PCR, specificity was also verified by melt curve analysis which yielded a single melting temperature per amplicon (78 °C for the three genes). Primer sequences are reported in Table 1.

Quantitative PCRs were run on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each reaction consisted of 2 μl cDNA, 10 μl SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus) (Takara, Kusatsu, Shiga, Japan), 0.4 μl ROX Reference Dye (Takara, Kusatsu, Shiga, Japan), 0.4 μl of both forward and reverse primers and 6.8 μl H_2O. Samples were run in triplicates and no RT controls were always included. The quantitative PCR reaction had the same steps for all three genes: 2 min at 95 °C,

followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 70 °C. Reactions were carried out in accordance with the manufacturer's protocol (SYBR® *Premix Ex Taq*™ (Tli RNaseH Plus), Product Manual, Protocol, 2016, Takara, Kusatsu, Shiga, Japan). At the end of the last cycle melt curves were run. In order to determine the amplification efficiency of our primer pairs, we ran standard curves for each gene. For each group of queens investigated (young virgin queens; flight ready virgin queens; freshly mated queens; haplometrotic founding queens: one day, 20 days, 40 days, 80 days and 1 year after mating; pleometrotic founding queens: one day, 20 days, 40 days, 80 days after mating as well as during the fight and one day after the fight), we measured gene expression for six replicate queens.

To control for inter-run variations, replicate samples were always run on the same plate as one of the young virgin queen replicates, so that for every group of queens, each of the six replicates could be calibrated relative to one of the six young virgin queen replicates. Relative expression ratios, between replicate queen samples and their corresponding young virgin queen calibrator sample, were calculated using the method devised by Pfaffl (2001).

2.4. Statistical analysis

Inotocin receptor relative expression ratios were transformed according to the method proposed by Willems et al. (2008). To control for the variation introduced by the fact that each replicate queen sample was calibrated relative to one of the young virgin queen samples, we used within subject statistical tests with the identity of the calibrator as the within subject factor.

For each time point common to haplometrotic and pleometrotic foundations, we used a within subject *t*-test to determine if *inotocin receptor* expression varied between the two modes of foundation.

We used three separate one-way within subject ANOVAs to test if *inotocin receptor* expression significantly varied between groups of queens sampled either before colony foundation (young virgin queens, flight ready virgin queens and freshly mated queens), during haplometrotic colony foundation (one day to one year after mating), and during pleometrotic colony foundation (one day to one year after mating).

To test whether *inotocin receptor* expression varied over the whole period of time investigated (from before mating to one year later), while avoiding losing too much statistical power due to the large number of queen groups tested (14 groups in total with 6 replicates per group), we assigned our data to four biologically relevant supergroups: virgin queens (young virgin queens and flight ready queens), newly mated queens (freshly mated queens and queens one day after mating), founding queens (haplometrotic and pleometrotic queens from 20 to 80 days after mating), and one year old queens. These supergroups are biologically meaningful as they each correspond to a different stage of queen life. Before mating, virgin queens live inside crowded colonies where they are constantly subject to interactions with their nestmates. During the first hours and days after mating, queens experience a radically different environment, as they have to find an adequate nest site to start their future colony. Once queens have settled in their new nests, they enter the colony foundation stage where they have to care for their brood and raise their first workers. Finally, one year after mating, queens will concentrate solely on egg-laying as they have produced a substantial worker force that will undertake the logistical tasks of the colony. We used the mean expression per young virgin queen calibrator in each supergroup as replicate data for a one-way within subject ANOVA.

P-values for the above tests were all Benjamini-Hochberg adjusted to account for the fact that we used the same data for several different tests. Because the Tukey HSD *post-hoc* test does not support within subject designs, we used multiple paired *t*-tests with Benjamini-Hochberg correction as *post-hoc* tests. We report effect sizes as classical Cohen's d (d) for within subject t-tests and as generalized eta squared (η_G^2) (Olejnik and Algina, 2003) for the one-way within subject ANOVAs. All statistical tests were carried out in RStudio (R Core Team, 2015).

3. Results

Inotocin receptor was expressed in queen heads from all the tested groups. Expression did not significantly differ between haplometrotic and pleometrotic queens (one day after mating: $t_5 = 0.92$, $P = 0.4$, d = 0.72; 20 days after mating: $t_5 = -0.28$, $P = 0.4$, d = 0.23; 80 days after mating: $t_5 = -2.44$, $P = 0.12$, d = 1.77), except 40 days after mating where pleometrotic queens had slightly higher expression levels than haplometrotic ones ($t_5 = -3.48$, $P = 0.047$, d = 2.68) (Fig. 1).

We found no significant difference in *inotocin receptor* expression between queen groups sampled before colony foundation (young virgin queens, flight ready virgin queens and freshly mated queens; $F_{2,10} = 1.54$, $P = 0.35$, $\eta_G^2 = 0.23$) (Fig. 1). For queens sampled during colony foundation, there was a significant overall difference in *inotocin receptor* expression between queen groups in haplometrotic foundations (queens one, 20, 40, 80 days and 1 year after mating; $F_{4,20} = 5.79$, $P = 0.01$, $\eta_G^2 = 0.53$). However, this difference did not survive the Benjamini-Hochberg correction applied to the *post-hoc* test results (Fig. 1). In pleometrotic foundations, *inotocin receptor* expression did not significantly vary between queen groups (queens one, 20, 40, 80 days and 1 year after mating, as well as during and one day after the fight; $F_{6,30} = 1.36$, $P = 0.35$, $\eta_G^2 = 0.21$).

Inotocin receptor expression significantly differed between the four supergroups (virgin queens, newly mated queens, founding queens and one year old queens; $F_{3,15} = 8.37$, $P = 0.01$, $\eta_G^2 = 0.59$). Virgin and newly mated queens had similar expression levels that were both significantly higher than those of founding queens (Fig. 1). Virgin queens also had higher expression levels than one year old queens (Fig. 1).

4. Discussion

The objective of this study was to measure *inotocin receptor* gene expression in ant queens engaging in reproduction, social interactions and overt aggression. We detected *inotocin receptor* expression in all tested *Lasius niger* queen heads. This result, together with the recent demonstration of the in vitro activation of the *L. niger* inotocin receptor by the inotocin peptide (Di Giglio et al., 2017), indicates that inotocin signaling might very well be functional in this ant species. Hereafter, we discuss the possible relationship between the level of *inotocin receptor* expression and queen behavior in a chronological fashion.

Before the nuptial flight, virgin queens spend the first weeks of their lives in their crowded mother colony and experience repeated interactions with their nestmates. Our data indicate that virgin queens had high levels of *inotocin receptor* expression compared to founding queens (Fig. 1). Oxytocin and vasopressin promote social recognition (recognition of familiar conspecifics) and social learning (copying behaviors) in rodents (Dore et al., 2013), while mesotocin, the oxytocin homolog in birds, favors familiar associations in zebra finches (Goodson et al., 2009). Inotocin could play a similar role in ants, as the high expression of the *inotocin receptor* in virgin queens could facilitate the establishment of social behaviors inherent to colony life. The level of *inotocin receptor* expression in newly mated queens (ca. 2 h and one day after mating) was comparable to that of virgin queens and higher than that of founding queens (Fig. 1). The maintenance of such a high expression around the time of mating, compared with the significant decrease in gene expression in founding queens 20 days after mating, implies that inotocin could play a part in copulation behaviors in insects, as it is the case for oxytocin/vasopressin-like peptides in other invertebrates (Gruber, 2014) and for oxytocin in vertebrates (Borrow and Cameron, 2012; Goodson, 2013).

Once they have mated, *L. niger* queens drop to the ground, start

searching for a nesting site, and found their colonies alone or with the help of a co-foundress. No overall differences in *inotocin receptor* expression were observed between haplometrotic and pleometrotic queens (Fig. 1). In vertebrates, oxytocin/vasopressin signaling is involved in pair bonding (prairie voles (Young and Wang, 2004;), zebra finches (Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012)) and promotes trust, empathy and cooperation in humans (Carter et al., 2009; Hurlemann et al., 2010; Kosfeld et al., 2005; Rilling et al., 2012). The lack of consistent *inotocin receptor* up-regulation in co-founding compared to solitary queens suggests that inotocin does not play a role in social bonding between *L. niger* queens. Co-founding, in this species, serves as a means of increasing nest survival chances during early foundation by enhancing worker production and ability to resist interspecific brood raids (Sommer and Hölldobler, 1995). Co-founding queens might therefore not engage in social interactions, but instead tolerate each other in order to benefit from the higher survival success associated with pleometrosis.

We found no change in brain *inotocin receptor* levels of pleometrotic fighting queens (Fig. 1). This finding was unexpected as vasopressin and oxytocin seem to modulate a variety of aggressive behaviors in rodents and humans (Pagani et al., 2013). The lack of association between the stable *inotocin receptor* expression and queen fighting does not preclude inotocin from being involved in the mediation of aggressive behaviors. Indeed, stable *inotocin receptor* expression in queen heads during foundation might be permissive, thus allowing the onset of queen-queen fighting.

5. Conclusion

In conclusion, our work suggests that the *inotocin receptor* is expressed in *Lasius niger* queens. Together with the finding of potent receptor activation by the inotocin ligand in this species (Di Giglio et al., 2017), our results indicate that the inotocin signaling system may have an important functional role in ant physiology. However it is important to keep in mind that we did not investigate the full inotocin signaling system. To understand details and possible roles of this signaling system in ant behavior it is imperative to further study and dissect the system, for instance by measuring inotocin precursor expression, as well as conducting genomic knock-down combined with behavioral and physiological experiments.

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