

# Inotocin: The Key to Ant Compassion?

By

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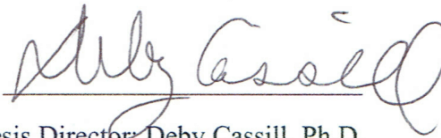
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Honors Thesis

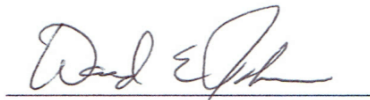
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## **Abstract**

In an ant colony, eggs are continuously laid by the queen and gently cared for by her sterile worker daughters. The queen and her sterile worker daughters care for the helpless brood (eggs, larvae and pupae), cleaning, feeding and protecting in a way analogous to a human mother caring for her helpless, newborn child. For humans and other mammals, oxytocin and vasopressin neurotransmitters promote social bonding, including maternal caretaking. The oxytocin/vasopressin neurotransmitters have been found in a number of invertebrates as well. In ants, the neuropeptide, inotocin, an analog to oxytocin, has been found. However, the function of inotocin in ant behavior is unknown. In this study, we explored the link between inotocin expression and maternal care behavior in the red fire ant, *Solenopsis invicta*. Our hypotheses were that inotocin would be expressed in mated queens and their sterile worker daughters, but not in their virgin (unmated) sons or daughters. Reverse transcriptase polymerase chain reaction (RT-PCR) and subsequent gel electrophoresis were used to determine if the inotocin gene in the fire ant was expressed. In a pilot study, we use unmated, winged males that do not participate in maternal care of the young. As hypothesized, inotocin was not expressed by unmated males. Although it has been confirmed that inotocin exists in ant species, including the fire ant, further research is required to determine if inotocin is expressed in mated queens and sterile worker daughters known to participate in maternal care of brood.

## **Introduction**

The red fire ant, *Solenopsis invicta*, is a eusocial insect species that lives in colonies composed of many individuals with specialized tasks. A mature colony can number to around

100,000 and 500,000 ants (Capinera, 2008). These many individuals work cooperatively to feed, protect, and ensure the future of the colony. Members of a fire ant colony are divided into a caste system with polymorphic differences between castes. The worker caste is divided into minor and major workers: minor workers are smaller, younger, and are tasked with tending to the brood and foraging for food, while the major workers are larger, older, and protect the rest of the colony (Mirenda and Vinson, 1981). As a worker ant develops, it undergoes morphological changes. The head and legs lengthen proportionately to the body, while the antennae lengthen at a slower rate. The head width increases to change the profile of the ant from a barrel-shape into a relative heart-shape. Major workers, also called soldiers, sport large mandibles that help them defend the nest and attack invaders, and they tend to have larger heads than minor workers. This allometric growth, where different parts of the body grow at different rates, characterizes the physiological difference between the worker types (Tschinkel, Mikheyev, and Storz, 2003).

Both minor and major workers are sterile females. The only members of the colony who are capable of reproduction are the mated queen, the virgin queens, and the male drones. Females and males differ in color: females are red with a brown or black gaster, and males have an entirely black body (Hedges, 1997). A mated queen is wingless, as are the workers. Meanwhile, both virgin queens and drones possess wings, which they use to fly from their colonies to engage in nuptial flight. This mating period can happen at any time of the year, but spring and early summer are favored for warmth. The ideal conditions of a nuptial flight also include a recent rain. As they fly, virgin queens emit attractant pheromones from their poison sacs to draw males to them (Capinera, 2008). A drone's one purpose in life is to deliver his sperm to a queen, and he dies soon after mating (Hölldobler and Wilson, 1990). A queen usually mates only once, and she

stores the sperm in an organ contained in her abdomen, the spermatheca, which she uses to fertilize her eggs all throughout her lifetime (Tschinkel, 1987). After mating, the queen lands and sheds her wings, proceeding to search for a spot to establish a new colony (Capinera, 2008).

In Hymenoptera species including the red fire ant, sex determination is determined by haplodiploidy. With this method of sex determination, unfertilized haploid eggs develop into males, and fertilized diploid eggs develop into females (Hölldobler and Wilson, 1994). Whether a female larvae will develop into a virgin queen or a worker is based primarily on environmental conditions such as the size of the colony, temperature, and food quality (Frohschammer and Heinze, 2009).

A newly mated queen lays between 10 to 15 eggs within the first 24 hours of mating. For this first batch of offspring, the queen is solely responsible for feeding the larvae regurgitated oils, trophic eggs, or salivary gland secretions. The muscles of her shed wings also break down to provide nutrients for the young (Vinson and Sorenson, 1986). The first workers are called minims, who are smaller than average workers due to being cared for by the queen alone with limited resources, instead of a ready team of nurse workers with previously accumulated resources. The queen releases a set of lactones called queen recognition pheromones, which are unique to her and draw in her daughter workers to encourage their caretaking and protection of her. One of her maternal behaviors is licking the eggs as grooming. She also coats them in venom, possibly to encourage care and protection like the queen recognition pheromones (Taber, 2000).

In this study, the fire ant equivalent of oxytocin and vasopressin is of interest. For mammals like humans, oxytocin has been popularized as the neurotransmitter responsible for

feelings of love and happiness. Discovered in 1906 by Sir Henry Dale, it is a substance made from the posterior pituitary gland that stimulates contractions during labor and lactation. The name oxytocin comes from the Greek words *oxus* and *tokos*, meaning “swift birth” (Viero et al., 2010). Previous studies have demonstrated that oxytocin signaling is closely associated with social cognition in mammals. Increased levels of oxytocin expression in the brain are shown to increase trust, recognition of other individuals, monogamy, and maternal behavior after childbirth (Shen, 2015). This social bonding is significant because it promotes fitness of the group, protection against predators and environmental changes, brain development, and cognitive abilities (Neumann, 2009).

A closely related neurotransmitter to oxytocin is vasopressin—also called arginine vasopressin, or antidiuretic hormone (ADH) (Dorland, 2011). Both oxytocin and vasopressin are exclusive to mammals, have nine amino acids each, and have a disulfide bridge constraining their respective structures (Choleris, Pfaff, and Kavaliers, 2013). As a physiological function, vasopressin is released into the bloodstream from the posterior pituitary and controls the constriction of blood vessels. It also controls the body’s ability to retain water by stimulating water re-absorption in the kidneys (Marieb, 2014). However, when released into the brain, vasopressin and oxytocin together have been shown to play a significant role in social functioning. As previously mentioned, the oxytocin/vasopressin signaling system is implicated in maternal behavior, trust and cooperation between individuals, and modulating fear and anxiety responses (Wójciak, Remlinger-Molenda, and Rybakowski, 2011). However, the functions of vasopressin are suggested to be more focused on body homeostasis, such as regulating blood pressure, water levels, arousal, and memory. Meanwhile, the functions of oxytocin are more

focused on outward homeostasis like behavioral maintenance, lactation, and the birthing process (Stoop, 2012). These differences in function could suggest that oxytocin and vasopressin act as complementary opposites to each other as an individual navigates through social interactions (Legros, 2011).

In a 1979 study by Cort Pedersen and Arthur Prange, female virgin rats that were given injections of oxytocin to the brain displayed maternal behavior towards unfamiliar pups. For a testing period of 10 days, the rats showed consistent maternal behavior, which included actions like nest building, grooming pups' fur, and returning lost pups to the nest. Meanwhile, injections of vasopressin did induce maternal behavior but not with the same consistency as oxytocin, and injections of saline did not induce the behavior. However, the female virgin rats who expressed prolonged maternal behavior from the oxytocin injections were also in varying stages of estrus, so elevated levels of estrogen could have also been a factor in activating the behavior. In a more recent study from Marlin et al. in 2015, female virgin mice were indifferent towards unfamiliar pups, but an injection of oxytocin to the brain similarly led to maternal behavior like picking up crying pups in their mouths. Analysis of the left auditory cortex of the brain showed that the oxytocin injection enhanced the female mouse's response to pups' cries.

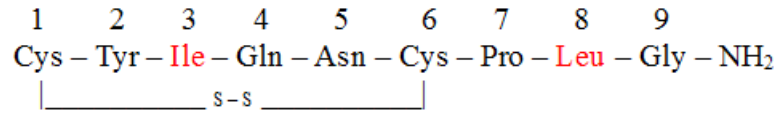
Other neuropeptides in non-mammalian species have previously been found that are structurally similar to the oxytocin/vasopressin signaling system. Segmented worms have annetocin, lungfish have mesotocin and vasotocin, and bony fish have vasotocin and isotocin, among others (Stoop, 2012). In invertebrates like roundworms, leeches, earthworms, and snails, these orthologs of the oxytocin/vasopressin system are thought to be involved in reproduction (Gruber, 2014). For example, conopressin in the great pond snail *Lymnaea stagnalis* expresses

oxytocin-like reproductive functions and vasopressin-like metabolic functions (Van Kesteren et al., 1995). The duck leech *Theromyzon tessulatum* expresses the mRNA of annetocin in the genital tract, ovary, and brain (Levoye et al., 2005). In the earthworm *Eisenia foetida*, injections of annetocin have been observed to induce egg-laying behavior like body shape changes, mucous secretion for cocoon formation, and the act of egg-laying itself (Oumi et al., 1996).

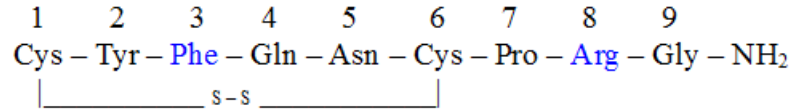
Inotocin, an insect-specific type of neuropeptide that is not found in all insects, has also been found to be structurally similar to the mammalian oxytocin/vasopressin signaling system (Stafflinger, 2009). Ants are among the insects that have been found to express inotocin. Recent studies have isolated inotocin from a select few ant species: the leaf-cutter ant *Atta cephalotes*, the carpenter ant *Camponotus floridanus*, the jumping ant *Harpegnathos saltator*, and the black garden ant *Lasius niger* (Gruber and Muttenthaler, 2012; Di Giglio et al., 2017). In the ants from the 2012 Gruber and Muttenthaler study, the precursor proteins of inotocin show structural similarities to the oxytocin/vasopressin equivalents of other species, as well as similar intron sites and overall lengths. Meanwhile, the ant inotocin sequences show amino acid variations on positions 2 and 4 (where Leu replaces Tyr and Thr replaces Gln, respectively). The 2017 Di Giglio et al. study isolated inotocin from *L. niger*; analyzing its pharmacological properties for use as a probe for human vasopressin receptors. While it has been confirmed that inotocin exists in these ant species, further research is required to determine exactly how ant behavior is affected by inotocin.



**Oxytocin:**



**Vasopressin:**



**Inotocin:**

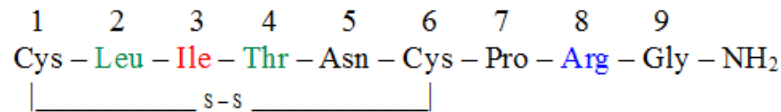
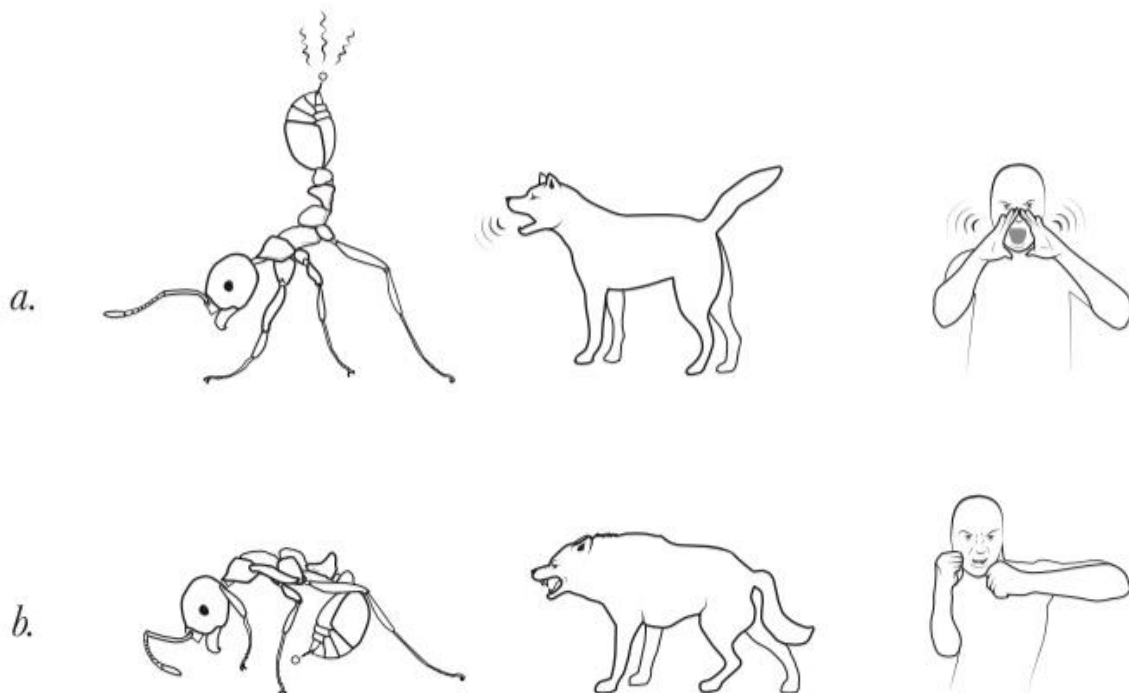


Figure 1: Comparison of oxytocin, vasopressin, and inotocin structures. All three are 9 amino acids in length with an amino group at the end and a disulfide bridge connecting the cysteines at positions 1 and 6. Red shows amino acids specific to oxytocin, blue for vasopressin, and green for inotocin.

The oxytocin/vasopressin system plays a significant role in reproduction and social behavior, and it shares structural and possibly functional similarities with inotocin. From this, it could be hypothesized that inotocin is the stimulant for social bonding in invertebrates that express such action. An oxytocin/vasopressin ortholog such as inotocin could serve as the internal trigger for bonding behaviors in social insects like ants, such as the maternal caretaking displayed by a queen ant carefully handling her eggs.

In the fire ant, movement of the abdominal section of the ant's anatomy has been observed while warning unfamiliar ants, eating, and tending to eggs, larvae, and pupae.

Abdominal movement in response to unfamiliar ants is termed “gaster flagging,” where the ant points its stinger upward as a warning and sprays venom when threatened (Drees and Oi, 2015). However, this behavior is distinctly different while eating or tending to the brood. In one study, the abdominal wagging of *S. invicta* did not attract or repel other individuals, did not involve the spraying of venom, and occurred longer and more often than in gaster flagging. This led to the speculation that abdominal wagging while eating or tending to the brood was a non-aggressive expression, possibly a behavior that expressed pleasure (Cassill, Ford, Huynh, Shiffman, & Vinson, 2016).



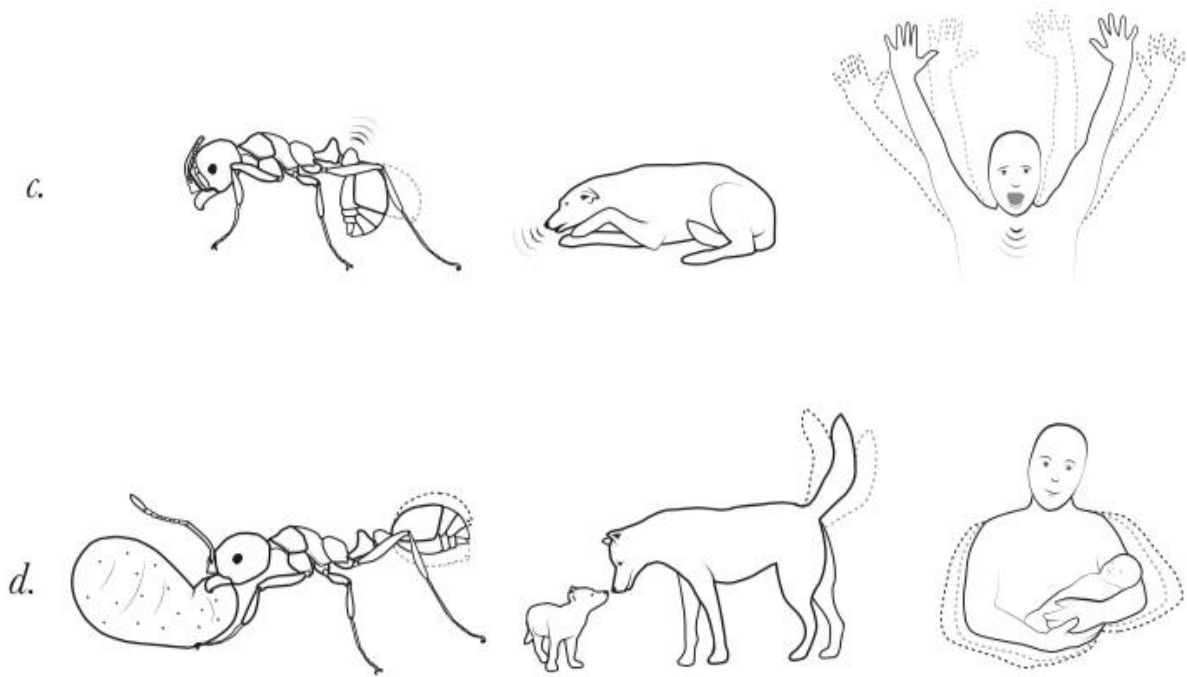


Figure 2: Postures displayed by fire ant workers are equivalent to those displayed by canines and humans. (a) Alerting family members of an invasion (respectively, emitting a chemical pheromone; vocalizing in the form of barking; vocalizing in the form of yelling, bugles or sirens). (b) Aggression in the form of a physical attack (respectively, stinging; biting; punching). (c) Call for help when buried or injured (respectively stridulating; whimpering; calling for help). (d) Expression of pleasure when interacting with young family members (respectively, abdominal wagging; tail wagging, arm rocking). Note: weapons such as the ant's stinger with venom, the dog's canines and the human's fists are not displayed during the call for help or the expression of pleasure. Graphic art by Elizabeth Bonert. Used with permission (Cassill et al., 2016).

This non-aggressive behavior, particularly while caring for the young, may be controlled by a chemical mechanism. With its relationship to the oxytocin/vasopressin system that is so closely involved in social bonding and maternal behavior, inotocin may be the mechanism that triggers maternal care expressed by the queen and worker ants that handle the young. The following experiment is a pilot study in identifying the presence of the inotocin gene in *S. invicta*. We hypothesized that inotocin would be expressed in mated queens and their sterile worker daughters, but not in their virgin (unmated) sons or daughters. This study and future experiments ultimately seek to support a link between inotocin gene expression and maternal care in red fire ants.

## Methods

We took an existing gene sequence for inotocin (CLITNCPRGGKR) from the red flour beetle, *Tribolium castaneum*, from previous research by Gruber and Muttenthaler in 2012. Using the red flour beetle gene sequence and the NCBI BLAST program, we designed forward and reverse primers for the inotocin receptors of the red fire ant. The primers were picked to give an approximately 350 base pair fragment from mRNA. We then ordered the primers from Eurofins. Their nucleotide base sequences are as follows:

Forward Primer (5' to 3'): TACCATGCCATCTGCCATCC

Reverse Primer (5' to 3'): AGGATCTCGATTTCGCTGGC

We re-suspended the dry-frozen primer stock to 100  $\mu$ M in nuclease-free water and made 10  $\mu$ M working dilutions. We ground up fire ant drones and extracted a RNA sample from them using the Qiagen Rneasy kit. Using the Access-Quick RT-PCR system from Promega, we applied

the process of reverse transcriptase PCR, or RT-PCR, to create and amplify complementary DNA from the RNA sample. We used this process because inotocin is a neuropeptide. When an ant encounters a stimulus such as a chemical signal or pheromone that would trigger maternal behavior, transcription factors bind to DNA to control the transcription of certain mRNA. The transcribed mRNA would presumably contain the expression of inotocin, which we can then change into cDNA and amplify for quantifiable results.

We placed a 50  $\mu$ L reaction sample into a Bio-Rad T100 thermal cycler to allow for the PCR amplification process to proceed. The process contains three steps: denaturation, annealing, and primer extension. Heat denatures the DNA helix into separate strands, which allows for the primers to bind to their target DNA sequences. Once the primers have become bound, DNA polymerase extends the sequence. The enzyme *Taq* polymerase from the thermophilic bacteria *Thermus aquaticus* was used for this experiment. The cycle then repeated itself 40 times. The thermal cycler settings were as follows:

Lid: 105°C		
Volume: 50 $\mu$ L		
Step	Temperature (°C)	Time (1:00=1 min)
1	45	45:00
2	95	2:00
3	95	0:30
4	58	0:45
5	72	0:30
6	Go to Step 3, 40X	
7	75	5:00
8	4	$\infty$

Table 1: Thermal cycler settings for PCR.

After RT-PCR, the sample underwent gel electrophoresis. With a solidified agarose gel submerged with a thin layer of tris acetate EDTA buffer at the gel surface, we loaded two wells in the gel using a 100  $\mu$ L pipette: one well was filled with 10  $\mu$ L of sample, and the other was filled with 7  $\mu$ L of DNA ladder. In gel electrophoresis, the well side of the gel is negatively charged, while the other end is positively charged. The buffer solution allows the charge to flow through the gel. The phosphate groups on a strand of DNA cause the DNA to have a negative charge, and DNA loaded into the wells would then be attracted to the positive charge of the opposite end of the gel. The agarose gel contains many pores through which DNA strands can travel towards the positive end. Like a sieve, the gel's pores separate the DNA strands by size: shorter strands travel farther, while longer strands travel shorter distances. With the system set on approximately 110.9 Volts, we ran the gel for approximately 30 minutes.

After this time period, we removed the gel from the electrophoresis system and placed it on a short-wave UV tray. We then illuminated the gel with UV light to read the bands that resulted from electrophoresis, comparing the bands made by the experimental sample to the bands of the DNA ladder.

## **Analysis**

The results of the gel electrophoresis are pictured in Figure 3 below. The left well represents the post-PCR sample, and the right well represents the DNA ladder of fragment sizes. The top band of the ladder is 2000 base pairs long, and the lowest band is 50 base pairs long.

We estimated the target inotocin gene sequence to be around 350 base pairs. The gray and yellow areas in the left well column are remnants of the loading dye, while the lower orange area

is the experimental sample. The band for the experimental sample is located lower on the gel than the ladder's 50 bp band. Instead of the target inotocin gene sequence, a primer-dimer was discovered. This means that the primers attached to each other and were amplified instead of the target inotocin DNA sequence. This suggests that little to no target DNA was present in the post-PCR sample, and the expression of the inotocin gene was not found in virgin males.



Figure 3: Experimental gel electrophoresis results (left photo) and DNA ladder values (right photo). The yellow and gray colorations in the experimental column (left well of gel results) are the loading dye. The orange coloration is the band for the experimental sample. When compared to the ladder (right well of gel results) and its corresponding bp lengths, the experimental sample represents a primer-dimer.

## Conclusion

Drones of *S. invicta* do not engage in the maternal care behavior shown by the queen and female workers who handle the eggs and larvae. After mating with a queen during nuptial flight, drones quickly die, and their purpose is complete (Hölldobler and Wilson, 1990). The experimental results of this study did not find evidence of inotocin expression, which corresponds to this absence of maternal care in drones. Further testing is required to support positive inotocin gene expression from mated queens and worker daughters who do engage in maternal care like feeding and grooming the young brood.

Errors in the experimental procedure could have been from a number of sources. The primer sequences or the volumes of components used in the working sample could have been incorrect. Other errors in procedure could have occurred, but more testing is needed to confirm that the primer sequences, volume amounts, and other steps in procedure will accurately isolate the inotocin gene from a sample that should contain the desired DNA expression.

Future studies will seek to troubleshoot this process and find a positive control that suggests positive inotocin gene expression in red fire ants that express maternal care.

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