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It has become increasingly evident that genomic recombination is an evolved trait that varies between and within species. The honey bee has an extremely high genomic recombination rate but the responsible mechanisms have not been studied. Based on the hypothesis that meiotic recombination and DNA damage repair share common mechanisms in honey bees, I predicted that oxidative stress leads to a further increase in recombination rate. This prediction was directly tested by subjecting honey bee queens to oxidative stress by paraquat injection and measuring the rates of genomic recombination in select genome intervals of their offspring before and after injection.

Of 27 intervals compared only 13 experienced an increase in the rate of recombination in the post-injection sample set as compared to the pre-injection set, suggesting no significant experimental effect. This result was confirmed when the analysis was restricted to the 16 intervals whose pre-injection recombination fractions were within ±8% of the value that was predicted based on a pre-existing linkage map. Intervals did not see consistent upregulation in all colony sample sets where upregulation occurred either. Overall the evidence does not support the hypothesis that oxidative stress induces an increase in the rate of genomic recombination.

TESTING THE EFFECTS OF OXIDATIVE STRESS ON GENOMIC RECOMBINATION IN THE HONEY BEE, *APIS MELLIFERA*

by

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CHAPTER I

INTRODUCTION

The honey bee has a high genomic recombination rate but the mechanisms that are responsible for this are not clear. I predict that oxidative stress leads to a further increase in recombination rate, based on the hypothesis that meiotic recombination and DNA damage repair share common mechanisms. My goal is to test this prediction by determining whether oxidative stress triggers an increase in genomic recombination in selected regions of the honey bee *A. mellifera*.

Honey Bee Importance and Biology

Honey bees are the most important crop pollinators known to man, and they are on the decline in many countries in Europe and North America (Morse & Calderone, 2000; Vanengelsdorp & Meixner, 2010). Knowing with what they interact in the environments to which they are exposed, and how these interactions affect their biological systems is central to developing a comprehensive understanding of what is driving their population decline. The honey bees' use as a model organism for various studies including aging, social evolution, phenotypic plasticity, and oxidative stress response due to the distinct differences between its three castes also makes it a very attractive organism to work with (Aamodt, 2009; Jemielity, Chapuisat, Parker, & Keller, 2005; Keller & Jemielity, 2006; Robinson & Weaver, 2006; Weinstock et al., 2006).

Honey bees are eusocial and live in colonies. The colony members can be divided into three castes. Drones are the haploid males, only having 16 chromosomes. They have the longest developmental period of the three castes at 24 days, a lifespan of about 50 days, and serve only a reproductive role, not contributing to hive productivity or construction (Stone, 2005). Workers and queens are both diploid females, but phenotypically they are very different. Workers typically emerge 21 days after the egg is laid, have a short lifespan of about one month during the summer, and perform all the essential maintenance functions necessary for the hive. From various glands they produce honey, royal jelly, wax, and propolis. The roles they serve in the colony vary depending on the age of the worker. The first four days after emergence are usually spent hardening their exoskeleton and activating their various secretory glands. The next seventeen days after that are spent on hive maintenance duties, such as feeding larvae, making wax, and regulating the temperature the hive. During the summer the last part of a worker's life is spent foraging for nectar, pollen, water, and propolis. During the winter the workers stay in the hive and cluster on the comb, attempting to conserve heat and food. Workers typically live months longer during the winter, partly due to their lack of foraging activities (Flottum, 2010; Stone, 2005). Queens are the egg layers of a colony, and there is usually only one queen per colony. They are the longest lived caste, typically having a lifespan of about 2-3years, and have the shortest developmental period at around 14 days. Immediately after emergence in her hive, a new queen will seek out any other queen cells and kill them. If the old queen has not left to find a new colony somewhere else before the new queen emerges, then the new queen will kill the old queen as well.

Within the first 5 to 15 days of emergence queens will go on orientation flights outside of the hive, after which they will go on mating flights and mate with up to 45 drones, storing the sperm in an organ called the spermatheca that will keep it viable for years. They are larger than workers and have longer abdomens, which coincides with having more developed ovaries and a greater number of ovarioles than workers (Flottum, 2010; Stone, 2005).

Both queens and workers develop from the same kind of egg. The phenotypic differentiation is triggered by feeding the young female larva with or without royal jelly (Flottum, 2010; Stone, 2005). Royal jelly is a vitamin and hormone rich secretion containing a histone deacetylase inhibitor called 10HDA, which alters the epigenetic programming of the larval DNA causing it to express the queen phenotype rather than the worker phenotype (Spannhoff et al., 2011). There are some key phenotypic differences between the queen and worker castes that are of significance for my study. An analysis of the proteomes of developing queen and worker larvae revealed that there are 120 differentially expressed proteins between queens and workers, 91 of which are up regulated in queens (Begna, Han, Feng, Fang, & Li, 2012). Another study showed that the second biggest group of differentially upregulated proteins in queens are the antioxidant proteins and that these proteins are critical in caste polymorphism (Li et al., 2010). One of the most significant of these proteomic differences is that queen bees produce a much higher amount of vitellogenin and a lower amount of juvenile hormone than workers. Vitellogenin is an egg yolk protein produced by the fat cells, which contributes to a queen's increased longevity by acting as an antioxidant and, specifically

in bees, by inhibiting intrinsic juvenile hormone levels (Corona & Robinson, 2006; Corona et al., 2007; Seehuus, Norberg, Gimsa, Krekling, & Amdam, 2006). At one week of age, vitellogenin levels are about 900-fold higher in queens than in workers (Corona et al., 2007). Queens also express an ortholog of mammalian NTH1 and a unique fusion protein consisting of a domain homologous to bacterial *mut*T and one domain homologous to mitochondrial ribosomal protein gene S23, both of which are thought to be involved in the prevention and repair of oxidative damage (Aamodt, 2009). Queens have much higher resistance to oxidative stress than workers as a result of these proteomic differences between castes (Aamodt, 2009; Corona, Hughes, Weaver, & Robinson, 2005; Corona & Robinson, 2006; Corona et al., 2007; Li et al., 2010; Seehuus, Krekling, & Amdam, 2006), which is important to consider when testing the effects of oxidative stress in bees.

Oxidative Stress

Oxidative stress is most broadly defined as being an imbalance between the production of oxidizing molecules and the production of cellular antioxidants, with the imbalance in favor of the pro-oxidants (Boelsterli, 2007). It is called oxidative stress because although there are many molecules that participate in oxidation and reduction reactions, the primary family of molecules that are involved in producing oxidoredutive stress are reactive oxygen species (ROS). Molecular oxygen (O_2) itself is a biradical with two unpaired electrons in their outer valence shells that have the same spin (Boelsterli, 2007). If one of these unpaired electrons takes up energy and changes its spin, it

becomes a ROS called singlet oxygen. Should molecular oxygen become reduced, gaining an electron, it becomes a reactive anion called superoxide (O_2^-). If superoxide gains another valence electron its net negative charge could attract positively charged protons, which upon binding to the reactive oxygen molecule turn it into hydrogen peroxide (H_2O_2). Should the hydrogen peroxide molecule interact with cellular ferrous iron before being detoxified, it would undergo a fenton reaction to become the highly reactive hydroxyl radical (HO^-). Each of these ROS, as the name implies, are highly chemically reactive. While this property makes them harmful, it also makes them necessary for aerobic life to function and they are produced as a normal by product of physiological processes (Boelsterli, 2007; Cooke, Evans, Dizdaroglu, & Lunec, 2003).

The main way in which oxidative stress is held in check in living cells is through the production of antioxidants. While there are a wide variety of antioxidant molecules, most of them perform the same basic functions. These functions include scavenging ROS, keeping cellular thiol redox status in the reduced form, preventing or repairing the oxidation of lipids, and sequestering redox-active metals like iron (Boelsterli, 2007). The honeybee genome encodes 38 different antioxidants, which are more highly expressed in queens than in workers, particularly old workers (Corona et al., 2005; Corona & Robinson, 2006; De Loof, 2011; Haddad, Kelbert, & Hulbert, 2007; Weirich, Collins, & Williams, 2002). These include, but are not limited to peroxidation-resistant membranes and the egg yolk protein vitellogenin. While it is well known that antioxidant genes are upregulated in response to oxidative damage, producing even more antioxidants as

needed, these systems can be overwhelmed (Boelsterli, 2007; Cooke et al., 2003; Mannuss, Trapp, & Puchta, 2012).

One of the most effective ways to overwhelm the antioxidant defense system is to introduce a xenobiotic that rapidly produces ROS. One such xenobiotic is a herbicide commonly used in America; a quaternary ammonium bipyridyl chemical by the name of N,N′-dimethyl-4,4′-bipyridinium, commonly known as paraquat (Bus & Gibson, 1984). Paraquat is known to induce oxidative stress in a variety of organisms and so is often employed as a model for oxidative stress. Among the many types of oxidative damage that this substance causes, it is well-established to induce DNA damage (Ali, Jain, Abdulla, & Athar, 1996; Bus & Gibson, 1984; Cooke et al., 2003; Keyer & Imlay, 1996; Lehmann, 2005; Mannuss et al., 2012; Pogozelski & Tullius, 1998; Ross, Block, & Chang, 1979; Singh, T., Tice, & L., 1988; Tokunaga, Kubo, Mikasa, Suzuki, & Morita, 1997) .

Paraquat's method of inducing oxidative stress is based on its redox cycling reactions that take place once it is inside a cell. Paraquat very quickly reduces oxygen, producing superoxide, which then gets processed by superoxide dismutase to become hydrogen peroxide, and hydrogen peroxide then reacts with cellular iron in what is known as a fenton reaction to become the highly reactive hydroxyl group. These free radicals can induce single and double strand breaks in the DNA chain, oxidize nucleotide bases, and trigger changes in the epigenome. Hydroxyl radicals cause DNA strand breaks by way of abstracting protons from the deoxyribose chain (Ali et al., 1996; Cooke et al., 2003; Pogozelski & Tullius, 1998).

The exact mechanism of hydrogen abstraction by hydroxyl radicals involves their net negative charge, which gives them an affinity for positively charged atoms and molecules. Multiple studies have shown that hydroxyl radicals attack deoxyribose and even DNA bases at all available positions, though it may favor some positions in the DNA chain over others due to structural availability (Cooke et al. 2003; Pogozelski and Tullius 1998). Pogozelski and Tullius elucidate biochemical pathways by which hydrogens at each position in the deoxyribose group on a nucleotide in B-DNA, except for the structurally inaccessible 2' hydrogen, can be abstracted by oxygen free radicals. They show that the pathway of cleavage is dependent on the helical structure of DNA. In the minor groove of B-DNA the most accessible, and most oxidized, hydrogens are those at the 4' and 5' positions on deoxyribose. In the major groove of B-DNA the most accessible hydrogen seems to be the one at the 3' position. Although a minor contributor to DNA strand scission, the 2' deoxyribose position can be made vulnerable to hydrogen abstraction through a base oxidation of guanine to 8-oxoguanine while guanine is still in the nucleotide pool. More often, the 2' hydrogen is attacked in ribose in RNA, in processes involving excited states, or by the presence of adjacent halogenated bases (Cooke et al. 2003). Incidentally, 8-oxoguanine can be used as a marker to confirm DNA damage, which can be detected via an ELISA assay (Tokunaga et al., 1997).

Strand breaks generated via hydrogen abstraction can either be single or double strand breaks, the frequency of which would depend on the relative concentration of hydroxyl radicals inside the nuclear envelope. It would be reasonable to assume, given the random nature of the hydrogen abstraction reaction that single strand breaks would be

more common. Double strand breaks (DSBs) trigger DNA repair mechanisms that involve the crossing over of DNA strands of neighboring sister chromatids (Mannuss et al., 2012; Slupphaug, 2003). Since many of the same mechanisms are active in meiotic recombination as in double strand break repair (see below) I predict that oxidative stress may cause an increase in the rate of meiotic recombination.

Meiotic Recombination

The process of meiotic recombination is one that is studied in model organisms such as the budding yeast *S. cerevisiae* (Neale & Keeney, 2006). The mechanisms by which meiotic recombination operates have not been confirmed in the honeybee *A. mellifera*. Therefore, the mechanisms described below are taken as a model of how meiotic recombination might occur in queen bees.

During the process of meiosis a eukaryotic organism generates gametes via two rounds of cell division and chromosome segregation to ensure that euploidy is maintained in each generation. Before homologous chromosomes are separated into daughter cells, a specialized pathway of homologous recombination takes place during prophase I, which is illustrated in Figure 1. During prophase I the newly replicated chromosomes condense to where they can be seen under a microscope and the homologues, each having two chromatids, pair up into a tetrad. Before the formation of a chiasma an enzyme called Spo11 cleaves double stranded DNA and binds to the DNA at the site of cleavage (Neale & Keeney, 2006). The Spo11-DNA complex is then removed by endonuclease. The 5' strands are degraded to yield 3' single stranded DNA tails, also known as sticky ends.

Recombinases Rad51 and Dmc1 bind to these sticky ends and facilitate the crossing over of DNA strands with the homologous chromatid on the paired homologous chromosome (Neale & Keeney, 2006; Szostak, Orr-Weaver, Rothstein, & Stahl, 1983). These sites at which strand crossover have occurred are called chiasma. After strand invasion forms a D-loop in the intact chromatid, DNA synthesis is primed from the invading 3' end (Neale & Keeney, 2006; Szostak et al., 1983). The displaced loop from the intact chromatid captures the other sticky end and primes a second wave of DNA synthesis. Ligation of both of these sticky ends yields a double Holliday Junction (dHJ). Resolution of the dHJ can happen in two different ways. One way is that both outer, strands of the dHJ are resolved by an endonuclease causing a crossover event leaving two chromatids with completely exchanged flanking DNA. Alternatively the inner strands of the dHJ could be resolved, leading to a non-crossover gene conversion event (Neale & Keeney, 2006; San Filippo, Sung, & Klein, 2008; Szostak et al., 1983).

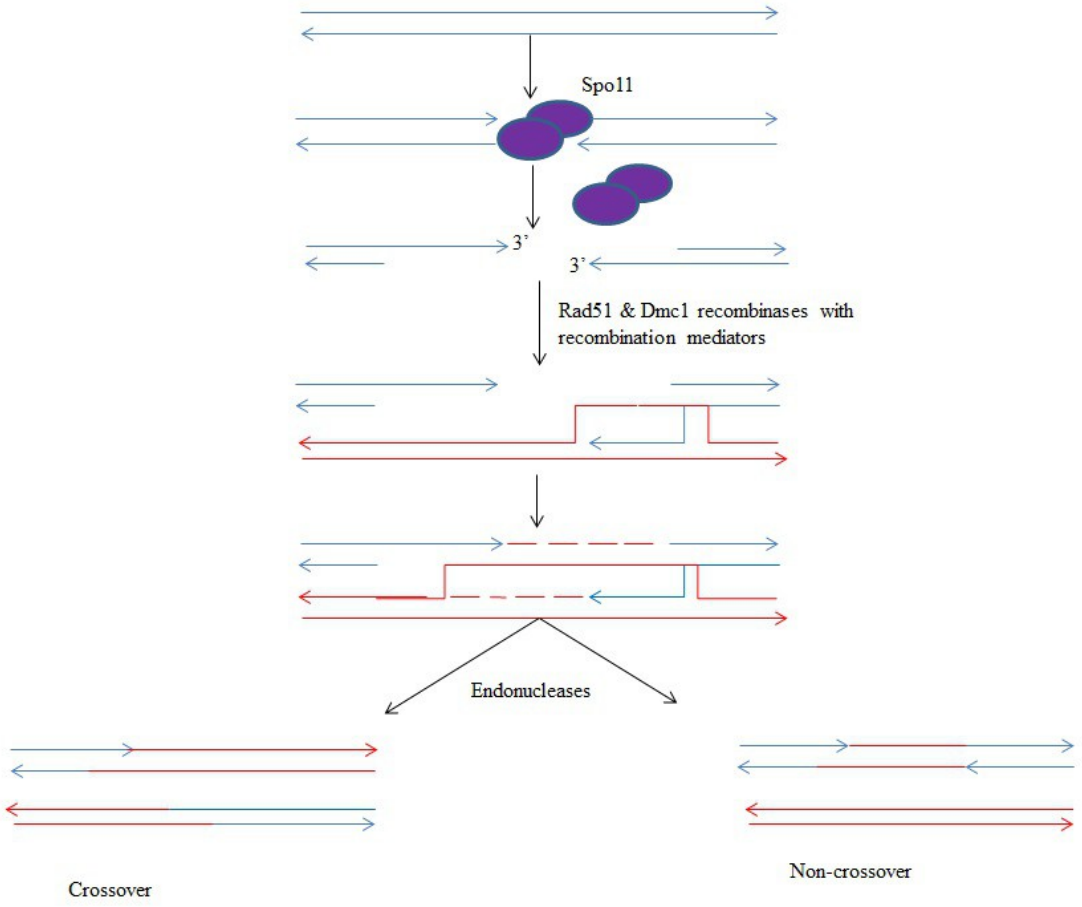

Figure 1. Diagram of how meiotic recombination may occur in the honeybee. DSBs are induced, probably by a Spo11 homologue. Endonuclease removes Spo11, allowing exonuclease to degrade 5' ends to yield 3' overhang. Recombinases and recombination mediator proteins bind to ssDNA and trigger strand invasion. DNA polymerase enters the D-loop and starts DNA synthesis. D-loop extends to 3' end on the other side of the break, forming dHJ. Endonucleases resolve the dHJ such that meiosis either results in a crossover event or a non-crossover event.

Honey bees have the highest meiotic recombination rate of any metazoan (Beye et al., 2006; Beye et al., 1999; Hasselmann & Beye, 2006; Meznar, Gadau, Koeniger, & Rueppell, 2010). This is unusual because genomic recombination rates are normally negatively correlated with size of an organism's genome because only one or two crossing over events per homologous chromatid pair occur in most species (Meznar,

Gadau, Koeniger, & Rueppell, 2010). This is not the case for *A. mellifera*, which averages over 5 crossing over events per homologous pair (Beye et al., 2006). Given 2n=32 chromosomes containing about 250Mb, this translates into a disproportionally high recombination rate of between 19 and 22cM/Mb (centiMorgans per megabase). In comparison consider that the pine tree *Pinus pinaster* has 2n=24 chromosomes and a recombination rate of 0.07cM/Mb, and the bumblebee *Bombus terrestris* has 2n=36 chromosomes and a recombination rate of 4.4cM/Mb (Chagné et al., 2002; Civetta, Wilfert, Gadau, & Schmid-Hempel, 2006; Meznar et al., 2010).

One of the few other social Hymenoptera to have a linkage map, the leafcutter ant *A. echinatior*, revealed a high recombination rate of 6.2cM/Mb with a chromosome number of 2n=36 (Sirvio et al., 2006). The common wasp *V. vulgaris*, another social Hymenoptera with a recently produced linkage map, has a recombination rate of 9.7cM/Mb and a chromosome number of 2n=50 (Sirvio, Johnston, Wenseleers, & Pamilo, 2011). While not as high as that of *A. mellifera*, these are still high compared to other insects. In comparison, six Diptera species all have recombination rates around 1.14cM/Mb (±1.03), three Lepidoptera species mapped at having 4.74cM/Mb (±1.84), and four Coleoptera species as having a recombination rate of 2.48cM/Mb (±1.31) (Wilfert, Gadau, & Schmid-Hempel, 2007).

Genetic control of meiotic recombination has been better characterized in model organisms other than *A. mellifera* and it is from these model organisms that most of our understanding of the regulation of meiotic recombination is derived. A previous study identified many genes critical to meiotic recombination in *Drosophila melanogaster* the

most statistically significant of which were *mei-P22* and *mei-P26*, in which high levels of nondisjunction occurred when these genes were mutated (Sekelsky et al., 1999). Sekelsky et al. had also identified novel meiotic phenotypes for the genes *CycE* (codes for CyclinE, which is important for the transition from G_1 phase to S phase), *Trl* (codes for a multi-purpose transcriptional activator and chromatin remodeling protein), *amn* (encodes a neuropeptide, PACAP38, the deficiency of which increases nod^{DTW} nondisjunction), *push* (the expression of which is important for male virility), *αTub67C* (encodes α-tubulin that is required for female meiosis and early embryonic division), and *ncd* (codes for kinesin-like motor protein needed for formation and stabilization of bipolar spindle). In the worm *C. elegans*, many genes were identified, including a Spo11 homologue, which induces double strand breaks, and *mnT12* and *meT7*, which seem to be involved in regulating chiasma formation (Dernburg et al., 1998; Hillers & Villeneuve, 2003). The most well-characterized organism for mechanisms of meiotic recombination, the yeast *S. cerevisiae*, has a multitude of characterized genes specific to regulating meiotic recombination including several RAD genes, SPOs, DMCs, MSCs, and many others that code for proteins involved in recombination and its regulation that also appear to be conserved in various degrees across several eukaryotic clades (San Filippo, Sung, & Klein, 2008; Szostak et al., 1983; Thompson & Stahl, 1999). These genes, having not been well-characterized in bees, are used as a model for which genes might regulate meiosis in *A. mellifera* since they appear to be conserved between species. It has been shown that genes involved in both DNA repair, chromatin remodeling, cell cycle, DNA

metabolism, and DNA replication are upregulated in response to DSBs, with genes related to HR being especially upregulated (Mannuss et al., 2012).

Another process similar to meiotic recombination is the DNA double strand break repair (DSBR) pathway of homologous recombination (HR). DSBR during mitosis involves a complete crossing over of the arms of sister chromatids in the stages of resectioning, strand invasion, the formation of a double Holliday Junction, and resolution by restriction endonuclease nicking of the junctions (San Filippo et al., 2008).

Given the mechanistic similarities between meiotic recombination and DSBR, and the fact that the two processes share some of the same proteins like the Rad51 recombinase and recombination mediator proteins like BRCA2 and Hop2, it is likely that in addition to its roles in promoting genetic diversity in offspring and ensuring proper chromosome segregation during the formation of gametes, meiotic recombination may also act as an improvised DNA repair mechanism (Dernburg et al., 1998; Gasior, Wong, Kora, Shinohara, & Bishop, 1998; Ghabrial, Ray, & Schupbach, 1998; Mao et al., 2011; Neale & Keeney, 2006; Pierce, Johnson, Thompson, & al., 1999; Szostak et al., 1983). In fact DSBR is considered a likely model for how meiotic recombination occurs (Szostak et al., 1983).

Even though it has been demonstrated that DSBs are needed in order for crossovers to occur not all DSBs result in crossover events (Dernburg et al., 1998; Pâques and Haber 1999; San Filippo, Sung, & Klein, 2008; Szostak et al., 1983). It is thought that the average frequency of crossover-associated gene conversions is 35% (Pâques and Haber 1999). There are three ways that a DSB gets resolved, two of which results in non-

crossover gene swapping. One method, as illustrated earlier, is if the outer DNA strands of the dHJ are resolved by endonucleases. Another method, not illustrated here, is synthesis-dependent strand annealing where the D-loop is unwound before the formation of a dHJ and the freed ssDNA strand anneals with the 3' overhang of the opposite end of the DSB. What seems to drive crossover events in *Drosophila* is a gene identified as REC (Blanton, Radford et al. 2005). REC is a MCM8 homologue that drives crossover events by acting during the repair synthesis step of meiotic recombination (Blanton, Radford et al. 2005). It is unknown whether honeybees also have a MCM8 homologue in their genome, or how such a homologue would be affected by oxidative stress.

Paraquat has been shown to be able to indirectly induce strand breaks in the double helix via the generation of ROS (Cooke et al., 2003; Pogozelski & Tullius, 1998; Singh et al., 1988). In the process of inducing strand breaks, double-strand-breaks (DSBs) have been shown to accumulate in the presence of ROS generated by paraquat (Salvo, Bracesco, Buccino, & Nunes, 1996). During meiotic recombination DSBs are induced by Spo11 to initiate the recombination process (Neale & Keeney, 2006). Therefore, DSBs induced as a result of paraquat exposure could lead to an upregulation of repair mechanisms, like Rad51, that would in turn lead to more recombination. It is important to note, however, that this has not been experimentally demonstrated in the honey bee or any other organism.

Hypothesis and Prediction

It is my hypothesis that meiotic recombination will be upregulated in response to DNA damage caused by oxidative stress. The basis for this hypothesis is that some of the same enzymes participate in both double strand break repair and meiotic recombination. Because of these shared mechanisms meiotic recombination might act as an improvised DSB repair pathway. Based on my hypothesis, I predict that the genotyping of paired markers in the offspring of honey bee queens before and after paraquat injection will reveal an increase in the rate of recombination after paraquat injection.

CHAPTER II

METHODS AND MATERIALS

One major experiment was conducted to test my prediction that oxidative stress increases recombination rates in queen honey bees. Oxidative stress was induced in the treatment group with paraquat injections. Offspring of the experimental queens were collected before and after the treatments and genotyped at several linked microsatellite loci to determine local recombination rates for each inter-marker interval. The necessary experimental steps are described in the following sections.

General Bee Keeping

The essential tools for general bee keeping are the smoker, hive tool, and protective gear (bee suit and veil). For the most part a protective veil was worn in combination with light colored cloths to reduce the risk of stinging, though a full body bee suit was employed after the light colored clothes proved to be insufficient protection. Most of the time thick elbow length gloves with elastic bands at ends to prevent bees from crawling into the cloths were worn to protect hands and wrists from being stung by workers. A hive tool is a small pry bar that was used to pry hive boxes apart from one another when the bees stuck sections of the Langstroth hive together with propolis. The hive tool was also used to aid in removing frames that were difficult to get out of the hive and in removing excess wax and propolis from hive. The smoker was used to mask

the presence of bee pheromones and confuse the bees in the interest of preventing the workers from attacking should a worker get crushed or attack the beekeeper (Flottum, 2010). On how to light a smoker and what to do with it after done working among beehives, I refer to "The Backyard Beekeeper: An Absolute Beginner's Guide to Keeping Bees in Your Yard and Garden" (Flottum, 2010).

To obtain a set of experimental queens, I produced a set of new queens by grafting. Grafting queens requires the presence of a queenless hive with many young workers, as well as a normal hive with an actively egg-laying queen. First a brood frame containing first-instar larvae was collected from the normal hive. Then, placing the brood frame on a grafting stand under a dissection microscope, first instar larvae were transferred into plastic queen cups with a grafting tool. Extra care was taken not to crush the larva in the process. The plastic queen cell cup with grafted larva was placed on a cell cup holding frame with the opening facing downward. The cell cup holding frame was placed into the queenless hive and the workers typically tended to the surviving larvae and fed them royal jelly. The cell cup holding frame was checked seven to eight days later to see which cell cups had been built into mature queen cells. The queen cells that were capped were placed in small three frame queenless nuclear (nuc) hives before emergence (Laidlaw Jr. & Page Jr., 1997). Each nuc and the queen and brood it contained was identified by an alphanumeric moniker either on the top of the lid or on a cover flap underneath it. Later on in the summer when successes from queen grafting became too few and progress in obtaining samples too slow, I supplemented my supply of queens by ordering pre-mated queens that were already laying from Miksa Bees. By

August of 2013, due to the mortality rate of the queens in the nucs and the oncoming of the fall season, I had started incorporating full-sized well-established hives into my study to maintain a high enough sample size for my study.

I used two methods for adding workers to nuc hives based on the presence or absence of a queen. To establish a new nuc, I took workers from another hive that was several miles away from the nuc hive at the Guilford College bee station and transplanted them to the nuc. A frame covered in workers was removed from the parent hive and the workers were brushed off the frame and into a mason jar through a funnel. The frame was checked before brushing to make sure that the parent hive's queen wasn't accidentally transferred. The jar was then capped with a lid with breathing holes in it and taken to the UNCG bee station where the worker bees were shaken out of the jar into their designated nuc hive. The distance between the nuc and the parent hive is critical in order to prevent the workers from returning to their parent hive. When transplanted far enough the workers were disoriented and would typically reorient themselves to their new home (nuc) and stay there. This method is good for placing a mix of foragers and nurse bees in the nuc hive, which is desirable when establishing a new nuc, but not when adding workers to an established nuc with an established queen as foragers transplanted from another hive will attack the queen and workers already present since they will smell different from their home hive. Workers established in the nuc react to new foragers as though they are robbing bees and attack them as well (Flottum, 2010).

Although the effects of adding foraging workers to an established nuc can be mitigated by spraying all the bees with sugar water, it is preferable only to transplant

nurse bees to an established nuc if the colony size needs to be increased. To transplant only nurse bees I took a brood frame with capped brood that were starting to emerge and, after brushing off all other workers, placed them in a single frame cage in a 37°C incubator overnight. The next day freshly emerged workers were transferred into nucs by the same method described above with a mason jar. Being freshly emerged from their cell, new workers have no home identity and immediately identify with the nuc hive they are transplanted in (Breed, Perry, & Bjostad, 2004).

Determination of Paraquat Dose

When performing paraquat injections a balance of survivability and oxidative stress must be maintained. If the queens die too early there will be nothing to study and if they experience insufficient oxidative DNA damage no experimental effect will be measurable. They must experience just enough stress to induce the oxidative DNA damage that would induce strand breaks which might trigger increased recombination during meiosis, but not so much paraquat that it kills the queen before she has the opportunity to lay eggs.

Two studies of experimental injections of paraquat suggested a-priori possible sublethal concentrations for my experiment: Corona et al. injected queens with 1µL of a 25.5µg/µL paraquat in PBS solution (Corona et al., 2007). The proportion of queens surviving to 33 days was 52%. Seehuus et al. injected workers with a paraquat dose of 150µg/g (Seehuus, Norberg et al. 2006), which caused their workers to die within three days. Accounting for body weight, this dose corresponds to 30µg/µL for queens at an

injection volume of 1μL. This is above Corona et al.'s established LD50 for queens. The dosages in these two studies are not directly comparable because queens are not only larger than workers, but also have a higher degree of resistance to oxidative stress due to their enhanced production of vitellogenin (Corona et al., 2007) and the high content of monounsaturated phospholipids in their cell membranes (Haddad, Kelbert et al. 2007). Therefore, I proceeded from the initial dosage used by Corona et al (2007) and determined empirically the best dosage for my experiment.

In my initial test of dosage determination I used three queens, and had injected them with 2μL of paraquat at a concentration 100μg/μL. All three queens died overnight, establishing this dose as a lethal upper limit. After a review of the above literature I proceeded to inject a second test cohort with 1μL of paraquat at a concentration of 10μg/μL. This dosage had visibly stressed the queens, causing behavioral changes including sluggish behavior, while still keeping them alive for over a week after injection, therefore for my experiment I had settled on a paraquat injection volume of 1μL at a concentration of 10μg/μL.

Experimental Procedure

Injections were made with a glass capillary needle between the 2nd and 3rd abdominal plates, counting from anterior to posterior, in order to introduce the paraquat as close to the ovaries as possible to ensure that oxidative stress occurs in them. Injections were made on the queens' left side and neither ventrally nor dorsally in order to

avoid puncturing the ventral nerve cord and aortic arch respectively. Bees were sedated by placing them in a closed chamber and flooding it with CO_2 prior to injection.

After injecting the queens they were placed back in their nuc hives within a queen cage plugged with queen candy, a dough-like mixture of confectioners' sugar and syrup. The cage prevented workers from immediately attacking the queen upon sensing that she was injured. It took days for the workers to eat away this plug and release the queen. This facilitated the re-acceptance of the queen by her workers by giving her time to recover and the workers time to reacclimate to her. The queens were then allowed to resume egg-laying. Those queens that did resume egg-laying and lived for more than three weeks were included in the main study.

Two groups of 200 larvae were collected per queen except the queens for nuc "29" and hive "O", whose queens died only after producing about 100 brood three weeks after injection. Before and after injections a frame with a sufficient number of capped brood cells was taken out of the hive and replaced with an empty frame. The collected brood frames were incubated at 33^{o}C in a cage in a brood incubator until emergence when the newly emerged bees were removed from the frame and placed in labeled individual 1.5mL snap cap centrifuge tubes. Each batch was placed in its own box, labeled according to the hive from which it was taken and stored at -80 ^{o}C. In some cases emerging workers and/or drones were collected from the frames along with their not yet emerged siblings. The pre-injection batches were collected before or shortly after the queen was subjected to injection, and served as the control group for the study. The post-injection batch was collected from brood derived from eggs that were laid after a period

of three weeks after treatment. This waiting time was to ensure that any brood the queens had laid before being injected, as well as brood produced from post-meiotic eggs that were in her ovaries at the time of injection, had been laid and were not included. Hypothetically, only the brood with DNA that was affected by the meiotic recombination rates that occurred while the queen was under oxidative stress was collected, reducing the chance for false negatives.

Thus, in total 400 pre- and post-treatment samples per queen were collected and stored for subsequent DNA extraction and genotyping. DNA was extracted from larvae or newly-emerged bees and then genotyped by sequencing PCR-amplified microsatellite markers on a Licor DNanalyzer™ genotyping platform.

DNA Extraction

DNA extraction was performed using a Chelex ™ extraction protocol (Walsh, Metzger, & Higuchi, 1991). The first step in extracting the DNA of the samples was to remove a small portion of the bee (such as the metathoracic leg or the head), cut it into 4-6 pieces, place those pieces in a well of a labeled 96-well plate, and record the sample location on a corresponding data sheet. The container of Chelex ™ solution was kept on a magnetic stir plate with a magnetic stir bar inside the Chelex ™ container, in order to maintain a homogenous suspension of glass beads within the solution. To each well 150µL of a 5% Chelex ™ solution and 5µL of proteinase K at a concentration of 10mg/µL was added. After sealing the plate it was incubated in a thermocycler using the following program: 1 hour at 55ºC, then 15 minutes at 99ºC, next 1 minute at 37ºC,

followed by 15 minutes at 99ºC (Walsh et al., 1991). When the thermocycler program ended, the plate was briefly vortexed, centrifuged and stored at -80ºC.

Genotyping

A microsatellite, otherwise known as a short tandem repeat, is a type of variable number tandem repeat motif about 2-6 base pairs long, and these loci are often used in genetic studies as neutral molecular markers (Turnpenny & Ellard, 2005). The loci chosen for this study have been selected based on their genomic location on the same chromosome that suggests that they are linked. However, individual genotypes are only linked unless recombination occurs between two loci. Linked genotypes were distinguished from recombinant paired genotypes based on which pairs of genotypes occur most often. Table 1 lists the primers that were used in the study, their approximate genomic location, and their sequences. These microsatellites have been used previously in linkage studies in honey bees (Graham et al., 2011; Rueppell et al., 2011; Solignac, Mougel, Vautrin, Monnerot, & Cornuet, 2007).

Table 1. Microsatellite Primer Pairs for Testing Recombination Rates in Chr. 3			
Locus	Primer sequence	Physical location (bp)	Recombination position (cM)
AC149	F_CGAGTCGAAACCTTTACACC	8591065-8591179	191.3
	R_CGATGAACTTAATTGGCTCC		
UN157T	F_AACCTCGAAAAAGCGTCTGG	9193051-9193158	201.7
	R_GCGTCCCACCTACTCTCAGC		
K0353	F_TTGTTGACATCGTGCGCC	9785478-9785660	215.8
	R_CGTCATCGACATTCGTTTCG		
K0311	F_AATTCCAGGCCAATGAATCA	9965958-9966254	221.3
	R_TGATAAGATTTCGCAACGATTC		
K0351	F_AGTGAAAATACGACAAAGCATCG	10537446-10537670	239
	R_CGCAATCGAAACGAGCAT		
AT066	F_CGTCCGTTATCCACTCGG	11525778-11526028	248.7
	R_GAGGAGCAGTTTCGCGAC		
UN295	F_CTCCATCGTCTACGGGCAG	11685349-11685505	265.6
	R_GGTTGGCGCCACTGAGTAG		
SV196	F_TCACGCAAAGTCAAACGAGC	12057293-12057464	270.3
	R_TTTCGAACCGTTTCGACAAAC		

PCRs were optimized to run in the following 15µL solution: 1µL of 10ng/µL template DNA added to a master mix containing 1.5µL of 2mM dNTPs, 0.75µL of 10µM forward primer, 0.75µL of 10µM reverse primer, 1.5µL of PCR buffer (20mM MgCl2, 100mM Tris–HCl [pH 8.3], 500mM KCl), 0.05µL of 5U/µL Taq DNA polymerase, and 9.45µL dH2O. This reaction mixture is modified from mixtures used previously (Meznar, Gadau, Koeniger, & Rueppell, 2010). The reaction mixture combined with the DNA of a single individual was then run on a temperature gradient (range: 45 to 60°C) to determine the optimal annealing temperature for PCR amplification. Multiplexing the primers was foregone in favor of mixing the post-PCR products in order to save time on optimizing the different microsatellite combinations for multiplexing compatibility.

Table 2 elucidates the sets of paired primers that were used to genotype the microsatellite markers on chromosome 3 of the honeybee genome. Primers that were selected for genotyping in a particular sample set are highlighted yellow. Note that each set of samples from each hive has a different set of primers that were genotyped to assess

recombination. The different sets were necessary because certain markers were monomorphic (=uninformative), in some colonies, yet polymorphic in others. No one set of markers was polymorphic in all of the colonies, so each colony was genotyped with a different set.

Table 2. Colony Screening Results for Chromosome 3

	Locus							
Colony	AC149	UN157T	K0353	K0311	K0351	AT066	UN295	SV196
N14	poly	Poly	poly	poly	Poly	poly	mono	poly
N26	poly	Poly	poly	poly	Mono	poly	mono	mono
N27	mono	Poly	poly	poly	Mono	poly	poly	mono
N29	mono	Mono	poly	poly	Poly	poly	mono	mono
A	mono	Poly	Maybe	poly	Maybe	poly	mono	mono
A10	poly	Mono	poly	poly	Mono	poly	mono	poly
A11	mono	Poly	Maybe	poly	Mono	poly	mono	mono
K	mono	Poly	mono	poly	Poly	poly	mono	poly
O	poly	Mono	mono	mono	Poly	maybe	mono	poly
U	poly	Poly	mono	poly	Poly	mono	poly	poly
Y	mono	Poly	mono	poly	Poly	poly	mono	mono
cM position	191.3	201.7	215.8	221.3	239	248.7	265.6	270.3

The markers were also chosen for optimal recombinatorial distance from each other whenever possible. The optimal window of distance between markers was between 20cM and 60cM. The further apart two paired markers are the closer the ratio of recombinant paired genotypes to non-recombinant paired genotypes gets to 1:1. This makes it impossible to tell which genotypes are the recombinants and which ones are the non-recombinants. The closer two paired markers are, the fewer number of recombination events exist between those two loci, until two loci come into complete linkage disequilibrium. Figure 2 graphically depicts the recombinatorial locations of these loci on chromosome 3.

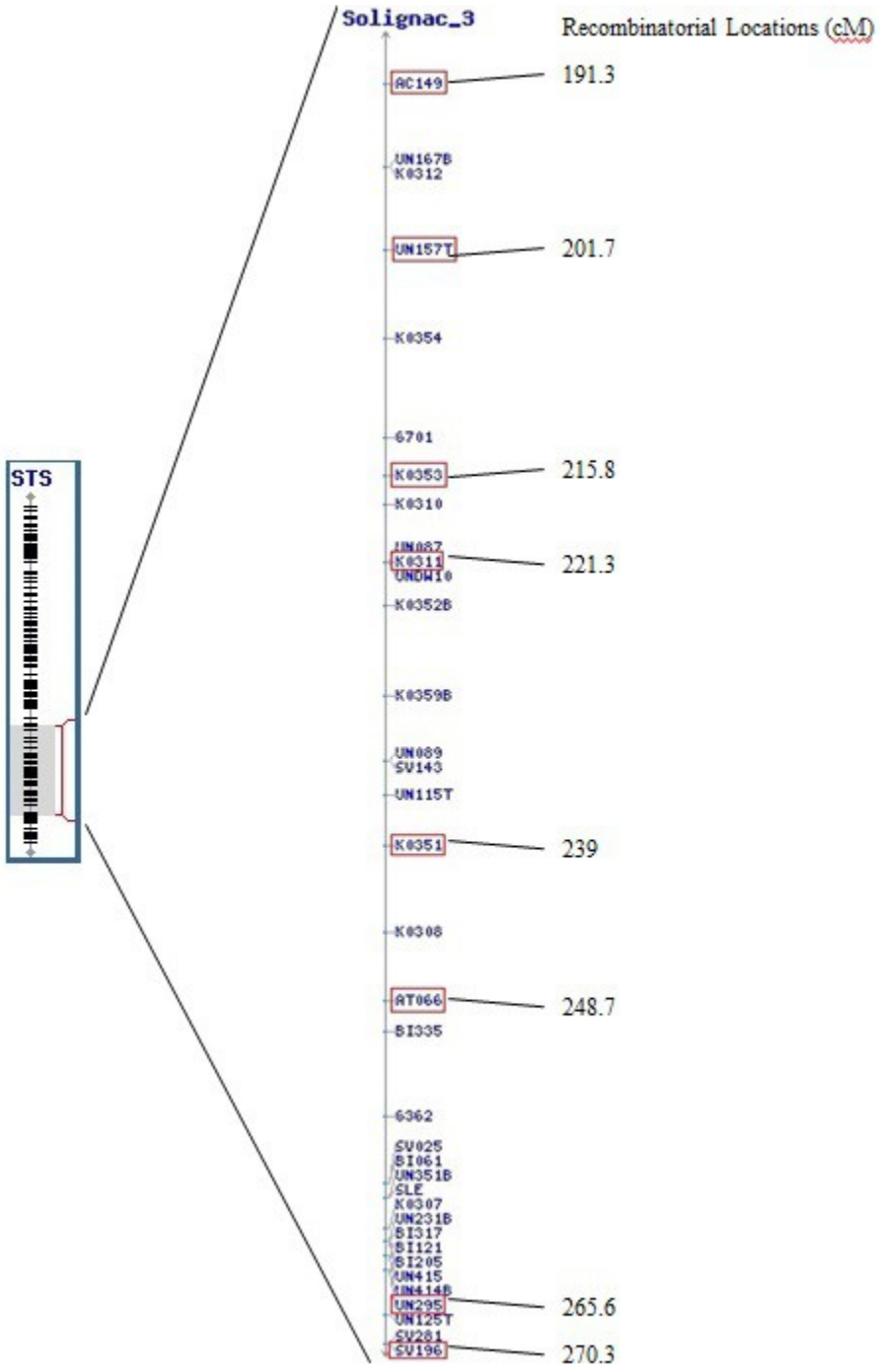

Figure 2. Recombinatorial position of loci on chromosome 3. The area on chromosome 3 indicated in brackets is blown up to indicate physical position of loci on the chromosome. Microsatellite markers used in this study are boxed in red with recombinatorial locations in cM indicated off to the side.

There were several intervals for which tested paired markers were closer together than 20cM. This is because in several sample sets it was not possible to select markers that were further away than 20cM. It would have been ideal for the study if each sample set had four to five markers to assess, giving us three to four intervals per sample set. However, some sample sets only had three markers that tested as being polymorphic during prescreening while others had more. Those markers that were genotyped were the ones deemed most useful given the circumstances.

Each PCR product was mixed with 2μL of stop buffer. Samples were then heated to 95^{o}C for three minutes and snap cooled on ice. Subsequently samples were loaded onto an ultra-thin 6% polyacrylamide gel and allele sizes determined by electrophoresis on a Licor DNA Analyzer 4300. Digital gel images were scored and multi-locus genotypes of all individuals per queen compared to detect and quantify recombination. Genotypes were called based on band size, with the band higher up on the gel being scored "B" and the band lower down on the gel being scored "A".

Statistical Analysis

Recombination in each interval was studied by comparing the genotypes of the flanking markers. First the recombinants were determined based on whether "AA + BB" or "AB + BA" were the most frequent allele pairing between the loci, with the least numerous of the pairings being labeled as the recombinants. Then the number of recombinants were divided by the total number of data points for their sample set and multiplied by 100 to get the percent recombination rate. For each interval in individual

queens, we determined whether the recombination rate was increased or decreased in the post-injection offspring, compared to her pre-injection offspring. Overall, statistical analysis was then performed using GraphPad QuickCals free online statistical calculator, using the number of intervals with increased recombination relative to the total number of intervals in a simple sign-test.

CHAPTER III

RESULTS

Out of 27 intervals the rate of genomic recombination increased in only 13 in the post-injection sample sets compared to the pre-injection sets. A sign-test of these values resulted in a one-tailed P value of 0.5. Figure 3 illustrates the percent change in recombination rate between the pre- and post-injection sample sets for all hives and all intervals. Given these results, I refrained from a further evaluation of the data with a paired student's t-test.

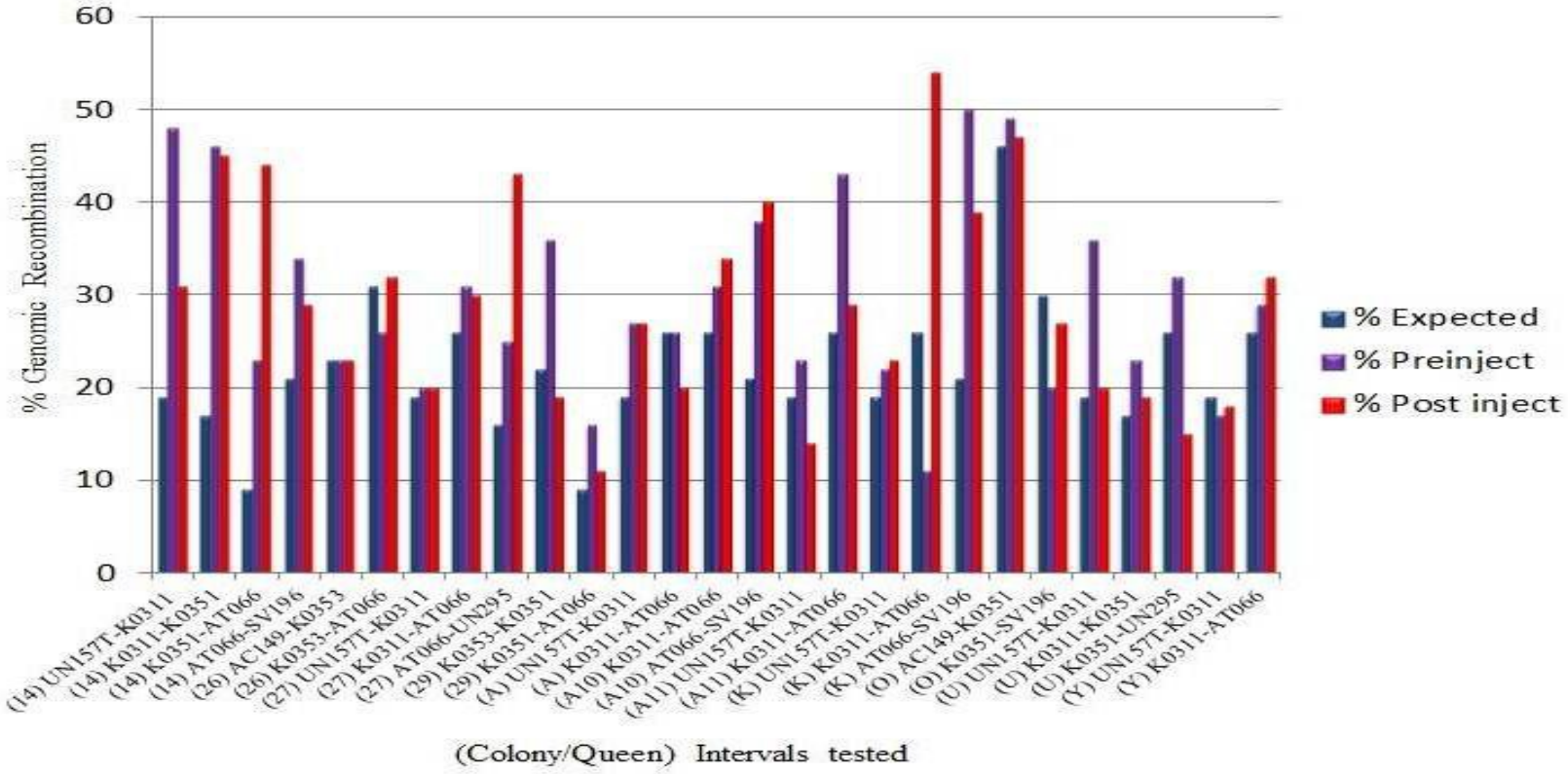

Figure 3. Percent recombination obtained from all sample sets at all intervals tested. No consistent pattern of effect was observed on recombination rate as a result of oxidative stress. The bars in blue represent the % recombination expected in the pre-injection set as determined by a third generation linkage map (Solignac et al., 2007). Purple and red bars represent the % recombination experimentally measured in the pre- and post-injection sets respectively.

The data from the AC149-K0311 interval in the hive "A10" sample sets could not be used because the number of "AA" + "BB" genotype pairings was equal to the number of "AB" + "BA" pairings, so the recombinant genotype pairs could not be determined for that interval in that sample set. In some cases, such as the UN157T-K0311 interval tested in nuc 27 and hive "A" (Fig. 3), the rate increase was only by fractions of a percent. Of the 27 usable intervals 14 showed an apparent decrease in recombination rate. In the intervals of UN157T-K0311 in nuc 14 and hive U, K0353-K0351 in nuc 29, K0311-AT066 in hive "A11", AT066-SV196 in hive "K", and K0351-UN295 in hive "U" the decrease in recombination rate was greater than 10%.

Three queens ("26", "A10", and "Y") showed a consistent increase of recombination in all intervals tested. In contrast the recombination fraction went down after paraquat injection in all the intervals tested in the queens "29", "A11", and "U". All other sample sets experienced inconsistent changes in recombination rates.

When comparing the recombination rate of the pre-injection sets to the expected rate based on a third-generation linkage map of the honeybee genome (Solignac et al., 2007), several instances were detected that showed large, unrealistic differences between the expected recombination rates versus the obtained pre-injection rates (Fig. 3). In some cases these rate differences were as much as 29%. When restricting the usable data set to those intervals whose pre-injection rates matched the expected rates from the Solignac linkage map, only 3 usable data points were left.

A margin of error of ±8% of the expected recombination rate in the pre-injection sets was therefore allowed to account for variation that might occur due to random

chance, individual queen effect, and small sample size, increasing the usable data points to 16. Among these 16 intervals, 9 experienced an increased rate of recombination in the post-injection set as compared to pre-injection. A sign-test of these results produced a one-tailed P-value of 0.4018.

CHAPTER IV

DISCUSSION

The honey bee *A. mellifera* has the highest meiotic recombination rate among metazoans, yet the molecular mechanisms underpinning this rate are not well studied. Much of what is already known of the molecular mechanisms of genomic recombination has been elucidated in the yeast *S. cerevisiae* (Neale & Keeney, 2006). Even still the mechanisms explored are of DSBR, and that is used as a model for meiotic recombination in both yeast and other eukaryotes (Szostak et al., 1983). This study was the first of its kind to try and study some of those mechanisms in *A. mellifera*. Given what is already known of genomic recombination in yeast, and using that as a model for how it occurs in honey bees, it was reasonable to conjecture that DSBs caused by oxidative stress might lead to an increase in recombination during meiosis.

However, contrary to my prediction, no statistically significant effect of paraquat injection into honey bee queens on genomic recombination was observed. These results did not support the hypothesis that oxidative stress triggers an increase in the rate of genomic recombination during meiosis. However, there are several issues that prevent these results from providing conclusive evidence against my original hypothesis.

One complication is that the post-injection wait period may have been too long or too short for most or all of the queens. Three weeks was an estimate of how long it would take for a queen to lay all post-meiotic eggs that were present in her ovaries at the

time of paraquat injection, and for all cells undergoing meiosis at the time of injection to become eggs that are moved posterior for laying. Perhaps the queens started laying the affected brood at one or two weeks after injection, or later than three weeks.

It could be that the wait time between injection and the collection of the second batch of brood had to be individualized per queen. In order for that to be considered it is necessary to take into account both the time between injection and when the queens started laying again, and the rate at which queens laid eggs post-injection. There was no consistent difference in the period of time between injection and resumption of egg laying between the individuals who experienced consistent upregulation versus all the individuals who did not. For example, "26", who was consistently upregulated, started laying eggs four days after injection, where as "29", who was consistently downregulated, started laying five days after injection. "14" and "27", who had inconsistent changes in post-injection recombination rates both started laying somewhere between four and six days after injection. The rate of egg-laying was not recorded.

None the less, the post-injection wait time may have been one factor that gave rise to some of these individual effects apparent in the study. Upregulation was consistent for all intervals in nuc "26", hive "A10", and hive "Y". Recombination was downregulated in all intervals for nuc "29", hive "A11", and hive "U". Inconsistent up or down regulation occurred for nuc "14", nuc "27", hive "A", and hive "K". Another illustration of the highly individualized and overall random effects is shown when looking at the pre-injection UN157T-K0311 interval in nuc "14", nuc "27", hive "A", hive "A11", hive "K",

hive “U”, and hive “Y”. The recombination fraction for this interval ranges between 17 and 48%.

It could also be that the lack of consistent effect is due to a lack of oxidative stress. Although the paraquat was injected right next to the ovaries in all queens to ensure efficient delivery to the target organs in which meiosis takes place, it is entirely possible that the increase in non-specific oxidative stress merely triggered the upregulation of the expression of antioxidant genes (Boelsterli, 2007). While in most cases the queens appeared to be visually agitated after injection, and in a few cases a queen's abdomen would darken and turn black, it could be that the queen bee's own antioxidant defense systems, which are already more robust than that of workers (Li et al., 2010), were upregulated and managed to scavenge sufficient ROS to protect the DNA in the nuclear envelope of the ovarian follicles, preventing the oxidative insults that might have triggered an increase in recombination rates. Vitellogenin, as an antioxidant is highly abundant in the ovaries (Corona et al., 2007). Being the largest contributor to a queen bee's antioxidant defense, and vital to proper egg development, vitellogenin may make the ovaries a naturally difficult location to induce oxidative insults.

Furthermore, no biomarkers for oxidative stress have been assayed in queens in response to paraquat injection. Previous studies have only measured mortality as a factor of paraquat toxicity in queen bees in order to establish a LD50 (Corona et al., 2007). Biomarkers have, however, been assayed in worker bees. Seehuus et al. measured the level of oxidative carbonylation in the proteins vitellogenin, apolipoprotein 1, and hexamerin in response to paraquat injection in worker bees. It was found that

vitellogenin was preferentially oxidized at a greater relative intensity per relative quantity of protein than the other two proteins tested (Seehuus, Norberg et al. 2006). Oxidative tissue damage in response to paraquat injection by immunohistochemical staining of bee brains for differential carbonylation and nitration damage was tested in workers (Seehuus, Norberg et al. 2006). So while it can be inferred that paraquat would cause oxidative stress in queen bees, it has not been molecularly demonstrated. A future study could measure biomarkers for oxidative stress in response to different dosages of paraquat in queens.

Alternatively, hydrogen abstraction, the mechanism by which oxidative stress produces single strand breaks, does so randomly, and as such there is only a random chance that two single strand breaks will appear on either side of the double helix opposite each other. Thus, while ROS does generate double-strand-breaks (Salvo, Bracesco, Buccino, & Nunes, 1996), they may be so statistically rare as compared to single strand breaks that even if the queens were sufficiently oxidatively stressed, the stress may not have produced enough double strand breaks to have an effect on the rate of meiotic recombination. This is further accentuated by the fact that a relatively small portion of DSBs result in crossover events (San Filippo, Sung, & Klein, 2008).

In order to test this it would have been necessary to perform an assay for DNA damage on the ovaries of the queens themselves to ensure that their DNA had, in fact, been oxidatively damaged such that double strand breakage occurred. For this the comet assay for DNA damage was attempted. However, since preliminary attempts at running this assay failed to yield reliable results it was abandoned. Without reliable comet assay

results it cannot be said for certain whether or not the developing gametes of the queens received sufficient genetic oxidative damage to affect recombination. Alternative assays for oxidative stress include a generalized oxidative stress test that uses fluorogenic probes for measuring generalized cellular oxidative stress, a lipid peroxidation assay, fluorescent protein-based redox sensors, GSH detection with fluorescent probes, and ELISA assays for 8-oxoguanine and 8-oxo-2'-deoxyguanosine. None of these assays were well established in our lab and we lacked the equipment to carry them out.

Also important is that REC, the MCM8 homologue that drives crossover events in *Drosophila*, could potentially be a meiosis-specific modification to DSB repair and not act in accidental breaks (Blanton, Radford et al. 2005). It is unknown whether such a gene, or homologue thereof, exists in the honeybee genome, or how it would be affected by oxidative stress. The rate of crossover events could be reduced in some cases should the expression of a REC-like gene be disrupted by oxidative stress. Alternatively, the lack of a statistically significant effect could be explained if a rec-like protein isn't recruited to stress-induced DSBs. The presence of a REC-like gene would first need to be verified in honeybees.

Another technical complication that made it harder to obtain conclusive results was that most of the brood that were collected before and after injecting their queens were workers instead of drones. Since workers are diploid and receive one set of alleles from their father, a drone, this often gives rise to two alleles showing up on the Licor gels for any give locus. In many cases the paternal alleles could be distinguished from the maternal alleles. There were, however, several instances in which a paternal allele was

indistinguishable from one of the maternal alleles. This caused samples to look as though they possessed both maternal alleles, resulting in that genotype technically dropping out of the study. This relatively frequent technical dropout lowered the number of effective data points were able to be obtained from the experiment and reduced the statistical power of this study. In the case of hive "A10" the sheer number of technical dropouts was so high for marker AC149 that for the AC149-K0311 interval only 80 data points in total were obtained out of almost 400 genotyped samples, pre- and post-injection. As noted earlier this not only reduced the statistical power for this interval, but masked the recombinant genotype pairs making it appear as though there were an even number of "AA" + "BB" and "AB" + "BA" genotype pairings, rendering that particular interval useless for the study.

It is possible that by testing on unmated drone-laying queens only that the presence of one maternal allele in the collected sample set would have all but eliminated the presence of technical dropouts, except in extraneous cases of sampling error. However, there is also the possibility that unmated drone-laying queens would have stood an increased chance of rejection by their workers. This would have limited the window during the summer in which samples could have been collected, thus possibly reducing the statistical power of the study further by reducing the number of colonies that would have been used in the experiment.

Yet another issue that limits the scope of the study is the fact that it was only carried out on one chromosome. The inconsistent amplification or decrease in recombination rate may be a chromosome-specific effect. Without looking at

recombination rates across several chromosomes it is impossible to tell whether this lack of a trend is genome-wide or isolated to one chromosome. Honeybees have a particularly elevated rate of recombination in chromosome 3, which contains the sex-determining locus (Beye et al., 1999). In humans chromosome-specific effects on recombination for chromosome X in the case of trisomy have been observed (Thomas, Ennis et al. 2001). In specific, though, the effects are age-related meiotic errors that increase non-disjunction and are not paraquat induced. Further study and characterization is needed to see if a non-specific stressor like paraquat could produce chromosome-specific alterations in recombination rate.

Genomic recombination is a process that is better characterized in model organisms than in *A. mellifera*. The results of this study being inconclusive only serve to illustrate, in conjunction with the honeybees' mysteriously high rate of meiotic recombination, the need for greater elucidation of the molecular mechanisms that could cause meiotic recombination to be so frequent and still produce such consistently viable offspring.

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