Measuring Expression of Antioxidant Genes in Spermathecae of *Culex pipiens*

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Introduction:

Spermathecae are receptacles used for storing sperm that are found in the reproductive tracts of female insects, such as honey bees and mosquitoes. Following mating, sperm is deposited in the bursa copulatrix of female insects and then the sperm migrates into the spermathecae. The female can then release the sperm to fertilize the eggs prior to oviposition, a decision she makes only when conditions are preferable. Certain antioxidant enzymes like catalase, glutathione S-transferase (GST), and superoxide dismutase (SOD2) promote sperm longevity and storage by protecting sperm from oxidative stress. Collins et al. (2004) measured the levels messenger RNA (mRNA) of *catalase, GST,* and *SOD2* in the spermathecae of queen honey bees. They found that the levels of each of these transcripts were higher in mated honey bees versus un-mated queen bees. Additionally, the levels of *catalase, GST* and *SOD2* were higher in older males and queens than in younger honey bees (Collins et al., 2004). These findings suggest that catalase, GST and SOD2 proteins play an important role in protecting sperm from oxidative stress.

Catalase, GST and SOD2 assume antioxidant roles within the spermathecae to extend the longevity and storage of sperm. Catalase, specifically, prevents cell oxidative damage by degrading hydrogen peroxide to water and oxygen ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) (Alfonso-Prieto et al. 2009). Glutathione S-transferase (GST) conjugates reduced glutathione resulting in transformation of compounds involved with oxidative stress (Nebert, Vasilious, 2004). Superoxide dismutase (SOD2) primarily destroys superoxide anion radicals (Fukai, Ushio-Fukai, 2011). The combination of these three anti-oxidative enzymes are critical for maintain an environment with reduced oxidative stress.

In order to survive the harsh conditions of winter females of the Northern house mosquito, *Culex pipiens*, enter a hibernation-like state called diapause. The absolute day lengths can cause mosquitoes to enter (short days) or avert diapause (long days; Spielman and Wong, 1973). When in diapause female of *Cx. pipiens* are less likely to take a blood meal or lay eggs, as they divert their resources from reproduction to survival (Denlinger, 2002). As male mosquitoes do not survive winters, it is essential that females store and protect their sperm (3-6 months) so that they can lay fertile eggs that following spring. Sim and Denlinger (2011) investigated the gene expression of *catalase* and *SOD2* in whole body diapausing mosquitoes compared to non-diapausing females. They found that both *catalase* and *SOD2* were upregulated in diapausing mosquitoes (Sim and Denlinger, 2011). No one has measured gene expression in the spermathecae of any diapausing insect. These investigations to measure the levels of *catalase, GST,* and *SOD2* genes will help to determine whether or not these genes are expressed at different levels in diapausing and non-diapausing mosquitoes, and will provide the first step in determining whether these enzymes might protect sperm from oxidative stress during diapause in females of *Cx. pipiens*. I hypothesize that antioxidant genes, *catalase, GST* and SOD2 are upregulated in the spermathecae of diapausing and older *Culex pipiens*.

The prevalence of insect-transmitted diseases like malaria, yellow fever and Dengue fever are widespread. Over one million people die from these diseases each year, and many of those are children (WHO, 2017). Adult female mosquitoes can lay 50-200 eggs per oviposition and produce over 800-1000 mosquito offspring in her lifetime (WHO, 2017). The reproductive capacity of mosquitoes creates the need to

control and kill insects to combat the spread of these death-causing diseases. With the growing use of insecticides, mosquitoes have become increasingly resistant to conventional insecticides that primarily target the insect nervous system. Therefore, limiting the reproductive capacity of mosquitoes could become a novel mode of control. Research about how females maintain and store sperm for long periods of time could therefore aid in the fight against vector-borne disease transmission.

Materials and Methods:

Insect rearing and maintenance

Mosquitoes were maintained in the lab as previously described (Meuti et al., 2015). Non-diapausing *Cx. pipiens* were generated by rearing larvae, pupae and adults in environmental chambers (Percival Scientific) with 16-hours of daily light exposure and 8 hours of darkness (L:D 16:8; Long Day or LD condition) at 18˚C. Diapausing females were generated by rearing larvae, pupae and adults under short day conditions (L:D 8:16; Short Day or SD condition) at 18 ˚C. Both diapausing and non-diapausing mosquitoes were given water and sugar water (10% sucrose solution). A sponge was dampened and placed a top cages and a plastic bag was placed around the cages to increase relative humidity.

Dissection and RNA extraction and cDNA synthesis

Ten days after adult emergence, the spermathecae from 20 female mosquitoes/sample and treatment (LD and SD) were dissected in an RNase-free, 0.9% NaCl solution (n = 5 biological replicates/treatment; 10 total). Spermathecae were

similarly dissected and prepared from 30-day-old, diapausing (SD) females (n = 3 biological replicates). After dissection, RNA was isolated from each sample using TRIzol Reagent (Invitrogen) according to a 1/5th proportion of the manufacturer's instructions (i.e 1000 uL reaction was reduced to 200 uL reaction). Complementary DNA (cDNA) was synthesized using 0.18 ug of the total RNA isolated from each sample with the qScript (Quantabio) cDNA synthesis kit. Genomic DNA was removed from each sample using TurboDNase (Fisher Scientific) and RNA was then treated with RNA Clean and Concentrate Kit (Zymo Research) to remove any contamination.

Quantitative Real Time PCR (qRT-PCR)

We designed qPCR primers to amplify a 100 bp fragment of *GST*. Previously published sequences for qPCR primers (Sim and Denlinger, 2011) were used to measure the levels of *catalase* and *SOD2* expression in non-diapausing (LD) and diapausing (SD) females. Standard curves of the exact primers used were performed to evaluate their efficiency and R^2 value of each gene is also included in Table 1.

All qPCR reactions for each biological sample were run in triplicate, using 20 ul reactions containing 10 ul of iTaq Sybr Green (BioRad), 0.8 ul of forward and reverse primers (400 nm total), 6.4 ul of water and 2 ul of sample cDNA in each well of a 96-well plate (BioRad). Samples were run on a BioRad iQ5 quantitative real time PCR machine for 40 cycles (94˚C for 20 sec, 59˚C for 12 sec) followed by a melt curve analysis to ensure reaction specificity. The expression level of *Catalase, GST and SOD-2* were normalized to the geometric average expression of *Ribosomal protein 49, RpL19* and *28S* genes, which served as internal controls.

Table 1*:* Primer Sequences of *catalase, GST* and *SOD2*

Gene of Interest	Forward Primer Sequence	Reverse Primer Sequence	Primer Efficiency	R^2
Catalase	CAAGTGATGACCTTCGAGCA	TTTTACCGACTGGGATCAGC	94.1%	0.994
GST	CCGACAACGAGAAGAAGATG	AAACTCAGATCCGCAATGG	104.1%	0.996
SOD2	GCATTGCGAAAACTTCCTTC	TGCCCAGATCATCAATTTCA	94.6%	0.994

Data Analysis

Relative expression of each antioxidant transcript was analyzed using the $2^{-\Delta CT}$ method as previously described (Meuti et al. 2015). A 1-way ANOVA test followed by a Tukey's Honest Significant Difference post-hoc test ($\alpha = 0.05$) was used to determine significant differences in the average expression of *catalase, GST and SOD-2* in non-diapausing (LD, day 10) and diapausing (SD, day 10 and day 30) female mosquitoes.

Results

Catalase expression

Expression levels of *catalase* differed significantly between day-10 diapausing and day-10 non-diapausing (1-way ANOVA test, Tukey's post-hoc test; $p<0.05$, $p = 0.0218$). Relative expression of *catalase* in the spermathecae of 10 day-old, short-day reared, diapausing females was five-fold higher than that in 10 day-old, long-day-reared, non-diapausing, mosquitoes (SD relative expression = 0.066 ± 0.009; LD relative expression = 0.021 ± 0.002). The relative level of *catalase* mRNA in the spermathecae of 30-day-old, diapausing females did not significantly differ from either

10-day-old diapausing or non-diapausing female spermathecae (1-way ANOVA test, Tukey's post-hoc test; $p > 0.377$).

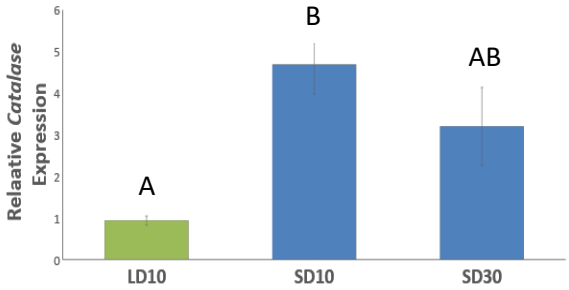

Figure 1: Relative *catalase* expression in mosquito spermathecae. All data were normalized to *Rp49*, *RpL19* and *18S*. Boxes represent average expression and bars indicate standard error. LD10 = spermathecae from long day, nondiapausing mosquitoes 10 days after adult emergence (N = 5); SD10 = spermathecae from short day, diapausing females 10 days after adult emergence (N = 5); SD30 = Short day, diapausing females 30 days post-emergence (N = 3). Different letters indicate statistically significant differences (1-way ANOVA, Tukey's post-hoc test; $p < 0.05$).

GST expression

Relative expression of *GST* differed significantly between day-10 diapusing and day-10 non-diapausing (1-way ANOVA test, Tukey's post-hoc test; $p = 0.040$). Relative *GST* expression in the spermathecae of 10-day-old, short-day reared, diapausing females was two-fold higher than that in 10-day-old, long-day-reared, non-diapausing mosquitoes (SD, day 10 relative expression = 0.384 ± 0.048; LD day 10 relative expression = 0.0179 ± 0.040). The level of *GST* mRNA in the spermathecae of 30-day-old, diapausing females did not significantly differ from either 10-day-old diapausing or non-diapausing female spermathecae (1-way ANOVA test, Tukey's post-hoc test; $p > 0.062$).

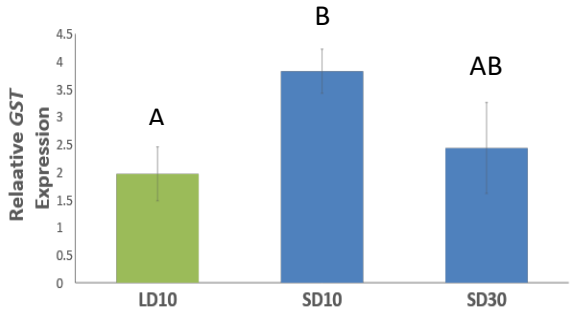

Figure 2: Relative *GST* expression in mosquito spermathecae. All data were normalized to *Rp49*, *RpL19* and *18S*. Boxes represent average expression and bars indicate standard error. LD10 = spermathecae from long day, non-diapausing mosquitoes 10 days after adult emergence (N = 5); SD10 = spermathecae from short day, diapausing females 10 days after adult emergence (N = 5); SD30 = Short day, diapausing females 30 days post-emergence (N = 3). Different letters indicate statistically significant differences (1-way ANOVA, Tukey's post-hoc test; $p < 0.05$).

SOD2 expression

Unlike *catalase* and *GST*, the relative expression of *SOD2* was the same in the spermathecae of diapausing and non-diapausing mosquitoes. The level of *SOD2* mRNA in the spermathecae of 10-day-old, diapausing females did not significantly differ from 10-day-old diapausing (1-way ANOVA, Tukey's post-hoc test; $p = 0.913$). The level of *SOD2* mRNA in the spermathecae of 30-day-old, diapausing females did not significantly differ from either 10-day-old diapausing or non-diapausing female spermathecae (1-way ANOVA test, Tukey's post-hoc test; $p > 0.4558$).

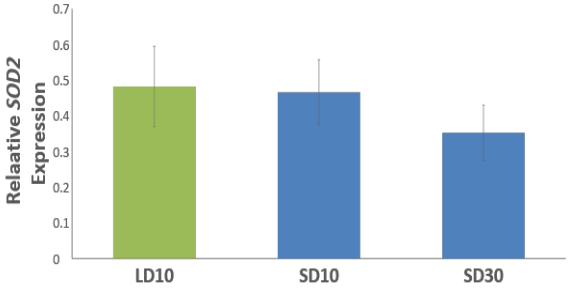

Figure 3: Relative *SOD2* expression in mosquito spermathecae. All data were normalized to *Rp49*, *RpL19* and *18S.* Boxes represent average expression and bars indicate standard error. LD10 = spermathecae from long day, non-diapausing mosquitoes 10 days after adult emergence (N = 5); SD10 = spermathecae from short day, diapausing females 10 days after adult emergence (N = 5); SD30 = Short day, diapausing females 30 days post-emergence (N =

3). There were no significant differences in *SOD2* expression across the treatments (1-way ANOVA, Tukey's post-hoc test; $p > 0.456$).

Discussion

Although similar levels of *SOD2* mRNA were present in the spermathecae of nondiapausing and diapausing mosquitoes, the relative expression of the antioxidant genes *catalase* and *GST* were significantly upregulated in early diapause (Day 10). These differences suggest that they play a role in sperm preservation and storage. Day-10 diapausing mosquitoes likely require elevated levels of *catalase* and *GST* to promote sperm longevity. Maintaining sperm and keeping it viable is imperative for diapausing females of *Culex pipiens* so that they can lay fertile eggs the following spring. This research is the first step in defining the environment within spermathecae and how genes like *GST* and *catalase* change depending whether a mosquitoes averts or enters diapause. The expression of *SOD2* was statistically equivalent between diapausing and non-diapausing mosquitoes and thus likely is not a primary enzyme used to maintain sperm within spermathecae for long periods of time.

Moving forward, increasing the sample size of day-30 mosquitoes would help to confirm whether antioxidant genes are upregulated at this time point. Previous work done in honey bees indicated that antioxidant genes, like *catalase, GST* and *SOD2,* were up-regulated in the spermathecae (Collin et al. 2004). However, I found that only *catalase* and *GST* were expressed at higher levels within the spermathecae of diapausing mosquitoes. Additionally, measuring protein levels within the speramthecae of diapausing and non-diapausing mosquitoes will allow us to validate that changes in

gene expression lead to actual protein production and its activity. Finally, future functional assays, such as knocking down the level of *catalase* and *GST* transcripts with RNA interference (RNAi) and then examining the viability of sperm within spermathecae, will confirm whether these genes are necessary for long-term sperm storage and maintenance.

This research, while preliminary, lays the foundation for more work on the mechanisms by which diapausing, females of *Culex pipiens* are able to preserve sperm 3-6 times longer than the lifespan of their non-diapausing sisters. Antioxidant genes like *catalase* and *GST* minimize the oxidative stress within spermathecae to keep sperm alive. Dramatically suppressing the expression of these genes would likely reduce the viability of sperm. Manipulating antioxidant gene expression may therefore help to reduce the reproductive potential of mosquitoes, and lead to better control of mosquito populations, a major issue affecting many regions of the world. My hope is that understanding the mechanisms of sperm preservation within spermathecae may one day help us combat the deadly army of mosquitoes that exist world-wide.

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