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Antioxidants in Atlantic Salmon on a Diurnal Basis

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ANTIOXIDANTS IN ATLANTIC SALMON ON A DIURNAL BASIS

by

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Abstract

Atlantic salmon is a common aquaculture species that is now greatly impacted by sea lice and amoebic gill disease. Currently, one of the treatments uses hydrogen peroxide (H_2O_2) because it breaks down safely in water, leaving no toxic residues like some of the previous treatments. Hydrogen peroxide was an effective treatment previously, but now resistance seems to be developing amongst the disease organisms and, if too high a dose is used, it can harm the salmon. Antioxidants, specifically superoxide dismutase (SOD), catalase (CAT), and both independent and dependent glutathione peroxidase (GPx), are present in organisms to break down reactive oxygen species (ROS) like H_2O_2. The goal of this study was to determine if antioxidants follow a daily rhythm, so that an ideal treatment time and a higher dose of H_2O_2 can be used to kill harmful organisms without causing damage to the salmon, since there are clear indications that many physiological processes vary on a circadian rhythm.

Three fish were sampled every four hours for fifty-two hours to determine if the levels of SOD, CAT, and GPx varied over time. Although this data showed some evidence of a daily rhythm, no statistical significance was found except in the GPx dependent levels. This experiment should be repeated using more than one tank and measuring cortisol levels to determine if stress was a possible contributing factor to the lack of statistical significance, or if human error caused the large amount of variance observed.

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Table of Contents

List of Figures:

List of Tables:

Background

Hydrogen Peroxide Treatment

Hydrogen peroxide (H_2O_2) is used throughout aquaculture for various topical skin and gill infections, including the treatment of various ectoparasites important in Atlantic salmon (*Salmo salar*) aquaculture today (Rach et al., 2000; Arvin & Pedersen, 2001; Adams et al., 2011). These diseases include sea lice and amoebic gill disease, both considered to be widespread diseases significant in fish health, especially farmed salmon (Kiemer and Black, 1997; Powell et al., 2008; Vera and Migaud, 2016; etc). Sea lice are (*Lepeophtheirus salmonis*) are currently considered responsible for multiple disease outbreaks and large economic losses to salmon farmers throughout the United States. The sea lice browse on the surface of salmon eating their mucus, epidermal cells and blood, which can ultimately lead to erosion on the surface of the fish. In some cases, the sea lice erode enough of the fish to expose underlying tissue and the skull leading to osmoregulatory problems, secondary bacterial infections, an increase in cortisol and glucose, indicative of a stress response, and ultimately death (Mustafa et al., 2000).

Amoebic gill disease, caused by the organism *Neoparamoeba perurans*, is also considered significant to salmon aquaculture especially because of evidence of its role in co-infections with other gill diseases (Powell et al., 2008). The disease first appears as raised white mucous patches and, ultimately, lethargy and rapid ventilation can be seen prior to mortality (Adams et al., 2011). In a study by Powell et al. (2008), they found that gas transfer limitations caused by the loss of functional lamellae and a reduced gill surface area can develop, which may lead to the impedance of swimming performance and lethargy. In a previous study, Powell et al. (2000), also found evidence of respiratory

acidosis occurring in fish experiencing a hypoxic condition. In chronically infected fish, altered heart morphology possible due to a reduction in blood flow has also been seen (Powell et al., 2008), although it appears that only the gills are the site of infection (Leef et al., 2005).

Hydrogen peroxide is currently one of the few effective treatments for both diseases. It appears to work by causing a temporary paralysis in the causative organism by the formation of bubbles, detaching them from the fish and causing them to float to the water surface (Bowers et al., 2002; Bravo et al., 2010). In the past, dichlorvos and emamectin benzoate (SLICE®; Merck Animal Health) have been used for the treatment of ectoparasites but are not used as frequently due to an increase in resistance, possibly health risks and environmental effects (Kiemer and Black, 1997; Treasurer et al., 2000). Dichlorvos is an organophosphate that has been shown to be very toxic to fish, through impacting their metabolism, and can cause both sub-lethal and lethal effects, including erratic swimming, excess mucus secretion, and equilibrium loss. It has also been shown to be a neurotoxin, inhibiting AChE, the enzyme that degrades acetylcholine, causing nerve disruption and ultimately death. It also may be carcinogenic after repeated exposures (Das, 2004). Emamectin benzoate was the treatment of choice for many years because it was effective against all life stages of sea lice, had a prolonged effect, and was easy to administer in the feed. However, it has now been used to the point of increased resistance in the parasite, meaning that the treatment is not currently very effective. Jones et al. (2012) found that the abundance of sea lice present after treatment was completed rose between 2004 and 2008, from 0 to 16 ineffective treatments. They also found that the successful treatments decreased from 100% in 2004 to only 51% in 2008. In addition,

Saksida et al. (2013) found that sea lice on farms that had previously treated with emamectin benzoate required a significantly higher treatment amount to detach.

Unlike dichlorvos, H_2O_2 is an environmentally safe treatment option. It is a powerful oxidizer but leaves behind no toxic residues (Vera & Migaud, 2016). It readily breaks down into water and oxygen, which are very nontoxic byproducts (Kiemer & Black, 1997; Bowers et al., 2002; Bravo et al., 2010; Adams et al., 2011). In fact, fishes naturally have antioxidants meant to break down H_2O_2 and other reactive oxygen species (ROS) (Trenzado et al., 2009; Cullen & Weydert, 2010; Barim-Oz & Yilmaz, 2016; etc).

Antioxidants

Since all organisms live in an aerobic environment, they are susceptible to damage by ROS, including superoxide (O_2^-), hydroxyl radical (OH^-), singlet oxygen (O_2) and hydrogen peroxide (H_2O_2) (Paller, 1991), produced through oxidative metabolism (Trenzado et al., 2009; Clotfelter et al., 2013). If an imbalance occurs, ROS can damage unsaturated fatty acids in cellular membranes, lipids, proteins, and DNA leading to cell death and mutations (Grant et al., 1998; Cullen & Weydert, 2010; Barim-Oz & Yilmaz, 2016). They have also been shown to contribute to postischemic renal injury (Paller, 1991). This imbalance can occur based on the water temperature, an increase in activity and metabolic rate, greater oxygen availability, or even lower oxygen availability (hypoxia). Body condition has also been shown to have an impact on overall susceptibility to ROS, with fish in better body condition able to breakdown ROS more effectively (Clotfelter et al., 2013).

Through evolution, antioxidant defenses have developed to keep ROS at low levels to prevent oxidative stress and the damages described above (Barim-Oz & Yilmaz, 2016). The main antioxidants are superoxide dismutase (SOD), catalase (CAT), and

glutathione peroxidase (GPx). These antioxidants transform the ROS to protect the cells from oxidative damage through detoxifying ROS, which is very important when treating with H_2O_2 because it can cause a larger imbalance in ROS (Tort, 2012). Although all three antioxidants catalyze the decomposition of different ROS, all three work together to prevent cellular damage and organism death.

Superoxide Dismutase

Superoxide dismutase (SOD) consists of a family of metalloenzymes containing copper and zinc or manganese or iron that breaks down the superoxide radical into H_2O_2, which can then be further broken down by catalase and glutathione peroxidase (Beyer & Fridovich, 1987). The manganese and iron containing forms are mostly found in prokaryotes, although the manganese form is located in mitochondria and the iron form can be found in some plants. The copper and zinc SOD (CuZnSOD) , on the other hand, is found in the cytosol of eukaryotic cells and is, therefore, the SOD the present study is concerned with (Fridovich, 1989). For CuZnSOD, the copper and zinc work together to allow the SOD enzyme to perform catalysis, with the copper reducing superoxide (O_2^-) to O_2 and the zinc assisting by increasing the reduction potential. If copper is limiting in the diet, SOD does appear to still function without its cofactors, but the overall activity will decrease over time (Harris, 1992).

Although present throughout cells, SOD tends to be higher in gill tissues possibly because of the need to destroy superoxide produced during respiration in the gills (Barim-Oz & Yilmaz, 2016). In solution, SOD requires the formation of the superoxide radical to be measured (Cullen & Weydert, 2010; Flohé & ötting, 1984). In most cases, including in this experiment, xanthine oxidase is used to generate the superoxide radical. Cytochrome c is then used as an indicator because xanthine oxidase uses electron transfer mediators to

reduce cytochrome c. SOD competes with the cytochrome c to breakdown superoxide, with a 50% inhibition equal to one unit of SOD (Beyer & Fridovich, 1987). Because of its role in superoxide dismutation, SOD is highly conserved among vertebrates but requires the function of catalase and glutathione peroxidase to further breakdown the H_2O_2 produced to prevent harmful impacts to the fish (Maral et al., 1977).

Catalase

Catalase (CAT) is a heme containing enzyme found in subcellular organelles, such as peroxisomes of liver or microperoxisomes found in various other cells (Regoli et al., 2012; Tort, 2012). CAT works through the reactions: CAT + H_2O_2 → CAT-H_2O_2 and then CAT-H_2O_2 + H_2O_2 → CAT + $2H_2O$ + O_2 (Wheeler et al., 1990). Hydrogen peroxide, if not detoxified, can become the toxic hydroxylradical-3-amino-1,2,4-triazole, so detoxification through the irreversible inactivated catalase is essential for proper cellular function. After forming the inactivated catalase, the CAT-H_2O_2 goes through an NADPH-dependent process to regenerate active catalase (Paller, 1991). Like SOD, it is found in the gills in higher amounts, especially after repeated low level exposures to H_2O_2 (Tort, 2012). For this determination, the rate of H_2O_2 decay is measured and is proportional to the amount of CAT present in the sample (Regoli et al., 2012). Although CAT seems to be more important in catalyzing the decomposition of H_2O_2, it shares this role with glutathione peroxidase (GPx) and GPx may be more sensitive to H_2O_2 in some cases (Grant et al., 1998; Barim-Oz & Yilmaz, 2016).

Glutathione Peroxidase

Glutathione peroxidase comes in both selenium-dependent and selenium-independent forms, and works to protect cells from oxidative damage caused by H_2O_2 and organic hydroperoxides. Specifically, the selenium-dependent form reacts with

hydroperoxides, including H_2O_2 and organic peroxides. The selenium-independent form, on the other hand, reacts only with organic hydroperoxides (Regoli et al., 2012). Unlike catalase, GPx requires co-factors, including glutathione reductase (GR) and NADPH. It also works through two intermediates, glutathione (GSH) and glutathione disulfide (GSSG) (Cullen & Weydert, 2010). The reactions are: ROOH+ 2GSH $\overset{GPx}{\rightarrow}$ ROH+ GSSG+ H_2O and then GSSG+ NADPH + H^+ $\overset{GR}{\rightarrow}$ 2GSH + $NADP^+$(Wheeler et al., 1990). GPx seems to be most important in membranes and preventing auto-oxidation of lipids, although it has also been found to be important in protecting hemoglobin from oxidative degradation (Cullen & Weydert, 2010; Barim-Oz & Yilmaz, 2016). There are two different ways to measure GPx activity, depending on if one wants to measure total GPx activity or only the dependent form. In both cases, the amount of GPx is measured through a reduction in the co-substrate NADPH as the H_2O_2 (for the measurement of the selenium-dependent form) or cumene hydroperoxide (for both Se-dependent and independent form) is broken down (Cullen & Weydert, 2010).

Circadian Rhythm

Through the rotation of the Earth, there is a daily light and dark cycle that affects organisms and their internal clocks, which impacts many physiological and behavioral processes (Duffield, 2003; Reebs, 2011). There is still debate as to how a circadian rhythm functions in fish, although they have been shown to exhibit a circadian rhythm when in constant conditions. In mammals, there is a master clock, called SCN, but none has been found in fish yet. Instead, the pineal gland seems to be a major contributor to the circadian clock because it is directly photosensitive, even when in culture. When the pineal gland is removed, however, a circadian rhythm still exists so there is a possibility

of a complicated network of both central and peripheral clocks that may be linked to create the overall circadian rhythm impacting the whole body (Reebs, 2011). Melatonin, which is synthesized from tryptophan in the pineal glands, appears to be the main hormone impacting the circadian rhythm. Melatonin levels rise through the night and are low during the day through the activation by the pineal photoreceptor cell in low light situations. This melatonin is then able to circulate throughout the circulatory system to impact locomotor activity, thermal preference, rest, food intake, vertical migration, skin pigmentation, osmoregulation, and metabolism (Falcón et al., 2010). Specifically, melatonin seems to work through G-protein coupled receptors, which brings about a change in the target cell. However, there is great variance in the number and location of these receptors making it more difficult to determine the full extent of melatonin's function in fish (Pévet, 2004).

Since the natural circadian rhythm can impact absorption, distribution, metabolism and elimination, there is evidence that the time of day of drug administration can have an impact on the efficacy and possibly toxicity of medications, including H_2O_2 (Bruguerolle, 1998; Vera & Migaud, 2016). Bruguerolle (1998) found that "… circadian variations in gastric acid secretion and pH, motility, gastric emptying time, gastrointestinal blood flow, drug protein binding, liver enzyme activity and/or hepatic blood flow, glomerular filtration, renal blood flow, urinary pH and tubular resorption may play a role in such kinetic variations." In a recent study, Vera & Migaud (2016) examined the effect of time on drug side effects, specifically H_2O_2, to help improve the treatment effectiveness. They found that in the liver, the gene expression for antioxidant enzymes displayed a daily rhythm, which correlated to different effects based on the time

of day H_2O_2 was given. They also found a significant difference in stress and toxicological response depending on the time of day, specifically an increase in sublethal toxic effects during the first half of the day.

Antioxidants and Circadian Rhythm

Although initially proven to be effective by several studies (Treasurer et al., 2000; Arvin & Pedersen, 2001, etc), the H_2O_2 treatment is beginning to lose effectiveness against many of the organisms originally treated. A decrease in the effectiveness indicates that organisms are beginning to develop resistance, possibly through genetic selection of resistant organisms or organisms are developing detoxifying enzymes. On farms that have previously used H_2O_2, only a 63% reduction was found at a higher dosage than recommended. Though the organisms are removed, many can recover from the treatment, some in as little as thirty minutes and then resettle on the salmon (Treasurer & Grant, 1997; Treasurer et al., 2000). The progeny of these organisms then show a reduced sensitive to H_2O_2, confirming the theory of genetic selection towards resistant organisms (Helgesen et al., 2015). To combat this problem, an increase in treatment dosage would be needed. However, this can cause many negative consequences for the fish.

There has been evidence of toxicity effects based on temperature and the dose of H_2O_2 given, which seems consistent with the narrow safety margin typically observed (Bowers et al., 2002; Adams et al., 2011). There appears to be a significant correlation between dosage and gill damage in fish, especially when the temperature increases (Kiemer & Black, 1997). In addition, mortalities have been shown to occur at doses above 2.5g/L, with most mortalities within thirty hours of treatment (Kiemer & Black, 1997; Gaikowski et al., 1999; Treasurer et al., 2000). So, although a higher treatment

dosage is needed to combat the development of resistance, these would typically result in an increase in fish mortality.

The goal of this experiment is to identify whether antioxidants, specifically SOD, CAT, and GPx, appear to run on a diurnal rhythm. Running on a diurnal rhythm, they should have a time which they are at the highest level. If this time can be indicated, a larger dose of H_2O_2 could be used to treat for ectoparasites, especially sea louse and amoebic gill disease. To accomplish this, Atlantic salmon exposed to a 12-hour light/dark cycle will be sampled every four hours over a 52-hour period. There levels of SOD, CAT, and GPx will then be measured using spectrophotometry and averaged for each time point.

Materials and Methods

Sample Collection

The fish were kept in an enclosed room in one, 650-liter tank filled with fresh water in a flow through system. The water was kept at 10°C. The fish were fed to satiation with size appropriate standard salmon pellets, about 3 mm. The room was kept on a 12-hour light-dark cycle, with light measured 36 inches below the light source, directly above the water surface. Fish use was approved by IACUC.

Sampling was done for 52 hours to have an overlap of each time point, to determine if there is a repeating cycle in the antioxidant amounts. Sampling was done every 4 hours, with the light and temperature of the tank being measured at each sampling point. Three fish were collected at each time point and put into a MS222 solution with a concentration of approximately 500mg/L for euthanasia. Each fish was then weighed and their length measured, which was recorded on a grid sheet. After measurements were taken, each fish had part of its liver and gill removed with a sterile

scalpel and scissors. All samples were then put into pre-weighed tubes, which were labeled with the time point (1, 2, 3, etc), the sample part (either l for liver or g for gill), and which fish it is (either a, b, or c to correspond to the measurements recorded on the sample sheet). The board used was wiped down with an ethanol solution and the scalpel and scissors put into a separate ethanol solution for sterilization. After each time point, the samples collected were brought into the lab and stored at -80°C to prevent degradation. After all samples were collected, the collection tubes were reweighed to determine the weight of each sample.

Sample Storage

Each sample was stored in 600 microliters of potassium phosphate (KPi) buffer at pH 7.5 at -80°C (Regoli *et al.,* 2012). After the addition of buffer, each sample was homogenized using a plastic pestle. The samples were then centrifuged to bring all solid material to the bottom. Each sample was then separated into three new tubes, labeled with the same time point, sample part, and fish but also labeled with roman numerals to distinguish the three tubes. Two hundred microliters of the samples were put into each tube.

Total protein Assay

A Bradford assay was used for each sample to determine the protein content, which was needed for the antioxidant calculations. A deionized water (DI) blank and seven standards were used, with the standards having protein concentrations of 0.125, 0.25, 0.50, 0.75, 1.0, 1.5, and 2.0μg/mL. A microplate was used for the protein assay. Two hundred microliters of Coomassie Brillant Blue G-250 was then put into each well using a multi-channel pipette. The plate was then mixed gently for 30 seconds by sliding the plate back and forth. Incubation at room temperature then occurred for 10 minutes

before the absorbance was read at 595nm using a microplate reader. The protein assay results were then saved in Excel and the average of the 2 recordings calculated. Each average was multiplied by 10, the dilution factor, to obtain the protein in μg/μL.

Antioxidant Determination

The procedure for the superoxide dismutase, catalase, and glutathione peroxidase determination was adapted from Regoli *et al*'s *Spectophotometric Assays of Antioxidants* (2012). Each spectrophotometric assay was done in duplicate for a replication of all results. All solutions were made per Regoli *et al's* results and reagent amounts used were adjusted as needed based on initial results. All tests were done through spectrophotometric analysis. Potassium phosphate (KPi) buffer was used for all three antioxidant measurements. Dissolving 0.680g of KH2PO4 in 50mL of distilled water made this buffer. The pH was then adjusted with concentrated potassium hydroxide to get it to the pH needed for that specific antioxidant test.

Superoxide Dismutase

Superoxide dismutase (SOD) was the first antioxidant test performed. For the KPi buffer, the pH was adjusted to 7.8. Dissolving 3.7224g of EDTA in 100mL of distilled water made the 100mM EDTA solution. Xanthine oxidase(XO) had a concentration of 300mU/mL, and was diluted to this concentration with cold distilled water. The working buffer for superoxide dismutase consisted of 5 mL KPi buffer, 100μL EDTA, 0.68mg hypoxanthine and 12.3mg cytochrome c. This test was performed using a plastic cuvette at a wavelength of 550nm. First, a reference reaction was performed, which consisted of 500μL of working buffer, 330μL of 100mM KPi buffer, and 170μL of XO. After the addition of XO, the cuvette was mixed and the increase in absorbance was read for three minutes. For the first duplicate, three different sample amounts were used, each at a 1:9

dilution. For the second duplicate, four sample amounts were used at either a 1:11 or 1:21 dilution depending on the results from the first duplicate. For all readings, 500μL of working buffer was used and 170μL of XO was added last, followed by mixing and reading the absorbance. For the first duplicate, the first cuvette contained 314.5μL of KPi buffer, 1.5μL of sample and 15μL of DI water; the second contained 308μL of KPi buffer, 2.0μL of sample and 20μL of DI water; and the third contained 297μL KPi buffer, 3.0μL sample and 30μL of DI sample. For the second duplicate, the first cuvette contained 319.5μL KPi, 0.5μL sample and 10μL DI water. The second cuvette contained 309μL KPi, 1μL sample, and 20μL DI water. The third cuvette contained 288μL KPi, 2μL sample, and 40μL DI water. The fourth sample contained 267μL KPi, 3μL sample and 60μL DI water. After all samples were read, the change in absorbance for each sample and the volume of sample was plotted. The line of best fit was used to determine an equation for each sample, either linear or polynomial depending on the graph. For the determined equation, x was the value corresponding to a 50% change in absorbance when compared to the reference reaction. The y-variable was the sample volume needed to reduce the reference by 50%, which is equivalent to 1 unit of SOD. This value needed to be normalized with the protein content. In order to accomplish this, the equation used was: SOD (units/ mg protein) = (1000/Vol)*(sample dilution)/proteins where "Vol" is the y-variable found above. After all these calculations were performed, the averages for each time point were calculated and graphed.

Catalase

For catalase, the KPi buffer had a pH of 7.0. A 1.2M H_2O_2 solution was made by adding 100μL of H_2O_2 stock to 900μL of distilled water. To check the concentration of

the stock solution, prior to creating the 1.2M solution, three different dilutions of the H_2O_2 stock were made and the absorbance measured at 240nm in a UV cuvette. The dilutions used were 1:20, 1:40, and 1:50. They contained 50µL H_2O_2 with 950µL DI water, 25µL H_2O_2 with 975µL DI water, and 20µL H2O2 with 980µL DI water respectively. Once the absorbance was measured, each absorbance was divided by the extinction coefficient, $0.04mM^{-1}$ cm^{-1}. This was then multiplied by the dilution factor and all three were averaged to determine the H_2O_2 concentration of the stock solution. To measure the catalase activity, a plastic cuvette was used and 970µL KPi buffer was added and the spectrophotometer zeroed at 240nm. Then, 10µL of H_2O_2 was added and the absorbance read, which needed to be about 0.48. This absorbance was read until it stabilized at this value. Finally, 10µL of sample was added and the absorbance read for 3 minutes. Each sample absorbance was then graphed and the change in absorbance per minute was calculated using the linear equations for each graph. The catalase activity was calculated through the equation: (change in absorbance per minute/-0.04) * (sample dilution)/proteins. The average for each time point was calculated and the averages graphed.

Glutathione Peroxidase

For glutathione peroxidase, the KPi buffer was at a pH of 6.97. One hundred micromolar EDTA solution was also used, as described above. The GSH working solution was also at 100mM and was freshly made by dissolving 0.0307g of glutathione reduced in 1mL of distilled water. NADPH working solution was also freshly prepared by dissolving 2mg of NADPH into 100µL of distilled water. Glutathione reductase was diluted to 100U/mL in cold distilled water to make the GR working solution, also freshly

prepared. The cumene hydroperoxide (CHP) working solution was freshly prepared by adding 38μL of a 5.2M CHP stock solution into 962μL of methanol. This was only used for the determination of Se-dependent and Se-independent forms. For the determination of the Se-dependent and Se-independent forms, a plastic cuvette was used at a wavelength of 340nm. For the blank, 946μL KPi buffer, 10μL EDTA, 20μL GSH working solution, and 10μL GR working solution was added to the cuvette. The instrument was then zeroed and then 10μL of NADPH working solution added. The absorbance was read and needed to be in between 0.9 and 1.2. Next, 4μL of CHP working solution as added and then the absorbance read for 2 minutes. For the sample, instead of 946μL of KPi buffer, only 846μL was added and a 1:5 dilution of the sample was added as well. This consisted of 20μL of sample and 80μL of DI water. Five of the samples (1a, 1b, 1c, 2a, and 6b) needed to be assayed at a 1:20 dilution because they contained too much protein and did not give linear graphs. This dilution consisted of 5μL sample and 95μL of DI water. Two of the samples (5a and 7b) were done at a 1:50 dilution for the same reason as outlined above. This consisted of 2μL sample and 98μL DI water.

Instead of CHP, the Se-dependent form involved sodium azide (NaN_3) and H_2O_2. Sodium azide was made to 100mM by dissolving 6.5mg into 1mL of distilled water. A 100mM H_2O_2 solution was made by adding 16.6μL of a 12M H_2O_2 stock solution into 1983.4μL of distilled water. Into the plastic cuvette, 835μL KPi buffer (actual pH= 6.95), 10μL NaN_3 working solution, 10μL EDTA, 20μL GSH working solution, and 10μL GR working solution was added. For the blank, 100μL of KPi buffer was added. For the samples, 20μL of sample and 80μL of DI water was added. After these additions, the

instrument was zeroed. Then 10μL of NADPH working solution was added and the absorbance read, which should have been between 0.9-1.2. Lastly, 5μL H_2O_2 working solution was added and the absorbance was read for 2 minutes. As in the glutathione peroxidase Se-dependent and Se-independent forms above, five of the samples (1a, 1b, 1c, 2a, and 6b) needed to be assayed at a 1:20 dilution because they contained too much protein and did not give linear graphs. This dilution consisted of 5μL sample and 95μL of DI water. Two of the samples (5a and 7b) were assayed at a 1:50 dilution for the same reason as outlined above. This consisted of 2μL sample and 98μL DI water. The sample 2a was also assayed at a 1:50 dilution for the duplicate.

For both glutathione peroxidase measurements, the calculation was the same. First, each sample was graphed and the line of best fit added. The slope from the line of best fit corresponded to the change in absorbance of the sample. This was also done for the blank, and corresponded to the change in absorbance of the blank. The absorbance of the final sample was calculated by subtracting the change in absorbance of the blank from the change in absorbance of the sample. The equation to calculate the glutathione peroxidase activity is: (change in absorbance final sample/-6.22) * (sample dilution) *1000/proteins. As with the other calculations, the average for each time point was calculated and graphed.

Statistical Analysis

After all the antioxidant calculations were completed, statistical analysis was done using the statistical program R. Specifically, ANOVA tests were performed to examine for statistical significance. The test was done against the time point and values were considered significant if $p<0.05$.

Results

Sample Collection

The salmon were sampled from 12pm on 6/9/16 to 4pm on 6/11/16. The sampling data is given in Appendix A. The average fish length was 18.14cm with a standard error of 5.03cm. The average fish weight was 59.02g with a standard error of 15.77g. The average temperature was 9.85°C with a standard error of 2.63°C. The lights were on for sampling at 8am, 12pm and 4pm. They were off for 8pm, 12am, and 4am. The light level had an averge of 7991.84 lumens during the light cycle and 0 lumens during the dark cycle.

Total Protein Assay

A Bradford Assay was conducted for each sample in duplicate, and redone on 9/13/16 to determine if protein degradation occurred during the experiment and storage, which involved freezing and thawing. The average protein content, including sample 13b is 14.558μg/μL. Without sample 13b, which is not included in the antioxidant averages due to its low protein content of 0.5915μg/μL, the average protein content is 14.898μg/μL. Not including 13b, the protein content ranges from 7.735μg/μL, in sample 13a, to 19.72μg/μL, in sample 5c. The median protein content is 15.71μg/μL. To see all protein contents, see Appendix B.

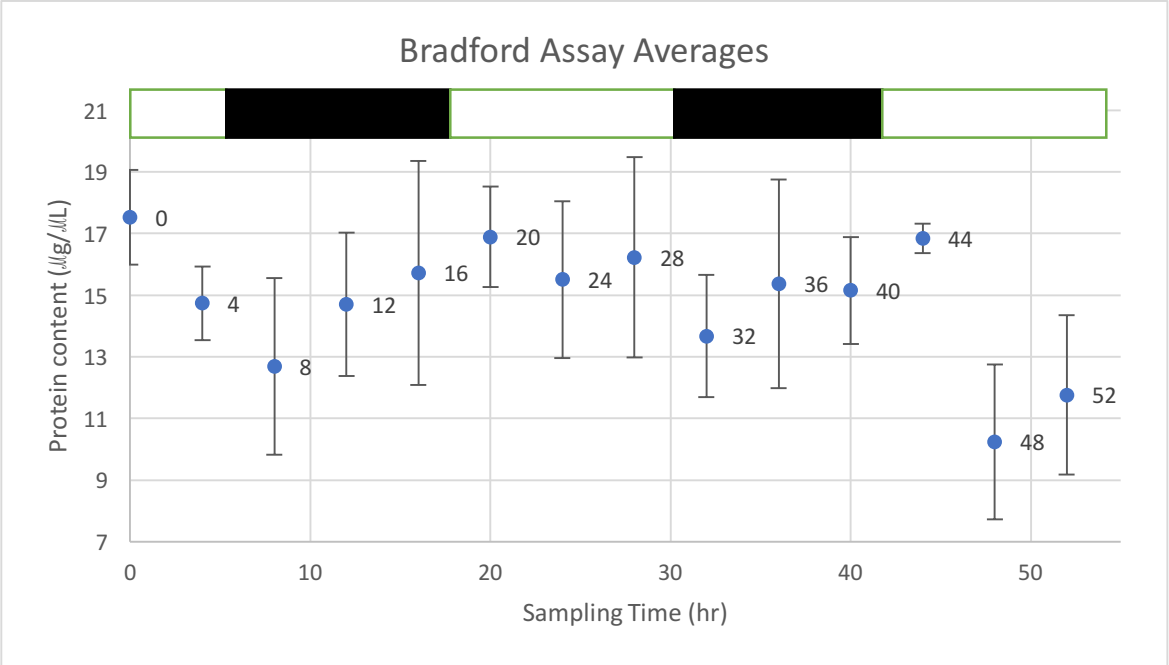

Figure 1: Average protein content in µg/µL as determined in the Bradford Assay with standard error shown. Dark squares indicate the times when lights were turned off, white squares when the lights were turned on.

Antioxidants

Superoxide Dismutase

The average SOD content for each sample ranged from -3.025U/mg protein, for sample 12b, to 13.937U/mg protein, for sample 2c. The average SOD content is 2.049U/mg protein and the median is 1.322U/mg protein. The average SOD content for each time point is displayed in Table 1. A graph of these values is showed in Figure 2.

Table 1: Average superoxide dismutase in U/mg protein for each sample time and its accompanying standard error

Sample Time	Average SOD (U/mg protein)	Standard Error
0	0.71396667	0.15098516
4	5.423967	5.213908
8	3.504283333	1.331506352
12	2.110317	0.850152
16	1.072467	0.184768

20	2.19655	1.034975
24	0.1932	0.700128
28	3.616767	2.490278
32	0.697933	0.643413
36	1.49205	0.674977
40	1.565983	1.324946
44	-0.043133	1.877791
48	3.082125	0.350775
52	3.0121	1.234331

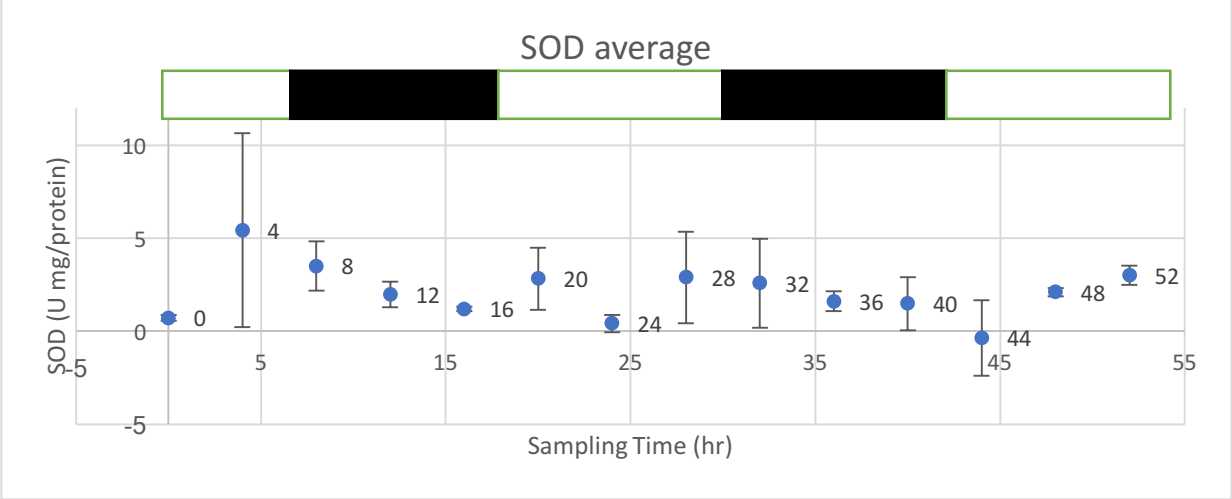

Figure 2: Average superoxide dismutase activity for each sampling time, with standard error shown. Dark squares indicate the times when lights were turned off, white squares when the lights were turned on.

Catalase

Catalase values ranged from $6.656*10^{—3}$ µmol/min* mg protein, for sample 5c, to $3.984*10^{-2}$µmol/min* mg protein, for sample 5a. The average CAT content is $2.061*10^{-2}$µmol/min* mg protein and the median is $2.039*10^{-2}$µmol/min* mg protein. All average values are shown in Table 2 and displayed in Figure 3.

Table 2: Average catalase in μmol/min mg protein for each sample time and its accompanying standard error*

Sample Time	Average CAT (μmol/min* mg protein)	Standard Error
0	0.02162994	0.00192135
4	0.018203	0.005557
8	0.013022829	0.001699263
12	0.015434	0.003064
16	0.022755	0.011748
20	0.023752	0.002385
24	0.020054	0.010039
28	0.01277	0.001268
32	0.02476	0.006214
36	0.03012	0.006214
40	0.022196	0.002663
44	0.022905	0.003036
48	0.021431	0.003036
52	0.019792	0.00669

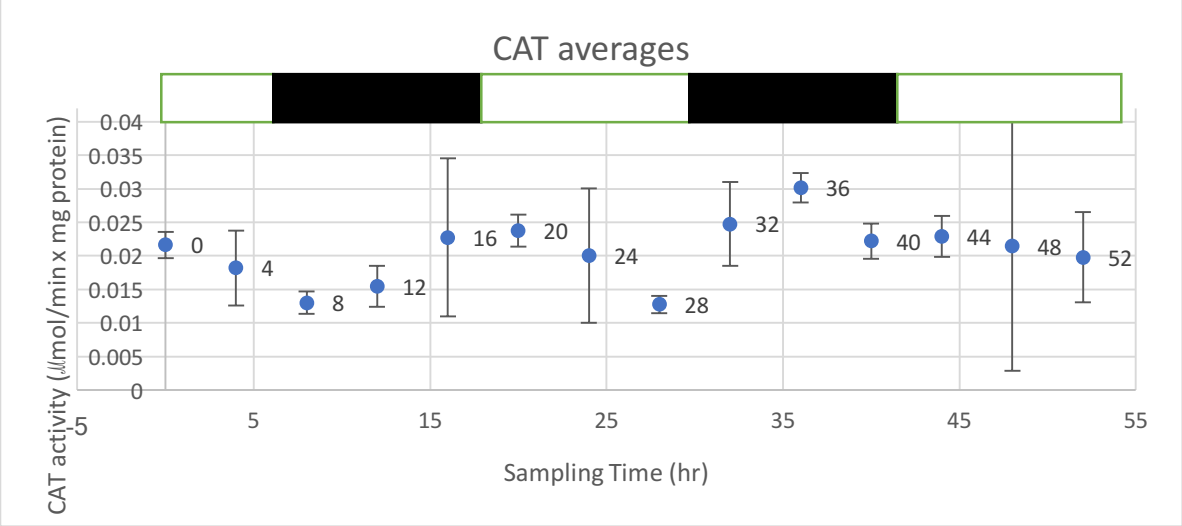

Figure 3: Average catalase activity for each sampling time, with standard error shown. Dark squares indicate the times when lights were turned off, white squares when the lights were turned on.

Glutathione Peroxidase Dependent

GPx dependent has a minimum value at sample 7b of $8.069*10^{-5}$nmol/min*mg protein and a maximum at sample 2c with a value of $4.110*10^{-3}$nmol/min*mg protein. The average is $1.738*10^{-3}$ nmol/min*mg protein and the median is $1.640*10^{-3}$ nmol/min*mg protein. The average value at each sampling time is displayed in Table 3 and Figure 4.

Table 3: Average glutathione peroxidase dependent activity, in nmol/min mg protein, and its accompanying standard error*

Sampling Time	Average GPx activity (nmol/ min*mg protein)	Standard Error
0	0.00040093	0.000044548
4	0.002221	0.001345
8	0.003967372	0.0000543436
12	0.001285	0.000231
16	0.000779	0.000550
20	0.001975	0.001182
24	0.001114	0.000634

28	0.00196	0.000213
32	0.001531	0.000463
36	0.00204	0.000217
40	0.001786	0.000344
44	0.002286	0.001018
48	0.001859	0.00022
52	0.001175	0.0000339

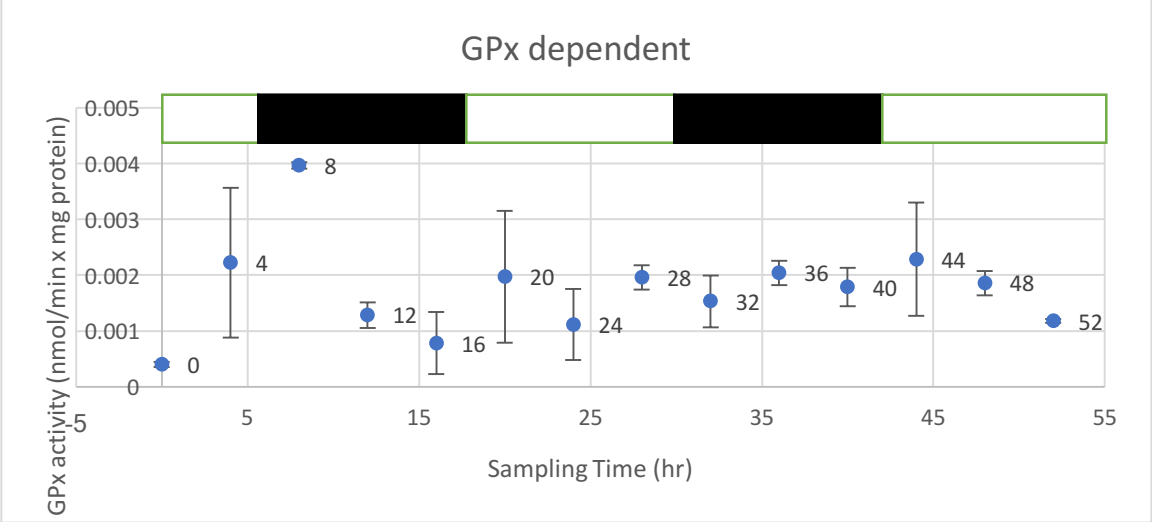

Figure 4: Average glutathione peroxidase dependent activity for each sample time, with standard error shown. Dark squares indicate the times when lights were turned off, white squares when the lights were turned on.

Glutathione Peroxidase Dependent and Independent

The smallest GPx dependent and independent value is $1.619*10^{-5}$nmol/min*mg protein, from sample 5a, and the largest is $3.603*10^{-3}$nmol/min*mg protein, from sample 12a. The average is $1.350*10^{-3}$nmol/min*mg protein, and the median is $1.321*10^{-3}$ nmol/min*mg protein. The average for each sample time is in Table 4 and displayed in Figure 5.

*Table 4: Average glutathione peroxidase dependent and independent activity, in nmol/min * mg protein, and its accompanying standard error*

Sampling Time	Average GPx activity (nmol/ min*mg protein)	Standard Error

0	0.00012923	0.000060291
4	0.000834	0.000408
8	0.001248495	0.000461554
12	0.001186	0.000285
16	0.000716	0.000553
20	0.001503	0.00095
24	0.001153	0.000765
28	0.001579	0.00018
32	0.001656	0.0006
36	0.001561	0.000408
40	0.001984	0.000596
44	0.00213	0.000904
48	0.001367	0.0000574
52	0.001855	0.00031

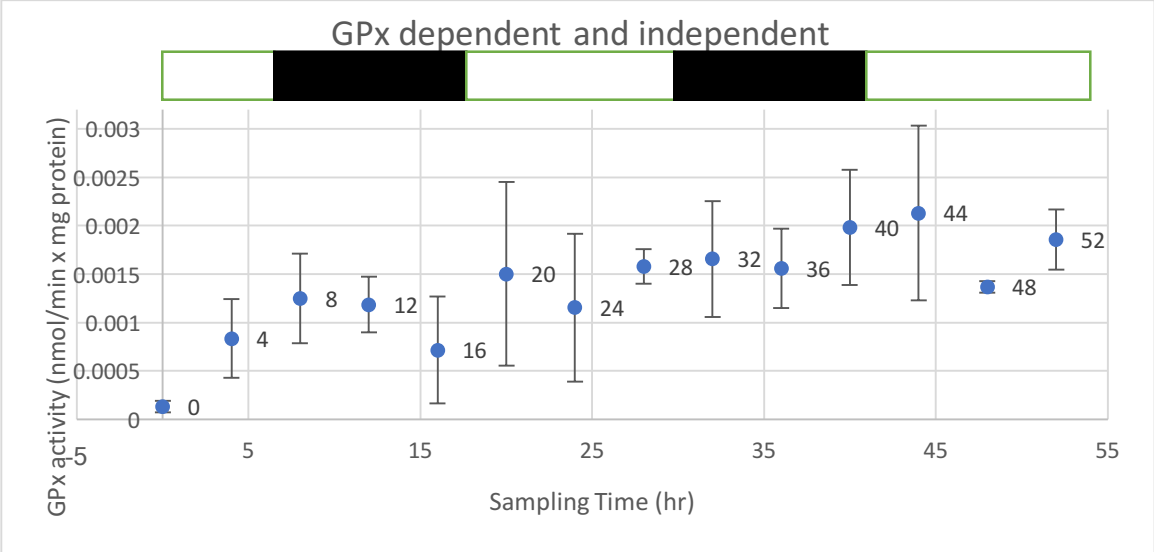

Figure 5: Average glutathione peroxidase dependent and independent activity for each sampling time, with standard error shown. Dark squares indicate the times when lights were turned off, white squares when the lights were turned on.

Statistical Analysis

With a series being considered significant with a $p<0.05$, only one sample set is statistically significant, GPx dependent with a $p=0.02$. SOD has $p=0.68$, CAT $p=0.41$, and GPx independent and dependent $p=0.20$, so none were statistically significant.

Discussion

Unfortunately, the results do not appear to be statistically significant for the antioxidants, except GPx dependent, although viewing the graphs a rhythm does seem to appear. For SOD, a cycle appears to be developing for the first 24 hours, with the average at time 0 and time 24 similar in value with an increase after these points. The signal does appear to dampen out after 28 hours however, which will be discussed later. In addition, there is a large standard error (>2.0) present at time 4 and 28. CAT also appears to have a cycle that seems to repeat from time 0 to time 20. This cycle does not seem to dampen out but does not repeat perfectly, with the cycle not consistent after time 28. After this time point, the CAT levels appear to remain high. For CAT, a large standard error ($>1.0*10^{-2}$) is present at times 16, 24, and 48. GPx dependent and independent does not appear to have much of a cycle, instead rising for most of the time points except for a slight decrease between time 8 and 12 and between time 32 and 36. A large decrease occurs between times 12 and 16 and between times 44 and 48. There also appears to be more variation, since larger standard errors ($>5.0*10^{-4}$) can be seen at times 16, 20, 24, 32, 40, and 44. GPx dependent was considered statistically significant when run against time point, although the cycle appears less strong after hour 28. A large standard error ($>2.00*10^{-3}$) is apparent at time 4, 20, and 44.

A possible cause for the finding of no statistical significance is the variance within the samples. As is apparent by the occasionally large standard errors, at some time

points there is a larger difference between the salmon than at others, which could indicate that the sample average is not actually indicative of the time point average. A possible explanation for this is human error during testing. Inaccurate amounts could have been added to the cuvettes and, since the reactions are dependent on the amounts added, this would alter the amount of antioxidant calculated based on the spectrophotometric data and, therefore, alter the overall time point average.

As described above, the possible rhythm that can be seen in the graphs above appears to dampen out after a few hours. This is a common problem with fish because, as described by Reebs (2011), fish kept in constant conditions will only exhibit a circadian rhythm for typically several days, sometimes several weeks. He also found that the rhythm can vary based on when the animal was captured or if it is in groups or alone. In the wild, fish synchronize based on daily environmental cycles, which would not be present in an indoor aquaculture setting. This could also contribute to the dampening out or overall lack of a circadian rhythm.

Another possible contributing factor to the variation in results and lack of statistical significance is stress. Stress could have occurred because of the sampling procedure, which had all fish in one tank that was sampled every four hours. This means that every four hours, a net was dropped into the tank, three fish were captured and brought to another room where they were put into MS222 for euthanasia. Barton & Iwama (1991) describe stress as "…caused by physical disturbances encountered in aquaculture, such as handling and transport." Stress, they found, could the alter physiological functions, hormones, or cellular mechanisms. Overall, the stress can alter the fish's homeostatic state especially if the stress is chronic. This stress is caused by a

combination of cortisol, adrenocorticotrophic hormone, adrenaline, and several other hormones.

Since stress can alter hormone levels, it is possible that an increase in cortisol could alter the amount of melatonin or antioxidants present within the salmon. If the melatonin was impacted, this would cause alterations in the circadian rhythm, which as discussed previously is dependent on melatonin levels increasing at night. Antioxidants could also be impacted, although it is currently unknown if they are affected and, if they are, if it causes an increase or decrease in their overall levels.

A recent, similar study by Vera & Migaud (2016) did show a significant daily rhythm in antioxidant enzymes through gene expression in the liver, again leading to the question of why the current study did not show a significant rhythm. This is also not the first time that genes encoding these enzymes have been found to have a daily rhythm (Duffield, 2003). In the case of Vera & Migaud (2016), they treated the fish with H_2O_2 at various times of the day and determined that the time of administration did have a significant impact on the overall stress response in the fish. Specifically, they measured the level of cortisol and saw a significant daily rhythm. This leads to more questions about whether stress had an impact on the antioxidant levels in the current experiment or if something else in the current study impacted the levels of antioxidants to cause a change in the antioxidant levels leading to the large variation and lack of statistically significant rhythm.

If this experiment were to be repeated, more than one tank should be used to house the fish and the tanks cycled through to combat a possible stress response. Great care also needs to be taken to avoid as much human error as possible, since this could

have been a contributing factor to the lack of significant results. It might also be interesting to perform the GPx dependent experiment without the sodium azide to include CAT in the overall reaction. This could help confirm the CAT results since the two work together to breakdown H_2O_2. The length of the experiment could also be reevaluated to examine for the possible dampening affect, both increasing and decreasing the length of the experiment to examine if the cycle continues past 52 hours or if the results are statistically significant for a shorter length of time.

In closing, more work needs to be done to study antioxidants and their possible circadian rhythm to allow for a higher dose of H_2O_2 to be used for treatment, especially for sea lice and amoebic gill disease. More tanks should be used to house the fish and cortisol levels should be measured to account for a possible stress effect altering antioxidant or melatonin levels. Additional studies should focus on antioxidant times with increased levels to determine if a higher dose of H_2O_2 can be used without some of the negative affects currently observed in treatment.

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Appendix A: Sample Fish Length and Weight, Recorded Temperature

Time	Fish A Length (cm)	Fish B Length (cm)	Fish C Length (cm)	Fish A Weight (g)	Fish B Weight (g)	Fish C Weight (g)	Temperature (°C)
12pm	-	-	-	54	31	43	10.2
4pm	19	18.5	11.7	68	63	12	10
8pm	19.2	19.6	16	64	73	51	9.8
12am	20	20.8	17.9	65	81	57	9.6
4am	17.2	14.4	18.1	60	33	45	9.9
8am	22.5	19.7	17	92	74	59	9.8
12pm	19	20.5	18	62	81	61	10
4pm	16	22	20	36	96	82	9.5
8pm	19.5	20	20.2	70	83	72	9.8
12am	20	20.4	18.6	73	67	57	9.9
4am	17.5	18	17.5	55	65	54	9.8
8am	19.4	19.9	11.1	67	78	12	9.9
12pm	14.1	12.5	18.2	24	22	59	9.9
8pm	19	18.5	16	63	61	54	9.8

Appendix B: Bradford Assay

Sample	Trial 1 (μg/μL)	Trial 2 (μg/μL)	Average (μg/μL)	Protein Content (μg/μL)
1a	1.569	1.472	1.5205	15.205
1b	1.872	2.027	1.9495	19.495
1c	1.913	1.66	1.7865	17.865
2a	1.346	1.971	1.6585	16.585
2b	1.149	1.714	1.4315	14.315
2c	1.367	1.293	1.33	13.3
3a	0.873	1.28	1.0765	10.765
3b	1.646	1.823	1.7345	17.345
3c	1.129	0.864	0.9965	9.965
4a	1.851	1.849	1.85	18.5
4b	1.309	1.268	1.2885	12.885
4c	1.293	1.255	1.274	12.74
5a	0.645	1.341	0.993	9.93
5b	1.629	1.874	1.7515	17.515
5c	1.967	1.977	1.972	19.72
6a	1.585	1.568	1.5765	15.765
6b	1.907	2.000	1.9535	19.535
6c	1.677	1.397	1.537	15.37
7a	1.181	1.904	1.5425	15.425
7b	0.869	1.522	1.1955	11.955
7c	1.892	1.938	1.915	19.15
8a	1.929	1.931	1.93	19.3
8b	1.79	1.898	1.844	18.44
8c	1.25	0.938	1.094	10.94
9a	1.016	1.184	1.1	11
9b	1.231	1.453	1.342	13.42
9c	1.695	1.624	1.6595	16.595
10a	1.075	0.909	0.992	9.92
10b	1.853	1.929	1.891	18.91
10c	1.745	1.706	1.7255	17.255
11a	1.572	1.638	1.605	16.05
11b	1.159	1.317	1.238	12.38
11c	1.699	1.706	1.7025	17.025
12a	1.706	1.801	1.7535	17.535
12b	1.673	1.681	1.677	16.77
12c	1.665	1.573	1.619	16.19
13a	0.722	0.825	0.7735	7.735
13b	0.712	0.471	0.5915	0.5915
13c	1.242	1.309	1.2755	12.755
14a	1.074	1.14	1.107	11.07
14b	0.738	0.962	0.85	8.5
14c	1.49	1.652	1.571	15.71

Appendix C: Superoxide Dismutase

Sample	Duplicate 1	Duplicate 2	Average
1a	0.7747	1.1414	0.95805
1b	0.3293	0.7942	0.56175
1c	0.827	0.4172	0.6221
2a	1.277	0.806	1.0415
2b	0.908	1.6788	1.2934
2c	0.939	26.935	13.937
3a	1.9502	8.025	4.9876
3b	1.2282	1.5435	1.3858
3c	5.73	2.5489	4.13945
4a	6.341	0.4176	3.3793
4b	1.6934	2.2335	1.96345
4c	0.898	1.0784	0.9882
5a	0.6397	1.6601	1.1499
5b	0.662	0.9004	0.7812
5c	1.7527	0.8199	1.2863
6a	0.7295	0.4303	0.5799
6b	0.2661	6.5973	3.4317
6c	4.1352	1.0209	2.57805
7a	1.2877	1.1512	1.21945
7b	-2.6194	1.1067	-0.75635
7c	-1.5293	1.7623	0.1165
8a	0.5842	2.06	1.3221
8b	-1.969	17.3123	7.67165
8c	1.3593	2.3538	1.85655
9a	1.6171	1.442	1.52955
9b	1.7204	1.6475	1.68395
9c	-0.873	0.9458	0.0364
10a	2.4525	2.7355	2.594
10b	0.5847	1.3407	0.9627
10c	1.3169	0.522	0.91945
11a	6.09	0.9008	3.4954
11b	-0.98	0.4867	-0.24665
11c	1.5729	1.3255	1.4492
12a	0.4931	1.161	0.82705
12b	-7.734	1.6847	-3.02465
12c	2.2281	1.9083	2.0682
13a	3.4534	3.4124	3.4329
13b	404.8	3.597	204.1985
13c	1.8609	3.6018	2.73135
14a	2.4711	4.1214	3.29625
14b	3.6264	5.57	4.5982
14c	0.7655	1.5182	1.14185

Appendix D: Catalase

Sample	Duplicate 1	Duplicate 2	Average
1a	0.030993094	0.018497205	0.02474515
1b	0.030200051	0.009297256	0.019749
1c	0.017422334	0.023369717	0.020396026
2a	0.017636418	0.012360567	0.014998
2b	0.036849	0.017464	0.027157
2c	0.011278	0.013628	0.012453
3a	0.004877	0.015676	0.010276
3b	0.009297	0.018809	0.014053
3c	0.01154	0.017938	0.014739
4a	0.024865	0.015946	0.020405
4b	0.01038	0.016492	0.013436
4c	0.014914	0.010008	0.012461
5a	0.043807	0.035876	0.039841
5b	0.020197	0.023337	0.021767
5c	0.002219	0.011093	0.006656
6a	0.017602	0.023073	0.020338
6b	0.023675	0.023995	0.023835
6c	0.022528	0.031636	0.027082
7a	0.014182	0.007131	0.010656
7b	0.03074	0.042033	0.036386
7c	0.01423	0.01201	0.01312
8a	0.008484	0.017034	0.012759
8b	0.009829	0.012134	0.010982
8c	0.015425	0.013711	0.014568
9a	0.016136	0.013977	0.015057
9b	0.032787	0.031576	0.032181
9c	0.02802	0.026062	0.027041
10a	0.055192	0.010837	0.033014
10b	0.028689	0.032324	0.030506
10c	0.030354	0.023327	0.02684
11a	0.021184	0.021573	0.021379
11b	0.02393	0.028675	0.026303
11c	0.020264	0.017548	0.018906
12a	0.023168	0.023169	0.023168
12b	0.018485	0.018485	0.018485
12c	0.027949	0.026174	0.027061
13a	0.042502	0.008728	0.025614
13b	0.038039	0.054945	0.046492
13c	0.024108	0.010388	0.017248
14a	0.01897	0.022019	0.020495
14b	0.007794	0.012206	0.01
14c	0.03485	0.022915	0.028883

Appendix E: Glutathione Peroxidase Dependent and Independent

Sample	Duplicate 1	Duplicate 2	Average
1a	0.00021147	0.00021147	0.00021147
1b	0.0000412	0.0000412	0.0000412
1c	0.000179985	0.0000899926	0.000134989
2a	0.000194	0.000145	0.00017
2b	0.001348	0.000898	0.001123
2c	0.001451	0.000967	0.001209
3a	0.001493	0.00239	0.001942
3b	0.002225	0.0000927	0.001159
3c	0.000645	0.000645	0.000645
4a	0.001043	0.001043	0.001043
4b	0.000998	0.000749	0.000873
4c	0.001767	0.001514	0.001641
5a	0.0000648	-0.0000324	0.0000162
5b	0.002019	0.001101	0.00156
5c	0.000489	0.000652	0.000571
6a	0.002448	0.003059	0.002753
6b	0.0000823	0.0000823	0.0000823
6c	0.001883	0.001464	0.001674
7a	0.000834	0.001668	0.001251
7b	0.0000269	0.0000269	0.0000269
7c	0.001679	0.002687	0.002183
8a	0.001499	0.001666	0.001583
8b	0.001176	0.001918	0.001831
8c	0.001176	0.00147	0.001323
9a	0.001462	0.000877	0.00169
9b	0.002156	0.003115	0.002636
9c	0.000969	0.001356	0.001163
10a	0.000648	0.001297	0.000972
10b	0.002381	0.00187	0.002125
10c	0.001677	0.001491	0.001584
11a	0.001262	0.001202	0.001232
11b	0.003195	0.002597	0.002896
11c	0.001945	0.0017	0.001823
12a	0.004273	0.002934	0.003603
12b	0.0014	0.001534	0.001467
12c	0.001648	0.000993	0.001321
13a	0.001788	0.000831	0.001309
13b	0.47022	0.298984	0.384602
13c	0.002092	0.000756	0.001424
14a	0.00183	0.000871	0.001351
14b	0.002761	0.001513	0.002137
14c	0.002517	0.001637	0.002077

Appendix F: Glutathione Peroxidase Dependent

Sample	Duplicate 1	Duplicate 2	Average
1a	0.00026434	0.00058155	0.00042294
1b	0.000289	0.000371	0.00033
1c	0.00035997	0.000539955	0.000449963
2a	0.0000776	0.000533	0.00305
2b	0.001797	0.002695	0.002246
2c	0.001934	0.006286	0.00411
3a	0.000896	0.00687	0.003883
3b	0.001298	0.006674	0.003986
3c	0.001613	0.006453	0.004033
4a	0.001564	0.000869	0.001217
4b	0.001747	0.00025	0.000998
4c	0.002019	0.001262	0.001641
5a	0.0000971	0.00013	0.000113
5b	0.002387	0.000918	0.001652
5c	0.000978	0.000163	0.000571
6a	0.003059	0.001224	0.002142
6b	0.000247	0.000206	0.000226
6c	0.004602	0.00251	0.003556
7a	0.001668	0.001668	0.001668
7b	0.000134	0.0000269	0.0000807
7c	0.001511	0.001679	0.001595
8a	0.002499	0.001666	0.002083
8b	0.002964	0.001395	0.00218
8c	0.002351	0.000882	0.001617
9a	0.002046	0.001754	0.0019
9b	0.002156	0.001677	0.001917
9c	0.001356	0.000194	0.000775
10a	0.001621	0.001945	0.001783
10b	0.00153	0.003231	0.002381
10c	0.002423	0.001491	0.001957
11a	0.002003	0.001202	0.001603
11b	0.002597	0.002078	0.002338
11c	0.002078	0.000755	0.001416
12a	0.003851	0.003484	0.003667
12b	0.003068	0.001726	0.002397
12c	0.000794	0.000794	0.000794
13a	0.002494	0.001663	0.002078
13b	0.516426	0.190262	0.353344
13c	0.001513	0.001765	0.001639
14a	0.001452	0.001765	0.001639
14b	0.001452	0.001765	0.001639
14c	0.001637	0.000819	0.001228

Author's Biography:

Alexa Grissinger was born on Mary 17, 1995 in Montgomery, Pennsylvania. She grew up in Elkins Park, Pennsylvania with her parents and two cats, Patrick and Henry. In 2013, Alexa graduated from Mount Saint Joseph Academy. She attended the University of Maine, studying Animal and Veterinary Science with a concentration in Pre-Veterinary Studies and a minor in Equine Studies. Alexa was president of the Maine Animal Club, a sister of Gamma Sigma Sigma, national service sorority, a team member of the Equestrian Team, and participated in numerous other clubs on campus.

After graduation, Alexa plans on attending Colorado State University College of Veterinary Medicine in the DVM program with a focus on mixed-animal medicine. She will also be marrying her fiancé, Joshua Schappert, and, after veterinary school, moving with him to his duty station as part of the Navy.