2011

The impact of tricaine methanesulfonate, 2-phenoxyethanol, and carvone-methyl salicylate on the innate immune response of zebrafish (Danio rerio)

Charles R. Wulff
Colby College

The impact of tricaine methanesulfonate, 2-phenoxyethanol, and carvone-methyl salicylate on the innate immune response of zebrafish *(Danio rerio)*

An Honors Thesis

Presented to the

Faculty of the Department of Biology

Colby College

In partial fulfillment of the requirements for the

Degree of Bachelor of Arts with Honors

By

Charles R. Wulff

Waterville, ME

May 2, 2011

Advisor: G. Russell Danner _____

Reader: Catherine R. Bevier _____

Reader: Paul G. Greenwood _____

Anesthesia plays a vital role in the maintenance of aquaculture species, where it is used to minimize stress during complex handling tasks such as transport, assessment, and harvesting. However, anesthetics have been shown to suppress the innate immune response, which could impact immunity and increase risk of infection. Tricaine methanesulfonate (MS-222) and 2-Phenoxyethanol (2-PE) represent two of the most commonly used anesthetics in aquaculture, with R-(+)-carvone, in the form of carvone-methyl salicylate (CMS) has recently been proposed as an alternative anesthetic for food fish. These three anesthetics were used to assess the influence of anesthetics on the immune system of zebrafish (*Danio rerio)*, a model organism for immunological study. Respiratory burst assays of the zebrafish kidney leukocytes were used to determine the impact of these anesthetics on the immune response. All treatments showed a significantly decreased immunological response, with no statistical differences among treatments. These results indicate that CMS does not suppress the innate immune response any less than the anesthetics 2-PE or MS-222. Further investigation is needed to better understand the influence of CMS impact on a fish's physiological response to confirm these findings.

1. Introduction

1.1 Anesthetics in Aquaculture

Aquaculture in the United States contributes approximately one billion dollars to the US economy, with US aquaculture representing less than 1% of the world's aquaculture production (1). Successful aquaculture depends upon healthy, properly cared for fish, and continuous cost versus growth analysis to determine profitability. Assessing the health and growth of aquacultured fishes usually requires anesthesia because fish become highly distressed when

emersed for examination. Fish are also highly susceptible to stress resulting from more complex handling events, such as transportation, harvesting, and surgery (2). This stress can in turn lead to acute immune suppression, physical injury, or death, while in the long term it can result in reproduction inhibition, growth reduction and an overall decrease in the life-span of the fish (3, 4). Anesthesia is used to reduce the stressfulness events. Chemical anesthesia, such as tricaine (MS-222), is a common method to altering the physiological conditions by raising the CO_2 concentrations or inducing hypothermia may be used to incapacitate the fish (3). An anesthetic's effectiveness is determined by how quickly fish reach the desired level of anesthesia, the rate the fish are revived, the mortality rate, and the amount of anesthetic required to achieve the desired level of anesthesia (3). The cost of the anesthetic must also be taken into consideration, as aquaculture is an industry.

In addition to considering the general aspects of anesthesia, different handling circumstances require varying levels and durations of anesthesia. For example, transportation requires no more than sedation of the fish, which can be achieved by either hypothermia or light anesthetics, or a combination of the two. This allows the fish to remain calm, and prevents the fish from settling to the bottom, crowding, and asphyxiating (3). Handling for emersion or surgery usually requires a much deeper level of anesthesia, both to prevent undue stresses to the fish and to also allow for ease of handling. However, prolonged exposure or improper dosing at high levels of anesthesia may kill the fish. Effective anesthetics also need to suppress the hypothalamo-pituitary-interrenal (HPI) axis. The HPI organs responsible for controlling a number of hormones, including cortisol. Cortisol is an anti-inflammatory and responsible for suppressing the innate immune response. If the HPI axis is not suppressed this will result in increased stress levels even when the fish receives anesthesia, as these organs continue to release

stress hormones (5). The size, activity level, metabolism, and gill-body mass ratio of the fish must all be taken into consideration when determining the optimal anesthetic and dosage. Larger fish, those with a low activity level or low gill to body mass ratios often require larger dosages to achieve anesthesia (2). Water chemistry and physical characteristics , such as lower temperatures and alterations of the pH, affect fish anesthesia (2, 3). These conditions vary from anesthetic to anesthetic, as they can increase the solubility of the anesthetic, serve as a secondary form of anesthesia, or reduce the efficacy of the anesthetic.

There are legal complications that must also be considered when determining which anesthetics may be used. Fish that are to be used for human food face tight restrictions so consumers do not ingest the metabolized anesthetics. The problem with these metabolites is two-fold. First, the use of these anesthetics may alter the quality of the fish, potentially providing consumers with food that is inferior or contains unknown compounds (3). Second, these metabolites may prove a health risk, and therefore are regulated to protect consumers. Currently, only MS-222, CO_2, and hypothermia are approved means of anesthesia for food fish, with MS-222 requiring a 21 day withdrawal period before the fish can be harvested for food products (3, 5).

Though anesthesia is used to reduce stress and prevent complications such as immune-suppression and death, applying anesthetics can result in similar physiological conditions to handling stress. These complications can vary, depending on both the level of anesthesia achieved and the type of anesthetic. For example, 2-PE reduces the effect of crowding stress when used to induce sedation in gilthead seabream (*Sparus aurata* L.), yet results in an increased negative immunological response if surgical anesthesia is induced under similar conditions(6). Tricaine MS-222 does not impede the HPI, allowing for stress hormones, such as cortisol, to

still be expressed under heavy anesthesia, yet it does not result in significant immune suppression (5, 7). The stress and immune suppression that can accompany anesthesia can result in an increased risk of infection, especially in the case of surgery, which potentially creates a vector for infection.

1.2 Tricaine methanesulfonate

Tricaine methanesulfonate (MS-222) is a commonly used anesthetic in aquaculture, especially as it is the only anesthetic legal for food fish in the United States. It is technically categorized as a local anesthetic; however, it is primarily used as a general anesthetic in aquaculture. Anesthesia is achieved by adding MS-222 in proper concentrations to the anesthetic bath to be absorbed by the skin and gills of the fish, though some form of buffering is usually required to prevent a significant reduction in the solution pH (3, 8). The rate of anesthesia and revival are directly linked to the dose administered, as are the chances of side effects (2, 8). Tricaine methansuflonate increases the risk of hypoxia and acidosis and does not decrease the basal levels of blood cortisol, since MS-222 does not inhibit the HPI (5, 8). This anesthetic is excreted through both the gills and urine, with non-metabolized MS-222 and polar metabolites filtered by the kidney; MS-222 is usually undetectable eight hours after administration (3, 8). Like other local anesthetics such as benzocaine, it acts by blocking Na^+ channels in neurons, reducing transmission of action potentials (7).

1.3 2-Phenoxyethanol

2-Phenoxyethanol (2-PE) is a general anesthetic. The active ingredient is ethylene glycol monophenyl ether when metabolized. Its exact mechanism is currently poorly understood, though it is one of the most frequently utilized anesthetics after MS-222 (2, 6). However, recent

research has indicated that it acts by reversibly suppressing *N*-methyl D-aspartate (NMDA) receptors (9). Though 2-PE has been shown to suppress the innate immune response, it is an anti-fungal and antibacterial compound, capable of reducing the risk of infection (2, 3, 6). Though low in cost, 2-PE carries the risk of inducing neuropsychological disorders in the human handlers (10).

1.4 Carvone-methyl salicylate (R-(+) carvone)

R-(+)-carvone, or simply carvone, is a monoterpene ketone that acts as a natural anesthetic. It is an essential oil, and it is derived from *Mentha spicata* var. *crispa lamiaceae* and is commonly used for spearmint flavoring (11). R-(+)-carvone decreases motor activity, and it significantly reduces the nerve transduction of mice (11, 12). These studies demonstrated that carvone most likely reversibly inhibits the Na^+ channels of nerves, suppressing transduction (12). Though carvone has not been widely used as an anesthetic in aquaculture, preliminary testing has shown that it is effective at achieving rapid anesthesia and quick revival (13). However, carvone's physiological impact in fish is mostly unknown. This study used R-(+) carvone in the form of carvone-methyl salicylate (CMS) as an anesthetic.

1.5 Zebrafish (Danio rerio)

The zebrafish is a frequently used model organism in immunology and genomics. The species is small, very easily maintained, relatively low in cost to acquire, and are an effective laboratory animal because of their short generational time and the large number offspring produced during each reproduction cycle (14). Another advantage is that all stages of life can be readily observed. As the embryo is transparent through the larval stages, internal development is also readily visible (15, 16). Zebrafish genetics have been well researched, with large portions of

its genome sequenced and readily available through various databases. Mutagenesis screens have been utilized both to determine gene functions, and to construct molecular models of other organisms (15). Zebrafish have the advantage of being similar to many other teleost fish, as well as other vertebrate species, making it a useful model for many different species (17).

The kidney is the primary immunological and hematopoietic organ of adult zebrafish. The head kidney portion is similar to bone marrow in mammalian species, and acts as the primary site for hematopoiesis (18). Unlike some other species, where the spleen also acts as a major site of hematopoiesis, the kidney acts as the sole source of blood and immune response cells (19). In addition to the creation of immune cells, the kidney also acts as the major site of the immune response. It is the primary location of non-circulating granulocytes, such as neutrophils and a cell type that acts as both an eosinophil and basophil (14, 16, 20). The kidney is also the site of a large number of macrophages and monocytes, and is one of the locations where T cells may be found in the zebrafish (14).

The neutrophils of zebrafish act in a similar manner to those of mammals (16). Like mammalian neutrophils, they are granulocytes that primarily destroy infectious agents through phagocytosis. The actual act of destruction is usually achieved through the release of reactive oxygen species (ROS) into the vesicle containing the phagocytosed bacteria, along with hydrolytic enzymes, in a process known as a respiratory burst (17). The production of ROS relies on phosphorylation of NADPH oxidase, which becomes active and then reduces molecular oxygen to produce superoxide. The superoxide is then converted to hydrogen peroxide by superoxide dismutase (21). The superoxide, hydrogen peroxide, and the reactive oxygen species derivatives of the molecules are then utilized for the respiratory burst (21). Neutrophils can also

release granules that contain antimicrobial and inflammatory proteins, resulting in further immune response (16).

1.6 Respiratory Burst Assay

Neutrophils release ROS when they phagocytose a bacterium or cellular debris. This response can also be triggered by specific chemicals, such as propylene glycol monomethyl ether acetate (PMA)(22). As the amount of ROS released is an indicator of the health of the innate immune response, analysis of ROS activity can reveal the influence of environmental factors on the organism. This analysis relies on fluorescence produced by the oxidation of select materials by the reactive oxygen species, with stronger fluorescence indicating a more significant innate immune response (21, 22). This fluorescence is gauged by a microplate reader using light transmission and optical density measurement at 485 and 530 nm. This allows for multiple replicates to be measured simultaneously, allowing for an effective comparison of treatments (21). The comparison of unstimulated and stimulated cells, such as those exposed to bacteria or PMA, can allow for the exclusion of background ROS release and cell counts, giving an overall measure of the organism's health. Because their kidneys are small, zebrafish require the pooling of kidneys from multiple individuals to achieve sufficient level of fluorescence (22).

1.7 Purpose of the study

Carvone has been investigated as a potential anesthetic in aquaculture, and understanding its influence on the physiological aspects of fish is important in determining its effectiveness. Through the use of respiratory burst assays, carvone's immunological impact can be compared to MS-222 and 2-PE, and to control samples. This study proposes that carvone may have a lesser impact on the zebrafish's innate immune response than MS-222 or 2-PE.

2. Material and Methods

2.1 Animals

Wild type zebrafish were maintained in community tanks that received a 16:8 light-dark cycle. They were allowed to acclimate to their tanks for 1 week before any procedures were performed to eliminate any stress response due to transport. Water temperature was held at 28^{o}C, and the fish received regular daily feedings. All fish were euthanized with a 0.4 mg/mL MS-222 solution preceding kidney removal (22). Kidney tissue from both male and female fish was used. All procedures were approved by the Colby College Institutional Animal Care and Use Committee.

2.2 Anesthetics

The concentrations of anesthetics that were used over the course of the experiment were determined during preliminary testing to ensure that a 1-hour exposure to the anesthetic could be achieved in which zebrafish suffered a loss of consciousness followed by recovery (7).

The MS-222 solution was prepared with 0.0161 g of 99.5% pure tricaine methanesulfonate (Ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich E10521, St. Louis, MO). This was dissolved directly in 200 mL of 28^{o}C aerated water for a 0.0805 mg/mL solution. This tricaine solution had an average pH of 6.9 at this concentration. The 2-PE solution was prepared with 65µL of a 99% solution of 2-PE (2-phenyloxyethanol, Sigma-Aldrich 60248 , St. Louis, MO). This was diluted in 200 mL of 28^{o}C aerated water for a 0.358 mg/mL solution with an average pH 7.8. The carvone solution was composed of carvone-methyl salicylate (Fish-eezzz; Maine Conservation Medicine Center, Waterville, ME). This was then diluted by adding 70µL of the solution to 200 mL of water for a solution with a final concentration of 95.09

mg/mL of carvone, and had an average pH of 6.9. Solutions were not buffered to minimize impact of buffering agents on immune system and due to their closeness to physiological pH. A control group was placed in 200 mL of water that had been aerated and was 28^{o}C, but received no other treatment.

2.3 Fish Selection and Anesthesia Exposure

For each trial, twelve fish were selected at random from the same community tank. These fish were immediately transferred to a 2 L tank matching the conditions of the community tank. A group of three zebrafish was transferred to 200 mL of either one of the anesthetic solutions ,or 200 mL of tank water for a control group. The time of initial exposure was recorded, and the fish were observed over a 1-hour period to ensure that they were both unconscious and alive (7). After 1-hour of exposure, the zebrafish were then revived by transferring them to four containers containing 200 mL of tank water, with each exposure group remaining isolated from the others (7). If any of the fish did not recover, or died during the one hour period, the procedure would be repeated and the current group of zebrafish discarded to ensure that there was no interference due to chemical changes caused by the individual's death. This was only done on only one occasion, with the two fish dying due to an overdose of 2-PE. After revival, 20 mL of 4mg/mL MS-222 solution was added to each of 200 mL of water, euthanizing each group (21). Tricaine MS-222 was used to anesthetize all sample groups as it has been indicated in literature to have the lowest level of suppression of the innate immune system, and to insure that the control group received the same means of euthanasia as treatment groups.

2.4 Kidney Extraction and Respiratory Burst Assay

Kidneys were extracted through microdissection of each fish and all kidneys from the same exposure group were pooled together in 700 mL of DMEM-F12 media (DMEM-F12, Hy Clone Laboratories, Logan, UT) and kept on ice. Single cell suspensions were produced by pipette aspiration. Each exposure group was distributed to six different wells on a black 96-well plate. Each well received 100 µL of cells and 0.1 µg of 3-dichlro-flourescein diacetate (2',7'-dichlorodihydrofluorescein diacetate, Invitrogen, Carlsbad, CA) dissolved in 50µL of DMEM-F12. Three wells of each exposure group received the unstimulated control treatment of 50 µl of DMEM-F12. The remaining three wells of each group received .04 µg of PMA (propylene glycol monomethyl ether acetate, Sigma-Aldrich, St. Louis, MO) dissolved in 50 µl of DMEM-F12 to stimulate the cells' respiratory bursts. Fluorescence was measured on an FLX800TB plate reader (BioTek) with excitation and emission filters of 485 and 530 nm. The respiratory burst assay was run for 3.5 hours, with samples taken every 15 minutes (22).

2.6 Data Analysis

The stimulation index (SI) of reactive oxygen species production was determined by calculating the mean relative fluorescence of the PMA stimulated wells and dividing this by the unstimulated control wells (22). This was used to indicate the relative level of oxidative bursts for each exposure groups, and the stimulation index was plotted over time to compare the different levels of suppression or excitation (22). The unstimulated fluorescence, or background fluorescence, will be utilized to determine the data validity, with excessively high or low background fluorescence acting to disqualify results. If the background fluorescence was either too high or too low, this was an indication of unsatisfactory kidney sample.

3. Results

3.1 Anesthetics Effects on ROS Levels

The SI was utilized to determine the differences, if any, that existed between the treatments for the kidney leukocytes. The peaks of the SI plots were utilized as these best represented the stimulation level of the kidney leukocytes. These peaks were taken from the 90 or 105 minute samplings, which were consistently the highest peaks across all anesthetics and trials (Fig. 1).

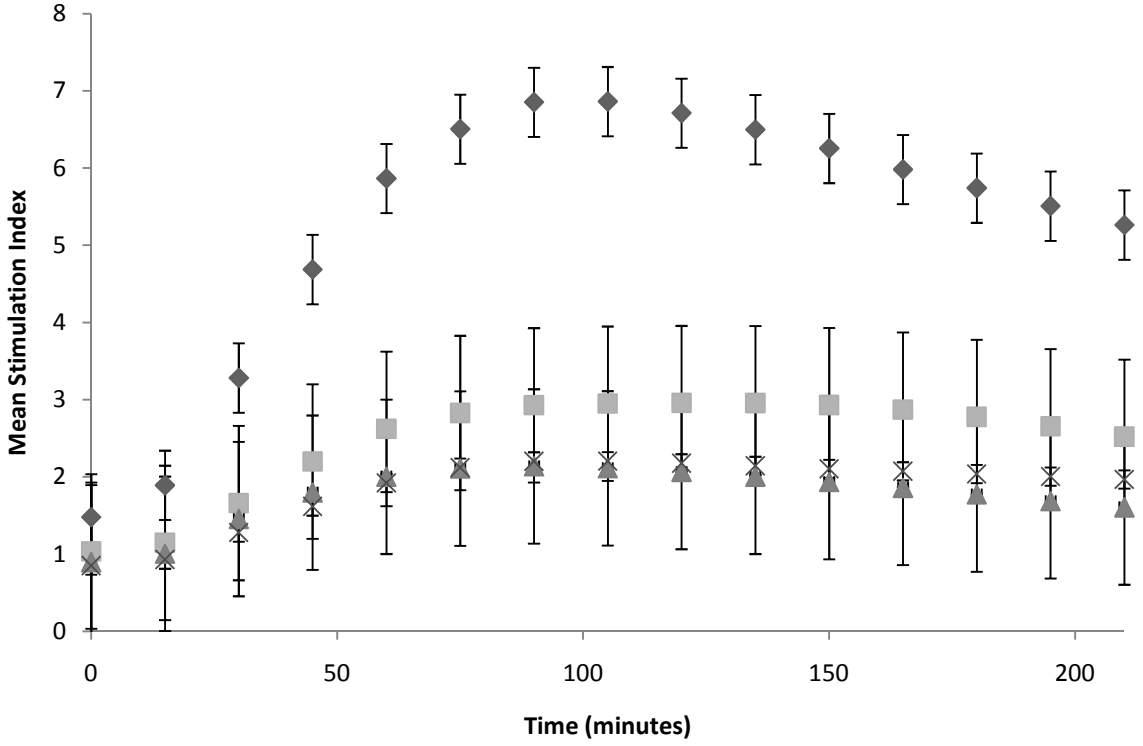

Figure 1. Mean fluorescence over time.
The mean values of the SI over a 210 minute period, with the fluorescence read at 15 minute intervals. The control group (♦, R^2=0.9972), CMS(▲, R^2=0.9962), MS-222 (■, R^2=0.9978), and 2-PE(X, R^2=0.9969) all showed maximum peaks at either 90 or 105 minutes. These were determined by H_2DCFA fluorescence. n=7.

The means of the trials were compared (Fig. 2), and statistical analysis by ANOVA showed that there was a significant difference between the trials ($p<0.005$). The control group showed a significantly stronger SI in comparison to the treatments groups. High SI values mean that the innate immune response of the zebrafish was not as suppressed. Furthermore, the SI for MS-222 appears to be slightly higher than that of the other two anesthetics. The MS-222 treatment's SI was 46.48% that of the control, versus the CMS treatment (36.17% of the control SI) and 2-PE (37.02% of the control SI). However, the difference was not statistically significant (ANOVA, $p > 0.05$).

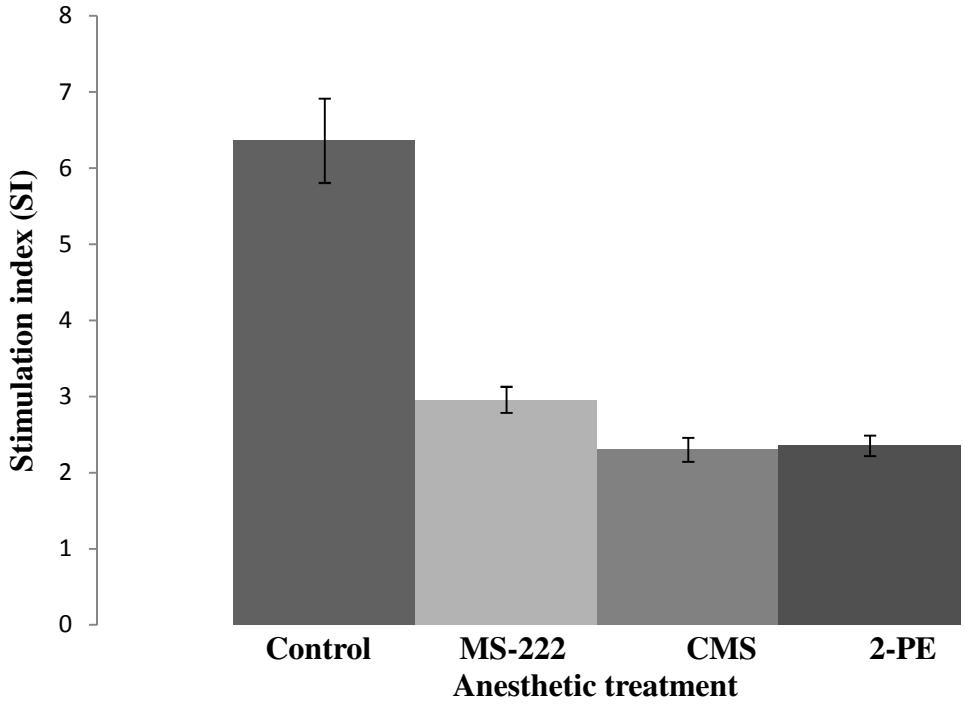

Figure 2. Comparison of the mean peaks of the control, MS-222, CMS and 2-PE SI treatment groups. Data represents the means of the peak stimulation indexes at either 90 or 105 minutes with the appropriate SE bars. n=7. P<0.005, determined by ANOVA test. The control showed a significantly higher SI than any of the anesthetics, indicative of these cells having a stronger immune response when stimulated.

3.2 Analysis of Background Fluorescence

The mean background fluorescence was plotted over time to determine if any of the treatments showed a significantly different fluorescence level, as this could indicate that the basal levels of expression could have already been elevated. It would also indicate if there were any fixed patterns of fluorescence levels.

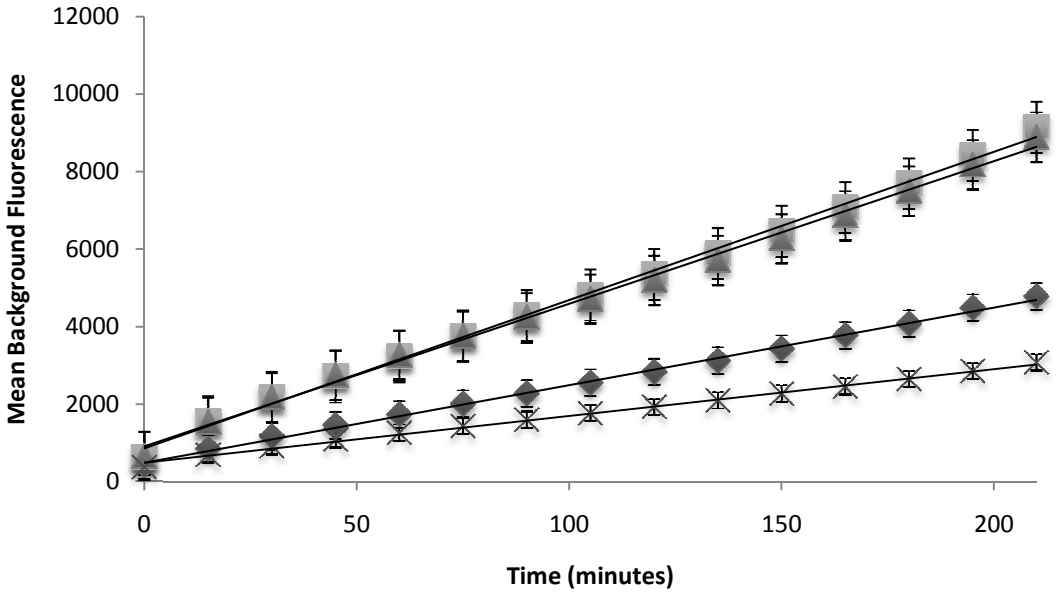

Figure 3. The mean background fluorescence over time by treatment groups.
The mean values of the background fluorescence over a 210 minute period, with the fluorescence read at 15 minute intervals. The control group (♦, R^2=0.9972), CMS(▲, R^2=0.9962), MS-222 (■, R^2=0.9978), and 2-PE(X, R^2=0.9969) all showed a linear fluorescence over time. These were determined by H_2DCFA fluorescence. n=7.

Background fluorescence positively and significantly increased over time, and followed a linear regression for fluorescence increase over the kinetics assay (Fig. 3). The mean background fluorescence had a near perfect fit ($R^2 > 0.99$). Mean fluorescence indicated that the CMS and MS-222 treatments significantly elevated the background fluorescence in each trial. However, analysis of the background fluorescence by each trial indicated that there was no significant difference among the individual treatments between each round of testing (ANOVA, $P > 0.05$)

(Fig. 4). Comparison of the background fluorescence peaks also indicates a high variability of the ranges of the data between each trial, with the minimal background fluorescence value of 1,062.67 and a maximum value of 21,183.67 over all of the trials. If the final fluorescence reading was less than 500 or greater than 25,000, than the sample was likely contaminated and the results would have been discarded. There also appeared to be a high variability within the trials themselves, most obvious in trial 6 (fig 4).

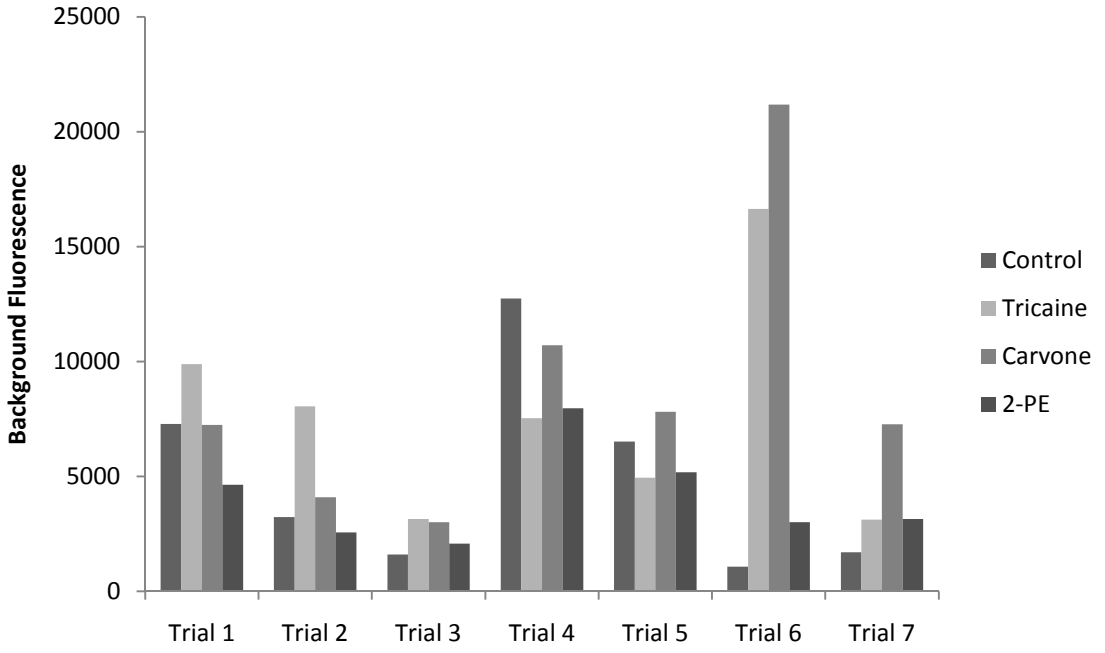

Figure 4. Comparison of raw background fluorescence peaks for the control, CMS, MS-222, and 2-PE groups by trial. n=7 for each group. There was no significant difference between the treatment groups (ANOVA, P >0.05). These are raw data from the trials, compared to determine the basal variance of the kidney leukocytes fluorescence.

4. Discussion

Anesthesia has been shown to suppress the innate immune response in many different species, and this was confirmed through analysis of the SI values of the respiratory burst analysis (Fig. 1) (2, 5-7). A larger SI indicates a much higher active release of ROS in a stimulated state

in comparison to the basal state of the samples, the results effectively demonstrate that the control group, which received no anesthesia, had twice the ROS release as the groups treated with anesthesia (Fig. 1). Furthermore, the data indicate that all of the anesthetics suppress the innate immune response equally. However, though all suppress the immune response relatively equally, the mechanisms of the suppression within leukocytes are not necessarily the same and cannot be determined without further experimentation. Previous literature has indicated that leukocyte suppression may be caused by the interference with adhesion of leukocytes to target sites, such as carbohydrate-lectin binding, which could be investigated in future studies (2, 6, 7).

Of interest is the level of the innate immune response suppression by the MS-222 treatment. Though difference in the mean SI values were not statistically different between treatments, the MS-222 mean SI percent difference was approximately 10% greater than the other two treatments, with a -53.5% suppression in comparison to a suppression of -63.8% by CMS and -63% by 2-PE (Fig. 4). Though previous studies indicate that 2-PE suppresses ROS while MS-222 shows minimal suppression, there may be no actual difference between the effects of the two anesthetics on the innate immune system (7). However, a larger data sample may allow for a more clear delineation of the results, and further testing should be pursued to determine if there is a difference.

The linear regression of the background fluorescence reflects good kidney leukocyte health and a successful protocol (Fig. 3). As there is no obvious plateau or deviation, this implies both that the fluorescent H_2DCFDA particles do not decrease significantly in fluorescence after oxidation, and that the cells continue to produce ROS at a relatively constant rate over the course of the trial. This finding is important, as a constant rate reflects that there is little cell death during the course of the trial. This would be detected a drop off in the

fluorescence. There are also no apparent external stimulating factors such as bacterial contamination, which would be noted by an upturn in the background fluorescence data. Both of these conditions would indicate a flaw in the experimental methods (20).

The noticeable variation of the background fluorescence among trials could have several sources (Fig. 4). The first is that the background fluorescence could have been affected by the amount of cells present, though the SI would not be impacted by the cell counts, as differences in the amount of cells present in an assay would affect the amount of ROS that could be released during a time period. As equalization of the cells caused the background fluorescence resulted in measurements that were too high to be read by the fluorescence reader. It also caused an exceptionally high variability within the triplicate wells. Thus, the cell concentrations could not be adjusted. The cell counts should not have affected SI values, as they are excluded by the calculation, and thus this variation is acceptable for the purpose of the assay. Revision of the protocol to minimize the variation in the results, which does not result in excessively high background fluorescence, should be sought in future studies. However, this variance did not seem to have any significant

Notably, while there was no significant difference between any of the treatment groups and the control, the mean background fluorescence did appear to be greater in the CMS and MS-222 treatments than in to the control and 2-PE samples. This indicates that the basal stimulation of these groups may in fact be elevated, which masks the stimulated results magnitude and thus resulted in a lower SI value. This may mean that CMS and MS-222 may in fact stimulate the immune response, which should be investigated in further studies. Also, a lethal dose of MS-222 was used to euthanize all the treatment and control groups. Though this may have acted as a confounding variable, MS-222 was used in other studies as a means of euthanasia without

noticeable interference with their results and the lack of noticeable suppression within the control groups indicates that there was little interference within this study (6, 22). Furthermore, it insured that the control group received the same euthanasia treatment, thus preventing any variation between the control and treatment groups beyond the anesthetics.

5. Conclusion

In conclusion, MS-222, CMS, and 2-PE all suppressed the innate immune response of the zebrafish kidney leukocytes, as reflected in signidicantly lower SI vlues compared to control treatments. There was no significant difference in the level of suppression of the immune response among the anesthetics, contrary to previous findings. Thus, CMS did not show any significant difference from the established anesthetics.

Acknowledgments

This research was made possible by grants through the Colby College Biology department and the Colby College Dean's Fund. The author thanks Austin Segel and Patti Easton for animal care assistance and Lynn Hannum for consultation on respiratory burst protocol and analysis. The author would like to extend additional thanks to Liam O'Brien for assistance with the study design and statistical analysis, as well as Nora McCall for assistance with editing. Finally, the author would like to thank Drs. G. Russell Danner, Catherine R. Bevier and Paul Greenwood for acting as advisors and mentors on this project and assisting with the review of this paper.

References

1. NOAA. 2010. Aquaculture in the United States. D. o. Commerce, and NOAA, eds, Silver Spring, Maryland.

2. Weber, R. A., J. B. Peleteiro, L. O. G. Martín, and M. Aldegunde. 2009. The efficacy of 2-phenoxyethanol, metomidate, clove oil and MS-222 as anaesthetic agents in the Senegalese sole (*Solea senegalensis* Kaup 1858). *Aquaculture* 288:147–150.

3. Coyle, S. D., R. M. Durborow, and J. H. Tidwell. 2004. Anesthetics in Aquaculture. South Regional Aquacultural Center, Jackson, Mississippi.

4. Ortuño, J., M. A. Esteban, and J. Meseguer. 2002. Lack of effect of combining different stressors on innate immune responses of seabream (*Sparus aurata* L.). *Veterinary Immunology and Immunopathology* 17-27.

5. Small, B. C. 2003 Anesthetic efficacy of metomidate and comparison of plasma cortisol responses to tricaine methanesulfonate, quinaldine and clove oil anesthetized channel catfish *Ictalurus punctatus*. *Aquaculture* 218:177–185.

6. Ortuño, J., M. A. Esteban, and J. Meseguer. 2002. Effects of phenoxyethanol on the innate immune system of gilthead seabream (Sparus aurata L.) exposed to crowding stress. *Veterinary Immunology and Immunopathology* 89:29-36.

7. Ortuño, J., M. A. Esteban, and J. Meseguer. 2002. Effects of four anaesthetics on the innate immune response of gilthead seabream (*Sparus aurata* L.). *Fish & Shellfish Immunology* 12:49-59.

8. Carter, K. M., C. M. Woodley, and R. S. Brown. 2011. A review of tricaine methanesulfonate for anesthesia of fish. *Reviews in Fish Biology and Fisheries.* 21:51-59.

9. Zahl, I. H., A. Kiessling, O. B. Samuelsen, and M. K. Hansen. 2009. Anaesthesia of Atlantic cod (Gadus morhua) -- Effect of pre-anaesthetic sedation, and importance of body weight, temperature and stress. *Aquaculture* 295:52-59.

10. Neiffer, D. L., and M. A. Stampe. 2009. Fish Sedation, Anesthesia, Analgesia, and Euthanasia: Considerations, Methods, and Types of Drug. *Institute for Labratory Research Journal* 50:343-360.

11. Buchbauer, G., W. Jäger, A. Gruber, and H. Dietrich. 2005. R-(+)- and S-(−)-Carvone: influence of chirality on locomotion activity in mice. *Flavour and Fragrance Journal* 20:686–689.

12. Sousa, D. O. P. D., F. F. D. F. N. Brega, and R. N. B. D. Almeida. 2007. Influence of the chirality of (R)-(+)- and (S)-(-)-carvone in the central nervous system: A comparative Study. *Chirality* 19:264-268.

13. Danner, G. R. 2011. Carvone as an Anesthetic in Aquaculture.

14. Phelps, H. A., and M. N. Neely. 2005. Evolution of the zebrafish model: From development to immunity and infectious disease. *Zebrafish* 2:87-103.

15. Briggs, J. P. 2002. The zebrafish: A new model organism for integrative physiology. *American Journal of Physiology- Regulatory, integrative and comparative physiology.* 282:R3-R9.

16. Lieschke, G. J., A. C. Oates, M. O. Crowhurst, A. C. Ward, and J. E. Layton. 2001. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* 98:3087-3096.

17. Hermann, A., and C. Kim. 2005. Effects of arsenic on zebrafish innate immune system. *Marine Biotechnology* 7:494-505.

18. Bennett, C. M., J. P. Kanki, J. Rhodes, T. X. Liu, B. H. Paw, M. W. Kieran, D. M. Langenau, A. Delahaye-Brown, L. I. Zon, M. D. Fleming, and A. T. Look. 2001. Myelopoiesis in the zebrafish, *Danio rerio*. *Blood* 98:643-651.

19. Davidson, A. J., and L. I. Zon. 2004. The `definitive' (and `primitive') guide to zebrafish hematopoiesis. *Oncogene* 23:7233-7246.

20. Palic´a, D. a., C. B. Andreasenb, D. E. Frankc, B. W. Menzeld, and J. A. Roth. 2005. Gradient separation and cytochemical characterisation of neutrophils from kidney of fathead minnow (*Pimephales promelas* Rafinesque, 1820). *Fish & Shellfish Immunology* 18:263-267.

21. Hermann, A. C., P. J. Millardb, S. L. Blakea, and C. H. Kima. 2004. Development of a respiratory burst assay using zebrafish kidneys and embryos. *Journal of Immunological Methods* 292:119-129.

22. Kaplan, J. E., R. D. Chrenek, J. G. Morash, C. M. Ruksznis, and L. G. Hannum. 2008. Rhythmic patterns in phagocytosis and the production of reactive oxygen species by zebrafish leukocytes. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 151:726-730.