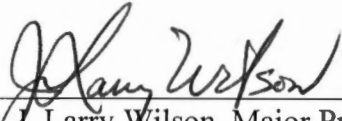
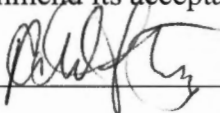


To the Graduate Council:

I am submitting herewith a thesis written by Jamillah Z. Johnson entitled "Efficacy of Clove Oil as an Anesthetic and Stress Reducer in Yearling Rainbow Trout". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Wildlife and Fisheries Sciences.


Dr. J. Larry Wilson, Major Professor

We have read this thesis and
recommend its acceptance:




Accepted for the Council:


Associate Vice Chancellor &
Dean of The Graduate School

**Efficacy of Clove Oil as an Anesthetic and Stress Reducer in
Yearling Rainbow Trout (*Oncorhynchus mykiss*)**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Jamillah Z. Johnson

August 2000

AG-VET-MED.

Thesis
2000
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Dedication

This thesis is dedicated to my family.

Acknowledgments

I would like to express gratitude to Dr. J. Larry Wilson for his guidance and motivation and to the members of my committee, Drs. Richard Strange and Tom Hill, for the time they have taken to review my work. Thank you, Dr. Arnold Saxton, for assistance and statistical support.

I also want to thank the departmental staff, especially to Regina, for always going the extra mile. Thanks to the students who got down and dirty in fish juice (Aggy Spicer, Amanda Smith, Billy Goodrich and Jeff Highfill).

Finally, thank you to friends and family that were supportive in my decision to pursue the M.S. degree at the University of Tennessee.

Abstract

The demand for a safe, economical, easy-to-use anesthetic is becoming increasingly necessary to keep up with the demands of aquaculture. Over the past five years, clove oil has become an often-used anesthetic in fisheries. It is inexpensive, safe to use, and has no stringent regulations imposed on it.

We examined the use of clove oil as a stress reducer and as an anesthetic in yearling rainbow trout. When exposed to a 25 mg/L dosage of clove oil, trout lost equilibrium and became anesthetized within approximately one minute (range of 45-90 seconds) after exposure. They recovered and were swimming normally within 5-10 minutes after being placed in fresh water.

When blood cortisol levels (an indicator of stress) were measured in trout exposed to an anesthetizing dose of clove oil for a 60-minute period, there was a significant increase in cortisol concentration (166.2 ng/mL) after 10 minutes in the anesthetizing solution. Cortisol concentrations then decrease to levels lower than that of Time 0 fish after 30, 40, 50, and 60 minutes in the clove oil solution.

The mean cortisol concentration level (163.5 ng/mL) in fish from the “standard” handling practice used in culture operations was significantly higher after 15 minutes in clove oil than the mean cortisol concentration level (93.1 ng/mL) of fish with no exposure to clove oil (control), and to the mean cortisol level (65.7 ng/mL) of undisturbed fish (sneak) being exposed to clove oil.

Clove oil is readily available, easy to use, and safe to humans. Its advantages are that it induces a quick induction time and an adequate recovery time in rainbow trout. It

is inexpensive when compared to MS-222 and has no FDA regulations. One potential disadvantage is that at concentrations of 25 mg/L clove oil stimulates a cortisol stress response. There is relatively little information on the effects of clove oil on the physiology of fish. The examination of clove oil as an inducer of stress should be investigated more closely.

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Chapter I

Introduction

Aquaculture is a science that has been developing for many years. It is a growing field that demands continuous progress towards trouble-free care taking. The culturist aims for the least amount of problems and the maximum amount of healthy fish. The fish may be used for food, by-products, research or just pleasure. Increases in the demand for fish pushes for advancements to be made for improved equipment and chemicals to meet the production needs.

A healthy fish will exhibit maximum growth and reproductive capabilities. A stressor can be defined as "stimulus acting on a biological system" (Pickering 1981). There are numerous things that can be categorized as a stressor. Some of the more common stressors that have been associated with aquacultural production systems include: grading, tagging, egg stripping, weighing or measuring length, surgery, confinement, overcrowding, abnormal temperature or fluctuations in temperature, poor water quality, pollutants, aggressive tank mates and transportation (Pickering 1981; Stuart 1981; Andrews 1988; Anderson et al. 1997).

When the stressor (stimulus) acts on the biological systems, the fish reacts with an attempt to escape the situation or adapt and regain homeostasis (Pickering 1981; Andrews 1988). The first response to a stressor is to escape the situation. The fish releases a hormone called adrenaline, which gives energy to the muscles and allows them to move quickly to escape. Unfortunately, the release of this chemical disturbs the osmoregulation (ion regulation) of the fish (Figure 1).

Osmoregulation in freshwater fishes

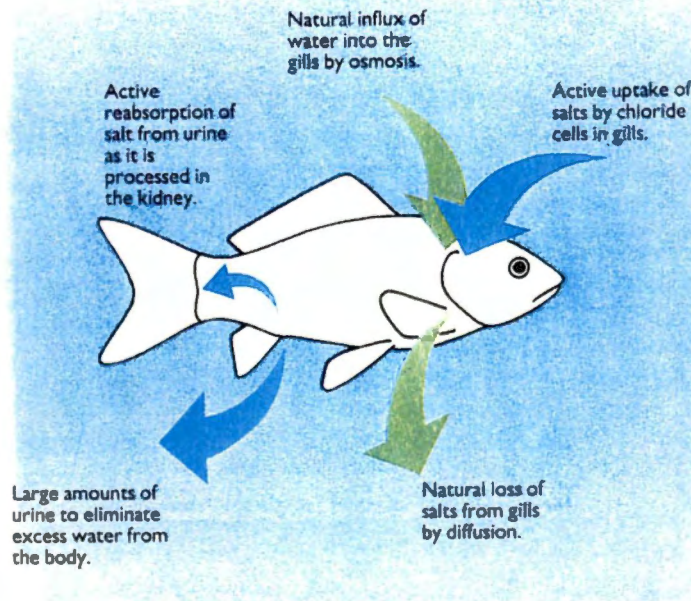


Figure 1. Illustration of osmoregulation in freshwater fishes. The body fluid of freshwater fishes has a higher concentration of salt than the surrounding environment; therefore, water flows in and salt will be lost from the tissues. To counteract the first process, freshwater fish use the kidneys to excrete water rapidly while salt is reabsorbed from the urine before it is excreted.

Source: Andrews, C., A. Exell, and N. Carrington. 1988. *The Manual of Fish Health*. Tetra Press, Morris Plains, New Jersey.

Another hormone involved in the escape reaction is cortisol. Its release can affect the white blood cell count and decrease their effectiveness in the immune system (Andrews 1988). Cortisol is a steroid secreted by the adrenal cortex. It is effective in anti-inflammatory activity and blood pressure maintenance. Also, cortisol is involved in gluconeogenesis, calcium absorption, and secretion of gastric acid and pepsin. The major functions of cortisol are energy metabolism, osmoregulation, and stress response (Evans 1998). When the fight or flight response activates the hypothalamic pituitary inter-renal

(HPI) axis, it will produce an increased cortisol secretion. The hypothalamus then secretes corticotrophic releasing hormone (CRH), which travels to the back of the pituitary gland. The pituitary then stimulates the release of adrenal corticotrophic hormone (ACTH) into the blood. There is no adrenal gland in fish; therefore, the ACTH goes to the adrenal tissue of the fish and then signals the release of cortisol back into the blood. The cortisol goes to the liver and then stimulates gluconeogenesis. Glucose is released into the blood and becomes a source of energy to the cells to speed up the biological systems (Evans 1998). The activity of the adrenal tissue is directly related to how stressed the animal has become; cortisol acts as an indicator of the adrenocortical function. Measurement of blood cortisol levels is useful in diagnostics of stress responses to stimuli (Diagnostic Products Company 1997).

Stress can cause decreased immunity, decreased growth, and decreased reproduction. All of these detrimental results can leave one with unhealthy or dead fish. These results of stress can cause great losses for the researcher, culturist, or hobbyist. The reduction of stress when working with fish culture systems can cause great benefits and fewer losses. The use of anesthesia while handling fish is the most widely practiced method of minimizing stress. An anesthetic can be defined as an agent that induces: 1) a calming effect, 2) a loss of equilibrium, and 3) a loss of mobility, consciousness, and reflex action (Keene et al. 1998). A list of compounds that historically have been used as anesthetics for fish is presented in Table I.

Table I. Anesthetics used in aquaculture. The asterisk (*) denotes anesthesia not recommended for use because more suitable drugs have become available. The diamond (♦) denotes injectable anesthesia that pose no harm either to the handler or to the fish.

Amytal sodium	Chloral hydrate*	Epontol ♦
Benzocaine	Chloretone*	Saffan ♦
Carbon dioxide	Methyl pentynol*	Xylocaine ♦
Electricity	Tertiary amyl alcohol*	
Etomidate	Tribromoethanol*	
Eugenol (FA-100)	Urethane*	
Metomidate	2- Phenoxyethanol*	
MS-222		
Quinaldine sulphate		
Rabonal		
Sodium Bicarbonate - acetic acid		

Sources:

Stuart, N. C. 1981. Anaesthetics in Fishes. *Journal of Small Animal Practice*. 22(1): 377-390.

Munday, P. L., and S. K. Wilson. 1997. Comparative Efficacy of Clove Oil and other Chemicals in Anaesthetization of *Pomacentrus amboinensis*, a coral reef fish. *Journal of Fish Biology* 51:931-938.

Peake, S. 1998. Sodium Bicarbonate and Clove Oil as Potential Anesthetic for Nonsalmonid Fishes. *North American Journal of Fisheries Management* 18:919-924.

MS-222 (3-aminobenzoic acid ethyl ester methanesulfate) is the most commonly used anesthetic for aquatic purposes. It is the only fish anesthetic approved for use by the Food and Drug Administration (FDA) and licensed by the Medicines Commission (Stuart 1981). During the past five years the mention of clove oil in aquatic anesthesia has

increased. The use of clove oil is still growing in spite of the limited information and research done on its efficacy and safety (Taylor 1999).

Clove oil is a plant compound derived from distilling the buds, leaves and stalks of the clove tree (*Eugenia aromatica*) (Anderson et al. 1997). Clove oil has a warm, spicy, sweet odor and a dark brown transparent color (Anderson et al. 1997). Eugenol (4-allyl-2-methoxyphenol) is the active ingredient that can make up to 70-90% of the clove oil (Soto 1995; Keene et al. 1998). The basic components of clove oil are phenol eugenol, eugenol acetate, and kariofilen (Soto 1995). Clove oil is FDA approved and has been reported to be antibacterial, anti-fungal, non-carcinogenic, non-mutagenic, and an antioxidant (Anderson et al. 1997; Keene et al. 1998). Cloves have also been used as a flavor additive in kretek cigarettes (Keene et al. 1998). The Expert Committee on Food Additives has established an adequate daily intake level for a human as 2.5 mg/kg (Anderson et al. 1997). Clove oil has traditionally been used as an analgesic or local anesthetic in professional dentistry.

Due to the fact that clove oil is a naturally derived anesthesia and is considered to be a GRAS (“generally recognized as safe”) substance by the FDA, it is an ideal candidate as an anesthesia for fish science (Anderson et al. 1997). There is a criterion for anesthesia approval by the FDA. An ideal fish anesthetic should have a short induction and recovery time. Clove oil can anesthetize in less than three minutes and recovery can be from five to ten minutes depending on the concentration used. The ideal anesthetic must be non-toxic to fish and humans. Clove oil is a plant-derived anesthetic and poses no harm to either the handler or to the fish. Single or multiple exposures must have no

effect on the fish's behavior or physiology. The anesthetic must also be metabolized from the body quickly; however, clove oil has an inhibitory effect. Since respiration and heart rate decrease, it takes longer for the excess to be removed from the system (Soto 1995; Anderson et al. 1997; Keene et al. 1998).

When using an anesthetic, the ease of administration is also an important factor. Clove oil can easily be dissolved in a small amount of ethanol and introduced into the water source. It is readily available at any health food store or pharmaceutical company and is also inexpensive when compared to MS-222. Pharmaceutical grade clove oil can be purchased at a cost of \$24.90 for 100 milliliters; this corresponds to a cost of \$0.25 per milliliter of clove oil used. When one considers MS-222 at a cost of \$125.70 for 100 grams, the corresponding cost for 1.0 gram (one gram = one milliliter) of MS-222 is \$1.26. (Sigma 1999). Therefore, the MS-222 is five times more expensive than the clove oil.

In this study, we assume that clove oil has an anesthetic effect, and not a tranquilizing effect, as stated by Keene et al. (1998). He characterized a tranquilizer as a central nervous system depressant, i.e., it decreases the arterial blood pressure and slows the heart rate to give a sedative effect. In addition, tranquilizers calm the animal and relieve fear. On the other hand, anesthetics inactivate the central nervous system, and thereby cause a total or partial loss of sensation and consciousness (Frank 1964; Gourley and Vasseur 1985; Upson 1985)

The primary purpose of this study was two-fold: (1) to determine if clove oil is an effective anesthetic for rainbow trout, and (2) to evaluate the effectiveness of clove oil in

minimizing stress when handling and confining yearling trout. We determined its efficacy by measuring knockdown and recovery times as well as survival of fish over time while under the anesthetic. Concentrations of cortisol in the blood of yearling trout were measured to evaluate stress of fish in various handling and confinement situations. In these trials, we compared stress in fish both under the anesthetic and without anesthesia. By meeting the criteria set by the FDA (as an ideal anesthetic), clove oil could become an economical and safe substitute for the commonly used synthetic compounds without having the stringent regulations that go along with them. This would be a tremendous economical value to hatcheries and other culture systems where handling fish is commonplace.

Chapter II

Literature Review

In the past 30 years, there have been increasingly more studies done on fish anesthetics. The demand for more effective products has fueled the search for better understanding of fish anesthetics. Over that period of time, the original fish anesthetics have become outdated and in their place are more effective products. In recent times, the most commonly used anesthetics for rainbow trout (and many other fishes) are MS-222 and etomidate. There continues to be in-depth investigations on the physiology of fish and the biochemistry of these and other anesthetics (Stuart 1981).

Strange and Schreck (1978) anesthetized yearling chinook salmon (*Oncorhynchus tshawytscha*) with MS-222. With exposure up to 180 minutes, they tested various concentrations (0, 25, 50, or 100 mg/L) to determine cortisol concentrations at interval times. The group receiving the 25 mg/L had highest cortisol concentrations. The 100 mg/L was determined to be lethal after 30 minutes because the group had no respiration and did not recover from anesthesia. In a separate experiment, chinook salmon were anesthetized at 50 mg/L before being transferred by dip net. These fish had lower cortisol levels than fish with no anesthetic. The third experiment consisted of 4 treatments, unbuffered and buffered MS-222 (50 mg/L) before confinement, buffered MS-222 after confinement, and 0.5% NaCl after confinement. Confinement and transfer were used as the two stressors. The results indicated reduced mortality rates when buffered and unbuffered MS-222 was administered before capture. Strange and Schreck (1978) reported that the 25 mg/L concentration only depresses fish, while the 50 mg/L level

produced a quick immobilization and less stress. The 100-mg/L level was determined to be a lethal dose.

Munday (1997) reported use of clove oil in anesthetizing a coral reef fish (*Pomacentrus amboinensis*). There was a calm reaction from the fish to the induction of the clove oil and a recovery period two to three times longer than with the known anesthetic, MS-222. Munday also reported excellent survival rates.

Soto (1995) used a clove oil and seawater solution on juvenile rabbitfish (*Siganus lineatus*). He investigated the effect of clove oil on various sizes and observed the health and growth after being repeatedly anesthetized. The concentration levels of clove oil were from 33 to 100 mg/L; only the 100-mg/L concentration was effective in anesthetizing the rabbitfish. The induction times were less than three minutes for all sizes and recovery periods ranged between 20 to 160 seconds when left in the solution for up to five minutes. Even with repeated exposure, there seemed to be no inhibition of growth, no onset of disease, and no mortalities.

Keene et al. (1998) documented slight coughing at various concentrations (0, 1, 2, 5, 15, and 30 ppm) when juvenile rainbow trout were placed in eugenol solutions. The coughing stopped in 20 seconds in all fish. They also reported that fish exposed to clove oil took a significantly longer amount of time to recover from being anesthetized than when anesthetized with MS-222. The difference in the recovery times between MS-222 and clove oil may be attributed to the “stimulatory” effect MS-222 has on the respiratory system and heart. This increase aids in eliminating excess chemicals from the fish’s system, which produces a more rapid recovery. Eugenol, like most anesthetics, has an

“inhibitory” effect on the respiratory system resulting in a decreased respiratory rate and an impaired ability to remove excess anesthesia from the system, thus a longer recovery time. Toxicity levels for eugenol were determined to be at an estimated 9 ppm.

Another study was performed to observe the effects of clove oil on the swimming performance of juvenile and adult rainbow trout. When clove oil was compared to MS-222, there seemed to be no significant difference in the induction time of juvenile trout at a concentration of 40 mg/L (Anderson et al. 1997). They did report a faster induction time for adult fish from clove oil when compared to MS-222; although, recovery seemed to take longer for fish exposed to clove oil than fish exposed to MS-222. The swimming performance of fish in both concentration groups (40 and 120 mg/L) was no different from non-anesthetized “control” fish. Anderson (1997) also reported a standard concentration of 100-120mg/L for heavy sedation and 20-40mg/L for light sedation in rainbow trout. Moreover, growth rates were not inhibited following exposure to clove oil in this study (Anderson et al. 1997).

Peake (1998) used sodium bicarbonate and clove oil to anesthetize several species of nonsalmonid fish. The optimal concentration was determined in preliminary tests and was stated to be 2.66 g/L for sodium bicarbonate that produced an induction time of seven minutes and a recovery period of five minutes. Clove oil at 60mg/L had an induction time of four minutes and recovery at 11 minutes. Peake also found these clove oil concentrations suitable for use in walleyes (*Stizostedion vitreum*), small mouth bass (*Micropterus dolomieu*), northern pike (*Esox lucius*), and lake sturgeon (*Acipenser fulvescens*).

Taylor and Roberts (1999) examined the toxicity of clove oil in various species to determine a safe concentration (1999). The lethal concentrations at 10 minutes of exposure was 62 mg/L for chinook salmon, 96 mg/L for coho salmon (*O. kisutch*), 250 mg/L for rainbow trout, and 526 mg/L for white sturgeon (*A. transmontanus*). They dosed all species at 25 mg/L for 120 minutes with no mortalities and reported this level would be a safe concentration.

Chapter III

Methodology

For this study, yearling rainbow trout (Figure 2) ranging in fork length from 20.5 to 28.7 cm and weighing between 0.13 and 0.25 kilograms were obtained from the Tennessee Wildlife Resources Agency Eagle Bend Hatchery in Clinton, Tennessee. They were acclimated for a minimum of three weeks prior to treatments in one of two sizes of fiberglass circular tanks (1.2 and 1.8 meters in diameter). They were held at approximately 19 degrees Celsius under normal photoperiod (12:12 light: dark) conditions and fed Purina's (Purina Feeds, St. Louis, MO) commercial trout feed at 2% body weight per day. Water quality parameters (dissolved oxygen, temperature, and chlorine) were monitored daily during the trial period.

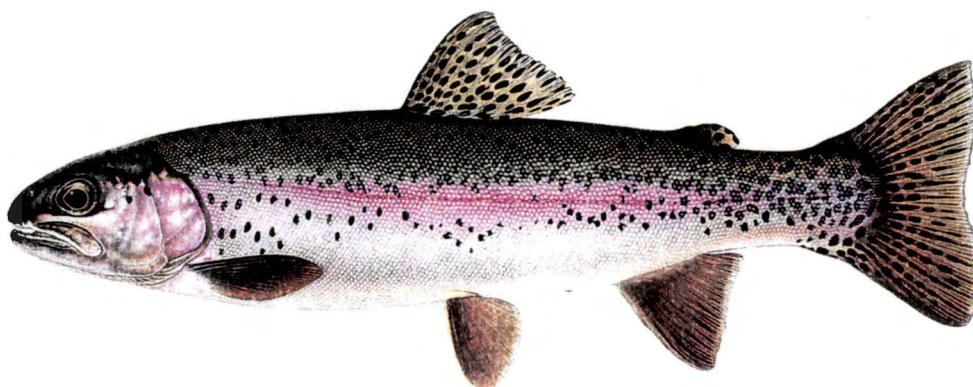


Figure 2. Illustration of Rainbow trout (*Oncorhynchus mykiss*)

Source: Behnke, R.J. 1992. Native Trout of Western North America. American Fisheries Society Monograph 6. Bethesda, Maryland.

Project Design

The design of this project consisted of two separate experiments (Figure 3) that were conducted during the period from 28 July to 12 August 1999. Experiment I was designed to evaluate the cortisol levels of rainbow trout exposed to clove oil over a period to time. On the morning of 12 August, 68 trout were held undisturbed in a 1.8-meter diameter circular tank and were subjected to a 25 mg/L concentration of clove oil.

Anderson et al. (1997) suggested a concentration of 20 to 40 mg/L for light sedation of rainbow trout. Taylor and Roberts (1999) reported a concentration of 25 mg/L safe for rainbow trout up to 120 minutes. The water flow was cut off during the induction to assure maintenance of the specified concentration levels. Once the fish began to lose equilibrium and appeared to 'belly up' (average of one minute), a sample of 10 fish was removed from the tank and bled within five minutes; this first sample was defined as Time 0. Every 10 minutes thereafter, an additional 10 fish were removed from the tank and also bled within five minutes. A total of 68 fish were sampled in Experiment I.

Experiment II consisted of three distinct treatment groups; each treatment was administered in a circular tank that held 20 trout and had been acclimated for a period of at least three weeks. The treatments simulated the extremes in conditions that might occur in hatchery situations:

- (1) Treatment 1 – a maximum handling situation with no anesthetic used, and
- (2) Treatment 2 – a no-handling situation with the anesthetic introduced without disturbing the fish. There was also an "intermediate" situation, i.e., Treatment 3, where

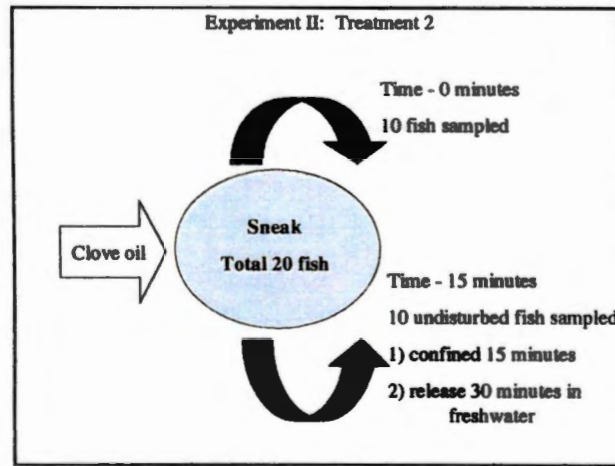
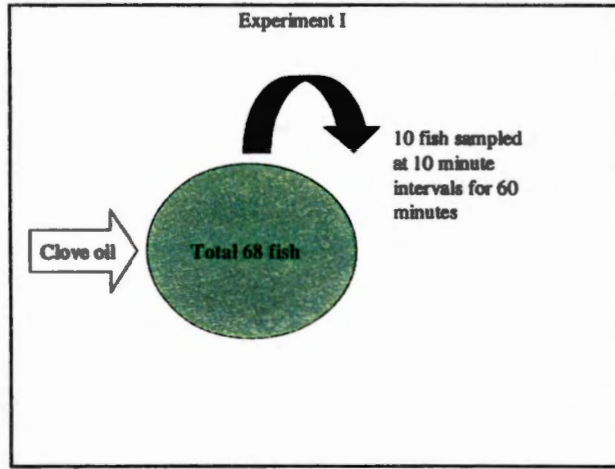
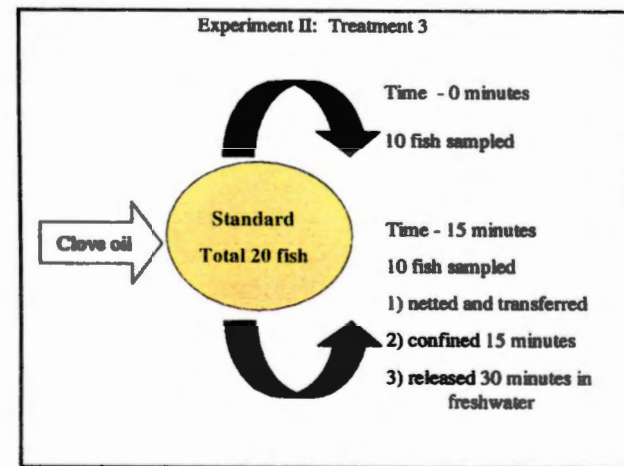
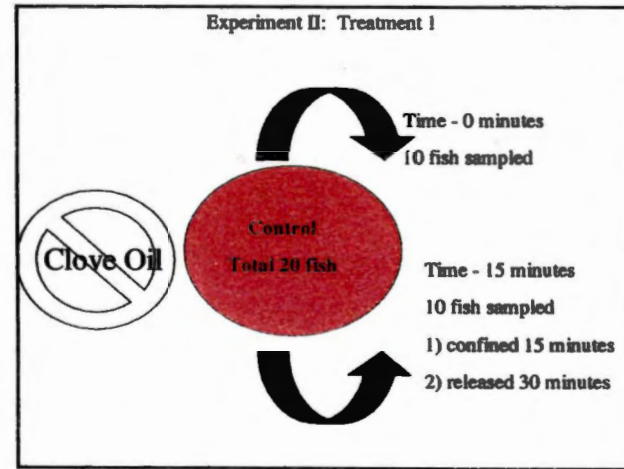


Figure 3. Illustration of experimental design of the two clove oil studies.

fish were dipped from the holding tank and placed in a container containing the anesthetic.

Treatment 1 was designed as the control; no clove oil was used on these fish. On 28 July, 10 fish were removed from the 1.2-meter diameter tank, euthanized with MS-222, and bled within five minutes. Simultaneously, the remaining 10 fish were closely confined in a net just below the water surface for 15 minutes. They were released into freshwater for 30 minutes, then recaptured, euthanized, and bled. A total of 20 fish were used; the first 10 removed were designated as Time 0 and remaining 10 were designated as Time 15 fish.

In Treatment 2, fish were held and sampled from a 1.8-meter diameter circular tank. On 12 August, a 25-mg/L concentration of clove oil was introduced without disturbing the fish; the water flow was shut down to maintain the specified clove oil concentration. When the trout ‘bellied up’ after approximately one minute, 10 fish were removed to represent Time 0, euthanized, and bled. Simultaneously, the remaining 10 anesthetized fish were confined in a net for 15 minutes in the tank having the 25-mg/L clove oil concentration. They were allowed to recover in freshwater for 30 minutes, recaptured, euthanized with MS-222, and bled. This treatment also used 20 trout with collection times of Time 0 and Time 15. Additionally, this treatment was designated the “sneak” treatment.

In Treatment 3, clove oil was used as the anesthetic and fish were held in a 1.8-meter diameter tank. Treatment 3 was included to simulate the common handling practice found in most culture operations. Fish are usually removed from a tank with a

net and placed in a bucket of water containing the anesthetic. In this treatment, 10 fish were removed immediately, euthanized in MS-222, and bled; there was no exposure to clove oil. Concurrently, the remaining 10 more fish were netted from the tank and placed in another tank containing water with a clove oil concentration of 25 mg/L. They were held in the anesthetic for 15 minutes, then removed and allowed to recover in freshwater for 30 minutes. They were then recaptured, euthanized with MS-222, and bled. A total of 20 fish were used with collection times of Time 0 and Time 15 minutes; this treatment was described as the “standard” handling practice in many culture facilities.

Clove oil was obtained from Sigma (St. Louis, MO) at a cost of \$94.60/ 500 milliliters. Due to clove oil’s partial solubility in water, the specified dosage had to be dissolved in 200-proof ethanol as a 10% solution. The density of clove oil is 1.04 (Sigma Chemical Company 1999) and must be kept in the dark to prevent breakdown of the product by light (Keene et al. 1998). The 25-mg/L solution was made fresh on the days the experiments were performed.

MS-222 was used as the euthanizing agent in all trials; it was dissolved in water and also made fresh on the days of use. The euthanizing concentration was calculated to be 200 mg/L.

Cortisol Analysis

Samples of blood were obtained from treated fish within five minutes of euthanasia. The caudal peduncle was severed and blood was collected from the vein with heparinized micro-hematocrit capillary tubes. The blood was then transferred to 0.5-ml micro-centrifuge tubes and spun at 300,000 rpm in an IEC Micro-MB centrifuge for 10

minutes. The plasma supernatant was siphoned off the top and placed in Corning 1.2-ml disposable cryogenic vials, labeled and frozen until further analysis.

Radioimmunoassay (RIA) was used for the determination of cortisol concentrations. Cortisol is obtained from a living subject and injected into a human. The human body perceives the cortisol as a foreign antigen and produces antibodies to destroy the foreign cortisol. The antibodies produced are then extracted from the human blood and isolated. Tubes in the diagnostic kit are then coated with the human antibody, producing sites with which the cortisol antigens bind. The radioactive ^{125}I (iodine) is highly competitive for the binding sites. When the sample is added, the cortisol and radioactive iodine bind to the walls of the tube. The gamma counter is a machine that measures the amount of radioactive iodine in counts per minute. If high values are produced, the amount of ^{125}I is high in the tube and cortisol counts are low. If low values are produced, then the amounts of ^{125}I is low and cortisol levels are high. At this point the counts per minute (cpm) of ^{125}I are known, but the exact cortisol concentration is still unknown. Included in the diagnostic kit are several calibrators with known cortisol concentrations. These are run in the gamma counter to obtain the counts per minute of the ^{125}I ; a calibration curve can then be made to determine the unknown cortisol concentrations of the rainbow trout blood samples.

Statistical analyses were performed using SAS (SAS Institute, Cary, NC). In Experiment I, the mean cortisol levels of fish subjected to different lengths of time in clove oil were tested for significant differences ($P = 0.05$) using a one-way analysis of

variance (ANOVA) procedure. A two-way ANOVA was used in Experiment II to test for significant differences in mean cortisol concentrations due to time and treatment effects.

Chapter IV

Results and Discussion

Experiment I

When exposed to a 25 mg/L dosage of clove oil, yearling rainbow trout lost equilibrium and became anesthetized within approximately one minute (range of 45-90 seconds). They recovered and were swimming normally within 5-10 minutes after being placed in fresh water. These times correspond to those in studies by Peake (1998) and Anderson (1997). The consensus in most studies is that the knockdown time for clove oil is somewhat quicker than MS-222 but the recovery time is longer.

The mean blood cortisol levels for trout subjected to a 25 mg/L concentration of clove oil over a 60-minute time period are illustrated in Figure 4. The abnormally high mean cortisol level (71.7 ng/mL) of fish at Time 0 (initial reading) may be attributed to several factors. At the onset of the experiment, the density of fish in the tank was at its highest point than at any other point in the trial. The normal morning activities of the persons in the lab could also have caused an elevation in cortisol levels as a result of stress.

An extremely high cortisol concentration value (166.2 ng/mL) occurred in the trout after 10 minutes in the anesthetizing solution. This high value is a definite indicator that there was a stress reaction to the clove oil. The rainbow trout were subjected to the 25 mg/L concentration of clove oil until they 'bellied up'. Since it took the fish approximately one minute to become anesthetized, the trout may have responded to the

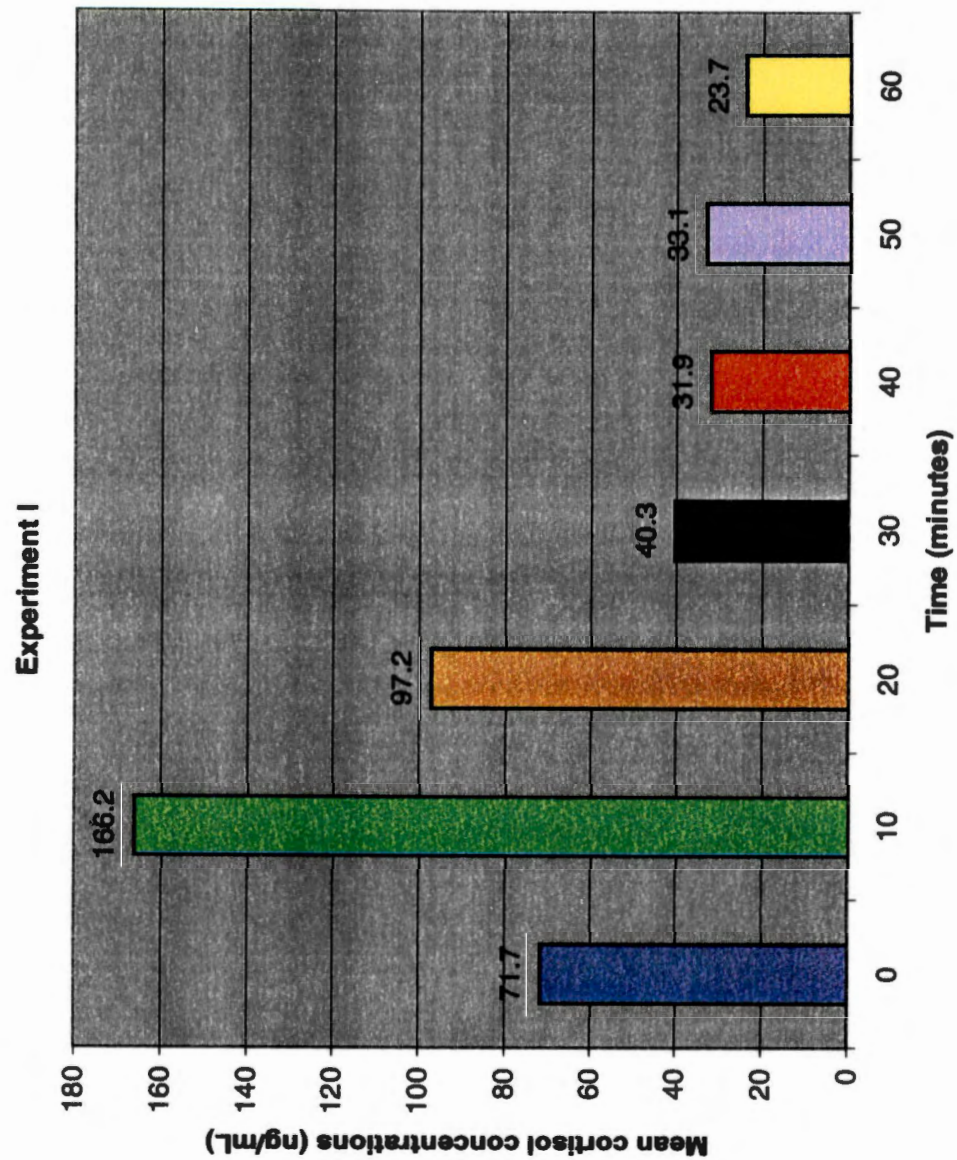


Figure 4. Experiment I: Mean blood cortisol concentrations of rainbow trout subjected to a 25 mg/L concentration of clove oil. Fish were sampled at 0 minutes when the first fish succumbed to the anesthesia and at succeeding 10-minute intervals.

clove oil. Fishes have well-developed chemoreceptors; the taste receptors (gustatory organs) and smell receptors (olfactory organs) are located on the skin, fins, lips, mouth cavity, throat, and gills of fishes (Etnier and Starnes 1993). Odorants such as clove oil are considered olfactory stimulants (Evans 1998). Since clove oil has been characterized as having a warm, spicy, sweet property (Anderson et al. 1997), the fish may have perceived the clove oil as an irritant and may have responded by emitting an “odiferous chemical” or pheromone. This alarm pheromone may have initiated a stress reaction in the other fish in the tank. If this were the case, however, this reaction time would be exceptionally fast when compared to the normal reaction time to produce cortisol levels of that magnitude.

The mean cortisol concentration at 10 minutes was significantly higher than the mean concentration values at 0, 20, 30, 40, 50, and 60 minutes. Cortisol values for 0 minutes and 20 minutes were essentially the same, but were significantly different from concentrations after 30, 40, 50, and 60 minutes. Cortisol levels began to decrease slightly after the initial “spike” and fell below the Time 0 level at 30, 40, 50, and 60 minutes. This was probably due to a decrease in the interrenal production of cortisol coupled with an increased elimination of cortisol by the liver. The values for 30, 40, 50, and 60 minutes were significantly lower than the Time 0 but were not significantly different from each other. The statistical data are presented in Table II.

Table II. Statistical output for Experiment I. Mean cortisol concentrations (ng/mL) are reported as a mean $\pm$ standard error. The probability level is set at 0.05; values without a common letter are significantly different.

Time (minutes)	Mean Cortisol Concentrations (ng/mL)	Pr > t
0	71.8 $\pm$ 36.5b	≤ 0.0001
10	166.2 $\pm$ 86.3a	≤ 0.0001
20	97.2 $\pm$ 67.6b	≤ 0.0001
30	40.3 $\pm$ 40.5c	0.0003
40	31.9 $\pm$ 15.9c	0.0042
50	33.1 $\pm$ 30.2c	0.0030
60	23.7 $\pm$ 13.0c	0.0549

Experiment II

In Experiment II, Treatment 1 served as the “control” and represented handling without anesthesia; this procedure produced the expected cortisol levels for those conditions. The value at 0 minutes was very low (2.8 ng/mL) and increased significantly after 15 minutes of confinement (93.1 ng/mL).

Treatment 2 (“sneak”) was designed to anesthetize the fish with no disturbances prior to them knocked out. Mean cortisol values for both 0 and 15 minutes were essentially the same. The Time 0 value seemed to be elevated due to some pre-existing stressful factor. There was also a great deal of human activity around the tank the morning of the study. The value after 15 minutes of confinement in clove oil yielded a cortisol concentration slightly lower than the initial 0 minute reading (Figure 5).

Treatment 3 was designed to be the “standard” handling method, i.e., being netted out of the tank, transferred to a 25 mg/L concentration of clove oil, and confined. Initial

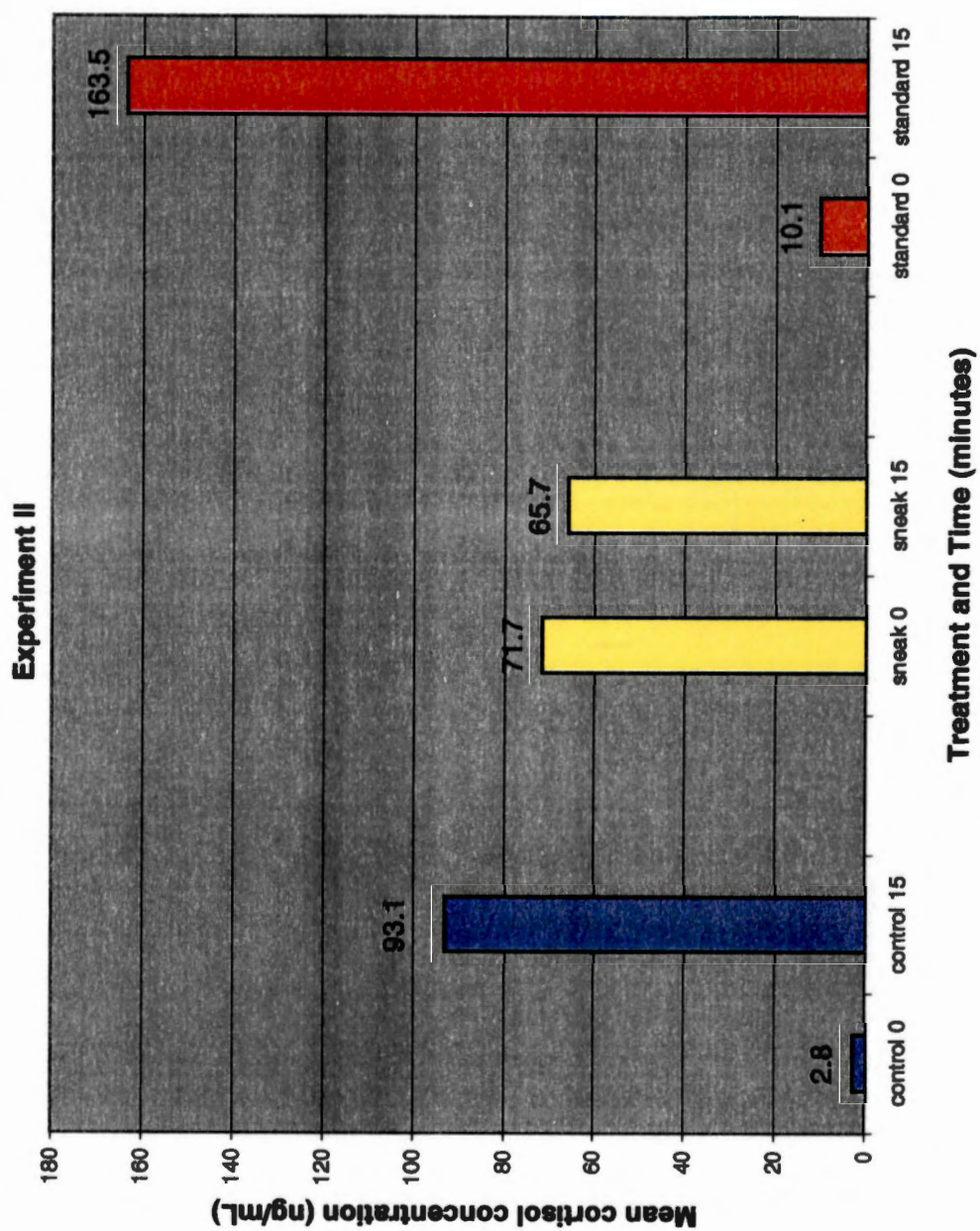


Figure 5. Experiment II: Mean cortisol concentration (ng/mL) levels. Treatment 1 (control) used no clove oil. Treatment 2 (sneak) and Treatment 3 (standard) subjected rainbow trout to a dosage of 25 mg/L of clove oil.

cortisol levels were low at Time 0 (10.1 ng/mL) and were substantially elevated at 15 minutes (163.5 ng/mL). The elevated level could be attributed to the stress of being netted without any anesthesia and being transported (as done in standard fishery practice). In addition to the stress of being handled and transferred, the fish's response to the sweet clove oil stimulus (odor) could have initiated a stress response.

The statistical procedures used in this experiment also analyzed the effects of time, treatment, and the combination of time and treatment effects on mean cortisol concentrations in the different treatment groups. The analyses indicated there was a significant difference ($P < 0.05$) in the mean cortisol concentrations levels measured at Time 0 and Time 15 (Table III).

The control treatment group had mean concentration values significantly different than the cortisol concentration values in the sneak and standard treatment groups. The sneak treatment group was essentially the same as the standard treatment group (Table IV) with respect to mean cortisol concentrations.

The cortisol concentration value at 15 minutes in the standard treatment was significantly higher than the values of the sneak and control treatment groups. The cortisol concentration values for control at 0 minutes and standard at 0 minutes were significantly lower than the values at control 15 minutes and sneak at 0 and 15 minutes. There were no differences in cortisol concentrations the control at 15 minutes and sneak at 0 and 15 minutes are significantly the same values for the cortisol concentration levels (Table V).

Table III. Statistical output for Experiment II: Time. Mean cortisol concentrations are reported as a mean $\pm$ standard error. The probability level is set at 0.05; values without a common letter are significantly different.

Time (minutes)	Mean Cortisol Concentrations (ng/mL)	Pr > t
0	28.17 $\pm$ 5.99b	≤ 0.0001
15	107.47 $\pm$ 5.99a	≤ 0.0001

Table IV. Statistical output for Experiment II: Treatment. Mean cortisol concentrations are reported as a mean $\pm$ standard error. The probability level is set at 0.05; values without a common letter are significantly different.

Treatment	Mean Cortisol Concentration (ng/mL)	Pr > t
Control	47.95 $\pm$ 7.33b	≤ 0.0001
Sneak	68.70 $\pm$ 7.33a	≤ 0.0001
Standard	86.83 $\pm$ 7.33a	≤ 0.0001

Table V. Statistical output for Experiment II: Treatment and Time. Mean cortisol concentrations are reported as a mean $\pm$ standard error. The probability level is set at 0.05; values without a common letter are significantly different.

Treatment	Time (min)	Mean Cortisol Concentration (ng/mL)	Pr > t
Control	0	2.8 $\pm$ 6.2c	0.7905
Control	15	93.1 $\pm$ 81.4b	≤ 0.0001
Sneak	0	71.7 $\pm$ 36.5b	≤ 0.0001
Sneak	15	65.7 $\pm$ 57.2b	≤ 0.0001
Standard	0	10.1 $\pm$ 5.4c	0.3312
Standard	15	163.5 $\pm$ 40.3a	≤ 0.0001

Chapter VI

Summary and Conclusions

Clove oil was evaluated as an anesthetic for rainbow trout and as an agent to minimize stress during handling. The major findings are:

1. Clove oil will anesthetize yearling rainbow trout within 45-90 seconds of the introduction of a dose of 25 mg/L; the recovery time in fresh water ranges from five to ten minutes.
2. When blood cortisol levels (an indicator of stress) are measured in trout exposed to an anesthetizing dose of clove oil for a 60-minute period, there is a significant increase in cortisol concentration after 10 minutes in the anesthetizing solution. Cortisol concentrations then decrease to levels lower than that of Time 0 fish after 30, 40, 50, and 60 minutes in the clove oil solution.
3. The mean cortisol concentration level in fish from the “standard” handling practice used in culture operations was significantly higher after 15 minutes in clove oil than the mean cortisol concentration levels of fish with no exposure to clove oil (control), and to the mean cortisol levels of undisturbed fish (sneak) being exposed to clove oil.
4. The initial mean cortisol concentration level of the undisturbed fish (sneak) was significantly higher than the initial mean cortisol concentration levels of the fish with no clove oil (control) and the fish subjected to the “standard” handling practice used in culture operations.

5. The initial mean cortisol concentration level of the undisturbed fish was not significantly different from the mean cortisol concentration level of the undisturbed fish exposed to clove oil for 15 minutes.
6. When assessing clove oil effects among treatment groups, the mean cortisol concentration level of the treatment with no clove oil was significantly different from the mean cortisol levels of undisturbed fish and fish handled like common culture operations.

Clove oil is readily available, easy to use, and safe to humans. Its advantages are that it induces a quick induction time and an adequate recovery time in rainbow trout. It is inexpensive when compared to MS-222 and has no FDA regulations. One potential disadvantage is that at concentrations of 25 mg/L, clove oil stimulates a cortisol stress response. A good anesthesia should cause a minimal amount of disturbance to the fish. In this study, undisturbed fish have lower cortisol levels than fish that are handled as in common practices of culture operations. There is no information on the effects of clove oil on the physiology of fish. The examination of clove oil as an inducer of stress should be investigated more closely. More studies should be done in the future to examine the effectiveness and safety of clove oil on the growth and reproduction of other species.

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Appendix

Raw Data

Table VI. Raw data for Experiment I. Final assay concentration (ng/mL), data for 68 rainbow trout used in Experiment I. Ten observations were used for each treatment in duplicate (A, B) blood samples were assayed. Assay concentrations equaling zero values have more than 100% binding of cortisol.

Experiment I

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 0	50.3
1B	Time 0	57.3
2A	Time 0	10.7
2B	Time 0	13.3
3A	Time 0	32.8
3B	Time 0	35.9
4A	Time 0	71.4
4B	Time 0	42.1
5A	Time 0	108.1
5B	Time 0	122.5
6A	Time 0	86.4
6B	Time 0	100.7
7A	Time 0	72.7
7B	Time 0	87.0
8A	Time 0	78.0
8B	Time 0	77.5
9A	Time 0	59.0
9B	Time 0	57.0
10A	Time 0	139.4
10B	Time 0	131.3
	Time 0 Mean	71.7

Table VI. (continued)

Experiment I

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 10	213.7
1B	Time 10	262.8
2A	Time 10	134.9
2B	Time 10	141.1
3A	Time 10	171.2
3B	Time 10	143.2
4A	Time 10	94.0
4B	Time 10	109.0
5A	Time 10	244.9
5B	Time 10	139.3
6A	Time 10	59.3
6B	Time 10	58.5
7A	Time 10	95.4
7B	Time 10	94.0
8A	Time 10	314.7
8B	Time 10	332.4
9A	Time 10	105.8
9B	Time 10	82.4
10A	Time 10	265.9
10B	Time 10	260.8
	Time 10 Mean	166.2

Table VI. (continued)

Experiment I

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 20	169.6
1B	Time 20	157.6
2A	Time 20	158.9
2B	Time 20	171.6
3A	Time 20	23.3
3B	Time 20	0.0
4A	Time 20	86.9
4B	Time 20	105.4
5A	Time 20	129.6
5B	Time 20	137.4
6A	Time 20	105.0
6B	Time 20	109.5
7A	Time 20	189.7
7B	Time 20	216.2
8A	Time 20	11.7
8B	Time 20	14.0
9A	Time 20	32.8
9B	Time 20	29.2
10A	Time 20	49.9
10B	Time 20	45.4
	Time 20 Mean	97.2

Table VI. (continued)

Experiment I

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 30	26.5
1B	Time 30	24.4
2A	Time 30	7.4
2B	Time 30	10.8
3A	Time 30	156.0
3B	Time 30	136.2
4A	Time 30	45.3
4B	Time 30	36.7
5A	Time 30	0.0
5B	Time 30	3.2
6A	Time 30	38.5
6B	Time 30	44.0
7A	Time 30	27.6
7B	Time 30	23.3
8A	Time 30	6.3
8B	Time 30	12.0
9A	Time 30	60.8
9B	Time 30	54.8
10A	Time 30	47.4
10B	Time 30	44.2
	Time 30 Mean	40.3

Table VI. (continued)

Experiment I

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 40	15.1
1B	Time 40	22.0
2A	Time 40	15.0
2B	Time 40	15.6
3A	Time 40	33.7
3B	Time 40	28.7
4A	Time 40	20.8
4B	Time 40	23.5
5A	Time 40	28.0
5B	Time 40	34.8
6A	Time 40	21.1
6B	Time 40	17.8
7A	Time 40	67.6
7B	Time 40	66.6
8A	Time 40	23.4
8B	Time 40	28.1
9A	Time 40	37.7
9B	Time 40	40.0
10A	Time 40	51.3
10B	Time 40	47.9
	Time 40 Mean	31.9

Table VI. (continued)

Experiment I

Observation	Treatme (minutes)	Assay Concentration (ng/mL)
1A	Time 50	48.6
1B	Time 50	54.8
2A	Time 50	45.1
2B	Time 50	31.0
3A	Time 50	14.6
3B	Time 50	12.7
4A	Time 50	3.7
4B	Time 50	4.8
5A	Time 50	19.3
5B	Time 50	23.0
6A	Time 50	12.4
6B	Time 50	14.2
7A	Time 50	104.2
7B	Time 50	94.2
8A	Time 50	3.2
8B	Time 50	5.9
9A	Time 50	23.2
9B	Time 50	16.2
10A	Time 50	66.6
10B	Time 50	64.8
	Time 50 Mean	33.1

Table VI. (continued)

Experiment I

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 60	21.1
1B	Time 60	12.4
2A	Time 60	29.6
2B	Time 60	34.9
3A	Time 60	0.0
3B	Time 60	0.0
4A	Time 60	27.3
4B	Time 60	27.4
5A	Time 60	34.6
5B	Time 60	38.0
6A	Time 60	16.8
6B	Time 60	16.7
7A	Time 60	15.3
7B	Time 60	23.3
8A	Time 60	39.1
8B	Time 60	43.3
	Time 60 Mean	23.7

Table VII. Raw data for Experiment II. Final assay concentration (ng/mL), for 60 rainbow trout used in Experiment II. Ten observations were used for each treatment and duplicate (A, B) blood samples were assayed. Assay concentrations equaling zero values have more than 100% binding of cortisol.

Experiment II - Treatment 1 (control)

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 0	0.0
1B	Time 0	0.0
2A	Time 0	0.0
2B	Time 0	0.0
3A	Time 0	0.0
3B	Time 0	0.0
4A	Time 0	0.0
4B	Time 0	0.0
5A	Time 0	16.3
5B	Time 0	22.8
6A	Time 0	0.0
6B	Time 0	0.0
7A	Time 0	0.0
7B	Time 0	0.0
8A	Time 0	0.0
8B	Time 0	0.0
9A	Time 0	8.5
9B	Time 0	5.3
10A	Time 0	2.4
10B	Time 0	0.1
	Time 0 Mean	2.8

Table VII. (continued)

Experiment II: Treatment 1(control)

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 15	117.9
1B	Time 15	145.2
2A	Time 15	251.8
2B	Time 15	195.2
3A	Time 15	106.2
3B	Time 15	144.9
4A	Time 15	46.7
4B *	Time 15	45.4
5A	Time 15	17.6
5B	Time 15	13.9
6A	Time 15	232.6
6B	Time 15	233.4
7A	Time 15	26.2
7B	Time 15	24.4
8A	Time 15	62.8
8B	Time 15	51.6
9A	Time 15	4.3
9B	Time 15	4.7
10A	Time 15	68.4
10B	Time 15	69.6
	Time 15 Mean	93.1

Table VII. (continued)

Experiment II - Treatment 2 (sneak)

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 0	50.3
1B	Time 0	57.3
2A	Time 0	10.7
2B	Time 0	13.3
3A	Time 0	32.8
3B	Time 0	35.9
4A	Time 0	71.4
4B *	Time 0	42.1
5A	Time 0	108.1
5B	Time 0	122.5
6A	Time 0	86.4
6B	Time 0	100.7
7A	Time 0	72.7
7B	Time 0	87.0
8A	Time 0	78.0
8B	Time 0	77.5
9A	Time 0	59.0
9B	Time 0	57.0
10A	Time 0	139.4
10B	Time 0	131.3
	Time 0 Mean	71.7

Tabel VII. (continued)

Experiment II: Treatment 2 (sneak)

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 15	38.5
1B	Time 15	36.1
2A	Time 15	19.7
2B	Time 15	32.6
3A	Time 15	19.2
3B	Time 15	19.0
4A	Time 15	115.8
4B	Time 15	86.0
5A	Time 15	147.4
5B	Time 15	138.3
6A	Time 15	49.8
6B	Time 15	32.8
7A	Time 15	28.8
7B	Time 15	24.1
8A	Time 15	29.2
8B	Time 15	26.0
9A	Time 15	45.5
9B	Time 15	50.8
10A	Time 15	172.9
10B	Time 15	202.0
	Time 15 Mean	65.7

Table VII. (continued)

Experiment II - Treatment 3 (standard)

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 0	5.8
1B	Time 0	8.3
2A	Time 0	15.0
2B	Time 0	11.4
3A	Time 0	1.2
3B	Time 0	5.9
4A	Time 0	4.4
4B	Time 0	4.1
5A	Time 0	7.9
5B	Time 0	16.2
6A	Time 0	20.6
6B	Time 0	17.7
7A	Time 0	10.0
7B	Time 0	7.4
8A	Time 0	3.6
8B	Time 0	6.8
9A	Time 0	13.6
9B	Time 0	13.9
10A	Time 0	16.6
10B	Time 0	12.1
	Time 0 Mean	10.1

Table VII. (continued)

Experiment II: Treatment 3 (standard)

Observation	Treatment Time (minutes)	Assay Concentration (ng/mL)
1A	Time 15	168.3
1B	Time 15	213.9
2A	Time 15	162.8
2B	Time 15	162.6
3A	Time 15	175.1
3B	Time 15	161.9
4A	Time 15	104.1
4B	Time 15	116.4
5A	Time 15	139.4
5B	Time 15	127.5
6A	Time 15	182.3
6B	Time 15	210.9
7A	Time 15	107.4
7B	Time 15	277.6
8A	Time 15	186.1
8B	Time 15	156.1
9A	Time 15	134.3
9B	Time 15	165.9
10A	Time 15	170.4
10B	Time 15	147.8
	Time 15 Mean	163.5

Vita

Jamillah Zakia K. Johnson was born in Orange, New Jersey on September 21, 1975. At the age of 12, she relocated with family to Stone Mountain, Georgia where she graduated from Clarkston High in 1993. She continued on to earn a B. S. degree in Veterinary Science at the Fort Valley State University Veterinary Science Department in June of 1997.

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