Evaluation and Application of Presumptive Tests for Blood for Fish Epithelial Injury Detection

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DEDICATION

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ABSTRACT

Any time a fish interacts with any structure, anthropogenic or natural, or other animal; there is an opportunity for injury. This thesis compared several presumptive tests for blood commonly used in crime scene analysis (fluorescein, Bluestar©, phenolphthalein, Hemastix®) and their ability to detect and quantify fish epithelial injury. Through a qualitative literature review and comparative field study, fluorescein was found to be the best (i.e., low rate of false positives, detected highest proportion of true positives). Based on this information, fluorescein was investigated further to refine its application in fisheries research. Using fluorescein, injuries could be detected up to 5 hours after the injury occurs and once fluorescein is applied, there is significantly less detectable fluorescein after one hour. Potential sources of injury from recreational angling gear and handling methods were then investigated on northern pike (*Esox lucius)* and largemouth bass (*Micropterus salmoides*) using fluorescein. It was determined that carpeted surfaces and knotted nylon landing nets caused the greatest injury for northern pike and overall injury from fishing tournaments was associated with the greatest injury detected for largemouth bass. Overall, fluorescein can be used to assess the injury from a variety of sources and can be used to educate anglers on proper handling methods and the least harmful gear choices.

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CO-AUTHORSHIP

Chapter 2: Application of forensic techniques to enhance fish conservation and management: Injury detection using presumptive tests for blood. A.H. Colotelo, K.E. Smokorowski, and S.J. Cooke.

While this study is my own, the research was undertaken as part of a collaborative effort, and each co-author played a valuable role in its completion. All writing was conducted by Colotelo. All co-authors provided comments and feedback on the manuscript. The manuscript is published in *Endangered Species Research*; Special Issue: Forensic Methods in Conservation Research 00:000-000 doi: 10.3354/esr00178.

Chapter 3: Evaluation of forensic presumptive tests for blood for the detection of injury in fish. A.H. Colotelo, K.E. Smokorowski, T. Haxton, and S.J. Cooke.

While this study is my own, the research was undertaken as part of a collaborative effort and each co-author played a valuable role in its completion. The project was conceived by Colotelo, Smokorowski, and Cooke. All computer and data analysis was conducted by Colotelo. Data were interpreted by Colotelo and Cooke. All writing was done by Colotelo. All co-authors provided comments and feedback on the manuscript. This manuscript is in preparation for submission to *Diseases of Aquatic Organisms.*

Chapter 4: Evaluation of Common Sources of Physical Injury to Popular Sport Fish during Recreational Angling Events. A.H. Colotelo and S.J. Cooke.

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manuscript is in preparation for submission to *North American Journal of Fisheries Management*.

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CHAPTER 1: General Overview

Fish can sustain injury from a variety of anthropogenic and natural structures as well as interaction with other animals (including humans). This injury can immediately influence the individual through mortality, or can have sublethal impacts (i.e. behaviour, physiology) that may lead to delayed mortality. Currently, the standard for assessment of injury on fish is gross macroscopic examination. This method is qualitative and lacks objectivity, which emphasizes the need for a new technique to enhance the detection and quantification of injury on fish. This thesis examines presumptive tests for blood, commonly used in crime scene investigation, for their ability to detect and quantify latent injuries to fish epithelium. Chapter 2 summarizes the current literature available regarding fish epithelial injury and outlines several presumptive tests which show promise in their ability to be used for the detection and quantification of fish epithelial injury. Chapter 3 outlines a field test which was done to compare 4 prospective presumptive tests for blood (fluorescein, Bluestar©, Hemastix® and Hemident™) in their ability to detect and quantify several types of injury commonly seen in fish (abrasion, punctures, cuts, and fin fray). Based on these results, further investigation was conducted to examine the capabilities of fluorescein. Chapter 4 uses the fluorescein method to detect epithelial injury in fish enabling a comparison of different gear and handling methods that are commonly used in recreational angling. Fluorescein was used to detect epithelial injury on largemouth bass and northern pike, two popular freshwater species commonly targeted during recreational angling events. Finally, Chapter 5 summarizes the findings of the above studies and outlines the management implications and future research that is needed to make this technique more readily available to fisheries

professionals. Overall, this thesis attempts to identify a presumptive test for blood that can easily be used to detect and quantify fish epithelial injury in both laboratory and field settings and apply the technique to a recreational angling issue to emphasize the potential utility of these tools in fisheries conservation and management.

CHAPTER 2: Application of forensic techniques to enhance fish conservation and management: Injury detection using presumptive tests for blood

ABSTRACT

The detection of injury on the skin of fish has generally been limited to gross macroscopic examination, which has numerous limitations, including researcher subjectivity and a lack of quantitative analytical capacity. The use of chemical enhancers, such as those used by forensic analysts, can aid in the detection and quantification of skin injuries in fish, which can arise from fish interactions with humans and anthropogenic infrastructure (e.g. recreational and commercial fishing, research sampling, fishway passage or guidance, turbines). In this review, we examine several presumptive tests for blood and evaluate their potential usefulness for detecting and quantifying injury in fish. Our evaluation was based on sensitivity, specificity, cost, carcinogenicity and ease of use. Fluorescein and Bluestar© offer the ability to perform whole body detection, but require low-light conditions and a digital camera to capture the emitted light. Several tests (i.e. Hemastix®, Hemident ™, phenolphthalein) yield rapid results and do not require large or expensive pieces of equipment, which makes them ideal for field use, although further research is needed to validate these tools for use on different fish species and in different contexts. Collectively, these tools show promise for a variety of fish research, conservation and management applications, including hydropower assessment, commercial fisheries bycatch evaluation, and analysis of the practices and gear regulations associated with recreational angling.

INTRODUCTION

Forensic techniques encompass those procedures employed during crime scene analysis by identification officers and forensic scientists. Increasingly, these tools are being

applied to conservation-related research and monitoring. Most notably, forensic techniques based on molecular genetics have been used in wildlife management cases, in which the animal poached is often visually unidentifiable, making it difficult to distinguish a threatened or protected species from a legal kill. DNA analysis can help identify the species in question, as well as help link an individual to the scene of the infraction (Wan & Fang 2003). Likewise, forensic genetic tools have been used to identify the illegal international trade of protected wildlife tissues (e.g. sturgeon and paddlefish caviar [Ludwig 2006]; sea turtles [Bowen & Avise 1996]). However, there are many other underutilized tools in the field of wildlife forensics that have the potential to contribute to animal conservation and management, especially with regards to fish.

Presumptive tests for blood utilize a variety of chemicals to identify the presence of blood through a reaction with the haemoglobin molecule (Spalding 2006). They are described as presumptive because there are substances other than haemoglobin which may cause a false positive reaction, and in forensic settings further testing is required to confirm the result. These are rapid tests that are used to identify whether an unknown substance is likely blood and to identify areas of a crime scene that should be investigated in more detail. The benefit of utilizing these tests is the rapidity of results and the ease of interpretation.

Presumptive tests for blood show promise for documenting injury to fish that have interacted with humans or anthropogenic infrastructure. For example, hydroelectric dams (including fishway facilities and guidance technologies) and irrigation intakes are potentially a cause of external injuries for fish interacting with the infrastructure (Cada 1990). Similarly, when fish are discarded as bycatch in the commercial fishing sector, or

through catch-and-release practices (voluntary or mandatory) in the recreational fishing sector, they can be exposed to dermal injuries from fishing line, hooks, nets and handling (e.g. Davis 2002, Barthel et al. 2003). Although basic capture techniques, such as the use of pot traps for the enumeration of fish, may appear to be benign, research has revealed that fish can experience significant abrasion and scale loss during the holding and handling processes (Cooke et al. 1998). Even minor abrasions to the skin of fish can increase the incidence of infection by opportunistic pathogens such as *Saprolegnia* spp. (Van West 2006), which could lead to delayed mortality or alterations in fish health and stress levels. There is now a suite of tools available for evaluating the physiological condition of fish in the field (summarized in Iwama et al. 1995), yet there are comparatively fewer tools available for detecting and quantifying physical injury. Quantifying the extent and severity of sublethal injuries is important for determining the consequences of human interactions with fish, and for developing strategies and/or infrastructure to reduce such injuries and promote fish welfare, which is currently becoming of increasing concern (Huntingford et al. 2006).

The objective of the present paper is to review a suite of presumptive tests for blood used in forensic analysis, and to identify their potential applicability to fish conservation and management. Such tests are necessary, as existing approaches for quantifying the sublethal injury of fish are qualitative and rely on crude indicators such as visual inspection of fin fraying (e.g. Barthel et al. 2003), scale loss (e.g. Chopin & Arimoto 1995) and macroscopic wounds (e.g. Davis 2002). Only in recent years have forensic tools been applied in a fisheries context to investigate epithelial injury. In this review, we first summarize the use and basis of presumptive tests in forensic science, and

then briefly discuss the integument, scales and mucus present on fish and reveal the associated challenges of using presumptive tests. Next, we summarize the various presumptive tests that have been developed and provide an overview of their actual or potential utility for research or monitoring of fish. We conclude by discussing how these presumptive tests have or could be applied to address a range of fish conservation and management problems and identify key research needs.

PRESUMPTIVE TESTS: PREMISES AND USES IN FORENSIC SCIENCE

Often at crime scenes there are stains composed of unknown substances that may be confused with blood. Identifying whether the substance is indeed blood allows further analyses to confirm species, and, if necessary, the individual (Spalding 2006). Alternatively, if blood was cleaned in an attempt to remove evidence, chemical enhancers are required to detect whether blood is present (even in miniscule amounts) and/or visualize any patterns (Spalding 2006). Presumptive tests are used to initially identify areas of high priority in crime scene investigation, and substances are later confirmed to be blood through microscopic tests (Spalding 2006). Presumptive tests have also been used in biomedical applications. For example, fluorescein, a common chemical for blood pattern visualization, has been adopted in ophthalmology to stain the corneal epithelium and identify lesions on the outer surface of the eye (Göbbels & Spitnaz 1989). Although presumptive tests for blood are currently used primarily in a human context, the composition of blood is highly conserved among vertebrates, making these tests theoretically useful for fish. Furthermore, the epithelium of fish has been compared to the composition of the human cornea (Noga & Udomkusonsri 2002), and so the use of

fluorescein in corneal lesions appears to indicate the potential for use in fish epithelial lesions.

Regardless of the taxa being investigated, there are 2 basic concepts that are important when comparing presumptive tests for blood, namely, sensitivity and specificity. Sensitivity refers to the lowest dilution of blood that can be detected (Cox 1991), and is typically tested in a laboratory with blood samples diluted with distilled water (Tobe et al. 2007). The most sensitive presumptive blood test is luminol, with a recorded sensitivity of 1:1 000 000 (Proescher & Moody 1939), allowing the potential detection of a miniscule amount of blood. The majority of presumptive tests have a sensitivity of 1:100 000 (Tobe et al. 2007), although, depending on the surface tested and the preparation methods, sensitivity can vary. For all of the tests outlined in this review, the sensitivity is such that they have the ability to detect blood that would not be identified by gross macroscopic examination alone.

Specificity refers to the ability of the chemical to accurately detect blood and identify substances that may present false positives or false negatives. Substances that may be visually mistaken for blood, or those that contain oxidizing agents (such as peroxidases), are tested as potential sources of false positives (Tobe et al. 2007). Generally, those substances that produce false positives take detectably longer to react and, therefore, may be eliminated through observational interpretation (Tobe et al. 2007). There is limited information in the forensic literature about false negatives, as it is difficult to prove that a false negative reaction has occurred (Ponce & Pascual 1999). It is thought that reduction compounds compete for oxygen in oxidation-reduction reactions and are thus capable of causing false negative reactions.

In the comparison of presumptive tests for blood in a forensic context, a number of surface types are tested (e.g. carpet, cotton, linoleum). For example, Dilbeck (2006) compared luminol and Bluestar© on 4 different surfaces and revealed that surface material influenced their chemiluminescence strength. In the context of fish research, fish skin is a surface on which there has been very little comparative work related to presumptive tests for blood. It is therefore imperative to review the characteristics of fish skin and mucus, and their potential impact on the results and applicability of different forensic tools.

FISH SKIN CHARACTERISTICS

Vertebrate skin is the largest organ of the integument system and consists of multiple layers of epithelial tissue, acting as a barrier protecting the internal structures from the external environment. Skin structure is based on the vertebrate's environment and therefore can vary drastically, especially among taxa (Flaxman 1972). Fish are unique from other vertebrates as the living cells of the integument are in close proximity to the ambient environment (Hawkes 1974). Other vertebrates have a layer of keratinized cells which protect the living tissue from the environment (Flaxman 1972), which, for fish, is done by the mucus layer. Fish mucus consists of glycoproteins or mucins and a high concentration of water and is produced by goblet cells present in the epidermal tissue, particularly those located in the gills (Shephard 1994). The role of fish mucus is to protect the fish from dermal abrasion, disease and infection, to provide lubrication within water, and to aid in ion and osmotic homeostasis. Removal of the protective layer of mucus and underlying epidermis can result in opportunistic pathogenic infection and

even death. The dynamic nature of fish mucus and its ability to be non-lethally sampled makes it a promising material to test for blood using presumptive tests.

CHALLENGES OF USING PRESUMPTIVE TESTS FOR BLOOD ON FISH

The use of forensic presumptive tests for blood in a fish context poses a number of challenges, including the unknown effect of the aquatic environment that may interfere with the correct interpretation of results. Although most presumptive tests for blood cannot distinguish between human and animal blood, there have not been any extensive studies on the sensitivity or specificity of these tests with respect to fish blood, particularly due to the unique substances present in an aquatic environment. Smith & Ramos (1976) tested the sensitivity of Hemastix® for haemoglobin taken from butterfly fish *Chaetodon miliaris*, and they found strong positive reactions for dilutions of up to 1 part haemoglobin to 4 parts fish mucus. There is a need to investigate the sensitivity of these tests to enable accurate interpretation of the results.

In forensic science, substances that may potentially cause false positive reactions are generally substances that resemble blood or contain similar properties to blood and may be found at a crime scene. Substances that have been tested and may cause false positives in some trials include tomatoes, red kidney beans, horseradish and saliva (Tobe et al. 2007). There may be substances that are common to aquatic settings or fish that have not been investigated for their potential to cause false positive reactions, and therefore further research is necessary. Even less is known about the causes of false negatives, and investigations into the potential sources of error are necessary.

In addition to the environmental and physiological limitations involving the use of presumptive tests for blood on fish, each test presents its own set of benefits and

limitations. The decision as to which tests should be further evaluated is based on the context of the research and the monitoring of fish condition and injury. The detection of haemoglobin in fish mucus is a non-lethal sampling procedure that can be conducted quickly to minimize the amount of time the fish is removed from its natural environment. There may also be reasons to employ these techniques in a laboratory situation or on dead fish.

OVERVIEW OF PRESUMPTIVE TESTS FOR BLOOD

Since many forensic tests have yet to be applied to research on fish, we provide an overview of the different presumptive tests for blood, discuss their actual or potential use on fish, and summarize their limitations and advantages. We base our assessment of potential fisheries applicability on a suite of 'ideal' characteristics.

An ideal presumptive test on fish for blood would:

• produce rapid, robust and repeatable results which can be quantified;

• be environmentally safe to use directly on fish and in natural environments;

• require little personal protective equipment for the user;

• have a low cost, enabling repeated sampling;

• be easy to use in field settings without cumbersome equipment;

• have few interfering compounds.

Catalytic colour tests

Catalytic colour tests characterize the largest group of presumptive tests for blood available to researchers. These tests react with the heme group in blood, specifically by chemically oxidizing the chromogen, to produce a visible colour reaction. An oxidizing

agent must be added to catalyze the reaction, generally hydrogen peroxide. A colour change characteristic of the chromogen that appears within seconds constitutes a positive reaction (Spalding 2006). All of the prescribed indicators have a reported sensitivity of at least a 1:10 000 dilution of human blood, but can be as high as 1:100 000 (Tobe et al. 2007). The most commonly known catalytic colour tests are benzidine, o-toluidine, tetramethylbenzidine, phenolphthalein, leuchomalachite green, Hemident™ and Hemastix®. Table 2-1 provides a summary of these tests and their potential applicability in fish research, but most have carcinogenic or other properties that eliminate their potential for use in fish research. The only catalytic colour tests that show potential practical application in fish research are phenolphthalein, Hemastix® and Hemident ™, which will be explored further.

Phenolphthalein (Kastle-Meyer test)

The Kastle-Meyer test is one of the most common presumptive tests used in forensic laboratories. It uses reduced phenolphthalein in alkaline solution. A positive reaction is denoted by a colour change of colourless to pink and is immediate (Spalding 2006). This test is one of the most sensitive catalytic colour tests (1:10000), although false positive reactions have been documented for substances, including 1 M ascorbic acid (Tobe et al. 2007). Phenolphthalein is listed by the International Agency for Research on Cancer (IARC) as a possible carcinogen to humans, and the agency has declared that there is sufficient evidence to support that phenolphthalein is carcinogenic to animals (IARC 2000). Therefore, there is a potential source of harm to the researcher. However, safety measures (e.g. wearing personal protective equipment) can be implemented, which would

decrease these risks (Phenolphthalein Material Safety Data Sheet 2005; www.sciencelab.com/msds.php?msdsId=9926469).

Hemastix®

Hemastix® were designed to detect blood in urine (for biomedical and veterinary applications), but have been adopted by the forensic community to test stains of unknown origin (Spalding 2006). Hemastix® are unique in their application and design. The kit contains test strips with reagent-treated filter paper attached to one end. The pre-treated filtered paper contains the reagent tetramethylbenzidine (TMB), along with all other chemicals required for the analysis (Tetramethylbenzidine Material Safety Data Sheet 2005; www.sciencelab.com/msds.php?msdsId= 9925220). This allows ease of use, and the materials required for this test are minimal and readily available.

The manufacturer supplies a scale corresponding to the amount of human haemoglobin in each sample based on the colour change, but generally a positive reaction is illustrated by a colour change from yellow to green (Spalding 2006). Hemastix® have a reported sensitivity of 1:100 000, although different users report different sensitivities (Tobe et al. 2007). Also, there have been substances reported to cause false positives, including 10% cupric sulphate and 10% ferric sulphate (Tobe et al. 2007). Cupric sulphate would be a potential false positive for fish injury detection, as it is a known aquatic algaecide that is commonly used in aquaculture (Schlenk et al. 1998).

Hemident™

Hemident™ was created in 1981 for the purpose of aiding in criminal investigations (Spalding 2006). The reagent is MacPhail's reagent (leuchomalachite green), which was

previously used as a fungal treatment for infected fish until its carcinogenic nature was revealed (Culp et al. 1999). Hemident™ is a self-contained chemical reaction, and so the hazards to the user are limited. The instructions and all materials needed for correct use of this test are provided by the manufacturer. The benefit of this test is that the container can be kept and brought back to a laboratory, where further testing can be done if required (Tobe et al. 2007). Another highlight is that this test was designed for field use, and so its simplicity and ease of use are beneficial to fisheries scientists. All that is required for sampling fish mucus is a swab from a pre-selected area of the fish. The tested sensitivity for Hemident™ has been recorded as 1:10 000 for blood (animal or human) (Tobe et al. 2007). With regards to specificity, there are no reported substances that stimulate a valid false positive reaction (Tobe et al. 2007).

Fluorescence and chemiluminescence tests

Fluorescence and chemiluminescence tests are typically used in situations where it is suspected that blood was once present and has subsequently been cleaned up (Spalding 2006), and they are used in liquid form for crime scene investigation. Chemical solutions are sprayed over the area, and, if blood is present, the bloodstain pattern will become evident through the production of light by either luminescence or fluorescence (Spalding 2006). This light can be detected visually and can be photographed for further reference and for quantification and localization of injury.

Fluorescein

Fluorescein is a commonly used presumptive test for blood for large areas and/or areas where no blood is present visually. It reacts with haemoglobin, similarly to the catalytic

colour tests, but the reaction is based on the production of light (Tobe et al. 2007). Fluorescein is prepared by reducing it in an alkaline solution and then applying it to the test area. Hydrogen peroxide is then applied to accelerate the oxidation of the heme group. Under ultraviolet light (UV) the reaction will fluoresce at 520 to 530 nm and can be photographed. This test requires an alternate light source (ALS), typically set at 450 nm, as well as a yellow filter for photography. The photography of the sample must be completed in a lower no-light environment, which poses issues for field usage (Spalding 2006). The sensitivity of fluorescein has been reported as 1:100 000, and there are some substances that may cause false positives, including copper and hypochlorite (Tobe et al. 2007).

This test has previously been used to detect physical injury in fish in a laboratory setting and showed promising results (Noga & Udomkusonsri 2002). The study used the rainbow trout *Oncorhynchus mykiss*, channel catfish *Ictalurus punctatus*, goldfish *Carassius auratus* and hybrid striped bass *Morone saxatilis* male × *M. chrysops* female). Fish were intentionally injured by removal of skin using a scalpel and by acute confinement, or by puncture with a needle. Fish were then euthanized and were completely submerged in the solution with the visible injuries photographed under UV light. Although fluorescein is documented as an irritant (Fluorescein Material Safety Data Sheet 2005; www.sciencelab.com/xMSDS-Fluorescein-9927171), there did not seem to be any adverse effects to live hybrid striped bass tested in high concentrations (Noga & Udomkusonsri 2002), although this may be species-specific.

The use of fluorescein to detect fish injury has been applied in commercial fishery bycatch studies. Davis & Ottmar (2006) used fluorescein to analyze the injury patterns in

fish that were dragged in a net during a laboratory study meant to simulate commercial fishing practices. The proportion of the body surface injured from the study was examined using computer analysis software. Results of the afore-mentioned study showed a link between mortality and the proportion of abrasion for walleye pollock *Theragra chalcogramma*, but not for other species examined.

Luminol

Luminol, like fluorescein, is another blood detection chemical that produces light as a positive reaction. However, the production of light is through luminescence, not fluorescence, and does not require an ALS (Barni et al. 2007). The reaction occurs when the alkaline solution is sprayed over a test area, followed by an oxidizing agent. The reaction is best observed in a light-absent room and can be photographed, albeit the reaction only lasts for 30 s (Barni et al. 2007). Luminol is regarded as one of the most sensitive presumptive blood tests. Proescher & Moody (1939) claimed that luminol could detect blood at a dilution of 1:10 000 000, although the sensitivity depends on the preparation method.

Luminol has been used previously on trout, to identify areas of injury on the skin and to the gills associated with recreational angling (Dedual & Shorland 2006). The fish mucus had to be removed to conduct this test (using a damp cloth), because it is believed that the mucus creates a barrier between the blood and the luminol. This poses issues for sampling of fish using this product, since removal of the mucus would also potentially injure the epithelium, which is what is being detected using the test. It would therefore be difficult to distinguish between injury caused by the treatment and injury caused by the

mucus removal. As well, the removal of the mucus from fish epithelium puts the fish at risk of infection, which can lead to stress and potentially to mortality after release.

Bluestar©

Bluestar© is the newest presumptive test for blood. It is based on the chemical formula for luminol, but has been altered to increase its ease of use (Barni et al. 2007). Bluestar© produces a positive result through luminescence; however, the reaction lasts longer than that of luminol and does not require complete darkness (Tobe et al. 2007). The reported sensitivity is 1:10 000, although laboratory tests have shown a sensitivity of 1:100 000 (Tobe et al. 2007). Literature from the manufacturer claims that false positives are easily identified by an experienced eye based on colour, duration and intensity of the light production. However, Tobe et al. (2007) report false positives on a number of substances, including tomatoes, bleach and 1 M ascorbic acid. A further benefit of Bluestar© is the ease of preparation, which involves the addition of 2 Bluestar© tablets to a prescribed amount of water (Dilbeck 2006), eliminating any complicated chemical processes prior to field use. The prepared solution can be kept for weeks, in contrast to luminol, which must be mixed moments prior to each use. Bluestar © is highly basic, and so its potential uses may be limited to dead fish (Bluestar© Material Safety Data Sheet 2005; www.bluestar-Forensic.com/pdf/en/MSDS_tablets_working_solution.pdf).

DISCUSSION

While some presumptive tests for blood have been applied to fish, there is potential that more of these tests may be applicable in fish health assessments and research. Fluorescein and luminol have been used to enhance patterns of injury on a variety of fish species

(Noga & Udomkusonsri 2002, Dedual & Shorland 2006), although these only proved to be helpful in visualizing injury patterns. Indeed, Dedual & Shorland (2006) published photographs of the illuminated (and thus injured) regions in a fishing magazine to provide a dramatic illustration for anglers on how poor handling practices can affect fish health. Bluestar© should also be investigated for use on dead fish, as it has been shown to be favoured over luminol in forensic science (Dilbeck 2006). Digital photographs of the fluorescein and Bluestar©-treated fish can be analyzed using computer software to determine the percentage of area covered by haemoglobin (Davis & Ottmar 2006, Dauble et al. 2007). None of the catalytic colour tests have been previously used in the context of enhancing injury patterns, although Hemastix® have been used to show early signs of stress in fish (Smith & Ramos 1976). Phenolphthalein is regarded as a very specific presumptive test and should be investigated for potential field and laboratory use on fish.

Although we have outlined which tests appear to have the greatest potential application in fish research, further testing is required. All of the tests have been evaluated for sensitivity with respect to human blood; however, it is unknown whether blood from fish or other animals will yield different results. Testing of sensitivity for fish blood can be done by using diluted blood samples, as shown in Tobe et al. (2007), but dilution should be done using fish mucus to achieve accurate sensitivity measurements. The minimum concentration of haemoglobin necessary for a positive reaction can then be determined.

The specificity of the presumptive tests should not change when comparing human and animal blood; however, the aquatic environment contains possible contaminants that may cause false positive reactions that have not been explored in

forensic science research, including biological peroxidises. Before these tests can be accurately applied in fish biology or research, investigations of possible causes of false positives should be completed. False negatives also should not change when comparing human and animal blood. One substance that has already been found to inhibit the fluorescence of fluorescein is tricaine (Davis et al. 2008). Tricaine is a commonly used fish anaesthetic, so when utilizing fluorescein other anaesthetics are necessary.

Identification of the most valuable presumptive tests for use in fish injury research would require controlled experimentation to determine which tests perform most accurately. As in Noga & Udomkusonsri (2002), fish should be anaesthetized and then inflicted with injuries commonly experienced, such as scale loss, abrasion and pinpoint ulcerations. The selected methods should then be compared. Furthermore, a comparison between the 2 methods of detection (catalytic colour vs. fluorescence and luminescence) should be conducted, to identify the benefits and limitations. Catalytic colour tests should be done by swabbing a selected area of mucus and applying reagents to the swab. Swabs from multiple pre-selected areas of the fish may help to outline general patterns of injury. Fluorescein, luminol and Bluestar© will require spraying or complete submersion of the fish in the appropriate solution. Digital photographs of these fish can be analyzed using computer software, to show the proportion of injury based on the proportion of the fish that is emitting a specific wavelength of light (i.e. 520 to 530 nm for fluorescein). Fluorescein and Bluestar© could be used to test swabs of fish mucus; however, this would eliminate the capability to use digital analysis to quantify the proportion of injury. In all instances, confirmation of lesions should be completed using histology.

The goal of these evaluations will be to identify tests that could be used in both laboratory and field settings, which will also require an assessment of the safety of these chemicals to fish. A safety assessment has already been done for fluorescein for hybrid striped bass (Noga & Udomkusonsri 2002), and similar methods could be used for luminol and Bluestar©. Since the catalytic colour tests do not come into contact with the fish, these chemicals do not pose a direct threat to the fish. Nonetheless, for all tests, it would be valuable to determine if there are any sublethal or lethal consequences from any of the chemicals or procedures needed to quantify injury. This could be done through telemetry (to evaluate behavioural impairments or post-release mortality; Donaldson et al. 2008), reflex assessment for predicting delayed mortality (Davis & Ottmar 2006), or holding fish for a period of time and monitoring delayed mortality.

A general assumption in fish injury assessments is that increased injury results in increased stress and threat to fish survival. The identification of presumptive tests for blood that can assist in quantifying the area of injury on fish would stimulate further research linking external injury to the physiological and behavioural responses, as well as delayed mortality due to injury. Physiological responses to adverse conditions can be investigated by non-lethal blood sampling and testing for a number of biomolecules, including cortisol, aspartate aminotransferase and lactate dehydrogenase (Grizzle et al. 1992, Wendelaar Bonga 1997).

POTENTIAL APPLICATION TO FISH CONSERVATION AND MANAGEMENT

Presumptive tests for blood show potential for use in fish conservation, including use in assessing sublethal injury from interaction with hydropower infrastructure, recreational and commercial fishing, research and handling, and general health monitoring. Before

using these tests in fish conservation and management, limitations related to result confirmation apply and must be eliminated or validated as described above. However, once validated, these tools could become a simple component of routine fish monitoring when there is interest in documenting injuries to fish that arise from various human or non-human interactions.

Hydropower

Hydropower structures can have numerous effects on fish and the aquatic environment. Hydropower infrastructure often creates an impassable physical barrier, which most notably alters fish movement (e.g. Cada 2001). Several solutions for upstream fish passage have been implemented, including the installation of fishways, or trapping fish and physically moving them around the dam (Gowans et al. 1999). To date, no study has focused on the possible physical damage caused by hydropower infrastructure during upstream migration, although such studies would aid in the conservation and management of especially fragile species.

Non-migratory and migratory fish travelling downstream are also at risk of physical damage from hydroelectric dams. Entrainment is avoided through the use of fish screens and dam bypass structures, while downstream fish passage is often facilitated by the installation of ‘fish friendly’ turbines. Passage through the hydroelectric turbines has been documented as a significant cause of mortality and physical damage (Cada 1990). Generally, the injury assessments of fish that pass through turbines are made by visual inspection, which at best can detect gross-scale injuries and trauma. The use of fluorescent or chemiluminescent presumptive tests would be able to help identify patterns of injury resulting from turbine passage, especially at the cellular level, which would

likely be overlooked using visual inspection and has been linked to the behaviour and physiology of sublethally injured fish (Davis 2002). Catalytic colour tests would also be useful in locating the areas of the skin injured by turbine passage through sampling of pre-selected areas.

Fluorescein has already been used to assess injury in salmonids passing through a turbine. It was helpful in quantifying the injury (using computer software analysis), as well as in determining the types of injury (Dauble et al. 2007). The researchers found this method to be highly efficient, as it did not cause any more injury than would have occurred through visual assessments of anaesthetized fish, and fish could be released back into the water after the treatment was complete.

Fish residing downstream of dams or passing through dams can also be subjected to gas bubble disease (from pressure changes and/or gas supersaturation; Weitkamp & Katz 1980). One of the signs of gas bubble disease is haemorrhaging from skin and fins, which could be quantified using forensic techniques.

Recreational and commercial fishing

Recreational and commercial fishing are practised on a global scale and involve the capture of billions of fish annually (Cooke & Cowx 2004). One of the current concerns surrounding recreational fishing is the biological impact this has on individual fish. In some areas, catch-and-release angling is widely practised, but the fates of those released fish are often unknown (Cooke & Cowx 2004). As with the gas bubble disease issues described above for hydropower facilities, fish captured from deep water can suffer from similar symptoms. A recent study of smallmouth bass *Micropterus dolomieu* used qualitative metrics (presence and absence of reddened tissue indicating haemorrhaging)

to characterize barotraumas incidences at a fishing tournament (Gravel & Cooke 2008). The use of presumptive tests for blood in the assessments of barotrauma could provide more robust and reliable estimates of injuries resulting from fish being captured from deep water.

In commercial fisheries, bycatch can be a significant proportion of the total catch, and individual fish are released due to species, size, quantity, or sex (Davis 2002). The post-release (discard) mortality of these individuals has been linked to wounding, measured using fluorescein in a laboratory setting (Davis & Ottmar 2006), and techniques exist to assess whether released fish will survive (Davis 2002). The evaluation of other methods of injury detection would be useful in both recreational and commercial fisheries, especially in relation to gear and handling method comparison. New techniques and equipment could be compared to standard practices for injury and associated delayed mortality.

Research and handling

Research often involves confinement and handling of fish, including containment in trap nets, hoop nets, live pot traps and holding tanks (Chopin & Arimoto 1995). The effects of this equipment on injury, physiology, behaviour and survival are largely unknown. Cooke et al. (1998) performed post-mortem examinations to study the injuries on greenside darters *Etheostoma blenniodes* resulting from live pot traps. They observed scale loss and abrasions around the caudal area, which resulted in fungal growth and the eventual death of 74% of the fish, but quantifying injury would have been helpful in this investigation. The testing of all capture and confinement methods would be beneficial to identify those

techniques with the least negative impacts on fish in order to maintain their condition and enhance survival.

Research involving individual fish almost always requires some form of handling. Handling is also an issue in catch-and-release angling, as fish are often held during hook removal, measurement and photography (Cooke & Cowx 2004). Previous research demonstrated that fish held for a longer period of time have higher rates of post-release mortality (Pickering et al. 1982, Schramm et al. 1987). Handling can cause significant damage to fish, and has been documented using luminol and fluorescein (Noga & Udomkusonsri 2002, Dedual & Shorland 2006). Noga & Udomkusonsri (2002) detected injuries that were not a result of the induced injury and concluded that these came from the handling of the fish during the investigation. Safe handling practices have been suggested for use by anglers and researchers, but visualizations of the injuries and quantifications of the extent of the trauma could aid in developing a standard protocol for fish handling and emphasize the negative consequences of poor handling.

General health monitoring

A technique that could rapidly assess the general health and injury level of fish without being a hazard to the fish or environment would be ideal and would provide valuable information. Areas that would benefit from these rapid techniques for health assessment, for example, include aquaculture endeavours and other areas in which there is interest in minimizing injury from handling. This is particularly relevant when using hatcheries to enhance endangered fish populations. Obviously, any methods used on potential food-fish would need to be screened for their human impacts.

CONCLUSION

Assessment of non-visible injury in fish has rarely been conducted. Typically, macroscopic visual examination is used to identify injuries, but this approach is subjective and based on the severity of the trauma detectable to the naked eye. In the current paper, we presented an alternative approach to identifying and quantifying fish injury through blood detection techniques used in forensic science. The identification of a relevant and robust presumptive test for blood on fish would benefit many areas of fisheries research, including assessing the impacts of hydropower infrastructure, evaluating recreational and commercial fisheries, enhancing handling practices, and performing general health assessments. Collectively, such data should facilitate the conservation of a wide range of fish species.

TABLES

Table 2-1. Summary of Presumptive Tests for Blood and their Applicability to Fish Research

Test	Sensitivity	Price ($CAD)	Carcinogen	Tested on Fish	Potential Field Use	Potential Lab Use	Rationale
Benzidine	1:100,000	N/A	Yes	No	None	None	Class I Carcinogen (IARC 1987)[A]
o-Tolidine	1:100,000	$32.95/kit	Yes	No	None	None	Class 2A Carcinogen (IARC 1972)[B]
Phenolphthalein	1:10,000	$34.00/kit	Possible	No	Medium	High	Class 2B carcinogen (IARC 2000)[C]
Leuchomalachite Green	1:10,000	N/A	Yes	Yes	None	None	Hazardous to fish and humans (Fessard et al. 1999)
TMB	1:10,000	$36.00/kit	No	No	Low	Low	Hemastix® contain the reagent
Hemastix®	1:100,000	$35.00/50 strips	No	No	High	High	Easy to use; test strips are pre-treated with reagent
Hemident™	1:10,000	$16.65/10 tests	No	No	High	High	Easy to use; self contained reaction
Fluorescein	1:100,000	$36.50/250mL	No	Yes	High	High	Has already been tested and used on fish for injury detection
Luminol	1:1,000,000	$18.25/236mL	No	No	Low	Low	Reaction only lasts 30 seconds; more applicable methods available

Bluestar©	1:100,000	$89.00/500mL	No	No	Medium	High	Easier to use than luminol; reaction lasts longer and is brighter

[A] Class 1 carcinogens are known carcinogens to humans
[B] Class 2A carcinogens are probable carcinogens to humans
[C] Class 2B carcinogens are possible carcinogens to humans

CHAPTER 3: Evaluation of forensic presumptive tests for blood for the detection of injury in fish

ABSTRACT

Current methods of fish epithelial injury detection are limited to gross macroscopic examination which has a subjective bias as well as an inability to quantify reliably the degree of injury. Fluorescein, a presumptive test for blood developed for use in forensic science, has been shown to have the capability to detect and quantify fish epithelial injury. However, there are several other presumptive tests for blood (i.e. Bluestar©, phenolphthalein, Hemastix®) which may have benefits over the use of fluorescein, particularly for field research on wild fish. This study investigated the capabilities of these four tests to detect and quantify a variety of injuries commonly encountered by fish (i.e. abrasion, cuts, fin frays, and punctures). Fluorescein was consistently found to be the most reliable (i.e., detected the highest proportion of true positive results and rarely detected false positive reactions) of the four presumptive tests for blood compared, so the capabilities of fluorescein were investigated further. By 24 hours after an injury was inflicted, it was no longer detectable by fluorescein and when fluorescein was applied to an injured fish, it was no longer detectable 3 hours after application. In a comparison of two common anaesthetics used for testing, there was no significant difference in the proportion of injury detected when MS-222 was used, when compared with clove oil and ethanol (1:9) solution. In summary, fluorescein was determined to be a reliable method for the detection and quantification of fish epithelial injury. Its ease of use, low cost and objective nature make it an ideal method for detection in both field and lab settings.

INTRODUCTION

Fish can acquire external injuries from a variety of different sources. For example, fish can experience injuries from interaction with their environment (e.g., substrate, woody debris), conspecifics (e.g., during spawning, competitive interactions; Hutchings & Meyers 1987, Neat et al. 1998), and predators (Harmon et al. 1994). In addition, fish also have the potential to experience injuries as a result of human activities (e.g., commercial fishing [Davis & Ottmar 2006], recreational fishing [Cooke et al. 2002], aquaculture [Ashley 2007], boating [Killgore et al. 2001]) or interaction with anthropogenic structures (e.g., hydroelectric turbines [Cada 2001], water intake structures [Larinier & Travade 2002], fishways [Larinier 2002]). These injuries can vary in severity and detriment to the fish. In some cases the injuries are so severe that they result in immediate death such as might be experienced in a turbine strike (Cada 2001), or when an angled fish is attacked by a predator (Danylchuk et al. 2007). However, there are undoubtedly more instances where fish experience injuries that are not lethal in the short term. For example, damage to the epithelium that covers the surface of the fish can result in infections from a variety of different opportunistic pathogens including bacteria, fungus and other parasites (Ventura & Grizzle 1987; Van West 2006). The injuries themselves or the associated pathogenic infections can lead to sublethal impairments such as physiological disturbance and behavioural alterations (Cooke & Sneddon 2007) and may even lead to delayed mortality (Davis 2005).

As part of routine fish monitoring practices or in fisheries research, injuries are often noted qualitatively through examination of scale loss (Main & Sangster 1988) and fin fraying (Barthel et al. 2003) via visual macroscopic evaluation. These techniques are

highly subjective as the degree of injury is based on researcher opinion and has little quantitative rigor. In addition, there may be injuries, such as abrasions, that may not be macroscopically detectable but can lead to later infection or death. Fluorescein, a non-toxic dye commonly used in crime scene investigation, has been shown to enhance the detection of epithelial injury in fish (Noga & Udomkusonsri 2002) and has been used in the quantification of injury for comparison of commercial fishing techniques (Davis & Ottmar 2005) as well as hydroelectric turbine passage evaluation (Dauble et al. 2007).

Presumptive tests for blood are used to locate latent blood stains and to differentiate blood from other substances (Spalding 2006). They interact with haemoglobin in blood to cause a positive reaction which is denoted by a change in colour or the production of light (Spalding 2006). Under ultraviolet light, fluorescein produces a green light when blood is present. There are several other presumptive tests for blood which may be useful for fish epithelial injury detection (reviewed in Colotelo et al. 2009). Similar to fluorescein, Bluestar© also produces light in the presence of blood, but does not require a UV light source. With both tests, photographs can be taken of this light production to calculate the proportion of the fish that is injured, based on the light produced (Davis & Ottmar 2006). Hemastix® and phenolphthalein can be applied with cotton swabs to test a specific area of the fish to determine which parts of the body are injured. These tests may be considered ideal for field use, since fish do not have to be anaesthetized, the chemical never touches the fish and the reaction occurs within minutes. Other presumptive blood detection techniques have been considered for application in a fisheries context (e.g., benzidine, o-tolidine, leuchomalachite green, tetramethylbenzidine, Hemident™, luminol) but are regarded as having too many

limitations to make them broadly useful for injury detection in field settings (Colotelo et al. 2009).

This study was conducted to evaluate several different presumptive tests for blood for injury detection in fish. Comparative efforts focused on four tests (i.e., fluorescein, Bluestar©, Hemastix® and phenolphthalein) that were identified by Colotelo et al. (2009) in a recent synthesis where ten different tests were qualitatively screened for their potential use in freshwater fish injury detection in field settings. An adaptive research approach was used in this study, whereby research efforts were adjusted and refocused through time. In the early phases of research tests were systematically excluded when appropriate to enable more detailed evaluation of those tests that showed the most promise. The first phase of research involved quantitative evaluation of the performance of the four tests in the detection of different types of experimentally-inflicted injury in a field setting. Based on findings from the first phase, an additional suite of investigations were conducted to further clarify the performance of fluorescein with respect to potential interaction with other chemicals (e.g., anaesthetics), to determine the "age" of injury upon detection, and to investigate how long fluorescein is detectable in the mucous after treatment. For the purpose of this study, bluegill (*Lepomis macrochirus*), a small freshwater fish, were used. Bluegill serve as an excellent model given their abundance and ease of capture and handling to obtain "uninjured" fish to use for experimentation. The tools evaluated and validated in this study will provide fisheries biologists and scientists with additional capacity for the detection and quantitative description of injury in freshwater fish. With the increasing potential for fish interaction with humans and

human infrastructure, there is a need for tools that can be incorporated into environmental monitoring and fisheries research.

METHODS

Comparative Analysis of Injury Detection

Bluegill were angled from Lake Opinicon, located in southeastern Ontario, Canada, using size 8 barbless hooks and standard angling gear. Fish within the size range of 130mm to 150mm were collected while fish outside this size range were released. Fish that were deeply hooked or exhibited any macroscopic evidence of injury were also released. Fish were handled only by gently, but firmly, gripping them by the lower lip using padded pliers. Fish were immediately placed in a water bath (4 L) in a plastic cooler with rounded edges containing 50ppm clove oil anaesthetic (clove oil emulsified in ethanol, 1:9; Sigma Aldrich, Toronto, ON) and remained there until fish reached stage 4 of anaesthesia noted by a loss of equilibrium and coordinated fin movements (Summerfelt & Smith 1990). Fish were then removed from the anaesthetic bath by grasping them by the lower lip with the padded pliers and those treated with fluorescein and Bluestar© were dragged across a 5mm nylon mesh net (18cm in length) 3 times on their right side. All fish were then inflicted with standard injuries on their left side (Figure 3-1a). The upper or lower lobe of the caudal fin was frayed by cutting with a scalpel 30 times in the anterior-posterior plane from the base of the fin to the end. A puncture wound was made in the left cheek and posterior and dorsal to the left eye using a dental pick. A scalpel scrape (1cm x 3 cm) was induced above or below the lateral line. Finally, a shallow cut was made from the posterior end of the soft dorsal fin to the posterior end of the anal fin, across the left side of the caudal peduncle using a scalpel. These injuries were inflicted to

replicate injuries that might occur in the natural environment or after interaction with anthropogenic structures. Fish were then treated with one of the four presumptive tests for blood, as outlined below.

Fluorescein

Immediately following injury infliction, fish were sprayed with 0.2mg/mL solution of fluorescein (Fluorescein, Disodium Salt; Aldon Corp., Avon, NY) in distilled water, which was left unrinsed for 6 minutes (Noga & Udomkusonsri 2002). Fish were then placed in an anaesthetic bath containing 120ppm clove oil (clove oil emulsified in ethanol at 1:9) for 6 minutes to euthanize them. Fish were then photographed in complete darkness using a digital SLR ELIXIM Pro EX-F1 camera (Casio Computer Co., Ltd., Tokyo, Japan) under incandescent light, long UV light (365 nm) and short UV light (254 nm) under various exposures (10, 13, 15 and 20 seconds) and at an ISO of 100. Fish were photographed in complete darkness, against a black background, with the camera positioned 42cm directly above the fish and the UV light source (Mineralight® UVGL-48; UVP Inc., Upland, CA) at a 45^{o} angle to the fish, 22cm above, so that the entire organism was illuminated by the UV light (Figure 3-2). It was determined that short UV light and 20 second exposure was the best settings for examining the injury patterns and was used for the remainder of the study with fluorescein.

Bluestar©

Immediately following injury infliction, fish were sprayed with Bluestar© solution (Bluestar© Forensic Mini Kit; Bluestar, Monte Carlo, Monaco) which was prepared following the instructions of the manufacturer. Since the Bluestar© reaction is time sensitive, fish were immediately photographed in complete darkness with a digital camera (described above) positioned 42cm directly above the fish, with an exposure of 30 seconds, and following the photography directions outlined by the manufacturer. Fish were then euthanized as described above.

Hemastix®

A moistened cotton swab was applied to $1cm^2$ predetermined areas of the fish, representing areas which were inflicted with injury and those which were not (Figure 1b & c). Each cotton swab was then touched to the pre-treated reagent pad of the Hemastix® (Hemastix Reagent Strips for Urinalysis; Bayer HealthCare LLC, Elkhart, IN) and the colour change reaction was compared to a scale provided by the manufacturer and the result was recorded after 60 seconds. Fish were then euthanized as described above.

Phenolphthalein

A moistened cotton swab was applied to $1cm^2$ predetermined areas of the fish, representing areas which were inflicted with injury and those which were not (Figure 1b & c). A series of reagents were then applied to each cotton swab, following the phenolphthalein manufacturer's instructions (WARD'S Phenolphthalein Blood Test Kit;

WARD'S Natural Science Establishment, LLC, Rochester, NY). The reaction results were recorded after 60 seconds and 4 minutes. Fish were then euthanized as described above.

Computer Analysis of Photographs

Fluorescein and Bluestar© cause a positive reaction through the production of green and blue light, respectively. Photographs were analyzed, using ImageJ software (http://rsb.info.nih.gov/ij/; National Institute of Health, Bethesda, MD), by tracing the areas of green or blue and measuring the number of pixels in that area. All analyses were conducted by the same individual. The proportion of injury on the entire body of the fish was then calculated by dividing the number of green or blue pixels by the total number of pixels of the fish. This process was done twice and the average number of pixels was calculated and used for statistical analysis.

Comparison of Presumptive Tests for Blood

The results of the injury evaluations were tabulated and compared graphically for difference in rates of true positives (area was injured and tested positive), false positive (area was not injured and tested positive), true negatives (area was not injured and tested negative) and false negatives (area was injured and tested negative). Areas that were injured and then tested with fluorescein or Bluestar© were denoted as positive for any proportion of injury higher than zero. Hemastix® were accompanied with a scale that categorized the colour change, into proportions of haemoglobin detected. Results that were classified as "large" or "medium", based on a scale provided by the manufacturer, were denoted as positive, while all others were considered negative. A positive result

from phenolphthalein was denoted as a change from colourless to pink as described by the manufacturer.

Focused Research on Fluorescein

Based on the comparative analysis we determined that fluorescein had strong potential for field application (see below), but required additional validation to determine its field applicability and to refine its use.

Latency of Detection Capability

Bluegill (n=10 for each time group) were angled from Lake Opinicon using the capture method described above. Fish were individually marked with small numbered anchor tags (Floy manufacturing, Washington) placed in their dorsal musculature. They were anaesthetized in a water bath containing a 50 ppm clove oil and ethanol solution (1:9) until fish reached stage 4 of anaesthesia as described above. A scalpel scrape (1cm x 3cm) was induced below the lateral line, on either the left or right side of the body. Fish were then submerged in a 0.2mg/mL solution of fluorescein in distilled water for 6 minutes and were then placed in a holding tank which was constantly provided with fresh lake water. Fish were examined for the presence of fluorescein at multiple times after injury infliction and fluorescein treatment. After 1, 3, 8 and 15 hours the same fish were anaesthetized with a 50ppm clove oil and ethanol solution (1:9) and photographed in complete darkness under incandescent and short UV light (20 sec exposure) as described above. Computer analysis to calculate the proportion of green in each photograph was conducted as described above.

Temporal Patterns in Injury Detection

Bluegill (n=10 for each time group) were angled from Lake Opinicon using the capture method described above. Fish were anaesthetized in a water bath containing 50ppm clove oil and ethanol (1:9) solution until stage 4 of anaesthesia as described above. A scalpel scrape (1cm x 3cm) was inflicted below the lateral line, on either the left or right side of the body. Fish were then held for 1, 5 or 24 hours in a tank that was constantly provided with fresh lake water. After the set time fish were removed and anaesthetized in a water bath containing 50 ppm clove oil and ethanol (1:9) solution followed by 6 minutes in 0.2mg/mL fluorescein solution in distilled water and then 6 minutes in a water bath containing 50ppm clove oil and ethanol (1:9) solution. Fish were then photographed in complete darkness under incandescent and short UV light (20 second exposure) as described above. Computer analysis to calculate the proportion of green in each photograph was conducted as described above.

Influence of Anaesthetics on Injury Detection

Bluegill (n=10 for each anaesthetic group) were angled from Lake Opinicon using the capture method described above. Fish were anesthetised in a water bath containing either 50ppm clove oil and ethanol (1:9) solution, or 50ppm buffered 3-aminobenzoic acid ethyl ester methanesulfate (MS-222 or tricaine; Western Chemical, Inc., Ferndale, WA) until equilibrium was lost. A scalpel scrape (1cm x 3cm) was inflicted below the lateral line, on either the left or right side of the body. All fish were then treated with a 0.2mg/mL solution of fluorescein in distilled water for 6 minutes. Fish anesthetised with the clove oil and ethanol (1:9) solution were then rinsed in a water bath containing 50ppm clove oil

and ethanol (1:9) solution for 6 minutes. Fish anesthetised with buffered MS-222 were placed in a rinse of pure lake water for 6 minutes and then lethally sampled via cerebral percussion. Fish treated with MS-222, therefore, must be euthanized using means other than an MS-222 overdose, following treatment with fluorescein in order to obtain photographs; they cannot be re-submerged in the anaesthetic (Davis et al. 2008). All fish were then photographed under incandescent and short UV light (20 second exposure). Computer analysis to calculate the proportion of green in each photograph was conducted as described above.

Statistical Analysis

Chi Square contingency table analyses were used to determine if there were significant differences in the number of true positive reactions for each test for each type of injury inflicted. To test for differences between the proportion of injury detected by fluorescein and Bluestar©, a one-way ANOVA was conducted on inverse transformed data. As well, a one-way ANOVA was used to test for differences in the proportion of injury detected when using clove oil and MS-222 as an anaesthetic on natural log transformed data. The time required to eliminate fluorescein from a fish's epithelium was analyzed using a repeated measures ANOVA, followed by a Tukey's HSD *post hoc* test. A one-way ANOVA was used to test for significance in the time after injury was inflicted that the injury could still be detected using fluorescein on square root transformed data. This was also followed by a Tukey's HSD *post hoc* test. All transformations were done to meet the assumptions of normality and homogeneity of variance required for parametric tests. SPSS software was used for all statistical tests and significance was assessed at $\alpha = 0.05$ (Zar 1984).

RESULTS

Comparative Analysis of Injury Detection

For all tests, there was a significant difference in the observed and expected values for true positives (Figure 3-3; Table 3-1). A *post hoc* test for independence was done for each analysis (Table 3-1) to determine the presumptive tests which contributed to the statistical significance of the difference in the observed and expected values for true positives. Bluestar© contributed to the rejection of the null hypothesis for all χ^2 tests, indicating that its values had the greatest difference between observed and expected frequencies. However, fluorescein was a contributing factor for the detection of punctures and fin fraying and phenolphthalein was a significant contributing factor for the detection of fin fraying. Hemastix® was not a significant contributing factor for any of the injury detection.

For the detection of abrasion fluorescein had the highest proportion of true positive results when compared to all presumptive tests, and for fin frays had a greater proportion of true positives than Bluestar© (Figure 3-4a & b). When compared to fluorescein, Bluestar© had higher rates of true negative reactions for both abrasion and fin fray injuries (Figures 3-4a & b). Fluorescein also had a higher proportion of true positive reactions, when compared with Bluestar©, for the detection of cuts and punctures (Figure 3-5a & b). Hemastix® had a higher proportion of false positive reactions when comparing Hemastix® and phenolphthalein in their ability to detect cuts and punctures. It was also found that Hemastix® had a higher proportion of false positive reactions, however, phenolphthalein had the highest proportion of true negative reactions (Figure 3-6a & b).

Computer Analysis

The proportion of total area of green or blue to the total area of the fish, for fluorescein and Bluestar© respectively, was calculated twice and an average value was used for all statistical analyses. The mean difference between the two calculations was 0.1 ± 0.7 % (mean ± S.D.). The minimum difference between the two calculations of proportion of injury was 0.1%, while the maximum difference was 2.0%.

Comparison of Fluorescein and Bluestar©

A one-way ANOVA was conducted to compare the proportion of injury detected when using fluorescein or Bluestar©. There was no significant difference in the proportion of injury detected between fluorescein (mean ± S.E.; 2.9 ± 0.6 %) and Bluestar© (mean ± S.E.; 9.7 ± 5.2 %) (ANOVA: $F_{1,28}$=1.7; p=0.198).

Latency of Detection Capability

There were significantly different proportions of fluorescein detected when fish were held for different periods of time after injury was inflicted (Figure 3-7; Repeated measures ANOVA: $F_{1,9}$=16.4; p=0.003; with Tukey's HSD *post hoc*). Specifically, there was significantly less detectable proportion of injury at 1, 3, 8 and 15 hours than at time of treatment (0 hour).

Temporal Patterns in Injury Detection

The ability of fluorescein to detect injuries that are more than 24 hours old was significantly reduced, when compared to testing fish immediately after (0 hours) or at 1

and 5 hours after injury occurred (Figure 3-8; one-way ANOVA: $F_{3,36}=20.7$; $p<0.001$; with Tukey's HSD *post hoc*).

Influence of Anaesthetics on Injury Detection

Fish anaesthetized in a water bath with 50ppm clove oil and ethanol (1:9) solution (mean ± S.E.; 9.6 ± 14.7 %) did not have significantly different levels of fluorescein detected than fish treated with MS-222 (mean ± S.E.; 25.6 ± 7.8 %).

DISCUSSION

There is a need to develop a method of detection and objective quantification of fish epithelial damage. Presumptive tests for blood have the ability to detect small amounts of blood that may not be visible (Spalding 2006). The rapid results and ease of use of these chemicals makes them ideal for field use (Colotelo et al. 2009). This study compared four presumptive tests for blood in their ability to detect and quantify several different types of injury. Based on our results for the first part of the study, we examined specific characteristics of fluorescein in a series of follow-up experiments.

Comparative Analysis of Injury Detection

Fluorescein was the most consistent and reliable method of injury detection in this study. Fluorescein detected the highest proportion of true positive results except for fin fraying, (Figure 3-4a & b; Figure 3-5a & b), and rarely detected false positive reactions, which supports its usefulness as a tool for a conservative estimate of injury. These results are consistent with previous studies where fluorescein has been used for the detection of fish epithelial damage in commercial fisheries and hydroelectric turbine passage research

(Davis & Ottmar 2006; Dauble et al. 2007). With its quantitative ability, low cost and field applicability, fluorescein is an ideal tool for the detection of fish epithelial damage.

Bluestar©, although possessing the ability to evaluate injury quantitatively, was not an effective test for fish epithelial damage. It demonstrated the lowest proportion of true positives when compared with all other tests (Figure 3-4a & b; Figure 3-5a & b) and consequently had a high proportion of false negative reactions. Dedual and Shorland (2006) demonstrated positive results when working with luminol (Bluestar© is a derivative of luminol) but had to remove the mucous layer for it to be effective. This may explain the negative results observed as the mucous layer was not removed for this study. Removal of mucous has the potential to alter findings as it could smear blood stains and preclude the ability to identify the precise location of an injury.

Hemastix® demonstrated a high proportion of true positives detected for all injury types in this study. However, it was not a significant contributor to the detection of any of the injuries as seen in Table 3-1. This shows that Hemastix® may be an effective method of epithelial damage detection, however, it also demonstrated a high proportion of false positive reactions, especially in the detection of cuts and fin fraying (Figure 3-4a & b; Figure 3-6a & b). These results indicate that before Hemastix® can be readily utilized for epithelial damage detection, more work needs to be conducted corresponding to the specificity, or the ability of the chemical to detect blood accurately and identify substances that may present as false positives or false negatives.

Phenolphthalein was not effective in the detection of the different injuries inflicted. The maximum proportion of true positives that were detected was 35% for punctures (Figure 3-4a & b; Figure 3-6a & b). For the detection of fin fraying,

phenolphthalein was a significant contributing factor (Table 3-1). The positive reactions were never strong reactions, and there is a level of familiarity with the chemical necessary for effectively interpreting the results. Also, the chemicals must be applied to the swab independently, which creates more steps and organization in a field situation.

Based on the statistical comparison of the presumptive tests as well as the ability to utilize these tests in the field, fluorescein was determined to be the most effective test for the detection and quantification of epithelial damage to fish. Additional experimentation was conducted to further understand the effectiveness and capability of fluorescein for the detection of injury in fish.

Latency of Detection Capability

Fish can be released after treatment with fluorescein, and so it is important to know how long the fluorescein stays in their epithelium. Fluorescein is a non-toxic dye and is used in the medical field on humans to detect corneal lacerations and in fluorescein angiography; however, it can cause some stomach discomfort when ingested (Lipson & Yannuzzi 1989). For any fish that may be consumed post-sampling it is necessary to ensure that there is no harm inflicted on the consumer. Although we did not conduct a formal food safety investigation, we did determine that at 1 hour after treatment with fluorescein the proportion of fluorescein detected, 3.8%, in the epithelium had decreased significantly from that of fish which were photographed immediately after treatment, 25.6% (Figure 3-7).

Temporal Patterns in Injury Detection

Another concern with the detection of injury is to clarify the source of the injury. In this study, the ability of fluorescein to detect an induced injury at different times after infliction was investigated. Twenty-four hours after infliction of injury, the proportion of injury detectable, 2.9%, was significantly less than that detected at 0, 1 and 5 hours (25.6%, 14.6% and 14.7% respectively) (Figure 3-8). These results suggest that at least 24 hours after injury is inflicted, fluorescein can no longer detect significant injury to the fish epithelium. This is important for understanding that the source of injury is from the treatment and not an outside source.

Influence of Anaesthetics on Injury Detection

In this study there was no difference between the proportion of injury detected when using MS-222 and clove oil as an anaesthetic. There have been previous concerns with using MS-222 as an anaesthetic when using fluorescein, as it has been shown to inhibit the fluorescence and may be a source of false negative results (Davis et al. 2008). Fish treated with MS-222, therefore, must be euthanized, using means other than an MS-222 overdose, following treatment with fluorescein to obtain photographs; they cannot be re-submerged in the anaesthetic. Clove oil was also examined as an anaesthetic, and there was no observable interference with the fluorescein, which poses a benefit over MS-222 as the fish do not have to be lethally sampled to be photographed.

Implications and Future Considerations

Fluorescein has been shown to be an effective method of detecting and quantifying fish epithelial injury (Noga & Udomkusonsri 2002). In this comparison with other presumptive tests for blood, it was shown to be the most useful and reliable in the

detection of multiple types of injury. Its low cost, ease of application in the field and non-toxic nature make it a valuable tool for field research investigating different sources of injury. Fluorescein can be used to visualize latent injuries, eliminating the subjectivity associated with conventional macroscopic evaluations and will be valuable for the comparison of different handling methods and gear that is used in the capture and holding of fish. This can be especially useful in public outreach and education, where visual presentations are essential to conveying scientific results. For example, there is the opportunity to inform anglers and commercial fishers of the potentially negative consequences that may arise from poor handling methods of fish which are to be released. Dedual & Shorland (2006) have shown that photographs of injured fish due to poor handling methods can be an effective conservation tool.

Although the benefits of this tool are numerous, fluorescein is a relatively new technique and has been used in a limited number of studies. Overall, the proportion of injuries detected were lower than expected, therefore further research is needed into the depth of injury needed to be detected and interference with other natural and anthropogenic chemicals (including anaesthetics). Fluorescein is capable of injury detection and while the potential negative effects of epithelial damage, including infection, can be quite severe, the long term consequences of detectable levels of injury have yet to be determined (Colotelo et al. 2009). Future research should focus on the sublethal disturbances which develop from injuries detectable using fluorescein, including physiological stress and infection, as well as mortality rates that result from these disturbances. Mortality rates can impact population levels, which is important especially when dealing with conservation of sensitive species (Simberloff 1988).

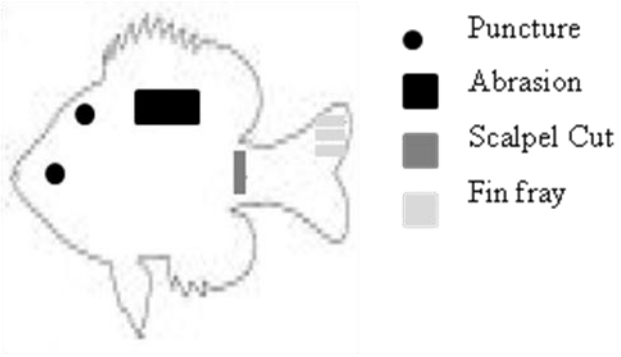
As MS-222 has shown to impede the fluorescence of a positive reaction with fluorescein, the use of other anaesthetics needs to be investigated (Davis et al 2008). Clove oil is an effective anaesthetic which has been used instead of MS-222 due to its less harmful properties (Anderson et al. 1997). It is necessary to investigate any potential effects in the enhancement or inhibition of the reaction of fluorescein to detect injury associated with any anaesthetic. Clove oil shows promise but there clove oil may also be a source of inhibition for fluorescence.

TABLES

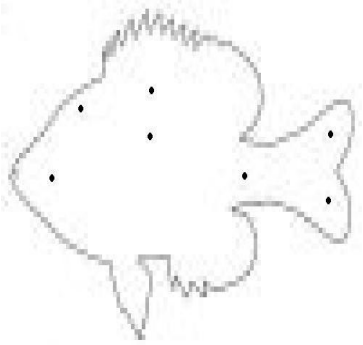
Table 3-1. Chi-square post hoc analysis for the proportion of true positives detected by each presumptive test for blood.

Injury	**χ^2 Result**		**Presumptive Test**	**Standardized Residual**	**p-value**
	$\chi^2_{0.05,3}$	***p-value***			
Puncture	14.88	0.0019			
			Fluorescein	-2.32	<0.05
			Bluestar©	-2.84	<0.05
			Hemastix®	-0.29	>0.05
			Phenolphthalein	-1.15	>0.05
Abrasion	17.08	0.0006			
			Fluorescein	-0.51	>0.05
			Bluestar©	-3.61	<0.05
			Hemastix®	-0.86	>0.05
			Phenolphthalein	-1.73	>0.05
Cut	18.15	0.0004			
			Fluorescein	-1.29	>0.05
			Bluestar©	-3.61	<0.05
			Hemastix®	-1.15	>0.05
			Phenolphthalein	-1.44	>0.05
Fin Fray	21.42	$9e10^{-5}$			
			Fluorescein	-2.06	<0.05
			Bluestar©	-3.61	<0.05
			Hemastix®	0	>0.05
			Phenolphthalein	-2.02	<0.05

FIGURES



A



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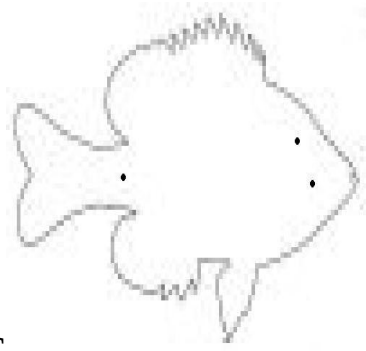


C

Figure 3-1. Diagrams of bluegills to illustrate:
(A) pattern of injury used for comparison of four different presumptive tests for blood for fish injury detection and quantification.
(B) sampling sites on the left side of the specimen.
(C) sampling sites on the right side of the specimen.

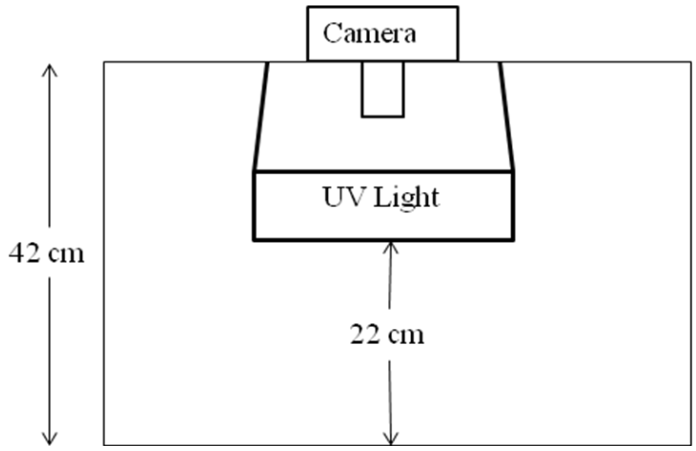


Figure 3-2. Diagram of container used to photograph fish treated with fluorescein.

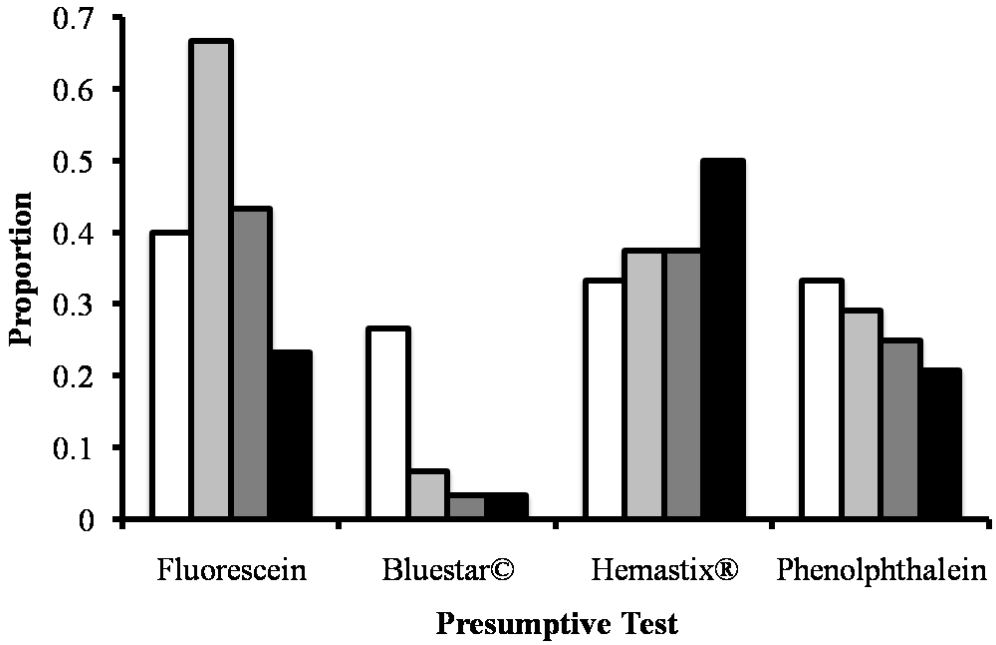


Figure 3-3. Proportion of true positives for all four presumptive tests, for punctures (white bars), cuts (light grey bars), abrasion (dark grey bars) and fin frays (black bars). For fluorescein and Bluestar© n=15 for the punctures and cuts, and n=30 for the abrasion and fin frays. For Hemastix® and phenolphthalein n=24 for all injury types.

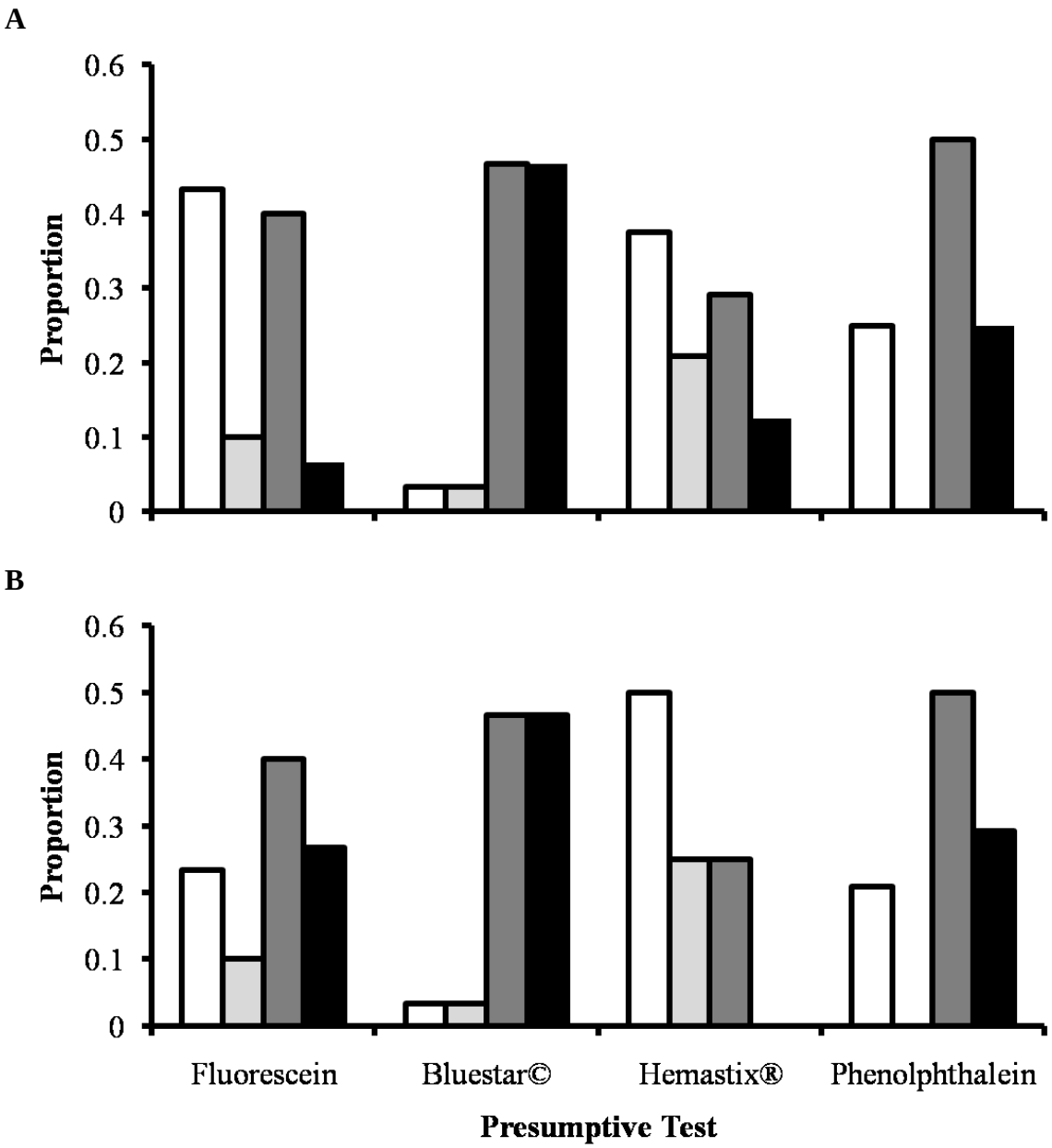


Figure 3-4. The proportion of true positives (white bars), false positives (light grey bars), true negatives (dark grey bars) and false negatives (black bars) recorded for testing for presence of abrasions (A) and fin frays (B) using fluorescein (n=30), Bluestar© (n=30), Hemastix®(n=24) and phenolphthalein (n=24).

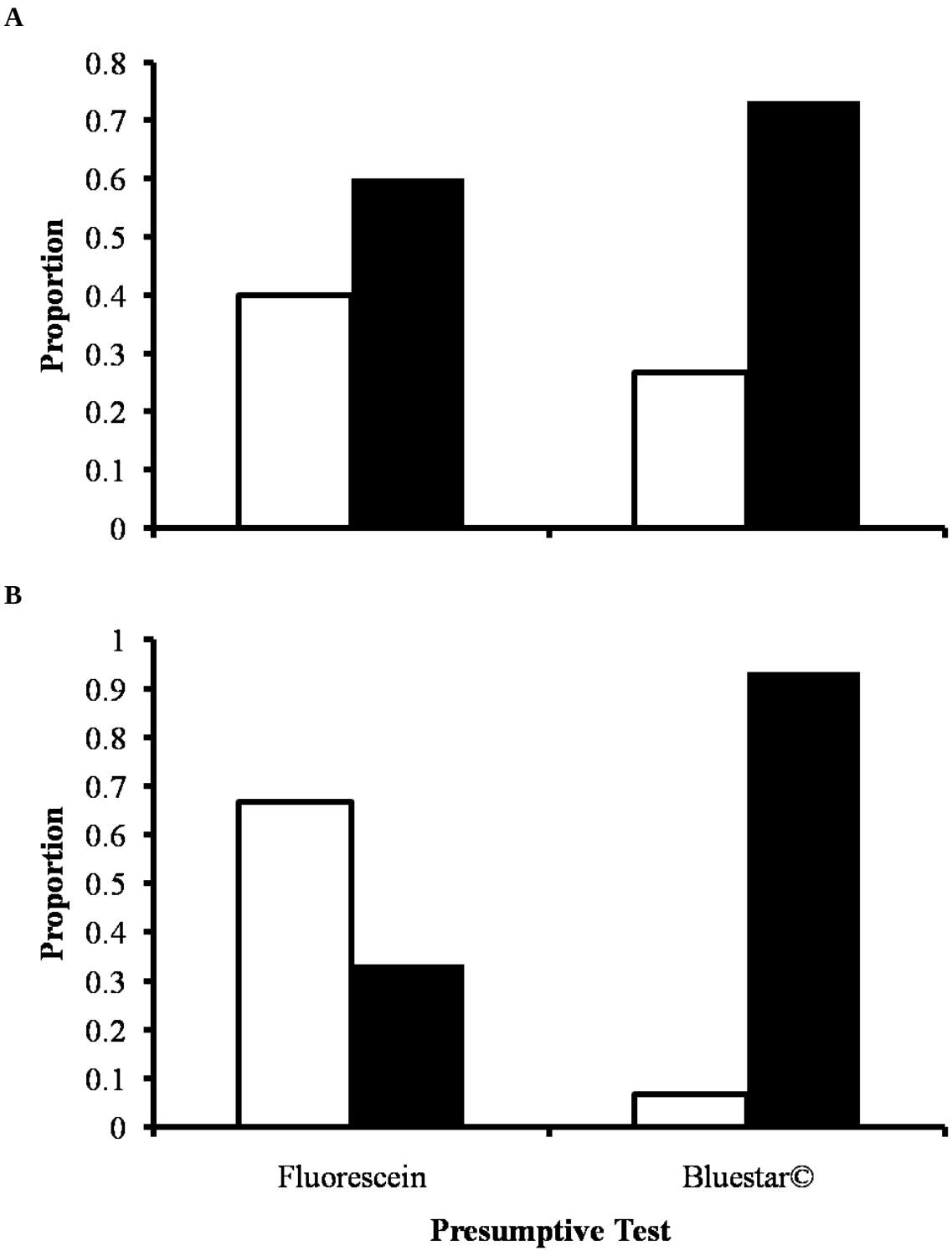


Figure 3-5. The proportion of true positives (white bars) and false negatives (black bars) recorded for testing the presence of puncture (A) and cut (B) wounds using fluorescein (n=15) and Bluestar© (n=15).

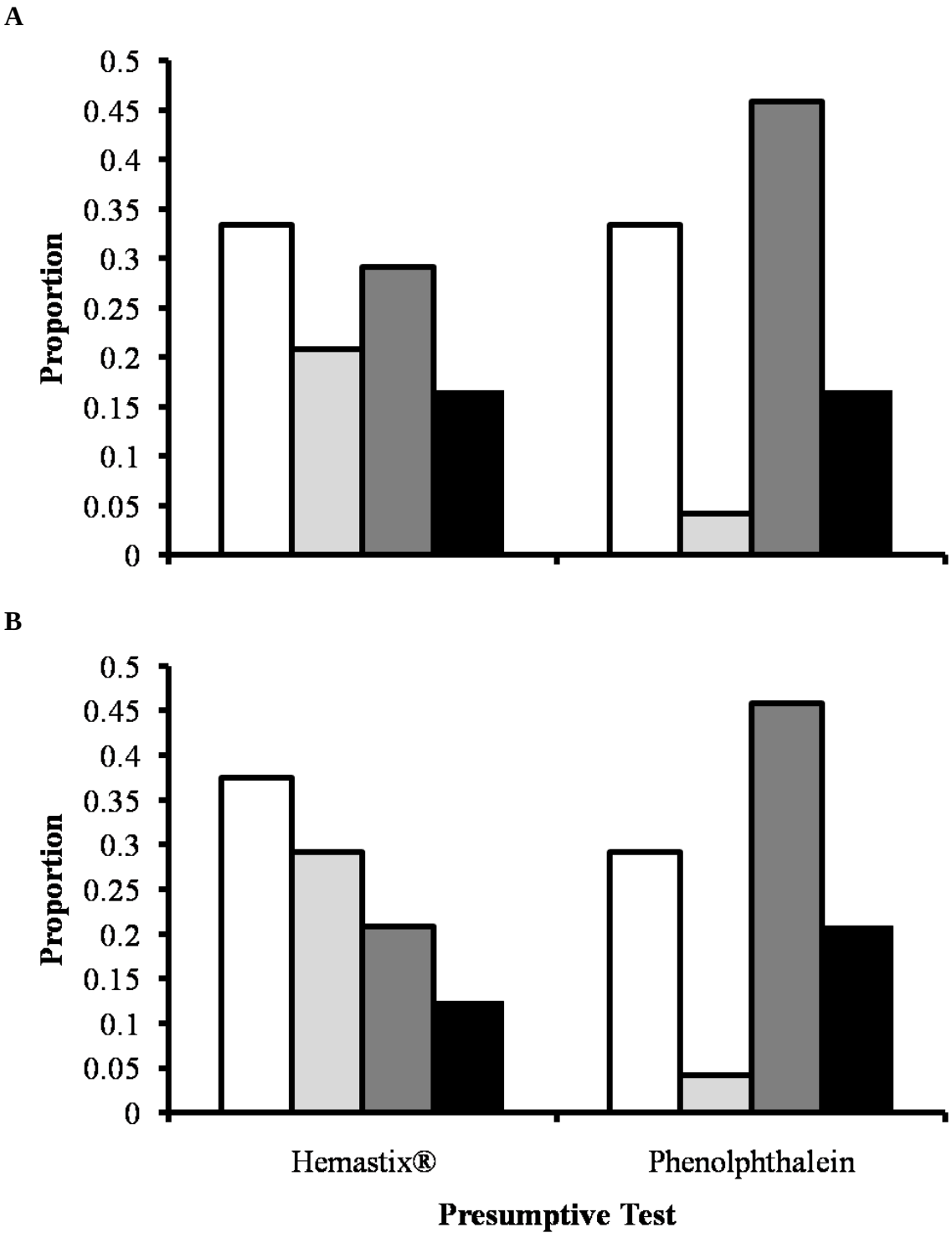


Figure 3-6. The proportion of true positives (white bars), false positives (light grey bars), true negatives (dark grey bars) and false negatives (black bars) recorded for testing for presence of puncture (A) and cut (B) wounds using Hemastix® (n=24) and phenolphthalein (n=24).

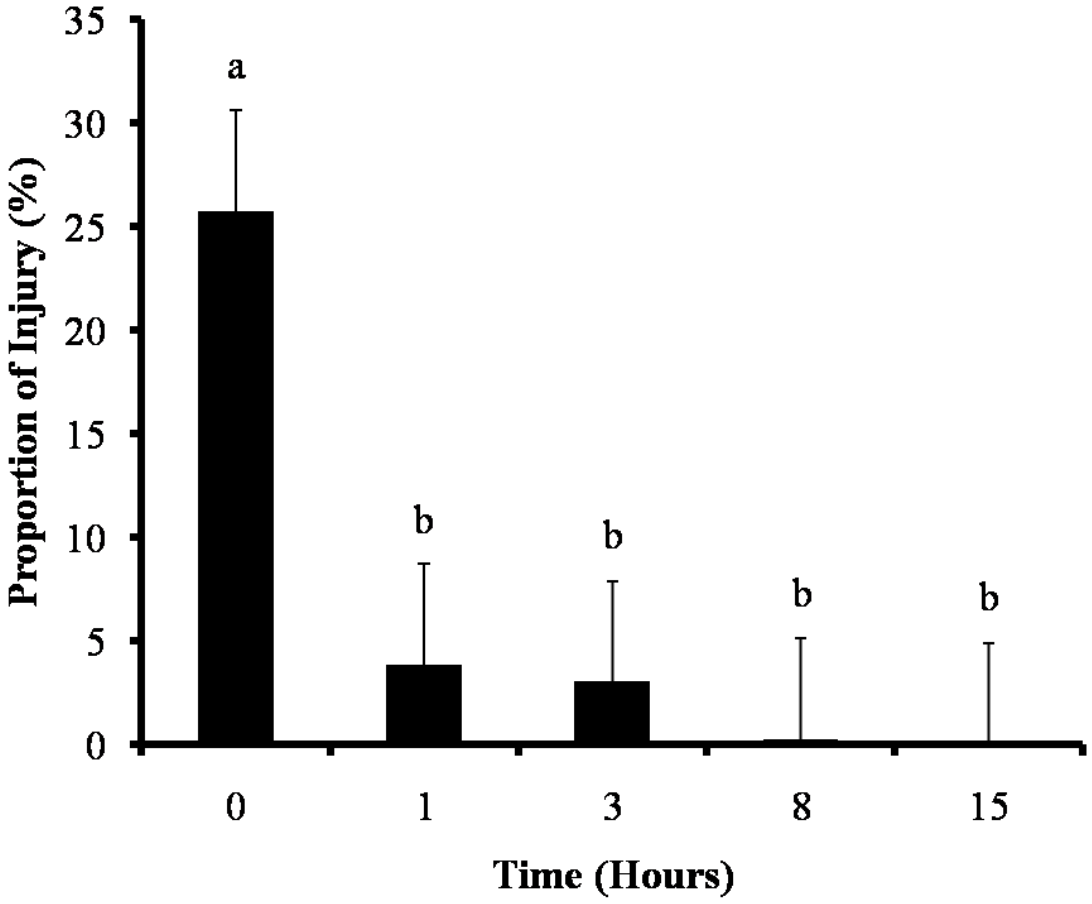


Figure 3-7. The proportion of fluorescein detectable after treatment (±S.E.), showing the latency of the detection capability of fluorescein. (N=10 for each time interval). Dissimilar letters indicate significant differences ($P < 0.05$) in the proportion of fluorescein detected.

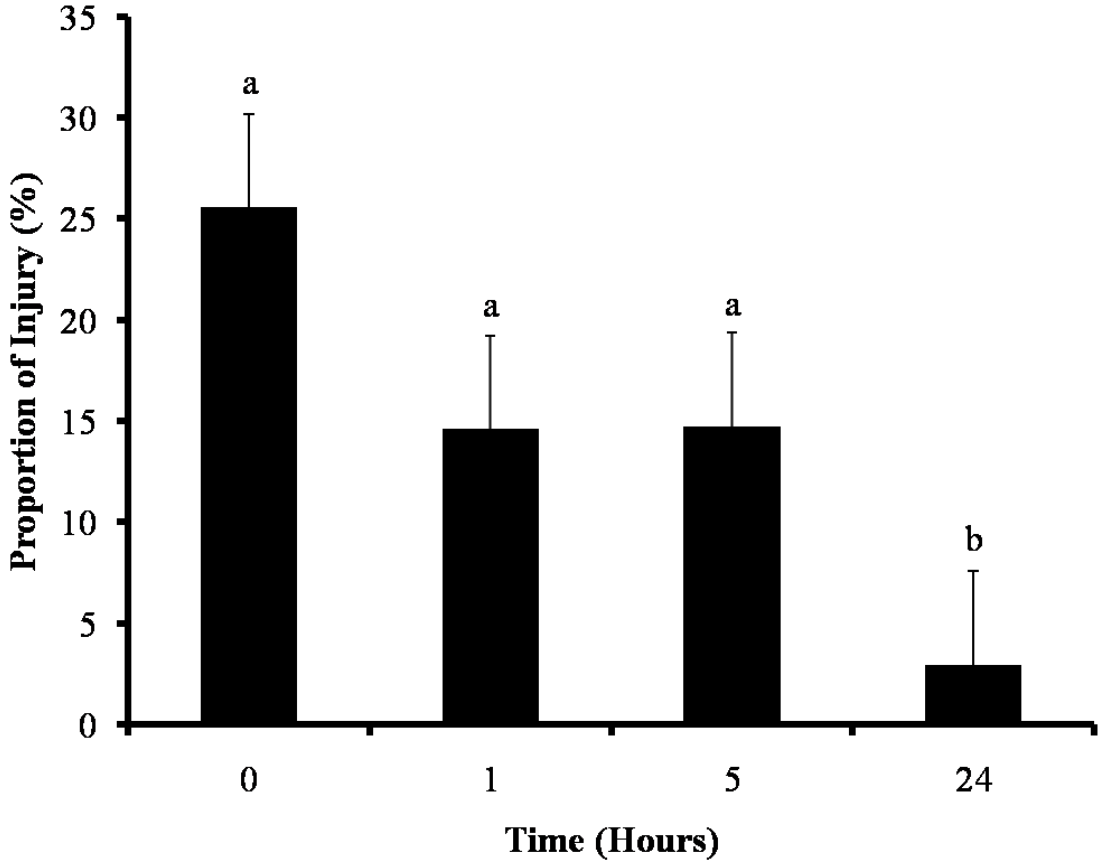


Figure 3-8. The proportion of injury detectable at various times after injury occurred (±S.E.), showing the temporal patterns of injury detection. (N=10 for each time interval). Dissimilar letters indicate significant differences ($P < 0.05$) in the proportion of fluorescein detected.

CHAPTER 4: Evaluation of common sources of physical injury to popular sport fish during recreational angling events using forensic injury detection tools

ABSTRACT

Angling is a popular recreational activity across the globe and a large proportion of fish captured by anglers are returned to their environment due to voluntary or mandatory catch-and-release practices because they are undersized or non-target/non-desirable species. The handling associated with hook removal and return of the fish to their environment can cause physical damage to the epidermal layer of the fish which may lead to infection (e.g., *Saprolegnia* spp.) and which compromises the health, condition, and potentially to the survival of released fish. This study investigated possible sources of injury associated with several different handling methods (i.e. landing net types, interactions with different boat floor surfaces, tournament procedures) used commonly in recreational angling for two popular freshwater sport fish species, largemouth bass (*Micropterus salmoides*) and northern pike (*Esox lucius*). Injury was examined by using fluorescein, a non-toxic dye, which has been shown to detect latent epithelial damage. Northern pike exhibited extensive injury after exposure to several of the induced treatments (i.e. interaction with a carpeted surface, knotted nylon net, and line rolling) but relatively little injury when exposed to knotless nets, smooth boat surfaces, or lip gripping devices. Largemouth bass did not show significant injury for any of the treatments, with the exception of fish caught in a semi-professional live release tournament. The detection of latent injuries using fluorescein can be an important management tool as it provides visual examples of potential damage that can be caused by different handling methods. Such visualizations can be used to encourage fish-friendly angler behaviour and enhance the survival and welfare of released fish. It can

also be used to test new products that are intended to or claim to reduce injury to caught and released fish.

INTRODUCTION

Recreational angling is a popular leisure activity world wide, with an estimated 47 billion fish caught annually (Cooke & Cowx 2004; Arlinghaus et al. 2007). The majority of these fish caught, approximately two-thirds, are released because they are non-desired/non-target species through voluntary angler actions or harvest regulations (Cooke & Cowx 2004). Whenever these fish interact with angling gear there is always some level of injury (Cooke & Sneddon 2006). At a minimum, there is the physical injury caused by the hook puncture(s). However, injury can also arise from other components of the angling event including the fight (e.g., line rolling), landing (e.g., net damage), and handling (e.g., dropping, holding). In general, most of the existing literature on injury arising from catch-and-release events is focused on the relationships between injury and mortality associated with different hook types (Meka 2004; Cooke et al. 2003), sizes (Cooke et al. 2005) and hooking locations (Pelzman 1978; Lyle et al. 2007; Fobert et al. 2009). Few studies have investigated injury associated with use of other angling gear such as landing nets (Barthel et al. 2003) and mechanical gripping devices (Danylchuk et al. 2008), or different handling practices.

Injuries resulting from recreational angling events, such as those caused by landing nets, may not be visually recognizable as they damage the epithelial layer which covers the entire surface of the fish as a protector against pathogens, UV light and dessication (Shephard 1994). Although these types of injuries do not tend to result in immediate mortality, they may put fish at risk of infection from a variety of different

opportunistic pathogens (Ventura & Grizzle 1987; Svendsen & Bøgwald 1997; Van West 2006) and these diseased states have the potential to cause sublethal disturbances in physiology, health and behaviour (Cooke & Sneddon 2006) and eventual delayed mortality (Steeger et al. 1994; Svendsen & Bøgwald 1997; Howe & Stehly 1998; Davis 2005). Fluorescein, a non-toxic dye, has been shown to identify these latent injuries through a chemical reaction with damaged epithelium (Noga & Udomkusronsi 2002). This technique allows latent injury to be identified and quantified in an objective and quantitative manner, which has not been previously possible (Chapter 2, 3; Colotelo et al., 2009).

This study investigated injury associated with several different handling methods used commonly in recreational angling for two popular freshwater sport fish species, largemouth bass (*Micropterus salmoides*) and northern pike (*Esox lucius*). This analysis used fluorescein, a non-toxic dye, which produces a green fluorescence when it is applied to injured epithelium (Noga & Udomkusronsi 2002). Because this forensic technique visualizes the injury, we discuss the potential utility of the tool for educating anglers about different handling practices.

METHODS

All fish were angled from Lake Opinicon, located in southeastern Ontario, Canada, via standard angling practices. All work took place in late June and early July at water temperatures of 23-26^{o}C. To avoid inflicting non-experimental injury, largemouth bass (*Micropterus salmoides*) were landed by firmly grasping the lower lip with the thumb and forefinger unless otherwise stated. Northern pike (*Esox lucius*) cannot be handled this

way because of their teeth and all were landed using a wetted rubber mesh net. Once each fish was successfully landed, it was randomly assigned to a treatment group, except for fish caught in the tournament. To avoid any influence on our results, fish with visible signs of injury at time of capture were excluded from the study.

Treatments

Treatments were differentially applied to largemouth bass and northern pike as documented in Table 4-1. Descriptions of the treatments are as follows:

A) Rubber Landing Net

Fish were held in a rubber landing net for removal from the water and hook removal, which lasted approximately 30 seconds.

B) Knotted Nylon Landing Net

Fish were held in a knotted nylon landing net for removal from the water and hook removal, which lasted approximately 30 seconds.

C) Wet Weigh-In

Individual fish were placed in a weigh-in plastic container (a plastic laundry basket which permitted the rapid flow of water in and out of the container) which was immersed in and removed from water a series of 3 times, simulating the weigh-in process at angling tournaments . For each cycle, the container was dipped into the water for 10 seconds and then removed from the water for 30 seconds. Following this, the container was immersed for 30 seconds to simulate the weight of the fish being recorded.

D) Dry Weigh-In

Individual fish were placed in a weigh-in plastic container (a plastic laundry basket which permitted the rapid flow of water in an out of the container) which was immersed in and

removed from water a series of 3 times, simulating the weigh-in process at angling tournaments . For each cycle, the container was dipped into the water for 10 seconds and then removed from the water for 30 seconds. Following this, the container was removed from the water for 30 seconds to simulate the weight of the fish being recorded.

E) Real Tournament

Largemouth bass were angled and weighed in a tournament on Big Rideau Lake, located in southeastern Ontario, Canada, as part of a semi-professional live-release bass fishing tournament. We had no control of how fish were handled in this portion of the study. The injury examined in this treatment was likely the combined result of angling, handling, livewell confinement and the weigh-in procedure. Fish caught in this tournament are confined to a livewell with up to 4 other fish for up to six hours. The weigh-in procedure was a wet weigh-in format and all 5 fish were weighed together.

F) Holding by Gills for Hook Removal

For the removal of the hook, each fish was held under the operculum, causing the gills of the fish to be in contact with the angler's hands. This is a common way of holding fish such as northern pike which can be difficult to control. Following the fluorescein treatment fish were euthanized using an overdose of 120ppm clove oil anaesthetic (clove oil emulsified in ethanol (1:9); Sigma Aldrich, Toronto, ON) and the opercula were removed.

G) Line Rolling

Angled fish had the hook removed, and were then wrapped in a 28cm steel leader to simulate line rolling, which can occur when angling northern pike. The fish was submerged in water with the leader taught around them for 30 seconds.

H) Interaction with Carpeted Surface

After fish were angled, they were placed in a plastic container which was lined with artificial outdoor carpeting, similar to that found in some fishing boats. Fish were able to move freely on this surface for 30 seconds.

I) Interaction with Boat Surface

Fish were able to move freely on the smooth metal surface of the floor of a boat for 30 seconds.

J) Gripping Device

For hook removal and handling of the fish, a mechanical device which grips the lower lip of a fish was used (see Danylchuk et al. 2008). These tend to be used for fish which are difficult to handle because of size or dentition.

A group of control fish were also examined for both species. Largemouth bass controls were removed from the water by grabbing the lower lip and immediately placed in the anaesthetic solution for injury detection. Northern pike controls were angled using standard angling gear and were landed using a rubber landing net. Upon capture, they were immediately placed in the anaesthetic solution for injury detection (see below).

A separate group of controls were also used to compare to those northern pike which were held by the gills for hook removal. These fish were held behind the base of the head for hook removal. Following the fluorescein treatment, the fish were euthanized by an overdose of 120ppm clove oil anaesthetic (clove oil emulsified in ethanol (1:9); Sigma Aldrich, Toronto, ON) and the opercula were removed prior to injury detection (see below).

Injury Detection

Following treatment all fish were placed in a 50ppm clove oil anaesthetic (clove oil emulsified in ethanol, 1:9; Sigma Aldrich, Toronto, ON) and remained there until fish reached stage 4 of anaesthesia noted by a loss of equilibrium and coordinated fin movements (Summerfelt & Smith 1990) and were then treated with fluorescein. Fish were submerged in a 0.2mg/mL solution of fluorescein (Fluorescein, Disodium Salt; Aldon Corp., Avon, NY) in distilled water for 6 minutes and were then placed in an anaesthetic bath containing 50ppm clove oil (clove oil emulsified in ethanol at 1:9) for 6 minutes to rinse and keep fish anaesthetized (Noga & Udomkusonsri, 2002). Fish were photographed in complete darkness, against a black background, using a digital SLR ELIXIM Pro EX-F1 camera (Casio Computer Co., Ltd., Tokyo, Japan) at ISO 100, F6.7, and a 20 second exposure. The camera was positioned 80cm directly above the fish and the shortwave (254nm) UV light source (Mineralight® UVGL-48; UVP Inc., Upland, CA) at a 45^{o} angle to the fish, 60cm above, so that the entire organism was illuminated by the UV light. Following treatment fish were placed in a container of fresh lake water until equilibrium was regained, and fish were released.

Fluorescein causes a positive reaction through the production of green light. Photographs were analyzed, using ImageJ software (http://rsb.info.nih.gov/ij/; National Institute of Health, Bethesda, MD), by tracing the areas of green and measuring the number of green pixels. This process was done twice and the average number of green pixels was calculated and used for statistical analysis. All analyses were conducted by the same individual. The proportion of injury on the entire body of the fish was then

calculated by dividing the number of green pixels by the total number of pixels of the fish.

Statistical Analysis

The proportion of the body that was injured was compared for all treatments using one-way ANOVAs and were followed by a Tukey's *post hoc* test when necessary. Data transformations were conducted as needed to meet the assumptions of normality and homogeneity of variance required for parametric tests. SPSS software was used for all statistical tests and significance was assessed at $\alpha = 0.05$ (Zar 1984).

RESULTS

Landing Net Material

Landing net material interactions elicited significant injury relative to control fish for northern pike (Figure 4-1; One-way ANOVA, $F_{1,21}=28.353$, $p<0.001$); however, there was no significant difference in injury among control fish and different net materials for largemouth bass (Mean ± S.E., Control 0.86 ± 0.74 %, Rubber Landing Net 0.17 ± 0.08 %, Knotted Nylon Landing Net 0.77 ± 0.33 %; One-way ANOVA, $F_{2,25}=0.387$, $p=0.683$). When comparing the proportion of injury detected for the injury caused by the knotted nylon net on northern pike and largemouth bass, northern pike exhibited significantly higher levels of detectable injury (Figure 4-2; One-way ANOVA, $F_{1,17}=57.764$, $p<0.001$).

Tournament and Weigh-In Practices

Largemouth bass exposed to simulated weigh in practices did not exhibit injury relative to controls; however, fish sampled from a semi-professional tournament experienced injury that was higher than both controls and the individual components of the

tournament (i.e. landing net interactions, handling, confinement in livewell, weigh-in) (Figure 4-3; One-way ANOVA; $F_{3,33}$=2.953, p=0.047; with Tukey's HSD *post hoc*). The overall impact of a largemouth bass being exposed to a live release tournament elicited higher levels of injury than we detected from any other treatments on this species.

Holding By the Gills for Hook Removal

There was a significant difference in the level of injury detected when comparing northern pike held by the gills for hook removal and those which were treated as controls. Control fish had a significantly higher level of injury than those held by the gills (Figure 4-4; One-way ANOVA, $F_{1,14}$=5.989, p=0.028).

Line Rolling

Northern pike which were exposed to line rolling showed a significantly higher proportion of injury than those fish which were not exposed to line rolling (Figure 4-5; One-way ANOVA, $F_{1,17}$=4.696, p=0.045). The majority of the injury was in the vicinity of the pelvic and pectoral fins.

Interaction with Boat Floor Surfaces

Northern pike were placed on either a smooth metal boat floor or a carpeted surface, and the carpeted surface treated fish showed a significantly higher proportion of injury than the smooth metal boat floor treated fish as well as the controls which did not interact with a boat surface (Figure 4-6; One-way ANOVA, $F_{2,30}$=12.301, p<0.001). There was no significant difference in injury levels between largemouth bass that interacted with a carpeted surface (Mean ± S.E., 2.4 ± 1.8 %) and those which did not interact with any surface (Mean ± S.E., 0.9 ± 0.7 %) (One-way ANOVA; $F_{1,25}$=0.435, p=0.516). There was a significant difference in the proportion of injury detected on the two species when

treated with the same potential injury source, with northern pike demonstrating a higher proportion of injury than largemouth bass (Figure 4-7; One-way ANOVA, $F_{1,25}$=31.009, $p<0.001$).

Mechanical Gripping Device

Northern pike that were handled with a mechanical gripping device for hook removal (Mean ± S.E., 0.0276 ± 0.101) did not show a significantly higher proportion of injury on the lower jaw than those handled as controls (Mean ± S.E., 2.6 ± 10.7) (One-way ANOVA, $F_{1,19}$=0.010, p=0.922).

DISCUSSION

Landing and keep net material, and its potential source of injury for retained fish, has prompted studies that qualitatively investigate this injury (Cooke & Hogle 2000; Barthel et al. 2003; De Lestang et al. 2008). While these studies noted visual differences in the injury (i.e., fin fraying and scale loss) resulting from interaction with netting materials, there have been no attempts to rigorously and objectively quantify the level of injury. Fluorescein serves as a tool that enables the quantification of injury. The results of current study revealed that higher levels of injury were detected on northern pike which were landed using a knotted nylon net when compared with a rubber net (Figure 4-1), although there was no significant latent damage detected under the same comparison with largemouth bass. Using a more subjective approach, Barthel et al. (2003) used bluegill (*Lepomis macrochirus*) as a model and revealed that knotted netting materials elicited higher levels of injury (i.e., fin fraying and scale loss) and subsequent mortality than rubber nets. Interestingly, largemouth bass and northern pike differed in their response to nets indicating that not all species may be equally sensitive to dermal

disturbance. In general, the netting assessments are consistent with the growing body of evidence that desirable netting materials are soft and non-abrasive and should only be used when it is necessary to control the fish to prevent injury to the fish or the angler.

Largemouth bass are commonly targeted in live release tournaments (Schramm et al. 1991). These fish are captured via hook, typically landed using nets, held in a live-well for up to eight hours, and weighed in with multiple individuals before they are released. Tournaments expose fish to a range of stressors (Suski et al. 2004) and mortality rates arising from such events can be high (Wilde 1997; Cooke et al. 2002). To date, however, there have been few studies that have quantified injury arising from the entire tournament or the various components of the tournament event. This study revealed that the injury present at the end of the tournament was cumulative and represented a combination of injuries experienced throughout the event (Figure 4-3). Although not statistically significant, there is some evidence that the wet weigh-in (0.1% dermal injury) may reduce injury relative to the conventional dry weigh-in (0.4% dermal injury). Wet weigh-in procedures were developed as a means of reducing stress associated with the air exposure during the weigh-in (see Tufts & Morlock 2004). Data from parallel physiological studies had revealed that as long as fish are provided with adequate water quality while being retained in the livewell, it tends to be the weigh-in at the end of the day that determines the physiological status of fish at time of release (Suski et al. 2004). Conversely, from an injury perspective, fish are not able to "recover" from injuries experienced in early components of the tournament such that the condition of the fish at time of release reflects all components of the handling event. Indeed, the only injury that was statistically significant from control levels was the tournament as a whole rather than

its components. Disease issues including direct transmission have already been identified as important in bass tournaments (e.g., Steeger et al. 1994; Schramm et al. 2004; Grant et al. 2005). Steeger et al. (1994) observed pathogens in 55% of tournament caught largemouth bass. Schramm et al. (2004) reported that there was increased occurrence of largemouth bass virus (LMBV) in fish that were caught in tournaments and held when compared to fish captured via electrofishing. This increased rate of infection was linked to transmission of the disease from infected to non-infected fish while being held in livewells during the tournament (Schramm et al. 2004). It is also suspected that immunosuppression increases expression of LMBV due to elevated stress levels as a result of angling, handling and confinement of the fish. Regardless of the proportion of injury detected, any efforts to reduce injury, even if quite minor, could be of immense benefit to tournament caught fish (Siepker et al. 2007).

Holding fish by the gills for hook removal and photographs is a common handling practice for large fish, or those difficult to hold on to such as northern pike. There is suggestion that this handling method is detrimental to the fish as the gills provide a thin barrier to the blood stream where gases are exchanged and damage to this tissue could result in inability of efficient transfer of these gases (Hughes 1984). As this tissue is very thin, it also suggests that this area would be more susceptible to injury. Interestingly, use of fluorescein failed to document significant injury arising from this handling method. Indeed, the control fish had significantly higher levels of injuries to the gills when compared with fish held by the gills for hook removal (Figure 4-4). It is unclear whether the gills are simply not injured from this handling method or if fluorescein is ineffective at documenting injury on the gills. Given that the gills are an active surface where gases

and ions are transferred and where water is passed even while anaesthetized, it is not unreasonable to suggest that fluorescein may be cleared more rapidly on gill tissue than on the epithelium on skin of the fish.

While angling for large fish such as northern pike, steel leaders are commonly used to prevent the line from being cut by their dentition. When these predatory fish strike, they may roll their bodies in an attempt to free themselves and they may become tangled in the line. Fish are often caught with visible scars indicating the fish has previously been tangled in a line. An important feature of the leader is that it can reduce break-offs where lures are left in the mouth of fish which creates welfare impacts on fish (Arlinghaus et al. 2007). Interestingly, the current study found significant damage resulting from the fish being manually wrapped in a coated steel leader taught for 30 seconds (12.0%; Figure 4-5a). It is unclear if different fishing line types or leader materials could be used to reduce injury arising from line wrapping.

Boats used during angling events are designed based on function and aesthetics. A popular flooring material used in boats is all-weather carpeting, which provides traction under wet conditions, and adds protection to the boat frame. When angling, it is a common occurrence for fish to interact with the flooring of the boat (e.g., when dropped or placed on the floor to facilitate hook removal) and the material on that surface may impact the injury caused to the fish before it is released. This study revealed that interacting with a carpeted surface caused the highest proportion of injury of all treatments for both largemouth bass and northern pike (2.4% & 31.4% respectively; Figure 4-6). Conversely, when the fish interacted with a flat metal surface, common in jon boats, there was relatively little injury (9.8% for northern pike; Figure 4-6). These

data emphasize that fish should be handled in a manner that reduces the chances of them being dropped in the boat, regardless of the surface, and that it is important to pick the fish up quickly if dropped, particularly in boats with rough interior surfaces. Ideally fish would be handled over water to minimize injury risk.

Mechanical gripping devices have been introduced as an alternative to landing nets for use in fish which are difficult to hold on to and to enhance fish condition when they are released by limiting scale loss and epithelial damage (Danylchuk et al., 2008). Danylchuk et al (2008) reported extensive damage to the lower jaw of bonefish (*Albula vulpes*) which were handled using the mechanical gripping device. External and internal injuries have also been reported on barramundi *Lates calcarifer* handled with lip gripping devices (Gould 2008 cited in De Lestang et al. 2008). This current study, however, did not detect significant injury on northern pike which were handled using the mechanical gripping device. The physical differences in the mouth of various species may explain the differences seen in the two studies. Bonefish are benthic feeders with down-turned fleshy mouths, and northern pike are toothy predators with large jaws and teeth, with reasonably little fleshy tissue on the jaw. More research is needed to understand the full range of injuries arising from use of these devices as there is evidence of skeletal damage resulting from the use of these devices (Gould 2008 cited in De Lestang et al. 2008). If these devices can prevent dropping the fish and avoid use of nets, there may be benefit of using lip gripping devices if they do not yield significant mouth injuries as observed by Danylchuk et al. (2008).

It is evident that northern pike and largemouth bass do not respond in the same manner to the treatments applied in this study. In both treatments which were consistent

across species, interaction with carpeted surface and knotted nylon net, northern pike had significantly higher proportions of injury than largemouth bass (Figures 4-2 & 4-7). Northern pike exhibited 31.4% and 29.1% injury for the two treatments respectively, while largemouth bass only displayed 2.4% and 0.1% injury. This suggests that there is a difference in the susceptibility to injury between the two species or in the way the fluorescein treatment reacts with their specific blood constituents. Indeed, the extent of epithelial injury could be influenced by fish size, body morphology, their behaviour while interacting with angling gear, and epithelial anatomy. All fish are covered by a protective epithelial layer, but the thickness and composition may differ between species which may affect both their sensitivity to injury but also the efficacy of fluorescein as a tool for the quantification of such injury (Shephard 1994). Disruption to this epithelial layer, as indicated by a positive reaction with fluorescein, creates susceptibility to infection, regardless of quantity of injury detected, however certain species may be affected by the same proportion of injury differently. Although there were differences in the proportion of injury detected in largemouth bass and northern pike, the sublethal and lethal consequences may not differ. It is unknown what the threshold of injury is for each species, which reinforces the need for research to focus on the long term consequences of epithelial injuries. Further research is also needed to identify which species are the best candidates for the fluorescein treatment, as it may not be appropriate for all species. Further research also needs to focus on the survival of fish which have injuries detectable with fluorescein. The threshold of tolerable injury may vary depending on species and this can influence gear choice and handling practices used. Davis and Ottmar (2006) found that walleye pollack (*Theragra chalcogramma*) were the only species, of four

tested, which showed a sigmoid curve relating injury detected and delayed mortality rates, suggesting an increased susceptibility to injury.

Management Implications and Future Considerations

Fluorescein is capable of quantitatively and objectively differentiating between levels of injury arising from different gear types which provides direction to managers and anglers for reducing the impacts of fishing on individuals that are released. Photographs of fish handled using different techniques can be used to encourage fish-friendly handling practices for anglers (Figure 4-2b & c; Figure 4-7b & c). Such an approach was used by government researchers in New Zealand to illustrate handling injuries in rainbow trout (*Oncorhynchus mykiss*) and was published in a magazine that was accessible to stakeholders (Dedual & Shorland 2006). Management agencies and even anglers (with appropriate training and permits) could conduct their own visual assessment of the injury caused by different handling practices and this will encourage conservation oriented practices. Also, as new conservation-oriented products are introduced, they can be tested with fluorescein to validate the improvements over current handling practices. Collectively, this study reveals that fluorescein is an effective tool for quantifying epithelial injury in fish. Moreover, common activities and gears employed by anglers yield different levels of injury that could be reduced or eliminated through education and/or innovations in gear design. Such research is consistent with the need to maintain the welfare status of fish (Cooke and Sneddon 2006) and has the potential to reduce mortality among fish that are angled and released. In addition, the use of fluorescein to investigate potential sources of injury is also highly relevant among other situations in

which fish are handled (i.e., aquaculture, ornamental trade, research, zoos and aquaria) and discarded (i.e. commercial fisheries, research) (Colotelo et al. 2009).

TABLES

Table 4-1. The treatments that were applied to largemouth bass and northern pike.

Treatment	***Largemouth Bass***	***Northern Pike***
Rubber Landing Net	✓	✓
Nylon Netted Landing Net	✓	✓
Dry Weigh In	✓	
Wet Weigh In	✓	
Real Tournament	✓	
Holding by Gills for Hook Removal		✓
Line Rolling		✓
Interaction with Carpeted Surface	✓	✓
Interaction with Metal Boat Surface		✓
Gripping Device		✓

FIGURES

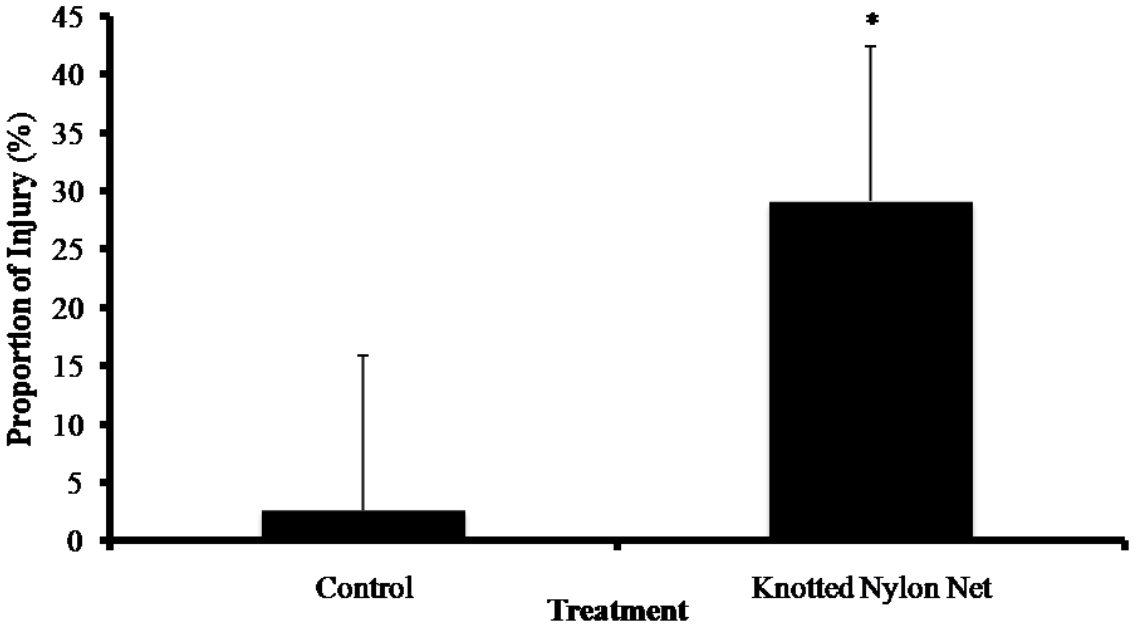
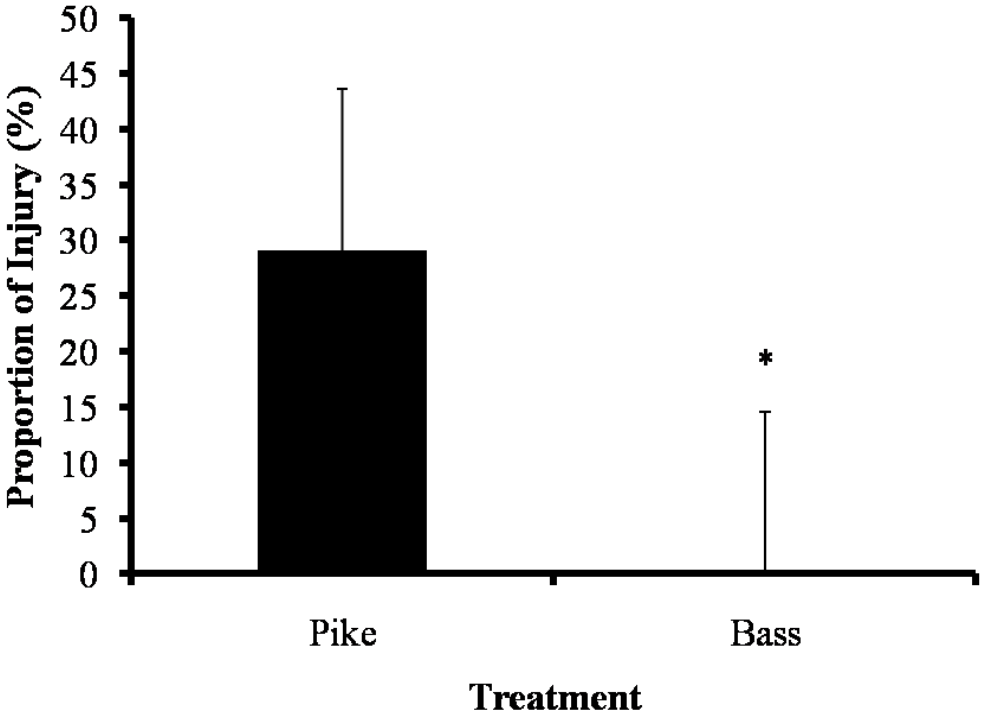
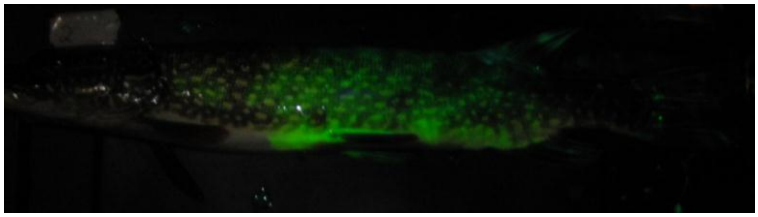


Figure 4-1. The proportion of injury (±S.E.) detected on northern pike treated with landing nets made of rubber (Controls; n=12) or knotted nylon (n=11).

A



B



C

Figure 4-2.

(A) The proportion of injury (±S.E.) detected on northern pike (n=11) and largemouth bass (n=9) when treated with a knotted nylon landing net.
(B) Photograph of northern pike handled with a knotted nylon landing net for 30 seconds and treated with fluorescein.
(C) Photograph of largemouth bass handled with a knotted nylon landing net for 30 seconds and treated with fluorescein.

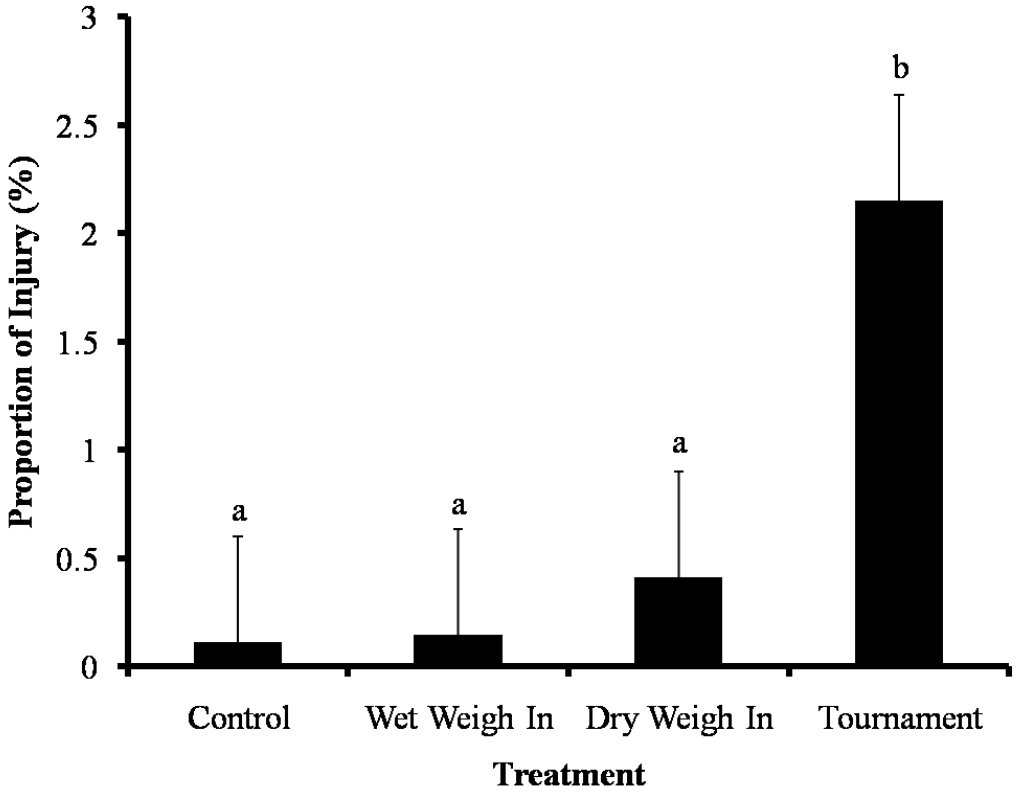


Figure 4-3. The proportion of injury (±S.E.) detected on largemouth bass when treated by wet (n=8) and dry (n=8) weigh in procedures as well as overall tournament effects (n=11). (N=10 for control fish)

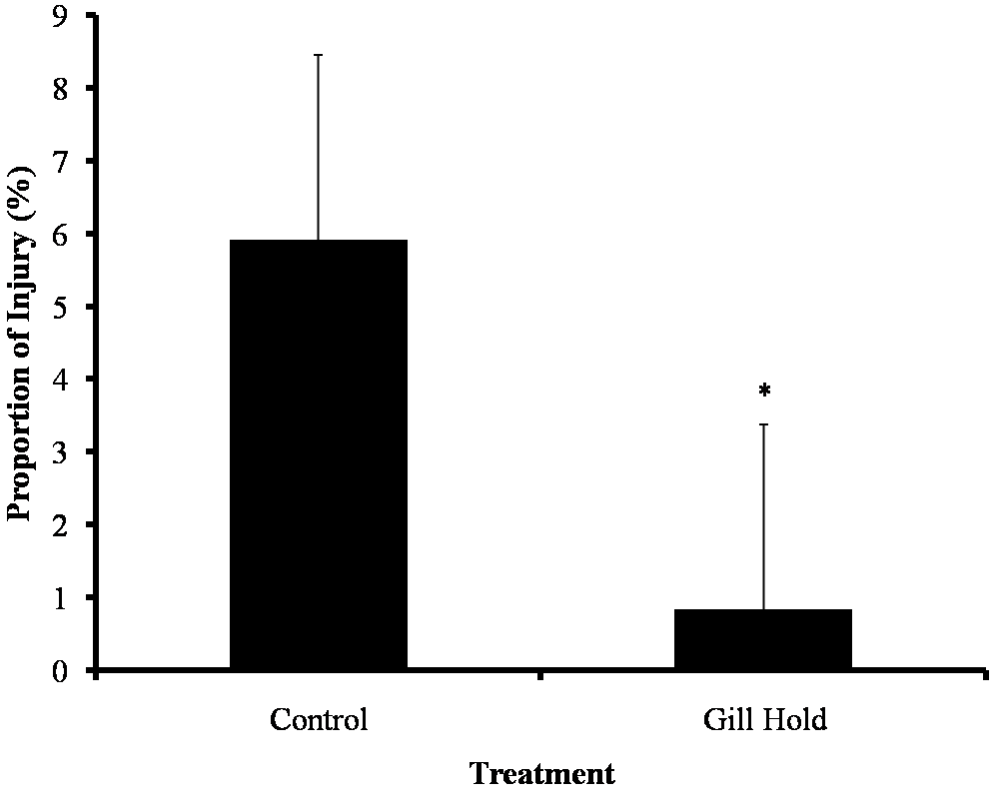


Figure 4-4. The proportion of injury (±S.E.) detected on northern pike which were held by the gills (n=8) and treated as controls (n=8).

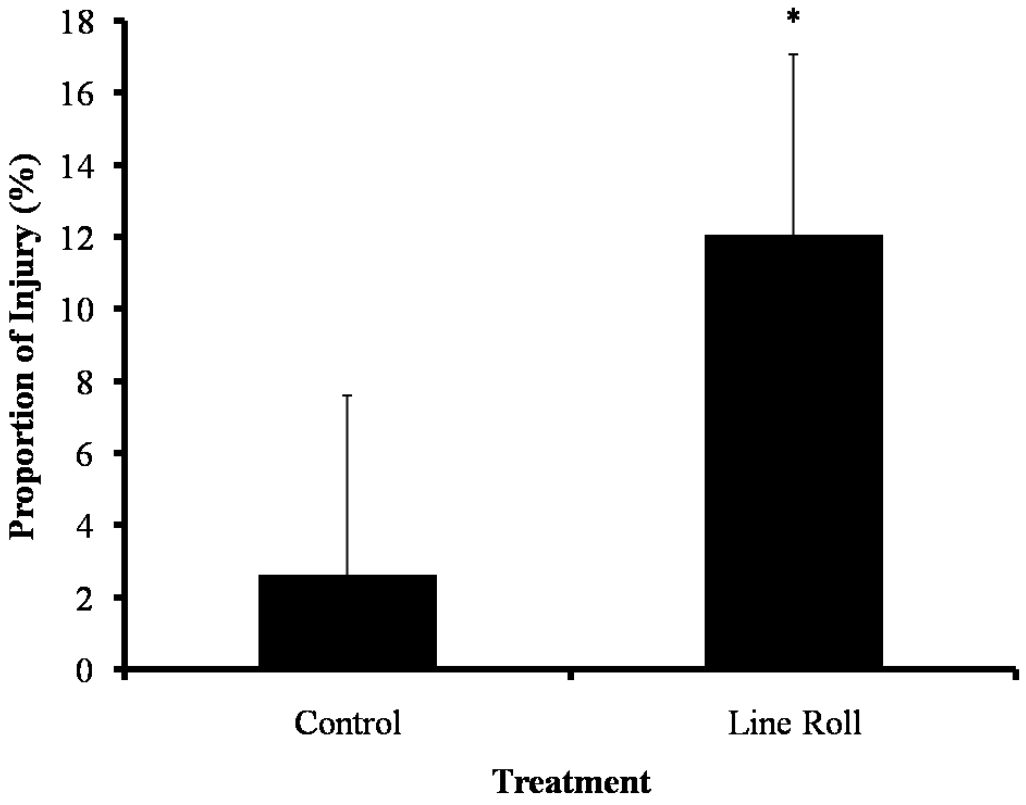


Figure 4-5. The proportion of injury (±S.E.) detected on northern pike wrapped in a steel leader (n=7) and treated as controls (n=12).

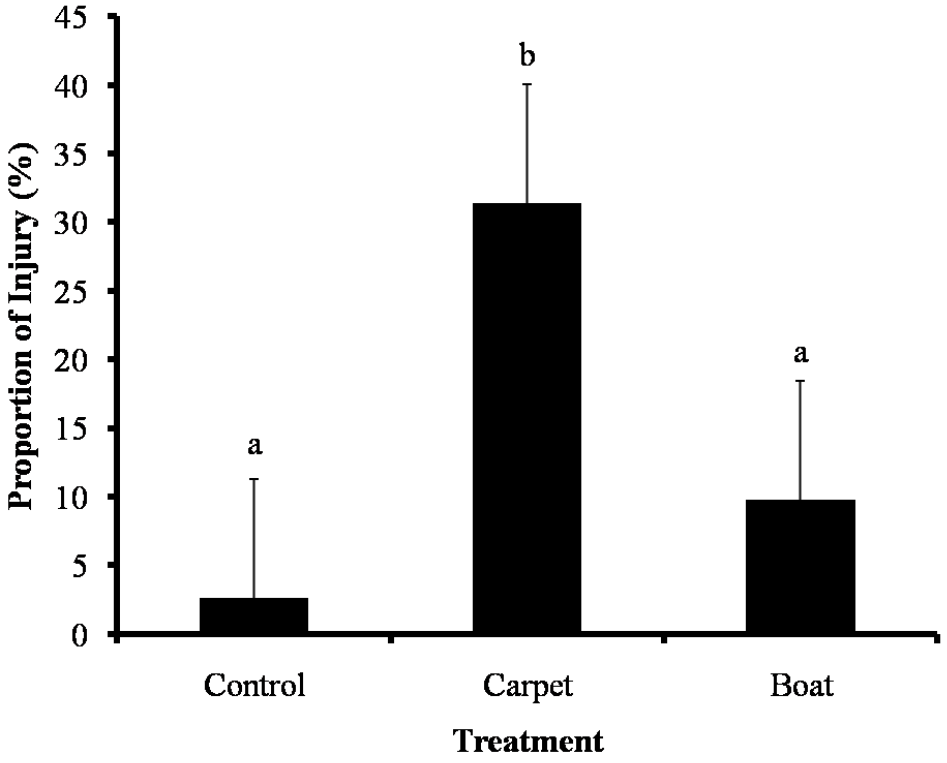
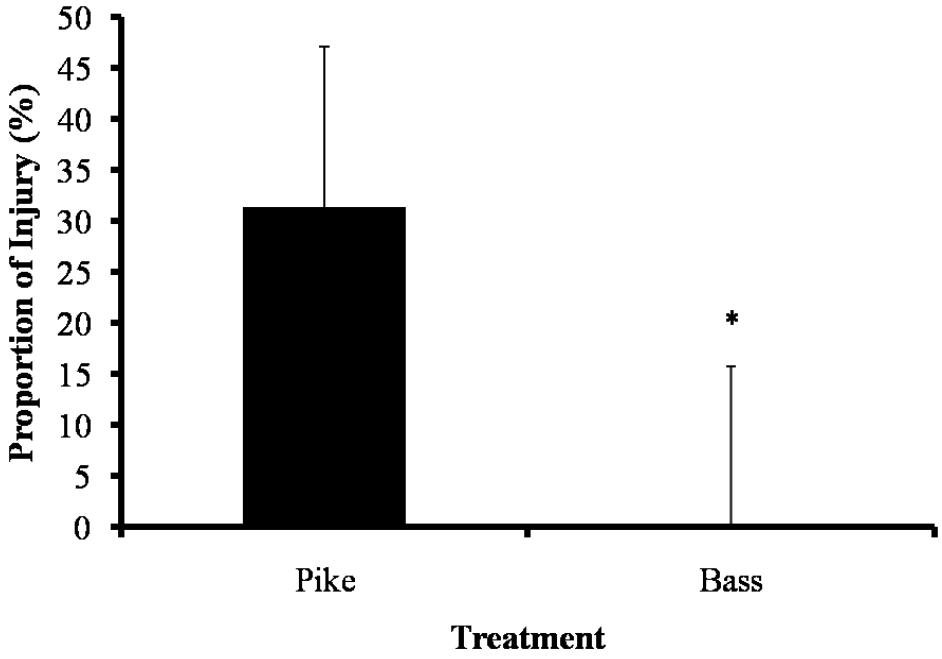
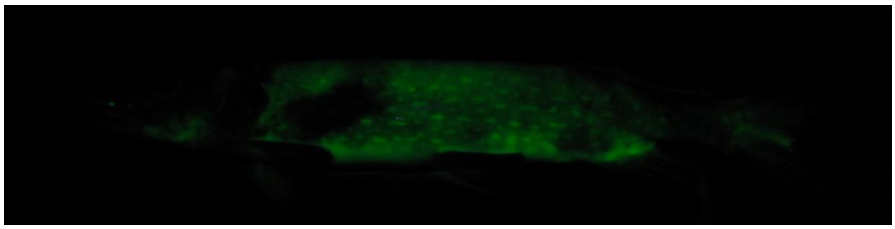


Figure 4-6. The proportion of injury (±S.E.) detected on northern pike which interacted with a carpeted surface (n=11) and smooth metal boat floor (n=10). (N=12 for control fish). Dissimilar letters indicate significant differences ($P < 0.05$) in the proportion of fluorescein detected.

A



B



C

Figure 4-7.

(A) The proportion of injury (±S.E.) detected on northern pike (n=11) and largemouth bass (n=17) that interacted with a carpeted surface.
(B) Photograph of northern pike placed on a carpeted surface for 30 seconds and treated with fluorescein.
(C) Photograph of largemouth bass placed on a carpeted surface for 30 seconds and treated with fluorescein.

CHAPTER 5: General Discussion

Fish can acquire injury from interactions with a variety of anthropogenic and natural structures as well as other animals including conspecifics and humans. These injuries are generally observed through gross macroscopic examination. However, recently it has been determined that fluorescein, a presumptive test for blood commonly used in crime scene analysis, can be used to quantify and detect latent injuries to the epithelium. The purpose of this thesis was to identify a presumptive test for blood that is ideal for detection of fish epithelial injury and to apply it to identify injuries related to gear and handling methods commonly used in recreational angling.

FINDINGS AND IMPLICATIONS

In chapter 2, this thesis reviewed the literature with respect to the detection of fish epithelial injury and how presumptive tests for blood have been previously used in this area. It also evaluated ten presumptive tests for blood for their applicability and usefulness in this field. The tests examined were compared based on cost, carcinogenity, ease of use in the laboratory and field, as well as sensitivity and specificity. It was concluded that fluorescein, Bluestar©, Hemastix®, Hemident, and phenolphthalein showed promise for this task and should be evaluated further in a comparative study.

Chapter 3 outlined a field study which based on Chapter 2 compared 4 presumptive tests for blood (fluorescein, Bluestar©, Hemastix®, phenolphthalein) in their ability to detect 4 commonly encountered types of injury (abrasion, fin fray, punctures, cuts). It was found that fluorescein was the most reliable test, with the highest proportion of true positive reactions and rarely detected false positive reactions. Based on these results fluorescein was further investigated on its capabilities. It was found that at 1

hour after treatment, the proportion of fluorescein detected was significantly less than when initially applied. Injuries can no longer be detected with fluorescein with the same reliability 24 hours after they occur. These results encourage the use of fluorescein as a method for detecting and quantifying epithelial damage in a variety of fish conservation and management applications.

Chapter 4 used fluorescein to compare common handling methods and gear used in recreational angling events targeting largemouth bass and northern pike. Fish were angled and injuries were inflicted through experimental exposure to a variety of different treatments including interaction with different boat surfaces, comparison of landing net mesh material, and examination of different tournament weigh in procedures. It was concluded that carpeted surfaces and knotted nylon landing nets caused the highest proportion of injury for northern pike, while largemouth bass were most sensitive to the overall tournament effects and interaction with a carpeted surface. The results of this chapter can be used to encourage handling methods which minimize the injury caused to released fish.

In general, fluorescein is a quantitative method of detecting latent injury to fish epithelium. Its ease of use and low cost make it ideal for field studies, and the computer analysis creates an objective comparison of different treatments. Although this study focused on the recreational angling applications, it can be used in a variety of different contexts including general health assessments, evaluation of commercial fishing and hydropower practices, and to improve husbandry in aquaculture and research settings. The positive chemical reaction produces a green light, and when photographed it can be readily used as educational material for anglers, commercial fishers and researchers to

demonstrate the potential injury that can be caused through use of different gear types and handling methods. Collectively, findings generated from studies using fluorescein have the potential to improve fish welfare and promote the sustainable use and management of fisheries resources.

FUTURE RESEARCH

Although there are numerous potential uses for fluorescein in fisheries conservation and management research, there is still further investigation required. Although injuries to the epithelium may result in infections that may alter the fish's behaviour and/or physiology and can result in mortality, no studies to date have outlined what proportion of injury is detrimental to the long term impact to the fish. Telemetry or laboratory experiments may be able to aid in the understanding of the long term impacts of injuries resulting from recreational angling. Indeed, understanding the relationship between injury and mortality is of fundamental importance to fisheries management and conservation.

The degree of injury detectable by fluorescein is also an important research topic. This thesis focused on superficial damage to the epithelium, and did not penetrate the flesh. Future studies should investigate varying depths of injury, to examine the sensitivity of fluorescein to epithelial damage.

As in forensic science, the specificity of presumptive tests for blood is important as there may be interfering compounds which can cause false positive or false negative reactions. Little is known about possible interfering compounds in aquatic settings which emphasizes the need to investigate this further. Knowledge of interfering compounds will lead to greater capability of interpretation of the results when using fluorescein for fish injury detection.

SUMMARY

1. Fluorescein is the most applicable presumptive test for blood that was compared in this study. Previous studies identified the applicability of fluorescein but failed to compare other chemicals which may have other benefits for fish injury detection. This thesis outlined four other potential presumptive tests for blood that may have been applicable and executed a comparative study to identify that fluorescein was indeed the most reliable.

2. Further investigation into the capabilities of fluorescein-identified limitations and guidelines for use. This thesis has further investigated the capabilities of fluorescein including the latency of detection, temporal patterns in injury detection and the influence of different anaesthetics.

3. Investigation of different handling methods and gear commonly used during recreational angling events on largemouth bass and northern pike. Previous studies have evaluated different handling methods and gear types used in recreational angling but have not done so in a quantitative manner. Fluorescein will enable similar studies, such as this thesis, to evaluate sources of injury in a quantitative and objective manner. This thesis also provides information that can be translated to anglers and fisheries managers on the least harmful handling methods.

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