Articles

Comparative Evaluation of Four Presumptive Tests for Blood to Detect Epithelial Injury on Fish

Alison H. Colotelo,* Karen E. Smokorowski, Tim Haxton, Steven J. Cooke

A.H. Colotelo, S.J. Cooke
Fish Ecology and Conservation Physiology Laboratory, Department of Biology and Institute of Environmental Science, Carleton University, Ottawa, Ontario, Canada K1S 5B6
Present address of A.H. Colotelo: 902 Battelle Boulevard, P.O. Box 999, MSIN K7-70 Richland, Washington 99352

K.E. Smokorowski
Great Lakes Laboratory for Fisheries and Aquatic Sciences, Fisheries and Oceans Canada, 1 Canal Drive, Sault Ste. Marie, Ontario, Canada P6A 6W4

T. Haxton
Aquatic Science Unit, Southern Science and Information Section, Ontario Ministry of Natural Resources, 300 Water Street, 4th Floor S., Peterborough, Ontario, Canada K9L 8M5

Abstract

Current methods of fish epithelial injury detection are limited to gross macroscopic examination that has a subjective bias as well as an inability to reliably quantify the degree of injury. Fluorescein, a presumptive test for blood, has been shown to have the capability to detect and quantify fish epithelial injury. However, there are several other presumptive tests for blood (Bluestar©, phenolphthalein, and Hemastix®) that 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 (abrasion, cuts, fin frays, and punctures) using the freshwater bluegill *Lepomis macrochirus* as a model. 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. Further testing was conducted to examine the reliability of fluorescein. By 24 h after an injury was inflicted, the injury was no longer detectable by fluorescein, and when fluorescein was applied to an injured fish, the fluorescein was no longer detectable 3 h after application. In a comparison of two common anaesthetics used in fisheries research, there was no significant difference in the proportion of injury detected when 3-aminobenzoic acid ethyl ester methanesulfate (tricaine) was used compared with a clove oil and ethanol (1:9) solution. In summary, fluorescein was the most reliable presumptive test for blood examined in this study for the detection and quantification of recent (hours) fish epithelial injury.

Keywords: fish injury detection; fluorescein; presumptive tests for blood

Received: January 16, 2012; Accepted: September 13, 2013; Published Online Early: October 2013; Published: June 2014

Citation: Colotelo AH, Smokorowski KE, Haxton T, Cooke SJ. 2014. Comparative evaluation of four presumptive tests for blood to detect epithelial injury on fish. *Journal of Fish and Wildlife Management* 5(1):26–34; e1944-687X. doi: 10.3996/012012-JFWM-007

* Corresponding author: alison.colotelo@pnnl.gov

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 and woody debris), conspecifics (e.g., during spawning, competitive interactions; Hutchings and Meyers 1987; Neat et al. 1998), and predators (Harmon

et al. 1994). In addition, fish have the potential to experience injuries as a result of human activities (e.g., commercial fishing [Davis and 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 and Travade 2002], fishways [Larinier 2002]). These injuries 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 other instances where fish experience injuries that are not lethal in the short term but may cause long-term harm. For example, damage to the epithelium that covers the surface of the fish may not result in immediate death, but it can result in infections from a variety of different opportunistic pathogens, including bacteria, parasites, and fungi (Ventura and Grizzle 1987; Van West 2006). The injuries themselves or the associated pathogenic infections can lead to sublethal impairments such as physiological disturbance and behavioral alterations (Cooke and Sneddon 2007) and may even promote delayed mortality (Davis 2005).

As part of routine fish monitoring practices or fisheries research, injuries are often noted qualitatively through examination of scale loss (Main and 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 visually detectable but can lead to later infection or death. Fluorescein, a nontoxic dye commonly used in crime scene investigation, has been shown to enhance the detection of epithelial injury in fish (Noga and Udomkusonsri 2002) and has been used in the quantification of injury for comparison of commercial (Davis and Ottmar 2006) and recreational (Colotelo and Cooke 2011) fishing techniques, as well as hydroelectric turbine passage evaluation (Dauble et al. 2007).

Presumptive tests for blood are commonly used in forensic science to locate latent blood stains and to differentiate blood from other substances (Spalding 2006). These tests interact with hemoglobin in blood to cause a positive reaction that is denoted by a change in color or by the production of light (Spalding 2006). Under ultraviolet (UV) light, fluorescein produces a green light when blood is present. Although fluorescein has been used to detect latent injuries to the epithelial layer of fishes, there are several other presumptive tests for blood that also may be useful for fish epithelial injury detection. In a literature review, Colotelo et al. (2009) compared 10 different presumptive tests for blood on their sensitivity, specificity, cost, carcinogenicity, and ease of use. Based on that review, it was determined that in addition to fluorescein, Bluestar©, Hemastix®, and phenolphthalein may be useful for the detection of epithelial injury on fish. Bluestar was selected because it produces light in the presence of blood, similarly to fluorescein, but it does not require a UV light source, making this method simpler than fluorescein. Photographs can then be taken of this light production to calculate the proportion of the fish that is injured (Davis and Ottmar 2006). Hemastix and phenolphthalein were selected because they would be ideal for field use, because fish do not have to be anaesthetized and the reaction occurs within minutes. In addition, these chemicals would be applied to cotton swabs that had removed small amounts of mucous from the fish and the chemicals are never applied directly to the fish. Other presumptive blood detection techniques have been considered for application in a fisheries context (e.g., benzidine, *o*-tolidine, leuchomalachite green, tetramethylbenzidine, Hemident™, and luminol), but they 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 compare the use of fluorescein, a proven detection method for epithelial damage on fishes, with several other presumptive tests for blood (i.e., Bluestar, Hemastix, phenolphthalein) for the detection of punctures, abrasions, cuts, and fin fray on fish. 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. An additional suite of experiments was 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, we focused our research efforts on bluegill *Lepomis macrochirus*, a small freshwater fish. 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 fish. With the increasing potential for fish interaction with humans and human infrastructure, there is a need for additional tools that can be incorporated into environmental monitoring and fisheries research.

Methods

Comparative analysis of injury detection

Bluegill were angled from Opinicon Lake, located in southeastern Ontario, Canada, using size 8 barbless hooks and standard angling gear. Fish within the total length range of 120–190 mm were collected; fish outside this size range were released. Bluegill that were deeply hooked or exhibited any macroscopic evidence of injury also were 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 50 ppm clove oil anaesthetic (clove oil emulsified in ethanol, 1:9; Sigma-Aldrich, Toronto, Ontario) and remained there until fish reached stage 4 of anaesthesia noted by a loss

of equilibrium and coordinated fin movements (Summerfelt and Smith 1990). Fish were then removed from the anaesthetic bath by grasping them by the lower lip with the padded pliers, and those fish to be treated with fluorescein and Bluestar were dragged across a 5-mm nylon mesh net (18 cm in length) three times on their right side. All fish were then inflicted with standard injuries on their left side (Figure 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 (1 × 3 cm) was induced above or below the lateral line to simulate a large section of mucous loss. 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 to simulate a deep wound that may occur due to a predation attempt (e.g., animal bite). 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 after injury infliction, fish were sprayed with a 0.2 mg/mL solution of fluorescein (fluorescein, disodium salt; Aldon Corp., Avon, NY) in distilled water; the solution was left unrinsed for 6 min (Noga and Udomkusonsri 2002). Fish were then placed in an anaesthetic bath containing 120 ppm clove oil (clove oil emulsified in ethanol at 1:9) for 6 min to euthanize them. Fish were then photographed using a digital SLR ELIXIM Pro EX-F1 camera (Casio Computer Co., Ltd., Tokyo, Japan) under white light, long UV light (365 nm), or short UV light (254 nm) under various exposures (10, 13, 15, and 20 s) and at an ISO of 100. Fish were photographed in complete darkness, against a black background, with the camera positioned 42 cm directly above the fish and the UV light source (Mineralight® UVGL-48; UVP Inc., Upland, CA) at a 45° angle to the fish, 22 cm above, so that the entire organism was illuminated by the UV light. It was determined that short UV light and 20-s exposure were the best settings for examining the injury patterns; thus, these settings were used for the remainder of the study with fluorescein.

Bluestar. Immediately after injury infliction, fish were sprayed with Bluestar solution (Bluestar Forensic Mini Kit; Bluestar, Monte Carlo, Monaco) that was prepared following the instructions from the manufacturer. Because the Bluestar reaction is time sensitive, fish were immediately photographed in complete darkness with a digital camera (SLR ELIXIM Pro EX-F1; Casio Computer) positioned 42 cm directly above the fish, with an exposure of 30 s, and following the photography directions outlined by the manufacturer. Fish were then euthanized as described above.

Hemastix. A moistened cotton swab was applied to 1-cm^2 predetermined areas of the fish, representing areas that were inflicted with injury and areas that were not (Figures 1B and 1C). Each cotton swab was then touched to the pretreated reagent pad of the Hemastix (Hemastix Reagent Strips for Urinalysis; Bayer HealthCare LLC, Elkhart, IN), and the color change reaction was compared with a scale provided by the manufacturer; the result was recorded after 60 s. Fish were then euthanized as described above.

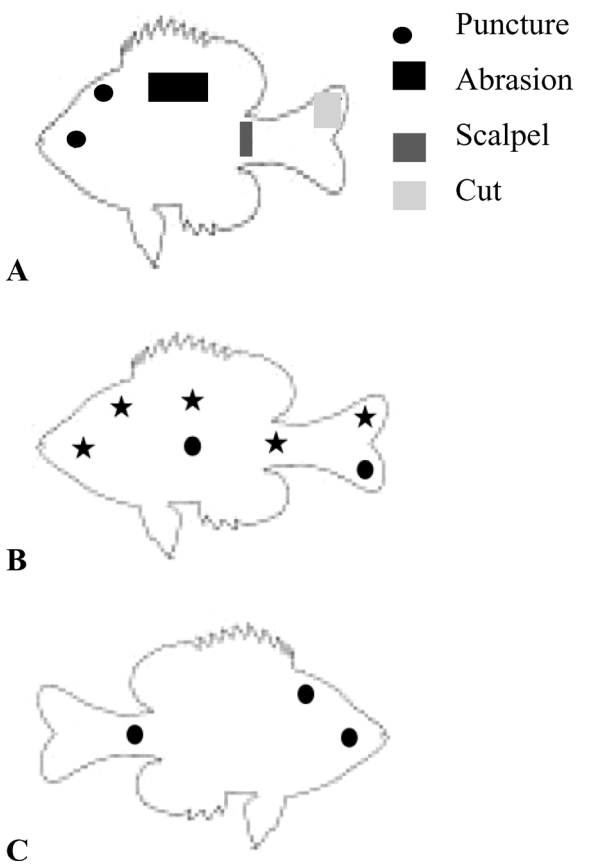

Figure 1. Diagrams of bluegill *Lepomis macrochirus*. (**A**) Pattern of injury used for comparison of fluorescein, Bluestar, Hemastix, and phenolphthalein in their ability to detect punctures, abrasions, scalpel cuts, and fin fray on bluegill. (**B**) Sampling sites used on the left side of each bluegill body for testing for epithelial damage using Hemastix and phenolphthalein. Stars indicate sites where injury was experimentally inflicted, and circles indicate sites where injury was not experimentally inflicted (control sites). (**C**) Sampling sites used on the right side of each bluegill body for testing for epithelial damage using Hemastix and phenolphthalein. Circles indicate sites where injury was not experimentally inflicted (control sites). Data were collected from fish collected from Opinicon Lake, southeastern Ontario, Canada, during 2008 field trials.

Phenolphthalein. A moistened cotton swab was applied to 1-cm^2 predetermined areas of the fish, representing areas that were inflicted with injury and areas that were not (Figures 1B and 1C). 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 s and 4 min. 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 Institutes of Health, Bethesda, MD), by tracing the areas of green or blue and measuring the number of pixels in that area. This process was done twice by the same individual, and the average number of pixels was calculated and used for statistical analysis. 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. There was a strong positive correlation between the two measurements recorded by the researcher, suggesting a high level of accuracy (Correlation: $r = 0.951$; $n = 16$; $P < 0.001$).

Comparison of presumptive tests for blood

The results of the injury evaluations were tabulated and compared for difference in rates of true positives, that is, area was injured and tested positive; false positives, that is, area was not injured and tested positive; true negatives, that is, area was not injured and tested negative; and false negatives, that is, 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 tests were accompanied with a scale that categorized the color change into proportions of hemoglobin detected. Results that were classified as ''large'' or ''medium,'' based on the scale provided by the manufacturer, were denoted as positive, whereas all other results were considered negative. ''Low'' classifications were not denoted as positive because lake water showed some form of contamination, resulting in a ''low'' reading. A positive result from phenolphthalein was denoted as a change from colorless to pink as described by the manufacturer.

Reliability of fluorescein

Detection of residual fluorescein posttreatment. Bluegill ($n = 10$ for each time group) were angled from Opinicon Lake using the capture method described in the Comparative analysis of injury detection section. Fish were individually marked with small numbered anchor tags (Floy Tag & Manufacturing Inc., Seattle, WA) 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 (1 × 3 cm) was induced below the lateral line, on either the left or right side of the body. Next, fish were submerged in a 0.2mg/mL solution of fluorescein in distilled water for 6 min and were then placed in a holding tank that was constantly provided with fresh lake water. Fish were examined for the presence of fluorescein at 1, 3, 8, and 15 h after injury infliction and fluorescein treatment by anaesthetizing fish in a 50 ppm clove oil and ethanol solution (1:9) and photographing fish in complete darkness under white and short UV light (20-s exposure) as described in the Comparative analysis of injury detection section. Fish were allowed to recover after each anaesthesia application in a holding tank of freshwater. 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 Opinicon Lake using the capture method described above. Fish were anaesthetized in a water bath containing 50 ppm clove oil and ethanol (1:9) solution until stage 4 of anaesthesia as described above. A scalpel scrape (1 × 3 cm) 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 h 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 min in 0.2 mg/mL fluorescein solution in distilled water and then 6 min in a water bath containing 50 ppm clove oil and ethanol (1:9) solution. Fish were then photographed in complete darkness under white and short UV light (20-s 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 Opinicon Lake using the capture method described above. Fish were anesthetized in a water bath containing either 50 ppm clove oil and ethanol (1:9) solution or 50 ppm buffered 3-aminobenzoic acid ethyl ester methanesulfate (MS-222 or tricaine; Western Chemical, Inc., Ferndale, WA) until stage 4 of anaesthesia was reached as described above. A scalpel scrape (1 × 3 cm) was inflicted below the lateral line, on either the left or right side of the body. All fish were then treated with a 0.2 mg/mL solution of fluorescein in distilled water for 6 min. Fish anesthetized with the clove oil and ethanol (1:9) solution were then placed in a water bath containing 50 ppm clove oil and ethanol (1:9) solution for 6 min to rinse excess fluorescein from the body and euthanize the fish. Fish anesthetized with buffered MS-222 were placed in a rinse of pure lake water for 6 min and then lethally sampled via cerebral percussion. Davis et al. (2008) determined that MS-222 suppressed fluorescein light production, so fish cannot be resubmerged in a MS-222 solution after exposure to fluorescein. Fish treated with MS-222 therefore needed to be euthanized using means other than an MS-222 overdose after treatment with fluorescein, to obtain photographs. All fish were photographed under white and short UV light (20-s exposure). Computer analysis to calculate the proportion of green in each photograph was conducted as described above.

Statistical analysis

A χ^2 contingency table analyses were used to determine whether 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 1-way ANOVA was conducted on inverse-transformed data. A 1-way ANOVA also was used to test for differences in the proportion of injury detected when using clove oil and MS-222 as an anaesthetic on natural

Table 1. The χ^2 post hoc analysis for the proportion of true positives detected by each presumptive test for blood (fluorescein, Bluestar, Hemastix, and phenolphthalein) for each injury type (punctures, abrasions, cuts, and fin fray) investigated on bluegill *Lepomis macrochirus* in this study. Data were collected from fish collected from Opinicon Lake, southeastern Ontario, Canada, during 2008 field trials.

	χ^2 result				
Injury	**χ^2 0.05, 3**	***P***	**Presumptive test**	**Standardized residual**	***P***
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	9.00×10^{-5}	Fluorescein	−2.06	<0.05
			Bluestar	−3.61	<0.05
			Hemastix	0	>0.05
			Phenolphthalein	−2.02	<0.05

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 honestly significant difference post hoc test. To test for the significance of the time after injury was inflicted that the injury could still be detected using fluorescein, a one-way ANOVA followed by a Tukey's honestly significant difference post hoc test was conducted. These data were square root–transformed to meet the assumptions of parametric tests. Transformations were completed as necessary to meet the assumptions of normality and homogeneity of variance required for parametric tests. All statistical tests were completed using SPSS software (IBM Corporation, Armonk, NY) and significance was assessed at $\alpha = 0.05$ (Zar 1984).

Results

Comparative analysis of injury detection

For all presumptive tests for blood, there was a significant difference in the number of true positive reactions. A test for independence was conducted to determine the presumptive tests for blood that determined a significantly different number of true positive reactions for each injury type investigated in this study (i.e., punctures, abrasions, cuts, fin fray; Table 1). For the detection of punctures, cuts and abrasions, fluorescein had the highest percentage of true positive detections (40.0, 66.7, and 43.3%, respectively; Table 2), whereas Hemastix detected the highest percentage of true positives for fin fray (50.0%). Hemastix also detected the highest percentage of false positives for all injury types investigated in this study (20.8% for punctures, 8.3% for cuts, 20.8% for abrasions, and 25.0% for fin fray), and phenolphthalein detected the lowest number of false positives for all injury types investigated (4.2% for punctures, 4.2% for cuts, 0.0% for abrasions, and 0.0% for fin fray). There was no significant difference in the proportion of injury detected between fluorescein (mean ± SE, 2.9 ± 0.6%) and Bluestar (mean ± SE, 9.7 ± 5.2%; ANOVA: $F_{1,28} = 1.7$; $P = 0.198$). Detailed information on the treatments, locations and types of inflicted injuries, and measured results using presumptive tests for blood are available in Table.S1.

Reliability of fluorescein

There were significantly different proportions of fluorescein detected when fish were held for different times after injury was inflicted (Figure 2; repeated measures ANOVA with Tukey's honestly significant difference post hoc: $F_{1,9} = 16.4$; $P = 0.003$). Specifically, there was significantly less detectable proportion of injury at 1, 3, 8, and 15 h than at time of treatment (0 h).

The ability of fluorescein to detect injuries that are more than 24 h old was significantly reduced compared with testing fish immediately after (0 h) or at 1 and 5 h after injury occurred (Figure 3; 1-way ANOVA with Tukey's honestly significant difference post hoc: $F_{3,36} = 20.7$; $P < 0.001$).

Fish anesthetized in a water bath with 50 ppm clove oil and ethanol (1:9) solution (mean ± SE, 25.6 ± 7.8%) did not have significantly different levels of fluorescein detected than fish anesthetized with MS-222 (mean ± SE, 9.6 ± 1.5%; Mann–Whitney *U* test: $U_{10,10} = 26.0$; $P = 0.076$).

Discussion

Comparative analysis of injury detection

Based on the comparison of the presumptive tests for blood in this study, fluorescein was determined to be the

Table 2. Number (and percentage relative to the total number of tests performed) of true positive, false positives, true negatives, and false negatives detected with fluorescein, Bluestar, Hemastix, and phenolphthalein applied to experimentally induced punctures, cuts, abrasions, and fin fray on bluegill *Lepomis macrochirus*. Data were collected from fish collected from Opinicon Lake, southeastern Ontario, Canada, during 2008 field trials.

Injury	Presumptive test	Total no. of tests	No. of true positives		No. of false positives		No. of true negatives		No. of false negatives	
			n	%	*n*	%	*n*	%	*n*	%
Punctures	Fluorescein	15	6	40.0	0	0.0	0	0.0	9	60.0
	Bluestar	15	4	26.7	0	0.0	0	0.0	11	73.3
	Hemastix	24	8	33.3	5	20.8	7	29.2	4	16.7
	Phenolphthalein	24	8	33.3	1	4.2	11	45.8	4	16.7
Cuts	Fluorescein	15	10	66.7	NA[a]	NA	NA	NA	5	33.3
	Bluestar	15	1	6.7	NA	NA	NA	NA	14	93.3
	Hemastix	24	14	58.3	2	8.3	0	0.0	8	33.3
	Phenolphthalein	24	7	29.2	1	4.2	11	45.8	5	20.8
Abrasions	Fluorescein	30	13	43.3	3	10.0	12	40.0	2	6.7
	Bluestar	30	1	3.3	1	3.3	14	46.7	14	46.7
	Hemastix	24	9	37.5	5	20.8	3	12.5	7	29.2
	Phenolphthalein	24	6	25.0	0	0.0	12	50.0	6	25.0
Fin fray	Fluorescein	30	7	23.3	3	10.0	12	40.0	8	26.7
	Bluestar	30	1	3.3	1	3.3	14	46.7	14	46.7
	Hemastix	24	12	50.0	6	25.0	6	25.0	0	0.0
	Phenolphthalein	24	5	20.8	0	0.0	12	50.0	7	29.2

[a] NA = not applicable.

most effective test for the detection and quantification of epithelial damage to fish. Fluorescein resulted in the highest percentages of true positive reactions and rarely detected false positives. 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 quantitatively evaluate injury, was not an effective test for fish epithelial damage. It demonstrated the lowest proportion of true positives compared with all other tests (Table 2) 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 herein because the mucous layer was not removed. Removal of mucous has the potential to alter findings because it could smear blood stains and preclude the ability to identify the precise location of an injury. In addition, this method would not be recommended for fish that are not lethally sampled, because the removal of the mucous layer would compromise the health of the fish.

Hemastix demonstrated a high proportion of true positives detected for all injury types in this study and detected the highest percentage of true positive reactions for fin frays (Table 2). As such, 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 fin fraying (25.0%), abrasions (20.8%), and punctures (20.8%). These results indicate that before Hemastix can be readily used for epithelial damage detection, more work needs to be conducted corresponding to the specificity or the ability of the chemical to accurately detect blood and identify substances that may present as false positives or false negatives.

Phenolphthalein was not effective in the detection of the different injuries inflicted in this study. The maximum proportion of true positives that were detected was 33.3% for punctures (Table 2). However, the positive reactions were never ''strong,'' and there is level of familiarity with the chemical necessary for effectively interpreting the results. Also, the chemicals must be applied to the swab independently, thereby creating more steps and organization in a field situation.

Reliability of fluorescein

Residual detection of fluorescein posttreatment. Fluorescein is a nontoxic 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 and Yannuzzi 1989). For any fish that may be consumed postsampling, 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 h after treatment with fluorescein the proportion of fluorescein detected (i.e., 3.8%) in the epithelium had decreased significantly from that of fish that were photographed immediately after treatment (i.e., 25.6%; Figure 2). Fish treated with fluorescein to detect epithelial damage also must be treated with an anesthetic (clove oil is recommended). Researchers should consult with their regional or national regulatory bodies if clove oil is used

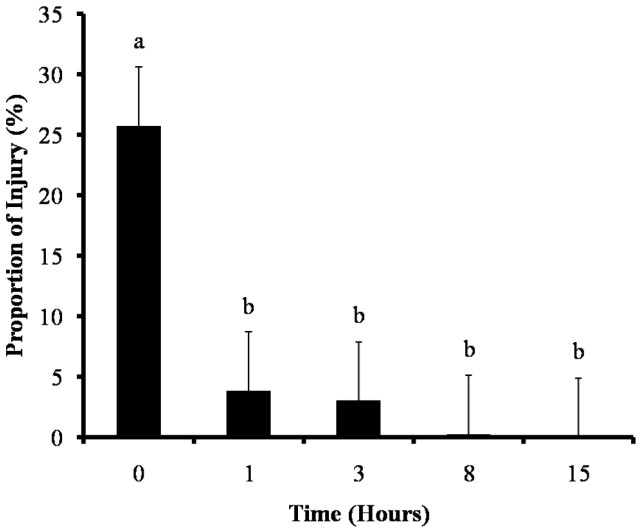

Figure 2. Proportion of fluorescein detectable after treatment (±SE), showing the residual detection of fluorescein on bluegill *Lepomis macrochirus* administered with abrasions to the epithelial layer (n = 10 for each time interval). Dissimilar letters indicate significant differences ($P < 0.05$) in the proportion of fluorescein detected. Data were collected from fish collected from Opinicon Lake, southeastern Ontario, Canada, during 2008 field trials.

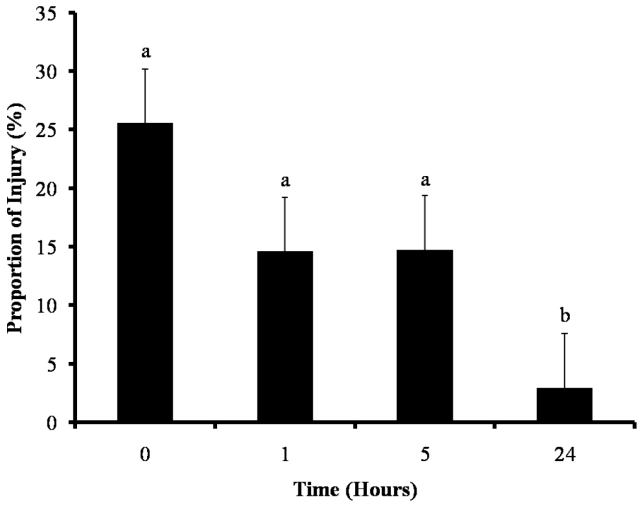

Figure 3. Proportion of injury detectable at various times after injury occurred (±SE), showing the temporal patterns of injury detection using fluorescein on bluegill *Lepomis macrochirus* administered with abrasions to the epithelial layer (n = 10 for each time interval). Dissimilar letters indicate significant differences ($P < 0.05$) in the proportion of fluorescein detected. Data were collected from fish collected from Opinicon Lake, southeastern Ontario, Canada, during 2008 field trials.

on fish that have the potential to enter the human food supply to ensure compliance with relevant legislation and policies.

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 h (25.6, 14.6, and 14.7% respectively; Figure 3). These results suggest that at least 24 h 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 (if measured as part of an experiment) and not an outside source.

Influence of anaesthetics on injury detection. In this study, there was no difference between the proportion of injury detected with fluorescein when using MS-222 and clove oil anaesthetics. However, there was substantial variability in the data. There have been previous concerns with using MS-222 as an anaesthetic when using fluorescein for injury detection, because 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 by an alternative means after treatment with fluorescein to obtain photographs; they cannot be resubmerged in the MS-222 anaesthetic. Clove oil also was examined as an anaesthetic, and there was no observable interference with the fluorescein, thereby posing a benefit over MS-222 because the fish do not have to be lethally sampled to be photographed. Although clove oil shows promise as an anaesthetic, it also may be a source of inhibition for fluorescence so additional research is needed to evaluate other anaesthetics such as CO_2 and electrical current that would be unlikely to inhibit fluorescence.

Implications and future considerations

Fluorescein has previously been shown to be an effective method of detecting and quantifying fish epithelial injury (Noga and Udomkusonsri 2002; Colotelo and Cooke 2011). In this comparison with other presumptive tests for blood, fluorescein demonstrated the lowest rate of false positive detection for multiple types of injuries. Its low cost, ease of application in the field, and nontoxic 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 it is valuable for the comparison of different handling methods and gear that are 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 that are to be released. Dedual and Shorland (2006) and Colotelo and Cooke (2011) have shown that photographs of injured fish due to poor handling methods can be an effective education and conservation tool.

Although the benefits of this tool are numerous, fluorescein is a relatively new tool and has been used in a limited number of studies. Overall, the proportion of injuries detected in this study was lower than expected. Therefore, further research is needed to evaluate the

severity (e.g., depth of epithelial damage) of injury that can be detected using fluorescein. Moreover, there is still little known about the potential interference of other natural and anthropogenic chemicals (including anaesthetics). Fluorescein is capable of epithelial injury detection and although 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 in wild fish (Colotelo et al. 2009). Future research should focus on the sublethal disturbances that develop from injuries detectable using fluorescein, including physiological stress and infection, as well as mortality rates that result from these disturbances. Mortality, if severe, can affect population levels and is important especially when dealing with conservation of threatened populations or species (Simberloff 1988). Evaluating sources of anthropogenic injury on fish and the development of strategies to reduce such injuries will benefit fish populations and also aid in the maintenance of the welfare status of individual fish.

Supplemental Material

Please note: The *Journal of Fish and Wildlife Management* is not responsible for the content or functionality of any supplemental material. Queries should be directed to the corresponding author for the article.

Reference S1. Dauble DD, Deng ZD, Richmond MC, Moursund RA, Carlson TJ, Rakowski CL, Duncan JP. 2007. Biological assessment of the advanced turbine design at Wanapum Dam, 2005. Pacific Northwest National Laboratory to U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Wind and Hydropower Technologies, under Contract DE-AC05-76RL01830: Richland, Washington.

Found at DOI: http://dx.doi.org/10.3996/012012-JFWM-007.S1; also available at http://www.pnl.gov/publications/abstracts.asp?report=228483 (1965 KB PDF).

Table S1. Data File. Work sheets that include the area of injury measured for bluegill used in the various analyses presented in this manuscript. Worksheet ''Comparison of tests'' includes the treatment type, description of injuries inflicted and the results collected for each fish. Worksheets ''Detection of residual fluoresce'', ''Temporal patterns'', and ''Influence of anesthetics'' include the area of injury detected for each fish included in these tests.

Found at DOI: http://dx.doi.org/10.3996/012012-JFWM-007.S2 (24 KB XLSX).

Acknowledgments

The authors thank members of the Cooke Lab (Carleton University) for assistance with data collection. We also thank the staff of the Queen's University Biological Station for logistic support. We appreciate the comments and suggestions to improve this manuscript made by the reviewers and Subject Editor. Research permits were provided by Ontario Ministry of Natural Resources and animal care approvals were granted by Carleton University and Queen's University on behalf of the Canadian Animal Care Council. This work was supported by Fisheries and Oceans Canada and Natural Resources Canada. Additional support was provided from the Ontario Ministry of Research and Innovation through the Early Researcher Award Program and the Canada Research Chairs Program.

Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

References

Ashley PJ. 2007. Fish welfare: current issues in aquaculture. Applied Animal Behaviour Science 104:199–235.

Barthel BL, Cooke SJ, Suski CD, Philipp DP. 2003. Effects of landing net mesh type on injury and mortality in a freshwater recreational fishery. Fisheries Research 63: 275–282.

Cada GF. 2001. The development of advanced hydroelectric turbines to improve fish passage survival. Fisheries 26:14–24.

Colotelo AH, Cooke SJ. 2011. Evaluation of common angling-induced sources of epithelial for popular freshwater sport fish using fluorescein. Fisheries Research 109:217–224.

Colotelo AH, Cooke SJ, Smokorowski K. 2009. Application of forensic techniques to enhance fish conservation and management: injury detection using presumptive tests for blood. Endangered Species Research 9:169–178.

Cooke SJ, Schreer DH, Wahl DH, Philipp DP. 2002. Physiological impacts of catch-and-release angling practices on largemouth bass and smallmouth bass. Pages 489–512 in Phillipp DP, Ridgeway MS, editors. Black bass 2000: ecology, conservation and management. Bethesda, Maryland: American Fisheries Society Symposium 31.

Cooke SJ, Sneddon LU. 2007. Animal welfare perspectives on recreational angling. Applied Animal Behaviour Science 104:176–198.

Danylchuk SE, Danylchuk AJ, Cooke SJ, Goldberg TL, Koppelman T, Philipp DP. 2007. Effects of recreational angling on the post-release behavior and predation of bonefish (*Albula vulpes*): the role of equilibrium status at the time of release. Journal of Experimental Marine Biology and Ecology 346:127–133.

Dauble DD, Deng ZD, Richmond MC, Moursund RA, Carlson TJ, Rakowski CL, Duncan JP. 2007. Biological assessment of the advanced turbine design at Wanapum Dam, 2005. Pacific Northwest National Laboratory to U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Wind and Hydropower Technologies, under Contract DE-AC05-76RL01830: Richland, Washington (see *Supplemental Material*, Reference S1, http://dx.doi.org/10.3996/012012-JFWM-007.S1); also available: http://www.pnl.gov/publications/abstracts.asp?report = 228483 (September 2013).

Davis MW. 2005. Behaviour impairment in captured and released sablefish: ecological consequences and

possible substitute measures for delayed discard mortality. Journal of Fish Biology 66:254–265.
Davis MW, Ottmar ML. 2006. Wounding and reflex impairment may be predictors for mortality in discarded or escaped fish. Fisheries Research 82:1–6.
Davis MW, Stephenson S, Noga EJ. 2008. The effect of tricaine on use of the fluorescein test for detecting skin and corneal ulcers in fish. Journal of Aquatic Animal Health 20:86–95.
Dedual M, Shorland J. 2006. Poor handling affects released trout. Target Taupo 52:5–13.
Harmon JR, Thomas KL, McIntyre KW, Paasch NN. 1994. Prevalence of marine-mammal tooth and claw abrasions on adult anadromous salmonids returning to the Snake River. North American Journal of Fisheries Management 14:661–663.
Hutchings JA, Myers RA. 1987. Escalation of an asymmetric contest: mortality resulting from mate competition in Atlantic salmon, *Salmo salar*. Canadian Journal of Zoology 65:766–768.
Killgore KJ, Maynord ST, Chan MD, Morgan RP. 2001. Evaluation of propeller-induced mortality on early life stages of selected fish species. North American Journal of Fisheries Management 21:947–955.
Larinier M. 2002. Fishways: general considerations. Bulletin Francais de la Peche et de la Pisciculture 364(Suppl 5):21–27.
Larinier M, Travade F. 2002. Downstream migration: problems and facilities. Bulletin Francais de la Peche et de la Pisciculture 364(Suppl):181–205.
Lipson BK, Yannuzzi LA. 1989. Complications of intravenous fluorescein injections. International Ophthalmological Clinics 23:200–205.
Main J, Sangster GI. 1988. Scale damage and survival of young gadoid fish escaping from the cod-end of a demersal trawl. In DeAlteris J, editor. Proceedings of Selectivity and Survivability Workshop. Narragansett, Rhode Island: University of Rhode Island Sea Grant Advisory Service Pp. 17-33.
Neat FC, Taylor AC, Huntingford FA. 1998. Proximate costs of fighting in male cichlid fish: the role of injuries and energy metabolism. Animal Behaviour 55:875–882.
Noga EJ, Udomkusonsri P. 2002. Fluorescein: a rapid, sensitive, nonlethal method for detecting skin ulceration in fish. Veterinary Pathology 39:726–731.
Simberloff D. 1988. The contribution of population and community biology to conservation science. Annual Reviews Ecological Systems 19:473–511.
Spalding RP. 2006. The identification and characterization of blood and bloodstains. Pages 237–260 in James SH, Nordby JJ, editors. Forensic science: an introduction to scientific and investigative techniques. 2nd edition. Boca Raton, Florida: CRC Press.
Summerfelt RC, Smith LS. 1990. Anaesthesia, surgery, and related techniques. Pages 213–272 in Schreck CB, Moyle PB, editors. Methods for fish biology. Bethesda, Maryland: American Fisheries Society.
Van West P. 2006. *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. Mycologist 20:99–104.
Ventura MT, Grizzle JM. 1987. Evaluation of portals of entry of *Aeromonas hydrophila* in channel catfish. Aquaculture 65:205–214.
Zar JH. 1984. Biostatistical analysis. New Jersey: Prentice Hall.