The Effect of Tricaine on Use of the Fluorescein Test for Detecting Skin and Corneal Ulcers in Fish

Michael W. Davis [a] , Jeana Stephenson [b] & Edward J. Noga [b]

[a] National Oceanic and Atmospheric Administration, National
Marine Fisheries Service, Alaska Fisheries Science Center, Hatfield
Marine Science Center, 2030 Southeast Marine Science Drive,
Newport, Oregon, 97365, USA

[b] Department of Clinical Sciences, College of Veterinary Medicine,
North Carolina State University, 4700 Hillsborough Street, Raleigh,
North Carolina, 27607, USA

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MICHAEL W. DAVIS

National Oceanic and Atmospheric Administration, National Marine Fisheries Service,
Alaska Fisheries Science Center, Hatfield Marine Science Center,
2030 Southeast Marine Science Drive, Newport, Oregon 97365, USA

JEANA STEPHENSON AND EDWARD J. NOGA*

Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University,
4700 Hillsborough Street, Raleigh, North Carolina 27607, USA

Abstract.—Fluorescein has been used for rapid and sensitive detection of fish skin and corneal ulceration. Effective use of the fluorescein test requires knowledge of conditions that might cause misleading interpretations or otherwise interfere with test reliability. Examination of fish health and the clinical workup often require tricaine as one of the most commonly used anesthetics. However, tricaine may interfere with correct interpretation of the fluorescein test and might also cause significant fish injury. The effects of tricaine exposure sequence on the fidelity of the fluorescein test was studied in Pacific halibut *Hippoglossus stenolepis*, walleye pollock *Theragra chalcogramma*, and northern rock soles *Lepidopsetta polyxystra* by examining the fluorescence of experimentally induced epidermal wounding. Tricaine can quench fluorescence that is emitted by fluorescein retained in skin ulcers, causing a false-negative reaction. Thus, for the fluorescein test to work properly, it is important to avoid the exposure of fluorescein-treated and rinsed ulcers to tricaine. The effects of exposure to buffered versus unbuffered tricaine on epidermal and corneal integrity were studied in Nile tilapia *Oreochromis niloticus* and channel catfish *Ictalurus punctatus* subjected to the fluorescein test and histological examination. Fluorescein could detect not only ulcers but also areas with only a partial loss of epithelium (i.e., erosion). The use of unbuffered tricaine to anesthetize these fish caused serious epidermal and corneal damage. If fish are euthanized with unbuffered tricaine for clinical workup, this severe epidermal or corneal damage could be misinterpreted as an antemortem lesion, leading to misdiagnosis. Even in water with alkalinity exceeding 50 mg/L as $CaCO_3$, it would seem prudent to always buffer tricaine with sodium bicarbonate to prevent a pH change that might lead to iatrogenic effects from unbuffered tricaine. Thus, current general recommendations suggesting that tricaine does not need to be buffered in waters with alkalinity greater than 50 mg/L might need to be modified.

Skin ulcers are one of the most common clinical manifestations of disease in fish. Loss of the protective skin barrier leads to a number of deleterious changes, especially impaired osmoregulatory capacity and the provision of a portal for pathogen colonization and invasion. Since virtually all fish skin is nonkeratinized, it is highly susceptible to damage, even from relatively minor trauma. To develop better means of assessing skin health, we developed a test for rapidly and sensitively detecting the presence of skin ulcers on fish. This test is based upon the use of fluorescein, an inexpensive, nontoxic, fluorescent dye that specifically stains ulcerated areas of any size, allowing the detection of even pinpoint ulcers (Noga and Udomku-sonsri 2002). We later discovered that this test was also a sensitive indicator of corneal ulceration (Udomku-

sonsri et al. 2004), which is understandable since the cornea is also made up of nonkeratinized epithelium and fluorescein has been used for many years to detect corneal ulceration in mammals (Bartlett et al. 1996). The fluorescein test has also been used to quantify the spatial extent of skin ulceration on fish by computer analysis of digital photographs (Davis and Ottmar 2006).

Fluorescein (3′,6′-dihydrospiro{isobenzofuran-1[3H],9′-[9H]xanthen}-3-one) is a yellow, relatively nontoxic, vital, hydroxyxanthene dye that produces an intense fluorescence in slightly acid to alkaline (pH > 5) solutions. Fluorescein exhibits a high degree of ionization at physiologic pH and thus neither penetrates intact epithelium nor forms a firm bond with (i.e., stain) vital tissue. However, fluorescein can rapidly penetrate a break in the epithelial barrier (Bartlett et al. 1996). When exposed to light, fluorescein absorbs light in the blue range of the visible spectrum with peak absorption at 480–500 nm. Fluorescein emits light from 500 to

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* Corresponding author: ed_noga@ncsu.edu

600 nm and the maximum intensity is at 520–530 nm (Berkow et al. 1991).

To effectively use the fluorescein test, conditions that might cause misleading interpretations or might otherwise interfere with the reliability of the test must be known. In this regard, we examined the interaction of tricaine with the fluorescein test. Tricaine (3-aminobenzoic acid ethyl ester methanesulfonate) is one of the most commonly used fish anesthetics. It is approved for food fish use in the USA and some other countries. In poorly buffered water, tricaine can cause a decline in pH to very low levels due to formation of methanesulfonic acid (Summerfelt and Smith 1990). Thus, tricaine solutions in poorly buffered waters (i.e., alkalinity < 50 mg $CaCO_3$/L of water) should be used with an appropriate buffer, such as a 2:1 (weight : weight) ratio of sodium bicarbonate : tricaine (Summerfelt and Smith 1990). Houston (1990) experimentally showed that unbuffered tricaine caused metabolic acidosis. Here, we describe serious fish skin and eye (corneal) damage associated with use of unbuffered tricaine and the effect of tricaine sedation and fluorescein treatment sequence on the reliability of the fluorescein test.

Methods

Effect of tricaine and pH on skin and corneal integrity.—Channel catfish *Ictalurus punctatus* (11.0–13.5 cm total length [TL]) and Nile tilapia *Oreochromis niloticus* (12.5–17.0 cm TL) were maintained in holding aquaria (160–760 L). Fish were fed a commercial feed at approximately 2% of their body weight daily and were maintained at a photoperiod of 12 h light : 12 h dark. During acclimation, dissolved oxygen was 6.8–7.5 mg/L, temperature was 27°C, pH was 6.65–6.87, un-ionized ammonia was less than 0.001 mg/L, nitrite was less than 0.10 mg/L, alkalinity was 0.054 mg/L, and hardness was 0.036 mg/L. Fish had been in the holding aquaria for over 2 months before experimentation.

We used the fluorescein test (Udomkusonsri et al. 2004) to determine the degree of skin or corneal ulceration in the fish. For each experiment, fish were examined for skin or corneal integrity both before and after the experimental treatment. Briefly, a single fish was gently placed in a plastic aquarium bag containing a solution of 0.20 mg of fluorescein (Akorn, Inc., Decatur, Illinois; AK-Fluor, 10% fluorescein sodium injection, 100 mg/mL) per milliliter of water and held in the bag for 6 min. The solution was then gently drained from the bag by inverting it while keeping the opening restricted to prevent the fish from leaving the bag. Freshwater was then immediately added to the bag. After 1 min, the water was gently replaced with

clean freshwater. After three freshwater rinses (3 min total), the fish was immediately examined under ultraviolet (UV) light (Mineralight, Upland, California; Model UVGL-58) for skin or corneal damage. Ulcers appeared as bright-green fluorescent areas. After the locations of any lesions were quickly (within 30 s) recorded, the fish was then exposed to one of the following treatments: unbuffered tricaine (pH = 3.5), low pH (water adjusted with 1-N HCl to a pH of 3.5), or buffered tricaine (1 part tricaine + 2 parts sodium bicarbonate; pH = 7.3). Buffered tricaine was used as the control. Before exposure to unbuffered tricaine, low pH, or buffered tricaine, each fish was examined with fluorescein to identify any small lesions; thus, each individual was used as its own control. Tricaine (Finquel; tricaine methanesulfonate) was obtained from Argent Chemical Laboratories (Redmond, Washington). Channel catfish were exposed to a 200-mg/L solution of tricaine, whereas Nile tilapia were exposed to 300-mg/L tricaine (i.e., the highest nonlethal dose for each species). All fish were exposed individually, and all were alive at the end of treatment. After exposure to the experimental treatment for a specific time period (25 min for channel catfish; 35 min for Nile tilapia), the fish was rinsed in untreated water, immediately placed back in fluorescein solution, and re-examined for skin and corneal damage. Fish were then euthanized by decapitation and pithing; fluorescein-positive areas (eyes and skin) were fixed in 10% neutral-buffered formalin (NBF) and examined histologically for epithelial loss. Each group contained four fish.

Effect of various chemical milieu on ulcer fluorescence.—Based upon preliminary observations, we hypothesized that the fluorescein response would be inhibited in the presence of tricaine and that such inhibition would depend upon the order in which fish were exposed to tricaine and fluorescein in the testing procedure. Pacific halibut *Hippoglossus stenolepis* (32.1–39.9 cm TL), walleye pollock *Theragra chalcogramma* (13.7–22.8 cm TL), and northern rock soles *Lepidopsetta polyxystra* (8.8–13.7 cm TL) were maintained in holding tanks (3,140 L) with flow-through seawater at 8–9°C. Fish were fed a commercial pelleted feed at approximately 2% of their body weight daily, and photoperiod was maintained at 12 h light : 12 h dark. During the experiments, dissolved oxygen was 9.53–9.81 mg/L, salinity was 30–32‰, temperature was 8–9°C, pH was 8.00–8.10, un-ionized ammonia was less than 0.001 mg/L, and nitrite was less than 0.10 mg/L. After at least 2 months of acclimation, fish were used for experiments.

Replicate fish were experimentally wounded to create an ulcer by scraping a scalpel blade along 2

cm of one flank. The fish was then exposed to one of the following six treatment sequences (all were performed in seawater): (1) seawater rinse (R), fluorescein (F), and another R; (2) buffered tricaine (T), F, and R; (3) F, T, and R; (4) F, R, and T; (5) F, R, and acidic seawater (ASW; pH = 6.7); and (6) F, R, T, and R.

These treatments were designed to systematically investigate the effects of seawater rinse, buffered tricaine, and ASW on fluorescein fluorescence in a skin ulcer. The standard order of baths in the fluorescein test for detecting skin ulcers is to expose fish to fluorescein, followed by water rinse and then euthanasia by tricaine (Noga and Udomkusonsri 2002). If tricaine inhibits fluorescein fluorescence in a skin ulcer, then use of tricaine after water rinsing in the test could produce false-negative results for skin ulcer detection. In all treatments, the null hypothesis was that fluorescein fluorescence was not inhibited (a positive response), while the alternative hypothesis was that fluorescence was inhibited (a negative response). Treatment 1 tested the null hypothesis that seawater rinses did not inhibit fluorescein fluorescence. This treatment was a control to establish the presence of ulcer fluorescence in the system of treatments. Treatments 2 and 3 tested the null hypothesis that tricaine exposure either before or after fluorescein did not inhibit fluorescence. These treatments tested whether tricaine irreversibly inhibited fluorescein, regardless of final seawater rinses. Treatment 4 tested the null hypothesis that fluorescein was not inhibited when tricaine was the final bath exposure after excess fluorescein was rinsed off with seawater. Treatment 5 tested the null hypothesis that fluorescein was not inhibited when ASW that matched the acidity of buffered tricaine was the final exposure after excess fluorescein was rinsed off with seawater. This treatment tested whether acidity was a cause of fluorescein inhibition. Treatment 6 tested the null hypothesis that fluorescein was not inhibited by rinsing of excess fluorescein, exposure to tricaine, and a final rinse with seawater. This treatment tested whether a final seawater rinse could reverse the effect of tricaine on fluorescein that was not present in excess.

Buffered tricaine consisted of tricaine at 400 mg/L and sodium bicarbonate at 800 mg/L. The tricaine concentration was that normally used for euthanasia in Pacific halibut, northern rock soles, and walleye pollock (Davis and Schreck 2005; Davis and Ottmar 2006). The pH of the seawater after adding the buffered tricaine was 6.7. The ASW was adjusted to a pH of 6.7 by adding 1.0-N HCl. Fluorescein was used at 200 mg/L. An initial seawater rinse (treatment 1) was for 5 min. Buffered tricaine exposures (treatments 2–4 and 6)

were for 5 min each. Fluorescein exposures (treatments 1–6) were for 6 min. Longer seawater rinses to remove fluorescein (treatments 1–6) were for 15 min. After the last step in the sequential treatment, the fish was examined for fluorescence under 254-nm illumination as described previously. The fish was then euthanized by decapitation, and the experimentally damaged skin was fixed in NBF and processed routinely for histopathology (as described in the next section) to confirm the presence of the scraping-induced ulcer. The intent of this histological examination was to confirm that an ulcer was produced by skin scraping, thereby confirming that any lack of fluorescence was due to the method of treatment rather than the failure to produce a wound. For each species, six replicate fish were used in each treatment. The presence or absence of fluorescence was noted for each fish after treatment. A sign test was used to determine statistical significance ($P < 0.05$) of fluorescence absence in each treatment.

Microscopic evaluation.—To confirm the presence of epithelial damage in Nile tilapia and channel catfish, we examined skin (body and fins) and eye tissues for epithelial loss via histopathology. Single sections of skin and both eyes from each treated fish and similar samples of nonfluorescing tissue taken from the same body areas in control fish were processed. Skin from Nile tilapia was decalcified in 100-g/L EDTA and 0.1-M phosphate buffer (pH = 7.2) before processing, whereas no other tissues were decalcified. After routine processing, all tissues were embedded in paraffin and processed routinely for light microscopy. All sections were stained with hematoxylin and eosin. Tissues were oriented in the longitudinal plane and then evaluated for pathological changes, including erosion (i.e., loss of some or all of the epithelium but retention of the basement membrane) and ulceration (i.e., complete loss of the epithelium, including the basement membrane). In some cases, an aliquot of the water that had been used to treat a fish with tricaine or low pH was gently centrifuged at approximately $2,000 \times$ gravity to generate a pellet from particles in the water; the pellet was then fixed in NBF for later microscopic examination to check for the presence of sloughed epithelial cells.

For confirmation of the presence of ulcers in experimentally wounded Pacific halibut, walleye pollock, and northern rock soles, the wounded area was dissected out, fixed in NBF, and processed as described previously.

Photography.—For Nile tilapia and channel catfish, photographs under UV light were taken in complete darkness at an International Standards Organization (ISO) setting of 125 at F11 (selection with the best

TABLE 1.—Effect of unbuffered tricaine (300 mg/L), buffered tricaine (300-mg/L tricaine + 600-mg/L sodium bicarbonate), and low pH on skin and corneal integrity in Nile tilapia. Fish were exposed to each treatment for 35 min. Corneal and skin damage was recorded both before and immediately after experimental exposure. All responses refer to the amount of bright yellow-green fluorescence detected (i.e., ulceration: − = none; + = some; ++ = more severe; +++ = most severe; NC = no change compared with pretreatment score; L = left eye; R = right eye; and LR = both eyes).

Treatment	pH	Fish number	Cornea		Skin	
			Before	After	Before	After
Unbuffered tricaine	3.5	05-2117	−	+ (LR)	++	+++
		05-2118	−	+ (LR)	+	++
		05-2119	−	+ (R)	+	++
		05-2120	−	+ (R)	++	+++
Low pH	3.5	05-2137	−	+ (R)	+	++
		05-2138	−	+ (LR)	+	++
		05-2139	−	+ (R)	+	++
		05-2140	−	+ (LR)	+	++
Buffered tricaine	7.3	05-2121	−	−	+	NC
		05-2122	−	−	+	NC
		05-2123	−	−	+	NC
		05-2124	−	−	+	NC

depth of focus) using a digital camera (Nikon, Tokyo, Japan; D1x) with a 60-mm macro lens. Digital photographs were taken in daylight mode. The images shot with the filter were set on open bulb mode while the shutter was manually held open for 60–90 s. The UV light source (Mineralight; Model UVGL-58) was held at a 45° angle to the fish at a distance that provided optimum fluorescence without interfering with image quality. Photographs were taken using either the long (365-nm) or short (254-nm) wavelength setting depending on which displayed the effects most clearly.

Digital images of Pacific halibut, walleye pollock, and northern rock soles were taken under 254-nm illumination with a Canon PowerShot S50 digital camera at an ISO setting of 100 at F6.3 and using a 10-s exposure without a yellow barrier filter. A VANOX AHS-3 photomicroscope and an Olympus C-35AD-4 camera were used to capture light micrograph images of histological sections; light micrographs of formalin-fixed particles from the treatment water were photographed with an inverted phase-contrast microscope (Nikon; Diaphot) and a Nikon D1x camera.

Results

Effect of Unbuffered Tricaine on Epidermal and Corneal Integrity

All Nile tilapia and channel catfish were alive at the end of the standard exposure period for all treatments (i.e., unbuffered tricaine, low pH, buffered tricaine); thus, the anesthetic doses used were nonlethal. Exposure of Nile tilapia to 300-mg/L unbuffered tricaine for 35 min caused significant corneal damage (Table 1). The eyes of all four fish had varying degrees of corneal ulceration that typically involved a significant area of the cornea (Figure 1A). Both eyes were

affected in two fish, and only one eye was affected in two fish. Exposure to low pH also caused a similar amount of corneal ulceration (Table 1). Exposure to buffered tricaine (300-mg/L tricaine + 600-mg/L sodium bicarbonate) did not cause corneal damage (Figure 1B).

All Nile tilapia used in the experiment had some minor skin ulceration before being exposed to any treatment (Table 1). However, all four fish exposed to unbuffered tricaine clearly had more severe damage after this treatment. This included ulcers in areas that previously were not ulcerated, especially on the tips of the fins and the posterior edge of some scales; some pre-existing ulcers (i.e., present before exposure to unbuffered tricaine) were also larger after exposure to unbuffered tricaine. Exposure to low pH also caused skin ulceration; the damage appeared to be as severe as that caused by exposure to unbuffered tricaine. When Nile tilapia were exposed to either unbuffered tricaine or low pH, we observed particles in the treatment solution, which were microscopically identified as rafts of detached epidermal epithelium (Figure 1C). Exposure of Nile tilapia to buffered tricaine did not cause skin damage. No skin particles were observed in water of fish treated with buffered tricaine.

Exposure of channel catfish to 200-mg/L unbuffered tricaine for 25 min caused visible fluorescence in two of four fish; one eye was affected in each of these fish (Table 2). However, the fluorescence was a relatively light yellow-green coloration (Figure 1D) and was not as strong as that typically seen with the Nile tilapia ulcers or in previous cases of frank ulceration that we have documented in other fish (Noga and Udomkusonsri 2002; Udomkusonsri et al. 2004, Udomkusonsri and Noga 2005). All four fish also had light yellow-
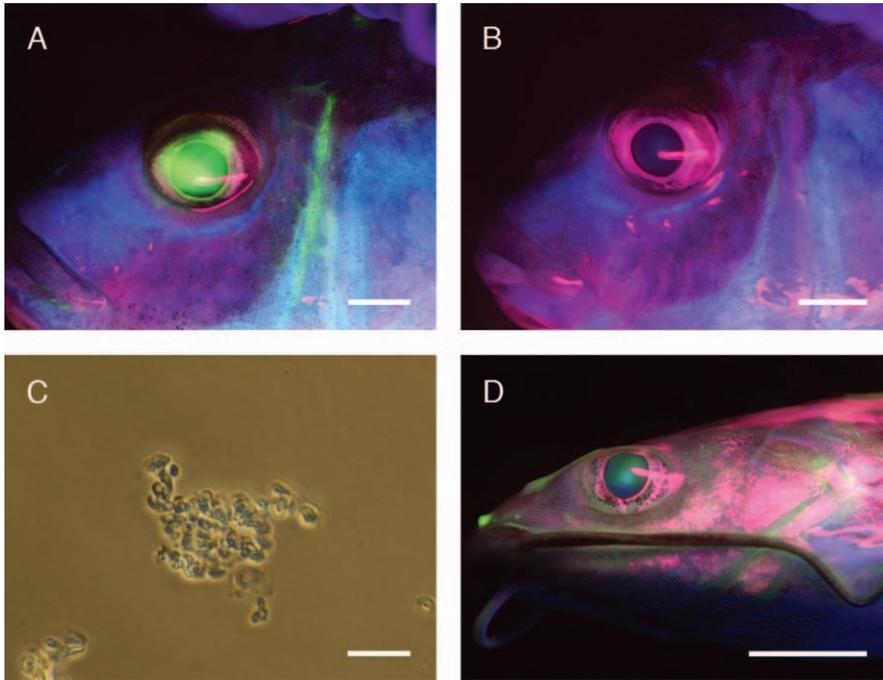
FIGURE 1.—Effect of exposure to unbuffered tricaine on the cornea and epidermis of fish subjected to the fluorescein test: **(A)** photograph of a Nile tilapia exposed to 300-mg/L unbuffered tricaine, exhibiting a strongly green fluorescent (ulcerated) area in the cornea (bar = 1 cm); **(B)** photograph of a Nile tilapia exposed to 300-mg/L buffered tricaine, lacking any corneal damage (bar = 1 cm); **(C)** wet mount of a skin particle observed in unbuffered tricaine solution used to sedate a Nile tilapia (formalin fixed; bar = 20 μm); and **(D)** photograph of a channel catfish exposed to 200-mg/L unbuffered tricaine for 25 min, showing areas of weaker green fluorescence than that in panel (A), especially evident on the cornea (bar = 1 cm).

green fluorescence on parts of the skin. Exposure to low pH did not cause corneal damage detectable by fluorescence, but a similar degree of mild fluorescence (as seen with unbuffered tricaine) appeared to be present on the skin of all four fish (Table 2). Histological examination of corneas and skin from areas that displayed the lighter green fluorescence indicated the presence of erosion but not ulceration

TABLE 2.—Effect of unbuffered tricaine (200 mg/L), buffered tricaine (200-mg/L tricaine + 400-mg/L sodium bicarbonate), and low pH on skin and corneal integrity in channel catfish, as measured by presence of erosions. Fish were exposed to each treatment for 25 min. Corneal and skin damage was recorded both before and immediately after experimental exposure. All responses refer to the amount of light-green fluorescence (i.e., erosion: − = none; + = some; ++ = more severe; +++ = most severe; NC = no change compared with pretreatment score; L = left eye; R = right eye; and LR = both eyes).

Treatment	pH	Fish number	Cornea Before	Cornea After	Skin Before	Skin After
Unbuffered tricaine	3.5	05-2105	−	+ (R)	+	+++
		05-2106	−	−	−	++
		05-2107	−	+ (L)	−	++
		05-2108	−	−	+	++
Low pH	3.5	05-2141	−	−	−	++
		05-2142	−	−	−	+++
		05-2143	−	−	+	+++
		05-2144	−	−	+	+++
Buffered tricaine	7.3	05-2109	−	−	+	NC
		05-2110	−	−	−	−
		05-2111	−	−	+	NC
		05-2112	-	-	+	NC
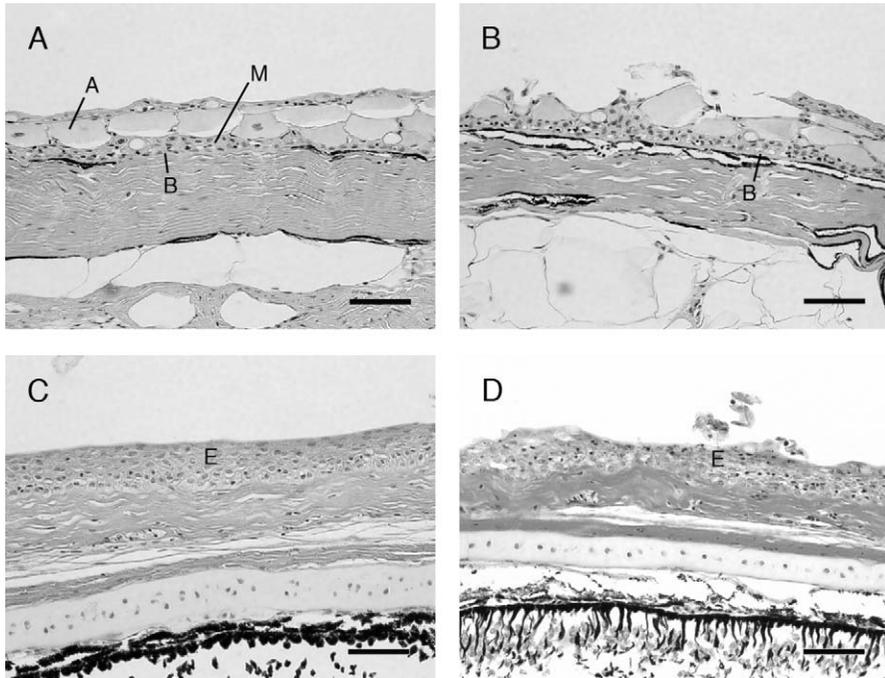
FIGURE 2.—Histopathology of skin and cornea from channel catfish exposed to a 200-mg/L concentration of buffered or unbuffered tricaine (bars = 100 μm; M = Malpighian cells; A = alarm cells; B = basement membrane; E = epithelium): **(A)** skin after exposure to buffered tricaine, showing M and A cells present in E; **(B)** skin after exposure to unbuffered tricaine, exhibiting a loss of E that does not extend to the basement membrane (thus, the skin is not ulcerated); **(C)** cornea after exposure to buffered tricaine; and **(D)** cornea after exposure to unbuffered tricaine, exhibiting an E layer that is eroded rather than ulcerated.

(Figure 2). Buffered tricaine (200-mg/L tricaine + 400-mg/L sodium bicarbonate) did not cause any apparent corneal or skin erosion.

The same channel catfish that were scored for erosions (Table 2) were also scored for ulcers (Table 3). Most had a few small skin ulcers before being exposed to any treatment (Table 3). However, no channel catfish exposed to unbuffered tricaine had more severe ulceration after exposure (Table 3). However, in all four fish exposed to low pH, some of

TABLE 3.—Effect of unbuffered tricaine (200 mg/L), buffered tricaine (200-mg/L tricaine + 400-mg/L sodium bicarbonate), and low pH on skin and corneal integrity in channel catfish, as measured by presence of ulcerations. Fish were exposed to each treatment for 25 min. Corneal and skin damage was recorded both before and immediately after experimental exposure. All responses refer to the amount of bright yellow-green fluorescence (i.e., ulceration: − = none; + = some; ++ = more severe; +++ = most severe; and NC = no change compared with pretreatment score). These are the same fish described in Table 2.

Treatment	pH	Fish number	Cornea		Skin	
			Before	After	Before	After
Unbuffered tricaine	3.5	05-2105	−	−	+	NC
		05-2106	−	−	−	−
		05-2107	−	−	−	−
		05-2108	−	−	+	NC
Low pH	3.5	05-2141	−	−	+	++
		05-2142	−	−	+	++
		05-2143	−	−	+	++
		05-2144	−	−	+	++
Buffered tricaine	7.3	05-2109	−	−	+	NC
		05-2110	−	−	−	−
		05-2111	−	−	+	NC
		05-2112	−	−	+	NC

TABLE 4.—Effect of tricaine (T) exposure sequence on the ability of fluorescein (F) to detect experimentally induced ulcers in three fish species. Fish (6 fish/species for each treatment) were exposed to various baths in the sequences shown (R = seawater rinse; ASW = acidic seawater, pH 6.7). A positive (+) detection of ulceration was indicated by green fluorescence under 254-nm illumination (− = no fluorescence). The use of buffered T after rinsing of excess F inhibited ulcer detection (treatments 4 and 6; sign test: $n = 6$, $P = 0.031$).

Treatment	Exposure sequence	Pacific halibut	Walleye pollock	Northern rock sole
1	R→F→R	+	+	+
2	**T**→F→R	+	+	+
3	F→**T**→R	+	+	+
4	F→R→**T**	−	−	−
5	F→R→ASW	+	+	+
6	F→R→**T**→R	−	−	−

the small focal ulcers present before exposure became larger. Exposure to buffered tricaine did not cause any corneal or skin ulceration.

Effect of Tricaine Exposure Sequence on Fidelity of the Fluorescein Test

All Pacific halibut, walleye pollock, and northern rock soles were experimentally wounded with a scalpel blade and had ulcers that were clearly evident via histopathology (data not shown); thus, all fish were expected to be fluorescein positive. As expected, fish exposed to fluorescein without tricaine treatment resulted in a clearly visible ulcer (treatment 1; Table 4; Figure 3A). Similarly, fish that were exposed to

fluorescein either before or after exposure to tricaine but before the rinsing of excess fluorescein were positive (treatments 2 and 3). However, exposure of a fluorescein-treated wound to buffered tricaine after the excess fluorescein had rinsed resulted in the total abrogation of the fluorescence response (treatment 4; Figure 3B). This was not attributable to a decrease in pH caused by addition of tricaine to seawater, because adjusting the pH of seawater to the same level did not have any effect on fluorescence (treatment 5). Also, rinsing the wound to remove the tricaine (treatment 6) had no effect, suggesting that the reaction of tricaine with fluorescein was irreversible (at least in terms of the ability to detect skin ulcers).

Discussion

Effect of Unbuffered Tricaine on Epidermal and Corneal Integrity

We were unable to photographically record the same fish before and after an experimental treatment because photographing the fish required it to be out of the water for at least several minutes. The time delay involved in taking the photograph, the stress associated with additional handling, and the fact that acute stress can cause skin and corneal ulceration in as little as 15 min (Udomkusonsri and Noga 2005) meant that photography before treatment would have added a potentially significant confounding variable to the response. The aggressive nature of Nile tilapia was especially evident from the presence of many small ulcers on the body and fins of all fish used in the experiments. Most channel catfish also had small ulcers before experi-
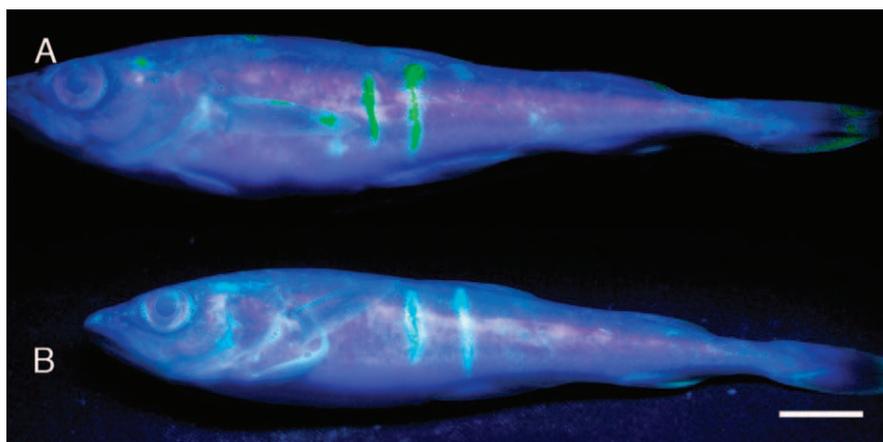
FIGURE 3.—Detection of skin ulcers in walleye pollock after exposure to fluorescein and tricaine in varying treatment sequences (bar = 1 cm): **(A)** experimentally induced ulcers (green vertical lines) on a fish exposed to tricaine and then fluorescein (treatment 2) and some additional ulcers (green foci) present on the caudal fin and other areas; and **(B)** experimentally induced ulcers (blue vertical lines, an artifact of tissue autofluorescence during photography) on a fish exposed to fluorescein, rinsed in seawater, and then exposed to tricaine (treatment 4); these ulcers were not visible under normal light.

mental treatment (Tables 1, 3). These findings were similar to our previous observation of skin damage in presumably healthy fish (Noga and Udomkusonsri 2002) and probably reflect damage that may occur as part of daily behavior. Netting the fish or other manipulations during the experimental procedures is unlikely to have caused these lesions, because there was no change in the lesions in the buffered tricaine group. Fish were sampled from populations that had been clinically normal for over 2 months; thus, such damage probably heals without consequence in otherwise healthy individuals.

Despite the presence of some skin damage on most fish before the experimental treatments, it was very clear that both unbuffered tricaine and low-pH treatments caused epithelial damage (Tables 1–3; Figure 1), whereas buffered tricaine caused no grossly observable effect. Channel catfish exhibited milder damage than did Nile tilapia, but channel catfish were exposed to a lower dose of tricaine for a shorter time period (200 mg/L for 25 min) than were Nile tilapia (300 mg/L for 35 min). Both doses were higher than typical recommended doses, yet were still nonlethal. This is probably due to the variable response to tricaine that can be influenced by water quality and fish species, size, and density. For this reason, we used the clinical response of the fish to determine the maximum nonlethal dosage to employ in experiments. The amount of spontaneous skin ulceration resulting from acute confinement stress also varies considerably among different fish species (Udomkusonsri and Noga 2005). While we did not quantify the relative severity of this quite complex pattern of skin and corneal damage, it was clear from our qualitative observations that the damage caused by low pH was generally similar to that observed for exposure to unbuffered tricaine in Nile tilapia. In channel catfish, corneal erosions were only observed in unbuffered tricaine (Table 2), while skin ulcers were exacerbated only in low pH (Table 3). We previously found that unbuffered tricaine (euthanizing dose of 1,000 mg/L for 10 min at pH 3.3) caused rapid detachment of the flagellate ectoparasite *Ichthyobodo necator* from the skin of sunshine bass (male striped bass *Morone saxatilis* × female white bass *M. chrysops*), while exposure to low pH only (pH = 3.3) had no detectable effect on the parasites (Callahan and Noga 2002). In that study, we did not observe any skin damage via histological examination due to unbuffered tricaine, but fluorescein was not used to assess damage. In Nile tilapia, skin ulcers were highly focal and thus might have been easily missed during routine light microscopy, which can only examine a very small area. This illustrates a

major advantage of using the fluorescein technique instead of histological evaluation of skin integrity.

Our qualitative evaluation of the effects of unbuffered tricaine or low pH showed that both treatments caused various degrees of epithelial damage in both Nile tilapia and channel catfish. A more detailed quantitative evaluation of the damage caused by tricaine versus pH alone is required to determine whether tricaine plays a direct role in skin damage (rather than simply causing damage due to its low pH). However, Rabinovitch and DeStefano (1974, 1975a) showed that cationic anesthetics inhibit cell-to-substrate adhesion and spreading of cultured cells. A local anesthetic, lidocaine, has been used to detach cells in tissue culture (Rabinovitch and DeStefano 1975b). Lidocaine, like other typical local anesthetics (e.g., benzocaine, which is closely related to tricaine), blocks sodium influx and thereby prevents nerve conductance. Both benzocaine (Summerfelt and Smith 1990) and lidocaine have also been used as fish anesthetics. Lidocaine has been used at a concentration of 250–350 mg/L (Carrasco et al. 1984). At a concentration of 12 mM (2,800 mg/L), lidocaine detached several types of mammalian cells within 5–15 min at room temperature (Rabinovitch and DeStefano 1975b). However, this lidocaine concentration was about 10 times higher than the tricaine concentration at which epithelial sloughing was observed in our study.

A number of other studies have shown that exposure to low pH causes skin erosion or ulceration, but all of these studies exposed fish to the acid stress for much longer periods than used here. Exposure of juvenile brown trout *Salmo trutta* to progressively lower pH (reduction to a pH of 4.2 over 5 d) caused epithelial sloughing (Segner et al. 1988). Daye and Garside (1976) described epidermal necrosis and sloughing in fingerling brook trout *Salvelinus fontinalis* exposed for 7 d to pH 3.2 or 2.2. Even a relatively mild decrease in pH (from 7.5 to either 6.0 or 5.0) damaged the skin in common carp *Cyprinus carpio* (Iger and Wendelaar Bonga 1994). This damage began as degeneration and necrosis of the upper epithelial layers. Later, after the first few days, apoptosis was more prominent. While the initial response was thinning of the epithelium (loss of pavement and mucous cells), it was followed after 14 d by a significant thickening of the epithelium (Iger and Wendelaar Bonga 1994).

Exposure of Arctic char *Salvelinus alpinus* to acid stress (pH of 4.5 for 2 weeks) resulted in infections of water mold *Saprolegnia* spp. in many fish within 4 d of return to normal pH (Jones et al. 1987). A natural acute acid stress (pH reduction from 6.2 to 3.8 over 7 d) that occurs in some Australian estuaries is associated with severe epidermal hyperplasia, dermatitis, and second-

ary water mold infection (Callinan et al. 2005). Callinan et al. (2005) did not mention the presence of primary ulceration in empire gudgeon *Hypseleotris compressa* exposed to this acidic water, but they did observe severe focal epidermal hyperplasia that was usually most pronounced at scale margins and that involved most of the scaled body surface. This reactive hyperplasia might be a sequel to the epidermal ulceration we commonly observed at the edges of scales in Nile tilapia.

We do not know whether epithelial loss in the presence of unbuffered tricaine or acid pH was due to direct toxicity to epithelial cells. Acute stress, such as that caused by acute confinement, can induce rapid and severe loss of corneal and epidermal epithelium (Udomkusonsri et al. 2004). This response appears to be at least partly hormonally mediated (Noga et al. 1998). Acclimation probably plays a very important role in response to both tricaine and pH stress. For example, short-term depression in pH may cause mortalities in exposed nonacclimated fish (Mount et al. 1990), while acclimated fish can sometimes tolerate pH levels as low as 2.0 for at least several days (Callinan et al. 2005).

Effect of Tricaine Exposure Sequence on Fidelity of the Fluorescein Test

The quenching effect of unbuffered tricaine on fluorescence was first observed in a study on fish wounding caused by net capture (Davis and Ottmar 2006). Problems with the use of unbuffered and buffered tricaine in the fluorescein test prompted us to investigate tricaine effects in more detail. The sequence in which fluorescein was used when sedating fish with buffered tricaine had a dramatic effect on the ability to detect ulcers. Buffered tricaine caused a total loss of fluorescence in ulcers that had been rinsed to remove excess fluorescein. Rinsing to remove excess fluorescein is necessary to eliminate fluorescein carryover, which can cause fluorescence on skin without ulceration. When fluorescein rinsing was not performed, no quenching was observed; this indicates that the quenching reaction can be overcome by the presence of excess fluorescein. Buffered tricaine in seawater is mildly acidic, but ASW alone did not induce fluorescence quenching, thus eliminating acid conditions as a cause for quenching. Also, seawater of normal pH did not cause quenching. Possible effects of bicarbonate on fluorescein were not studied. Since the loss of fluorescence associated with tricaine could not be reversed by rinsing with seawater, quenching may have been caused by a chemical interaction between tricaine and fluorescein. The chemistry for loss of fluorescein fluorescence and all possible interactions of

physical and chemical conditions that would enhance this loss are not known. Further testing of the quenching effects of unbuffered and buffered tricaine and bicarbonate are required to positively identify the causes of fluorescence loss. While quantitative in vitro chemical tests could be performed to study fluorescein quenching, it will also be necessary to consider in vivo tests, since interactions with ulcers and rinsing are important. Our results clearly show that the fluorescein test for skin ulcers can be compromised by the use of buffered tricaine for anesthesia or euthanasia.

Recommendations

One practical consideration drawn from our findings is that for the fluorescein test to work properly, the fluorescein-treated and rinsed wounds must not be exposed to tricaine, since this can quench the signal and cause a false-negative result. Another important finding is that the use of unbuffered tricaine to sedate fish can cause serious skin and corneal damage. Severe epidermal and corneal changes occurred at nonlethal tricaine concentrations. Such erosions and ulcerations might provide a ready portal of entry for opportunistic microbes, especially bacteria and water molds. Acute skin ulceration can greatly increase susceptibility to severe, lethal skin infections (Udomkusonsri and Noga 2005). Use of unbuffered tricaine to euthanize fish for clinical workup could also lead to significant misdiagnoses, such as reporting the presence of severe epidermal or corneal damage that is not actually part of the case's pathology. The severe damage to these tissues might also result in significant loss of skin-dwelling pathogens, especially ectoparasites; since the skin is one of the most common sites for parasitosis, such a loss could severely compromise a diagnosis.

Another consideration is that the general recommendations for buffering tricaine might require modification. Some have recommended buffering of tricaine solutions whenever alkalinity is lower than 50 mg/L (Ohr 1976; Summerfelt and Smith 1990). We did not examine the effect of unbuffered tricaine solutions in more buffered waters and do not know whether similar effects occur at less drastic pH changes, but it seems prudent to always buffer tricaine with sodium bicarbonate to prevent pH changes that lead to iatrogenic effects. Our data also suggest that relatively transient exposure to suboptimal pH can cause significant epidermal and corneal damage; therefore, even very short-term pH changes should be avoided.

We further substantiated that clinically normal fish can have a significant amount of focal skin ulceration. Determining the "acceptable" versus "infection-prone" levels of skin damage might prove useful in the monitoring of population health. Since the fluorescein

test appears to also detect skin damage that is milder than frank ulceration (i.e., lesions in channel catfish were due mainly to erosion and had a much lower intensity of fluorescence than ulcerated areas), this extends the potential sensitivity of the fluorescein test in assessing epithelial integrity.

Acknowledgments

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