

Short communication

## Tricaine dramatically reduces the ability to diagnose protozoan ectoparasite (*Ichthyobodo necator*) infections

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Tricaine (tricaine methanesulphonate), also known as MS-222, is a widely used anaesthetic and euthanasia agent for fish and amphibians. Tricaine is particularly effective in fish because it is highly water- and lipid-soluble and readily crosses the gill membrane (Hunn & Allen 1974; Treves-Brown 2000). Additionally, tricaine moves bidirectionally across the gills, allowing rapid removal from the body and a quick post-anaesthetic recovery (Hunn, Schoettger & Willford 1968). For euthanasia, tricaine is often used unbuffered, but when used as an anaesthetic, tricaine is commonly buffered because it causes a significant decrease in pH at effective dosages.

Tricaine is advocated as a chemical restraint for clinical evaluation of fish (Post 1987; Brown 1993; Noga 1996), including the collection of gill and skin samples for diagnosing common ectoparasites. One of the most common ectoparasites of cultured fish is the kinetoplastid flagellate, *Ichthyobodo necator* (Hennequy 1883) (Robertson 1985; Urawa, Ueki & Karlsbakk 1998). *Ichthyobodo necator* (costia) primarily infects young fish, causing high mortalities if left untreated (Urawa, Ueki, Nakai & Yamasaki 1991; Grignard, Melard & Kestemont 1996). While clinically evaluating fish for the presence of this parasite, it was discovered that

unbuffered tricaine caused the rapid detachment and mortality of *Ichthyobodo* trophonts from the skin after 5 min of immersion. This suggested that the use of tricaine has a serious impact on the clinical evaluation of skin and gill infections for this parasite. To understand this phenomenon, the effect of varying concentrations of both buffered and unbuffered tricaine on the motility and attachment of *I. necator* was examined.

Hybrid striped bass (*Morone saxatilis* male × *M. chrysops* female), subclinically infected with *I. necator*, were obtained from a local producer. Fish were housed in 300 L aquaria at low temperature (14 °C) and high density (one fish, 70–85 mm length, per 5 L water); they were fed a restricted diet to inhibit growth. Outbreaks of ichthyobodosis occurred spontaneously in the laboratory during spring and autumn, but could be induced at other times of the year by either increasing or decreasing the temperature several degrees for a 24-h period before returning to 14 °C. All fish used for this study were approximately 1 year in age.

Sick fish with typical signs of ichthyobodosis (lethargic, disoriented, dark pigmentation, clamped fins, reddening at the base of the dorsal fin) were screened for severe *I. necator* infections. Infections on the body primarily occurred around the base of the dorsal fin. Therefore, samples were removed from this region for each experiment. Because of the small size of the fish, the number of scales removed with each sampling varied between two and five. Infection intensity was assessed by placing scales in aquarium water on a microscope slide, covering with a plastic coverslip, and examining the sample

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at 100× using a Nikon inverted phase contrast microscope. Only fish with severe infections [ $>1000$  parasites  $(\text{mm}^2)^{-1}$ ] were used for experiments. Because of the variability in infectivity between fish, a split plot experimental design was employed.

Solutions of unbuffered tricaine ( $1000 \text{ mg L}^{-1}$ ) were made by dissolving and diluting tricaine in aquarium water ( $0.036 \text{ mg L}^{-1}$  hardness,  $0.054 \text{ mg L}^{-1}$  alkalinity, pH 6.3). An equivalent stock solution of tricaine ( $1000 \text{ mg L}^{-1}$ ) was buffered with  $2000 \text{ mg L}^{-1}$  sodium bicarbonate and also diluted in aquarium water. Final test concentrations for both tricaine dilution series were 1000, 250, 100, 50, 25 and  $0 \text{ mg L}^{-1}$ , respectively. The diluted solutions were then coded by another investigator as either U1–U6 or B1–B6 so that observations were carried out blindly. Aliquots ( $50 \mu\text{L}$ ) of the randomly assigned tricaine dilutions were added to the bottom of  $75 \text{ cm}^2$  Corning™ tissue culture flasks (top side removed). Each treatment was replicated five times (Table 1).

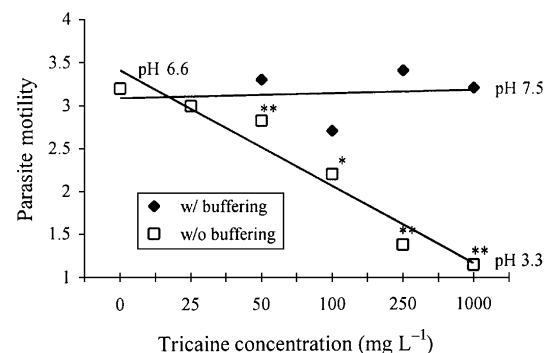
Samples containing two to five infected scales were added to each tricaine aliquot. The scales were

incubated at room temperature for 5 min and then covered with a plastic coverslip. Parasite motility (percentage of visible parasites moving) was quickly scored as follows: 1 = 0–25% motility, 2 = 25–50% motility, 3 = 50–75% motility, 4 = 75–100% motility. Half values were used when a 1 could not be distinguished from a 2, a 2 from a 3, and so on. For consistency, only the area around one infected scale was scored for parasite motility. Infected scales from 12 fish were scored, and the differences among the test dilutions for both the buffered and unbuffered tricaine dilution series were log-transformed and analysed using ANOVA.

In preliminary experiments, it was determined that as the unbuffered tricaine concentration increased, the pH decreased to as low as 3.3 at  $1000 \text{ mg L}^{-1}$  tricaine. In contrast, all buffered tricaine solutions remained between pH 6.6 and 7.5 (Fig. 1). To determine the effect of pH on parasite motility, aquarium water was adjusted to either pH 7.5 or 3.3 using  $0.1 \text{ N HCl}$ . Duplicate,  $50 \mu\text{L}$  drops of each pH solution were placed on a flask as described before. Scales from the fish were added to each replicate pH solution and parasite motility was assayed as described. Parasite motility at the two pHs was compared using the Wilcoxon rank test (Steel, Torrie & Dickey 1997). The pHs in the second set of replicate solutions were measured to confirm that they did not substantially change after the addition of scale tissue.

**Table 1** The split plot design used for examining the effect of tricaine on *Ichthyobodo necator* motility. Each tricaine dose from the buffered (B) and unbuffered (U) dilution series was replicated five times. Dosages were randomly assigned numbers (U1–U6 or B1–B6) and aliquoted as indicated below

Flask number	Treatments
1	U1, U2, U5 U2, U5, U6
2	U1, U2, U4 U1, U4, U6
3	U4, U5, U6 U2, U3, U4
4	U1, U3, U5 U3, U4, U5
5	U1, U3, U6 U2, U3, U6
6	B1, B2, B5 B2, B5, B6
7	B1, B2, B4 B1, B4, B6
8	B4, B5, B6 B2, B3, B4
9	B1, B3, B5 B3, B4, B5
10	B1, B3, B6 B2, B3, B6



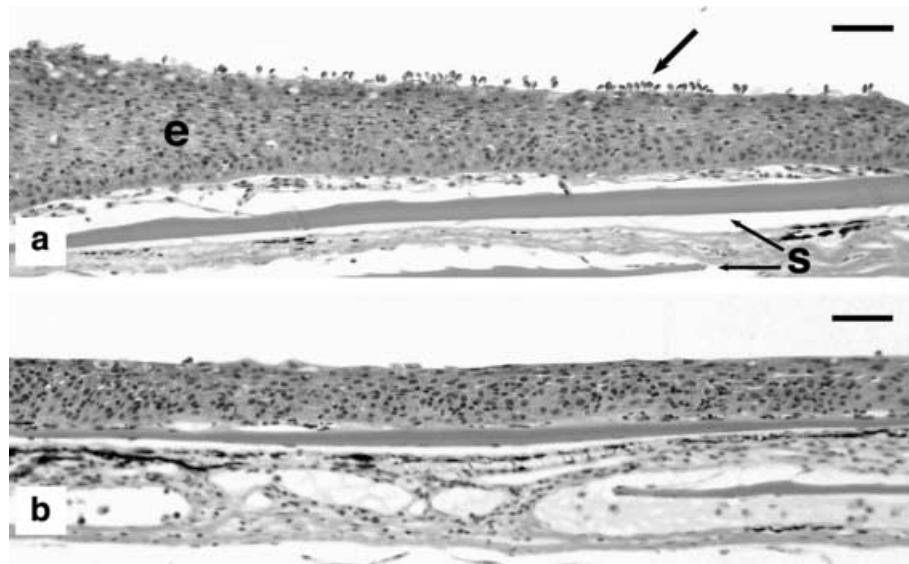
**Figure 1** The motility of *Ichthyobodo necator* after exposure to different concentrations of buffered or unbuffered tricaine solutions, where 1 = 0–25%, 2 = 25–50%, 3 = 50–75%, 4 = 75–100% motility. Motility scores between the same buffered and unbuffered concentrations were compared using ANOVA. Each point on the graph represents the mean score from 12 fish. (\*) Denotes significance at  $<0.01$ , (\*\*) denotes significance at  $<0.0001$ . The pH of the 0 and  $1000 \text{ mg L}^{-1}$  concentrations are indicated above the corresponding point.

To determine how euthanizing fish in buffered and unbuffered tricaine affected the attachment of *I. necator* to the skin, four heavily infected hybrid striped bass were placed into a solution of either 1000 mg L<sup>-1</sup> tricaine with 2000 mg L<sup>-1</sup> sodium bicarbonate or 1000 mg L<sup>-1</sup> tricaine alone. After 10 min, the fish were fixed in 10% neutral buffered formalin. After 7 days, the fish were decalcified in ethylenediamine tetraacetic acid for an additional 7 days. All fish were sectioned at the posterior border of the dorsal fin using standard histological procedures. Slides were photographed with an Olympus VANOX AHS-3 photomicroscope using an Olympus C-35AD-4 camera.

There was a dramatic, dose-dependent decrease in *I. necator* motility with increasing unbuffered tricaine concentrations (Fig. 1). In contrast, parasite motility in buffered tricaine solutions remained unchanged. The decreased motility with unbuffered tricaine was significantly different from that of buffered tricaine at all concentrations greater than 25 mg L<sup>-1</sup> (Fig. 1). Similarly, when fish were euthanized in 1000 mg L<sup>-1</sup> buffered tricaine, *I. necator* remained attached to the skin (Fig. 2a), but completely detached when fish were euthanized in unbuffered tricaine (Fig. 2b). The low pH alone was not responsible for this effect, as there was no difference in the motility of *I. necator* incubated in

aquarium water at pH 3.3 versus 7.5 ( $P=0.125$ ,  $n=13$ ).

The recommended sedative dose of tricaine for most fish ranges from 10 to 40 mg L<sup>-1</sup>, while the anaesthetic dose is 50–250 mg L<sup>-1</sup> for immersion or 1000 mg L<sup>-1</sup> sprayed on the gills of large fish (Summerfelt & Smith 1990; Ross & Ross 1999). Concentrations commonly used for euthanasia range from 150 to 1000 mg L<sup>-1</sup> (Noga 1996). Our results demonstrated that unbuffered tricaine concentrations as low as 50 mg L<sup>-1</sup> significantly affect parasite motility and at higher doses cause *I. necator* to rapidly detach from the host tissue and become immobilized. Our results also suggest that this is not directly caused by the drop in pH, but rather by a decrease in the unionized form of tricaine. Tricaine methansulphonate is a weak base with a p*K*<sub>a</sub> of 3.5 (Treves-Brown 2000). In unbuffered media, a much higher proportion of tricaine would be in the ionized form. As the concentration of unbuffered tricaine increases, the pH of the solution approaches the p*K*<sub>a</sub>, which would decrease the amount of drug that can pass through lipophilic cell membranes (Allen & Hunn 1986). As a result, tricaine uptake would need to be via an active transport mechanism (ion channel) across the membrane. It appears that *I. necator* may be able to prevent the passive transport of the lipophilic (unionized) form of the drug, but may



**Figure 2** Histological cross-section of the skin of hybrid striped bass. (a) Attached *Ichthyobodo necator* trophonts (arrow) after fish were killed in buffered tricaine (1000 + 2000 mg L<sup>-1</sup> sodium bicarbonate). The epidermis (e) and scales (s) are indicated. (b) The absence of *I. necator* trophonts after fish were killed in unbuffered tricaine (1000 mg L<sup>-1</sup>) (H&E, bar = 40 µm).

have an active transport mechanism that allows the ionized form to enter into the cell.

Many guidelines for the collection of clinical samples from fish include appropriate tricaine concentrations and buffering requirements (Summerfelt & Smith 1990; Noga 1996; Ross & Ross 1999). However, some others do not (Post 1987; Brown 1993; Reimschuessel 1997). As a result, tricaine may be used in inappropriately large concentrations without buffering. Our findings indicate that tricaine should always be buffered when fish are to be clinically evaluated, particularly in water with low alkalinity. Buffering is less important when assaying parasites of marine fish because of the higher buffering capacity of seawater.

As with all skin and gill ectoparasites, the severity of clinical signs and outcome for recovery is directly related to the number of parasites present. Thus, it is essential that an accurate estimate of parasite load be determined during the clinical examination. If tricaine is unbuffered, ectoparasites might be reduced in number or totally lost, providing an incorrect assessment of their clinical importance. To our knowledge, this is the first report that tricaine can affect protozoa. However, it has been known for some time that tricaine can narcotize metazoans (Delly 1985). Tricaine has previously been reported to anaesthetize the rotifer, *Branchionus calyciflorus* (Nogrady & Keshmirian 1986) and the nematode, *Caenorhabditis elegans* (McCarter, Bartlett, Dang & Schedl 1999). We have also narcotized leeches (*Myzobdella*) with tricaine (E. J. Noga & R. A. Bullis, personal communication) and have observed that a high concentration of unbuffered tricaine narcotizes *Trichodina*, another common ectoparasite of fish, although they are not completely immobilized like *I. necator* (H. A. Callahan, personal communication). Future work should examine how the ionized form of tricaine anaesthetizes *I. necator* and if tricaine affects other protozoan parasites as well.

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