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Comparative efficacy of MS-222 and benzocaine as anaesthetics under simulated transport conditions of a tropical ornamental fish *Puntius filamentosus* (Valenciennes)

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Abstract

There is a growing commercial interest in the fish, *Puntius filamentosus*, in the ornamental fish trade in India and elsewhere. The trade is, however, hampered by severe mortalities during transport of the fish owing to insufficient data available on the use of anaesthetics. To resolve this problem, we evaluated the efficacy of two anaesthetics, MS-222 and benzocaine, in sedating *P. filamentosus* in simulated transportation experiments and used stress response parameters such as cortisol and blood glucose levels to perform assessments. We observed that MS-222 at 40 mg L^{-1} and benzocaine at 20 mg L^{-1} were sufficient to induce sedation for 48 h. Above these concentrations, both the anaesthetics adversely affected the fish and resulted in mortalities. Both anaesthetics significantly lowered the blood cortisol and glucose levels compared with the unsedated controls. Importantly, the anaesthetics treatment significantly lowered the post-transport mortality in the fish. The results of the study show that MS-222 and benzocaine could be used as sedatives to alleviate transport-related stress in *P. filamentosus* to improve their post-transport survival and hence reduce economic loss.

Keywords: ornamental fish, *Puntius filamentosus*, simulated transport, stress, anaesthetics, post-transport survival

Introduction

Ornamental fish export in India is a growing industry in tune with the increased interest for ornamental fish-keeping all over the world. The export of ornamental fish from India mainly focuses on freshwater indigenous ornamental species (Ramachandran 2002). It is an inevitable part of modern aquaculture practices to stress the animals by containment, handling, sorting, transportation, periodic low oxygen or high ammonia, etc., and the associated post-transport mortality (Donaldson 1981; Wendelaar Bonga 1997; Carneiro & Urbinati 2001; Pavlidis, Angellotti, Papandroulakis & Divanach 2003; Southgate 2008). The transport logistics involved inevitably stress the animals, causing post-transport mortality, and exporters are expected to compensate customers for losses exceeding 5% death on arrival (DOA) industry standard (Lim, Dhert & Sorgeloos 2003). The bulk of post-transport stress-mediated mortality occurs during the 1-week recovery period and enhancing the stress resistance of the ornamental fish is insufficiently addressed. In aquaculture operations, anaesthetics are commonly used to minimize stress and reduce the physical injury to fish during the various handling procedures. The choice of anaesthetics is often dependent on considerations such as availability, cost-effectiveness, ease of use, nature of the study and user safety (Cho & Heath 2000; Mylonas, Cardinaletti, Sigelaki & Polzonetti-Magni 2005). Before recommending the use of a particular anaesthetic, a range of stress-response indices must be measured to assess its efficacy. Until recently, the packing and live transport of ornamental fish did not involve the use of any chemical anaesthetics. However, the increased concern for fish health and product quality makes the use of anaesthetics inevitable to reduce the stress during handling and transportation procedures. To date, much of the information on the use of anaesthetics in fish has been derived from studies on salmonids (Pickering 1992; Iversen, Finstad, McKinleyc & Eliassen 2003; Pirhonen & Schreck 2003; Iversen, Eliassen & Finstad 2009) and other temperate species (Mattson & Ripple 1989). Investigations on high-latitude species have also yielded valuable information (Wells, Mcintyre, Morgan & Davies 1986), and except for a few reports, the use of anaesthetics in the transport of ornamental fish remains largely unaddressed (Teo, Chen & Lee 1989; Guo, Teo & Chen 1995a, b; Kaiser & Vine 1998; Crosby, Hill, Watson & Yanong 2006).

In the international ornamental fish market, the Indian tiger barb *Puntius filamentosus* is very popular, known by different names such as Filament barb, DMK fish, Long fin barb, Feather fin barb, etc. The export figures reveal that the contribution of Indian tiger barb to the total ornamental fish export from India is increasing (Pramod, Mini & Ramachandran 2002). In this study, we compared the efficacy of the anaesthetics, MS-222 (tricaine methanesulphonate) and benzocaine (ethyl aminobenzoate), in *P. filamentosus*, to alleviate stress during a simulated transportation experiment. Biochemical stress indices such as plasma cortisol level and glucose levels were used to assess the effect of the anaesthetics.

Materials and methods

Experiment I: optimal dose of MS-222 and benzocaine as anaesthetics

Indian tiger barb *P. filamentosus* (average body length 126 ± 8 mm and body weight, 12 ± 1 g), collected from Chalakudy river systems, were brought to the laboratory and acclimatized for 1 week in FRP tanks with a 5000 L capacity. The fish were fed a commercial pellet feed for 1 week and feeding was terminated 24 h before the experiment. Two commercial anaesthetics viz., MS-222 and benzocaine (Sigma Chemicals, St Louis, MO, USA), at concentrations of 5, 10, 20, 30, 40, 50 and 60 mg L^{-1}, were added to water in low-density polyethylene bags (LDPE) of 22×60 cm size. Since it is readily soluble in water, MS-222 was mixed directly into the transporting medium, whereas benzocaine, being insoluble, was dissolved in a few drops of ethanol before mixing into the transporting medium. Six fish (approximately 72 g L^{-1}) were transferred into each polyethylene bag filled with 1 L water containing the anaesthetics at different concentrations as described earlier. It was then inflated with medical-grade oxygen and the top of the bag was tied and made airtight. A control group without the anaesthetics was also similarly maintained. All treatments were in triplicates. The experiment was conducted at a temperature of 22 ± 1 °C to simulate the air shipment conditions for 48 h. The fish were observed carefully at 30-min intervals and their behavioural responses were noted. The highest concentration of anaesthetics providing sedation, but the lowest mortality at the end of 48 h was chosen as the optimal dose for transportation.

Experiment II: biochemical analysis for stress indices

Seventy-two LDPE bags (22×60 cm) containing six fish each in 1-L water were divided into three groups ($n = 24$). One group served as the control while the anaesthetics MS-222 and benzocaine were added to the other two at the optimum concentration determined in the earlier experiment. The bags were filled with medical-grade oxygen, made airtight and each group was placed together in a styrofoam box ($60 \times 40 \times 55$ cm size) for thermal insulation to prevent sudden changes in the temperature of the transport water. All the boxes were kept at a controlled temperature of 22 ± 1 °C for 48 h.

Plasma cortisol and glucose levels were analysed as stress indices in fish sampled at 6-h intervals from each group during the experiment. Before sampling, fish from both control and anaesthetic-treated groups were euthanized with MS-222 at a lethal concentration of 200 mg L^{-1} following Cho and Heath (2000), where such exposure did not produce changes in the cortisol levels (Barton, Schreck & Sigismondi 1986). Blood was collected through a cardiac puncture using a heparinized syringe and transferred to 1.5 mL tubes. The plasma was separated by centrifugation at 4500 g for 10 min at 4 °C and stored at −20 °C until further analysis. Baseline values for all parameters were obtained from blood samples were taken from fish just before packing. Cortisol levels in the plasma were quantified using a commercially available direct ELISA kit (CAN-C-270, Diagnostics Biochem Canada, ON, Canada), whereas the plasma glucose level was measured using a glucose colorimetric assay kit (Sigma-Aldrich, St Louis, MO, USA).

Post-transport survival

After 48 h of the experiment, the remaining fish in the experimental bags were released into FRP tanks containing aerated water. Separate tanks were maintained for all the three experimental groups for observing post-transport mortality for 7 days after simulated transport. The water temperature in the tanks was 28 ± 1 °C with an average dissolved oxygen level of 12 mg L^{-1}, and the fish were fed with pellet feed.

Statistical analysis

All results were analysed using a one-way analysis of variance and Duncan's multiple comparison of the means using SPSS 10.0 for Windows. Differences were considered to be significant when $P < 0.05$.

Results

The safe and optimal anaesthetic dosage required for inducing sedation with least mortality after 48 h in *P. filamentosus* was determined. Fish exposed to MS-222 at concentrations $\leq$ 40 mg L^{-1} experienced sedation with no mortality at the end of 48-h experiments but not at the higher dosages tested (Fig. 1). Therefore, the concentration of 40 mg L^{-1} was judged to be optimal and safe and the concentrations below this were found to be suboptimal. In benzocaine-treated groups, sedation and no mortality were observed at concentrations $\leq$ 20 mg L^{-1}, above

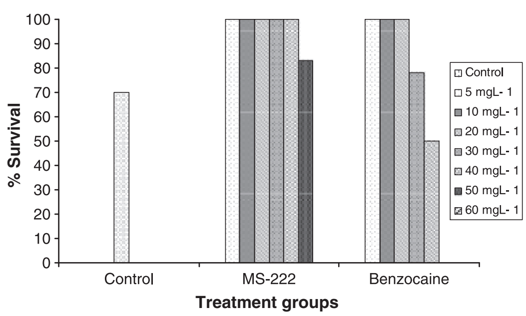

Figure 1 Percentage survival of *Puntius filamentosus* with different doses of MS-222 and benzocaine at the end of 48 h of simulated transportation.

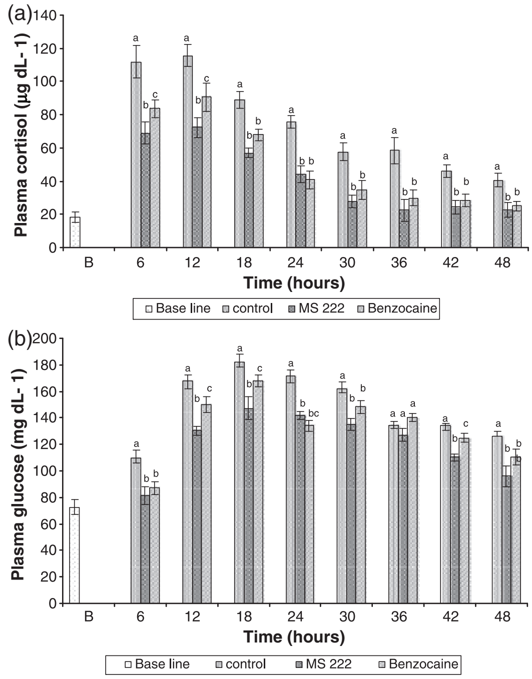

Figure 2 (a) Plasma cortisol level (μg dL^{-1}) and (b) glucose level (mg dL^{-1}) of *Puntius filamentosus* during 48-h simulated transportation with 40 mg L^{-1} MS-222 or 20 mg L^{-1} benzocaine. Values with the same exposure time with different superscripts are significantly different ($P < 0.05$)

which the fish displayed adverse effects like loss of equilibrium and death. Overall, anaesthesia-induced mortality was more pronounced in benzocaine-treated fish, than in MS-222 treatments.

When the efficacy of the anaesthetics was tested, the lowest increase in plasma cortisol levels was in the MS-222-treated group, followed by the benzocaine treatment compared with the untreated control within the first 6 h of packing (Fig. 2a). This increase was significantly lower ($P < 0.05$) in the sedated fish than in the control group, which indicated that the anaesthetics used were effective in lowering the stress response in the animals. The mean plasma cortisol levels in fish treated with the anaesthetics gradually declined after 6 h and almost reached the baseline within 48 h. Plasma cortisol concentrations in the control group remained significantly higher than that of the other treatments throughout the experiment ($P < 0.05$). However, there was no significant difference between the two anaesthetics treatments in the study.

The blood glucose levels increased steadily in all treatment groups up to 18 h and declined thereafter (Fig. 2b). Herein too, the increase was significantly

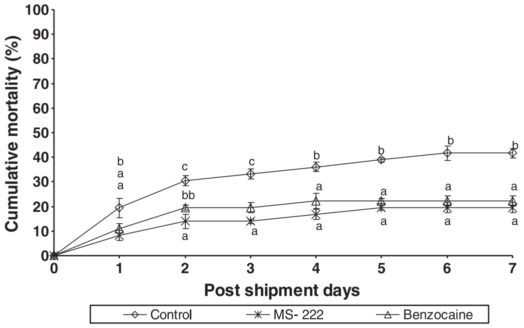

Figure 3 Post-transport mortality for 7 days in *Puntius filamentosus* after 48 h of a simulated transport experiment with 40 mg L^{-1} MS-222 or 20 mg L^{-1} benzocaine. Values with different superscripts are significantly different ($P < 0.05$).

lower in the sedated groups compared with the controls but not between the anaesthetics tested. The blood glucose levels, however, did not reach baseline levels at the end of 48 h in all the groups. Expectedly, the post-transport mortality at the end of 7 days was significantly higher in the unsedated fish than in the sedated groups (Fig. 3). This indicated that both the anaesthetics tested effectively reduced the stress during transport.

Discussion

Handling of fish out of their natural environment for transportation always results in considerable stress to the animal. Struggling of the fish has detrimental effects on their physiology and behaviour, leading to large-scale mortality and loss in product quality during the subsequent period of transportation (Ross & Ross 1999, 2008). Losses due to DOA and dead after arrival are significant problems in ornamental fish trade, which is unequivocally attributed to the stress associated with packing and transportation of fish (Schmidt & Kunzmann 2005). This severely impedes the ability of suppliers to meet the customers' needs for high-quality fish. Use of modern packaging technology for air transport to increase the survival and product quality is a principal factor in ornamental fish trade. Therefore, current packaging practices focus on minimizing the stress imposed on the fish by controlling the metabolic rate and removal of metabolic waste from the transport water (Lim *et al.* 2003; Harmon 2009). Although the use of anaesthetics to reduce the stress associated with handling, transport, confinement, etc. is well established in aquaculture, their use in ornamental fish trade is little studied.

The proper dosage of anaesthetics required is critical and varies widely between the species and the size of fish (Coyle, Durborow & Tidwell 2004). While low concentrations reduce activity and metabolic rate, higher dosages are routinely specified during procedures that are deemed stressful or painful for the fish. There are many instances where light sedation is sufficient and in fact desirable over deeper sedation, to facilitate handling of fish for different husbandry practices or especially for the transport of fish (Wedemeyer 1997; Golovanova, Nikonorov & Moise 2006). Notably, while increasing the concentration of anaesthetics decreases the induction time, it also significantly increases the recovery period (Gullian & Villanueva 2009). In this study, we found that 40 and 20 mg L^{-1} of MS-222 and benzocaine, respectively, were optimal in *P. filamentosus* to impart light sedation, above which significant loss of equilibrium and mortality resulted. Although benzocaine (ethyl aminobenzoate) is an effective fish anaesthetic with the desirable characteristics of rapid induction and recovery times (Ross & Ross 2008), the structurally similar MS-222 (tricaine methanesulphonate) is the most frequently used and preferred anaesthetic for fish. A marked difference between these two local anaesthetics was the delayed response to the visual stress stimulus after benzocaine anaesthesia. It was also observed that benzocaine concentrations above 20 mg L^{-1} resulted in partial or complete loss of equilibrium within 5 min after application and with subsequent mortalities. This emphasizes that benzocaine is a more potent anaesthetic than MS-222 requiring lower concentrations and that with a quicker action, there is a narrower margin of safety. The greater activity of benzocaine can be attributed to its higher lipid solubility compared with MS-222, permitting easier passage through the blood–brain barrier, resulting in more pronounced central nervous system effects (Kiessling, Johansson, Zahl & Samuelsen 2009).

Plasma catecholamines and cortisol levels are an indices of stress response in fish (Gamperl, Vijayan & Boutilier 1994) and acute stressors cause a rapid increase in these hormones, which in turn increase blood glucose levels through rapid breakdown of glycogen (Barton & Iwama 1991). Herein, we showed that anaesthetizing *P. filamentosus* before transport resulted in significantly lower plasma cortisol and glucose levels, indicating effective sedation. Although the plasma cortisol and blood glucose levels increased initially in the anaesthetized groups, they quickly returned to the baseline level compared with the unanaesthetized controls. The MS-222-treated group recovered faster from the stress as evidenced from the reduced levels of plasma cortisol and blood glucose indices in this group. The initial elevation in cortisol and glucose levels is probably due to the handling stress during the capture of the fish for experiment. Once the fish were transferred to transporting bags and tranquillized, their levels reduced considerably and reached the basal level in the subsequent hours. Recently, Iversen *et al.* (2009) reported a similar observation, which shows the stress-reducing potential of clove oil anaesthetics in Atlantic salmon. The studies by Tomasso, Davis and Parker (1980) and Carmichael, Tomasso, Simco and Davis (1984) reported a decrease in stress response in hybrid striped bass and largemouth bass when the fish were anaesthetized before capture and kept sedated during transport. Similarly, Wagner, Arndt and Hilton (2002) observed that when adult rainbow trout were anaesthetized with MS-222 or CO_2, the cortisol level returned to the initial level within 7 and 24 h after handling.

The reduction in post-transport mortality plays a central role in the management of ornamental trade and there is a strong interest, motivated by sound economic and conservational reasons, to avoid such mortalities (Schmidt & Kunzmann 2005). Presently, exporters are expected to compensate the customers for the loss exceeding 5% DOA (Lim *et al.* 2003). These post-transport mortalities are presumably due to osmoregulatory dysfunction or stress-mediated diseases, occurring during the first week after transport. In the present study, the post-transport mortality was markedly reduced in anaesthetized groups of *P. filamentosus*, which shows that their use alleviated the stress. Teo *et al.* (1989) reported zero mortality in a guppy packaging experiment using 2-phenoxyethanol at 0.11 and 0.22 g L^{-1} after a 20-h simulated shipment. Similarly, Guest and Prentice (1982) reported that in blue back herring, *Alosa aestivalis*, the anaesthetized fish had higher survival than non-dosed fish after transportation.

In conclusion, the low stress indicators and low post-transport mortalities associated with the use of anaesthetics would be desirable in the ornamental fish trade to reduce the economic loss and increase product quality. The present result implies that the use of anaesthetics during packing and transportation would help to reduce the transportation stress and improve the post-transport survival of *P. filamentosus*. In ornamental fish trade, the use of anaesthetics at a desired and optimal level should be encouraged and, because the dose of anaesthetics is species specific; more studies are warranted in adopting this technology, particularly for high-value species.

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