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Effects of anaesthesia with MS-222, clove oil and CO_2 on feed intake and plasma cortisol in steelhead trout (*Oncorhynchus mykiss*)

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Abstract

We examined the effects tricaine methanesulfonate (MS-222), clove oil and CO_2 on feed intake and cortisol response in steelhead trout, *Oncorhynchus mykiss*. Even though a body of literature exists about the effects of different anaesthetics on fish, no comparative information seems to be available about their effects on feed intake after anaesthesia, which would be important to know especially in aquaculture research. We anaesthetised juvenile steelhead trout with these three anaesthetics, and then sampled them 4, 24 and 48 h later. Fish in all groups ate relatively well already 4 h after anaesthesia. However, feed intake in fish treated with clove oil or MS-222 was lower than in the controls. There were no differences in feed intake among anaesthetised groups. Plasma cortisol concentrations were elevated 48 h after anaesthetisation, but the treatment means were equal throughout the experiment. Our results support previous findings that clove oil is a reasonable alternative to MS-222.

Keywords: Anaesthesia; Feed intake; Clove oil; CO_2; Cortisol; MS-222

1. Introduction

Several different chemicals are in use to anaesthetise fish for handling, transportation and reducing trauma during more invasive operations. When selecting an anaesthetic for

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a particular purpose, the user may want to consider properties such as the convenience for use, safety for the fish, humans and the environment, effectiveness, physiological perturbations and the cost. The most commonly used fish anaesthetic is tricaine methanesulfonate (MS-222) (Marking and Meyer, 1985). However, this anaesthetic is regarded as a carcinogenic and also a 21-day withdrawal period is required if the fish is intended for human consumption. Also, MS-222 is relatively expensive. One option to anaesthetise fish is clove oil, which is relatively new as fish anaesthetic. Clove oil is extracted from buds, leaves and stems of clove tree (*Eugenia aromatica*; the active compound is eugenol) and it has traditionally been used as topical anaesthetic for toothaches, headaches and joint pain (Soto and Burhanuddin, 1995). It is also used as food additive, and as organic substance does not require any withdrawal period. Clove oil is readily available, for example, from health food stores and it is inexpensive when compared to MS-222 (Keene et al., 1998); however, it is light sensitive (Cho and Heath, 2000). Recovery time from clove oil anaesthesia has usually been reported to be longer than with MS-222 in rainbow trout (*Oncorhynchus mykiss*) (Keene et al., 1998) and in carp (*Cyprinus carpio*) (Hikasa et al., 1986). Anderson et al. (1997) reported that recovery time for juvenile rainbow trout following exposure to clove oil was longer than to MS-222; however, there was no difference between these two substances with adult trout.

Carbon dioxide has been used to immobilise fish; it was first described as a fish anaesthetic by Fish (1942). Carbon dioxide can be produced by bubbling CO_2 gas or by the soda-acid technique (Post, 1979; Summerfelt and Smith, 1990). It is safe for humans and its use is unrestricted (Summerfelt and Smith, 1990). CO_2 may also be only partly effective at immobilizing fish, slow acting, and lethal after repeated exposures (Marking and Meyer, 1985); CO_2 does not induce analgesia and hence has a "shallow effect" when compared to MS-222 (Anderson et al., 1997). Physiological responses to CO_2, such as no decrease in blood P_{O_2} and lowered plasma pH during anaesthesia, differ from those of benzocaine, 2-phenoxyethanol, MS-222 and metomidate (Iwama et al., 1989).

Several studies describe physiological effects of different anaesthetics (e.g. Soivio et al., 1977; Iwama et al., 1989; Cho and Heath, 2000), their efficacy (Keene et al., 1998; Munday and Wilson, 1997; Peake, 1998) and effects on swimming performance after anaesthesia (Anderson et al., 1997). Invariably, in any study with clove oil, it has been regarded as an effective and acceptable alternative to other anaesthetics. However, no comparative studies are available concerning the effects of anaesthesia on feed intake. Soto and Burhanuddin (1995) did, however, observe most rabbitfish (*Siganus lineatus*) feeding a few hours after anaesthesia with clove oil and Prince and Powell (2000) reported that adult rainbow trout were feeding actively 1 week after anaesthesia. Our main objective was to compare the effects of MS-222, clove oil and CO_2 on feed intake after anaesthesia in steelhead trout. Blood samples were also taken to verify the effects of these anaesthetics on plasma cortisol, used as an indicator of stress (Wedemeyer et al., 1990). We wanted to do the evaluation of anaesthetics within the context of how they might actually be applied by a fishery biologist, so we used fin clipping as part of the anaesthetisation experience. In addition, this also served the useful purpose of allowing us to identify treatment groups.

2. Materials and methods

2.1. Fish and rearing conditions

The experiment was carried out at the Fish Performance and Genetics Laboratory of the Oregon State University in Corvallis, OR, between 3 and 5 January 2001. The fish were zero-age hatchery steelhead trout of the Alsea River (Oregon) stock. Thirty-two individuals were counted into each of the 12 circular experimental tanks (60 cm wide, water depth 40 cm) supplied with 12.6 °C flow-through well water at 6.5 l min^{-1}. Photoperiod was 8L:16D. During the 6-week acclimation period, fish were fed by hand twice a day (once during the weekends) to apparent satiation, as suggested by the cessation feeding and uneaten pellets remaining on the tank bottom. Feeding the fish in all the tanks to satiation took about 15–20 min. The fish were fasted for 24 h before the start of the experiment. At the time of sampling, fish weight varied between 26 and 79 g (mean ± S.E., 47.4 ± 0.45 g, $n = 379$).

2.2. Determination of feed intake

The feed was a commercial dry feed (Bio Dry 1000 by Bio-Oregon, Warrenton, OR). For the experiment, the feed was ground into powder and repelleted. A small batch of the ground feed was mixed with X-ray dense lead glass beads (2% by weight of the feed; ballotini size 9, average diameter 0.355 mm, Jencons, Leighton Buzzard, UK) before repelletising for the estimation of feed intake (Jobling et al., 1993). Known amounts of labelled feed were then X-rayed and standard curve for the relationship between numbers of ballotinis (B) and feed's weight (g) were calculated ($g = 0.0134 \times B - 0.036$, $r^2 = 0.98$, $N = 15$). All the feed had to be repelleted to ensure that the texture, colour and size in regular feed fed during the acclimation period and in labelled feed during the test were identical. After the anaesthetisation (time 0 h), the fish in four tanks were fed to satiation at each sampling time (4, 24 and 48 h) with the X-ray dense feed; that is, the fish in each group were fasted for the entire period after anaesthetisation until they were refed and sampled (e.g. the 48 h group was not fed at 4 and 24 h). About 20 min after feeding, all the fish in each of the sampled tanks were killed by an overdose of buffered MS-222 (200 mg l^{-1}), sampled for blood, weighed, wrapped in a labelled tissue paper and placed in a ziplock plastic bag in which they were later X-rayed (Faxitron cabinet X-ray machine, Agfa Structurix D4 film). The number of ballotinis present in the gut of each fish was counted from the X-ray plates and the amount of feed eaten by each individual was estimated based on the standard curve.

2.3. Anaesthetisation and sampling procedures

A fresh solution of three anaesthetics was prepared in 5 l of water for each tank. Concentrations were based on a preliminary experiment so that the exposure time for different anaesthetics was 3 min. Pure clove oil (obtained from a health food store) was first dissolved in 95% ethanol (to which no additives had been added) at 1:10 (Cho and Heath, 2000). Two milliliters of this was added into the bath water giving a final

concentration of 40 ppt. Buffered (with $NaHCO_3$ at 160 mg l^{-1}) MS-222 was used at 80 mg l^{-1}. CO_2 anaesthesia was induced by soda-acid method as described by Post (1979). We used 40 ml of both 6.75% (w/v) $NaHCO_3$ and 3.95% (w/v) H_2SO_4 in the anaesthetising bath. pH in these baths for MS-222, clove oil and CO_2, respectively, was 7.1, 6.8 and 6.2; pH in untreated well water was 6.6.

At time 0 (designated for each tank at the time of anaesthetisation), eight fish were netted from each tank into each of the three anaesthetics; eight fish were left in the tank as untreated control fish (i.e. eight fish × four treatments per tank). At the point when total loss of equilibrium was obtained and opercular movements became irregular (i.e. at 3 min), the fish were uniquely marked to identify the particular anaesthetic that they received by fin clipping about one third of either left or right pelvic fins or the adipose fin; fish in CO_2 often became startled when they were lifted to air. Respective fin clips varied by treatment from tank to tank. After fin clipping, the fish were first placed into a bucket with pure well water to recover; when all fish had been fin clipped and recovered, they were carefully netted back to their original tank. At 4, 24 and 48 h after anaesthetisation, respectively, all of the fish in four of the tanks (i.e. three sampling times × four replicated tanks with four treatments in each) were killed with an overdose (200 mg l^{-1}) of buffered ($NaHCO_3$ at 400 mg l^{-1}) MS-222 and four individuals per treatment from each tank were sampled for blood by caudal severance. Blood was taken into heparinized capillary tubes, transferred into microcentrifuge tubes and centrifuged. Plasma was separated and stored frozen for later analysis of cortisol by radioimmunoassay (Redding et al., 1984). All values below the lowest standard (3.9 ng ml^{-1}) were designated to contain this amount of cortisol.

Possible differences between treatments were tested by using two-way ANOVA (time vs. treatment); to avoid pseudoreplication, treatment mean value within each tank was used as observation (i.e. $n=4$). Post hoc comparisons of means were done with Tukey's HSD test. Variances in cortisol concentrations between treatments were unequal and therefore the values were log transformed before computations.

3. Results

There was a tendency for nonanaesthetised control fish to ingest about 15–20% more food than what their anaesthetised counterparts ingested, irrespective of the sampling time. The fish anaesthetised with MS-222 and clove oil ate significantly less than the control fish (two-way ANOVA post hoc comparisons: $P=0.03$ for MS-222 and $P=0.01$ for clove oil). In all treatment groups, feed intake increased from 0.8–1% of body weight during the 4 h sample up to about 1.5–1.9% of weight during the 24 and 42 h samples (Fig. 1A); feed intake was significantly lower at 4 h than at 24 and 48 h samples ($P=0.000$) There was no statistically significant interaction between time and treatment in the two-way ANOVA.

Mean plasma cortisol concentrations were relatively low (averages between 5.8 and 15.8 ng ml^{-1}) in all groups during the first two sampling times, but in the 48 h sample, concentrations were elevated ($P=0.000$) irrespective of the treatment; average values varied between 29.8 and 35.8 ng ml^{-1} (Fig. 1B). The effect of treatment was not significant and there was interaction between time and treatment. We also tested for

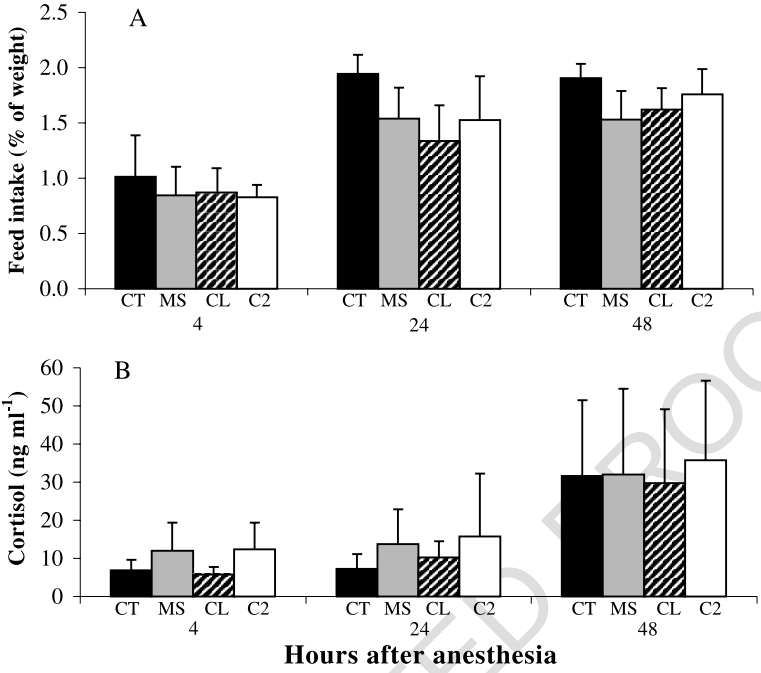

Fig. 1. (A) Feed intake (percentage of weight per feeding) and (B) plasma cortisol concentrations (ng ml^{-1}) of *O. mykiss* 4, 24 and 48 h after anaesthesia. Data presented as means ± S.D. ($n = 4$). Treatments: CT = control, MS = MS-222, CL = clove oil, C2 = CO_2.

possible correlation between feed intake and cortisol concentrations at individual level within tanks and within treatments, but no clear correlation between the two variables could be demonstrated.

We also estimated recovery times from anaesthesia to the stage when the fish turned upright. Recovery required about 1 min 30 s with MS-222, about 2 min with CO_2 and 3 min 30 s with clove oil.

4. Discussion

MS-222 and clove oil have similar depressive effects on feed intake after anaesthesia in *O. mykiss*. Fish anaesthetised with CO_2 did not differ from any other group in terms of feed intake. Our results suggest that even if feeding can be resumed soon after anaesthesia, it may measurably impact rates of food consumption. This is an important result relative to experiments concerning feeding, nutrition and growth, when only a sample of a tank can be taken (e.g. for weight measurement). Control fish ate about 15–20% more than the treated fish at each sampling time, which could inevitably lead to growth differences. For how long this depression in feed intake would persist should be tested. Fish in our experiment were fasted for 24 h before anaesthetisation, which may have reduced the

effects of handling stress (Barton et al., 1988) to the extent that the fish were able to eat relatively well already at the 4 h sample. The fish were fasted before anaesthetisation because if anaesthetics had an impact on appetite, then one could have expected these to be more pronounced in previously fasted fish. The increase in feed intake from 4 to 24 and 48 h samples was likely because of the increase in the length in fasting time.

Even though recovery from anaesthesia with clove oil may take slightly longer than with MS-222 (e.g. Keene et al., 1998), it does not seem to be deleterious for fish, because the feed intake between the anaesthetised groups of fish did not differ. The recovery time recorded for clove oil (3 min 30 s) was much shorter than that reported for rainbow trout by Keene et al. (1998) but similar to that reported by Anderson et al. (1997) and Prince and Powell (2000). This relatively slow recovery should be taken into account in the field to ensure total recovery of anaesthetised fish before release. Because some variation seems to exist between experiments, it is advisable to confirm optimum concentrations before using clove oil or any other anaesthetic with test fish. There apparently are also large differences between species in sensitivity to clove oil (Pirhonen and Hoskonen, unpublished).

Plasma cortisol values measured from our test fish were low in the 4 and 24 h samples, but for unknown reason significantly higher in the 48 h sample. Most likely this elevation in cortisol was not due to fasting for 3 days (1 day before and 2 days after anaesthetisation) because fasting has not been shown to affect cortisol concentrations (Milne et al., 1979; White and Fletcher, 1986; Pirhonen et al., in press). Cho and Heath (2000) reported cortisol values around 200 ng ml^{-1} in chinook salmon (*Oncorhynchus tshawytscha*) 6 h after anaesthesia with MS-222 and clove oil; their preanaesthetic cortisol concentrations were about 30 ng ml^{-1}. Wagner et al. (2002) observed that adult rainbow trout anaesthetised with clove oil (in the form of AQUI-S) had lower cortisol concentrations at 1 or 7 h postimmersion than fish treated with MS-222 or CO_2, but were elevated at 24 h. They reported that cortisol in MS-222- and CO_2-treated fish returned to the initial level within 7 and 24 h after handling. Strange and Schreck (1978) instead measured cortisol values c. 40 and 20 ng ml^{-1} in chinook salmon 2 and 7 h after MS-222 anaesthesia, respectively. The method of killing the fish by the overdose of MS-222 can be regarded safe to obtain unbiased cortisol values (Barton et al., 1980; 1985a,b).

Fin clipping likely did not stress the fish as indicated by the lack of statistical difference in cortisol between treated (fin-clipped) and control fish. While chronically elevated cortisol concentrations have been shown to negatively affect appetite in rainbow trout (Gregory and Wood, 1999), we did not observe any correlation between cortisol and feed intake at individual level. In conclusion, all three test compounds did not appear to stress steelhead trout to significant degree relative to controls as estimated by cortisol, but despite that, feed intake was significantly decreased when fish were treated with MS-222 or clove oil.

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