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The Efficacy of Clove Oil As An Anesthetic for the Zebrafish, *Danio rerio* (Hamilton)

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ABSTRACT

The anesthetic effects of clove-oil-derived eugenol were studied in the zebrafish, *Danio rerio* (Hamilton). Acute lethality and the effects of exposures to various dosages of eugenol were measured. The estimated 96-h LC_{50} for eugenol was 21 ppm. Times to induction and recovery from anesthesia were measured and compared with MS-222 under similar conditions. Eugenol induced anesthesia faster and at lower concentrations when compared to MS-222. The recovery times for fish exposed to eugenol were generally longer compared to similar concentrations of MS-222. Doses of 60–100 ppm eugenol produced rapid anesthesia with an acceptably short time for recovery. These findings suggest that eugenol could be an effective anesthetic for use with this species, and when compared to MS-222, its benefits include a lower cost, lower required dosage, improved safety, and potentially lower mortality rates.

INTRODUCTION

FISH BIOLOGISTS USE VARIOUS types of anesthetics to aid in the capture, handling, and transport of fish.[1] An emerging and efficacious anesthetic for use in fish is clove oil, containing the active ingredient eugenol.[2] Though eugenol has not yet gained approval for use in food fishes in Canada or the United States, the Japanese have legalized the use of FA-100 (10% solution of eugenol) for anesthetic purposes.[3]

Eugenol (4-allyl-2-methoxyphenol), the active ingredient of clove oil, constitutes 70–95%[4,5] of the total weight of the base clove oil. Clove oil is distilled from the flowers, flower stalks, and leaves of the clove tree *Eugenia aromatica*, and is used throughout the world in the dentistry profession as a topical anesthetic,[6] and also as a food flavoring.[7] Eugenol is considered to be a noncarcinogenic, nonmutagenic,[8] "generally recognized as safe" (GRAS) substance by the FDA.[7,9] In fact, it may even be an anticarcinogenic agent.[10] It has also been found to have potentially beneficial antiviral,[11] antimicrobial,[4,12] and antifungal[13,14] properties. Clove oil, in the form of FA-100 (10% commercial preparation of eugenol), has been examined for its anesthetic characteristics in common carp, *Cyprinus carpio*,[3] juvenile rainbow trout, *Oncorhynchus mykiss*; medaka, *Oryzias latipus*; and crucian carp, *Carassius auratus*.[15] More recent studies have also shown eugenol to be an effective alternative to 3-aminobenzoic acid ethyl ester methanesulfate (MS-222) for the anesthetization of rainbow trout.[16–19] Clove oil has also been found to be effective for the anesthetization of the marine reef fish *Pomacentrus amboinensis*;[1] channel catfish, *Ictalurus punctatus*;[20,21] sockeye salmon, *Oncorhynchus nerka*;[22] and rabbitfish *Siganus lineatus*.[23]

The zebra danio, *Danio rerio*[24] is a tropical Cypriniform (family Cyprinidae), and has become a widely popular fish species for modeling studies in developmental biology, toxicol-

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ogy, and transgenic techniques.[25] As a consequence, it is essential to evaluate new compounds that may represent alternative anesthetics for zebrafish used for these procedures. Therefore, the purpose of the present study was to compare the effectiveness of clove oil to that of the commonly employed MS-222, as an anesthetic for zebra danios. Zebrafish were exposed to varying doses of clove oil (eugenol) to determine the 96-h LC_{50}, as well as to both eugenol and MS-222 to observe differences in anesthesia onset and recovery times.

METHODS AND MATERIALS

Experimental organisms

Approximately 1200 sexually immature, 1-month-old zebrafish were used, with an average weight of 0.62 ± 0.01 g and a mean fork length of 3.68 ± 0.1 cm. The fish population was distributed equally among ten 50–L holding tanks, each maintained at 24 ± 2°C. Fish were maintained on a lighting regimen representative of the local natural environment (13 L:11 D) and fed twice daily to satiation with a diet consisting of a combination of 2 PT trout pellets (Martin Feed Mills, Elmira, Ontario, Canada) and commercially available flaked tropical fish food. Tanks were siphoned once every second day and approximately 2 L of water were exchanged during each cleaning.

Preparation of the anesthetics

Clove-oil-extracted eugenol (Hilltech Canada Inc., Vankleek Hill, Ontario, Canada) and MS-222 (Argent Chemical Laboratories, Redmond, Washington, USA) were the two anesthetics investigated. To reduce the amount of photodegradation, the clove-oil stock solution was kept in a sealed metal container on the lab bench at approximately 19°C.[26] Since clove oil does not readily mix with water, all samples were first mixed with ethanol to create a stock solution of 1:10 (eugenol:ethanol) prior to use. The maximum amount of ethanol used in any experiment was 1400 ppm. MS-222 was prepared by measuring the appropriate amount of anesthetic powder into a clean plastic container and then adding it directly to the experimental tanks per normal protocols.

96-h LC_{50} clove oil

Six glass aquaria (50 cm × 26 cm × 30 cm) were filled to a volume of 20 L each and maintained at a temperature of 24 ± 2°C throughout the 96–h experimental period. Water in these experimental tanks was heated to the test temperature and saturated with oxygen for 24 h prior to the introduction of the zebrafish, and oxygen levels were maintained above 85% saturation throughout the experiment. For each treatment, the clove-oil stock solution was mixed with the experimental tank water to produce final concentrations of 0, 1, 2, 5, 15, and 30 ppm, respectively, in the six experimental tanks. Fish were netted from the 50-L holding tanks, with small subsamples of fish taken to add to the experimental tanks. Two fish at a time were placed into each tank until there was a total of ten fish per tank. The total mortalities, behaviors, temperature, and oxygen saturation were recorded every hour for the first 12 h of the experiment, every 3 h for the next 12 h, and every 6 h for the remaining 72 h. Fish were considered dead when there were no opercular beats observed for 15 continuously monitored min. This complete experimental protocol was replicated three times.

Onset and recovery from anesthesia

The observations of stages 4 and 5-anesthesia onset[18] were made using MS-222 and clove oil under the same experimental conditions. A 20-L experimental aquarium was maintained at a temperature of 24 ± 1°C with an oxygen saturation greater than 85%. Ten zebrafish were randomly selected from the holding population and placed into the experimental tank at one of the treatment concentrations of either MS-222 or clove oil. The tested concentrations of MS-222 were 100, 120, 140, 160, 180, and 200 ppm, while the experimental concentrations of clove oil used were 60, 80, 100, 120, and 140 ppm. The times to stage 4 and stage 5 anesthesia onset were also recorded. Once an individual fish had reached the onset of stage 5 anesthesia, a dip net was used to immediately remove it from the tank. The fish was then transferred to a 20-L,

well-oxygenated 'recovery' tank (i.e., no anesthesia present) maintained at 24 ± 1°C and observed until it fully recovered. During this recovery period, fish behavior was observed and times to stage 3 and 4-anesthesia recovery[18] were recorded. Three replicates of ten fish each were used for every individual anesthetic concentration treatment of MS-222 and clove oil. Once a fish had been used for a treatment, it was left in the recovery aquarium for approximately 1 day prior to being transferred back to a 50-L recovery holding tank for the remainder of a 14-day observational recovery period. Any abnormal behavior or mortalities were recorded during this 14-day recovery period.

Ethanol exposure effects

Three glass aquaria (50 cm × 26 cm × 30 cm) were filled to a volume of 20 L each. The water in each tank was again maintained at a temperature of 24 ± 1°C and an oxygen saturation greater than 85%. Zebrafish were randomly selected from the population as per the procedures described above, and distributed into each of three replicate experimental tanks until there were ten fish per tank. Ethanol was then added to the tanks to make a final concentration of 1400 ppm of ethanol (the maximum concentration required to dissolve the highest concentration of eugenol). Fish were then observed for mortality over a 96-h period, removed and placed into a 50-L recovery tank, and observed for morbidity and mortality for the following 14 days.

RESULTS

96-h LC_{50} clove oil

Zebrafish placed into the higher concentration treatments of 5, 15, and 30 ppm initially exhibited irritation, as evidenced by rapidly darting about the aquaria, displaying a coughing behavior, and a regurgitation reflex.

All fish in the 0, 1, and 2-ppm treatments survived the 96-h trial, with only one mortality occurring in the 5-ppm treatment after 42 h of exposure. Fish treated with 15 ppm of eugenol exhibited a mean mortality rate of approximately 10%. These occurred as early as 4 h and as late as 96 h into the treatment. Fish exposed to 30-ppm treatments exhibited a 100% mortality rate, with the majority of fish dying within the first 2 h, and the remainder by 4 h. A large number of observed 0 and 100% mortality rates confounded the 96-h LC_{50} results. Therefore, a Berkson adjustment factor was applied and the LC_{50} was estimated using a logistic regression scaled to the deviance. The LC_{50} values were modeled as responses to time and trial using a multiple regression approach. The final result was a $LC_{50} = \text{time}^{-1}$ relationship that revealed a mean LC_{50} value of 21.35 ppm for zebrafish using clove oil (Fig. 1).

Onset of anesthesia

The effective anesthetic dosage range was lower for eugenol than for MS-222, with some similarities in effects at concentrations of 100, 120, and 140 ppm. The times to achieve stage 4 (Fig. 2) and 5 (Fig. 3) onset for clove oil and MS-222 both exhibited a negative exponential response to dose. Stage 5 was induced faster and at lower concentrations for eugenol than for MS-222. The response to clove oil at each experimental concentration was less variable than for MS-222, exhibiting a smaller standard error of the mean for the experimental dosages tested (Fig. 3).

Recovery from anesthesia

Fish exposed to eugenol anesthesia took longer to achieve recovery stages 3 (Fig. 4) and 4 (Fig. 5) than those fish exposed to MS-222. Fish exposed to the highest concentration of MS-222 required > 6 min to achieve stage 4 recovery. Regardless of eugenol concentration, stage 3 recovery occurred at approximately 5 min. Stage 4 recovery was longer for eugenol over all concentrations except 140 ppm. No relationship was found between mean time to stage 4 recovery and concentration for eugenol.

Mortality

During the 14-d recovery from an experimental MS-222 anesthetic bath, a total of three mortalities occurred at each of the 100 and 180-ppm treatments at 5 and 7 days, respectively. One mortality also occurred in each of the 160 and 200-ppm treatments. In contrast, fish ex-

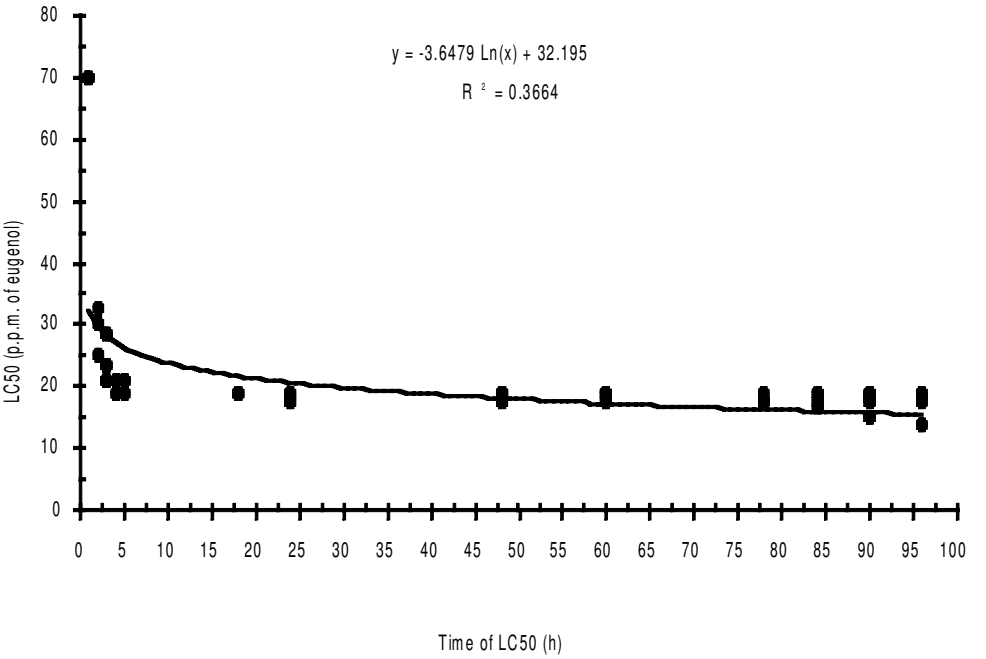

FIG. 1. The LC_{50} over time for zebrafish exposed to clove oil. Data points represent the mean response of ten fish.

posed to eugenol experienced no mortalities during the 14-day recovery period.

Ethanol exposure effects

Fish exposed to the 1400-ppm solution of ethanol all survived the 96-h trial. There were no observed behavioral changes in fish subjected to the ethanol solution and only one mortality was observed 8 days after transfer to the recovery tank during the 14-day recovery period.

DISCUSSION

Currently, 3-aminobenzoic acid ethyl ester methanesulfate (MS-222) is the most commonly used anesthetic in fisheries science, and was

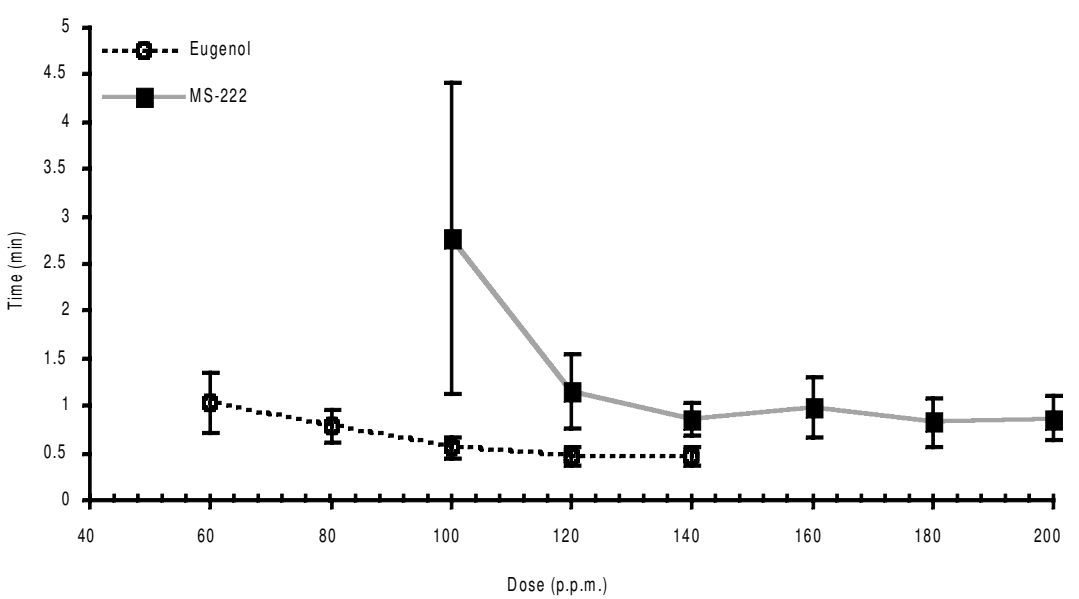

FIG. 2. Time required for zebrafish to achieve stage 4-anesthesia at various concentrations of clove oil and MS-222. Data points represent the mean ± SEM, $n = 10$.

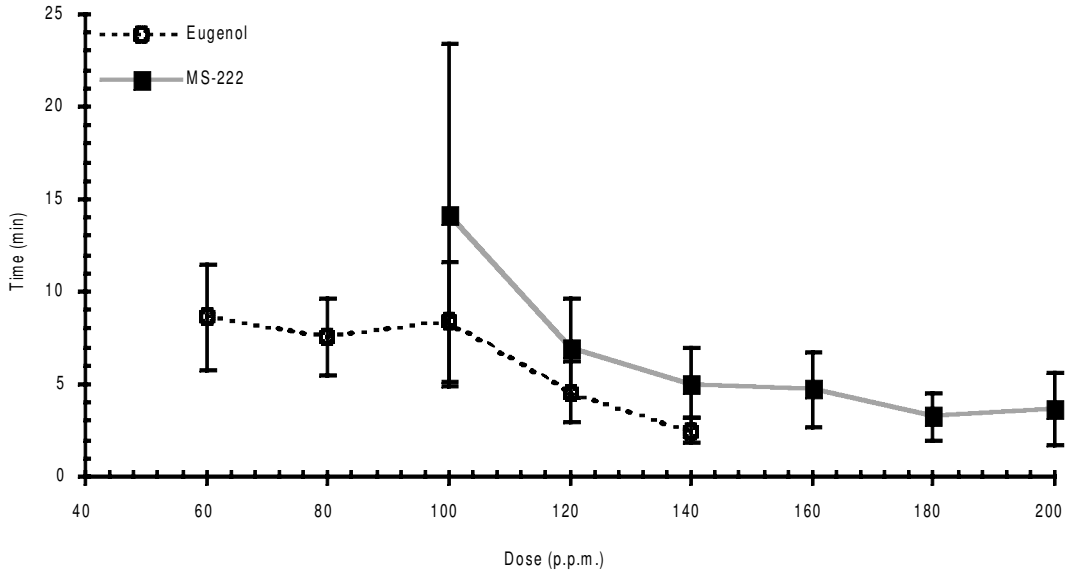

FIG. 3. Time required for zebrafish to achieve stage 5-anesthesia at various concentrations of clove oil and MS-222. Data points represent the mean ± SEM, $n = 10$.

therefore a suitable candidate for the current comparison to clove oil. The results of these experiments show that zebrafish can be successfully anesthetized using appropriate concentrations of clove oil. The observed progression through the various stages of anesthesia was consistent with the descriptions by Keene et al.,[18] modified from the original descriptions by Jolly et al.,[27] and Hikasa et al.[3] for respective onset and recovery states from chemical anes-

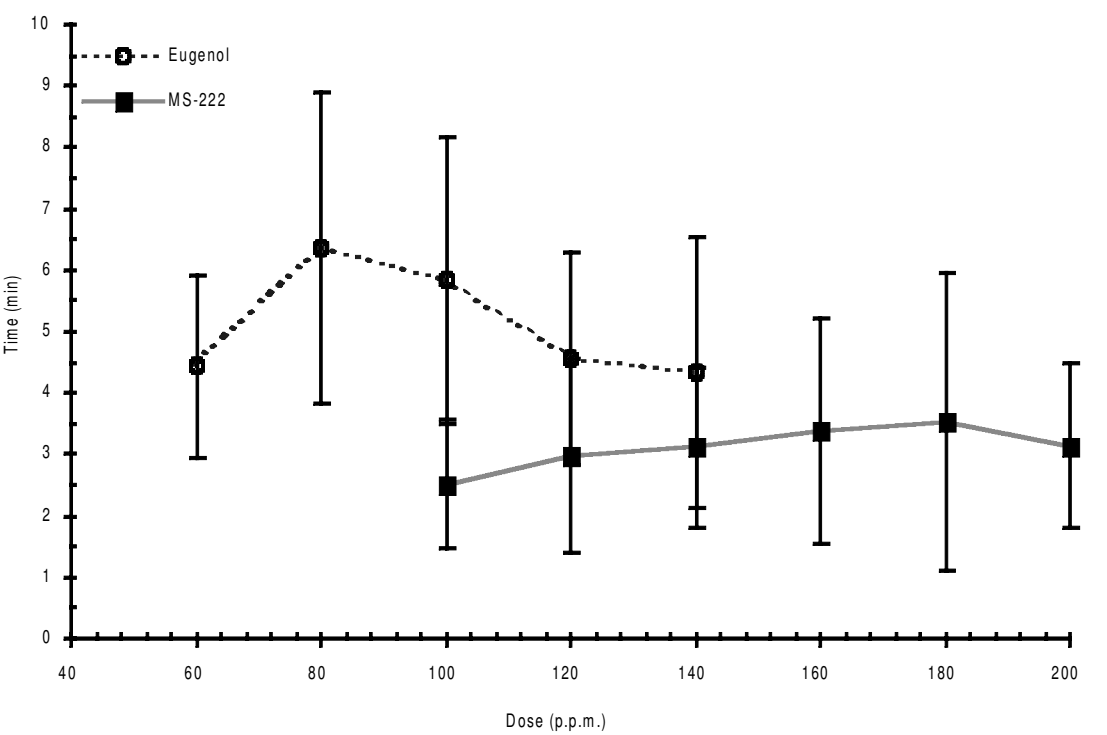

FIG. 4. Time required for zebrafish to achieve stage 3-recovery from anesthesia at various concentrations of clove oil and MS-222. Data points represent the mean ± SEM, $n = 10$.

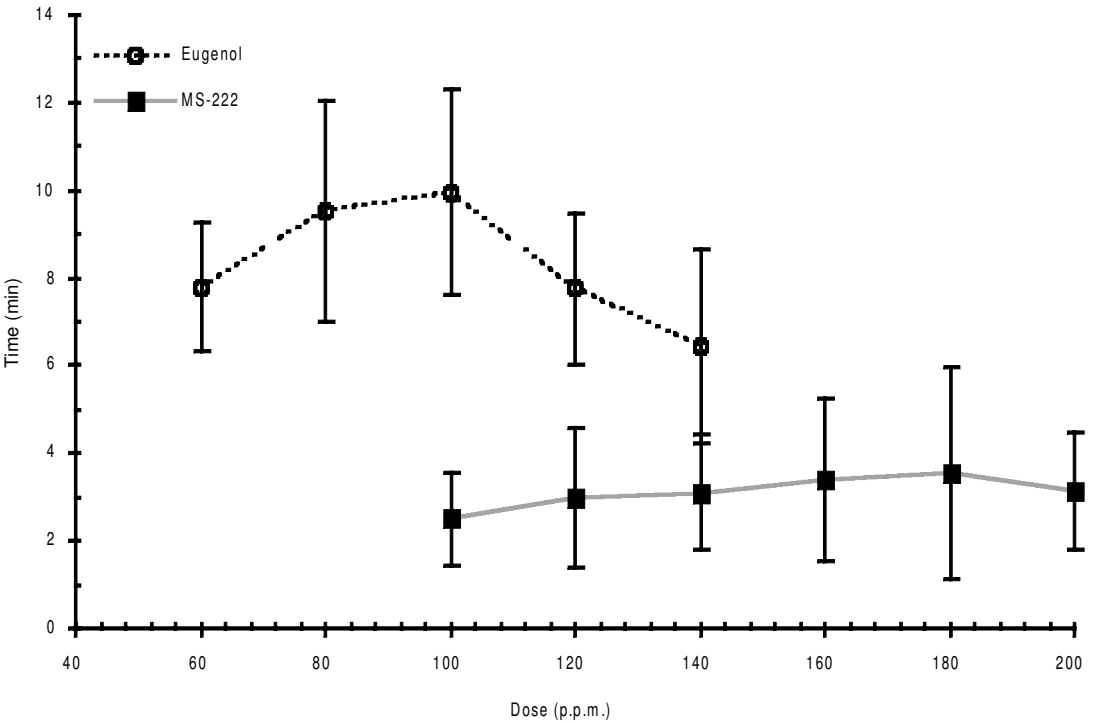

FIG. 5. Time required for zebrafish to achieve stage 4-recovery from anesthesia at various concentrations of clove oil and MS-222. Data points represent the mean ± SEM, $n = 10$.

thesia. Ethanol, used to administer the eugenol, appeared to have no confounding deleterious effects upon the zebrafish.

96-h LC_{50} clove oil

The 96-h LC_{50} for zebrafish was 21.35 ppm, however a comparison with MS-222 was not possible due to logistics at the time of the experiment. Also, there are no comparable data for MS-222 (96-h LC_{50}) available from previous reports. It is notable, however, that toxicity tests[28] indicated that zebrafish were more tolerant than rainbow trout to a number of xenobiotic chemicals. Similarly, if the eugenol results of the current study are compared with those for rainbow trout,[18] zebrafish, in general, were more tolerant of eugenol. For example, a 100% mortality rate was observed[18] in rainbow trout at eugenol concentrations of 15 and 30 ppm. In comparison, a 100% mortality rate for zebrafish was observed only at 30 ppm, while a value of 30% mortality was observed at a concentration of 15 ppm. Similarly, all rainbow trout exposed to 30 ppm died within 2 h of exposure,[18] while zebrafish in the current study required as long as 4 h to expire at the same concentration. These comparisons suggest that zebrafish, when compared to rainbow trout, are more tolerant of slightly higher concentrations of clove-oil-derived eugenol.

Anesthesia onset

Anesthesia was generally achieved more quickly and at a lower dosage for clove oil than for MS-222 in the zebrafish. During the onset of anesthesia, the times to stages 4 (loss of equilibrium) and 5 (loss of reflex reactivity) were concentration dependent. However, both were achieved in a relatively short period at the investigated concentrations. Davidson et al.[17] used the recommended dosage of 17 ppm of AQUI-S™, a related compound containing 20 ppm of isoeugeniol, and found that rainbow trout all reached stage 4 anesthesia within 10 min, longer than it took zebrafish on average in the current study. As well, differences were observed between MS-222 and eugenol, with eugenol requiring lower concentrations to achieve both stages. Keene et al.[18] also found that fish anesthetized with eugenol could

achieve stage 5 at lower concentrations and more rapidly than those subjected to MS-222. Hikasa et al.[3] suggested that the differences in the effect of eugenol and MS-222 on *Cyprinus carpio* were due to the influence that each anesthetic had on blood-gas and acid-base balance during anesthesia onset and recovery.

In all cases, low doses of clove oil produced effects similar to higher doses, suggesting that clove oil has a larger safety margin than does MS-222, which showed a significantly greater dose response (Figs. 2 and 3). Similar effects have been demonstrated in rainbow trout,[18] medaka, goldfish, and Crucian carp.[15]

Anesthesia recovery

Recovery of equilibrium and fear response times in the current study were consistently longer for eugenol than for MS-222. Keene et al.[18] also observed longer recovery times when eugenol was compared to MS-222 in rainbow trout. Eugenol has greater effects on the respiratory and cardiac system than MS-222[29] resulting in slower heart rate and respiratory rates. Keene et al.[18] suggested that these systemic effects may result in a longer retention of eugenol in the bloodstream. An interesting observation in the current study was made during the recovery of the fear, or fright response (stage 4 recovery) in fish anesthetized with eugenol. Though a positive relationship was observed for the mean fear response recovery time (i.e., between dosage concentration and time to recovery) for concentrations of 60, 80, and 100 ppm, responses for concentrations of 120 and 140 ppm showed a negative relationship (i.e., increasing concentrations shortened time to recover the fear response, Fig. 5). Thus there was no clear relationship between dosage concentration and time to recovery of the fear response for zebrafish anesthetized with eugenol.

CONCLUSION

Clove oil meets at least six of the eight criteria for an ideal anesthetic,[30] and is an effective anesthetic for zebrafish. Recovery times were slightly longer than the required 5 minutes; however, they were close to this standard, with variances suggesting that eugenol produces an acceptable recovery time for zebrafish. Given the cost benefits previously demonstrated when compared to MS-222 use in rainbow trout,[18] and the demonstrated efficacy at lower dosages in the current study, clove oil appears to be a viable alternative for anesthetic use with zebrafish. The main advantages of clove oil are its low cost and relative safety to both fish and humans. The recommended dose to elicit stage 5 anesthesia in zebrafish is 60–100 ppm eugenol for 6–12 min. Levels of 2–5 ppm may be used safely to sedate zebrafish for extended periods (less than 8 h) to facilitate procedures such as transport.

However, as with the administration of any anesthetic, consideration should be given to any cumulative or specific physiological effects upon zebrafish, and these were not assessed in the current study. This may be of particular importance with zebrafish, as they are commonly used for medical research and the potential effects upon research variables, such as blood hormone levels, have not been investigated. Small[20] found that cortisol levels were suppressed by a dose of 100 ppm of clove oil. Similarly, Holloway et al.[31] demonstrated differential perturbations in various blood hormone levels that are commonly measured in fisheries research, depending on which anesthetic was utilized. Researchers should therefore ensure that suitable controls are incorporated within their experimental designs when utilizing eugenol (or any other anesthetic) in zebrafish models, in order to elucidate any potential confounding effects of this alternative anesthetic. Finally, all users are encouraged to fully comply with provincial, state and national regulations concerning anesthetic use in experimental or food animals.

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