



Behavioral and physiological assessment of low concentrations of clove oil anaesthetic for handling and transporting largemouth bass (*Micropterus salmoides*)

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Received 5 October 2003; received in revised form 21 June 2004; accepted 25 June 2004

Abstract

Clove oil has become a popular fish anaesthetic for invasive fisheries research procedures, but few studies have examined the use of low concentrations of clove oil to achieve sedation for aquaculture procedures such as fish handling and transport. In this study, we used largemouth bass as a model species to examine the behavioral and physiological responses of fish to a gradation of clove oil concentrations (0 to 20 mg l⁻¹) while exposed to truck transport. Concentrations of clove oil ranging from ~5 to 9 mg l⁻¹ elicited a sedative effect resulting in loss of reactivity and reduced cardiac output while maintaining equilibrium. Fish sedated by 5 to 9 mg l⁻¹ clove oil achieved that level of anaesthetization rapidly and recovered behaviorally more quickly than at higher concentrations. During transportation, videography revealed that fish in deep sedation (stage 2

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induction) experienced the least opportunity for physical damage from the tank or conspecifics and had reduced activity relative to other concentrations. Cardiovascular assessments indicated that when exposed to clove oil of any concentration, cardiac output and heart rate rose following an initial bradycardia. Fish exposed to low levels of clove oil recovered rapidly when returned to fresh water, but those exposed to higher concentrations (usually stage 4 or 5 induction) exhibited protracted cardiovascular recovery. Recovery occurred more rapidly for fish that were exposed to stage 2 anaesthesia than nonanaesthetized controls. Low levels of clove oil (5 to 9 mg l⁻¹) yielded rapid induction and maintenance of stage 2 anaesthesia in subadult largemouth bass and was effective for mitigating the effects of fish transport stress. The results from this study could be useful for aquaculturists and other handling related husbandry practices that require sedation of fish.

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Keywords: Clove oil; Eugenol; Anaesthetic; Heart rate; Behavior; Hauling; Handling

1. Introduction

Modern aquaculture practices frequently expose fish to a variety of acute stressors that have the potential to negatively affect fish performance and survival (Barton, 1997; Barton, 2000). One method commonly used to minimize or mitigate the effects of stress on fish is the use of anaesthetics (McFarland, 1959; Berka, 1986). Anaesthetics are used to aid in the handling of fish during practices that include enumeration, pathological analyses, hormonal implants or injections, vaccinations, stripping, transfer, and hauling (Carmichael and Tomasso, 1988; Brown, 1993). In general, anaesthetics calm or sedate fish, although the specific pharmacodynamics and pharmacokinetics of fish anaesthetics are poorly understood (Ross and Ross, 1999). In the last several years, clove oil has been recognized as an effective anaesthetic for sedating fish for a number of invasive and noninvasive fisheries management and research procedures (Soto and Burhanuddin, 1995; Anderson et al., 1997; Keene et al., 1998; Prince and Powell, 2000; Srivastava et al., 2003). More recently, efforts have been devoted to testing the efficacy of clove oil for use in the fish culture industry (Taylor and Roberts, 1999; Wagner et al., 2002). Clove oil is also the main constituent in the commercially available product Aqua-S that is marketed extensively towards culturists (Davidson et al., 2000).

Building on the initial studies of Endo et al. (1972) and Hikasa et al. (1986), a suite of research has been conducted that characterizes the dose responses to clove oil for a variety of cultured fishes (e.g., Asian sea bass *Lates calcarifer*; Afifi et al., 2001; rainbow trout, *Oncorhynchus mykiss*; Anderson et al., 1997; Keene et al., 1998; Taylor and Roberts, 1999; white sturgeon, *Acipenser transmontanus*; Taylor and Roberts, 1999; Atlantic salmon, *Salmo salar*; Chanseau et al., 2002). Clove oil generally compares favorably with other common anaesthetics such as tricaine methanesulfonate (MS 222) or Quinaldine for induction and recovery times (e.g., Anderson et al., 1997). Several studies have also compared the physiological effects of using clove oil versus more conventional anaesthetics. Clove oil consistently yields similar levels of

physiological disturbance and minimizes responses to external stressors to that observed with MS 222 (Cho and Heath, 2000; Sladky et al., 2001; Wagner et al., 2003). Collectively, the range of studies available suggests that clove oil is an effective alternative for the sedation of fish and may in fact have several benefits over other methods including its low cost. Interestingly, of those studies conducted, most have assessed high clove oil concentrations that result in deep sedation, loss of equilibrium, and loss of reflex reactivity. These high concentrations and levels of sedation are ideal and necessary for invasive procedures such as surgery (e.g., Prince and Powell, 2000) or extensive biosampling (Taylor and Roberts, 1999). However, in aquaculture, there are many instances where light sedation is sufficient and in fact desirable over deeper sedation, such as to facilitate handling of fish for different husbandry practices or especially for the transport of fish.

Fish are transported for a number of reasons for husbandry purposes including collection and movement of broodstock, movement of hatchery fish to potential release sites (stocking and supplementation), movement of fish to market, or to share unique strains or species with other culture facilities (Berka, 1986). There is no doubt that the transport of fish can result in extensive stress and thus affect survival, and also is energetically costly (Chandross et al., in review). Anaesthetizing fish prior to transport (e.g., Carmichael et al., 1984) can reduce metabolic rate and hence oxygen demand, reduce general activity, increase ease of handling, and mitigate the stress response. The ideal level of sedation for fish transport is referred to as deep sedation and includes loss of reactivity to external stimuli, decrease in metabolic rate, but maintenance of equilibrium (McFarland, 1959). This level of anaesthesia is consistent with stage 2 anaesthesia as described by Summerfelt and Smith (1990). If fish are too heavily sedated, lose equilibrium, and cease swimming, they may die from suffocation if they all settle to the bottom, or experience mechanical injury from hitting the tank walls. Authors (e.g., Wagner et al., 2003) have suggested that low concentrations of clove oil may facilitate fish transport, but at present there is only one preliminary study that actually examines low levels of clove oil. Cooke et al. (2000) evaluated the response of adult rainbow trout transported using four clove oil concentrations by activity radio telemetry. The authors suggested that clove oil showed promise for this purpose, but most of the concentrations tested resulted in total or partial loss of equilibrium and thus hyperactivity or hypoactivity.

The objective of this study was to examine the efficacy of low concentrations of clove oil as a calming agent for fish transportation and handling using largemouth bass *Micropterus salmoides* as a model. In particular, we were interested in identifying the concentration of clove oil that resulted in deep sedation for fish, while permitting the maintenance of equilibrium. This level of sedation has been determined to be optimal for fish transport and general handling (McFarland, 1959; Berka, 1986). Our comprehensive approach examined the behavioral and physiological responses of fish to a gradient of concentrations up to 20 mg l⁻¹. Behavioral assessments involved visual observations of anaesthesia and recovery, as well as videographic observations during hauling by truck. Physiological assessments involved cardiovascular monitoring that encompassed the period of induction, transport, and recovery. Taken together, this study represents one of the first assessments of clove oil for transporting fish, and is

one of the first cardiovascular and in situ behavioral assays of fish responses to hauling.

2. Methods

2.1. Model species and experimental animals

Largemouth bass are one of the most popular recreational sport fish in North America and have been widely introduced around the globe. Many private and government hatcheries raise largemouth bass for eventual fisheries supplementation. These fish can be transported long distances and there are a series of papers that provide culturists with direction for transporting largemouth bass with the use of anaesthetics (e.g., McCraren and Millard, 1978; Carmichael, 1984; Carmichael et al., 1984). The large body of research on largemouth bass transport, combined with their economic importance, makes them an ideal model species for studies of this type.

Fish used for the experiment were obtained by draining a single 1349 m² pond on 10 April 2002 at the Sam Parr Biological Station, Illinois. Surface water temperature at the time of draining was 15.5 °C. Following draining, largemouth bass were transported 150 m from the pond to the Homer Buck Laboratory at the Sam Parr Fisheries Research Station and were held at low densities (<5 kg/m³) in multiple tanks (1.3 m deep and 1.1 m diameter) supplied with a continuous flow of aerated fresh water. Water temperatures varied on a slight diel basis similar to the natural fluctuations observed in the ponds and was similar to the warming trends expected in the spring. Fish were held for 1 week prior to experimentation during which time water temperatures rose and were stabilized at 21 °C. Fish were not fed during the 48 h holding period preceding surgery or transportation so that they would be in a post-absorptive state. Because Woody et al. (2002) documented size specific trends with induction time for sockeye salmon (*Oncorhynchus nerka*), we carefully standardized fish size to eliminate size as a covariate or factor in this study. We chose largemouth bass that were of a size typically used for stocking advanced fingerlings or subadult fish (Carmichael et al., 1984). All experiments described in this study were conducted under the authority of the Committee for Laboratory Animal Resources at the University of Illinois.

2.2. Experimental design

The experimental design involved introducing largemouth bass into tanks containing one of several different clove oil concentrations to examine the behavioral and physiological response of largemouth bass to transport. Two days prior to experimentation, eight fish were affixed with cardiac output devices (i.e., 'cardiac' fish) and permitted to recover. On the day of an experiment, 8 largemouth bass outfitted with cardiac output devices, along with eight additional 'companion' largemouth bass, were moved from the laboratory to the transport tanks containing the appropriate dose of clove oil, ranging from 0 to 20 mg ml⁻¹ (1:9, clove oil: ethanol). During the experiment 'companion' fish were observed for behavioral analyses, while largemouth bass affixed with cardiac output

devices (i.e., ‘cardiac’ fish) were used to quantify cardiac variables. The tanks used in the experiment consisted of eight rectangular plastic containers (approximately 70 l in volume, 60×40 cm), each with a tight-fitting lid, and the volume of water used for all experiments was 50 l. Tanks were oriented in the bed of the pickup truck (Ford F-150) such that the longest side was parallel to the front of the truck. Dissolved oxygen levels were maintained through the use of low fish densities and sloshing from transport. Verification with a dissolved oxygen probe revealed that oxygen levels remained above 6 ppm, a value that provides largemouth bass with adequate oxygen and does not invoke a cardiovascular alteration (Furimsky et al., 2003).

Fish were introduced to tanks already containing appropriate doses of clove oil (or controls) and were monitored visually for 30 min prior to transport to evaluate patterns of induction for behavioral analysis. We used the stages of anaesthesia and recovery outlined in Summerfelt and Smith (1990) (see Table 1). Clove oil concentrations ranged from 0 to 20 mg l⁻¹ and were mixed fresh daily with ethanol to assist with emulsification (1:9, clove oil: ethanol). Lids were applied to the transport tanks, and fish were then driven for 30 min on a paved, secondary highway at speeds of 80 km h⁻¹ and monitored with video equipment.

During hauling, largemouth bass were monitored using a video camera to obtain behavioral measurements. We then returned to the laboratory and monitored cardiac output for 10 min. We then drove for an additional 30 min on a winding gravel road in a State Park. We again monitored video during this period, prior to returning to the lab to monitor cardiac output for an additional 10 min. ‘Cardiac’ fish were then returned to darkened individual chambers containing fresh water and monitored for 4 h. ‘Companion’ fish were introduced to a large freshwater arena and monitored for patterns of behavioral recovery. All ‘companion’ fish were tagged with unique combinations of external anchor tags to identify individuals. Specific details on the behavioral assays and physiological assays can be found below.

Table 1
Stages of anaesthesia induction from Summerfelt and Smith (1990)

Stage of anaesthesia	Descriptor	Characteristics
0	Normal	Reactive to external stimuli; opercular rate and muscle tone normal
1	Light sedation	Slight loss of reactivity to stimuli; slight decrease in opercular rate; equilibrium normal
2	Deep sedation	Total loss of reactivity to all but strong stimuli; slight decrease in opercular rate; equilibrium normal
3	Partial loss of equilibrium	Partial loss of muscle tone; swimming erratic; increased opercular rate; reactive only to strong tactile or vibrational stimuli
4	Total loss of equilibrium	Total loss of muscle tone and equilibrium; slow opercular rate; loss of spinal reflexes
5	Loss of reflex reactivity	Total loss of reactivity; opercular movements slow and irregular; heart rate slow; loss of all reflexes
6	Medullary collapse	Opercular movements cease; cardiac arrest follows

2.3. Behavioral assays

To monitor fish during hauling, a 4-cm hole was cut in the side of each tank, and a sheet of transparent plastic was glued over the hole to provide a watertight seal. A high resolution 0 Lux black and white video camera with infrared illumination (AU 401, J.J. Communications, Englewood, NJ) was positioned outside of each tank, and fish were discretely monitored during treatments (Cooke and Bunt, 2004). A 2×2 grid dividing the tank into four equal quadrants was drawn on the inside of each tank to allow for the determination of relative position within the tank, and a video cassette recorder (SRT 7072, Sanyo, Tokyo) was used to record fish behavior for subsequent analyses.

During transport, each "companion" fish was video taped for at least 2 min during each of two treatments (smooth and rough roads) of which a 60-s period was used for transcription. A series of response variables were transcribed during play-back on a monitor at between normal and 1/10th speed after collection of data. The response variables measured in the study were caudal fin rate, pectoral fin rate, opercular rate, amount of time the fish were resting on the bottom (either with or without equilibrium), the number of times that the fish interacted with each other (an interaction resulted in a change in position or orientation of the "companion fish" and included nips and nudges), and collision rate (a collision involved an uncontrolled or controlled impact of the snout of a fish with a tank wall). In total, behavioral data were collected from 40 'companion fish' fish.

2.4. Physiological assays

Surgical procedures and the equipment used to measure cardiac output are described in detail elsewhere (Cooke et al., 2003b). Briefly, 'cardiac' fish were anaesthetized with 60 mg l⁻¹ clove oil until they lost equilibrium and were non-responsive. Water containing a maintenance concentration of anaesthetic (30 mg l⁻¹ clove oil) was pumped over the gills during surgery. A flexible silicone cuff-type Doppler flow probe (subminiature 20 MHz piezoelectric transducer: Iowa Doppler Products, Iowa City, IA, USA), sized to match the diameter of the vessel, was placed around the aorta and secured with a single suture. The lead wire from the probe was then sutured to the side of the fish in six locations to prevent shifting of the cuff. We used a flowmeter (545C-4 Directional Pulsed Doppler Flowmeter: Bioengineering, The University of Iowa, Iowa City, IA, USA) and a digital strip-chart recorder (LabVIEW, Version 4.0.1, National Instruments, Austin, TX, USA) to monitor cardiac variables including cardiac output and its two components, heart rate and stroke volume. Following surgery, individual 'cardiac' fish were placed immediately into blackened perspex boxes and acclimated to these conditions for ~48 h. Cardiac variables were recorded continuously for at least 1 h prior to the experiment (the basal period). Access to the laboratory was restricted during resting and recovery to prevent external disturbance.

Following experimentation, 'cardiac' fish were euthanized with an overdose of anaesthetic (200 mg l⁻¹ clove oil), and a post-mortem calibration was conducted to convert Doppler shift (in Volts) to actual blood flow (ml min⁻¹) (Cooke et al., 2003b). Data were averaged over a 10-min period to obtain basal values, as well as values for

both smooth and rough transport. To determine recovery times, cardiac traces were adjusted to resting (100%), plotted for each fish as 60-s means and evaluated visually (Schreer et al., 2001; Cooke et al., 2003b). A fish was considered to have recovered when values became stable and returned to within 5% of resting values (Schreer et al., 2001). In total, valid cardiac output data were collected for 30 of the 40 fish. Problems with calibrations and cuff slippage during transport resulted in the exclusion of some cardiac fish from analysis.

2.5. Statistical analyses

Differences in response variables were plotted versus the clove oil concentrations for both smooth and rough roads. Where possible, we used linear regression and analysis of covariance (ANCOVA) to assess the relationship between clove oil concentration and fish behavior and physiology. For instances when transformations did not linearize data, we were unable to use ANCOVA, so instead we used third order polynomials and plotted 95% confidence limits. For instances where confidence intervals overlapped and patterns were similar, we concluded no difference between smooth and rough treatments (Sokal and Rohlf, 1995). We also divided the gradient of clove oil concentrations into 4 categories (0–4.9, 5–9.9, 10–14.9, and 15–20 mg l⁻¹) for further analyses with ANOVA and Tukey HSD post-hoc tests. All statistical analyses were performed using JMP IN version 4.0 (SAS Institute), and the level of significance (α) for all tests was 0.05.

3. Results

3.1. Induction behavior

The mass or total length of fish used in behavioral assays did not vary across the gradient of concentrations (Regression Analyses, $P>0.05$) or among the four categorical concentrations (ANOVA, $P>0.05$). Also, the mean size of fish used in the behavioral assays (TL=206±4 mm, Mass=93±7 g) did not differ from the mean size of fish used in physiological assays (TL=209±5 mm, Mass=98±6 g; T -tests, P 's>0.05). Experimental water temperatures were 21.1±0.3 °C.

The maximum depth of anaesthesia increased significantly as clove oil concentration increased (Regression, $r^2=0.85$, $P<0.001$; Fig. 1A). Most fish achieved either stage 2 or stage 4 anaesthesia. Very few fish, however, achieved or maintained a maximum value of 3, which is indicative of partial loss of equilibrium. Stage 2 anaesthesia is regarded as an ideal value for fish transport and general handling. Control fish exhibited no indication of anaesthesia (Stage 0). When examined on a categorical basis, there was a consistent increase in stage of anaesthesia for each increasing clove oil concentration category (ANOVA, $P<0.001$; Tukey's HSD Test, P 's<0.05; Table 2). The time required to reach the maximal and stable stage of anaesthesia also varied significantly in a non-linear manner and was best described by a 3rd order polynomial equation (Regression, $r^2=0.22$, $P=0.033$; Fig. 1B). When examined on a categorical basis, the two lowest

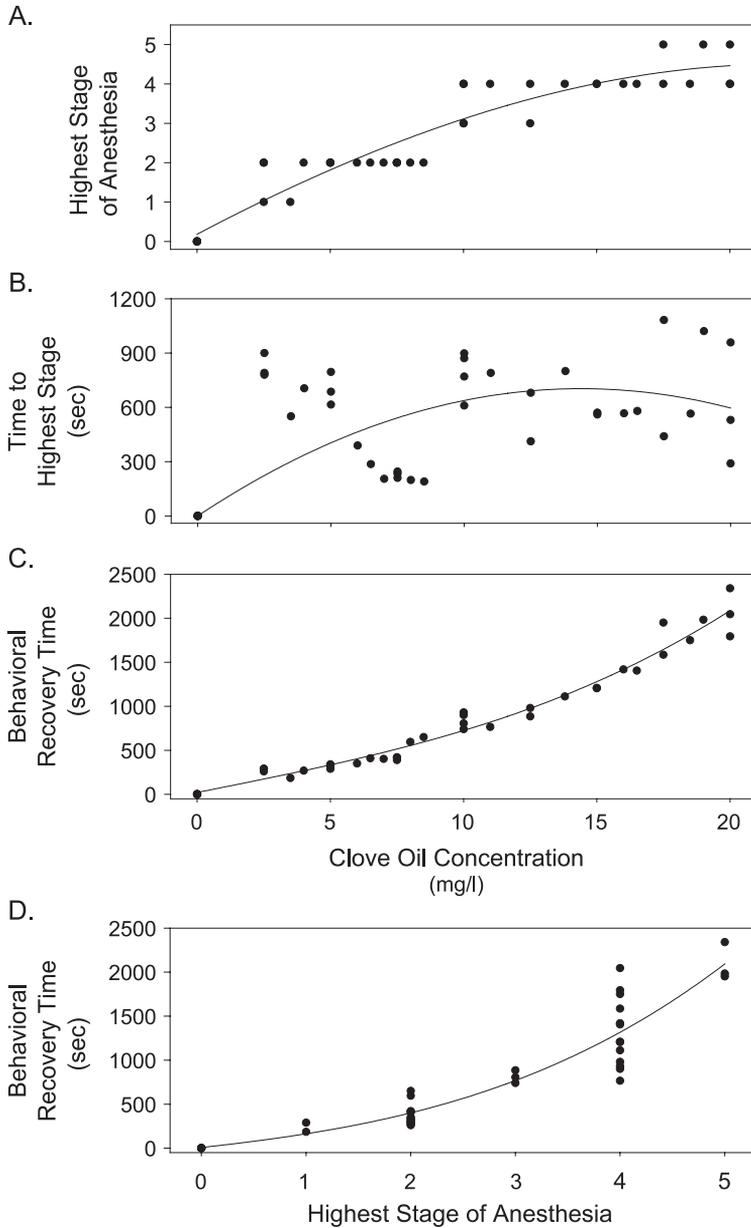


Fig. 1. Effects of a gradient of clove oil concentrations on the induction behavior and recovery of largemouth bass ($N=40$ fish). Visual observations included (A) the highest stage of stable anaesthesia, (B) the time to reach the highest stage of anaesthesia, (C) the time for fish to recover from anaesthesia, and (D) the behavioral recovery time for different stages of anaesthesia. Stage of anaesthesia and recovery were based upon the criteria set by Summerfelt and Smith (1990). All regressions are 3rd order polynomials.

Table 2
Behavioral values for fish exposed to four categorical concentrations of clove oil

Variable	Road type	Clove oil concentration category			
		0.0–4.9 mg l ⁻¹	5.0–9.9 mg l ⁻¹	10.0–14.9 mg l ⁻¹	15.0–20.0 mg l ⁻¹
<i>N</i>	NA	9	11	9	11
Maximum stage of anaesthesia	NA	0.8 ^a (0.3)	2.0 ^b (0.0)	3.6 ^c (0.2)	4.3 ^c (0.1)
Time to maximum stage of anaesthesia (s)	NA	414 ^{a,b} (134)	368. ^a (67)	729 ^b (56)	651 ^{a,b} (76)
Behavioral recovery time (s)	NA	144 (47)	417 (33)	889 (43)	1699 (110)
Caudal fin rate (beats/min)	Smooth	56.4 ^a (3.1)	39.4 ^b (1.8)	21.8 ^c (3.8)	4.7 ^d (2.3)
	Rough	62.2 ^a (1.2)	44.6 ^b (2.3)	26.1 ^c (4.6)	3.9 ^d (0.7)
Pectoral fin rate (beats/min)	Smooth	63.6 ^a (2.2)	46.3 ^b (2.1)	24.1 ^c (3.5)	7.2 ^d (3.2)
	Rough	66.3 ^a (1.8)	50.2 ^b (2.4)	26.2 ^c (4.9)	5.5 ^d (1.0)
Ventilation rate (vents/min)	Smooth	65.7 ^a (0.9)	57.2 ^b (1.1)	55.8 ^b (2.5)	39.5 ^c (1.6)
	Rough	66.5 ^a (0.9)	59.0 ^b (4.7)	57.1 ^b (2.1)	41.9 ^c (1.9)
Conspecific interaction rate (interactions/min)	Smooth	3.7 ^a (0.8)	0.1 ^b (0.1)	0 ^b (0)	0 ^b (0)
	Rough	3.9 ^a (0.7)	0.5 ^b (0.1)	0 ^b (0)	0 ^b (0)
Tank collision rate (collisions/min)	Smooth	0.1 ^a (0.1)	0.1 ^a (0.1)	4.6 ^b (0.9)	12.1 ^c (1.4)
	Rough	0.9 ^a (0.3)	1.2 ^a (0.3)	16.7 ^b (3.1)	27.6 ^c (2.4)
Time resting on bottom (s)	Smooth	1.6 ^a (1.3)	3.5 ^a (1.2)	31.3 ^b (8.9)	85.4 ^c (3.9)
	Rough	0.6 ^a (0.3)	2.8 ^a (1.4)	30.5 ^b (3.3)	50.2 ^c (4.1)

Values reported are means (\pm S.E.). Dissimilar superscript letters within a row indicate significant differences detected with Tukey HSD post-hoc tests at $P < 0.05$.

concentration categories achieved significantly lower stages of anaesthesia than the two highest categories (ANOVA, $P = 0.016$; Tukey's HSD Test, P 's < 0.05 ; Table 2).

3.2. Behavior during transportation

Significant negative linear relationships were observed for caudal fin rate and pectoral fin rate with clove oil concentration during transport (Regressions, r^2 's = 0.90 to 0.93, P 's < 0.05 ; Fig. 2A,B) and fish transported on both smooth and rough terrain exhibited similar patterns (ANCOVA, $P > 0.05$). Opercular rates also exhibited significant negative linear relationship with clove oil concentration for both smooth (Regression, $r^2 = 0.80$, $P < 0.001$) and rough roads (Regression, $r^2 = 0.79$, $P < 0.001$; Fig. 2C) and did not differ statistically (ANCOVA, $P > 0.05$).

While being transported, the number of interactions between "cardiac" and "companion" fish was greatest (ANOVA, P 's < 0.05 , Table 2) at low clove oil concentrations and decreased precipitously (Regressions, $r^2 = 0.96$, $P < 0.001$, Fig. 3A) at higher clove oil concentrations (> 5 mg l⁻¹) regardless of road type. Tank collision rate, indicating the amount of physical damage that was occurring to the fish, was very low at low concentrations, but significantly increased above 10 mg l⁻¹ (ANOVA, P 's < 0.05 , Table 2) for both road types (Regressions, r^2 's = 0.69 to 0.79, P 's < 0.05 , Fig. 3B). Regardless of road type, fish exposed to higher concentrations of clove oil spent a significantly greater percentage of time sitting on the bottom (ANOVA, P 's < 0.05 ; Table 2) than did fish exposed to lower concentrations of clove oil (Regressions, $P < 0.05$, Fig. 3C).

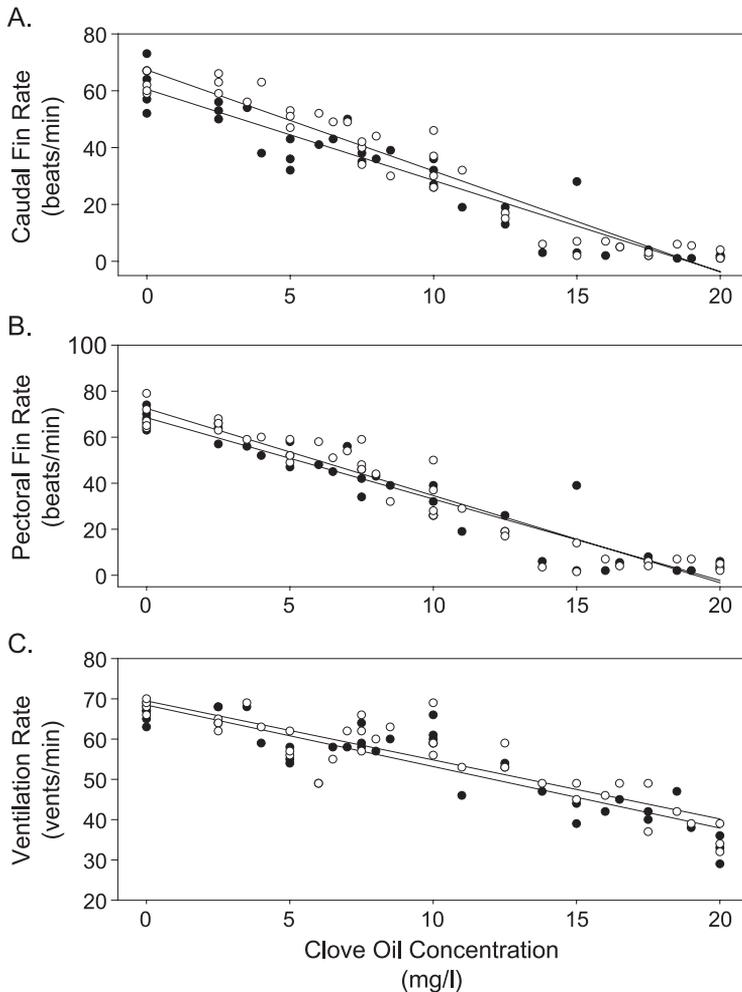


Fig. 2. Behavior and activity of largemouth bass during transport in different concentrations of clove oil. Filled circles are for the smooth road treatment and open circles are for the rough road treatment. Quantitative values were recorded for (A) caudal fin rates, (B) pectoral fin rate, and (C) ventilation rate. All line presented are linear regressions.

3.3. Behavioral recovery

Two hours after the introduction of the clove oil and the initiation of the transport experiment, behavioral recovery times varied extensively by concentration (Regression, $r^2=0.97$, $P<0.001$; Fig. 1C). As clove oil concentrations increased recovery times took longer (Regression, $r^2=0.97$, $P<0.001$; Fig. 1C) and also increased (ANOVA, $P<0.001$; Tukey's HSD Test, $P's<0.05$; Table 2). Control fish did not exhibit any indications of anaesthesia and were behaviorally considered recovered immediately after their introduction to the recovery tank. Behavioral recovery time was also positively correlated

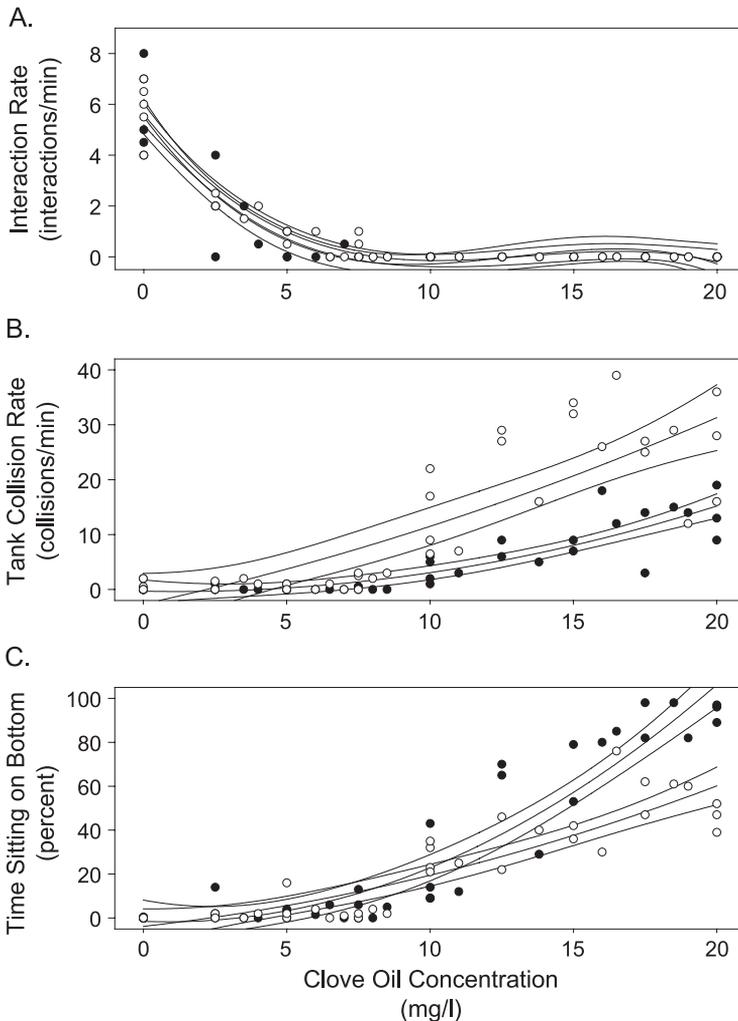


Fig. 3. Behavior and activity of largemouth bass during transport in different concentrations of clove oil. Filled circles are for the smooth road treatment and open circles are for the rough road treatment. Quantitative values were recorded for (A) interaction rate, (B) tank collision rate, and (C) time sitting on the bottom (percent). All lines presented are 3rd order polynomials, although (A) is an inverse fit. Confidence intervals (95%) are also plotted.

with the maximum stage of anaesthesia (Regression, $r^2=0.87$, $P<0.001$; Fig. 1D). All fish survived the experiments.

3.4. Physiological disturbance and recovery

The mass or total length of fish did not vary across the gradient of concentrations (Regressions, $P>0.05$) or among the four categorical concentrations (ANOVA, $P>0.05$)

for fish used for cardiac analyses. Similarly, basal cardiovascular variables (i.e., cardiac output, heart rate, and stroke volume) did not vary across the gradient of concentrations (Regression, $P>0.05$) or among the four categorical concentrations (ANOVA, $P>0.05$; Table 3). Overall mean basal cardiovascular variables during experiments conducted at 21.1 ± 0.3 °C were 30.9 ± 0.3 ml kg⁻¹ min⁻¹ for cardiac output, 40.2 ± 0.3 beats/min for heart rate, and 0.771 ± 0.003 ml kg⁻¹ for stroke volume. Although there was no evidence indicating that basal cardiovascular variables varied with treatment, there was sufficient individual variation that we transformed individual raw values for the smooth and rough driving treatments to percent change from basal.

When initially exposed to clove oil, fish experienced a brief bradycardia (seconds) prior to elevating cardiac output through increases in heart rate. Stroke volume typically decreased slightly. Interestingly, cardiac output and heart rate rarely decreased throughout the entire experiment. Cardiovascular responses to transport on smooth roads were quite variable across a gradient of clove oil concentrations. Both cardiac output and heart rate exhibited similar wave patterns, whereas stroke volume exhibited an inverse wave pattern relative to cardiac output and heart rate (Regressions, P 's <0.05 ; Fig. 4A,B,C) and differed among the clove oil concentrations (ANOVA's, P 's <0.05). The second lowest category that incorporated concentrations of 4.9 to 9.9 mg l⁻¹ consistently had less cardiovascular alteration than the other concentrations (Tukey HSD tests, P 's <0.05 ; Table 3).

Cardiovascular data for the rough driving treatment followed the same patterns observed for the smooth road as indicated by significant overlap of confidence intervals.

Table 3
Physiological values for fish exposed to four categorical concentrations of clove oil

Variable	Road type	Clove oil concentration category			
		0.0–4.9 mg l ⁻¹	5.0–9.9 mg l ⁻¹	10.0–14.9 mg l ⁻¹	15.0–20.0 mg l ⁻¹
<i>N</i>	NA	7	8	5	10
Basal cardiac output (ml kg ⁻¹ min ⁻¹)	NA	30.8 (0.5)	31.5 (0.4)	30.5 (0.4)	31.0 (0.3)
Basal heart rate (beats/min)	NA	40.3 (0.7)	40.3 (0.6)	39.4 (0.4)	40.5 (0.4)
Basal stroke volume (ml kg ⁻¹)	NA	0.767 (0.003)	0.776 (0.002)	0.773 (0.004)	0.769 (0.003)
Cardiac output (ml kg ⁻¹ min ⁻¹) during transport	Smooth	44.1 ^a (0.8)	32.6 ^b (1.6)	46.7 ^a (1.4)	43.5 ^a (1.8)
	Rough	45.2 ^a (1.1)	32.3 ^b (6.6)	46.5 ^a (3.4)	43.1 ^a (1.9)
Heart rate (beats/min) during transport	Smooth	66.6 ^a (1.1)	46.2 ^b (3.3)	84.6 ^c (4.3)	74.1 ^{a,c} (4.0)
	Rough	72.4 ^a (1.7)	47.0 ^b (4.7)	93.9 ^c (6.0)	74.0 ^a (3.9)
Stroke volume (ml kg ⁻¹) during transport	Smooth	0.664 ^a (0.006)	0.709 ^a (0.016)	0.555 ^b (0.017)	0.589 ^b (0.011)
	Rough	0.627 ^a (0.007)	0.698 ^b (0.018)	0.510 ^c (0.027)	0.589 ^{a,c} (0.015)
Cardiac output recovery time (min)	NA	82 ^a (2)	51 ^b (4)	114 ^c (5)	119 ^c (4)
Heart rate recovery time (min)	NA	81 ^a (3)	51 ^b (4)	114 ^c (5)	119 ^c (4)
Stroke volume recovery time (min)	NA	73 ^a (2)	45 ^b (4)	104 ^c (6)	107 ^c (4)

Values reported are means (\pm S.E.). Dissimilar superscript letters within a row indicate significant differences detected with Tukey HSD post-hoc tests at $P<0.05$.

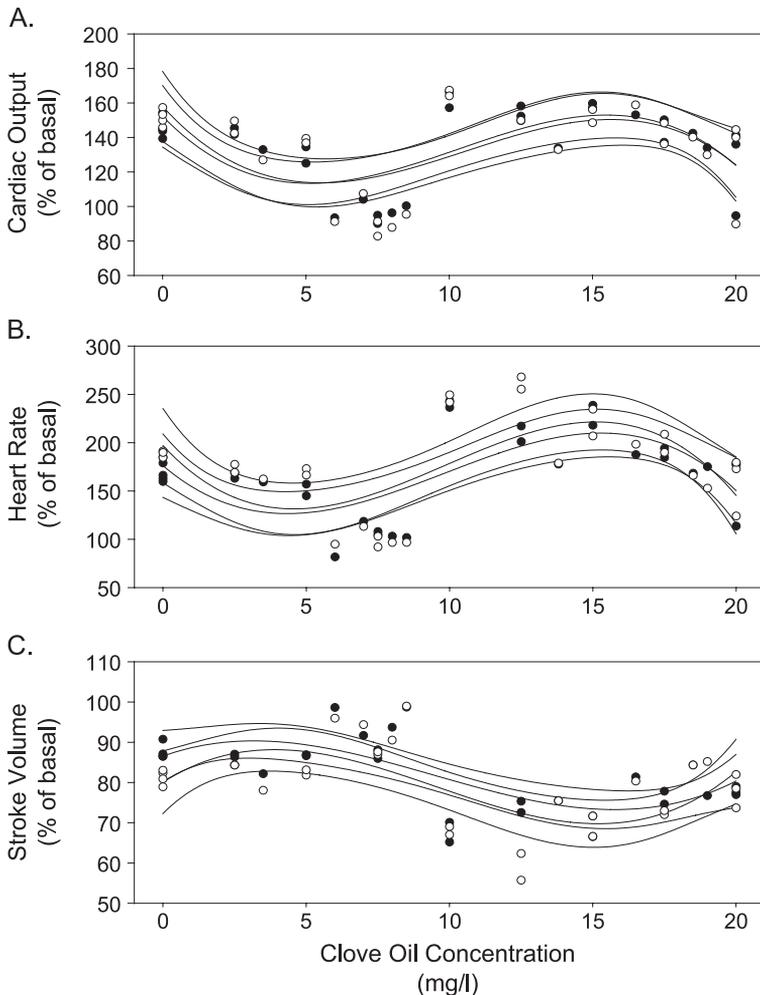


Fig. 4. Mean cardiovascular variables (% of basal) for largemouth bass during exposure to corresponding clove oil concentrations while being transported on different types of roads. Filled circles are for the smooth road treatment and open circles are for the rough road treatment. All lines presented are 3rd order polynomials with 95% confidence intervals.

All three cardiovascular variables varied among clove oil concentration categories (ANOVA, $P < 0.05$). The second lowest concentration of clove oil (4.9 to 9.9 mg l^{-1}) resulted in less cardiovascular disturbance (cardiac output, heart rate, and stroke volume) than the lower or two higher concentrations (Tukey HSD tests, $P < 0.05$; Table 3).

When returned to anaesthetic-free fresh water, cardiac output and heart rate typically increased. Although recovery times were variable across clove oil concentrations, there was a strong relationship between cardiovascular recovery time and clove oil concentration for all three cardiac variables (regressions, r^2 's = 0.47 to 0.49 , P 's < 0.05). Recovery time varied significantly among clove oil concentration categories, increasing with the

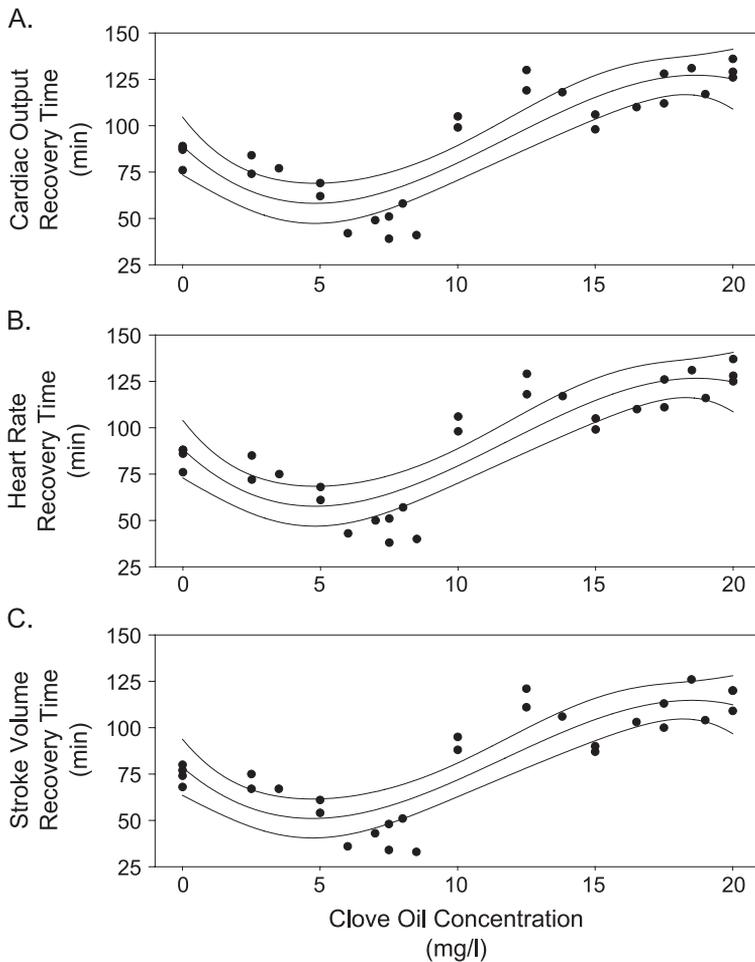


Fig. 5. Individual cardiac recovery times for largemouth bass exposed to a range of clove oil concentrations during transportation. Recovery values were recorded after fish were introduced to fresh water void of anaesthetic. All lines presented are 3rd order polynomials with 95% confidence intervals.

higher categories (Fig. 5A,B,C). The only departure from this pattern was the 5 to 9.9 mg l^{-1} category where recovery times were significantly faster (~ 45 min) than all other categories (range of ~ 70 to 120 min) including the lower category (0 to 4.9 mg l^{-1}) (Tukey HSD Tests, $P < 0.05$; Table 3).

4. Discussion

This study evaluated the use of low concentrations of clove oil as a tool for sedating fish for handling and transportation in aquaculture. Light anaesthesia that permits fish to maintain equilibrium, swimming activity, and breathing can be effective for mitigating

stress associated with fish handling and fish transport (McFarland, 1960; Piper et al., 1982). Collectively, our results indicate that low levels of clove oil can be used to induce anaesthesia ranging from subtle calming to complete immobilization and loss of equilibrium. Coupled with this variation in depth of anaesthesia, we observed substantial differences in physiological disturbance and behavior during transportation. In addition, behavioral and physiological recovery rates varied with level of anaesthesia. However, our results clearly identified a range of clove oil concentrations that are optimal for fish handling and transport. Specifically, concentrations of clove oil ranging from ~5 to 8.5 mg l⁻¹ yielded rapid and stable stage 2 anaesthesia (see Table 1; Summerfelt and Smith, 1990). During transport, fish anaesthetized at that level exhibited reduced activity and interaction with conspecifics, but were able to maintain equilibrium, swimming capacity, and avoid physical damage resulting from collision with the tank walls. Furthermore, the magnitude of cardiovascular disturbance during transportation at that level of anaesthesia was low, the cardiovascular recovery time was rapid, and the behavioral recovery was fast relative to largemouth bass anaesthetized at other levels, or nonanaesthetized controls. We discuss our findings in the context of using low concentrations of clove oil for fish handling and transportation.

The duration of time required to reach a stable level of anaesthesia was longer than previously documented when clove oil was used at higher concentrations in other species (e.g., white sturgeon, 100 mg l⁻¹, 246 s, Taylor and Roberts, 1999; rainbow trout, 30 mg l⁻¹, 3.7 min, Prince and Powell, 2000; red pacu, 50 mg l⁻¹, 290 s, Sladky et al., 2001; sockeye salmon, 50 mg l⁻¹, 84 s, Woody et al., 2002; Atlantic salmon, 50 mg l⁻¹, 360 s, Iversen et al., 2003). Although there are a number of factors including water temperature (Hamáčková et al., 2001; Walsh and Pease, 2002), fish size (Woody et al., 2002), and gender (Woody et al., 2002) that may affect induction time, our experience with using clove oil to anaesthetize largemouth bass for a number of surgical procedures indicates that at higher concentrations, induction of largemouth bass is rapid (Cooke, personal observations). For example, at similar water temperatures, largemouth bass that were both smaller (Cooke et al., 2003a) and larger (Cooke et al., 2003b) exposed to 60 mg l⁻¹ required less than 300 s to reach stage 5 anaesthesia. Stage 2 anaesthesia appears relatively easy to achieve relative to stage 3 anaesthesia. Stage three involves loss of partial equilibrium and most fish either maintain equilibrium and stay at stage 2 or lose equilibrium completely and progress to stage 4. At the higher end of concentrations that yielded stage 2 anaesthesia, induction was rather rapid, requiring less than 5 min. This timing is more consistent with the rapid induction times previously noted among many studies of clove oil. This also provides support that 5 to 9 mg l⁻¹ is an effective concentration for rapidly inducing stage 2 anaesthesia. Other researchers that have used low concentrations of clove oil indicated protracted induction times relative to higher concentrations, although no one else reports values as low as those reported here (e.g., Atlantic salmon, 10 mg l⁻¹, 720 s to reach stage 3a, Iversen et al., 2003; white sturgeon, 10 mg l⁻¹, 180–260 s to unreported stage, Taylor and Roberts, 1999; coho salmon *Oncorhynchus kisutch* and Chinook salmon *Oncorhynchus tshawytscha*, 10 mg l⁻¹, 240 s to unreported stage, Taylor and Roberts, 1999).

During transport, fish can become injured from physical interactions with conspecifics or from abrasion or concussion with the tank walls (McFarland, 1959).

In our study, we observed that interaction rates between fish were highest for unanaesthetized controls and dropped steadily for fish that had reached stage 2 anaesthesia. Conversely, rates of collision with the tank walls was low for unanaesthetized controls up to stage 2 anaesthesia, but increased rapidly at higher concentrations. This was particularly evident for fish that had lost equilibrium and were impacting the tank wall with wave motions. These findings are consistent with the belief that stage 2 is effective for minimizing fish damage during transport. Fish exposed to high levels of anaesthesia in our study ($10\text{--}20\text{ mg l}^{-1}$) spent much of their time sitting on the bottom, often on their side or upside down. Interestingly, differences between smooth and rough roads were only noted for tank collision rate and duration of time spent on the bottom. Rough roads resulted in more tank collisions and less time spent resting on the bottom for fish anaesthetized beyond stage 2. In a preliminary study, Cooke et al. (2000) used low levels of clove oil and monitored activity in adult rainbow trout during transport and determined that fish which loose equilibrium may expend significant energy attempting to right themselves.

There is a paucity of information on the cardiovascular responses of fish to anaesthetics. Using tench (*Tinca tinca*), Randall (1962) observed that the heart rate measured via electrocardiograms (ECGs) was markedly increased by anaesthesia with MS 222, particularly at higher concentrations. Consistent with the findings of Randall (1962), we documented consistent increases in heart rate for most concentrations. Afifi et al. (2001) noted focal subepicardial hemorrhage in the hearts of Asian sea bass exposed to 9 mg l^{-1} of clove oil indicating that a slight increase in heart rate may have occurred which is also consistent with our results. Although not assessed quantitatively, Sladky et al. (2001) indicated that red pacu exposed to high concentrations (i.e., 100 to 200 mg l^{-1}) of clove oil may have had their cardiac function compromised as evidenced by difficulty in obtaining blood samples. They further speculate that this may be a result of hemodynamic instability or insufficient oxygen loading or delivery at high concentrations. Hikasa et al. (1986) conducted preliminary examinations on the effects of clove oil on the heart rate of carp (*Cyprinus carpio*) and noted that it had an inhibitory effect at high concentrations. Clove oil concentrations used in our study were much lower and we observed no empirical evidence of cardiovascular compromise or collapse.

Our study is different from the aforementioned studies in that it is the first to report on the effects of a fish anaesthetic on cardiac output, the product of heart rate and stroke volume. Different species of fish respond to different stimuli through either regulation of heart rate (frequency modulation) or stroke volume (volume modulators; Farrell and Jones, 1992). In our study, we used largemouth bass that have been identified as frequency modulators (i.e., they regulate cardiac output principally through changes in heart rate; Cooke et al., 2003b). However, much research indicates that other species of fish including most salmonids, can maintain or reduce heart rate, but elevate stroke volume to maintain or elevate cardiac output (Farrell, 1991). For this reason, it is possible that reduced heart rate is not a global response as suggested in the criteria proposed by Summerfelt and Smith (1990). In our study, heart rate generally increased from low to intermediate concentrations, but then began to decrease slightly at values approaching 20 mg l^{-1} . It is possible that heart rate would have continued to decrease

yielding the response indicated by [Summerfelt and Smith \(1990\)](#). Indeed, our observations from anaesthetizing fish in clove oil at higher concentrations (i.e., $>40 \text{ mg l}^{-1}$) suggest that heart rate (and cardiac output) decreases after prolonged anaesthesia (Cooke, unpublished data). We suggest that reduced heart rate or ventilation rate should not be included in the criteria for anaesthesia, and instead, these criteria should be replaced by the more robust measure of cardiac output which should yield a more consistent response to anaesthesia, irrespective of whether fish are frequency or volume modulators. Although we did not record ventilation rate on the same fish for which we monitored cardiac output, there seems to be a clear decoupling of ventilation rate and cardiovascular variables further highlighting the need to monitor cardiac output.

Cardiac recovery times were used as an indicator of physiological recovery and fish exposed to anaesthesia generally exhibited increased recovery time with increasing concentrations of clove oil. Fish at lower concentrations of clove oil (i.e., $2.5\text{--}9 \text{ mg l}^{-1}$) recovered in ~ 60 min, even more quickly than unanaesthetized control fish (~ 75 min). Cardiovascular recovery time is important as it can indicate relative metabolic demand ([Farrell and Jones, 1992](#)). Fish that recover more rapidly have increased metabolic scope for engaging in other activities such as feeding, movement, predator avoidance, or preparation for successive stressors ([Priede, 1985](#)). Other studies examining the physiological disturbance of fish following exposure to clove oil indicate that biochemical indicators of stress in fish anaesthetized with clove oil are similar to fish anaesthetized with other anaesthetics ([Cho and Heath, 2000](#); [Sladky et al., 2001](#); [Iversen et al., 2003](#); [Pirohen and Schreck, 2003](#); [Small, 2003](#); [Tort et al., 2002](#)). Interestingly, our cardiovascular recovery times are much more rapid than the 4 to 24 h often required for biochemical indicators of stress such as cortisol and glucose to return to resting levels.

Behavioral recovery followed similar patterns to physiological recovery. Fish exposed to higher concentrations, yielding deeper levels of anaesthesia, exhibited slower behavioral recovery. In particular, those fish that reached level 4 and 5 anaesthesia required between 10 and 30 min to recover behaviorally. This period is substantially longer than recovery times reported for other fishes at higher concentrations (sockeye salmon, 50 mg l^{-1} , 330 s, [Woody et al., 2002](#); rainbow trout, 30 mg l^{-1} , 294 s, [Prince and Powell, 2000](#); white sturgeon, 50 mg l^{-1} , 186 s, [Taylor and Roberts, 1999](#)). In aquaculture settings, recovery of that duration would be problematic, particularly if fish were being stocked to supplement a fishery. Fish would be highly susceptible to predation and displacement by flow or currents during prolonged recovery so transport at these deep levels of sedation (i.e., $>$ stage 2) would be undesirable. Although behavioral recovery was longer for fish exposed to low concentrations than controls, physiological recovery was more rapid for low concentrations than controls. When combined with the reduced oxygen demand during transport (inferred from lower cardiac output), ease of handling, and reduced interaction with conspecifics during transport, the use of the low levels of clove oil appears to be more favorable than transporting unanaesthetized fish.

Although our study design and approach provided novel insights to the effects of anaesthetics on fish and the utility of clove oil for transport, there were several limitations. First, we applied the clove oil after an initial 10 s net transfer and although this was brief, we may have not derived the greatest potential value from sedation. A number of studies

have identified that the stress response in fish is initiated during the transfer and handling stages (Strange and Schreck, 1978; Barton et al., 1980). Thus, adding anaesthetic to the water after the initial stressor can be less effective. Nonetheless, our research clearly illustrates positive effects arising from anaesthesia. Furthermore, Wagner et al. (2003) determined that clove oil can also be effective for minimizing stress after the stressor has already been applied. Second, we used low hauling densities ($<0.02 \text{ kg/m}^3$) relative to actual hauling practices ($>100 \text{ kg/m}^3$). Our desire to use focal video sampling strategies combined with the presence of the cardiac output cuff wire precluded the use of higher densities. However, our control fish exhibited significant conspecific interactions indicating that our densities were effective for generating transport type stressors. One final limitation of our study was that fish affixed with cardiac monitoring apparatus had been anaesthetized with clove oil at 50 mg l^{-1} to achieve immobilization for surgery. There is no evidence that multiple exposure of individuals results in any alterations in how fish respond to subsequent exposures, however, evidence is limited to a single study (Afifi et al., 2001). We waited 48 h between surgery and experimentation in an attempt to ensure adequate clearance of clove oil residuals.

Previous research on largemouth bass determined that use of low levels of MS 222 (i.e., 25 mg l^{-1}) during transport was effective following an initial induction with 50 mg l^{-1} , when combined with withholding of food prior to transport, and use of cooled water with salt and antibiotic (Carmichael et al., 1984). In our study, we withheld food prior to transport, but did not employ the other prophylactic suggestions used by Carmichael et al. (1984). Their study provided little detail on the level of sedation achieved by the use of these anaesthetics. Based upon the positive results of our study using clove oil to transport largemouth bass, coupled with the growing body of literature indicating similar biochemical disturbances and mitigative effects between clove oil and MS 222 (e.g., Cho and Heath, 2000; Iversen et al., 2003; Pirohen and Schreck, 2003; Wagner et al., 2003), we suggest that clove oil should be an effective alternative transport anaesthetic, especially when combined with some of the other treatments that Carmichael et al. (1984) identified to alleviate stress. Using the concentrations identified here for eliciting stage 2 anaesthesia in largemouth bass at 21°C , it should be possible to determine appropriate concentrations for stage 2 anaesthesia for specific organisms and environmental conditions. Although our study focused on the use of clove oil for fish transportation, the concentrations required to induce stage 2 anaesthesia identified as optimal for fish transport should also be effective for the general handling of cultured fish for grading, marking, enumerating, inspection, and gamete stripping.

Acknowledgments

This study benefited from the expert field assistance of E. Osier, B.J. Bauer, M. Siepker, and C. Ostrodka. We also thank D. Philipp for supporting our research activities at the Sam Parr Biological Station. Financial support was provided by the Illinois Natural History Survey, Illinois Department of Natural Resources, University of Illinois, Queen's University, and the Natural Sciences and Engineering Research Council of

Canada. In addition, SJC received a Grant in Aid of Research from Sigma Xi to support this research.

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