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Brian R. Gause<sup>a</sup>, Jesse T. Trushenski<sup>a</sup>, John C. Bowzer<sup>a</sup> & James D. Bowker<sup>b</sup>

<sup>a</sup> Fisheries and Illinois Aquaculture Center, Southern Illinois University Carbondale, 1125 Lincoln Drive, Life Science II, Room 173, Carbondale, Illinois, 62901-6511, USA

<sup>b</sup> U.S. Fish and Wildlife Service, Aquatic Animal Drug Approval Partnership Program, 4050 Bridger Canyon Road, Bozeman, Montana, 59715, USA

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TECHNICAL NOTE

# Efficacy and Physiological Responses of Grass Carp to Different Sedation Techniques: I. Effects of Various Chemicals on Sedation and Blood Chemistry

Brian R. Gause, Jesse T. Trushenski,\* and John C. Bowzer

Fisheries and Illinois Aquaculture Center, Southern Illinois University Carbondale, 1125 Lincoln Drive, Life Science II, Room 173, Carbondale, Illinois 62901-6511, USA

James D. Bowker

U.S. Fish and Wildlife Service, Aquatic Animal Drug Approval Partnership Program, 4050 Bridger Canyon Road, Bozeman, Montana 59715, USA

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**Abstract**

Grass carp *Ctenopharyngodon idella* are commonly used as a low cost, biological control for aquatic vegetation in aquaculture ponds and other private and public waters. In order to minimize the risk of establishing self-sustaining populations in U.S. waters, many states now require grass carp be certified as triploid prior to sale and stocking. To facilitate ploidy testing, grass carp are typically sedated before collecting blood samples. Chemical sedatives such as tricaine methanesulfonate (MS-222) and carbon dioxide (CO<sub>2</sub>) are most commonly used to sedate fish, but there is increasing interest in other chemical sedatives such as benzocaine and eugenol. We evaluated time to induction to Stage IV sedation and recovery, survival, and postsedation blood chemistry of grass carp (301 ± 8 g, mean ± SE) sedated with MS-222 (150 mg/L), benzocaine (150 mg/L), eugenol (60 mg/L), or CO<sub>2</sub> (~400 mg/L). Induction times for all sedatives excluding CO<sub>2</sub> (14.9 min) were less than 2.4 min (range, 1.5–2.4 min). Average recovery time after induction was 5.8 min (range, 2.8–8.3 min) excluding benzocaine, which had a recovery time of 15.4 min. Survival was high and unaffected by sedative option. Plasma cortisol and lactate levels peaked between 0.5 and 1 h postinduction before returning to resting levels at 6 h postinduction. No obvious changes were observed in blood glucose or hematocrit. Each of the sedatives was effective in sedating grass carp, and though changes in blood chemistry indicated that an acute stress response occurred, the response was transient. Although each of the evaluated sedatives would facilitate ploidy testing, some strategies may be more appropriate than others based on FDA approval status and access to the sedative compound, handling time, withdrawal period, and on-site conditions and resources.

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Grass carp *Ctenopharyngodon idella* are commonly used as a low cost, biological control for aquatic vegetation in aquaculture

ponds and other private and public waters (Masser 2002). Concerns regarding the establishment of self-sustaining populations of this nonnative species have led to bans on stocking fertile, diploid grass carp in many states (Kelly et al. 2011). Triploid fish, rendered “functionally sterile” through the interfering effects of ploidy manipulation on gametogenesis (Benfey 1999; Zajicek et al. 2011), may be legally stocked in some states, but triploidy must be verified prior to sale and stocking in those states allowing such fish (Zajicek et al. 2011). A rapid triploidy verification test developed by Wattendorf (1986) requires only a small blood sample for analysis to verify ploidy state. Fish are typically sedated to facilitate blood sampling, but there are a limited number of drugs or chemical sedatives currently available for this purpose that are approved by the U.S. Food and Drug Administration (FDA) or are otherwise made available by the FDA for use.

The only drug currently approved by the FDA for the temporary immobilization of fish is tricaine methanesulfonate, most commonly referred to as MS-222. The use of MS-222 is limited to ictalurids, salmonids, esocids, percids, or other laboratory and hatchery fishes at water temperatures greater than 10°C. Users of MS-222 must adhere to a 21-d withdrawal period prior to fish being released or slaughtered for consumption. Fish must be fed and kept healthy during this holding period, and in the case of grass carp, must also be maintained in separate holding systems to maintain validity of the ploidy verification tests. Owing to limitations of suitable holding tanks, it is often impractical to hold segregated fish for an extended period of time and doing so probably contributes to additional costs for producers and

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\*Corresponding author: saluski@siu.edu

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customers alike. Carbon dioxide (CO<sub>2</sub>) is not currently approved by the FDA as a sedative for fishes, but is considered a drug of “low regulatory priority,” meaning that the FDA is unlikely to enforce regulations provided that CO<sub>2</sub> is administered according to the stipulations made in the FDA’s “Enforcement Priorities for Drug Use in Aquaculture” documentation (USFDA 2011). Although CO<sub>2</sub> has no withdrawal period, creating and maintaining intended sedative concentrations in the field can be difficult, and is not effective for all fishes or environments (e.g., hypercapnia tolerant species, marine environments). A number of crude and purified drugs (e.g., clove, spearmint, and wintergreen oils, quinaldine) are widely used to sedate fishes in the field and laboratory. These compounds, however, are not FDA-approved and can only be used in fish for research purposes, provided that all treated fish are destroyed via incineration, burial, or some other method to ensure they do not enter the food chain. As such, many fisheries professionals have desired FDA approval of a chemical sedative that is safe, effective, and affordable, and for which a lengthy withdrawal period is not required.

Two drugs that are not currently approved by the FDA, benzocaine (Benzoak; 20% benzocaine; manufacturer: ACD Pharmaceuticals AS, Leknes, Norway; U.S. distributor: Frontier Scientific, Logan, Utah) and eugenol (AQUI-S 20E [10% eugenol]; AQUI-S New Zealand, Lower Hutt, New Zealand), may be used as sedatives under an Investigational New Animal Drug (INAD) authorization held by the U.S. Fish and Wildlife Service. Although a 3-d withdrawal period is currently required under the INAD authorization, both of these drugs are being investigated as “immediate release” sedatives that would allow fish to be released immediately after sedation and could be used on fish intended for human consumption.

Although many studies have been conducted to evaluate the effectiveness of chemical sedatives to sedate or anesthetize fish (Gilderhus and Marking 1987; Pirhonen and Schreck 2003; Davis and Griffin 2004), few studies have compared chemical sedatives side by side in terms of their efficacy and effects on fishes (Sattari et al. 2009; Trushenski et al. 2012). Although there is considerable interest in the use of drugs or chemicals to sedate fish during some stage of production, the need for an effective sedative is particularly great in triploid grass carp production because of blood testing and ploidy verification requirements. Accordingly, we evaluated the effect of different chemical sedatives (MS-222, benzocaine, eugenol, and CO<sub>2</sub>) on induction and recovery times, and on postsedation survival and blood chemistry of grass carp.

## METHODS

*Sedation procedures.*—A reference population of triploid grass carp ( $301 \pm 8$  g and  $30.9 \pm 0.3$  cm total length, mean  $\pm$  SE) was held in an outdoor raceway configured as a partial flow-through system (static raceway, periodically flushed with screened surface water) with supplemental aeration at Keo Fish Farm, Keo, Arkansas. Feed was withheld for 24 h prior to

sampling. Groups of 15 fish were randomly collected from the reference population and transferred into a sedation chamber (114-L cooler) filled with 70 L of culture water (water depth of  $\sim 10$  cm) containing a sedative solution. Sedatives were applied under static conditions as follows: CO<sub>2</sub>,  $\sim 400$ -mg/L solutions prepared according to the sodium bicarbonate–sulfuric acid method described by Post (1979) (analytically verified as 360 mg/L); 150 mg/L benzocaine (Benzoak; 20% benzocaine; manufacturer: ACD Pharmaceuticals AS, Leknes, Norway; U.S. distributor: Frontier Scientific, Logan, Utah); 60 mg/L eugenol (AQUI-SE; 50% eugenol; manufacturer: AQUI-S New Zealand, Lower Hutt, New Zealand); and a 150-mg/L solution of MS-222 (Finquel; Argent Chemical, Redmond, Washington). Culture water used to prepare all baths was aerated before use, but baths were not aerated after the addition of the chemical sedative or during use.

Composite water samples, collected by combining aliquots collected from the sedative baths before and after use, were analyzed in duplicate along with water collected from the holding system for the following: temperature, dissolved oxygen (YSI 550 meter, Yellow Springs Instruments, Yellow Springs, Ohio) conductivity, pH, salinity (Multi-Parameter PCSTestr 35, Eutech/Oakton Instruments, Vernon Hills, Illinois), hardness, alkalinity (digital titrator and reagents, Hach, Loveland, Colorado), and total ammonia nitrogen, nitrite-nitrogen, and nitrate-nitrogen (DR 2800 spectrophotometer and reagents, Hach). All measured water quality characteristics were within ranges appropriate for grass carp (Masser 2002) at the start of the experiment (Table 1).

Fish were monitored during sedation to determine induction to Stage IV of “anesthesia” (Summerfelt and Smith 1990; although we have elected to use the term “sedation,” “anesthesia” is the term used by these authors). Stage IV is associated with the total loss of equilibrium, muscle tone, and responsiveness to visual and tactile stimuli, but maintenance of a slow, steady, opercular ventilation rate. After the loss of equilibrium, sedation was verified by slight manual pressure along the trunk and caudal peduncle as a tactile stimulus. Fish were considered sedated when they no longer responded to this stimulus, but the opercular ventilation rate remained steady, albeit reduced, relative to unsedated fish. After induction, blood samples were collected from three fish ( $t = 0$ ; see below) from the caudal vasculature using heparinized, evacuated, blood collection assemblies (Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey). The remaining 12 fish were then monitored to determine recovery of normal equilibrium and tactile responsiveness. When all fish were able to maintain equilibrium and were responsive to tactile stimulus, the group was considered fully recovered (i.e., recovery time = time for last fish to recover). Since assessment of induction and recovery can be somewhat subjective, bias was minimized by having the same observer apply all stimuli and assess when fish were sedated and recovered. Recovered fish were transferred to a second raceway configured in the same manner as the one housing the reference population; fish in different

TABLE 1. Water quality characteristics measured during the trial. Values represent means of water samples analyzed in duplicate.

Characteristic	Holding system	CO <sub>2</sub>	MS-222	Benzocaine	Eugenol
Temperature (°C)	16.1	15.8	16.0	15.9	15.8
Dissolved oxygen (mg/L)	9.62	8.73	9.57	9.66	9.46
Total ammonia nitrogen (mg/L)	0.00	0.02	0.04	0.09	0.71 <sup>a</sup>
Nitrite-nitrogen (mg/L)	0.004	0.003	0.003	0.004	0.008
Nitrate-nitrogen (mg/L)	0.75	0.95	0.95	0.9	1.6
Alkalinity (mg/L, as CaCO <sub>3</sub> )	228	248	208	230	226
Hardness (mg/L)	450	488	458	468	440
Salinity (‰)	0.425	0.834	0.432	0.426	0.426
Conductivity (μS/cm)	876	1,681	897	882	880
pH	8.22	6.32	7.24	8.27	8.18

<sup>a</sup> The presence of eugenol results in a yellow–green color to the water, which can interfere with the Nessler ammonia method used in this trial. Similar results were observed in previous trials using eugenol (Trushenski et al. 2012).

treatment groups were separated by means of raceway dividers—crowders positioned at ~1-m intervals along the length of the recovery raceway.

**Sample collection and analysis.**—Blood samples were then collected from three fish per group at  $t = 0.5, 1, 2,$  and  $6$  h postsedation (three fish per group per time point, individuals were only sampled once). To facilitate handling, all fish were immersed in a bath of metomidate hydrochloride (Aqualcalm, Western Chemical, Ferndale, Washington; ~5–10 mg/L for ~30 s), a fish sedative known to minimize corticosteroid increase during sampling (Olsen et al. 1995; Davis and Griffin 2004), before sampling; although additional sedation was not necessary for fish sampled at  $t = 0$ , these fish were also treated with metomidate hydrochloride to ensure consistent treatment of all fish. Once fish were sedated to a stage where they were easily handled, they were weighed (to the nearest gram) and measured (total length to the nearest 0.5 cm), and a blood sample was collected as described previously. Although metomidate hydrochloride was used to sedate fish before collecting blood samples, all samples were collected within 5 min of capture to minimize the possibility of other confounding responses of handling and blood sample collection as additional stressors. To establish resting blood chemistry characteristics of nonsedated fish, two fish from the reference population were sampled every hour during the course of the experiment ( $n = 14$ ). After blood collection, fish were returned to a separate area in the recovery raceway and monitored for 24 h.

Blood samples were kept on wet ice (<36 h) until analysis. Although this is a somewhat lengthy period of time to hold blood samples prior to analysis, some assays could not be immediately conducted in the field and samples had to be transported back to the Fisheries and Illinois Aquaculture Center, Carbondale, Illinois. It is possible that levels of metabolically relevant molecules (e.g., glucose and lactate) could have changed slightly during this holding period; however, all samples were treated in the same manner to ensure validity of comparisons among treatments. Hematocrit (Statspin cen-

trifuge, Fisher Scientific, Pittsburgh, Pennsylvania) and glucose (Freestyle Freedom Lite glucose meter, Abbott Laboratories, Abbott Park, Illinois) were determined using aliquots of whole blood, and then the remaining whole blood was centrifuged ( $3000 \times g, 4^{\circ}\text{C}, 45$  min). Resultant plasma was collected and stored at  $-80^{\circ}\text{C}$  until further analysis. Plasma samples were analyzed to determine lactate (Accutrend lactate meter, Roche, Mannheim, Germany), osmolality (Vapro 5520, Wescor, Logan, Utah), and cortisol (EIA 1887, DRG International, Mountaintside, New Jersey). Although portable lactate and glucose meters such as those used in this study can slightly underestimate metabolite levels in fish blood relative to laboratory methods, they are considered precise and reliable for use in generating comparative data (Wells and Pankhurst 1999; Venn Beecham et al. 2006). The cortisol kit used has a range of 0–800 ng/mL with a sensitivity of 2.5 ng/mL for human samples, and has been validated and used successfully to measure cortisol in samples from a variety of fish species including Nile tilapia *Oreochromis niloticus* (Delaney et al. 2005), tench *Tinca tinca* (Owen et al. 2009), common carp *Cyprinus carpio* (Sepici-Dinçel et al. 2009), cobia *Rachycentron canadum* (Trushenski et al. 2010), striped bass *Morone saxatilis* (Woods et al. 2008), and hybrid striped bass (female white bass *M. chrysops* × male striped bass) (Trushenski et al. 2012).

Although multiple fish were sampled from each treatment group at each time point, individuals were group-sedated and housed together after sedation. Therefore, it was determined that individuals did not represent independent observations. Since the experiment lacked true replication, no quantitative statistical analysis was performed and only qualitative comparisons were made from summary statistical values.

## RESULTS

All fish were successfully induced to Stage IV sedation; however, the observed induction and recovery times varied among sedatives (Figure 1). With the exception of CO<sub>2</sub> (induction

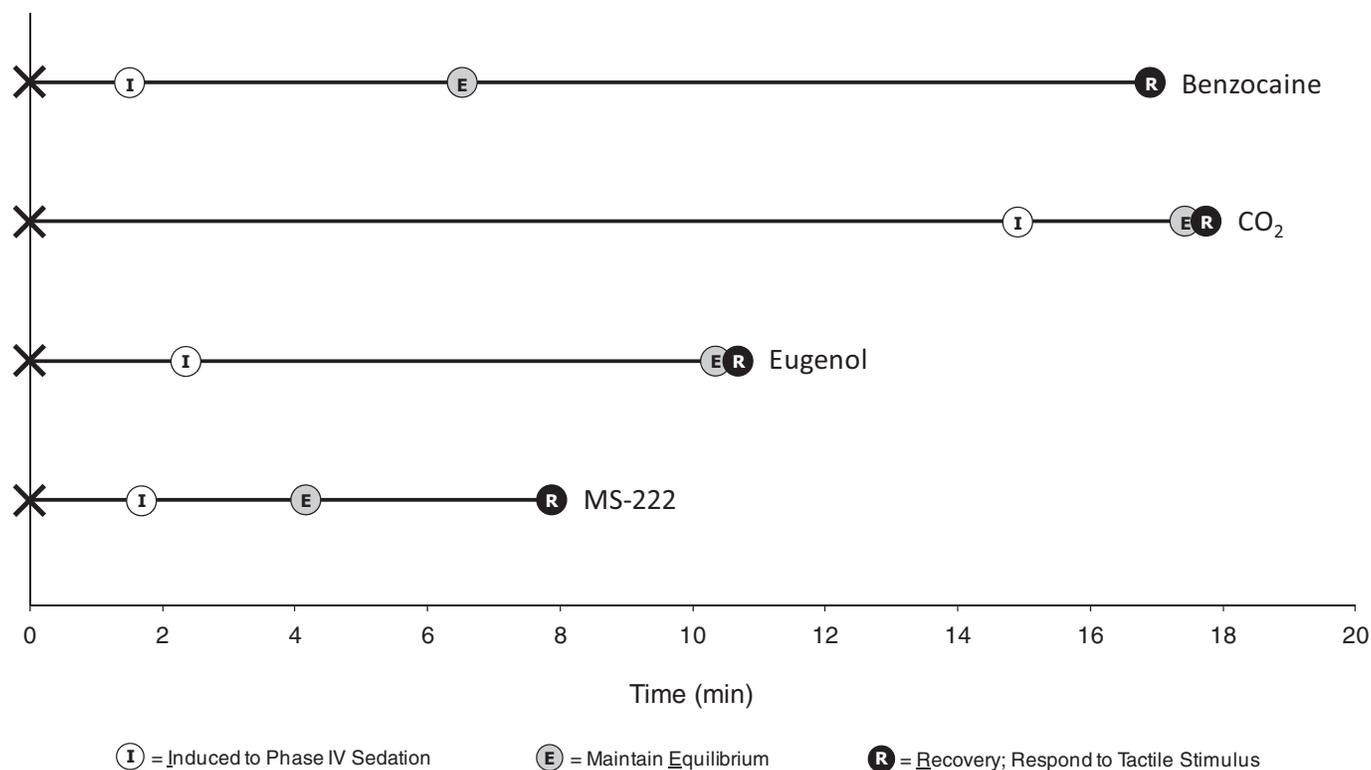


FIGURE 1. Schematic illustrating induction and various stages of recovery of grass carp sedated to Stage IV sedation using various chemical sedatives.

time = 14.9 min), all sedative treatments were successful in inducing Stage IV sedation within approximately 2 min (mean excluding CO<sub>2</sub> = 1.8 min; range, 1.5–2.3 min). Once sedation was achieved, time to regain equilibrium (mean = 4.5 min postinduction; range, 2.5–8.0 min) was less variable than time to recover tactile responsiveness. With the exception of benzocaine (regained tactile responsiveness = 15.4 min), fish regained tactile responsiveness within 5.8 min (range, 2.8–8.3 min). All fish ultimately recovered and no fish died during the 24-h postsedation observation period.

Physiological responses varied among the sedatives evaluated at different time points following sedation. Plasma lactate varied among treatments at each time point from  $t = 0$  to  $t = 2$  h by as much as 8.5 mmol/L ( $t = 2$  h,) with maximum concentrations observed at  $t = 0.5$  h for some sedatives (eugenol > MS-222) and  $t = 0$  h for the others (CO<sub>2</sub> > benzocaine). The range of plasma lactate concentrations at  $t = 6$  was considerably narrower (1.1–2.7 mmol/L) than that observed at other time points. Plasma osmolality varied at each time point with an overall range of 278–361 mOsm/kg (reference population = 296 mOsm/kg). Peak plasma cortisol concentrations occurred at  $t = 0$  for CO<sub>2</sub> (164 ng/mL) and at  $t = 0.5$  h for all other sedatives (73–166 ng/mL). Cortisol levels in sedated fish appeared to be approaching the levels observed in fish from the reference population (22 ng/mL) by  $t = 6$  h, except for those in fish sedated with MS-222, in which levels increased slightly from  $t =$

2 h to  $t = 6$  h. Hematocrit (range, 20–29%; reference population = 22%) and blood glucose (range, 61–98 mg/dL; reference population = 79 mg/dL) did not vary much among sedative treatments at any time point. No fish died during the study.

During sedation with CO<sub>2</sub>, fish were observed “piping” at the surface (appeared to be gasping for air) and had to be routinely pushed back down into the water to prevent attempts to avoid CO<sub>2</sub> narcosis via air breathing. Slight petechial hemorrhaging was observed along the lower flank and opercular area in a few fish before and after sedation, but the occurrence of these hemorrhages did not appear to be related to the sedative used.

## DISCUSSION

Induction times for three of the four sedatives were considered relatively rapid and would probably be considered acceptable to fisheries professionals for sedation of grass carp. Induction time for the CO<sub>2</sub> dose used was nearly seven times longer than that for the other chemical sedatives. This lengthy induction time was probably due to the low oxygen demands and metabolic rates of grass carp (Fu et al. 2009) and their ability to avoid CO<sub>2</sub> narcosis via air breathing. Furthermore, grass carp used in the current study were held at cooler water temperatures (15.8–16.1°C) than the water temperature that grass carp tend to prefer (21–30°C, Masser 2002). The cooler water temperature associated with the present study probably reduced

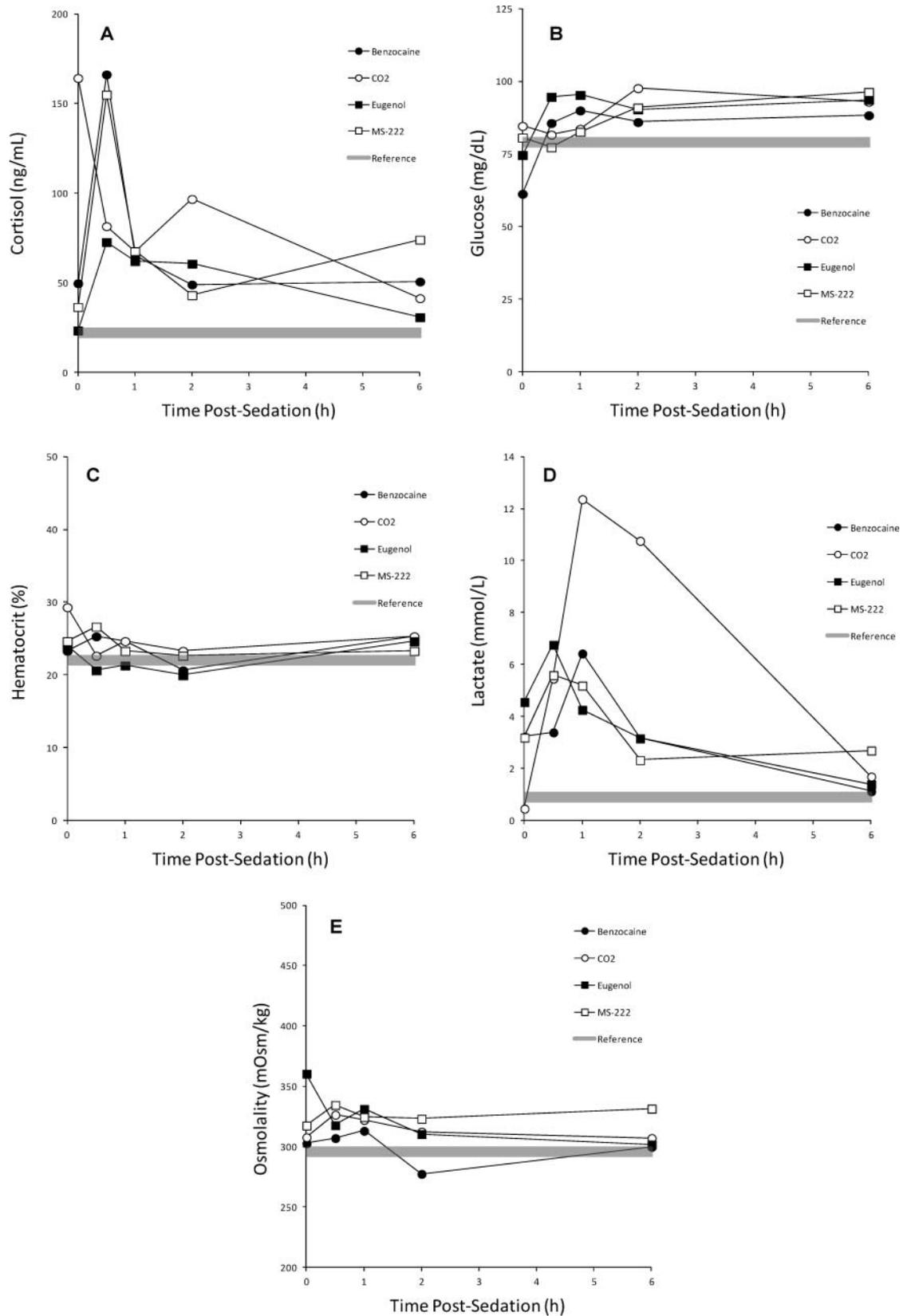


FIGURE 2. Time course of hematological responses (A = plasma cortisol, B = blood glucose, C = hematocrit, D = plasma lactate, and E = plasma osmolality) of grass carp following chemical sedation. Points represent means  $\pm$  SE; grey reference bars represent means of values observed for fish sampled from the reference population throughout the course of the experiment.

the resting metabolic rate and oxygen demand of our test fish even further. Reduced oxygen demand combined with the relatively high environmental oxygen concentrations ( $>8$  mg/L), may have reduced the need for respiratory gas exchange, opercular ventilation, or gill perfusion rates (Itazawa and Takeda 1978), which may have affected  $\text{CO}_2$  uptake and the subsequent times to sedation. Additionally,  $\text{CO}_2$  sedation times may have been slowed because fish were observed piping at the surface and had to be routinely pushed back below the surface of the water to ensure that fish were constantly exposed to the  $\text{CO}_2$ -treated water.

Grass carp treated with  $\text{CO}_2$  and eugenol regained tactile responsiveness and recovered quickly after first regaining equilibrium ( $<0.3$  min). Grass carp in the benzocaine and MS-222 treatments, on the other hand, required additional time (10.4 and 3.7 min, respectively) to recover from sedation after gaining equilibrium. The chemical similarity of benzocaine (ethyl para-aminobenzoate) and MS-222 (ethyl meta-aminobenzoate; Kiessling et al. 2009) may partially explain why grass carp sedated with these chemicals had similar patterns in which full recovery was preceded by regaining equilibrium by at least several minutes. Recovery results observed for benzocaine and MS-222 in grass carp were considerably longer than that observed in a previous study conducted by Trushenski et al. (2012) using hybrid striped bass, in which hybrid striped bass fully recovered in less than 2 min after regaining equilibrium. The pattern of recovery observed when fish were sedated with  $\text{CO}_2$  and eugenol, in which fish fully recovered within seconds of regaining equilibrium, was also observed in a study conducted concurrently with ours by Bowzer et al. (2012, this issue), in which grass carp were electrosedated with varying voltages and exposure durations. It is unclear whether the observed differences in recovery times between grass carp and hybrid striped bass were influenced by differences in resting metabolic rate or other intertaxonomic differences, differential rates of chemical sedative metabolism and excretion during recovery, or some combination of these factors.

Physiological responses generally followed the pattern of the generalized stress response (Barton 2002) suggesting that sedation should be considered as a stressor (Zahl et al. 2010). Plasma cortisol in fish sedated with all sedatives except  $\text{CO}_2$  peaked at 0.5 h postinduction, then dropped steadily over the next 6 h, but was still elevated compared with that in the reference population. Cortisol levels in fish from the  $\text{CO}_2$  treatment, however, peaked at  $t = 0$ , and were probably due to the lengthy time required to induce sedation before sampling at  $t = 0$ . Plasma lactate increased rapidly with all sedative options, peaking at 0.5 or 1 h postinduction and steadily decreasing after that. Peak lactate levels were somewhat lower than that observed in hybrid striped bass (Trushenski et al. 2012) and may be due to the decreased oxygen demand observed in grass carp. Grass carp have a resting oxygen demand of 56 mg  $\text{O}_2$ /kg per hour (Fu et al. 2009) while hybrid striped bass have a resting oxygen demand of 132 mg  $\text{O}_2$ /kg per hour (Tuncer et al. 1990; Brougher et al. 2005). Another

factor that may have contributed to the differences observed in our study and that reported by Trushenski et al. (2012) was that hybrid striped bass were sedated at higher temperatures ( $\sim 21^\circ\text{C}$ ) than were grass carp ( $\sim 16^\circ\text{C}$ ). We speculate that the higher water temperature under which hybrid striped bass were tested and their greater overall metabolic demands could have resulted in a more rapid depletion of available oxygen within the tissues, an increase in anaerobic respiration, and ultimately the greater increase in plasma lactate observed in hybrid striped bass (maximum observed value:  $17.2 \pm 0.2$  mmol/L in hybrid striped bass versus 12.4 mmol/L in grass carp). Conversely, the cooler water temperatures under which grass carp were tested in our study, combined with the lower overall metabolic demands of grass carp, may have resulted in lower peak lactate values. Blood glucose and hematocrit values of sedated fish were similar to those of the reference population regardless of sedative used or time of sample collection. Plasma osmolality appeared to vary somewhat among sedatives and over time; however, the observed values did not differ greatly from those observed in the reference fish. The lack of substantial change in these physiological characteristics—glucose, hematocrit, and osmolality—may suggest a relatively minor and brief acute stress response following sedation.

Although differences in the magnitude of blood chemistry responses were observed, the responses to sedation we noted are generally consistent with the results of concurrent work involving sedation of grass carp with pulsed DC electricity (Bowzer et al. 2012). Maximum plasma lactate observed in our study was slightly higher (12.4 mmol/L in  $\text{CO}_2$  treated fish) than that observed by Bowzer et al. (2012) when fish were sedated with pulsed DC electricity at 150 V for a 10-s exposure (9.4 mmol/L), which was probably due to the prolonged exposure to  $\text{CO}_2$  and the resultant increase in anaerobic metabolism. Peak plasma cortisol generally occurred 0.5 h postsedation in both studies ( $\text{CO}_2$  being the exception) with slightly higher peak cortisol occurring in electrosedated grass carp (162–288 ng/mL) than in chemically sedated grass carp (73–166 ng/mL). Although a noticeable peak in blood glucose concentration was not observed in either study, a slight increase in concentration between 2 and 6 h postsedation was detected. Overall, glucose levels were slightly higher in the electrosedation study (73–124 mg/dL) than in the current study (61–98 mg/dL) while hematocrit (current study: 20–29%; electrosedation study: 22–31%) and osmolality (current study: 278–361 mOsm/kg; electrosedation study: 301–375 mOsm/kg) did not appear to vary greatly from resting levels in either study.

In conclusion, each of the sedatives was effective in sedating grass carp with no apparent negative effects on selected blood chemistry characteristics or survival. Use of  $\text{CO}_2$  as a sedative option for grass carp at the concentration used in this study would not likely be recommended owing to the lengthy time required to reach sedation and the difficulty of maintaining steady and effective  $\text{CO}_2$  concentrations in water. Although benzocaine rapidly induced sedation, the lengthy recovery time should be

taken into consideration. Eugenol and MS-222 would probably be considered the most effective chemical sedatives (at the doses tested) owing to relatively rapid induction and recovery times. Lastly, since sedating fish can also act as a stressor, consideration should be given to reduce other potential stressors when conducting ploidy testing in grass carp.

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