

1 Chemical and Electrical Approaches to Sedation of Cobia: Induction, Recovery, and Physiological  
2 Responses to Sedation

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24 electroanesthesia, sedative, stress, *Rachycentron canadum*

25 **Abstract**

26 To support growing interest in marine fisheries research in areas such as biotelemetry, tagging, and  
27 tracking research we assessed the suitability of sedatives needed to facilitate this research in juvenile  
28 coxia (~300 g) using tricaine methanesulfonate (MS-222, 150 mg/L), carbon dioxide (CO<sub>2</sub>, ~750 mg/L),  
29 eugenol (60 mg/L), benzocaine (150 mg/L), or pulsed DC electrosedation [100 volts, 30 Hz, 25% duty  
30 cycle, 5 sec exposure]). Induction times (CO<sub>2</sub> [z] > benzocaine [y] > eugenol [y] > MS-222 [y] >  
31 electrosedation [x]), recovery of equilibrium (CO<sub>2</sub> [z] > eugenol [z] > MS-222 [y] > benzocaine [y] >  
32 electrosedation [x]), and responsiveness to tactile stimulus (eugenol [z] > MS-222 [y] = benzocaine [y] =  
33 CO<sub>2</sub> [xy] > electrosedation [x]) differed significantly among the sedative treatments (treatments with the  
34 same letter labels are not significantly different). Total handling time, from initial sedative exposure to  
35 recovery, differed among the sedatives as well (CO<sub>2</sub> [z] > eugenol [y] > benzocaine [x] > MS-222 [x] >  
36 electrosedation [w]) with a cumulative mean of 5.9 ± 0.2 min elapsed for fish sedated with CO<sub>2</sub>, 4.1 ± 0.2  
37 for eugenol, 2.7 ± 0.2 min for benzocaine and MS-222, and 1.0 ± 0.2 min for electrosedation.  
38 Physiological responses differed significantly over time, with transient increases in plasma cortisol,  
39 glucose, osmolality, and lactate that were resolved within 6 h. The overall magnitude of physiological  
40 responses differed among sedatives depending on the response variable; however, in each case, CO<sub>2</sub>  
41 elicited the greatest response. Variation in induction and recovery times were observed, however, it is  
42 likely that these differences could be reasonably accommodated within the context of typical research  
43 by adjusting the sedative treatments or allowing for longer induction and recovery times as needed.

#### 44 **Introduction**

45 The availability of safe and effective fish sedatives is crucial to fisheries researchers, managers, and  
46 aquaculturists. Fisheries professionals sedate or anesthetize fish for a variety of purposes, ranging from  
47 simple handling to invasive surgical procedures. Although the specific constraints differ from one  
48 situation to the next, ideally, a fish sedative is safe and easy to administer, is effective at low doses  
49 (minimizing the amount needed for field applications), sedates fish quickly and predictably, has a  
50 reasonable margin of safety with respect to over-sedation, can be used over a broad range of water  
51 chemistries, is inexpensive, and allows for rapid recovery from sedation and its effects (Bowker and  
52 Trushenski 2011). Additionally, in field research, it is particularly advantageous if sedative use does not  
53 require treated fish be held to complete a withdrawal period prior to release (i.e., “immediate release”).

54

55 At this time, there are few legal options available to sedate fish, and those that are available are not  
56 always ideal relative to their safety, efficacy, and practicality of use. Currently, there is only one  
57 sedative compound that is approved by the U.S. Food and Drug Administration (FDA) for temporary  
58 immobilization of fishes: tricaine methanesulfonate (commonly referred to as MS-222). Two MS-222  
59 products are currently approved in the U.S., but use of these products is restricted to ictalurid, salmonid,  
60 esocid, and percid fishes (approved for other fishes in laboratory or hatchery settings only) treated at  
61 water temperature > 10°C. Use of MS-222 is further restricted by a 21-d withdrawal period deemed  
62 necessary to allow for drug residue depletion prior to treated fish being released into the wild (or  
63 otherwise made available for human consumption). Although not approved by FDA, carbon dioxide  
64 (CO<sub>2</sub>) is considered a drug of “low regulatory priority” (FDA 2011) and its use allows for fish to be  
65 released immediately after sedation. However, CO<sub>2</sub> can be difficult to apply uniformly, is typically slow-  
66 acting, and adverse effects have been reported (Neiffer and Stamper 2009). There are at least two  
67 additional drugs currently being investigated for use as fish sedatives, specifically benzocaine and

68 eugenol. These drugs can currently be used under the authorization of Investigational New Animal Drug  
69 (INAD) exemptions held by the U.S. Fish and Wildlife Service with an associated 3-d withdrawal period.  
70 In the meantime, there is an effort by the drug sponsors and researchers to gain FDA approval of one or  
71 both of these compounds as immediate-release fish sedatives. Another option which is not subject to  
72 the rigors of FDA animal drug oversight is the use of electricity to sedate fishes. Electrofishing has been  
73 used for decades as a field technique in fisheries, but only recently has this approach been modified  
74 specifically for sedating/anesthetizing fishes and commercialized (Zydlewski et al. 2008; Hudson et al.  
75 2011; Trushenski et al. 2012a, 2012b).

76

77 Each of the aforementioned sedatives has positive and negative attributes associated with its use,  
78 including approval status (approved drug vs. low regulatory priority drug vs. Investigational New Animal  
79 Drug status), allowable use patterns (immediate-release vs. 3-d withdrawal period vs. 21-d withdrawal  
80 period), disposal considerations, cost, ease of use, and efficacy. Additionally, each of these sedatives  
81 have proven effective in numerous freshwater fish (Trushenski et al. 2012a, 2012b; J. Bowker [U.S. Fish  
82 and Wildlife Service], *unpublished data*). However, it is unclear whether these approaches can be  
83 effectively applied to marine species with the same degree of safety and efficacy. Furthermore, it is  
84 unclear whether the differences in physiological responses to sedation observed in freshwater taxa also  
85 extend to marine fishes. Traditionally, marine species have received less attention in terms of sedatives  
86 research: a recent review of MS-222, CO<sub>2</sub>, eugenol and related compounds, and benzocaine research  
87 reported studies in 10 freshwater taxa and only 5 marine taxa (Trushenski et al. 2012a). This is  
88 particularly true in the case of strategies for electrical immobilization of fishes, which is generally less  
89 effective in brackish and saltwater than freshwater. This is a particularly critical information gap, given  
90 the growing interest in biotelemetry and other tagging and tracking approaches to marine fisheries  
91 research and management efforts (Silbert and Nielsen 2001) and the concomitant demand for effective

92 sedatives to facilitate this type of research. Accordingly, we evaluated the effectiveness of Fiquel®  
93 (MS-222; 100% tricaine methanesulfonate; Argent Laboratories, Redmond, WA), AQUI-S E (50% eugenol;  
94 AQUI-S New Zealand, Ltd., Lower Hutt, New Zealand), Benzoak (20% benzocaine; Frontier Scientific, Inc.,  
95 Logan, Utah), CO<sub>2</sub>, or pulsed DC electrosedation in sedating juvenile cobia. Metrics measured included  
96 induction and recovery times, and physiological responses to sedation. Cobia were selected as a model  
97 species for this assessment because they are found in warm coastal waters throughout the world,  
98 excluding the eastern Pacific Ocean (Shaffer and Nakamura 1989), and are commonly targeted in  
99 assessments of commercial and recreational marine fisheries (Lucy and Bain 2000; Williams 2001; Smith  
100 et al. 2003; Mahon and McConney 2004).

101

102 The terms “sedation”, “anesthesia”, and “immobilization” are used somewhat interchangeably with  
103 respect to fishes, but the terms have distinct meanings: Ross and Ross (2008) define anesthesia as “a  
104 reversible, generalized loss of sensory perception accompanied by a sleep-like state induced by drugs or  
105 by physical means”, and sedation as “a preliminary level of anesthesia, in which response to stimulation  
106 is greatly reduced and some analgesia is achieved, but sensory abilities are generally intact and loss of  
107 equilibrium does not occur.” “Immobilization” generally refers to prevention of movement, and does  
108 not imply any status regarding the acuity of sensory perception. However, the definitions of Ross and  
109 Ross differ somewhat from the medical profession’s understanding of sedatives and anesthetics:  
110 according to the Medline Plus Medical Dictionary, a sedative is an agent or drug “tending to calm,  
111 moderate, or tranquilize nervousness or excitement”, whereas an anesthetic is a substance that causes  
112 the “loss of sensation and usually of consciousness without loss of vital functions”, specifically those  
113 substances that “block the passage of pain impulses along nerve pathways to the brain” (NLM 2012).  
114 Both sources appear to agree that sedation and anesthesia represent progressions in the loss of the  
115 ability to perceive and respond to stimuli, but they disagree regarding the issue of pain. Given the

116 controversy as to whether fishes are even capable of perceiving pain (Rose 2002; Braithwaite and  
117 Huntingford 2004), the use of definitions which rely on the relative ability to do so seems inappropriate.  
118 Although one could argue that none of these terms or definitions perfectly describe the processes we  
119 evaluated in the present work, “sedative” (at least as defined by the medical community), seems the  
120 best choice. Thus, for consistency, we have elected to use the term “sedation” throughout this  
121 manuscript.

122

123 We hypothesized that each of the sedatives assessed would be effective, but that cobia would respond  
124 differently to each in terms of induction and recovery times and physiological responses to exposure.

125

## 126 **Methods**

127 All procedures described below were conducted under the guidance and approval of the Southern  
128 Illinois University Carbondale Institutional Animal Care and Use Committee (IACUC, protocol # 10-028).

129

### 130 *Experiment 1: Induction and Recovery Times*

131 Juvenile cobia were obtained as eggs from a commercial vendor (Troutlodge Marine Farms LLC, Vero  
132 Beach, Florida), and cultured until they reached an advanced fingerling stage. Feed was withheld for 24  
133 h prior to the experiment. Individual fish ( $297 \pm 9$  g,  $38.0 \pm 0.5$  cm total length, mean  $\pm$  SE) were  
134 transferred from holding tanks in a brackish water (20 ppt salinity) recirculating aquaculture system  
135 (Table 1) and placed into a sedation chamber (142-L cooler for electrosedation, 30-L cooler for all  
136 others) filled to a depth of approximately 8 cm. Although the fish had been held in several separate  
137 tanks within the recirculation system, they were from the same population of fish that had been  
138 arbitrarily stocked among the holding tanks approximately 24 h prior to starting the experiment. The  
139 electrosedation chamber was filled with freshwater, whereas the chemical sedation baths were

140 prepared using aerated culture water from the holding system (see description of water quality testing  
141 below; Table 1). Sedation treatments were prepared as described in Table 2. To avoid potential  
142 variability associated with different sources of the chemical sedatives, a single lot was used for each  
143 product. Chemical sedative concentrations and electrosedation settings were chosen based on our  
144 previous experience to achieve a level of sedation appropriate for basic handling (see description of  
145 sedation procedures below) in less than 5 min. We chose concentrations of MS-222, eugenol, and  
146 benzocaine and an electrosedation protocol that have achieved the desired effect in freshwater taxa,  
147 though we targeted a higher concentration of CO<sub>2</sub> (~750 mg/L compared to ~400 mg/L) in this case to  
148 compensate for the reported difficulties in achieving sedation with CO<sub>2</sub> in saltwater. The chemical  
149 sedatives were not tested with cobia beforehand, but we tested the electrosedation protocol prior to  
150 experimentation to ensure the settings would yield appropriate level of sedation. Although the culture  
151 water used to prepare these baths was aerated prior to use, baths were not aerated following the  
152 addition of the chemical sedative or during use. Fresh chemical sedative baths were prepared after  
153 treating 5 individual fish; however, water in the electrosedation unit was not exchanged during the  
154 treatment of individual fish. After extended use, sedative baths can 'wear out' as the sedative agent is  
155 absorbed by the fish or is otherwise dissipated. Also, debris (e.g., mucus, scales, feces) and dissolved  
156 wastes (i.e., ammonia) can accumulate in the sedative bath and affect fish during sedation. Although it  
157 is unlikely that loss of sedative efficacy or substantial waste accumulation would have occurred after  
158 treating the relatively small numbers of fish used in our study (Trushenski and Bowker, *unpublished*  
159 *data*), we exchanged the bath treatments to avoid the possibility altogether. Dissolved oxygen (YSI-85  
160 dissolved oxygen/temperature meter, Yellow Springs Instruments, Yellow Springs, Ohio), conductivity,  
161 pH, salinity (Multi-Parameter PCSTestr™ 35, Eutech Instruments, Oakton®, Vernon Hills, Illinois),  
162 hardness, and alkalinity (digital titrator and reagents, Hach Inc., Loveland, Colorado) were maintained  
163 within ranges appropriate for cobia culture throughout the experiment (Table 1). Although freshwater

164 conditions in the electrosedation chamber would not be considered appropriate for culturing cobia, the  
165 fish were only exposed to freshwater for a short period of time associated with electrosedation (~30-45  
166 s elapsed from stocking to completion of induction)—fish were recovered in a brackish water bath  
167 identical to that used during recovery of fish sedated with the chemical sedatives.

168

169 During sedation, each fish was monitored to determine the time (from the time of sedative exposure) at  
170 which Stage IV of sedation (Summerfelt and Smith 1990) was achieved. Stage IV is associated with the  
171 total loss of equilibrium, muscle tone, and responsiveness to visual and tactile stimuli, but maintenance  
172 of a steady, though reduced, opercular ventilation rate. After the loss of equilibrium, fish were  
173 continually challenged with tactile stimuli (manual stimulation of the buccal cavity). Fish were  
174 considered induced to Stage IV when they no longer responded to this stimulus, but the opercular rate  
175 remained slow but steady. In the case of the electrosedative treatment, a tremor was observed  
176 following electrical exposure; although fish were not responsive during this tremor (and were perhaps  
177 temporarily in Stage V or VI of sedation), induction was considered complete after the tremor had  
178 ceased. After induction, fish were weighed (to the nearest 0.1 g) and measured to determine total  
179 length (to the nearest 0.5 cm) and then transferred to a static recovery tank filled with aerated culture  
180 water (water exchanged at the same time as sedative baths). In the recovery tank, fish were monitored  
181 using the same techniques mentioned above to determine time to recovery of normal equilibrium and  
182 tactile responses. When fish exhibited normal equilibrium and began responding to the tactile stimulus  
183 (by apparent attempts to dislodge the researcher's finger from the buccal cavity), they were considered  
184 fully recovered. Recovered fish were returned to a holding system and monitored for survival for 24 h.  
185 Since assessment of induction and recovery can be somewhat subjective, bias was minimized by having  
186 the same observers make all assessments.

187

188 *Experiment 2: Physiological Responses to Sedation*

189 In this experiment, sedative baths were prepared as previously described. Single working baths of  
190 benzocaine, eugenol, and MS-222 were used to sedate all groups of fish in Experiment 2. However,  
191 fresh baths of CO<sub>2</sub> were prepared to sedate each group of fish in this treatment because of the volatile  
192 loss of CO<sub>2</sub> likely to be exacerbated by fish movement during group sedation. As with the chemical  
193 sedatives, the freshwater used in the electrosedation chamber was not exchanged during Experiment 2.  
194 Water samples were prepared by collecting aliquots from the sedative baths before and after each use  
195 and combining these (50/50) to create a single composite water sample for each sedative treatment.  
196 Each of the composite water samples was analyzed in duplicate as described for Experiment 1 along  
197 with water samples collected from the holding recirculation system at the beginning and end of the  
198 study period. With the exception of the freshwater electrosedation bath (to which fish were only  
199 exposed temporarily, i.e., ~30-45 s), all measured values were within ranges acceptable for cobia culture  
200 (Rodrigues et al. 2007; Chen et al. 2009; Atwood et al. 2008; Benetti et al. 2008; Table 1). Additional fish  
201 from the same population described for Experiment 1 (same cohort) were used for Experiment 2 (fish  
202 were not reused in either experiment). Groups of five fish ( $286 \pm 7$  g,  $37.0 \pm 0.5$  cm total length, mean  $\pm$   
203 SE) were transferred from the same holding tanks in a brackish water recirculating aquaculture system  
204 previously described for Experiment 1 and placed into the sedation chamber and sedated *en masse*.  
205 Immediately after induction to Stage IV, one fish per group was transferred to a bath of metomidate  
206 hydrochloride (Aquacalm™, Western Chemical, Ferndale, Washington, USA, ~3-5 mg/L for ~30 sec).  
207 Although fish sampled at the start of the experiment did not require further sedation in order to collect  
208 blood samples, sedation was required to facilitate blood sampling at later time points in compliance  
209 with our IACUC-approved animal care and use protocol. Using a secondary sedative in addition to the  
210 other sedatives tested did present a potential confounding effect, i.e., our observations would  
211 essentially represent the responses of fish treated with two sedatives (metomidate hydrochloride plus

212 the sedative of interest). We considered several alternative approaches, including blood sampling  
213 without sedation or repeat use of the test sedative (e.g., eugenol, MS-222). However, these approaches  
214 were deemed unsuitable because they could likely have a confounding influence on the responses (i.e.,  
215 fish sampled under sedation at time = 0, but not subsequent data points; fish exposed to protocols that  
216 were inconsistent among treatments and through time). Using a distinct, secondary sedative for blood  
217 sampling was the preferred approach and would facilitate the most direct comparison among  
218 treatments. Metomidate hydrochloride is known to block corticosteroid synthesis in some fish species  
219 (Mattson and Ripley 1989; Olsen et al. 1995; Davis and Griffin 2004). Consequently, it can be a useful  
220 sedative for stress physiology experiments because it may minimize the effects of handling and sample  
221 collection on circulating cortisol levels. For consistency, all fish sampled, including those sampled  
222 immediately after sedation, were transferred to a solution of metomidate hydrochloride. After  
223 exposure to the metomidate hydrochloride bath for approximately 30 s, fish length and weight were  
224 measured, and a blood sample was collected from the caudal vasculature using heparinized, evacuated  
225 blood collection assemblies (Vacutainer<sup>®</sup>; Becton Dickinson and Co., Franklin Lakes, New Jersey, USA).  
226 Although metomidate hydrochloride was used, in part, as a potential corticosteroid blocker, all blood  
227 samples were collected within five min of capture (< 5 min elapsed from netting the fish to placing the  
228 blood sample on ice) to minimize the possibility of other confounding responses of handling and  
229 sampling via the caudal vasculature as acute stressors. The remaining four fish in each group were  
230 returned to a holding tank in the recirculation aquaculture system. One fish was then sampled from  
231 each group at 0.5, 1, 2, and 6 h post-sedation. After blood collection, fish were euthanized by  
232 immersion in an ice water bath until all voluntary and involuntary movement ceased, and disposed of in  
233 the local landfill. Every two hours during the sampling period, three fish were sampled from the  
234 reference population to represent untreated, resting conditions. These fish were also treated with  
235 metomidate hydrochloride to facilitate blood sampling. These fish did not represent true controls (not

236 treated with any sedative whatsoever), but were intended to provide a reference by which the effects of  
237 the test sedatives could be qualitatively assessed.

238

239 Necessary hematological testing equipment was not available at the Virginia Seafood Agricultural  
240 Research and Extension Center, so tubes containing blood samples were kept on wet ice during  
241 transport from Hampton, VA to Carbondale, IL (total time between collection and analysis < 36 h).

242 Subsamples of whole blood were used for the determination of hematocrit (Statspin<sup>®</sup> centrifuge; Fisher  
243 Scientific, Pittsburgh, Pennsylvania, USA). Whole blood samples were then centrifuged (3000 x gravity,  
244 45 min, 4°C) and the resultant plasma was stored at -80°C until further analysis. Plasma samples were  
245 analyzed to determine glucose (glucose test reagent, Pointe Scientific, Inc., Canton, Michigan; test  
246 adapted for 96-well plates using external standards), lactate (Accutrend<sup>®</sup> lactate meter, Roche,  
247 Mannheim, Germany), osmolality (Vapro 5520; Wescor, Inc.; Logan, Utah, USA), and cortisol (EIA 1887;  
248 DRG International, Mountainside, New Jersey, USA). Although the portable meters, such as the one we  
249 used to measure lactate, have been shown to slightly underestimate metabolite levels in fish blood  
250 relative to laboratory methods, they are considered precise and reliable for use in generating  
251 comparative data (Wells and Pankhurst 1999; Venn Beecham et al. 2006). The cortisol kit used has a  
252 range of 0-800 ng/mL with a sensitivity of 2.5 ng/mL for human samples, and has been validated and  
253 used successfully to measure cortisol in samples from a variety of fish species (Delaney et al. 2005;  
254 Woods et al. 2008; Owen et al. 2009; Sepici-Dinçel et al. 2009).

255

### 256 *Statistical Analyses*

257 For Experiment #1, individual fish were considered experimental units ( $n = 10$ ). Induction and recovery  
258 times were analyzed by one-way ANOVA (PROC MIXED) using SAS<sup>®</sup>, version 9.1 (SAS Institute, Cary,  
259 North Carolina, USA) to detect significant differences among the sedatives relative to induction and

260 recovery times. For parameters exhibiting significant treatment effects, post-hoc Tukey's HSD tests  
261 were used for pairwise comparisons of LS-means. Fish weight and length were assessed as potential  
262 covariates (PROC CORR), but no significant correlations between body size and induction/recovery times  
263 were observed. For Experiment #2, replicate groups of fish were considered the experimental unit.  
264 Although each sedative was applied to triplicate groups, each comprised of five fish, it was determined  
265 that groups, not individuals, should serve as experimental units. By definition, experimental units  
266 represent independent observations. We determined that individuals sedated in the same group could  
267 not be considered fully independent observations because the presence and/or position of other fish  
268 within the sedation chamber could affect the general behavior of the group or, in the case of  
269 electrosedated fish, alter the way in which the waveform was applied to individuals. Thus, to maintain  
270 a reasonably conservative statistical approach, sedation group was used as the level of replication or  
271 experimental unit for each statistical procedure ( $n=3$ ). Thus, fish sampled at each time point  
272 represented repeated observations made on the same experimental unit (i.e., sedation group or tank).  
273 Accordingly, physiological data were analyzed by one-way, repeated measures ANOVA (PROC MIXED;  
274 SAS® 9.1). For parameters exhibiting significant treatment effects, treatment LS-means were compared  
275 at individual time points using post-hoc Tukey's HSD tests for pairwise comparisons. In all cases,  
276 differences were considered significant at  $p < 0.05$  and no data were transformed prior to analysis.

277

## 278 **Results**

279 Induction times differed significantly among the sedatives evaluated ( $\text{CO}_2$  [z] > benzocaine [y] > eugenol  
280 [y] > MS-222 [y] > electrosedation [x], treatments with the same letter labels are not significantly  
281 different; Figure 1). Briefly, mean induction time using  $\text{CO}_2$  was  $2.7 \pm 0.1$  min, mean induction time for  
282 benzocaine, eugenol, and MS-222 ranged from  $1.2-1.4 \pm 0.1$  min, and mean induction time for  
283 electrosedation was  $0.2 \pm 0.1$  min (LS-means  $\pm$  SE). Recovery of equilibrium ( $\text{CO}_2$  [z] > eugenol [z] > MS-

284 222 [y] > benzocaine [y] > electrosedation [x]) and responsiveness to tactile stimulus (eugenol [z] > MS-  
285 222 [y] > benzocaine [y] > CO<sub>2</sub> [xy] > electrosedation [x]) also differed significantly among the sedative  
286 treatments. With the exception of fish treated with CO<sub>2</sub>, which exhibited a more protracted recovery  
287 and regained tactile responsiveness before equilibrium, the general recovery pattern was to regain  
288 equilibrium then tactile responsiveness in rapid succession. All benchmarks of recovery were achieved  
289 most rapidly in the electrosedation treatment: mean time to regain equilibrium and tactile  
290 responsiveness were  $0.6 \pm 0.1$  and  $0.8 \pm 0.1$  min post-induction, respectively. Equilibrium was regained  
291 among fish treated with benzocaine in  $1.2 \pm 0.1$  min, MS-222 in  $1.3 \pm 0.1$  min, eugenol in  $2.7 \pm 0.1$ , and  
292 CO<sub>2</sub> in  $3.2 \text{ min} \pm 0.1$  min post-induction. Tactile responsiveness was regained among fish treated with  
293 CO<sub>2</sub> in  $1.0 \text{ min} \pm 0.1$  min, benzocaine in  $1.4 \pm 0.1$  min, MS-222 in  $1.5 \pm 0.1$  min, and eugenol in  $2.9 \pm 0.1$   
294 min post-induction. Total handling time, from initial sedative exposure to recovery, differed among the  
295 sedatives as well: CO<sub>2</sub> [z] > eugenol [y] > benzocaine [x] > MS-222 [x] > electrosedation [w]) with a total  
296 of  $5.9 \pm 0.2$  min elapsed for fish sedated with CO<sub>2</sub>,  $4.1 \pm 0.2$  for eugenol,  $2.7 \pm 0.2$  min for benzocaine  
297 and MS-222, and  $1.0 \pm 0.2$  min for electrosedation.

298  
299 Physiological responses differed significantly among the sedatives evaluated, and over time (Figure 2,  
300 Appendix 1). Plasma cortisol concentrations increased within 0.5 h following sedation, but began  
301 returning to resting levels within 1 h post-sedation for all sedatives except CO<sub>2</sub>, which remained elevated  
302 through 2 h post-sedation. A similar response pattern was observed for osmolality and lactate, though  
303 lactate levels remained somewhat elevated 2 h after sedation with pulsed DC electricity or CO<sub>2</sub>. Plasma  
304 glucose levels increased following sedation, peaking in most cases between 0.5-1 h post-sedation,  
305 though a second, higher peak was observed among the electrosedated fish at 2 h post-sedation.  
306 Nonetheless, glucose gradually decreased following the peak response in each treatment, returning to  
307 near-resting levels within 6 h of sedation. The overall magnitude of the physiological responses differed

308 to a greater (cortisol, lactate, glucose) or lesser (glucose) degree among the sedatives tested; however,  
309 in each case, CO<sub>2</sub> elicited the greatest response. Although a significant time effect was observed for  
310 hematocrit, reflecting a generalized decline from 0-0.5 h to the end of the sampling period, differences  
311 were not observed between the sedatives.

312

313 Several anecdotal observations were made during the course of the experiments with respect to  
314 behavioral responses to the sedatives. Fish exhibited opercular flaring, fin extension, and body rigidity  
315 during electrosedation, but posture returned to normal after resolution of the post-exposure tremor.  
316 Blanching of the skin was observed among some fish sedated with CO<sub>2</sub> and was particularly evident  
317 among electrosedated fish. During exposure to CO<sub>2</sub>, fish were hyperactive and observed to pipe at the  
318 water surface. Although some hyperactivity was observed during sedation with benzocaine, it was less  
319 pronounced than that associated with CO<sub>2</sub> (not all fish exhibited hyperactive swimming and those that  
320 did were not as agitated as those exposed to CO<sub>2</sub>). There were no mortalities during the course of the  
321 two experiments involving sedation and handling of 125 individuals.

322

### 323 **Discussion**

324 Our results suggest that, despite taxonomic, biological, and physiological differences, cobia respond to  
325 chemo- and electrosedation in a manner broadly similar to that observed in largemouth bass  
326 *Micropterus salmoides* (Trushenski et al. 2012b), hybrid striped bass *Morone chrysops* x *M. saxatilis*  
327 (Trushenski et al. 2012a), walleye *Sander vitreus* (J. Bowker [U.S. Fish and Wildlife Service], *unpublished*  
328 *data*), and other species tested using similar sedation protocols (i.e., grass carp *Ctenopharyngodon idella*  
329 and shovelnose sturgeon *Scaphirhynchus platyrhynchus*; J. Trushenski [Southern Illinois University  
330 Carbondale], *unpublished data*). Although a relatively small number of individuals were involved in the  
331 present work ( $n = 10$  for Experiment #1,  $n = 3$  for Experiment #2), the results are nonetheless

332 compelling. The pattern of induction observed in our study was strikingly similar to the induction  
333 patterns observed for larger hybrid striped bass and largemouth bass (~500 g) sedated using similar  
334 sedative approaches (hybrid striped bass: 60 mg/L eugenol; 150 mg/L benzocaine; 150 mg/L MS-222;  
335 ~400 mg/L CO<sub>2</sub>; electrosedation = 60 volts, 30 Hz, 25% duty cycle, 3 sec exposure; largemouth bass: 60  
336 mg/L eugenol; 150 mg/L benzocaine; 150 mg/L MS-222; ~400 mg/L CO<sub>2</sub>; electrosedation = 100 volts, 30  
337 Hz, 25% duty cycle, 3 sec exposure): fish were sedated to Stage IV in 0.2 min using electrosedation; 1.3-  
338 1.9 min using eugenol, benzocaine, or MS-222; and 2.5-3.6 min using CO<sub>2</sub> (Trushenski et al. 2012a,  
339 2012b). Although the walleye tested were smaller (~50 g), similar protocols (60 mg/L eugenol; 150 mg/L  
340 benzocaine; 150 mg/L MS-222; ~400 mg/L CO<sub>2</sub>; electrosedation = 100 volts, 30 Hz, 25% duty cycle, 5 sec  
341 exposure) yielded similar induction times for this species as well: fish were sedated to Stage IV in 0.1  
342 min using electrosedation; 0.7-0.9 min using eugenol, benzocaine, or MS-222; and 2.1 min using CO<sub>2</sub> (J.  
343 Bowker [U.S. Fish and Wildlife Service], *unpublished data*). Similar times to induction of Stage IV  
344 (referred to as “Stage III” by the authors, but equivalent to Stage IV as defined by Summerfelt and Smith  
345 [1990]) were observed in a study by Gullian and Villanueva (2009). In their study, two size classes of  
346 juvenile cobia (~5 g and ~14 g) were sedated using various concentrations of clove oil (product  
347 contained ~88% eugenol, 20-100 mg/L) and MS-222 (40-120 mg/L). The authors found that, regardless  
348 of fish size, time to induction with MS-222 ranged from 1.15 to 1.25 min and from 1.70 to 2.22 min with  
349 60 mg/L clove oil, the latter being slightly longer (0.49-1.01 min) than that observed in our study. The  
350 slower induction times observed by these authors may be attributed to the difference in eugenol purity  
351 between AQUI-S® 20E and clove oil and the corresponding decrease in effective eugenol concentration  
352 (~53 mg/L vs. 60 mg/L). Taken together, all of these experiments represented different fish sizes (~5-  
353 500 g), temperatures (~19-27°C), salinities (~0-39 ppt), and a broad taxonomic range (Rachycentridae,  
354 Centrarchidae, Moronidae, and Percidae), thus it would appear that the sedative approaches we  
355 investigated in cobia yield relatively consistent results in terms of induction to Stage IV of sedation

356 across a range of scenarios. Consistency in apparent safety and efficacy across a range of conditions and  
357 taxa is encouraging for fisheries professionals attempting to perform routine handling procedures  
358 collecting biometric data, tagging, or noninvasive tissue harvest (e.g., fin clips, spines) in various  
359 research scenarios. Although sedative safety and efficacy have not been quantitatively demonstrated  
360 for all taxa, the data we have generated assessing these sedative approaches in cobia and various other  
361 taxa suggest that with a modicum of experience, researchers could apply the sedatives to most, if not  
362 all, fish without substantial risk of adverse effects. Nonetheless, when preparing to sedate an untested  
363 taxon or life stage, we advise researchers to conduct a preliminary test using a few individuals to  
364 determine appropriate sedation protocols.

365

366 Recovery of equilibrium and tactile responsiveness also differed among the sedatives evaluated, with  
367 complete recovery occurring most rapidly among electrosedated fish, followed by fish sedated with  
368 benzocaine or MS-222 and fish sedated with eugenol or CO<sub>2</sub>. With the exception of CO<sub>2</sub>-treated fish,  
369 which progressively regained tactile responsiveness and then equilibrium (2.2 min elapsed between  
370 benchmarks), sedated cobia regained equilibrium first, followed quickly by tactile responsiveness (12 s  
371 elapsed between benchmarks). Despite differences in the process and pattern of recovery of  
372 equilibrium and tactile responsiveness, the differences in induction times were essentially repeated in  
373 terms of recovery and total handling time: induction and recovery were fastest among electrosedated  
374 fish and slowest among fish sedated with CO<sub>2</sub>, with the other sedatives yielding intermediate times.  
375 The present results are somewhat different from those observed in previous evaluations of these  
376 sedatives: the current and previous studies differ in terms of the range of handling times observed (~1-7  
377 min, depending on sedative and taxon), which sedative was associated with the longest total handling  
378 time (eugenol or CO<sub>2</sub>, depending on taxon), whether equilibrium or tactile responsiveness were  
379 regained first (variable among sedatives and taxa), and whether benchmarks of recovery were achieved

380 slowly or in rapid succession (variable among sedatives and taxa)(Trushenski et al. 2012a, 2012b..  
381 Despite relatively consistent results in terms of induction times, it seems there is considerable variability  
382 in the pattern and process of recovery among sedative types and among different fishes sedated using  
383 these approaches. This variation may be attributed to biological differences among taxa, differences in  
384 fish body size, or differences in abiotic factors such as water temperature or pH (Ross and Ross 2008).  
385 However, given the circumstances under which fish sedatives are most likely to be used (i.e., by  
386 experienced fisheries professionals familiar with what is considered normal vs. abnormal behavior for  
387 different fishes), it is likely that variation among different taxa could be readily accommodated by  
388 adjusting sedative dose and/or the amount of time allowed for recovery prior to release.

389

390 The transient changes in circulating cortisol, glucose, lactate, and osmolality we observed indicate that  
391 cobia undergo an acute stress response following sedation. Depending on the sedative concentrations  
392 used, transient primary and secondary stress responses have been observed in fishes following sedation  
393 with MS-222, CO<sub>2</sub>, benzocaine, various clove derivatives, and pulsed DC electricity (Davidson et al. 2000;  
394 Wagner et al. 2002; Davis and Griffin 2004; King et al. 2005; Bolasina 2006; Zahl et al. 2010; Trushenski  
395 et al. 2012a, 2012b). Although sedatives are often used with the intention of reducing handling stress  
396 (Sandodden et al. 2001; Finstad et al. 2003; Iversen et al. 2003; Wagner et al. 2003; Cooke et al. 2004;  
397 Small 2004; Palić et al. 2006), sedatives can elicit mild to moderate stress responses, particularly if  
398 sedative application is accompanied by changes in water chemistry (i.e., pH shifts associated with CO<sub>2</sub>  
399 and MS-222; Trushenski et al. 2012a). The increases in cortisol, glucose, lactate, and osmolality  
400 occurring 0.5-2 hours post-sedation are consistent with induction of the generalized stress response in  
401 fishes, including both the primary (i.e., elevated cortisol) and secondary (i.e., elevated glucose, lactate,  
402 and osmolality) responses to stressor exposure (Mazeaud et al. 1977; Barton 2002). The time course of  
403 physiological responses is consistent with the responses of juvenile cobia exposed to other acute

404 stressors, such as a 1 min air-exposure challenge (Cnaani and McLean 2009; Trushenski et al. 2010) or 15  
405 min low-water challenge (Trushenski et al. 2010): in both cases, glucose and cortisol responses peaked  
406 within 2 h of stressor exposure, and were largely resolved within 6 h. Additionally, the range of peak  
407 cortisol, glucose, lactate, and osmolality responses observed following sedation shows considerable  
408 overlap with the range of responses reported by Trushenski et al. (2010) in association with acute air-  
409 exposure and low-water challenges (cortisol: ~190-450 vs. ~130-230 ng/mL; glucose: ~50-190 vs. ~130-  
410 190 mg/dL; lactate: ~4-13 vs. ~1-9 mmol/L; and osmolality: ~420-450 vs. ~400-450 mOsm/kg), though  
411 higher lactate responses were associated with CO<sub>2</sub> and higher cortisol responses were associated with  
412 CO<sub>2</sub>, electrosedation, and eugenol.

413

414 Generally, the magnitude of the physiological stress response is considered indicative of stressor  
415 severity. Therefore, the greater magnitude and duration of the cortisol, glucose, lactate, and osmolality  
416 pulses observed among cobia sedated with CO<sub>2</sub> suggests that this drug is the most stressful of those we  
417 evaluated. This is also anecdotally supported by the observation of skin blanching in this treatment,  
418 which has been associated with stress in fishes (Iger et al. 2001). This is not surprising, as the pH of the  
419 CO<sub>2</sub> sedative baths was markedly lower than that of the culture water (8.2 vs. 9.5). Induction times  
420 were also significantly longer for CO<sub>2</sub>, and slower-acting sedatives have been linked with greater stress  
421 responses (Chiba et al. 2006; Trushenski et al. 2012a). Additionally, higher lactate responses have been  
422 previously linked with stressors that interfere with gas exchange (i.e., air exposure; Trushenski et al.  
423 2010); given the inhibitory effects of environmental hypercapnia on CO<sub>2</sub> release and O<sub>2</sub> uptake at the  
424 gill, rapid transition to anaerobic respiration and lactate accumulation following sedation with CO<sub>2</sub> may  
425 be expected. Electrodesation was conducted in freshwater and was associated with the 2<sup>nd</sup> highest  
426 cortisol response. It is possible that exposure to fresh water might have exacerbated the cortisol  
427 response to electrodesation in the same manner that low pH likely induced a greater response among

428 fish sedated with CO<sub>2</sub>. However, it is possible that the extremely short induction times associated with  
429 electrosedation limited the effects that freshwater exposure might have otherwise had on the  
430 secondary stress response parameters. A control treatment in which cobia were exposed to freshwater,  
431 but not electrosedation, would be necessary to parse the physiological response of these fish into the  
432 distinct effects of exposure to pulsed direct current electricity and freshwater. Regardless, it is  
433 important to note that all measured physiological perturbations, including the more marked responses  
434 associated with CO<sub>2</sub>, were resolved within 6 h post-sedation. Consequently, it seems unlikely that  
435 singular or periodic sedation of juvenile cobia using any of the approaches we evaluated would be  
436 sufficiently stressful to elicit the tertiary effects of stress (e.g., decreased growth, survival, or  
437 reproductive capacity) or other negative consequences in the near- or long-term.

438

439 One short-coming of our study is that we did not assess fish for vertebral abnormalities or other internal  
440 lesions post-sedation, which have been observed following exposure to pulsed DC electrosedation in  
441 some (Gaikowski et al. 2001; Zydlewski et al. 2008) but not all fishes (Vandergoot et al. 2011). The  
442 occurrence of injuries such as vertebral compressions or fractures and hemorrhages appears to be  
443 highly dependent on the type and strength of the waveform used, as well as the morphology and size of  
444 the fish involved. We cannot say whether such injuries occurred in the cobia we electrosedated.  
445 However, those that have reported injuries associated with pulsed direct current electrosedation have  
446 generally concluded that these injuries are relatively minor (e.g., occurring in a relatively small  
447 percentage of individuals or not resulting in delayed mortality) or may be avoided by modifying the  
448 electrosedation protocol to suit the circumstances. A second short-coming is that we did not assess the  
449 behavior, physiological status, general performance, or survival of treated fish after completion of the  
450 24-h observation period. To unequivocally demonstrate these treatments do not negatively influence  
451 fish when used in an immediate-release context, it would be necessary to treat fish, release them, and

452 monitor their performance post-stocking. Given the withdrawal periods currently required for MS-222  
453 (21 d), benzocaine and eugenol (3 d), this was not readily possible. However, we anticipate that any  
454 adverse events associated with treatment are most likely to occur immediately or shortly following  
455 sedation and serious, long-term effects are less likely. In previous studies, we have held and observed  
456 fish for 2 d to several weeks following sedative treatment (Trushenski et al. 2012a, 2012b; J. Trushenski  
457 [Southern Illinois University] and J. Bowker [U.S. Fish and Wildlife Service], *unpublished data*). Excluding  
458 a few incidental mortalities, adverse effects of sedative treatment (e.g., abnormal behavior, histological  
459 pathologies, mortality) were not generally observed in these studies, and in no case were they observed  
460 to develop or increase after 24 h. Based on this information, we think using the sedatives tested in an  
461 immediate-release context is unlikely to yield long-term, adverse effects not quantified as part of our  
462 study.

463

464 Selecting an appropriate sedative can be challenging, particularly when several methods may be used to  
465 achieve the desired level of sedation. Choosing the appropriate sedative is generally a matter of the  
466 cost and logistics associated with the intended application. Although numerous scenarios exist in the  
467 fisheries profession requiring the use of sedatives, some generalizations can be made regarding practical  
468 use of the sedatives described in this paper. For example, chemical sedatives are inexpensive in the  
469 short-term compared to electrosedation, which requires a relatively high initial investment. However,  
470 purchasing an electrosedation unit is a one-time investment, and lower-cost alternatives to  
471 commercially available units may be an option for some users (Hudson et al. 2011). If large numbers of  
472 fish are being sedated regularly, an electrosedation unit may be more economical than chemical  
473 sedatives, but chemical sedatives may be more appropriate and cost-effective for small numbers of fish  
474 or infrequent sampling. Electrodesation is uniquely suited for field applications because it reliably and  
475 quickly sedates a variety of taxa without concerns regarding chemical disposal or withdrawal periods.

476 Chemical sedatives may be more suited for research and hatchery facilities due to the need for proper  
477 chemical disposal and holding fish during the currently required withdrawal periods. By considering the  
478 effect of the different types of sedatives on the fish along with costs and the intended application,  
479 fisheries professionals can make more informed decisions concerning which sedative to use. However,  
480 all of these generalizations are subject to change as costs change, new sedatives become available or  
481 are approved, or withdrawal periods are modified.

482

483 In conclusion, benzocaine, MS-222, eugenol, CO<sub>2</sub>, and pulsed DC electrosedation were all effective in  
484 sedating juvenile cobia to Stage IV of sedation for the purposes of basic handling and morphometric  
485 measurement. Variation in induction and recovery times and physiological responses to sedation were  
486 observed. However, these differences could be reasonably accommodated within the context of typical  
487 field or laboratory research, though further research would be necessary to assess the relative suitability  
488 of the different sedatives for more invasive procedures. Although CO<sub>2</sub> and electrosedation may be  
489 tenable immediate-release options for some scenarios, these options may not be practical or advisable  
490 in other circumstances. We recommend that a greater range of immediate-release sedatives be made  
491 available to fisheries professionals so that they may select the sedative option best suited to their  
492 application and collect the highest quality data possible.

493

494

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643

644 **Figure Captions**

645 Figure 1. Schematic illustrating mean times to induction and various stages of recovery of cobia sedated  
646 to Stage IV of anesthesia using various chemical sedatives or electrosedation ( $n = 10$ ).

647

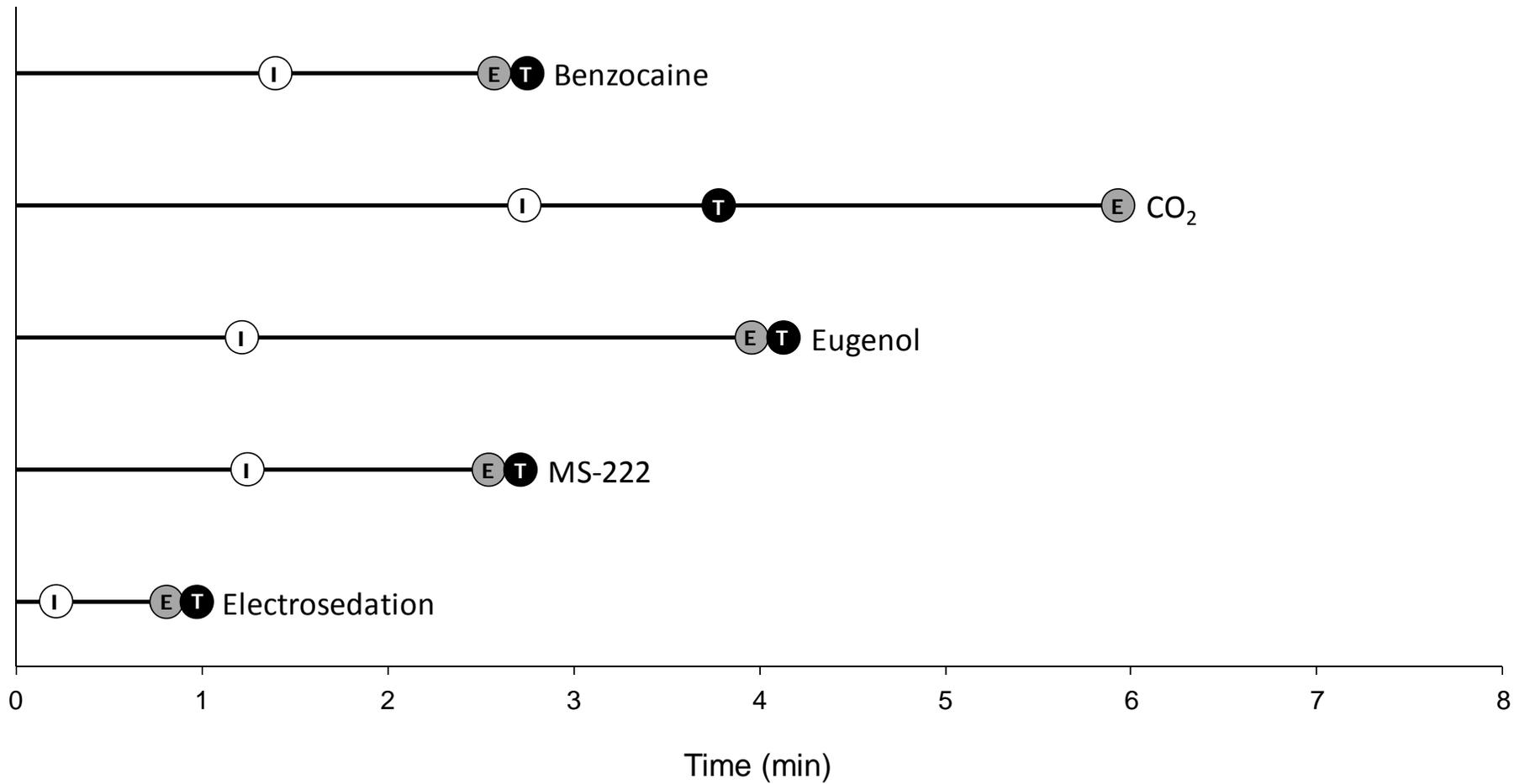
648 Figure 2. Time course of physiological responses (A = cortisol, B = glucose, C = hematocrit, D = lactate,  
649 and E = osmolality) of cobia following sedation to Stage IV of anesthesia using various chemical sedatives  
650 or electrosedation. Points represent LS-means reported in Appendix 1 ( $n = 3$ ); grey reference bars  
651 represent LS-means of values observed for fish sampled from the reference population throughout the  
652 course of the experiment.

Table 1. Water quality measured in Experiments #1 and #2. Values represent means of composite samples collected by combining aliquots collected from the sedative baths before and after use, and analyzed in duplicate along with water samples collected from the holding recirculation system at the beginning and end of the study period.

Parameter	Holding System	Sedative				
		Eugenol	Benzocaine	CO <sub>2</sub>	MS-222	Electrosedation
Temperature (°C)	27	27	27	27	27	27
Dissolved Oxygen (mg/L)	6.2	6.2	6.2	6.2	6.2	6.2
Alkalinity (mg/L)	88	97	103	104	98	45
Hardness (mg/L)	3650	3390	3245	3520	3540	280
Salinity (ppt)	20	20	20	20	20	0
Conductivity (mS)	>1999 (over range)	700				
pH	9.5	9.2	9.0	8.2	8.9	8.9

Table 2. Description of sedatives treatments applied in Experiments #1 and #2.

Sedative	Preparation Details
Eugenol	120 mg/L solution of AQUI-S® E (60 mg/L eugenol)
Benzocaine	750 mg/L solution of Benzoak® (150 mg/L benzocaine)
CO <sub>2</sub>	~750 mg/L solutions prepared according to the sodium bicarbonate/sulfuric acid method described by Post (1979) (analytically verified as 736 ± 21 mg/L, mean ± SE of replicate baths)
MS-222	150 mg/L solution of Finquel® (150 mg/L tricaine methanesulfonate)
Electrosedation	pulsed direct current (100 volts, 30 Hz, 25% duty cycle, 5 sec exposure) delivered via Portable Electroanesthesia System® (Smith-Root, Inc.; Vancouver, WA)



ⓘ = Induced to Phase IV Sedation

ⓔ = Maintain Equilibrium

Ⓣ = Respond to Tactile Stimulus

Figure 1.

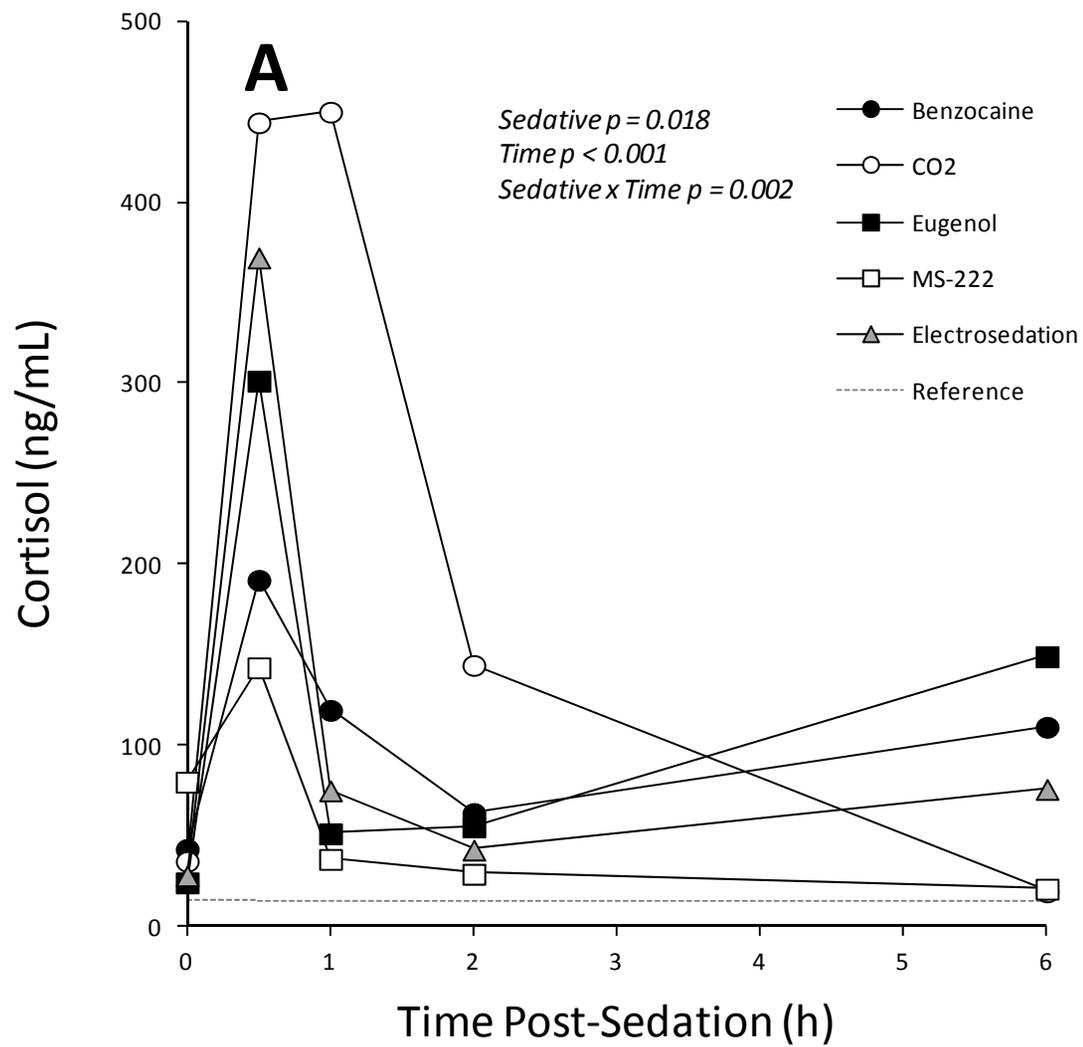


Figure 2A.

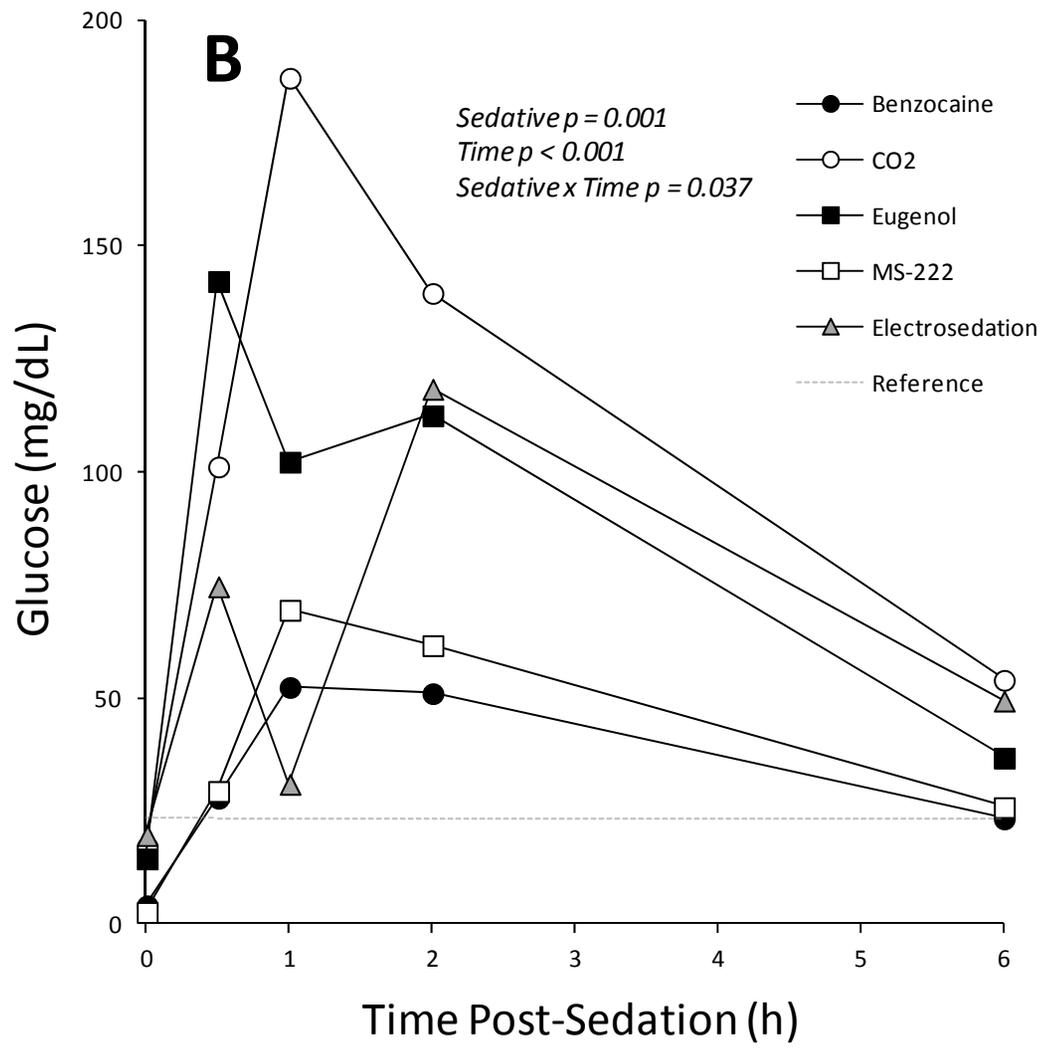


Figure 2B.

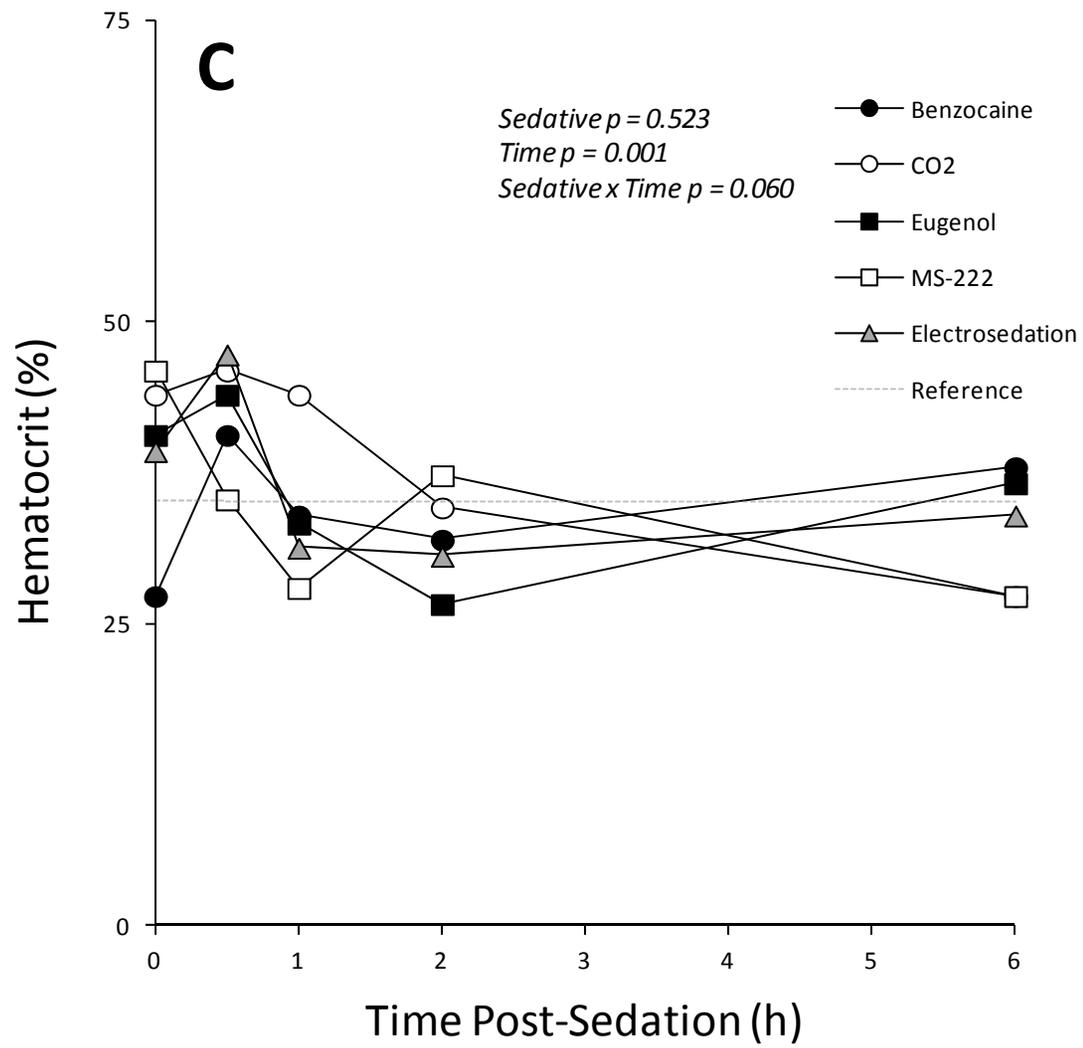


Figure 2C.

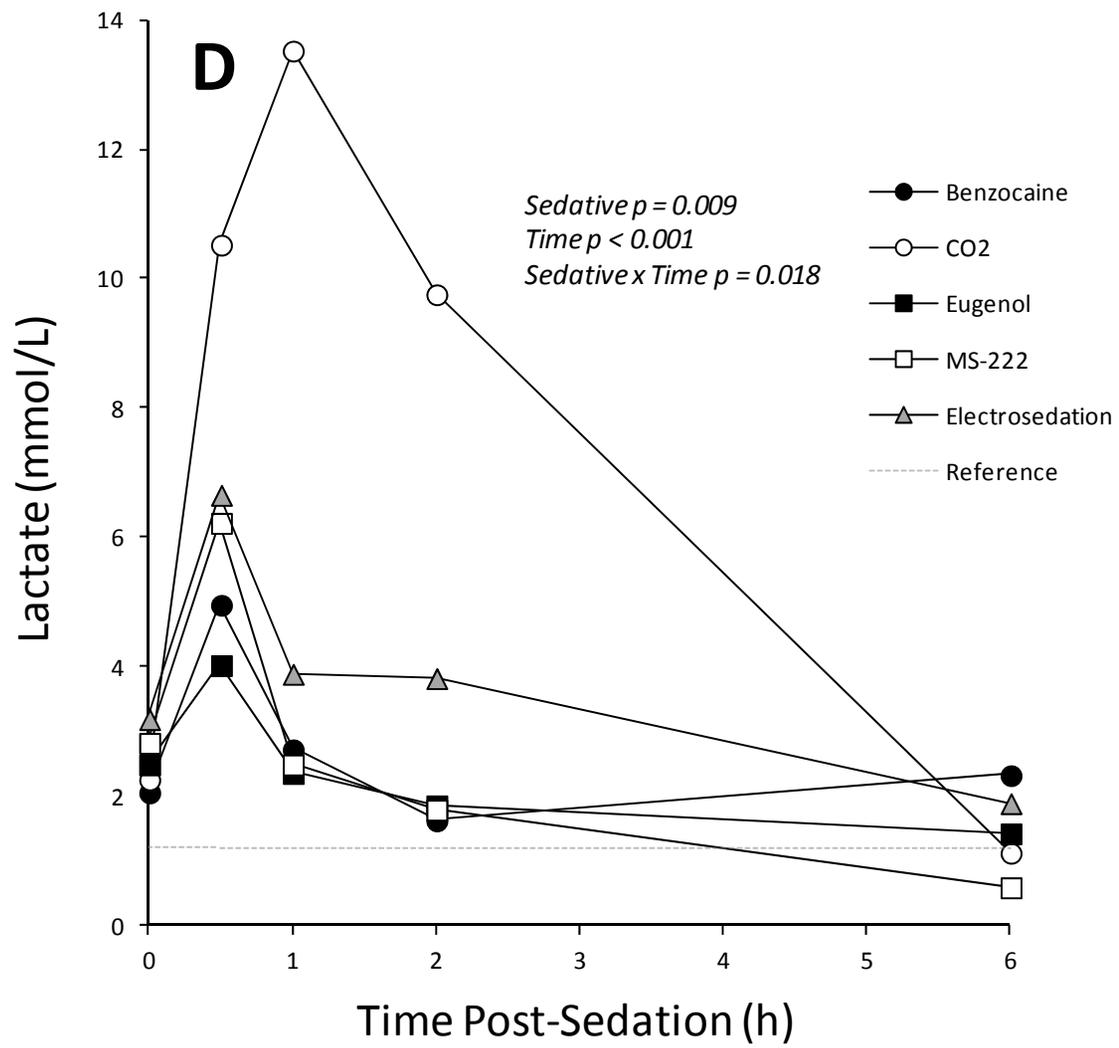


Figure 2D.

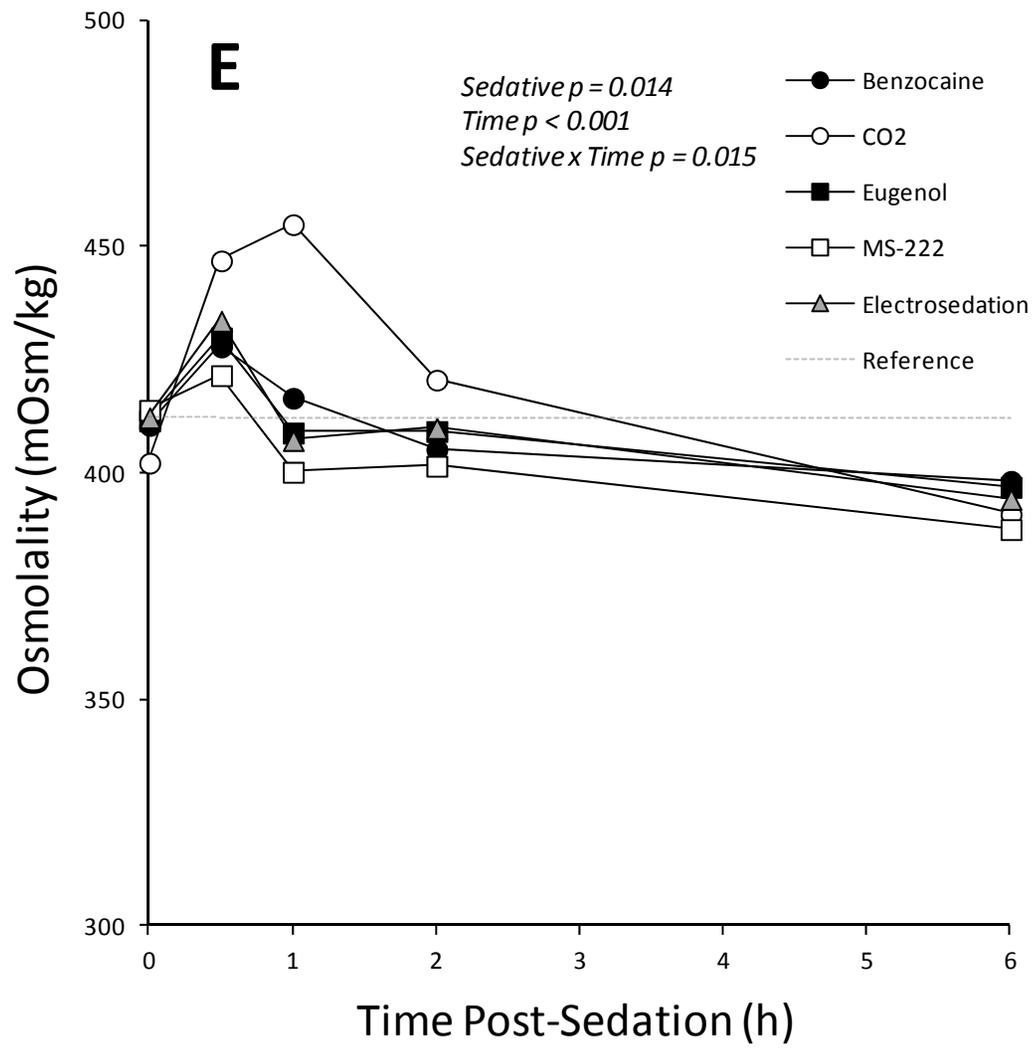


Figure 2E.