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**James D. Bowker, Jesse T. Trushenski, David C. Glover, Daniel G. Carty & Niccole Wandelaar**

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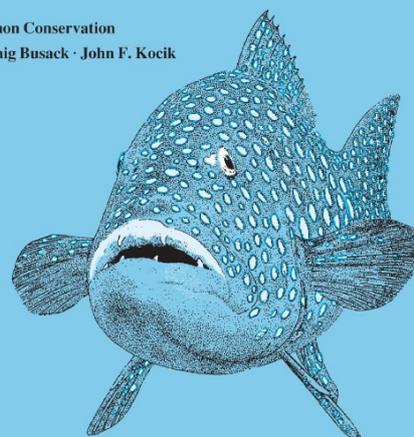
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# Sedative options for fish research: a brief review with new data on sedation of warm-, cool-, and coldwater fishes and recommendations for the drug approval process

James D. Bowker · Jesse T. Trushenski ·  
David C. Glover · Daniel G. Carty ·  
Niccole Wandelaar

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**Abstract** The absence of a suitable sedative allowing treated fish to be released immediately after recovery constrains research and poses a risk to fish and those handling them. The U.S. Food and Drug Administration's reliance on multi-taxon datasets represents a major hurdle in the approval process. Experiments were conducted with twelve freshwater taxa to assess time to induction and recovery of fish sedated with different doses of AQUI-S 20E (10 % eugenol), Benzoak (20 % benzocaine), or MS-222 (99.5 % tricaine methanesulfonate) administered under various conditions. A retrospective analysis

was conducted to determine whether sedative dose, water temperature, dissolved oxygen concentration, and fish length or weight contributed to variation in induction and recovery times. A subsequent experiment with eugenol was conducted to further assess time to sedation as a function of water temperature and sedative dose. Generally, higher doses and warmer temperatures were associated with faster inductions. Warmer temperatures were also associated with more rapid recoveries, however, high doses tended to delay recovery. Positive relationships linking estimated respiration rates and times to induction and recovery suggest the effects of temperature and body size on sedation timing may be a function of oxygen consumption. Collectively, our results demonstrated that the response of fish to chemical sedatives is primarily a function of sedative dose and water temperature, and, to a lesser extent, fish size and dissolved oxygen, not taxonomic classification. Accordingly, we suggest that as much information could be gained from a single taxon evaluated under different conditions as experiments involving multiple fishes. We recommend those establishing data requirements for fish drug approvals review these findings and consider alternative experimental designs as means of addressing regulatory requirements more efficiently and with greater rigor.

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J. D. Bowker · D. G. Carty · N. Wandelaar  
Aquatic Animal Drug Approval Partnership Program,  
U.S. Fish and Wildlife Service, 4050 Bridger Canyon  
Road, Bozeman, MT 59715, USA

J. T. Trushenski (✉)  
Center for Fisheries, Aquaculture, and Aquatic Sciences,  
Southern Illinois University Carbondale, 1125 Lincoln  
Drive, Life Sciences II, Room 251, Carbondale,  
IL 62901-6511, USA  
e-mail: [saluski@siu.edu](mailto:saluski@siu.edu)

D. C. Glover  
Aquatic Ecology Laboratory, The Ohio State University,  
1314 Kinnear Road, 230 Research Center, Columbus,  
OH 43212-1156, USA

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## Introduction

In the United States, drugs used on animals must be approved by the U.S. Food and Drug Administration (FDA) and must be used in accordance with the Federal Food, Drug, and Cosmetic Act (FFDCA, USC 2010). The FDA's definitions of "drug" and "food animal" are broad, and can be interpreted to include nearly any product applied to virtually any fish. Even ice and salt (NaCl) are considered animal drugs when applied to fish, if the intent is to alter their condition in any way (e.g., to reduce their metabolic rate or ease osmoregulation). Unless a drug is explicitly approved for a specific use in a particular species, its use, marketing, and distribution for that purpose are prohibited by the FFDCA and those engaged in these activities are subject to misdemeanor (no requirement for knowledge or intent) or felony charges (requires demonstration of intent to defraud or mislead, including evasion of detection) of violating the FFDCA (DoJ 1997). The same is true for products that are approved for other uses in animals or humans, active ingredients or generic versions of approved products, and products that are "Generally Recognized As Safe" (GRAS): unless a product is FDA-approved for a particular use, it is illegal to use it in that way.

The fisheries and aquaculture professions would benefit from greater access to FDA-approved drugs, including sedatives for transporting, handling, and harvesting fish, as well as for surgical and other procedures. Ideally, a fish sedative is easy to administer, safe to use, effective at low doses, provides quick and predictable sedation, offers some analgesia, elicits a state of sedation that is easily managed, has a reasonable margin of safety with respect to over-sedation, can be used over a broad range of water chemistries, allows for rapid recovery from sedation and physiological responses to the sedative, and is inexpensive (e.g., Marking and Meyer 1985; Summerfelt and Smith 1990; Trushenski et al. 2012a, b, c, d). For certain applications, releasing fish into public waters or harvesting them for human consumption immediately after sedation is particularly useful. Unfortunately, there are currently no FDA-approved 'immediate-release' sedatives available for use in the United States. The FDA approval process for fish drugs is considered to be among the most conservative in the world, though access to fish sedatives is nonetheless a constraint in many developed countries. It appears that

only a handful of tricaine methanesulfonate, benzocaine, carbon dioxide, chlorbutanol, and isoeugenol products are approved as fish sedatives throughout the world, and only AQUI-S (50 % isoeugenol) appears to have any approved claims (i.e., New Zealand) as an immediate-release sedative (Ross and Ross 2008).

Lack of approved, immediate-release sedatives is a consequence of many factors, including complexities of the approval process, the substantial human and monetary resources involved in obtaining an approval, and the specialized nature of the work. In the United States, sufficient data must be generated to complete "technical sections" that demonstrate the safety and effectiveness of an animal drug before it can be approved. Oversight bodies in other countries have similar requirements to demonstrate drug safety and effectiveness (Treves-Brown 2000). Although FDA guidance suggests that technical sections may only require data for two representative freshwater taxa for an 'all freshwater fish' approval (FDA 2008), previous experience has indicated demonstration of target animal safety or effectiveness requires generation of acceptable data in studies conducted with two representative cold-, cool-, and warmwater fishes (typically exclusive of ornamental fishes), i.e., data must be provided for six representative freshwater taxa (*personal communication*, David Erdahl, U.S. Fish and Wildlife Service, Bozeman, Montana).

Efforts are underway to complete data requirements for an all freshwater fish, immediate-release sedative claim; however, pursuing such an approval within the current framework will consume substantial public and private resources and will take years to complete (Trushenski et al. 2012a, b, c, d). Early reports suggested new aquaculture drug claims required a minimum investment of US\$3.5 million (Schnick et al. 1996) over a decade. However, recent estimates indicate the figure is actually much higher and could exceed \$40 million, depending on the claim (Storey 2012). Various types of 'meta-analyses' have been suggested as a strategy to assess variation within and among fishes to reduce the number of taxa and studies required to demonstrate the safety and effectiveness of aquaculture drugs. For example, systematic review (a quantitative literature review that synthesizes results from multiple works and provides a "weight of evidence"—based assessment of the information available) has been suggested as one means of

satisfying technical section requirements for aquatic animal drug approvals (Storey 2012). In most instances, there are not enough data to support this approach, but there is considerable data available regarding the effectiveness of sedatives.

We have conducted many experiments to demonstrate the effectiveness of candidate immediate-release sedatives, including previously published works (Bowzer et al. 2012; Gause et al. 2012; Trushenski et al. 2012b, c, d) and additional data presented herein. The objective of these experiments was to demonstrate the effectiveness of two candidate immediate-release sedatives, AQUI-S 20E (10 % eugenol; AQUI-S New Zealand, Ltd., Lower Hutt, New Zealand) and Benzoak (20 % benzocaine; ACD Pharmaceuticals AS, Ålesund, Norway), in comparison with an approved (though not for immediate-release applications) and widely used sedative, Tricaine-S/Finquel (99.5 % tricaine methanesulfonate; Western Chemical, Inc, Ferndale, Washington/Argent Chemical, Redmond, Washington). Most of these experiments were conducted in accordance with FDA-concurred protocols such that the data generated would be accepted by FDA in fulfillment of the effectiveness technical sections for the proposed use of AQUI-S 20E and Benzoak as immediate-release fish sedatives (Bowker et al. 2010, 2011). The others were conducted in a manner generally consistent with the FDA-concurred protocols, but tested additional sedative doses, water temperatures, and different taxa or life stages. These additional experiments were conducted following consultation with FDA to address the agency's likely concerns regarding the broad-spectrum efficacy of AQUI-S 20E and Benzoak.

The primary objective in the present work was to demonstrate the effectiveness of AQUI-S 20E and Benzoak in sedating a variety of cold-, cool-, and warmwater fishes to handleable under a range of environmental conditions in support of FDA approval of the use of these products as immediate-release sedatives. We defined effectiveness such that treated fish would be induced within 2 min and recover within 5 min. The wealth of data generated to address our primary objective offered the opportunity to address a secondary objective: to determine whether a data-synthesis approach would be viable means to fulfill data requirements with empirical data from fewer taxa. Accordingly, we analyzed the effectiveness data a posteriori to determine whether factors such as

sedative dose, water temperature, dissolved oxygen concentration, and fish length or weight could be used to explain variability in induction and recovery times for a range of freshwater taxa. Based on the results of this retrospective analysis, we also conducted a factorial experiment with eugenol to further test the hypothesis that sedative dose and water temperature are the primary drivers of variation in induction and recovery times. Herein, we present a synthesis of these related experiments and analyses, i.e., experiments conducted to demonstrate sedative effectiveness under various conditions, retrospective meta-analysis of induction and recovery times, and factorial experiment to further test the effects of water temperature and sedative dose. In light of these results, we also offer some commentary on the United States drug approval process as it applies to drugs for aquatic animals and ways in which it could be made more efficient. Although this commentary is primarily focused on the drug approval process in the United States, strategies to improve the efficiency of the drug approval process and FDA approval of an immediate-release sedative will ultimately benefit users in many countries. As noted above, the FDA approval process is rigorous and acceptance of a drug as safe and effective by FDA can help to inform and streamline evaluation of the same product by other oversight bodies. Additionally, FDA approval of a drug facilitates access to American markets. Although the FFDCAs, as amended by the Animal Drug Availability Act (USC 2010), provides a basis for legally marketing fish treated with unapproved animal drugs, such seafood can only be imported if drug residues in the tissues fall below established "import tolerances" (FDA 2014), which have not been established for any fish sedatives. If a drug has been approved by the FDA, however, such marketing restrictions do not apply. Accordingly, approval of an immediate-release sedative in the United States would allow fisheries professionals throughout the world to safely and effectively sedate fish without restricting their access to the second largest seafood export market in the world (FAO 2014).

## Methods

In the following subsections, we describe a series of experiments conducted at four locations from May through November 2011 that were used to determine

the general effectiveness of sedatives. Data resulting from these experiments were also used in the retrospective meta-analysis. Although specific elements such as conditions, test species, and personnel vary across these experiments, the general experimental designs and procedures were similar. The experiments were conducted according to procedures outlined in FDA-concurred pivotal protocols for generating effectiveness data for AQUI-S 20E and Benzoak as immediate-release fish sedatives (Bowker et al. 2010, 2011) or in a manner generally consistent with these protocols. In the following sections, a brief description of the on-site conditions for each study location, detailed accounts of the individual experiments, and a summary of general methods applied to all experiments are provided.

#### Experiment locations, taxa, and water sources

Experiments with Rainbow Trout *Oncorhynchus mykiss* (RBT) and Cutthroat Trout *Oncorhynchus clarkii* (CTT) were conducted at the U.S. Fish and Wildlife Service Bozeman Fish Technology Center (BFTC; Bozeman, Montana). Taxa were housed separately in indoor tanks supplied with flow-through water. Cold- and warm spring water sources were mixed to achieve desired temperatures.

Experiments with hybrid Striped Bass (female White Bass *Morone chrysops* × male Striped Bass *M. saxatilis*, (HSB), Blue Catfish *Ictalurus furcatus* (BCF), Channel Catfish *Ictalurus punctatus* (CCF), and Nile Tilapia *Oreochromis niloticus* (TIL) were conducted at the Center for Fisheries, Aquaculture, and Aquatic Sciences (CFAAS) at Southern Illinois University Carbondale (Carbondale, Illinois). Taxa were housed separately in indoor recirculation aquaculture systems equipped with biological and mechanical filtration units and provided with supplemental aeration. Although all systems were originally filled and maintained (i.e., water added to compensate for losses due to evaporation and routine filter backflashes) using dechlorinated municipal water, individual recirculation systems served as the water source for each experiment, except for the factorial experiment (see below). For the factorial experiment, the recirculation system was filled with dechlorinated municipal water and treated with Ammo-Lock (Mars Fishcare, Inc.; Chalfont, Pennsylvania) to detoxify ammonia and remove residual chlorine and chloramines.

Experiments with Brown Trout *Salmo trutta* (BNT), Walleye *Sander vitreus* (WAE), Yellow Perch *Perca flavescens* (YEP), Lake Trout *Salvelinus namaycush* (LKT), Common Carp *Cyprinus carpio* (CMC), and Fathead Minnow *Pimephales promelas* (FHM) were conducted at the U.S. Geological Survey Upper Midwest Environmental Sciences Center (UMESC; La Crosse, Wisconsin). Brown Trout were housed in a single outdoor raceway. Other taxa were housed indoors in the UMESC wet lab facility in separate circular culture tanks. Flow-through well water was the water source for all experiments.

An additional experiment with WAE was conducted at the Iowa Department of Natural Resources Rathbun Fish Culture Research Facility (RFCRF; Moravia, Iowa). Fish were housed in indoor flow-through raceways supplied with screened, flow-through water from Rathbun Lake, which was the water source for the experiment.

#### Experimental designs

##### *Comparing sedatives in terms of general effectiveness*

To compare general effectiveness among sedatives, we conducted experiments as described in Electronic Supplementary Table 1 to assess times to induction and recovery of fish sedated in static baths with eugenol (25–60 mg/L), benzocaine (40–150 mg/L), or MS-222 (80–150 mg/L; Electronic Supplementary Table 1). Fish were held in their respective systems for at least 24 h before conducting the experiments. Sedative doses used in these experiments varied by taxon, and were selected based on preliminary testing (data not shown) to determine doses likely to yield induction times less than 2 min and recovery times less than 5 min. Sedatives were assessed using sets of 10–30 fish sedated individually in series (one fish at a time until all fish in the set had been sedated). Generally, sedative solutions were prepared in bulk for each set, and used to fill fresh sedative baths for each individual fish within the set (i.e., sedative baths were not reused). However, in the WAE experiment conducted at RFCRF, fresh sedative baths were prepared for each set of 10 fish and were not exchanged from fish to fish within each set (i.e., sedative solution was reused). Fish were allowed to recover in tanks supplied with flowing water.

### *Effect of sedative dose*

To determine the effect of sedative dose on induction and recovery times, additional experiments were conducted with RBT (two size classes), HSB (two size classes), and YEP. In each experiment, fish were sedated with benzocaine or eugenol at higher or lower doses than those used to assess general effectiveness (e.g., 48 and 72 vs. 60 mg eugenol/L for HSB; Electronic Supplementary Table 1). Each sedative dose was tested in the manner described for the general effectiveness experiments, except that each dose was tested with a single set of 10–30 fish.

### *Effect of water temperature*

To determine effect of water temperature on induction and recovery times, additional experiments were conducted with RBT (two size classes) and HSB (two size classes). In each experiment, fish were sedated with different doses of benzocaine and eugenol at cooler temperatures (within the thermal range for the relevant taxon) than those used for the general effectiveness and sedative dose experiments (e.g., 16–17 vs. 22 °C for HSB; Electronic Supplementary Table 1). Fish were allowed to acclimate to the cooler water temperatures for at least 12 h before conducting the experiments. Each dose-temperature combination was tested in the same manner as described for the general effectiveness experiments, except that each combination was tested with a single set of 10–30 individual fish. Sedative solutions were prepared in bulk with water of the appropriate temperature for each set of fish and then used immediately to fill fresh sedative baths for each fish within the set.

### *Retrospective meta-analysis*

Data from the aforementioned general effectiveness, sedative dose, and water temperature experiments were analyzed by regression tree analysis to determine whether explanatory variables such as sedative dose, water temperature, dissolved oxygen concentration, and fish length or weight could be used to predict induction and recovery times. See “[Statistical analyses](#)” below for a full description of the methods used.

### *Factorial experiment to further assess the effects of sedative dose and water temperature*

The aforementioned regression tree analysis indicated that sedative dose and water temperature influenced induction times, with higher sedative doses and warmer water temperatures generally yielding more rapid inductions. To test this result with greater rigor, a follow-up experiment was conducted with juvenile hybrid Striped Bass ( $32.7 \pm 7.8$  g,  $14.2 \pm 1.1$  cm total length; mean  $\pm$  SD) sedated with 20, 40, 60, 80, 100, or 120 mg eugenol/L at water temperatures of 10, 18, 24, or 28 °C. A small recirculation system was constructed to accommodate this experiment, consisting of an acclimation-holding tank; a raceway-water bath; and water aeration, heating, and cooling systems. Twelve, 10-L plastic containers were filled with 7.6 L of water and placed in the raceway/water bath to allow water temperatures to equilibrate; six of these containers were used for sedation and six were used for recovery (static water in all cases). All sedative baths were aerated before use, whereas recovery baths were aerated continuously. Water was recirculated continuously between the acclimation-holding tank and the raceway-water bath. Fish were acclimated to conditions in the system and were held in the acclimation-holding tank for 2–12 h before the experiment was conducted. A fresh sedative bath was prepared for each dose-temperature combination but the baths were not exchanged between fish. Fish were sedated and allowed to recover in static baths of fresh, aerated water.

### General methods

#### *Preparation of sedative solutions*

For all experiments, we used commercially available sedatives containing the active ingredients MS-222 (Tricaine-S or Fiquel), eugenol (AQUI-S 20E), or benzocaine (Benzoak). In most of the experiments, samples collected from the bulk sedative solutions or sedative baths were analyzed for eugenol or benzocaine to verify sedative doses administered. MS-222 doses were not verified because the purpose of the studies were to generate effectiveness data to support approvals of AQUI-S20E and Benzoak; MS-222 dose verification was not necessary according to the FDA-concurred pivotal protocols (Bowker et al. 2010,

2011). Eugenol doses were verified spectrophotometrically by measuring the absorbance of samples at 279 nm and calculating eugenol concentration via a standard linear regression ( $r^2 \geq 0.9$ ) of absorbance values from known standards (0–150 mg eugenol/L) prepared using eugenol (99 % USP grade, dissolved in ~ 50 mL of ethanol) and the appropriate source water. In experiments conducted at the BFTC and CFAAS, benzocaine doses were verified spectrophotometrically by measuring the absorbance of each sedative solution sample (10- or 20-fold dilution) at a wavelength of 285 nm and calculating benzocaine concentration via the following equation,

$$\text{benzocaine dose (mg/L)} = 165.2 \times \left( \frac{\text{DF} \times A_{285 \text{ nm}}}{1 \times 17.03} \right)$$

where 165.2 represents the molar mass of benzocaine,  $A_{285}$  represents the absorbance of the solution, DF represents the dilution factor (i.e., 10 for tenfold dilutions or 20 for 20-fold dilutions), 1 represents the cell path length in cm, and 17.03 is a constant (personal communication; J. Bommer; Frontier Scientific, Inc.; Logan, Utah). In experiments conducted at UMESC, benzocaine doses were verified spectrophotometrically by measuring the absorbance of sedative solution samples at 285 nm and calculating benzocaine concentrations via a standard linear regression curve ( $r^2 \geq 0.9$ ) based on known standards (0–160 mg benzocaine/L) prepared using benzocaine (98 % USP grade ethyl 4-aminobenzoate, dissolved in methanol) and the appropriate source water.

#### *Determination of induction and recovery times*

Fish were sedated to “handleable” in all experiments. For our purposes, handleable was equivalent to stages 3–4 as described by Summerfelt and Smith (1990). A fish was determined to be handleable when it lost equilibrium and responsiveness to external stimuli, could be easily caught by hand, and did not struggle while being removed from the sedative solution and measured for length or weight. A fish was determined to be recovered when it regained equilibrium, resumed normal swimming behavior, avoided obstacles (e.g., a net handle) placed in its swimming path, and actively evaded the observer’s attempts to capture and handle the fish. Determining induction and recovery times is somewhat subjective; therefore, to maximize accuracy

and precision, the number of observers was kept to a minimum (1–3 per experiment), observers reviewed sedation criteria prior to each experiment and simultaneously observed general sedation behavior of the taxon involved at the beginning of each experiment.

Each fish was netted from the holding system, placed into the sedative bath, and observed until swimming ceased or appeared sluggish and fish were unable to maintain equilibrium. After loss of equilibrium, the fish was gently lifted from the bath to assess whether it responded to the tactile stimulus of handling or emersion. If a response (whole body movement or active fin movement) was observed, the fish was returned to the bath and reassessed a few seconds later. After the fish was sedated and measured, it was placed in a recovery tank and monitored until it recovered from sedation. Induction and recovery times were measured for each fish to the nearest second. General fish behavior was assessed during sedation and recovery, and observations of abnormal behavior (e.g., head shaking, agitation, piping) were recorded.

#### *Water quality assessment*

For each experiment, source water was analyzed for a suite of water quality parameters using the standard equipment and methods commonly used for water quality testing at each of the study locations. Commonly available water testing meters were used to determine water temperature and dissolved oxygen (DO) concentration [i.e., YSI 550 Temperature and Dissolved Oxygen Meter (YSI, Inc., Yellow Springs, Ohio) or HQ40d Meter (Hach Co., Loveland, Colorado)], and pH [Multi-Parameter PCSTest<sup>TM</sup> 35 (Eutech Instruments, Vernon Hill, Illinois), pHep 5 pH/Temperature Tester (Hanna Instruments, Smithfield, Rhode Island), or YSI EcoSense pH Pen (YSI)]. Water hardness and alkalinity were measured with commercially available reagents and a digital titrator according to the manufacturer’s instructions (Hach Co.); nitrite, nitrate, and ammonia levels were measured with commercially available reagents (Hach Co.) and a spectrophotometer (Genesys 2 Spectrophotometer, Thermo Electron Scientific Co., Madison, Wisconsin); note that source water hardness, alkalinity, nitrite, nitrate, and ammonia were not determined for the factorial experiment. The system used for this experiment was filled with dechlorinated municipal water and treated with Ammo-Lock prior to each use,

thus deterioration of water quality due to accumulation of nitrogenous waste was not considered. Results of the water quality analyses are summarized for each study location in Electronic Supplementary Table 2.

Temperature, DO concentration, and pH were also measured for bulk preparations of sedative solutions prior to distribution to individual sedative baths (Electronic Supplementary Tables 2 and 3). Additionally, water temperature and DO concentration were measured in each sedative bath and recovery tank immediately before use (Electronic Supplementary Tables 1 and 3).

### Statistical analyses

All statistical analyses were conducted with SAS Version 9.2 or 9.3 (SAS Institute, Cary, North Carolina), with the exception of regression tree analysis, which was conducted using the RPART (Recursive PARTitioning) library of tree routines in the program R version 2.15.2 (R Development Core Team 2012). In all cases, individual fish were treated as experimental units and a priori  $\alpha$ -level was 0.05.

### General sedative effectiveness

Mean and variance estimates were calculated as appropriate for the general effectiveness data with the PROC MEANS procedure. Data from all experiments were subjected to a two-sided binomial exact test (PROC FREQ) to test whether 80 % of eugenol- or benzocaine-treated fish were induced to a handleable state within 2 min.

### Regression tree analysis of variation in times to induction and recovery

Variation in times to induction and times to recovery was assessed for each sedative separately with regression trees, also known as recursive partitioning analysis (De'ath and Fabricius 2000). This approach simultaneously evaluates the distribution of a single response variable (i.e., induction time or recovery time) as a function of each independent variable included in the analysis and determines the numerical value of each independent variable that would minimize heterogeneity of the response on either side of a split or "node"; the independent variable that explains the most variation in the response, or leads to the

largest decrease in heterogeneity, is selected as the first node. Additional splits are performed on new data subsets until a specified threshold is met, thereby producing a "tree." We evaluated the effect of sedative concentration, fish length, and fish weight, water temperature, and dissolved oxygen concentration on time to induction and time to recovery. Although it was clear that some of these variables would be correlated, we allowed the regression tree approach to indicate which variables were most important for explaining and homogenizing variance in time to induction and time to recovery; the hierarchical nature of this test negates potential issues with multicollinearity that would arise with other statistical methods such as multiple linear regression. The 1-SE rule was used as our threshold to select the most parsimonious tree model (Breiman et al. 1984). Specifically, a tenfold cross-validation was used to estimate error rates of various tree sizes; the tree size that fell within 1-SE of the minimum error rate was selected as the best model.

### *Probability of induction and recovery within ideal time limits*

To further explore the factors influencing induction and recovery that were identified with the regression tree analysis, a logistic regression approach (PROC LOGISTIC) was used to assess data from the factorial experiment conducted with HSB. Specifically, we examined the main and interactive treatment effects of sedative dose and water temperature, as well as the effects of fish size and dissolved oxygen concentration, on the probability of induction within 2 min, the probability of recovery within 5 min, and the probability of both induction and recovery within these ideal time limits (limits were chosen based on generalized preferences of fisheries professionals). Similar to the 2-way ANOVA approach for the factorial experiment, dose and temperature were treated as class variables, whereas the main effects of fish weight and dissolved oxygen were treated as continuous covariates; all possible interactions among the five variables were also considered for inclusion into the model. A stepwise model-selection procedure was used to determine the best model, such that variables were entered into the model if they were significant at the  $\alpha = 0.05$  level and were retained in the model if they maintained that significance level following the

introduction of other explanatory variables. Lastly, we estimated respiration rates (mg O<sub>2</sub>/min) for individual fish using observed water temperature and body mass according to the Striped Bass respiration function defined by Hartman and Brandt (1995). We assumed fish activity to be negligible and thus only estimated standard respiration rates; we did not validate the respiration function for HSB as our intent was to generate relative information for comparisons between treatment groups. Given the error likely in estimates of standard respiration rates, we used these values to determine whether correlations (PROC CORR) existed between estimated respiration rates and the probabilities of induction within 2 min and recovery within 5 min.

## Results

Although some induction and recovery times exceeded the benchmarks we established for effectiveness, all fish were sedated to a handleable state and recovered from sedation. Induction times varied among the effectiveness experiments, ranging from 0.6 to 3.5 min, with standard deviations of 0.1–0.9 min (Electronic Supplementary Table 1). Recovery times were generally longer and more variable than inductions, with times as long as 14.4 min and standard deviations as high as 4.8 min. Some fish exhibited piping or mild agitation, but the majority of fish behaved normally during induction and recovery (data not shown). Data from the experiments conducted under pivotal protocols were accepted by FDA in support of approvals of AQUI-S20E and Benzoak.

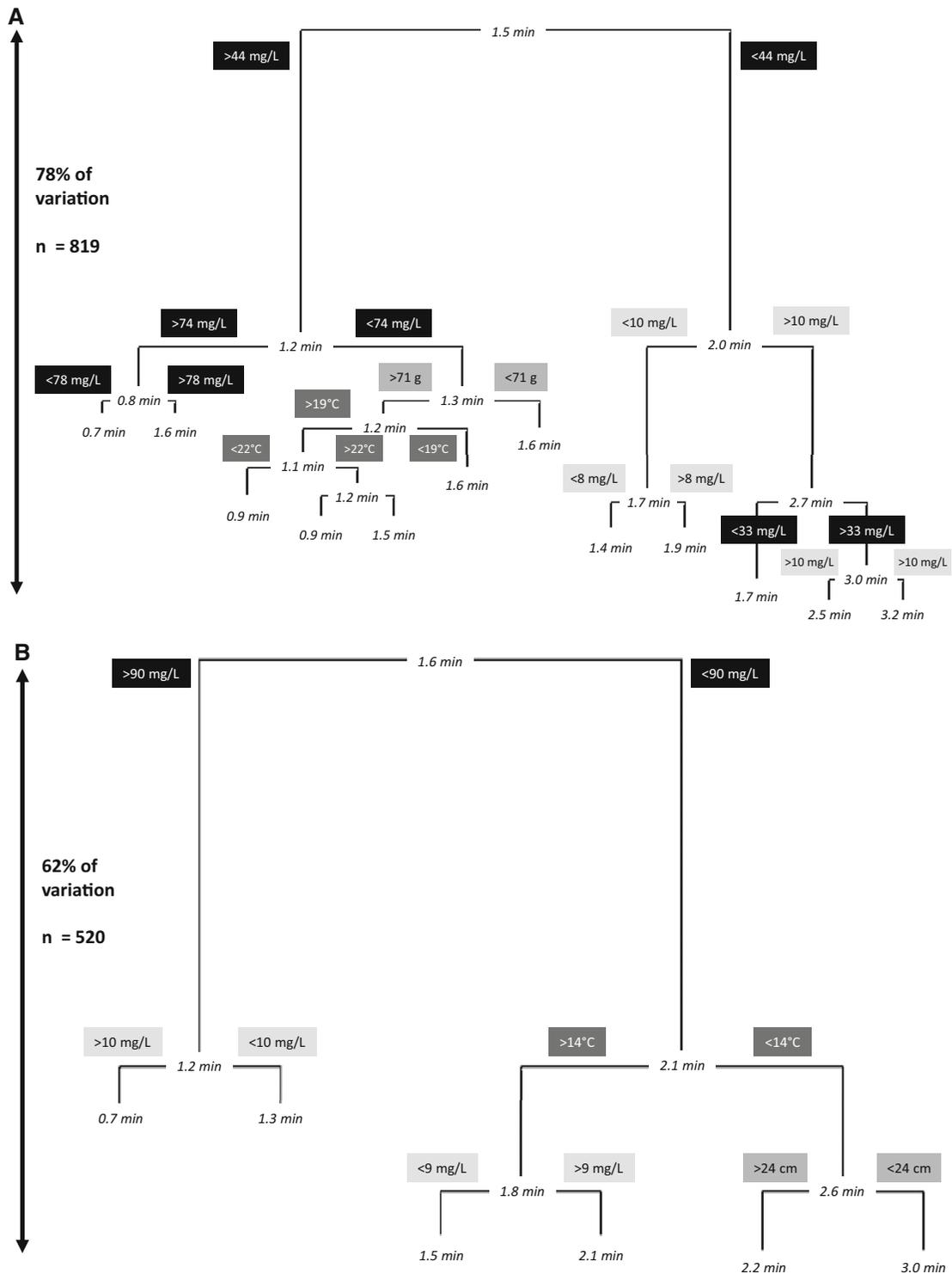
Regression tree analysis of the general sedative effectiveness data explained 78 and 62 % of the variation in induction times for fish treated with eugenol and benzocaine, respectively, with sedative dose as the primary explanatory variable for both sedatives (Fig. 1a, b). As the primary driver of variation, higher sedative doses were associated with shorter induction times. Regression tree analysis explained 71 % of the variation in induction times for MS-222-treated fish, but fish length was the primary explanatory variable in this dataset (Fig. 1c), albeit with a small range of dose-temperature combinations evaluated. As the primary driver of variation, larger fish sizes were associated with shorter induction times in the MS-222 dataset. Regression tree analysis

was slightly less successful in explaining variation in recovery times for eugenol (63 %), benzocaine (35 %), and MS-222 (53 %) (Fig. 2a–c). Dissolved oxygen concentration was the primary explanatory variable in the eugenol and MS-222 recovery datasets, and, in both cases, higher oxygen levels were generally associated with shorter recovery times. Water temperature was the primary explanatory variable in the benzocaine recovery dataset, with warmer temperatures associated with shorter recovery times.

Eugenol treatments administered in the factorial experiment were also effective in sedating fish to handleable (Electronic Supplementary Table 3). Induction (range 0.6–5.2 min) and recovery times (2.6–15.4 min) were significantly affected by sedative dose and water temperature, and a significant dose by temperature interaction effect was also noted (Fig. 3). Generally, higher doses and warmer water temperatures were associated with shorter inductions; however, at the lowest doses this pattern was less consistent and less readily apparent. Warmer water temperatures were also generally associated with more rapid recoveries, however, high doses tended to delay recovery (Electronic Supplementary Table 3). Despite these trends, in nearly all instances, induction and recovery were most rapid for fish sedated at 24 °C (Fig. 3).

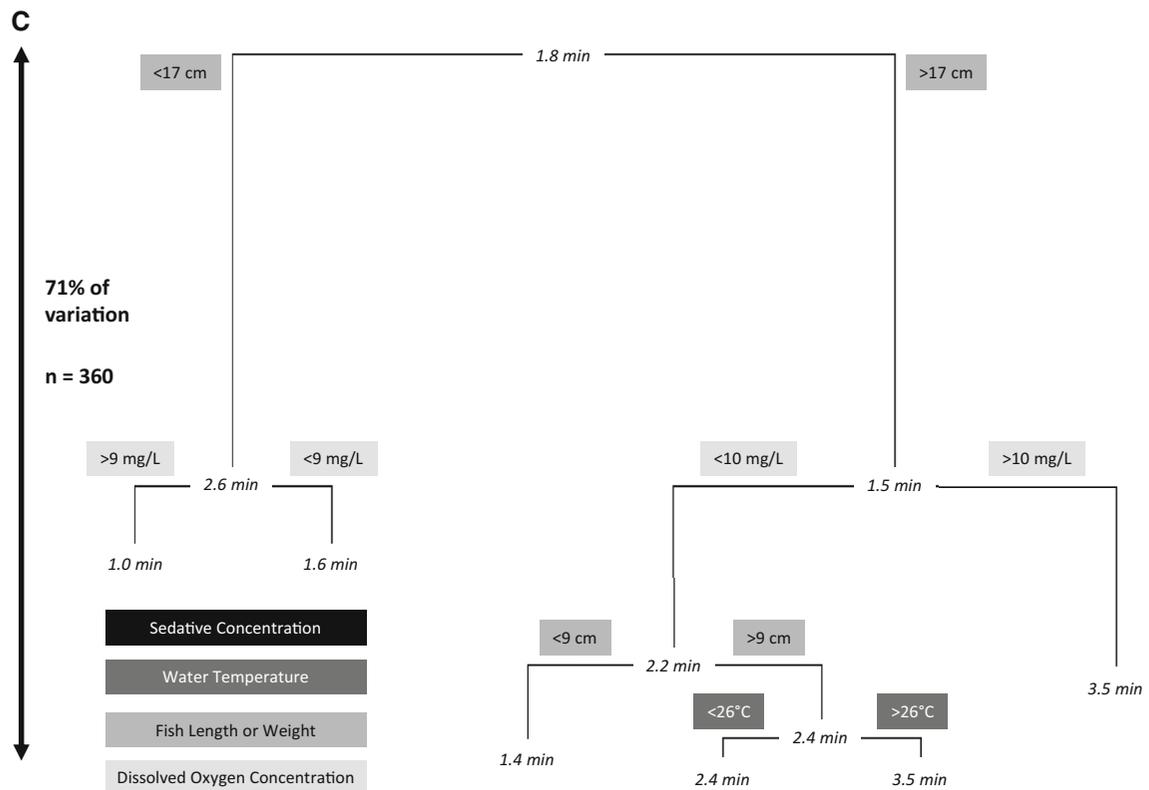
The best logistic regression model indicated that the probability of induction within 2 min was affected by the main effects of sedative dose and temperature, and explained 62 % of the variation (Electronic Supplementary Fig. 4A). A total of 75 % of the variation in the probability of time to a 5 min recovery was explained by the main effects of temperature, dose, and dissolved oxygen (Electronic Supplementary Fig. 4B). Across all treatments, a 1 mg/L increase in dissolved oxygen was predicted to increase the odds of recovery within 5 min by 7.3 times (Electronic Supplementary Fig. 4B). The probability of an individual fish being both sedated within 2 min and recovering within 5 min was affected by the main effects of dose and temperature, which explained 74 % of the variation (Electronic Supplementary Fig. 4C).

There were significant positive relations, albeit weak, between estimated respiration rate and the probability of induction within 2 min at all doses in the factorial experiment (20 mg/L,  $r^2 = 0.40$ ,  $P = 0.002$ ; 40 mg/L,  $r^2 = 0.45$ ,  $P < 0.001$ ; 60 mg/L,  $r^2 = 0.37$ ,  $P = 0.004$ ; 80 mg/L,  $r^2 = 0.42$ ,  $P = 0.001$ ; 100 mg/L,  $r^2 = 0.40$ ,  $P = 0.001$ ; 120 mg/L,



**Fig. 1** Regression trees describing effects of dissolved oxygen (light grey), fish size (medium gray), water temperature (dark grey), and sedative dose (black) on induction times for fish

sedated with eugenol (a), benzocaine (b), or MS-222 (c). Regressions trees were constructed using data described in Electronic Supplementary Table 1



**Fig. 1** continued

$r^2 = 0.40$ ,  $P = 0.002$ ). There were also significant positive relations between respiration rate and the probability of recovery within 5 min at all doses (20 mg/L,  $r^2 = 0.80$ ,  $P < 0.001$ ; 40 mg/L,  $r^2 = 0.82$ ,  $P < 0.001$ ; 60 mg/L,  $r^2 = 0.74$ ,  $P < 0.001$ ; 80 mg/L,  $r^2 = 0.76$ ,  $P < 0.001$ ; 100 mg/L,  $r^2 = 0.75$ ,  $P < 0.001$ ; 120 mg/L,  $r^2 = 0.76$ ,  $P < 0.001$ ).

In experiments in which dose verification was conducted, actual doses of eugenol administered ranged from 84 to 125 % of the intended dose. Analytically verified concentrations of benzocaine suggested ‘under-dosing’ was more common for this sedative (81–107 % of the intended dose), most likely because the Benzoak product does not dissolve or disperse in water as readily as the other sedative products evaluated.

## Discussion

### Sedative effectiveness

Final Study Reports summarizing results from each study were submitted to FDA with a request that no

additional data be required to support a claim of effectiveness of AQUI-S20E and Benzoak to sedate freshwater fish to handleable. A qualitative comparison showed that at a given water temperature, similar induction times were observed regardless of fish species tested. The FDA considered the data submitted for AQUI-S20E sufficient to satisfy data requirements and complete the effectiveness technical section for claims of sedation of freshwater finfish to a handleable condition (FDA 2013). The Benzoak effectiveness data were accepted, but the technical section complete request has been delayed pending submission of data to support use of the dose verification method by the sponsor.

### Influence of environmental conditions and fish size

Ours is the first attempt to quantitatively assess the effects of water temperature, sedative dose, and other variables on sedative effectiveness and the timing of induction and recovery using such a broad range of representative freshwater fishes. The retrospective

regression tree analysis of the effectiveness, dose, and temperature experiments and the subsequent factorial experiment indicate that, independent of taxon, the response of fish to chemical sedatives is primarily a function of sedative dose, water temperature, and, to a lesser extent, fish size and dissolved oxygen. Although the regression trees for the MS-222 datasets deviate somewhat from these generalizations, we believe this was the result of far fewer dose-temperature combinations being tested for MS-222 (i.e., most taxa were tested at a single MS-222 dose and temperature). As such, the narrow range of doses and temperatures tested limited the heterogeneity of variance across these factors, obscuring the full influence of sedative dose and water temperature on times to induction and recovery of fish sedated with MS-222. Thus, in light of this caveat and the broad similarities observed in the other datasets, it seems reasonable to conclude that the timing of sedation and recovery is influenced by sedative dose, water temperature, and fish size for fish sedated with any of the products tested and perhaps other chemical sedatives. These generalizations are also supported by the results of others who have assessed these variables, albeit in narrower contexts.

Regarding the effects of sedative dose, Afkhami et al. (2013) reported that increasing doses of 2-phenoxyethanol and clove powder (a crude source of eugenol, isoeugenol, and other compounds with sedative properties in fish) resulted in shorter induction times, but longer recovery times in Sobaity Sea Bream *Sparidentex hasta*. Bauquier et al. (2013) reported similar results for Goldfish *Carassius auratus* sedated with different concentrations of alfaxalone, as did Javahery et al. (2012b) for Caspian Rutilus *Rutilus frisii kutum* sedated with clove oil (another crude clove derivative), Shaluei et al. (2012) for Great Sturgeon *Huso huso* sedated with 2-phenoxyethanol, Small (2003) for CCF sedated with metomidate, and Mattson and Riple (1989) for Atlantic Cod *Gadus morhua* sedated with benzocaine. Akbulut et al. (2012) reported shorter inductions for Siberian Sturgeon *Acipenser baerii* when using higher doses of clove oil or benzocaine, and Iversen et al. (2003) reported the same relation between dose and induction time in Atlantic Salmon *Salmo salar* smolts sedated with metomidate, clove oil, benzocaine, or isoeugenol. Christiansen et al. (2013) used MS-222, benzocaine, eugenol, and metomidate to sedate Pacific Lamprey *Entosphenus tridentatus* and reported shorter

induction times and longer recovery times at higher doses, regardless of the sedative used; Öğretmen and Gökçek (2013) reported the same results for African Catfish *Clarias gariepinus* sedated with eugenol, clove oil, or 2-phenoxyethanol. The same general induction and recovery trends were reported for Silver Catfish *Rhamdia quelen* sedated with MS-222, propofol (Gressler et al. 2012), or *Ocimum gratissimum* oil (a crude source of eugenol and other compounds with sedative properties in fish) (de Lima Silva et al. 2012), Acumara *Algansea lacustris* sedated with xylocaine (Rivera Lopez et al. 1991), and White Sea Bream *Diplodus sargus* and Sharp Snout Sea Bream *D. puntazzo* sedated with 2-phenoxyethanol (Tsantilas et al. 2006). Induction was also more rapid among Freshwater Angelfish *Pterophyllum scalare* sedated with higher doses of clove oil; however, recovery times were less consistent in terms of dose effects, with intermediate concentrations yielding the slowest recoveries (Hekimoğlu and Ergun 2012).

Regarding the effects of temperature, Zahl et al. (2009) summarized the results of many authors who reported decreased induction and recovery times at increased water temperatures, including studies of Striped Bass, European Sea Bass *Dicentrarchus labrax*, Gilthead Sea Bream *Sparus aurata*, RBT, BNT, Atlantic Salmon, Whitefish *Coregonus lavaretus*, European Perch *Perca fluviatilis*, Roach *Rutilus rutilus*, and Atlantic Cod sedated with benzocaine, 2-phenoxyethanol, clove oil, isoeugenol, MS-222, or metomidate. Zahl et al. (2009) also reported shorter inductions occurring at warmer temperatures in CMC, FHM, and Atlantic halibut *Hippoglossus hippoglossus* sedated with MS-222 or benzocaine. In a factorial experiment similar to our own with Steelhead *Oncorhynchus mykiss* fry, Woolsey et al. (2004) reported that induction time decreased significantly with increasing temperature and clove oil dose, and that recovery times decreased significantly with increasing temperature. These authors reported a significant interaction effect, indicating that water temperature influenced induction times at the lower sedative doses; however, there was no significant interaction between sedative dose and water temperature observed for recovery. They also reported that mortality at 24 h postsedation was significantly higher among fish sedated with higher doses and at higher temperatures. In another temperature-dose factorial experiment, Küçük (2010) reported that higher doses of MS-222

and warmer water temperatures also yielded more rapid induction times for Sailfin Silver Mollies *Poecilia latipinna*. Curiously, neither temperature nor dose influenced recovery times in this experiment.

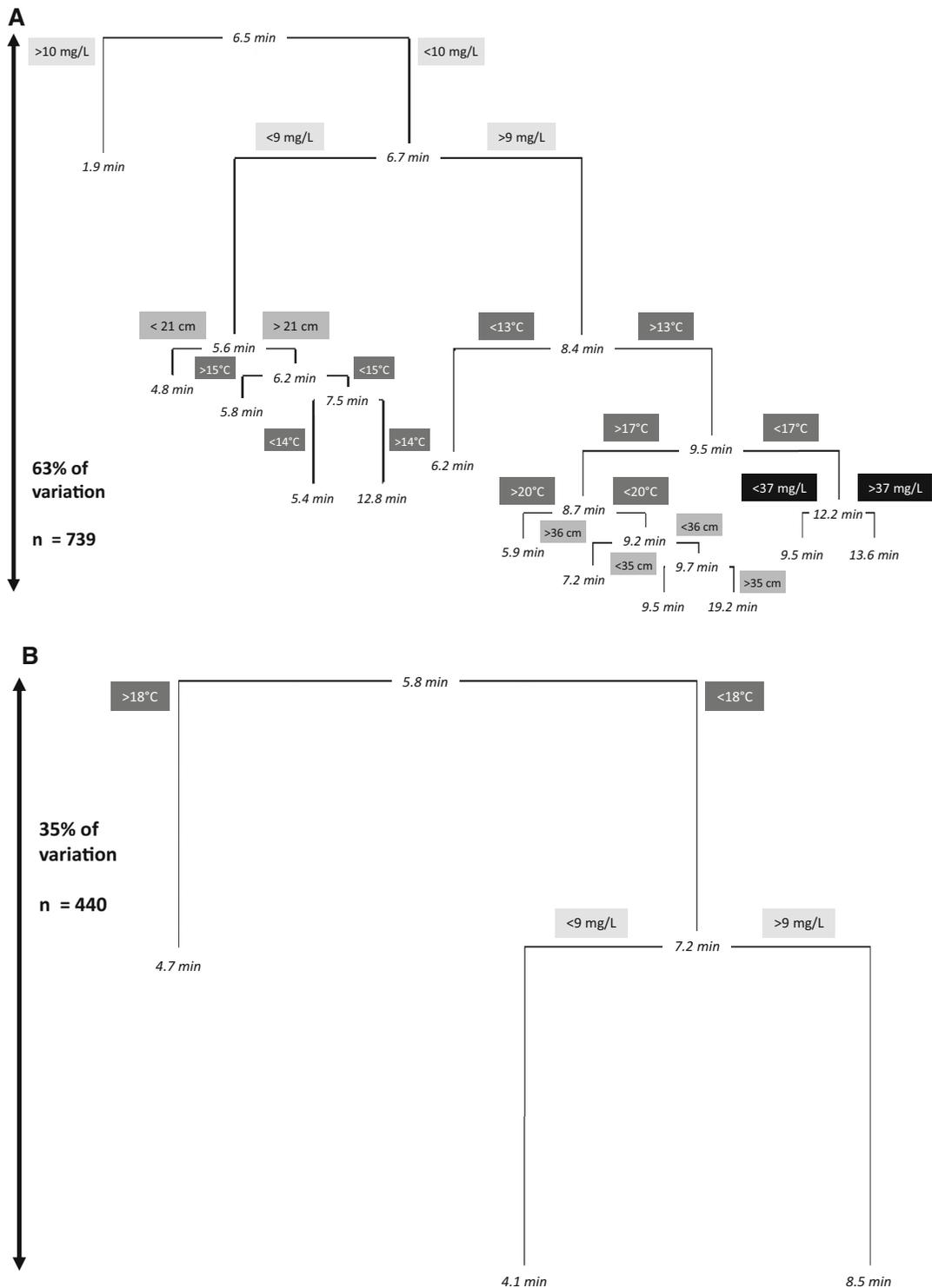
Regarding the effects of size, in an experiment evaluating 2-phenoxyethanol as a sedative for juvenile Tench *Tinca tinca*, Myszkowski et al. (2003) reported clear, positive relation between fish size and induction and recovery times, though the magnitude of the effect was greater for induction. These authors argued that fish age and developmental state (i.e., scale development), rather than absolute size, might also be a contributing factor. Small (2003) reported the same effect of fish size on induction times for CCF sedated with metomidate, but also reported significantly shorter recoveries for larger fish. In assessing MS-222, 2-phenoxyethanol, benzocaine, and metomidate-treated Atlantic Cod, Zahl et al. (2009) noted that induction and recovery times were generally longer for larger fish, but that these effects were somewhat inconsistent among the sedatives and water temperatures tested. Tsantilas et al. (2006) also reported that larger White Sea Bream treated with 2-phenoxyethanol exhibited longer induction times, but the relation between size and recovery was highly variable and dependent on sedative dose (Tsantilas et al. 2006). These authors also investigated the same relations in Sharp Snout Sea Bream, and found the relations between fish size and induction and recovery times to vary depending on 2-phenoxyethanol dose. Similarly, Gressler et al. (2012) found no consistent relationship between fish size and induction or recovery times in Silver Catfish sedated with MS-222 or propofol. Rivera Lopez et al. (1991) assessed xylocaine as a sedative for different size classes of Acumara *Algansea lacustris*, and although there was no clear influence of fish size on induction, recovery appeared to be slower among larger fish, particularly larger fish sedated with higher doses of xylocaine. Although these authors also reportedly repeated their experiment at different water temperatures, temperature-specific data were not shown or discussed.

In spite of the deviations and inconsistencies noted above, the trends relating sedative dose, water temperature, and fish size with the process and pattern of sedation we observed are considered credible. In their review of clove oil as a fish sedative, Javahery et al. (2012a) discussed the effects of dose, water temperature, and fish size on effectiveness and induction

times, generally supporting the relations we have described. Furthermore, these authors suggested that both induction and recovery times are inversely related to body size and also cautioned readers to consider other aspects of water chemistry that may affect fish physiological status and, in turn, sedative effectiveness. In a more comprehensive review addressing various fish sedatives, Zahl et al. (2009) reached the same conclusions about the effects of dose and water temperature on induction and recovery times, but were more circumspect in their assessment of the effects of body size, noting that both positive and negative relations have been reported and, in some cases, there appears to be no connection between fish size and the timing of induction and recovery. Other morphological or physiological attributes—such as metabolic rate, oxygen demand, gill perfusion, gill surface area-body mass ratio, and vascular dynamics; fish adiposity and sedative affinity for lipids; and modes and rates of sedative uptake and excretion—are known to influence the speed at which sedatives are absorbed, dispersed throughout the body, and ultimately cleared (Zahl et al. 2009). The positive correlation we observed between estimated respiration rates and probabilities of induction and recovery within ideal time periods suggests that factors affecting respiration (e.g., temperature and body size) are likely to influence uptake and elimination of sedatives and thus the process and pattern of induction and recovery from chemical sedation. This is also supported by evidence of dissolved oxygen availability influencing the odds of recovery within 5 min, suggesting respiration and ventilation rates may be a limiting factors for sedative clearance. That many of these morpho- and physiological attributes vary among fish of different sizes, but not always in a concerted fashion, may offer some insight as to why fish size is a less reliable predictor of induction and recovery times than sedative dose or water temperature.

Considerations regarding the drug approval process and its efficiency

Collectively, the available data suggest sedative dose, water temperature, and fish size are much more important factors than taxon in determining sedative effectiveness. Our results support the feasibility and relevance of synthesizing information from multiple datasets and experiments to support aquatic animal



**Fig. 2** Regression trees describing effects of dissolved oxygen (light grey), fish size (medium gray), water temperature (dark grey), and sedative dose (black) on recovery times for fish

sedated with eugenol (a), benzocaine (b), or MS-222 (c). Regressions trees were constructed using data described in Electronic Supplementary Table 1

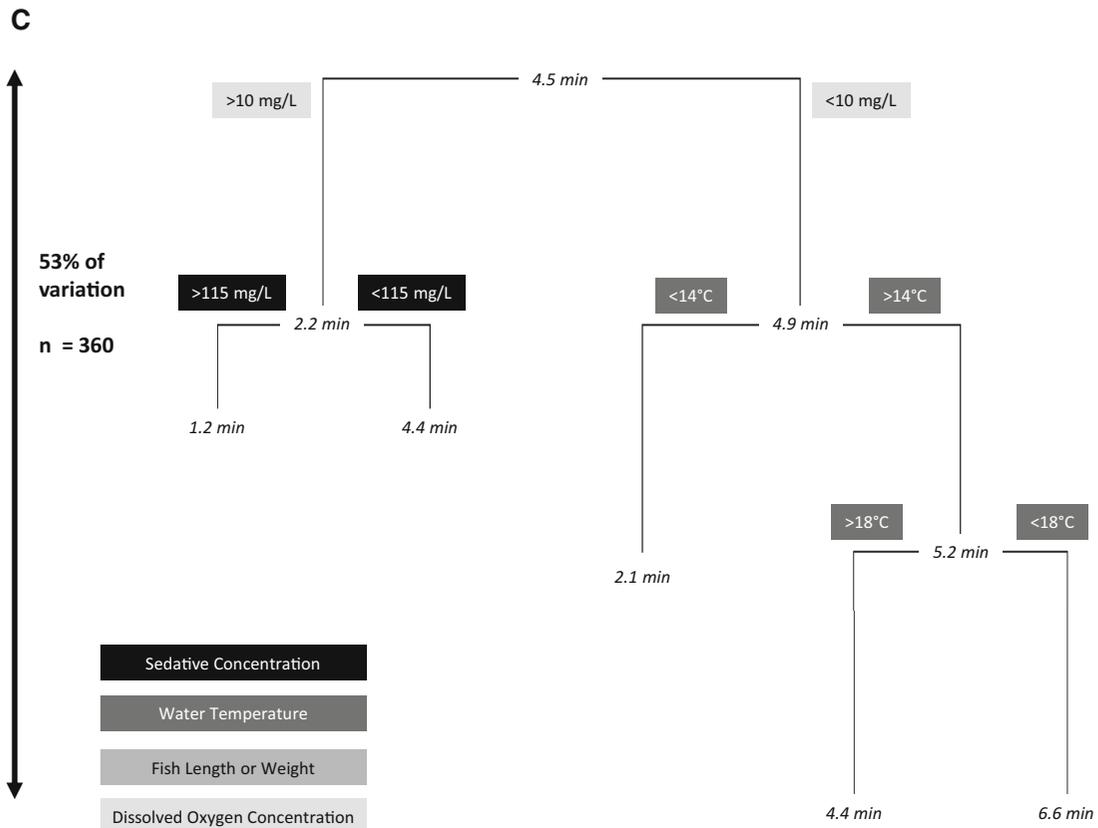
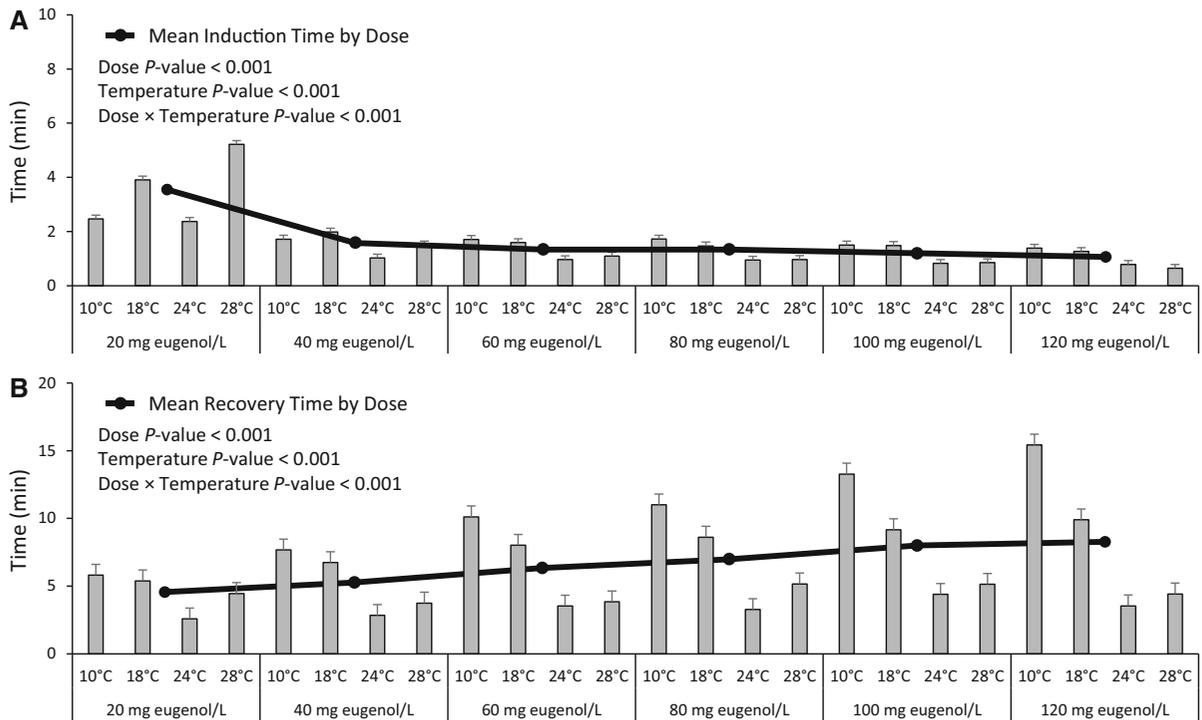


Fig. 2 continued

drug approvals, and the use of sedative dose, water temperature, and fish size as predictive criteria to extrapolate from tested to untested fishes. In this sense, effectiveness data from a single taxon held at multiple temperatures or exposed to multiple doses might have been equally or perhaps more relevant than data from multiple species tested at one dose or temperature. Yet such an approach, focused on attributes other than taxonomic groupings, is not generally considered a likely means for completing data requirements to support aquatic animal drug approvals, and alternative approaches to satisfying data requirements have not been widely embraced. The data presented here (excluding the sedative dose/water temperature factorial) were generated primarily in response to guidance from FDA regarding the number and type of experiments that were considered likely necessary to support all freshwater fish approvals for AQUI-S20E or Benzoak as immediate-release sedatives. Despite published guidance documentation that suggests experiments with two representative fish species is

generally considered adequate for an all freshwater fish drug claim (FDA CVM 2008), in practice, the rule of thumb for generating data in support of an all freshwater fish claim is 'two, two, and two': two representative coldwater taxa, two representative coolwater taxa, and two representative warmwater taxa. In light of these conventions and practices, we generated a wealth of data using numerous representative taxa in order to satisfy the implied needs of the regulatory agency. However, much of the existing literature on the subject already supported these notions, albeit in a less quantitatively rigorous fashion. Demonstrating sedative effectiveness in numerous taxa and investigating the interrelated effects of sedative dose, water temperature, and fish size has increased our knowledge of fish sedation, but this approach seems redundant in terms of providing regulators with the information needed to make determinations of drug effectiveness.

Our study indicates that experiments focused on sedative dose, water temperature, and fish size, not



**Fig. 3** Induction (a) and recovery (b) times of hybrid Striped Bass (HSB) sedated with different concentrations of eugenol at different water temperatures. Columns represent mean times for each dose/temperature combination; error bars represent pooled

standard error. The heavy black lines and data points represents mean times for sedative doses pooled across water temperatures.  $P$  values generated by two-way ANOVA are provided for each dataset

taxonomic diversity, are likely to provide equivalent or, in some cases, greater information for predicting broad effectiveness of drugs such as sedatives. Given the time, effort, and resources associated with these experiments and the critical need for sedatives in the fisheries disciplines (Trushenski et al. 2012a, b, c, d), we encourage decision-makers to consider the benefits of a pragmatic, regulatory science approach in lieu of the current paradigm.

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