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The use of tricaine methanesulfonate, clove oil, metomidate, and 2-phenoxyethanol for anesthesia induction in alewives (Alosa pseudoharengus)

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THE USE OF TRICAINE METHANESULFONATE, CLOVE OIL, METOMIDATE, AND 2-PHENOXYETHANOL FOR ANESTHESIA INDUCTION IN ALEWIVES *(ALOSA PSEUDOHARENGUS)*

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THESIS

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Anesthetics are widely used in routine aquaculture operations to immobilize animals for tagging, spawning, handling, and vaccination. A number of anesthetics are currently available for finfish, but their efficacy and optimal dosage is highly species-specific. The efficacy of the anesthetic agents (tricaine methanesulfonate (MS-222), clove oil, metomidate, and 2-phenoxyethanol (2-PE)) was studied in adult, juvenile (133.3 ± 1.5 mm, 27.5 ± 8.9 g), and larval Alewives (*Alosa pseudoharengus* Wilson). In an initial trial, wild-caught adults were anesthetized with doses of 87.5-112.5 mg/L MS-222, 25-40 mg/L clove oil 0.5-5.0 mg/L metomidate and 0.125-0.550 mg/L 2-PE. Optimal doses for anesthesia were similar for larvae and juveniles, and were identified as: 75-100 mg/L MS-222, 40 mg/L clove oil, 5-7 mg/L metomidate, and 500 mg/L 2-PE. All juvenile fish survived 48 hours post-exposure to each optimal dose. In a longer-term (24 hour) sedation experiment, juvenile alewives were netted

and exposed to low clove oil (2.5 and 5.0 mg/L) and metomidate (0.25 and 0.50 mg/L) doses, and plasma cortisol was measured. Fish exposed to the clove oil treatments exhibited a cortisol stress response that was prolonged in the higher dose treatment. No cortisol stress response was observed in the metomidate treatments. Overall, optimal acute anesthesia doses for alewives were similar to those reported for other species, and metomidate may be useful for longer-term sedation.

CHAPTER 1

INTRODUCTION

Alewife Life History

Alewives, *Alosa pseudoharengus* and blueback herring, *Alosa aestivalis*, are anadromous clupeid fishes found along the Atlantic coast of North America that are collectively referred to as river herring in the US and "gaspereau" in Canada (Monroe 2002). Their natural range is from North Carolina to Newfoundland (Scott and Crossman 1988). They spend the majority of their lives at sea but will return to their natal streams and utilize their olfactory sense to guide them to their natal rivers. They are reproductively considered iteroparous, characterized by multiple reproductive cycles over the course of its lifetime. A species is considered semelparous if it has a single reproductive episode before death. Spawning begins between May and June, and adult female Alewives (4-5 years old) will spawn between 60,000 and 100,000 eggs in freshwater lakes, ponds and streams. They grow to be 10-11" (25.4-27.9 cm) and ½ pound (0.22 kg) but can be as large as 14" (35.6 cm) and a pound (0.45 kg) or more.

General Species Importance

Alewives are ecologically important because, as juveniles in riverine environment, they serve as prey for several fish species, including large and small mouth bass *(Micropterus salmoides* and *Micropterus dolomieu),* yellow perch *(Perca flavescens)*, brown trout *(Salmo trutta)* and other salmonids. Upon entering the estuarine environment, they are preyed upon by striped bass *(Morone saxatilis),* cod *(Gadus morhua)* as well as numerous birds and mammals, and predation increases as they enter the marine environment (Loesch 1987;, Munroe 2002;

Walter et al. 2003). They are an essential keystone species in the freshwater, marine and terrestrial food webs.

Endangered Species

Historically, river herring were one of the most valuable anadromous fishes harvested commercially in New England, and were sold as food, commercial bait, and fertilizer (Belding 1921; Messieh 1977; Loesch 1987). River herring populations have been declining in the mid-Atlantic and Northeast coastal regions for several decades, particularly since the late 1990s. Alewives were highly abundant, but as is the case with Atlantic salmon *(Salmo salar)* and many other anadromous fishes, their numbers have declined because of anthropogenic causes, including dam construction and overfishing (Savoy and Crecco 1995; Atlantic States Marine Fisheries Commission 1999). According to a technical report published by the Massachusetts Division of Marine Fisheries (2011) that assessed river herring stocks, commercial landings increased from 5 million pounds in the early 1950's, to 33 million pounds in 1958. By the early 1980's, however, river herring landing were only a small percentage of the historical highs and regulations in 1989 limited harvest to 25 fish per day. This coincided with restoration programs in the Atlantic states to manage striped bass, an important recreational and commercial fishery.

The cooperative interstate fishery management of striped bass began in 1981, with the development of a fishery management plan by the Atlantic States Marine Fisheries Commission, an organization of Atlantic coastal states. Effective fishery management and additional research and monitoring contributed to a tenfold increase in abundance of striped bass stocks by the late 1990s. This dramatic increase resulted in increased predation on a variety of anadromous fish species, including American shad, blueback herring, and Alewives. (Grout 2006). The decline in

river herring is symptomatic of environmental problems that are impacting other fish, wildlife and whole ecosystems. Reasons for their decline include: barriers to fish passage, water withdrawals and diversion, loss of habitat, water pollution, poaching and predation. Fish stocks have declined along the east coast from Maryland's Chesapeake Bay (Fisheries Service 1997) to Massachusetts and Maine (Nelson et al. 2011).

Due to the population decline, several states have placed moratoria on taking or catching any river herring (Closed in RI, MA, CT, NC), but a limited harvest is still open in some states, including NH and ME. These states have met sustainable population and spawning criteria numbers and habitats to keep regulated fishing open. Current restoration efforts in New England are attempting to reverse this trend by removing dams and constructing fish ladders, to allow them to regain access to historical spawning grounds in streams and lakes.

In 2011, a petition to river herring listing them as endangered was submitted to the National Marine Fisheries Service. In August of 2013 NOAA released an update regarding river herring, stating that: "neither species is threatened or endangered so listing under the Federal Endangered Species Act is not warranted at this time. However, we still have concerns about the status and threats to these species. As a result, both species are still included on our species of concern list" (NEFSC 2013). One tool that is available for stock enhancement of these depleted fish populations is the release of hatchery-reared larvae and juveniles.

Species Culture

The earliest documentation of the culture of Alewives in laboratory settings date back to the 1950's and 1960's and were largely unsuccessful (Mansueti 1956; Norden 1967, 1968). Early work with alewife larvae estimated an optimum egg hatching temperature for egg hatching

was to be at 18^{o}C (Edsall 1970). The hatching early growth and feeding of Alewives to day 50 post-hatch was documented in the early 1980's (Heinrich 1981).

Detailed hatchery procedures were not available until a recent study documented procedures for spawning, larviculture and established environmental conditions and feeding regimes conducive for juvenile growth (DiMaggio et al. 2015). A very significant aspect of culture is the ability to safely handle and transport the fish. Transporting fish to their release sites can be stressful, and the addition of salts and buffers and low doses of anesthetics have proven useful in reducing stress and improving survival (Tomasso et al. 1980; Mazik et al. 1991; Harrell et al. 1992, David and Griffin 2004). In order to minimize stress and disease, a proper and effective anesthetic agent and dose must be determined.

Anesthetic Use and Function

Anesthetics are widely used in routine aquaculture operations to immobilize animals for transport, spawning, vaccination and handling. A fish anesthetic must meet several important criteria, such as short induction/recovery time, non-toxicity to fish and humans, no lasting physiological effects, rapid clearance from the body, high solubility in fresh and salt water, stability under normal hatchery conditions, readily available and cost effective (Schoettger and Julin 1967). Lethal overdoses of an anesthetic may also be used as an ethical way to euthanize fish (Stoskopf 1992). All of the previously mentioned hatchery procedures elicit a defense mechanism in the form of a stress response. All vertebrate organisms attempt to adapt to these stresses following the "General Adaptation Syndrome" or GAS (Selye 1950). This adaptation consists of three stages: an alarm reaction, stage of resistance and a stage of exhaustion. Following an initial stressor such as netting, handling, or vaccination, the physiological

responses may disappear during the stage of resistance, or become exacerbated if the stressor continues (Selye 1950).

Although common physiological responses are universal among vertebrate organisms, the fact that fish are poikilothermic and live in an aqueous environment renders them particularly sensitive to environmental changes and that elicit stress responses. When there is a disturbance outside the normal (or tolerable) range for that species of fish (e.g. a stessor) a series of neuroendocrine responses are initiated and stress hormones are released (catecholamines and cortisol). This primary response initially activates the sympathetic nervous system and causes the release of catecholamines (adrenalin and noradrenalin) from the chromaffin tissue (located in the head (anterior) kidney and adrenergic neurons distributed throughout the body. The release of catecholamines from these neurons affect many organ systems, including the cardiovascular and respiratory systems, that gives rise to the "fight or flight" response (Randall and Perry 1992). A stress response also stimulates the hypothalamic-pituitary-interrenal axis (HPI axis), that has slower, but longer-acting physiological consequences (Jobling 1995). Activation of the HPI axis causes corticotropin releasing hormone, a neuropeptide released from the hypothalamus, to stimulate production and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH travels systematically to the head kidney and stimulates the production and release of corticosteroid hormones, specifically cortisol.

The release of catecholamines and cortisol triggers a broad range of biochemical and physiological changes that alter a number of metabolic pathways, resulting in profound changes to blood chemistry. One common indictor of secondary (metabolic) response to stressors in fishes is an elevated plasma glucose concentration due to cortisol-stimulated glycogen catabolism (glycogenolysis). Increases in plasma glucose concentrations are thought to assist the

animal by providing energy substrates to tissues such as the brain, gills and muscles to cope with the increased energy demand. Although adrenaline is cleared rapidly from circulation after stress, plasma cortisol and glucose remain elevated for longer periods of time post-stress (Vijayan et al. 1996; 1997). These effects are of particular importance in the period of recovery from stress, when the fish attempts to maintain oxygen supplies to tissues and regain osmotic and ionic equilibrium.

Recovery to pre-stress concentrations occurs quickly after removal of a brief or mild stressor. Acute (short term) responses to stressors such as general hatchery handling and other anthropogenic activities may be beneficial to the fish and extend their normal adaptive ability (Davis 2006).

In aquaculture, chronic stress, such as that created by poor water quality, predisposes fish to a number of diseases resulting from pathogens (viruses, bacteria, fungi and parasites; Walters and Plumb 1980; Collins et al. 1976). In addition to disease and mortality, stress in aquaculture situations can reduce growth, thereby reducing profitability or prevent successful reproduction of broodstock fish (Noga 2000). Chronic elevation of circulating cortisol is speculated to decrease growth by increasing gluconeogenesis (Davis et al. 1985; Barton et al. 1987; Pickering 1990) and increase disease susceptibility by suppressing immune function (Maule et al. 1989).

Use of Anesthetics in Fish Culture

To minimize many stressors associated with aquaculture, it is common practice to use some form of anesthesia. By definition, anesthesia is a biologically reversible state, induced by a chemical or non-chemical external agent, which results in the partial or complete loss of sensation or neuromotor control (Summerfield and Smith 1990). Various anesthetic agents have been used with fish since the 1950's (McFarland 1959; McFarland and Klontz 1969; Schoettger

and Julin 1967) and were developed from human and terrestrial veterinary medicine practices. Anesthesia (from Greek: an- 'without' and aesthesis 'sensation') has several levels, but for fish culture the primary use is for sedation. Sedation is a reduction in sensitivity, which results in tranquility and calmness, two desirable characteristics for handling fish. Anesthetic agents are now widely used in fish culture, ranging from a light sedation during handling or non-invasive procedures to full anesthesia, to avoid inflicting pain during surgery (Ackerman et al. 2005; Neiffer and Stamper 2009; Ross and Ross 2008; Summerfelt and Smith 1990). A generally accepted anesthetic is one that produces a total loss of muscular tone within three minutes and a recovery time within ten minutes (Maricchiolo and Genovese 2011). Table 1 illustrates the various stages of anaesthesia and their classification according to Zahl et al. (2012). A broad range of anaesthestics are currently available for fish and among the most common are MS-222, benzocaine, isoeugenol, metomidate, 2-phenoxyethanal, quinaldine and clove oil (Ackerman et al. 2005; Neiffer and Stamper 2009; Ross and Ross 2008; Summerfelt and Smith 1990). The effective dosages of the various anesthesia agents are species-specific and are not well known for many of the more than 30,000 fish species (Froese and Pauly 2011). Additionally, effective doses will vary by life stage and environmental conditions (salinity, temperature, pH; Zahl et al. 2012). A compilation of many anesthetics used in aquaculture and their effective doses are provided in Table 2.

Fish size (weight) is one variable that influences anesthesia dose and induction time, but results have been variable among species. For instance, in Atlantic cod *(Gadus morhua)*, Zahl et al. (2009) found greater doses of MS-222 and Benzocaine were required for larger fish and this may be due to the relatively smaller gill surface present as fish increase in size (Oikawa and Itazawa, 1985). Similar results were also found for northern pike *(Esox lucius)* and striped bass

(Morone saxatilis) (Dawson and Gilderhus 1979; Gilderhus et al. 1991). In other species, however, such as Atlantic salmon (Olsen et al. 1995), rainbow trout *(Oncorhynchus mykiss;* Gilderhus and Marking 1987) and brook trout *(Salvelinus fontilalis;* Houston et al. 1976), increased efficacy and sensitivity correlated with larger body size.

In contrast to size or weight, the effects of temperature are more consistent, with shorter induction times at higher water temperatures. The rapid induction and recovery times associated with higher water temperature are likely due to the increases oxygen demand due to increases in basal metabolic rates. This leads to increased respiration and blood flow and accelerated absorption and/or elimination of the anesthetics (Zahl et al. 2009). Recent investigations have focused on using combinations of anesthetics to reduce doses (costs) and improve induction and recovery efficacy (Zahl et al. 2012).

Mechanism of Action

Numerous anesthetics are commonly used in research including, but not limited to: Tricaine methanesulphonate (TMS, MS-222), benzozaine (p-aminobenzoic acid ethyl ester), lidocaine {2-(diethylamino)-N-(2,6-dimethylphenyl) acetimide}, metomidate and etomidate {(1-phenylethyl-1H-imidazole-5-carboxylic acid methyl ester)}, 2-phenoxyethanol (2-PE 1-hydroxy-2-phenoxyethane), clove oil (*Eugenia* sp.) and its active ingredients, quinaldine sulfate (2-methylquinoline sulfate) (Ackerman et al. 2005). For the purpose of this thesis only, those chemicals that were tested (MS-222, 2-PE, clove oil and metomidate) will be discussed in detail. Appendix Table 3 shows a comparison of anesthetics and their water solubility.

Globally, very few compounds (tricaine methanesulfonate, benzocaine, chlorobutanol and isoeugenol) have gained approval as fish anesthetics and most have a withdrawal period restricting consumption or release of treated animals (Bowker et al 2015). In the US, MS-222

remains the only legally registered anesthetic and has been used extensively for a variety of purposes, including immobilization, suppression of sensory systems during invasive procedures, and euthanasia (Anderson et al. 1997; Woody et al. 2002; King et al. 2005; Carter et al. 2011). MS-222 is one of the most widely used anesthetic drugs in fisheries science worldwide, since its introduction as a local analgesic agent in humans in 1967 (Topic Popovic et al. 2012). The use of MS-222 in food fish is somewhat limited, however, as extended withdrawal periods are required in many countries, post-induction, before human consumption or release into the wild (reviewed by Carter et al. 2011; Topic Popovic et al. 2012). MS-222 has also been used with many fish species during laboratory and hatchery procedures. It is highly water-soluble (1.25 g/mL), exhibits rapid uptake via gills, and inhibits the initiation and propagation of action potentials in excitable cells. Therefore, it blocks most neurons and muscle cells and may cause paralysis in addition to blocking nociception (Zahl et al. 2012). The use of MS-222, however, is also associated with numerous side effects during prolonged exposure, including hypoxia, elevated catecholamine and cortisol levels, and possible effects on P450 enzyme activity in the liver (King et al. 2005; Carter et al. 2011; Topic Popovic et al. 2012). MS-222, therefore, is not widely used as a long term sedative, but rather for acute anesthesia induction.

Clove oil, a distillate of herbaceous portions of the clove tree *Eugenia aromatic* (Webster) has been used for many years as a food additive and a topical analgesic in dentistry. It is recognized as a GRAS (Generally Recognized As Safe) substance by the US Food and Drug Administration (FDA) for use in humans, but has not been approved for use with fish in North America. Clove oil or its active ingredient eugenol has been shown to be as effective as MS-222 for anesthesia in rainbow trout (Anderson et al. 1997). One mechanism for its analgesic effect is the inhibition of prostatglandin H synthase by eugenol (Keene et al. 1998).

Another active ingredient in clove oil, isoeugenol, has been commercialized as Aqui-S® (50% isoeugenol) and can be used in New Zealand and some other countries without a withdrawal period. Recently, the FDA granted amended authorization (Investigational New Animal Drug (INAD) 11-741) for the use of AQUI-S® for the immediate release of freshwater finfish sedated as part of field-based fisheries management activities. (USFWS-AADAP). The immediate-release provision is for field-use only, and a withdrawal period of 72 hours is still required for hatchery use (U.S. Food and Drug Administration 2015).

Metomidate is a methyl analogue of the imidazole derivate etomidate, which activates and modulates inhibitory gamma-aminobutyric acid type A ($GABA_A$) receptors, and thereby affects higher regions of the nervous system (Ashton and Wauquier 1985; Yang and Uchida 1996). Metomidate is classified as a non-barbiturate hypnotic that was developed from the mammalian analogue etomidate in the 1960s (Gooding and Corssen 1976; Preziosi and Vacca 1982: Fraser et al. 1984). In fishes, one of the effects of metomidate, in addition to respiratory and cardiovascular depression, is inhibition of the mitochondrial cytochrome P450 (CYP) dependent interenal enzyme 11-β-hydroxylase that catalyzes the production of cortisol from deoxycortisol (Wagner et al. 1984; Vanden Bossche et al., 1984). By preventing cortisol synthesis, metomidate mitigates stress responses and has been shown to be superior to other anesthetics in many fish species (Mattson and Riple 1989). It may also be effective in low doses for long-term fish transport.

The final anesthetic chosen for these studies was 2-phenoxyethanol (2-PE), which is another commonly used anesthetic agent in fish. 2-PE likely acts to decrease neuron excitability in the central nervous system by inhibiting the activity of excitatory N-methyl-D-aspartate (NMDA) receptors (Musshoff et al. 1999). Common 2-PE doses for acute anesthesia range from

200 to 600 mg/L, and results in rapid induction and recovery times (Mattson and Riple 1989; King et al. 2005). While 2-PE has been widely used in closed recirculating aquaculture systems (Velisek et al. 2007), caution should be used at high doses and long exposure times, because of the narrow margin between inductive and lethal doses (Ackerman et al. 2005).

Research Objectives

The purpose of this research was to establish effective anesthetic doses for use with Alewives. These efforts may improve stock enhancement by mitigating stress during handling and transport. In addition to culturing alewives for stock enhancement, they also offer great opportunity as a baitfish to support the lucrative marine recreational fishing industry. Baitfish production in the U.S. in 2005 was valued at over $38 million (USDA-NASS 2005). Current production (2013) numbers have declined to $29 million with Arkansas accounting for 62% of all U.S. production (Census of Aquaculture 2012). To date, the production of marine baitfish for the enormous recreational fishery in the northeast, including the striped bass *(Morone saxatilis)* fishery have remained untapped. The striped bass is one of the most sought species by recreational anglers, along with the blue fin tuna *(Thunnus thynnus)* that has gained popularity in recent years, as commercial harvests have been limited. Aquaculture production in the U.S. has grown from a $1.1 billion dollar a year industry in 2005 to almost $1.4 billion in 2013, a 21% increase (Census of Aquaculture 2012). Advancements in fish husbandry, such as those presented in this research, will enable this industry to continue to grow.

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Table 1. Stages of anaesthesia in fish.

Stage	***Plane***	***Description***	***Appearance***	***Swimming activity***	***Equilibrium***	***Response***	***Respiration***
0		Normal	Normal	Normal	Normal	Normal	Normal
I		Light sedation	Disoriented	Reduced	Normal	Slightly reduced	Normal
II		Excitatory Stage	Excited	Increased	Struggles to maintain balance	Normal or exaggerated	Irregular or increased
III	1	Light anaesthesia	Anaesthetised	Stopped	Lost	Reacts to strong tactile stimuli	Normal or decreased
	2	Surgical anaesthesia	Anaesthetised	Stopped	Lost	None	Shallow
	3	Deep narcosis	Anaesthetised	Stopped	Lost	None	Nearly absent
IV		Impending death	Moribund	Stopped	Lost	None	Stopped

Table 2. Fish species and anesthetic doses.

Fish Species	Anesthetic	Recorded Dosage	Reference
American Shad *(Alosa Sapidissima)*	Metomidate	0.5 mg/L	Ross et al. (1995)
Atlantic Cod *(Gadus morhua)*	Metomidate Benzocaine MS-222 Aqui-S	5 mg/L 40 mg/L 75 mg/L 17/mg/L	Mattson & Riple (1989) Ross & Ross (1984) Digre et al. (2011)
Atlantic Halibut *(Hippoglossus hippoglossus)*	Metomidate MS-222	10 mg/L 250-480 mg/L	Malmstrom et al. (1993)
Atlantic Salmon *(Salmo salar)*	Benzocaine MS-222 Metomidate Aqui-S	25-45 ppm 50-100 ppm 5 ppm 5 mg/L	Yesaki (1988) Stickney (2000) Iversen and Eliassen (2009)
Black Sea Bass *(Centropristis Striata)*	MS-222 2-Phenoxyethanol Clove Oil Metomidate	125 mg/L 100-300 mg/L 30mg/L 5.0 mg/L	King et al. (2005)
Black Spot Seabream *(Pagelus bogaraveo)*	MS-222 Clove Oil	100 mg/L 40mg/L	Maricchiolo & Genovese (2011)
Blueback Herring *(Alosa aestivalis)*	MS-222	20-25 mg/L	
Bluegill *(Lepomis macrochirus)*	MS-222 Quinaldine sulfate Aqui-S	260-330 mg/L 10-30 mg/L 20-30 mg/L	Tucker (1998) Schoettger & Julin (1968) Stehly & Gingerich (1998)
Channel Catfish *(Ictalurus punctatus)*	MS-222 Quinaldine sulfate Aqui-S	140-270 mg/L 30-70 mg/L 40 mg/L	Tucker (1998) Schoettger & Julin (1968) Small and Chatakondi (2005)
European Flounder *(Platichthys flesus)*	Clove Oil Aqui-S	753 mg/L 300 ppm	Akbulut et al. (2012) Norambuena et al. (2012)
European Sea Bass (Dicentrarchus labrax)	Clove Oil Aqui-S	40 mg/L 300 ppm	Mylonas et al. (2005) Norambuena et al. (2012)
Greater Amberjack *(Seriola dumerilii)*	MS-222 Clove Oil	100 mg/L 40 mg/L	Maricchiolo & Genovese (2011)
Hybrid Striped Bass *(Morone chrysops x Morone saxatilis)*	MS-222 Clove Oil Metomidate Aqui-S	25 mg/L 8.0 ul/L 1.5 mg/L 3.6 mg/L	Davis & Griffin (2004)
Marbled Spinefoot Rabbitfish *(Siganus rivulatus)*	MS-222 Clove Oil Benzocaine 2-Phenoxyethanol Aqui-S	100-125 mg/L 70 mg/L 60-70 mg/L 400 uL/L 100mg/L	Ghanawi et al. (2013) Soto & Burhanuddin (1995)

Table 2., Cont. Fish species and anesthetic doses.

Fish Species	Anesthetic	Recorded Dosage	Reference
Rainbow Trout *(Oncorhynchus mykiss)*	Metomidate Clove Oil Clove Oil 2 - phenoxyethanol Aqui-S	5 mg/L 25 mg/L 40-50 mg/L 200-500 ml/L 40 mg/L	Gilderhus & Marking (1987) Endo et al. (1972) Anderson et al. (1997) Keene et al (1998) Barton & Helfrich (1981) Bell & Blackburn (1984) Sehdev et al. (1963) Yesaki (1988) Anderseon et al. (1997) Keene et al. (1998)
Striped Bass *(Morone saxatilis)*	Quinaldine sulfate Aqui-S	25-55 mg/L 35 mg/L	Lemm (1993) Woods et al (2008)
Tilapia *(Oreochromis niloticus)*	MS-222 Benzocaine	80-100 mg/L 50-100 mg/L	Ferriera et al. (1979) Ross & Ross (1984)

* Dosage based on a general anesthetic for normal handling. Size of fish and water quality parameters (temperature, salinity and oxygen) will effect dosage.

Table 3. Water solubility of anaesthetics.

Anaesthetic	**Log P**	**pKa**	**Water Solubility (mg/L)**
MS-222	1.8	3.8	1.0×10^5
Benzocaine	1.9	2.5	1.3×10^3
Metomidate Hydrochloride	3.1	4.5	6.3 x 10
2-Phenoxyethanol	1.2	15.1	2.7×10^4
Isoeugenol	3.0	9.9	3.6×10^2
Quinaldine		5.7	5.0×10^2

CHAPTER 2

This chapter was submitted to North American Journal of Aquaculture

THE USE OF TRICAINE METHANESULFONATE, CLOVE OIL, METOMIDATE, AND 2-PHENOXYETHANOL FOR ANESTHESIA INDUCTION IN ALEWIVES *(ALOSA PSEUDOHARENGUS)*

Abstract

The efficacy of the anesthetic agents (tricaine methanesulfonate (MS-222), clove oil, metomidate, and 2-phenoxyethanol (2-PE)) was studied in adult, juvenile (133.3 ± 1.5 mm, 27.5 ± 8.9 g), and larval Alewives (*Alosa pseudoharengus* Wilson). In an initial trial, wild-caught adults were anesthetized with doses of 87.5-112.5 mg/L MS-222, 25-40 mg/L clove oil 0.5-5.0 mg/L metomidate and 0.125-0.550 mg/L 2-PE. Optimal doses for anesthesia were similar for larvae and juveniles, and were identified as: 75-100 mg/L MS-222, 40 mg/L clove oil, 5-7 mg/L metomidate, and 500 mg/L 2-PE. All juvenile fish survived 48 hours post-exposure to each optimal dose. In a longer-term (24 hour) sedation experiment, juvenile Alewives were netted and exposed to low clove oil (2.5 and 5.0 mg/L) and metomidate (0.25 and 0.50 mg/L) doses, and plasma cortisol was measured. Fish exposed to the clove oil treatments exhibited a cortisol

stress response that was prolonged in the higher dose treatment. No cortisol stress response was observed in the metomidate treatments. Overall, optimal acute anesthesia doses for alewives were similar to those reported for other species, and metomidate may be useful for longer term sedation.

Introduction

Anesthetics are widely used in routine aquaculture operations to immobilize animals for tagging, spawning, handling, and vaccination. These compounds depress the nervous system and reduce sensory perception, likely increasing the overall wellbeing of fish during handling procedures (Carter et al. 2011; Zahl et al. 2012). Repeated handling without proper anesthesia may induce severe physiological changes, such as a prolonged cortisol response, hyperglycemia, and immunosuppression that may ultimately lead to disease, decreased growth or death (Wendelaar Bonga 1997; Barton 2002; Carter et al. 2011). Although MS-222 is currently the only fish anesthetic approved for use in food fish in the US, considerable research has been conducted on other anesthetics including clove oil and its active ingredients, 2-Phenoxyethanol (2-PE), quinaldine and quinaldine sulfate, benzocaine and metomidate (Davis and Griffin 2004; King et al. 2005; Weber et al. 2009; Velíšek et al. 2009).

The desirable attributes of anaesthetics used for finfish include: short induction and recovery time, non-toxicity to fish and humans, no lasting physiological effects, rapid clearance from the body, high solubility in fresh and salt water, stable under normal hatchery conditions (light, heat), non-foaming, readily available, and cost effective (Schoettger and Julin 1967). As the efficacy and safety of anesthetic agents varies among species, life stages and environmental conditions considerable research has been conducted to identify proper anesthetics and their

doses, in many commercially important species, including salmonids (Olsen et al. 1995; Woody et al. 2002), numerous temperate and cold water marine species (Mattson and Riple 1989, King et al. 2005; Weber et al. 2009; Zahl et al. 2009; Zahl et al. 2011), and tropical reef fishes (Cunha and Rose 2006).

Alewives (*Alosa pseudoharengus* Wilson) are an anadromous fish found along the north Atlantic coast that serve as essential prey for many commercially important piscivorous fish species. The range of Alewives extends from Labrador to South Carolina, and often exists sympatrically with a congeneric species, Blueback Herring (*A. aestivalis* Mitchell), that are collectively known as river herring (Loesch 1987). River herring populations have exhibited drastic declines throughout much of their range (Hightower et al. 1996) and were listed as U.S. National Marine Fisheries Service "species of concern" in 2006. While the exact causes of these population declines are unknown, a number of state-specific fishing restrictions have been implemented, including harvest moratoria. One tool that is available for stock enhancement of these depleted fish populations is the release of hatchery-reared larvae and juveniles. Transporting fish to their release sites can be stressful, and the addition of salts and buffers and low doses of anesthetics have proven useful in reducing stress and improving survival (Tomasso et al. 1980; Mazik et al. 1991; Harrell et al. 1992, David and Griffin 2004). Practical hatchery culture methods for both alewives and blueback herring were recently developed (DiMaggio et al. 2015), but further research is needed to determine optimal anesthetic agents and their doses for handling and transporting these species.

The purpose of the present study was to assess effective anesthesia induction and recovery in alewives, using four, commonly used anesthetic agents: MS-222, clove oil,

metomidate, and 2-phenoxyethanol. Preliminary trials were conducted with adult alewives, and a range of effective doses were further evaluated in larval and juvenile fish. An additional experiment investigated a longer-term (24 hours) exposure of juveniles to low doses of clove oil and metomidate, and the cortisol stress response was evaluated.

Materials and Methods

Preliminary Adult Trials

Adult Alewives (~250 mm total length, 200 g) were collected from Lamprey River fish ladders (Newmarket, NH, USA) in May-June 2012 and immediately transported to six, 1,750 L tanks within an 11,000 L recirculating system at the University of New Hampshire (UNH) Aquaculture Research Center (Durham, NH). Salinity and water temperature were maintained at 0 g/L and 15-17°C, respectively, to simulate ambient conditions. To identify a preliminary range of anesthetic doses, individuals (n = 50) were transferred to 20 L containers and subjected by bath immersion to various doses of commonly used anesthetic agents. For all treatments, MS-222 and metomidate solutions were diluted in freshwater and buffered (pH 7.0-8.0) prior to experimental procedures. In addition, clove oil and 2-PE stock solutions (100 and 500 mg/L, respectively) were made using 100% ethanol, prior to dilution with freshwater to appropriate concentrations.

Following anesthetic administration, individual fish were monitored to observe the progress of induction, and timed using a stopwatch. Anesthetic induction times (seconds, sec) were recorded at two stages, following guidelines reviewed by Zahl et al. (2012): 1) the loss of equilibrium (Stage III-1), and 2) failure to respond to strong tactile stimuli (Stage III-2). To

evaluate tactile stimulation, fish were removed from the anesthetics, and if no immediate reaction occurred, time was recorded and floy tags (Floy Tag, Inc., Seattle, WA) were injected into the dorsal musculature using a tagging gun (Mark II Pistol Grip, Avery Dennison Corporation, Glendale, CA). After tagging, fish were placed into new, 20 L containers without anesthetic, and timed for progress of recovery to: 1) regain equilibrium (Stage I) and 2) resume normal swimming behavior (Stage 0) (Zahl et al. 2012).

Experiment 1: Juvenile Acute Anesthesia

Based on the effective doses of anesthetics observed in adult preliminary trials, a dose response experiment was conducted using juvenile Alewives. Juvenile fish (133.3 ± 1.5 mm, 27.5 ± 8.9 g) were collected by dip net from the outflow of Pawtuckaway Lake (Nottingham, NH, USA) in the summer of 2012, and immediately transported to three, 600 L tanks in a 2,000 L recirculating system at UNH. Fish were maintained at simulated ambient conditions, fed daily rations (Europa 15, 55% protein 15% fat, Skretting, Putten, Netherlands), and held in culture for one month prior to experimental procedures. Individual fish (n = 5 per treatment) were transferred to 3 L plastic tanks that contained various doses of each anesthetic: 1) 62.5, 75.0, 87.5, or 100 mg/L MS-222, 2) 20.0, 25.0, 30.0, 35.0, or 40.0 mg/L clove oil, 3) 3.0, 4.0, 5.0, 6.0, or 7.0 mg/L metomidate, 4) 300.0, 350.0, 400.0, 450.0, or 500.0 mg/L 2-PE. Progress to induction of anesthesia (Stages III-1, -2) was timed as described previously (without tagging), and fish were immediately transferred to additional 3 L freshwater tanks and timed for recovery (Stages I and 0).

To evaluate the effects of anesthetic treatment on juvenile survival, the optimal doses of each anesthetic agent (based on shortest Stage III-2 induction and Stage 0 recovery times) were used in an additional experiment. Juvenile Alewives (n = 10 per treatment) were treated with each anesthetic dose in 3 L tanks as described above, monitored for progression to Stage III-2, and quickly transferred to 300 L freshwater tanks in a 1,400 L recirculating system kept at simulated ambient conditions. The fish were allowed to recover from anesthesia (Stage 0), and survival was monitored in the tanks at 24 and 48 hours (hrs).

Experiment 2: Larval Acute Anesthesia

To evaluate effective anesthetic doses for larval Alewives, an experiment was conducted using the same four anesthetic agents and largely similar concentrations, as described above. To obtain larval fish, previously collected adult female Alewives (n = 28) were selected for hormonally-induced spawning, anesthetized in 100.0 mg/L MS-222, and implanted in the dorsal musculature with a 95% cholesterol: 5% cellulose pellet (Sherwood et al. 1988) containing 25 μg [D-Ala^6 Des-Gly^{10}]-LHRH ethylamide (LHRHa; Bachem, Belmont, CA, USA). After recovery, fish were returned to tanks with spermiating males for volitional spawning. Spawned eggs were collected, treated with tannic acid (150 mg/L for 10 min) for disinfection and de-adhesion, and incubated at 21-24°C with gentle aeration until hatch (DiMaggio et al. 2015).

Larval Alewives were maintained in 80 L static tanks, and kept at 5 g/L salinity with simulated natural temperature and photoperiod regimes. Larvae were fed three times daily S-strain rotifers (*Brachionus plicatilis*) and *Artemia* nauplii from 0-30 and 25-34 days post hatch

(DPH), respectively. At 34 DPH, individual larvae (12.3 ± 0.4 mm, n = 5 per treatment) were removed from tanks using plastic containers fitted with a mesh bottom, to avoid water transfer that would dilute anesthetic doses. The plastic containers with larvae were immediately submerged into 250 ml beakers containing experimental doses: 1) 37.5, 50.0, 62.5, 75.0, or 87.5 mg/L MS-222, 2) 20.0, 25.0, 30.0, 35.0, or 40.0 mg/L clove oil, 3) 3.0, 4.0, 5.0, 6.0, or 7.0 mg/L metomidate, 4) 300.0, 350.0, 400.0, 450.0, or 500.0 mg/L 2-PE. Anesthesia induction times (Stage III-1, -2) were recorded as described previously, and larvae were gently removed from anesthetic treatments using the plastic containers. Larvae were then transferred to a second beaker containing only tank water (5 g/L salinity) to remove excess anesthetic agents, prior to submersion in a third beaker for recovery from anesthesia. Recovery times (Stage I and 0) were recorded as described previously.

Experiment 3: Evaluation of Anesthetic Agents for Sedation

The efficacy of two anesthetic agents (clove oil and metomidate) for longer term sedation was also evaluated in juvenile Alewives. Previously collected juvenile fish (174.2 ± 0.1 mm, 39.5 ± 0.1 g) were quickly netted and removed from recirculating systems and transferred to eight, 200 L static tanks (17-18°C, 3 g/L salinity, 24:0 light:dark cycle) that contained four replicates of two clove oil concentrations (2.5 or 5.0 mg/L). Eight to 12 fish were stocked per tank, and an additional eight fish were immediately euthanized with 10 mg/L metomidate and sampled for plasma cortisol determinations (time = 0 hr). Briefly, blood was collected from caudal vessels using heparinzed syringes, centrifuged at 8,000 x g for 10 min at 4°C, and plasma was stored at -20°C. One hour after transfer to tanks (time = 1 hr), all fish in one static tank from each anesthetic concentration were euthanized, and plasma was collected. This procedure was

repeated for the remaining tanks at 3, 6, and 24 hrs. These experimental procedures were conducted two additional times, with 1) two metomidate concentrations (0.25 and 0.50 mg/L), and 2) a control experiment without anesthesia.

Plasma cortisol levels were measured using a direct enzyme-linked immunosorbent assay (ELISA), following protocols adapted from the Smithsonian's National Zoological Park endocrine workbook (Brown et al. 2013). Briefly, plasma samples (50 µl) were double extracted using 1.0 ml diethyl ether, dried at 37°C under a constant stream of N_2, stored at -20°C, and reconstituted in 0.15-1.2 ml assay buffer (0.1 M phosphate, 0.15 M NaCl, 0.1% bovine serum albumin, pH 7.0). NUNC 96-well Maxisorp plates (Thermo Fisher Scientific, Inc., Waltham, MA) were coated with 1:8500 diluted rabbit anti-cortisol antibody (University of California, Davis, CA) and incubated overnight at 4°C. Plates were washed with 0.15 M NaCl and 0.05% Tween 20 in a microplate washer (Thermo Labsystems, OPSYS MW, Thermo Fisher Scientific) and blotted dry. Samples, cortisol standards (0.078-20 ng/ml, Sigma Aldrich), and cortisol-horseradish peroxidase conjugate (University of California Davis) were added and incubated for 1 hr at room temperature. Plates were washed again, incubated with substrate buffer (0.05 M citric acid, 6.0 mM H_2O_2, 0.4 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), pH 4.0) for 15 min with shaking, and absorbance was read at 405 and 620 nm using a Synergy 2 Plate Reader (BioTek, Winooski, VT). Background absorbance (620 nm) was subtracted from 405 nm readings prior to analyses, and cortisol standard curves were generated using a 5 parameter logistic model in GEN 5 software (BioTek). Cortisol assays were also validated for parallelism using blood plasma pooled from >20 individuals, and assay quality was monitored using duplicate pool samples included on each plate. Intra- and interassay coefficients of variation for cortisol assays were 3.0 ± 0.7 and 12.5 ± 0.6 %, respectively.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc tests for multiple comparisons (JMP Pro 11, SAS Institute, Cary, NC, USA) were used to identify differences among anesthetic doses. Some data (anesthesia induction/recovery time, plasma cortisol concentrations) were either log or square root transformed prior to analyses to meet normality and equal variance assumptions. All data are represented as the mean ± standard error, and $P < 0.05$ was considered statistically significant for all analyses.

Results

Preliminary Adult Trials

Most adult Alewives anesthetized with low MS-222 doses (87.5 or 100.0 mg/L) exhibited extended Stage-III-2 induction and Stage 0 recovery times (~ 600 and 840 sec, respectively). The highest MS-222 dose (112.5 mg/L), however, effectively anesthetized all fish (n = 12, mean 341 ± 14 and 644 ± 43 sec). Similarly, the lowest clove oil (25.0 mg/L), metomidate (0.5 mg/L), and 2-PE (0.125 mg/L) doses were not effective anesthetics and resulted in strong responses to tactile stimulation (>600 sec). Rather, higher doses (35.0, 5.0, and 550.0 mg/L, respectively) exhibited rapid induction and recovery times, with 2-PE overall being a very rapid anesthetic agent for adults (n = 4, Stage III-2: 210 ± 4; Stage 0: 315 ± 27 sec).

Experiment 1: Juvenile Acute Anesthesia

Juvenile Alewives largely exhibited faster induction times with increasing concentration of each anesthetic agent (Table 4). The lowest doses of MS-222 (62.5 mg/L), clove oil (20.0 mg/L), and 2-PE (300.0 mg/L) were ineffective at inducing anesthesia in some fish (n = 2, 1, and

5, respectively). In contrast, recovery times were similar among most doses within an anesthetic treatment, with Stage I times exhibiting no significant differences. Stage 0 recovery times, however, exhibited significant increases in the MS-222, clove oil, and 2-PE higher doses. Based on the fastest Stage-III induction and Stage 0 recovery times, the optimal doses for each anesthetic agent for juveniles were: 100.0 mg/L MS-222, 40.0 mg/L clove oil, 5.0 mg/L metomidate, and 500.0 mg/L 2-PE. Higher optimal metomidate concentrations were not chosen due to the extremely high mean Stage 0 recovery time for 6.0 mg/L (696 ± 63 sec). Optimal anesthetic doses resulted in 100% survival in all treatments at 24 and 48 hrs post acute exposure.

Experiment 2: Larval Acute Anesthesia

Larval Alewives exhibited faster induction times with increasing concentration of each anesthetic agent (Table 5). All doses were effective at inducing anesthesia. Recovery times generally decreased or remained stable with increasing concentration, except among MS-222 doses, where the highest dose (87.5 mg/L) resulted in significantly longer Stage I recovery. The optimal doses for each anesthetic agent for larvae were: 75.0 mg/L MS-222, 40.0 mg/L clove oil, 7.0 mg/L metomidate, and 500.0 mg/L 2-PE. The highest MS-222 concentration was not chosen as optimal due to the elevated mean Stage I recovery time (138 ± 14 sec).

Experiment 3: Evaluation of Anesthetic Agents for Sedation

Juveniles exposed to longer term (24 hr) doses of either clove oil or metomidate exhibited different plasma cortisol profiles (Fig. 1). Control fish that were not immersed an anesthetic agent exhibited peak cortisol levels at 3 hr post handling, followed by a return to basal levels by 6 hr. In contrast, fish exposed to 2.5 mg/L clove oil exhibited peak cortisol at 1 hr, then a decrease at 3 hr (Fig. 1A). Juveniles in the 5.0 mg/L clove oil treatment also exhibited an

increase and decrease in plasma cortisol levels at 1 and 3 hrs, respectively, but was followed by gradual increases at 6 and 24 hrs. One individual in this treatment also exhibited extremely high cortisol levels at 24 hrs, and was above the assay's detectable limit (>504 ng/ml plasma).

Metomidate concentrations (0.25 and 0.5 mg/L), in comparison to the control, induced little increase in plasma cortisol concentration by 3 hrs (40 and 14 ng/ml, respectively), and remained near basal levels throughout the experiment (Fig. 1B). Among the anesthetic treatments, both metomidate concentrations also resulted in significantly lower plasma cortisol than either clove oil dose at 1 hr ($P < 0.0001$). At 3 hrs post handling, the 0.5 mg/L metomidate dose exhibited significantly lower cortisol than the other treatments ($P < 0.0001$). At 6 and 24 hrs, the 2.5 mg/L clove oil and 0.25 mg/L metomidate exhibited similarly low cortisol levels, while the 5.0 mg/L clove oil treatment was significantly elevated ($P < 0.0001$). The 0.50 mg/L metomidate dose, by comparison, was significantly lower than all other treatments at 24 hrs ($P < 0.0001$).

Discussion

Many biological and environmental factors, including stage of life cycle, size, water temperature, lipid content, and age can influence the metabolic rates of fishes, and therefore the pharmokinetics of anesthetic compounds to which they are exposed (Zahl et al. 2011). Members of the genus *Alosa* have been shown to be highly susceptible to stressors that can lead to high mortality rates during hatchery practices (Mylonas et al. 1995). In the present study, Alewives were effectively anesthetized using acute doses of four, commonly used anesthetic agents. Optimal doses of MS-222, clove oil, metomidate, and 2-PE were largely similar among larvae, juveniles, and adult fish, and were also similar to doses used in other species, including Black

Sea Bass (*Centropristis striata*), Atlantic Cod (*Gadus morhua*), Red Pacu (*Piaractus brachypomus*), salmonids, Senegalese Sole (*Solea senegalensis*), and many tropical reef species (Mattson and Riple 1989; Sladky et al. 2001; Woody et al. 2002; King et al. 2005; Roubach et al. 2005; Cunha and Rose 2006; Weber et al. 2009; Carter et al. 2011). Although no mortality was evident in juveniles 48 hrs after exposure to each optimal dose, longer term sedation with clove oil did elicit a prolonged cortisol response. Since some of these commonly used chemicals exhibit different modes of action, the choice of induction method in Alewives, as in other species, is dependent on several factors, including the invasiveness of the procedure, length of anesthetic exposure, cost and legal authorization.

MS-222 has been one of the most widely used anesthetic drugs in fisheries science worldwide, since its introduction as a local analgesic agent in humans in 1967 (Topic Popovic et al. 2012). It remains the only legally approved anesthetic for use in fish in the United States and Canada, and has been used extensively for a variety of purposes, including immobilization, suppression of sensory systems during surgical procedures, euthanasia, as well as many laboratory and hatchery procedures (Anderson et al. 1997; Woody et al. 2002; Carter et al. 2011). The use of MS-222 in food fish is somewhat limited, however, as extended withdrawal periods are required in many countries, post-induction, before human consumption or release into the wild (reviewed by Carter et al. 2011; Topic Popovic et al. 2012). It is water soluble, rapidly crosses the gill membrane, and suppresses neural transmission in both the peripheral and central nervous systems (Carter et al. 2011). A range of adverse effects are also associated with the use of MS-222, however, including hypoxia, elevated catecholamine and cortisol levels, depression of cardiovascular, osmoregulatory and respiratory function and possible effects on

hepatic P450 enzyme activity (Carter et al. 2011; Topic Popovic et al. 2012; Zahl et al. 2012). MS-222, therefore, is not recommended for prolonged sedation, but rather for acute anesthesia induction (Carter et al. 2011). In the present study, short term MS-222 exposure was highly effective in Alewives, at similar doses (75-112.5 mg/L) to those reported in numerous other teleosts, including salmonids and marine species (Sladky et al. 2001; Woody et al. 2002; King et al. 2005; Zahl et al. 2011). 2-PE is a colorless, oily liquid compound with antibacterial properties that is found in many dermatological products, perfumes, and insect repellent. It is also used as an anesthetic agent in fish, although the exact mechanism of action has not been reported (Zahl et al. 2012). Common 2-PE doses for acute anesthesia range from 200 to 800 mg/L, and result in rapid induction and recovery times (Mattson and Riple 1989; King et al. 2005; Weber et al. 2009; Mitjana et al. 2014). Adverse effects of 2-PE are similar to those reported for MS-222 and include reduced cardiovascular function, elevated cortisol levels, and immunosuppression (Iwama et al. 1989; Mattson and Riple 1989; Ortuño et al. 2002). All Alewives, irrespective of age, were effectively anesthetized at approximately 500 mg/L, and no negative impacts were observed on larval fish. For both larval and juvenile Alewives, the highest 2-PE dose tested (>500 mg/L) resulted in the most rapid anesthesia induction, but recovery at the higher doses (400-500 mg/L) was dose independent. Whereas both positive (Mitjana et al. 2014) and negative (Weber et al. 2009) dose-dependent recovery times have been reported, further research, exploring higher doses of 2-PE is warranted for Alewives.

Clove oil, derived from the *Eugenia aromaticum* tree, and its active ingredients eugenol and iso- eugenol, are other widely used fish anesthetics (Javahery et al. 2012; Zahl *et al.* 2012). Clove oil was historically used as a human dental analgesic and has been affirmed as a Generally

Recognized as Safe (GRAS) substance that can be used in human alimentation by the US Food and Drug Administration (FDA; Gullian and Villanueva 2009). Despite this fact, clove oil and its active ingredients are not approved as a fish anesthetics in the US, but iso-euganol, marketed as Aqui-S®, is approved for use in food fish in Australia, New Zealand, Costa Rica, Republic of Korea and Chile with no withdrawal period (Zahl et al. 2012). Clove oil is relatively inexpensive and has been shown to be highly effective in many freshwater and marine species (Cho and Heath 2000; Javahery et al. 2012). Determination of species-specific dosages is imperative, however, to avoid potentially severe adverse effects (Barton and Helfrich 1981; Sladky et al. 2001; Woody et al. 2002; Hill and Forster 2004; Woolsey et al. 2004; Javahery et al. 2012).

In the present study, total recovery times for juvenile Alewives were prolonged compared to MS-222 and 2-PE, and in larvae were greater than all the other anesthetics tested. Extended recovery from clove oil may be due to the persistence of oil on gill surfaces, which likely increases exposure times (Sladky et al. 2001). Clove oil has been shown to cause local inflammation and cellular necrosis (Sladky et al. 2001) and elevates cortisol levels in the absence of other stressors (Zahl et al. 2010). In the present study, fish quickly transferred to tanks containing clove oil at low doses (2.5 and 5.0 mg/L) experienced a cortisol stress response, similar to that of the controls. This suggests a need for pre-anesthetic sedation prior to transfer to mitigate the transfer stress. Juvenile fish exposed to the higher clove oil dose for a prolonged period (24 h) experienced a second cortisol elevation, which may have been due to a stress response elicited by tissue irritation.

Metomidate is a water-soluable, rapid-acting, non-barbituate hypnotic that has proven to be highly effective for immobilizing many fish species (Mattson and Riple 1989; Davis and

Griffin 2004; Zahl et al. 2012; Iversen et al. 2013). Metomidate has also been shown to block cortisol synthesis, prevent handling-related glucose elevation, and perhaps prevent the immunosuppressive effects cortisol has been shown to induce (Maule et al. 1989; Barton and Iwama 1991; Thomas and Robertson 1991, Olsen et al. 1995; Sandodden et al. 2001). It has been suggested that because of these properties, metomidate in low concentrations may be useful for mitigating the stress effects associated with fish transport (David and Griffin 2004). In the present study, Alewives anesthetized with metomidate underwent rapid induction, but the recovery of juveniles was somewhat longer than that required for the other anesthetics tested. Unlike the fish transferred to tanks containing low does of clove oil, however, those exposed to low metomidate concentrations for prolonged periods did not experience a cortisol stress response. Metomidate, therefore may be useful for alewife transfer to release sites, but will require regulatory clearance prior to adoption.

In summary, all of the anesthetic agents tested were effective for acute anesthesia in larval, juvenile, and adult Alewives with induction and recovery times similar to those found in many other teleost species. Prolonged exposure to low doses of clove oil, but not metomidate resulted in elevated cortisol levels. Further research is necessary to determine the effects of environmental conditions (e.g. temperature and salinity) on anaesthesia efficacy as well the benefits of pre-anesthetic sedation prior to netting and transport.

Acknowledgements

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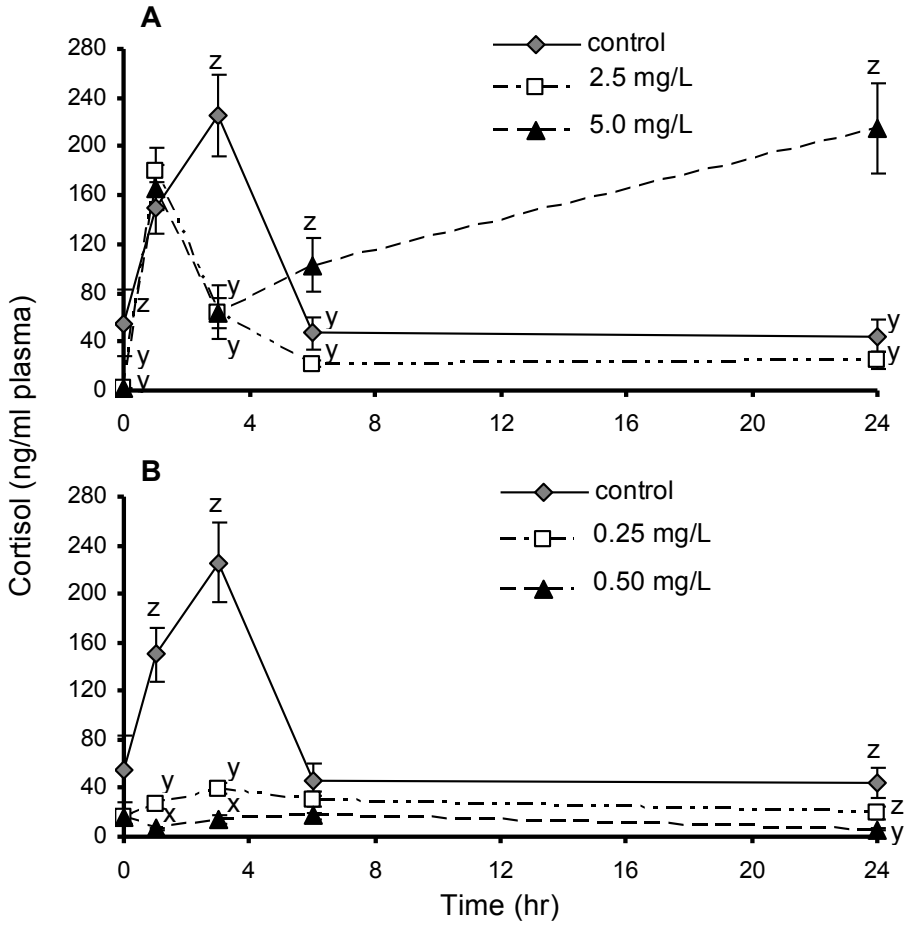
Table 4. Induction and recovery times (sec) of juvenile Alewives (*Alosa pseudoharengus* Wilson) exposed to various doses (mg/L) of anesthetics (treatment). Stages of anesthesia refer to those defined by Zahl et al. 2012, and sample sizes are indicated by n. Different letters denote significant differences among doses within one anesthetic treatment. Bolded text indicates the recommended optimal dose for each anesthetic. The 300.0 mg/L 2-PE treatment was removed from analyses due to poor efficacy to induce anesthesia.

Treatment	Dose (mg/L)	n	Stage III-1 (sec)	Stage III-2 (sec)	Stage I (sec)	Stage 0 (sec)
MS-222	62.5	3	292 ± 19^{z}	691 ± 81^{z}	73 ± 5	236 ± 2^{zy}
	75.0	5	252 ± 57^{z}	389 ± 29^{y}	158 ± 34	273 ± 20^{z}
	87.5	5	137 ± 20^{zy}	231 ± 12^{x}	82 ± 12	174 ± 15^{y}
	100.0	**5**	**69 ± 8^{y}**	**149 ± 19^{x}**	**95 ± 15**	**197 ± 12^{y}**
Clove oil	20.0	4	177 ± 25^{z}	417 ± 43^{z}	250 ± 28	430 ± 22^{z}
	25.0	5	91 ± 20^{y}	285 ± 30^{zy}	225 ± 34	318 ± 20^{zy}
	30.0	5	60 ± 3^{yx}	207 ± 23^{yx}	262 ± 39	364 ± 32^{zy}
	35.0	5	44 ± 2^{x}	166 ± 16^{x}	238 ± 43	371 ± 31^{zy}
	40.0	**5**	**54 ± 3^{yx}**	**143 ± 10^{x}**	**197 ± 22**	**310 ± 22^{y}**
Metomidate	3.0	5	103 ± 12^{z}	554 ± 47^{z}	424 ± 54	664 ± 76
	4.0	5	50 ± 4^{y}	237 ± 16^{y}	299 ± 58	480 ± 66
	5.0	**5**	**44 ± 4^{yx}**	**142 ± 9^{y}**	**340 ± 51**	**542 ± 51**
	6.0	5	31 ± 3^{x}	224 ± 56^{y}	478 ± 88	696 ± 63
	7.0	5	34 ± 6^{yx}	145 ± 11^{y}	262 ± 29	473 ± 41
2-PE	350.0	5	68 ± 7	452 ± 71^{z}	252 ± 80	363 ± 56^{z}
	400.0	5	67 ± 9	181 ± 26^{y}	122 ± 17	214 ± 12^{y}
	450.0	5	68 ± 9	153 ± 7^{y}	105 ± 22	216 ± 16^{y}
	500.0	**5**	**38 ± 4**	**107 ± 5^{y}**	**96 ± 16**	**214 ± 23^{y}**

Table 5. Induction and recovery times (sec) of larval Alewives (*Alosa pseudoharengus* Wilson) exposed to various doses (mg/L) of anesthetics (treatment). Stages of anesthesia refer to those defined by Zahl et al. 2012, and sample sizes are indicated by n. Different letters denote significant differences among doses within one anesthetic treatment. Bolded text indicates the recommended optimal dose for each anesthetic.

Treatment	Dose (mg/L)	n	Stage III-1 (sec)	Stage III-2 (sec)	Stage I (sec)	Stage 0 (sec)
MS-222	37.5	5	115 ± 3^{z}	334 ± 42^{z}	87 ± 8^{y}	218 ± 9^{zy}
	50.0	5	94 ± 11^{z}	214 ± 15^{zy}	85 ± 9^{y}	251 ± 25^{z}
	62.5	5	53 ± 8^{y}	144 ± 21^{yx}	76 ± 13^{y}	155 ± 18^{y}
	75.0	**5**	$\mathbf{47 \pm 9^{y}}$	$\mathbf{119 \pm 16^{xw}}$	$\mathbf{95 \pm 8^{zy}}$	$\mathbf{169 \pm 5^{y}}$
	87.5	5	30 ± 4^{y}	82 ± 6^{w}	138 ± 14^{z}	206 ± 13^{zy}
Clove oil	20.0	5	141 ± 11^{z}	355 ± 26^{z}	112 ± 10	254 ± 32
	25.0	5	136 ± 17^{z}	329 ± 42^{z}	127 ± 53	266 ± 56
	30.0	5	68 ± 12^{y}	175 ± 12^{y}	121 ± 25	278 ± 19
	35.0	5	58 ± 8^{y}	147 ± 6^{y}	151 ± 23	288 ± 32
	40.0	**5**	$\mathbf{47 \pm 9^{y}}$	$\mathbf{100 \pm 12^{x}}$	$\mathbf{106 \pm 14}$	$\mathbf{253 \pm 24}$
Metomidate	3.0	5	113 ± 22^{z}	487 ± 82^{z}	61 ± 14	413 ± 27^{z}
	4.0	5	121 ± 19^{z}	325 ± 78^{zy}	86 ± 26	298 ± 28^{zy}
	5.0	5	64 ± 11^{zy}	182 ± 31^{yx}	94 ± 27	285 ± 53^{zy}
	6.0	5	44 ± 3^{y}	158 ± 19^{x}	148 ± 29	276 ± 49^{zy}
	7.0	**5**	$\mathbf{50 \pm 11^{y}}$	$\mathbf{156 \pm 17^{x}}$	$\mathbf{84 \pm 15}$	$\mathbf{184 \pm 14^{y}}$
2-PE	300.0	5	105 ± 7^{z}	281 ± 52^{z}	32 ± 5	127 ± 14
	350.0	5	58 ± 6^{y}	209 ± 23^{zy}	45 ± 5	115 ± 13
	400.0	5	53 ± 8^{y}	170 ± 27^{zy}	60 ± 11	138 ± 16
	450.0	5	63 ± 13^{y}	183 ± 19^{zy}	57 ± 11	136 ± 14
	500.0	**5**	$\mathbf{42 \pm 5^{y}}$	$\mathbf{124 \pm 23^{y}}$	$\mathbf{57 \pm 16}$	$\mathbf{134 \pm 21}$

Fig. 1. Plasma cortisol levels ± standard error (ng/ml) of juvenile Alewives (*Alosa pseudoharengus* Wilson) exposed to an acute handling stressor, followed by 24 hr doses of clove oil (A) and metomidate (B). The control curve refers to fish exposed to the stressor but without anesthetic treatment. Different letters denote significant differences from controls at each time point.



CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

In summary, all of the anesthetic agents tested were effective for acute anesthesia in larval, juvenile, and adult Alewives, with induction and recovery times similar to those found in many other teleost species. Prolonged exposure to low doses of clove oil, but not metomidate, resulted in elevated cortisol levels. Further research is necessary to determine the effects of environmental conditions (e.g. temperature and salinity) on anesthesia efficacy as well the benefits of pre-anesthetic sedation prior to netting and transport. The literature cited indicates that temperature will have the most significant effect on the doses and recovery times due to the direct effect on the metabolic rate of poikilothermic species (Zahl et al. 2012). Combined anesthetic and temperature toxicity and increased efficacy could be measured at various temperatures now that suitable dosages have been determined for alewives.

The majority of hatchery management procedures, including sampling, grading, transferring and transportation, are often time consuming and result in cortisol stress responses in fish. As both restocking and baitfish production require handling and transportation, methods for reducing stress are imperative to improve long- term survival. Although continued research is required, these initial findings show metomidate to be a very useful anesthetic because it inhibits the cortisol stress response. Because metomidate is relatively expensive, however, future investigations may include using metomidate in combination with other anesthetics to reduce costs while still mitigating stressors.

Metomidate may be useful for Alewife transfer to release sites, but its widespread use will require regulatory approval prior to adoption. The fisheries and aquaculture professions would benefit greatly from greater to FDA-approved drugs. The lack of approved anesthetics for fish use is due to several factors, including complexities in the approval process, and the substantial human and monetary resources necessary for adequate testing (Bowker et al. 2015). In the United States, sufficient data must be developed to demonstrate the effectiveness and safety of any drug on an animal before it can gain approval. In the specialized case of fish, this process requires generation of acceptable data in studies with two representative cold, cool, and warm-water fishes (personal communication, David Erdahl, U.S. Fish and Wildlife Service, Boseman, Montana). Attempting to obtain approval within the current framework will require substantial resources, both public and private, and may take years to complete (Trushenski et al. 2012a, b, c, d). Early reports suggest new aquaculture drug claims require a minimum investment of $3.5 million dollars (Schnick et al. 1996) over a ten-year period. Recent estimates indicate that figure may exceed $40 million, depending on the claim (Storey 2012).

Once approved for use, another aspect important to any hatchery is cost. This is a reoccurring operating expense and depending on the size of the hatchery could be very costly. Table 4 shows a comparison of prices of fish anesthetics from commercial sources (Sigma Aldrich Co. LLC and Western Chemical Inc.). Prices vary due to quantities purchased so several cost/quantity are shown. Clove oil and 2-phenoxyethanol would have the lowest cost per dose based on determinations in this study ($0.18 and $0.47) respectively, whereas MS-222 and metomidate are at $1 per dose or more depending on purchase quantities. Ultimately, the choice

of anesthesia used in a hatchery should be based on overall efficacy including long-term benefits to the fish's well being, as well as relative costs.

Table 6. Cost effectiveness of anesthetics based on purchase price.

Anesthetic	Quantity sold	Price (US $)	Effective dose	# of Treatments	Approximate Cost/Treatment
2 PE	1 L	$93	500mg/L	~200	$0.47
MS-222	50g	$146	100mg/L	~50	$2.92
	300g	$327		~300	$1.09
Clove Oil	500mL	$227	40mg/L	~1250	$0.18
Metomidate	50g	$940	5mg/L	~1000	$0.94
	10g	$240		~200	$1.20

APPENDIX A: ANIMAL CARE AND USE APPROVAL DOCUMENTATION

University *of* New Hampshire

Research Integrity Services, Service Building
51 College Road, Durham, NH 03824-3585
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26-Apr-2012

Berlinsky, David L
Biological Sciences, Rudman Hall
Durham, NH 03824

IACUC #: 120404
Category: D
Project: River Herring Aquaculture and Population Assessment

The Institutional Animal Care and Use Committee (IACUC) has reviewed and recommended approval of the protocol submitted for this study contingent upon your response to the following:

1. In Section IV, A of the application, the researcher needs to add administration of GnRH as a possibility.
2. In the last sentence of the first paragraph of Section IV, A of the application, the researcher needs to change the amount to < 1000 as discussed at the meeting.
3. The researcher needs to complete Section VII, A, #4 of the application to reflect tagging of fish.

The IACUC made the following comment:
1. In Section V, Table 1 and Table 2 of the application, the IACUC changed the pain and distress classification for each species to D (the highest pain and distress level prevails for the protocol).

As soon as the IACUC receives an appropriate response to its concerns, above, it will issue you an approval letter for this protocol. **You may not commence activities in this protocol involving vertebrate animals until you have received IACUC approval**. Please respond to the IACUC within sixty days of this letter. If the IACUC does not receive a response within sixty days, your protocol will be withdrawn.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Robert C. Drugan, Ph.D.
Chair

cc: File