CONDITIONED PLACE AVOIDANCE OF ZEBRAFISH (*DANIO RERIO*) TO THREE CHEMICALS USED FOR EUTHANASIA

by

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Abstract

Zebrafish are increasingly used as a vertebrate model organism for developmental and biomedical research. These fish are commonly euthanized at the end of an experiment with an overdose of tricaine methanesulfonate (TMS), but to date little research has assessed if exposure to this or other agents meets the criteria of a "good death". Clove oil and metomidate hydrochloride are alternatives to TMS and have been approved for use in several countries. The aim of my thesis was to use a conditioned place avoidance paradigm to compare aversion to TMS, metomidate, and clove oil. Zebrafish showed a natural preference for the light environment (in a 900-s trial, they spend 95% of their time in the light compartment); by exposing them to anaesthetics in the light side of a light-dark box we were able to show a difference in preference after exposure. Conditioned place avoidance was less pronounced for fish exposed to metomidate and clove oil than for TMS; fish exposed to the former reduced the time spent in the preferred light side by 131 ± 68 s and 165 ± 97 s, respectively, versus a reduction of 591 ± 88 s for those exposed to the TMS. Complete rejection, where no attempted entries were made into the light side, were recorded after exposure to anaesthetics. Nine of 17 fish exposed to TMS did not re-enter the previously preferred side, versus 2 of 18 fish and 3 of 16 fish for metomidate and clove oil, respectively. These results suggest that the use of metomidate and clove oil are humane alternatives to TMS and should be considered when euthanizing zebrafish.

Preface

All research and associated methods were approved by the University of British Columbia Research Ethics Board (Certificate Number: A11-0252).

A version of Chapter 2 has been submitted for publication and is awaiting decision: Wong, D., von Keyserlingk, M.A.G., Richards, J.G. and Weary, D.M., 2013. Conditioned place avoidance of zebrafish (*Danio rerio*) to three chemicals used for euthanasia. PLoS ONE.

Leigh Gaffney and Devina Wong co-created the testing apparatus and supervisory committee members helped fine-tune the logistics. Leigh Gaffney also conducted the preliminary trials for TMS and clove oil in Chapter 2. All zebrafish were supplied by J.G. Richards, with help from Tammy Rodela. The execution of the experiment and data collection was conducted by Devina Wong. Drs. D. Weary, M.A.G. von Keyserlingk, and J.G. Richards supervised data analysis, interpretation and manuscript preparation.

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Chapter 1: General Introduction

1.1 The use of zebrafish in animal research

Beginning in the early 1980's research involving zebrafish began to increase, primarily in response to the work initiated by the molecular biologist, George Streisinger, who pioneered techniques to clone homozygous zebrafish (Streisinger *et al.*, 1981). Since then, the numbers of zebrafish used in research has grown rapidly; recent statistics from the Canadian Council on Animal Care on animal use for scientific purposes show that fish use (as a percentage of all animals used) has increased from 20 % in 2000 to 42% in 2010 (CCAC, 2000, 2010). This percentage equates to the use of approximately 1.6 million fish annually in Canada (CCAC, 2010).

Despite the myriad of research uses, nearly all fish are euthanized at the end of an experiment (Nuffield Council on Bioethics, 2005), indicating that refinements in euthanasia practices may benefit many animals. The CCAC recognizes that fish are "capable of behavioural, physiological and hormonal responses to stressors and noxious stimuli which can be detrimental to their well-being" (CCAC, 2005), and while a variety of anaesthetic agents are licenced for use on fish, it is unclear which one or combination, of these agents are most suitable for the purposes of sedation, transport, surgery, or anaesthesia prior to euthanasia. Induction and recovery rates, as well as the overall effectiveness of the anaesthetic agent, differs highly between fish species (Sneddon, 2012), therefore species-specific guidelines are required. This thesis seeks to assess various alternative methods of euthanasia for zebrafish with the aim of providing a scientific basis for recommended techniques. This introductory chapter sets the stage by briefly reviewing the natural history of zebrafish, the development of this species as a laboratory animal, the pros

and cons of various methods of fish euthanasia, and the behavioural methods of assessing the suitability of different agents.

1.2 Natural history

There appears to be little consensus as to what the exact natural geographic range of zebrafish is but there is general agreement that it is centred around north-eastern India and extends from Pakistan in the West to Myanmar (Burma) in the East, and from Nepal in the North to Sri Lanka in the South (Engeszer *et al.*, 2007; Spence *et al.*, 2008). Zebrafish are typically found in rivers, small streams and standing bodies of water (Engeszer *et al.*, 2007). The stagnant or slow-moving water bodies that zebrafish inhabit are often connected to rice paddies where high zooplankton growth (associated with fertilizer use) provides a major component of the zebrafish diet (Spence *et al.*, 2008). Aquatic vegetation is also common in these sites, with substrates varying from silt to cobble. Many sites are un-shaded, though vegetation may suffice as protection from the elements and predators. Water conditions vary, but generally visibility extends to approximately 30 cm, temperatures range from 6°C in the winter to 38°C in the summer (Spence *et al.*, 2008), and pH levels range from 6.6 – 8.2 (McClure *et al.*, 2006). This ability to tolerate a range of conditions, in addition to other attributes, contributes to the popularity of zebrafish as a laboratory animal (Grunwald and Eisen, 2002; Guo, 2004).

1.3 Use as a research model

Although reproduction is seasonal in the wild (Spence *et al.*, 2008), controlled conditions within the laboratory have enabled researchers to breed zebrafish throughout the year (Grunwald and Eisen, 2002). Hundreds of progeny are produced by an individual female, and each embryo can develop into swimming, self-feeding larvae in just 5 days (Guo, 2004). Gametes are fertilized externally, allowing genetic researchers the ability to directly manipulate fertilization

conditions, further contributing to the popularity of the zebrafish as a laboratory research animal for use in genetic and embryonic studies.

Adult zebrafish range from 3–5 cm in size and require relatively little space, allowing for low cost and easy maintenance. Zebrafish have translucent embryo and larval stages, facilitating the study of cellular and developmental processes in intact organisms (Lele and Krone, 1996). Zebrafish also provide many valuable characteristics of other vertebrate animal models, including a completely sequenced genome (Wellcome Trust Sanger Institute, 2013). Many genetic and cell-biological pathways are also fundamentally conserved in zebrafish (Grunwald and Eisen, 2002).

1.4 Euthanasia in fish

A recent consensus statement on humane euthanasia summarized the principles of anaesthesia as follows: "it is more important to avoid or minimise pain and distress than it is to ensure a rapid loss of consciousness...a gentle death that takes longer is preferable to a rapid, but more distressing death (Hawkins *et al.*, 2006)." Euthanasia is the most commonly conducted laboratory procedure in research. The majority of research to date on refinement of euthanasia methodologies has been on rodents. This research has employed a variety of approaches including preference testing, approach-avoidance testing, and aversion-avoidance testing (Kirkden *et al.*, 2008; Leach *et al.*, 2002a, 2002b; Makowska and Weary, 2009; Makowska *et al.*, 2008; Niel and Weary, 2007; Niel *et al.*, 2008a, 2008b; Wong *et al.*, 2013). The CCAC recommends that when euthanizing fish used for scientific purposes, a two-step procedure is used. First, induction of anaesthesia to the point of loss of equilibrium followed by a physical or chemical method causing brain death (CCAC, 2005). The first step of anaesthesia is the most important as a humane induction to an insensible state eliminates any welfare concerns.

Fish are euthanized in laboratories using a range of methods including exsanguination (a procedure causing extensive loss of blood), percussive stunning, electrocution, and chemical overdose (CCAC, 2005; Kreiberg, 2000; Robb and Kestin, 2002); smaller fish are often euthanized with a lethal dose of anaesthetic (Kreiberg, 2000). Only two chemicals are approved in Canada for veterinary use as a fish anaesthetic: tricaine methanesulfonate (TMS) and metomidate hydrochloride (metomidate). Alternatives approved in other countries include the eugenol-containing anaesthetics, such as clove oil and AQUI-S® (AQUI-S New Zealand LTD, Lower Hutt, New Zealand), as well as benzocaine, 2-phenoxyethanol, and quinaldine sulfate.

1.5 Anaesthetics

1.5.1 Tricaine methanesulfonate

Tricaine methanesulfonate, also known as MS-222 or TMS [3-aminobenzoic acidethyl ester methanesulfonate], is a white crystalline powder that was originally produced as a local analgesic alternative to cocaine and has been used as such in humans (Popovic *et al.*, 2012). However, its value as a fish anaesthetic was soon recognized and it is now exclusively used for this purpose.

TMS is generally prepared as a concentrated, water-based solution. However, dissolving TMS in freshwater reduces the pH of the solution (Ohr, 1976). Fish held in acidic environments have difficulty maintaining physiological functions, such as ionic and osmotic balance (Burka *et al.*, 1997; Iwama *et al.*, 1989). Furthermore, acidic conditions will positively charge the anaesthetic portion of TMS, reducing its potency (Ohr, 1976). To counterbalance the reduced pH, bases such as sodium bicarbonate or sodium hydroxide are often added to buffer the stock solution. TMS is administrated by immersing fish into a prepared solution of buffered TMS until cessation of vital signs, such as opercular movements.

1.5.1.1 Mode of action

TMS is absorbed through the gills into the bloodstream and distributed throughout the body (Hunn and Allen, 1974). TMS is lipid soluble and moves into the cell membrane to block voltage-sensitive sodium channels, inhibiting entrance of sodium ions (Na^+) into the nervous system. This decreases nerve membrane excitability, which leads to suppression from the periphery to the whole central nervous system (Burka *et al.*, 1997; Butterworth and Strichartz, 1990). TMS concentrates are metabolized rapidly by acetylation reactions and excreted back through the gills and kidneys (Burka *et al.*, 1997; Hunn and Allen, 1974). Beginning with a slight loss in reactivity, fish gradually loses other basic physiological functions, and although TMS is rapidly eliminated from the body, continual uptake through immersion ultimately causes asphyxiation and cardiac arrest.

1.5.1.2 Advantages and disadvantages

TMS is now one of the most widely used anaesthetics in fish (Ackerman *et al.*, 2005; Kreiberg, 2000; Marking and Meyer, 1985) and is approved in many countries including Canada (CCAC, 2005), the United States (U.S.; AVMA, 2013), and the United Kingdom (Close *et al.*, 1996). It is the only fish anaesthetic approved by the U.S. Food and Drug Administration Centre for Veterinary Medicine (http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/Aquaculture/ucm132954.htm), with a required 21-day withdrawal period if used in food fish (American Fisheries Society, 2011; Western Chemical, 2008).

TMS use in fish provides rapid induction and recovery from anaesthesia (Anderson and Mckinley, 1997; Ibarra-zatarain *et al.*, 2011), and has a high solubility compared to other commonly used anaesthetics (such as benzocaine and CO_2). This makes it suitable for use in

freshwater or seawater as well as under different temperatures (Coyle *et al.*, 2004; Ortuno *et al.*, 2002).

While TMS meets the basic criteria for efficacy and safety (Marking and Meyer, 1985), some studies report that TMS is less effective than other anaesthetics, with higher induction times (Grush *et al.*, 2004) and a lower safety margin (failed recovery following anaesthesia), likely due to reduced gill ventilation (Matthews and Varga, 2012; Mattson and Riple, 1989). Others report that TMS exacerbates stress responses (Small, 2003; Wagner *et al.*, 2003) and is stress-inducing, causing brief (Barton and Peter, 1982) or prolonged (Thomas and Robertson, 1991) responses during exposure.

1.5.2 Metomidate hydrochloride

Metomidate, also known as Aquacalm® [methyl 3-(1-phenylethyl) imidazole-4-carboxylate hydrochloride], is a white powder marketed for sedation and anaesthesia of aquarium and certain non-food fish species (Syndel Laboratories, Vancouver, BC). It is a methyl analog of the imidazole derivate etomidate, a sedative commonly used in human and veterinary medicine (Falk and Zed, 2004; Sams *et al.*, 2008). Metomidate is water soluble and is prepared by dissolving the dry powder into fresh or saltwater. Under the recommended sedation and anaesthesia concentrations (0.25 and 10 mg/L, respectively), the pH of the solution is neutral (Syndel Laboratories Ltd., 2011). However, required concentrations and effects vary for different species of fish at different life stages. For example, Siefkes *et al.* (2003) found that only 3mg/L of metomidate was required to anaesthetize adult lamprey, but Christiansen *et al.* (2013) found that juvenile lamprey required concentrations as high as 100mg/L for anaesthesia. At this high concentration, the pH was acidic and required light neutralization. These authors also reported that metomidate agitated lampreys during induction and that the fish were prone to involuntary

muscle twitching or spasms during anaesthesia. In comparison, lampreys that were anaesthetized using TMS and eugenol-based anaesthetics remained immobile during handling procedures (Christiansen *et al.*, 2013; Hajek *et al.*, 2006).

1.5.2.1 Mode of action

While the mode of action of metomidate is not completely understood, it is generally thought to have similar mechanisms as the methyl analog etomidate. Etomidate activates and modulates inhibitory gamma-aminobutyric acid type A ($GABA_A$) receptors, affecting the higher regions of the nervous system (Yang and Uchida, 1996). Activation of the $GABA_A$ receptors produces sedation and hypnosis, but only limited analgesia and immobilisation (Grasshoff *et al.*, 2006). Unlike TMS, metomidate has been shown to suppress cortisol production after injection with adrenocorticotropic hormone (ACTH) as compared to saline injections (Olsen *et al.*, 1995), suggesting that fish are less likely to experience a stress response when exposed to this agent. The mechanism by which this occurs is unknown for metomidate, but etomidate is known to disrupt the mitochondrial cytochrome enzyme that catalyzes the synthesis of cortisol (Bossche *et al.*, 1984; Wagner *et al.*, 1984).

1.5.2.2 Advantages and disadvantages

As stated previously, metomidate is water soluble and at most concentrations results in a solution of neutral pH. It is a recommended sedative for fish transport as dosages that suppress the cortisol stress response still allow for the maintenance of equilibrium (Ackerman *et al.*, 2005; Davis and Griffin, 2004). However, metomidate may be less popular for surgery and handling as fish sometimes exhibit muscle spasms (Christiansen *et al.*, 2013; Syndel Laboratories Ltd., 2011). Metomidate lacks analgesic properties and functions as a hypnotic (AVMA, 2013; Sneddon, 2012).

Metomidate is recommended for use in only six groups of non-food fishes, including fishes from order Siluriformes and families Cyprinidae, Poeciliidae, Pomacentridae, Cichlidae, and Centrarchidae (Syndel Laboratories, 2011). Exhaustive testing for any agent is unlikely as there are more than 30,000 species of fish (Froese and Pauly, 2011), but the lack of literature on other species may explain why metomidate remains less popular than TMS for fish anaesthesia.

The FDA has listed metomidate in the Index of Legally Marketed Unapproved New Animal Drugs for Minor Species (with a specified use for sedation and anaesthesia), meaning that use for euthanasia is off-label (unapproved and requires veterinary prescription). In Canada, metomidate is one of the two agents approved for veterinary use on all fish species.

1.6 Alternatives

Some alternative anaesthetics may be preferable to both TMS and metomidate. For example, clove oil has been found to suppress plasma cortisol levels (Small, 2003), allow swift induction and recovery from anaesthesia, and does not excessively disturb the physiological balance of the fish (Anderson and Mckinley, 1997; Cooke *et al.*, 2004).

Clove oil [4-allyl-2-methoxyphenol] is derived from the flowers, stems, leaves, and buds of the clove tree *Eugenia aromatic* or *caryophyllata*, and was previously used as an analgesic in human dentistry (Curtis, 1990). The main component of clove oil is eugenol, ranging between 85 – 95% (Briozzo *et al.*, 1989), with isoeugenol, methyleugenol comprising the remainder of the anaesthetic. Clove oil is also a common food additive and is considered Generally Recognized As Safe (GRAS) by the FDA. Aqui-S 20E (10% eugenol) is listed in the United States Fish and Wildlife Service's Investigational New Animal Drug (INAD) program (http://www.fws.gov/fisheries/aadap/national.htm) and is currently undergoing testing for FDA approval for aquaculture applications. FA-100 (10% eugenol) is an approved fish anaesthetic in

China and Japan and believed to have a zero-withdrawal period (Endo *et al.*, 1972; Peng *et al.*, 2011). In some countries, including Norway, Australia, Chile and Korea, AQUI-S (a eugenol-based product) is the only aquatic anaesthetic approved for use on fish harvested for human consumption (AQUI-S® New Zealand LTD, Lower Hutt, New Zealand). Many studies have suggested that clove oil is effective for the sedation of fish (Cooke *et al.*, 2004; Sladky *et al.*, 2001; Wagner *et al.*, 2003) and may be a better alternative than TMS (Anderson and Mckinley, 1997; Grush *et al.*, 2004).

Benzocaine is another anaesthetic that is often used in laboratories and fisheries research. Commercially this is marketed under the trademark Benzoak® [ethyl 4-aminobenzoate] (20% benzocaine; Frontier Scientific Inc., Logan, Utah). It is currently undergoing testing for FDA approval for aquaculture applications in the U.S., but is not approved for veterinary use for fish in Canada (CCAC, 2005). Benzocaine is used as a local anaesthetic for humans. It is the active ingredient in nearly 100 over-the-counter anaesthetics ointments (mainly for pain relief products; Trushenski *et al.*, 2013) for insect bites, minor burns and small wounds (de Araujo *et al.*, 2010).

Benzocaine is chemically similar to TMS but requires an organic solvent (Trushenski *et al.*, 2013). Unlike TMS, the final solution is neutral so no buffering is required. Benzocaine is effective for sedating a variety of freshwater and saltwater fish, and generally has faster induction times than TMS (Christiansen *et al.*, 2013; Ferreira *et al.*, 1979; Kiessling *et al.*, 2009; Munday and Wilson, 1997).

The above alternatives have several useful features as anaesthetics, including quick induction times, analgesic properties and immobility during anaesthesia. From the fish's perspective the most important property is likely the degree of unpleasantness associated with exposure; one way to assess this issue is preference testing.

1.7 Preference testing

Preference testing is used in animal welfare research to establish preferred and non-preferred environmental conditions (Fraser and Matthews, 1997). Such conditions include bedding types, ambient temperatures, or lighting conditions, etc. Animals are allowed to experience a range of conditions and then show their preference by choosing to be exposed to or avoid the condition. This choice takes into account all factors that affect an animal's preference, and funnels it into one response.

A recent study assessed preferences for different anaesthetics in zebrafish by using a flow-through chemotaxic choice chamber in which fish were able to swim freely between untreated water and water contaminated with an anaesthetic (Readman *et al.*, 2013). The fish avoided seven out of the nine anaesthetics tested. The authors ranked the degree of aversion using the time fish chose to spend in each of the nine agents.

This type of test shows preferences relative to untreated water, but is limited to the testing of certain agents that do not disrupt the laminar flow. For example, acetic acid and clove oil were rejected as potential test compounds as the disruption was too great to keep the lanes separate. Furthermore, the use of a simple preference test does not show us how strong the preference is (Fraser and Matthews, 1997). It is important to establish how strongly fish prefer to avoid an anaesthetic, as forced exposure to an anaesthetic (that fish are strongly adverse to) will have greater negative welfare consequences.

An example of a motivational test is the light/dark shuttle box test commonly used to assess anxiety in rodents (Bourin and Hascoët, 2003; Slawecki, 2005; Stern and Laties, 1998). This test harnesses the innate anxiety rodents feel towards light/dark conditions and compares it to another stressor (Bourin and Hascoët, 2003; Hascoët *et al.*, 2001). This provides a relatively

simple and non-invasive test, and has been widely applied in zebrafish studies involving anxiolytic and addictive drugs (Ali *et al.*, 2011; Gerlai *et al.*, 2000; Guo, 2004; Mathur *et al.*, 2011; Steenbergen P.J. *et al.*, 2011). Research into humane euthanasia methods in rodents has also made use of a light/dark shuttle box (Wong *et al.*, 2013).

There are conflicting reports on zebrafish preferences for light or dark environments. Several researchers found that larval and adult zebrafish are phototaxic (i.e. prefer light over dark; Ali *et al.*, 2011; Gerlai *et al.*, 2000; Steenberger *et al.*, 2011) perhaps because the fish use vision to capture prey and avoid predators in nature (Burgess and Granato, 2007; MacPhail *et al.*, 2009). Other researchers have found that zebrafish are scototaxic (i.e. prefer dark over light), perhaps because the fish use dark areas for visual camouflage (Blaser and Rosemberg, 2012; Guo, 2004; Maximino *et al.*, 2010a, 2010b, 2010c, 2007; Serra *et al.*, 1999). The reason for this discrepancy has not yet been resolved. However, anti-anxiety drugs have been found to reduce dark and light avoidance behaviours, supporting the hypothesis that these behaviours are indicative of anxiety and validating the use of light/dark preference tests to study anxiety in fish (Ali *et al.*, 2011; Gould, 2011; Steenbergen *et al.*, 2011).

1.8 Conditioned place preference testing

Conditioned place preference testing has been used to assess the reinforcing effects of rewarding or aversive experiences (Darland and Dowling, 2001; Mathur *et al.*, 2011; Mattioli *et al.*, 1998). These tests are often used in addictive substance testing and employ classical conditioning whereby an unconditioned stimulus becomes associated with a conditioned stimulus. In addiction tests, the drug acts as the unconditioned stimulus, and when paired with a previously neutral environment, the environment gains a secondary motivational property and acts as the conditioned stimulus (Tzschentke, 2007).

In rodent models of conditioned anxiety and fear, measures of avoidance and arousal include latency to enter the conditionally aversive environment, freezing behaviour and physiological indicators such as blood pressure and heart rates (Maximino *et al.*, 2010b). The validity of these measures and their limitations has been documented (Belzung and Griebel, 2001; Blanchard *et al.*, 2003). In zebrafish, dwelling time in non-preferred compartments is the most commonly used measure of anxiety, but this measure can also be associated with a wide range of behavioural measures including fast-starts, freezing, shoaling, thigmotaxis (tendency to stay close to walls), bottom-dwelling and erratic swimming. (Mathur *et al.*, 2011; Maximino *et al.*, 2010b; Tzschentke, 2007). Of these behavioural measures, freezing, thigmotaxis and erratic swimming behaviours are the most commonly analysed. However, behavioural tests of anxiety are relatively new in zebrafish and these measures are less well validated than those in rodents (Blaser *et al.*, 2010).

Freezing behaviour is defined as complete or partial immobility, with fish performing only opercular beats, oculomotor responses and fin movements necessary to maintain a dorsal posture (Maximino *et al.*, 2010b). This measure was found to be highly predictive in one study (Blaser *et al.*, 2010) but not in others (Bass and Gerlai, 2008; Egan *et al.*, 2009; Levin *et al.*, 2007). However, when Blaser *et al.* (2010) scored freezing using video-tracking equipment and by direct observation, they found that the automated video-tracking tended to overestimate the frequency of freezing behaviour.

Thigmotaxis, or "centrophobic" behaviour, occurs when animals spend the majority of their time near the walls of the apparatus (Maximino *et al.*, 2010b). This measure has been criticised as animals within the same experiment have been shown to display thigmotaxis in both dark and light compartments (Blaser *et al.*, 2010). The response was also found to be unreliable as

presentation of predators and predator stimuli failed to induce thigmotaxis (Bass and Gerlai, 2008). Thigmotaxis can be seen as an extension of white-avoidance with the fish avoiding the brighter centre of the tank (Blaser *et al.*, 2010).

Erratic swimming refers to an unpredictable pattern of "zig-zag" swimming behaviour in which fish move with changes in angular velocity (Maximino *et al.*, 2010b). Conflicting results were found in studies that used this measure as an indicator of anxiety (Bass and Gerlai, 2008; Blaser *et al.*, 2010; Gerlai *et al.*, 2009; Speedie and Gerlai, 2008).

Fortunately, measurements of latency to enter and dwell in environments associated with aversive stimuli have been found to be highly consistent and reliable in the studies reviewed (Blaser and Penalosa, 2011; Blaser and Rosemberg, 2012; Maximino *et al.*, 2010c; Serra *et al.*, 1999). In addition, measurement of time expenditure is simple, quick and can be measured objectively through video tracking, allowing it to be easily adapted to large-scale screening.

1.9 Aims

With virtually all fish euthanized at the end of an experiment, it is important that anaesthetic induction, the first step of euthanasia, is conducted as humanely as possible. Furthermore, the CCAC requires researchers to avoid subjecting animals to pain and distress (CCAC, 1989); it is the responsibility of the researchers to ensure a humane death for the animals at the end of a study.

The aim of my thesis was to use a light/dark box to test aversion-avoidance and conditioned place avoidance to three fish anaesthetics: TMS, metomidate, and clove oil. Baseline preference for light/dark environments was determined for each fish prior to any anaesthetic exposure. These fish were then tested with one of the anaesthetics added to the preferred

compartments. After exposure, fish were retested in the chamber (but without any anaesthetic) to assess the degree of conditioned avoidance to the previously preferred side.

Chapter 2: Conditioned place avoidance of zebrafish (*Danio rerio*) to three chemicals used for euthanasia

2.1 Introduction

Almost every fish used in research is euthanized at the end of the study with an overdose of anaesthetic. Fish are anaesthetized via immersion in water containing an agent such as the widely used tricaine methanesulfonate (TMS, also known as MS-222; Kreiberg, 2000; Sneddon, 2012). Alternative agents include metomidate hydrochloride (also known as Aquacalm™) and clove oil (approximately 95% eugenol).

Research into humane euthanasia methods in rodents has made use of a light/dark shuttle box (Wong *et al.*, 2013), commonly used to assess anxiety in rodents. There are conflicting reports on zebrafish preferences for light or dark environments. Steenbergen *et al.* (2011) found that adult zebrafish display preference for light, but other work has found a preference for dark (Maximino *et al.*, 2010c). Regardless of the direction, individual preferences allows for the application in the light/dark box to study relative preferences in fish.

Conditioned place avoidance is a behavioural paradigm that has been used to determine reinforcing effects of aversive experiences (Mathur *et al.*, 2011). The aversive experience is paired with a previously neutral environment and results in avoidance of the paired environment; behavioural parameters such as number of entries and time spent in the compartment can be used to assess the degree of avoidance (Maximino *et al.*, 2010b).

The aim of the current study was to use a light/dark box to test aversion-avoidance and conditioned place avoidance in response to exposure to TMS, metomidate, and clove oil. During baseline testing, fish were not exposed to either agent; behaviour during this stage was used to

establish the preferred location (light or dark side of the tank) for each fish. Each individual was then tested with one anaesthetic added to the preferred compartment. We predicted that the fish would respond by 1) spending less time in the previously preferred compartment during the period of exposure, and 2) avoid the previously preferred compartment when tested on subsequent days with no exposure to the anaesthetic.

2.2 Material and Methods

2.2.1 Animals and housing

Fifty-four 6-month old zebrafish (mixed sex) were used. Fish were group housed in two 12.0L, 300 × 195 × 205 mm acrylic tanks (Faunarium Plastic Terrarium, ExoTerra PT-2260, Montreal, Canada) and fed *ad libitum* once a day with fish flakes (Nutrafin Max Tropical Fish Flakes, Montreal, Canada). Water was filtered using mechanical (sponge) and chemical (activated carbon) filtration units (AquaClear Power Filter, Montreal, Canada). To ensure dechlorination and removal of toxic metals, tap water was mixed with a de-chlorinator and de-metalizer (Nutrafin® Aqua Plus Tap Water Conditioner, West Yorkshire, UK) and allowed to sit for at least 24 hours before use. Seachem Equilibruim™ (Seachem Laboratories, Inc., Madison, GA, USA) was added to raise the hardness of the water to at least 80 ppm, and Alkaline Buffer™ (Seachem Laboratories, Inc., Madison, GA, USA) was added to maintain hardness. The water temperature was maintained at 26°C (± 2ºC) and lights were on between 700 h and 1900 h. All testing and feeding took place during the light phase between 1100 h and 1500 h.

2.2.2 Experimental apparatus

During training and testing, fish were housed in pairs in the testing apparatus consisting of two 2.5 L acrylic tanks (Faunarium Plastic Terrarium, ExoTerra PT-2250, Montreal, Canada) connected with a 5.0-cm long, 2.8-cm wide PVC tube (Figure 2.1). The sides of both tanks were

covered with black, light-proof plastic. However, one side of the tank had a light-proof lid and the other side was lit with a 40W incandescent bulb. Water temperature was kept at 26 ± 2°C and both sides were aerated with an air stone. Plastic plants provided hiding places on both sides of the apparatus. Fish were allowed to habituate to the apparatus for two days before testing.

2.2.3 Solution preparation

To prepare solutions, clove oil (95% eugenol, Sigma-Aldrich, USA) was dissolved in 95% ethanol at a 1:9 ratio (clove oil:ethanol) and then further diluted with tank holding water (following Endo *et al.*, 1972). The resulting mixture had a pH between 6.5 – 7.0. Buffered TMS solution was prepared by dissolving ethyl 3-aminobenzoate methanesulfonate powder (Sigma-Aldrich, USA) into a sample of the tank holding water and was then buffered with sodium bicarbonate until the pH reached 7.0 (following Neiffer and Stamper, 2009). Metomidate was prepared by dissolving Aquacalm™ directly into a sample of tank holding water. The resulting pH was 7.0; no buffering was required. Temperatures of these solutions were equilibrated to that of the test tank. Aliquots of the stock solution were then used to achieve the desired concentrations in the test tank.

Concentrations of TMS, metomidate, and clove oil used to achieve anaesthesia vary widely in the literature (TMS: Ackerman *et al.*, 2006; Iversen *et al.*, 2003; Massee *et al.*, 1995; Neiffer and Stamper, 2009; Thomas and Robertson, 1991; metomidate: Massee *et al.*, 1995; Neiffer and Stamper, 2009; Thomas and Robertson, 1991; clove oil: Anderson and Mckinley, 1997; Hajek *et al.*, 2006; Holloway *et al.*, 2004. To allow direct comparisons between three anaesthetics, equipotent concentrations were used. In preliminary testing we exposed 10 zebrafish to three concentrations of TMS (100, 150, 200 ppm), clove oil (45, 55, 65 ppm), and metomidate (8, 12, 15 ppm) within the range reported in the literature and measured the latency

until the fish were unconscious (defined as loss of equilibrium and reflex in the water column). The concentrations that resulted in minimal incidences of aversive behaviours between the three anaesthetics, while rendering fish unconscious, were used. We identified concentrations to obtain similar times to loss of equilibrium in the water resulting in concentrations for TMS, clove oil, and metomidate of 150 ppm, 55 ppm, and 13.5 ppm, respectively.

2.2.4 Training

Before testing, fish were trained to cross through the PVC tube from one side of the apparatus to the other. Training occurred at feeding time for 3 to 5 days before testing. Before feeding, the light/dark conditions were switched by moving the light and switching the lid. The light/dark condition that the fish were found in before the conditions were switched was considered the "preferred side". Fish were consistently found in the "preferred side" every day for 3 days following introduction to the testing apparatus. Food was then dropped into the preferred side after reversing the conditions, prompting fish to re-enter the preferred side. Fish were considered ready for testing once they consistently crossed from one compartment to the other within 1 min of the switch. Training sessions occurred 5 times per day; within 10 days of training all fish met the learning criterion.

2.2.5 Testing

On the day of testing, time spent in each side of the apparatus was recorded for 15 min before testing to determine place preference before exposure to chemicals. Nine pairs of fish were exposed to equipotent concentrations of TMS, metomidate, and clove oil. Three trials were run per day, one for each anaesthetic, with the order of use determined by a 3x3 Latin square. At the beginning of the trial, a divider was used to block the tunnel entrance and the chosen anaesthetic was mixed thoroughly in the non-preferred side. The divider was then moved at the

same time that the light/dark conditions were switched, signalling that the fish could enter. Trials ended when fish lost equilibrium in the water column, or after 15 min, whichever occurred first. Fish were transferred to a recovery tank for 15 min to allow complete recovery from the anaesthetic. Fish were then returned to the non-preferred side of the cleaned testing apparatus with the divider again in place. After 5 min the divider was again removed, following the identical procedure used in training, and we recorded the time spent on each side of the apparatus for 15 min. All procedures were approved by the University of British Columbia Animal Care Committee (application number: A11-0252) and all efforts were made to minimize stress and suffering.

Each trial was video recorded from above to measure latency to cross from the non-preferred side to the preferred side, total time spent in the preferred side, the number of entries into the preferred side, and the number of attempted entries into the preferred side (defined as fish coming within 1 cm of tunnel entrance, with the head facing towards the tunnel, but without actually entering). Complete rejection was defined as no attempted entries into the preferred side post-exposure.

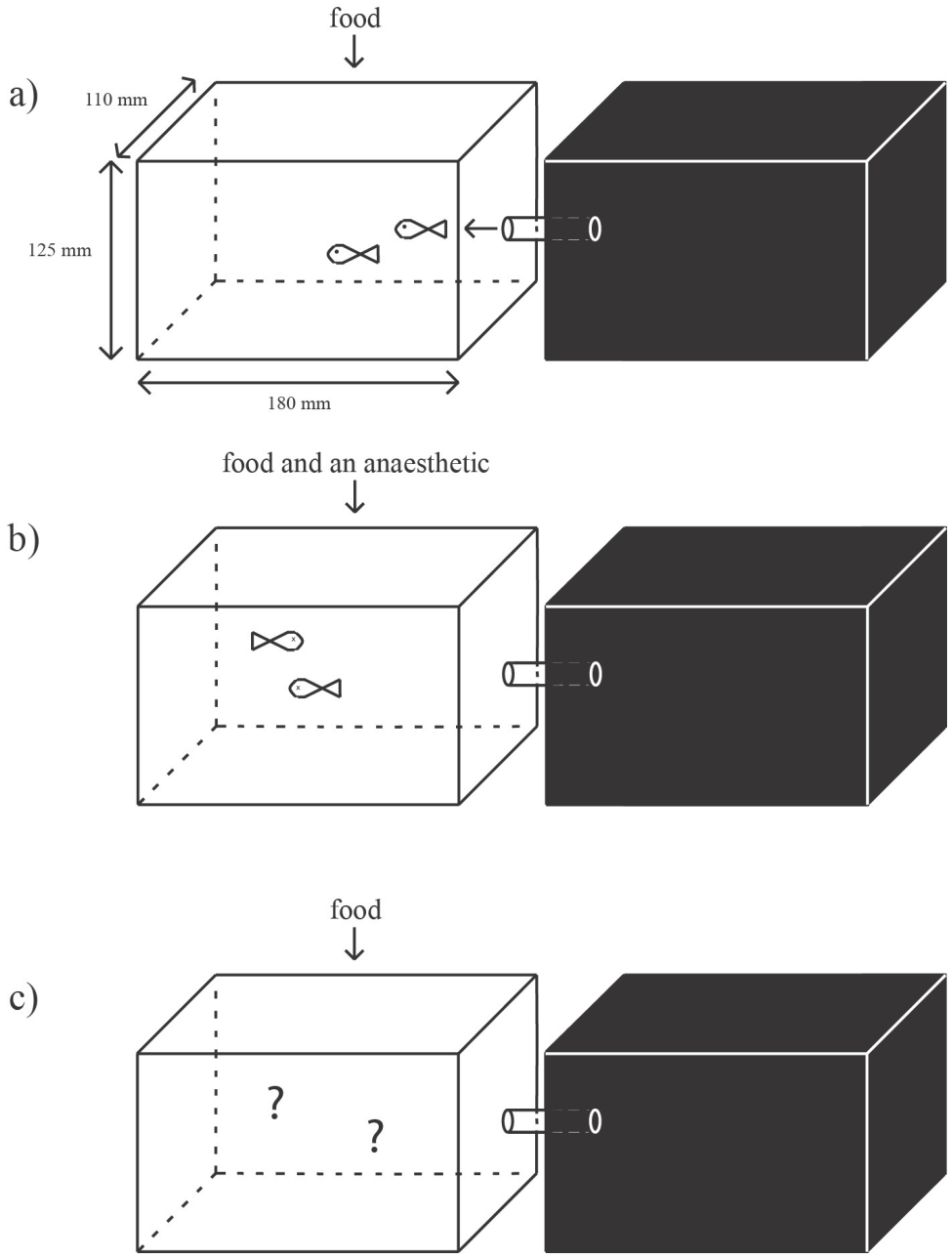

Figure 2.1 Schematic diagram of testing apparatus and training procedure: a) initial trials were used to establish which side the fish preferred and food was used to reinforce place preference, b) in a conditioning trial fish were exposed to one of the three anaesthetics while in the preferred side, c) preferences were then re-tested (in the absence of any anaesthetic) to test for conditional place avoidance.

2.2.6 Statistical methods

Differences in the time spent in the previously preferred side before and after exposure were tested using a Wilcoxon non-parametric test. Pairwise differences between treatments in number of complete rejections were tested using the Fisher's Exact test.

2.3 Result

Out of the 54 fish tested, 53 showed an initial preference for the light side. The one fish that preferred the dark side was removed and replaced with a 'filler' fish that also preferred the light side and acted as a pair-mate but was not included in the analysis.

During exposure testing, two fish did not enter the chemical compartment (one for TMS and one for clove oil). Of the remaining 51 fish, 50 entered the chemical compartment and lost equilibrium in the water column without leaving. There was no effect of chemical on latency to enter the test compartment (97 ± 25 s). One fish chose to leave the clove oil after 3 s, but re-entered after 16 s and stayed until loss of equilibrium.

After exposure testing, all but one of the fish tested with TMS spent less time in their previously preferred side; fish exposed to metomidate and clove oil showed less evidence of conditioned place avoidance ($P < 0.001$; Figure 2.2). Nine of 17 fish tested completely rejected the previously preferred side after exposure to TMS; in contrast only 2 of 18 and 3 of 16 showed complete rejection after exposure to metomidate and clove oil, respectively (Table 2.1, $P < 0.015$).

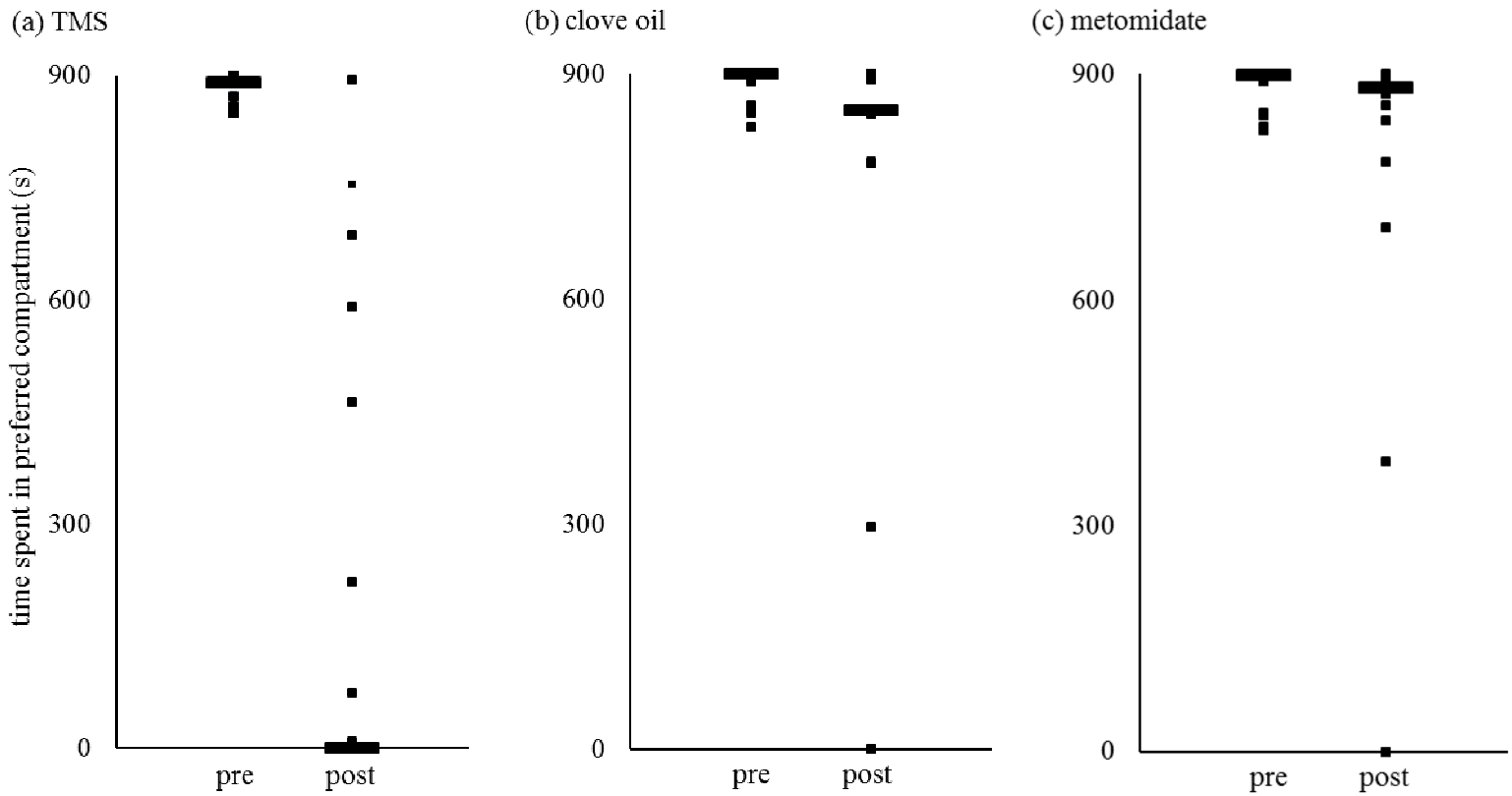

Figure 2.2 Total dwelling time spent in preferred compartment pre- and post-exposure to TMS (a; n =17), clove oil (b; n = 16), and metomidate hydrochloride (c; n = 18). The black bar shows the median. Each square dot shows the value of a single fish. Duplicate values are superimposed

	Complete Rejection	Re-entered
TMS	9/17	8/17
Clove oil	3/16	13/16
Metomidate hydrochloride	2/18	16/18

Table 2.1 Number of fish showing complete rejection (i.e. no attempted entries) into the previously preferred compartment after exposure to TMS (n = 17), clove oil (n = 16), and metomidate hydrochloride (n = 18)

2.4 Discussion

Marking and Meyer's (1985) define ideal fish anaesthetics as those that "produce anaesthesia within 3 minutes or less, cause no toxicity to fish at treatment levels, present no mammalian safety problems, leave low tissue residues after a withdrawal time of 1 h or less, and be reasonable in cost"; previous work assessing fish anaesthetics has used these criteria (Grush *et al.*, 2004). However, these criteria do not include the capacity of the anaesthetic to cause suffering during induction or recovery. The current study is the first to directly assess these welfare effects. We demonstrated that exposure to TMS causes conditioned place avoidance; fish spent less time in their previously preferred side after exposure to TMS than the other two chemicals. Conditioned place avoidance implies that the fish experienced an unpleasant state during induction and learned (in a single exposure) to associate this unpleasant state with the light side of the light/dark apparatus. There was much less evidence of conditioned avoidance following exposure to metomidate and clove oil, suggesting that these agents are less aversive to zebrafish.

It is possible that fish also may have experienced a negative affective state during recovery from TMS or the other agents. However, fish were allowed to recover in a neutral recovery tank, so fish could not learn to associate this experience with either side of the light/dark apparatus.

TMS enters the body via the gills and produces anaesthesia by impeding neuronal signal transmission peripherally to the central nervous system. During the initial phase of anaesthesia using TMS, hyperglycaemia (increased blood glucose level), increased heart rate and respiration were observed, followed by depression of heart rate and ventilation (Sneddon, 2012).

Anaesthesia using TMS failed to prevent elevation in plasma cortisol during stress-inducing experimental procedures, such as blood sampling (Keene *et al.*, 1998; Wagner *et al.*, 2003). Furthermore, an increase in plasma catecholamine values has been reported in fish exposed to TMS, consistent with a stress response to TMS alone (Iwama *et al.*, 1989; Wedemeyer, 1970). These results, in addition to those found in this study, agree with a recently conducted behavioural study showing avoidance to anaesthetic agents used for euthanasia of zebrafish (Readman *et al.*, 2013). Readman et al. (2013) demonstrated that zebrafish will chose to avoid exposure to TMS when given the option of escaping into untreated water. The results of the current study extend these findings by showing that exposure to TMS also causes conditioned place avoidance, and that zebrafish are more motivated to avoid the conditioned compartment than to approach a food reward and preferred light conditions.

Metomidate hydrochloride is a non-barbiturate hypnotic that acts centrally on the central nervous system and produces sedation and hypnosis in humans. Used as a clinical and veterinary sedative, it has been shown to inhibit production of cortisol and prevent handling-related glucose responses in fish (Iversen *et al.*, 2003). Readman *et al.* (2013) also found that zebrafish avoided exposure to etomidate, which is an analogue to metomidate. While this anaesthetic was not tested in my study, we found that the anaesthetic experience of metomidate was not sufficiently aversive enough to repel zebrafish from the previously preferred compartment.

Eugenol-based anaesthetics, including AQUI-S and clove oil, were also found to inhibit cortisol production and handling-related glucose responses (Iversen *et al.*, 2003). As the rate of eugenol passage through the gills is dependent on lipid solubility and degree of ionization (Hunn and Allen, 1974), Keene *et al.* (1998) speculated that eugenol-based anaesthetics are more efficient due to the oils' high lipid solubility. This results in rapid induction times even when

using low concentrations of clove oil. Sladky *et al.* (2001) speculate that the oily property of clove oil coats the gill epithelia and consequently prolongs anaesthetic effects. Eugenol has also been reported to have greater effects on the respiratory and cardiac system than does TMS, resulting in lower ventilation and heart rates (McFarland, 1959). For euthanasia purposes, higher recovery times may be beneficial as a longer buffer time is allowed for secondary euthanasia, reducing the risk of fish recovering.

The unit cost of TMS is lower than metomidate, but the cost of anaesthesia is higher for TMS as the concentration required is higher. Human safety concerns have been expressed for clove oil (US FDA, 2007), but we suggest that these risks can be minimized in laboratories with well-trained staff.

We conclude that metomidate hydrochloride and clove oil are humane alternatives to TMS for the euthanasia of laboratory zebrafish.

Chapter 3: General discussion

After exposure to TMS, zebrafish showed stronger conditioned place avoidance than when exposed to clove oil or metomidate. Zebrafish either completely rejected the compartment that was previously favoured, or spent more time away from that compartment than they did before conditioning. These results indicate that the zebrafish experienced an unpleasant affective state during exposure to TMS. However, there are limitations regarding the inferences that can be made and critiques in the use of the conditioned place avoidance paradigm. I address these limitations and critiques below and end this chapter with recommendations regarding study design for future research.

3.1 Drawing inferences regarding affect in zebrafish

As discussed earlier, fish are proving to be an invaluable species in research to the point where numbers of fish used surpasses that of rodents. Some scientists are skeptical about use of fish in behavioural or cognitive studies, even though zebrafish are increasingly being recognized as a valid animal model in such research. Kaleuff *et al.* (2012) discusses the validity of the use of zebrafish in "affective" paradigms, and argues for the existence of zebrafish emotionality; namely that zebrafish have mental states that give positive or negative values to a stimulus or experience.

Panksepp (2005) has proposed certain empirical criteria to determine existence of emotionality: 1) existence of neural circuits that can activate emotional behaviours, 2) artificial arousal of these systems should be sufficient to generate approach, escape, and/or avoidance response in animals, and 3) the key neurochemical processes and brain activation patterns of the systems should be correlated to the predicted type of affected change in humans. Zebrafish possess neural circuits that activate fear and anxiety-like behaviours (Jesuthasan, 2012; Lau *et*

al., 2011), as well as neurochemical pathways and brain activation patterns that correlate with fear or anxiety (Lau *et al.*, 2011; Lee *et al.*, 2010). The presence of these neural pathways make it difficult to attribute the display of conditioned approach, escape, or avoidance behaviours solely to reflex responses. Furthermore, zebrafish have been shown to demonstrate behaviours consistent with certain affective states, such as anxiety. Display of a strong fear response to what would normally be neutral or mild stimuli, is one way to define anxiety (Jesuthasan, 2012). Lee *et al.* (2012) found that zebrafish show this type of response when pairing a normally mild stimuli with inescapable electric shock. Rather than displaying typical escape behaviours, zebrafish displayed freezing behaviour indicative of learned helplessness, a failure to respond even though opportunities to avoid aversive stimuli are presented. This result indicates that zebrafish possess more than primitive escape responses and are capable of more complex anxiety-like behaviours (Cachat *et al.*, 2010; Stewart *et al.*, 2011).

3.2 Conditioned place preference testing

The conditioned place preference paradigm has rapidly gained popularity over the past 15 years, particularly in studies testing the reinforcing effects of drug use (Tzschentke, 2007). In recent years, several animal models have been developed to assess the emotional component of pain, including conditioned place avoidance. Avoidance behaviour produced by a painful stimuli paired with a distinct compartment is seen as an indicator of the affective component of pain (Tzschentke, 2007). Many studies have used conditioned place avoidance to study negative affect. The motivation to avoid the previously preferred area shows that this area is now associated with an unpleasant occurrence, as in the current study in which zebrafish preferred not to attempt another encounter even though the area was now anaesthetic-free.

Responses during exposure to anaesthetics can be difficult to interpret, as a lack of response may be due to general inhibition of the central nervous system and not to lack of aversion. The anaesthetic metomidate may be of particular concern as it is generally considered to be a hypnotic (Sneddon, 2012; Zahl *et al.*, 2012). If fish are rendered unable to react yet still retain the ability to perceive the unpleasant affect associated with exposure, a severe welfare problem is presented (Zahl *et al.*, 2012). The value of conditioned place avoidance studies is that the overall experience of the anaesthesia exposure is taken into account when a fish chooses whether or not to return to the previously preferred compartment. Conditioned place avoidance will not be effective if agents have amnesic effects, as the animal will have little or no memory of the aversive event. The strong conditioned place avoidance response to TMS therefore indicates that that 1) exposure to this agent is unpleasant for the fish, and 2) that any amnesic effects of TMS are sufficiently mild that the fish are able to associate the environment with this unpleasant experience. The weaker conditioned place avoidance to metomidate and clove oil may be because exposure to these agents is less aversive, or because the drugs have stronger amnesic properties.

3.3 Critique of study design

For conditioned place avoidance testing to become useful in high-throughput experiments, or to compare aversiveness of other anaesthetics, it should be possible to complete testing in a relatively short period of time. There are three steps in the procedure: determination of initial preference, conditioning sessions, and the determination of the final preference. The quickest reported protocol takes two days and requires only one conditioning session (Mathur *et al.*, 2010). However, the majority of other conditioned place protocols reported in the literature require more than one conditioning session, and often run from one to two weeks (Tzschentke,

2007). In the current study we were able to demonstrate conditioned avoidance after only a single session, suggesting that the negative experience was highly salient for the fish.

The duration of the current study was extended by the requirement of determining equipotent concentrations of the different anaesthetics before starting the experiment. Although time consuming, I argue that this step was essential due to the very different dose-response relationships amongst anaesthetics. For example, there was little effect over a range of lower doses and a sudden maximal effect at higher doses for metomidate.

For zebrafish that chose to avoid the previously preferred compartment, I inferred that the strength of aversion to anaesthetic exposure was greater than light/dark preferences combined with the motivation to feed. Similarly, fish that chose to re-enter or stay in the previously preferred compartment were likely more motivated to approach the food than to avoid any conditioned anxiety or fear associated with the compartment. Thus a lack of avoidance does not necessarily mean that the anaesthetic was not aversive, just that this aversion was less than the motivation for food. To distinguish between the two motivations the same test would need to be carried out in the same apparatus used in the current study but without food.

To facilitate individual identification of fish, only one pair of fish was tested at any one time. Zebrafish are naturally a shoaling species, and grouping them at low densities leads to the formation of dominance hierarchies that are stressful to subordinate fish (Pavlidis *et al.*, 2013). In the current study, I added plastic plants to the tank as a means of escape for the subordinate fish, but I did not document aggression in the tank. Even when kept as a pair, the natural shoaling tendencies of zebrafish may have affected the independence of their responses during the test; for example, the motivation of one fish to stay with its pair-mate may have been greater than any conditioned place avoidance. However, individuals within 11 of the 27 pairs showed

independent responses relative to their companion after exposure to anaesthetics, providing some evidence that the fish are indeed able to respond independently.

3.4 Variability in individual fish

The behavioural responses of fish to different anaesthetics vary greatly, within and among species (Zahl *et al.*, 2012). Although only a single strain of zebrafish was used in the study, behavioural responses varied among fish. For example, some fish learned quickly to cross through the tunnel and others required up to a minute before crossing. Genetics, individual life experiences, social position, etc., may influence the behavioural phenotype of a zebrafish. One advantage of the study design used in this thesis is that it allows each individual fish to act as its own control, thus minimizing the effect of this variation in behaviour on the results. Other studies have shown that treatments are experienced differently, resulting in variation in responses across multiple tests within an individual (Gerlai *et al.*, 2009; Gronquist and Berges, 2013; Lange *et al.*, 2013). Given this collection of evidence I highly recommended the use of study designs that reduce variability among and within zebrafish due to seasonal changes, temperature fluctuations, feeding schedules, etc.

3.5 Future directions

The study of affect can be approached in several ways: behaviourally, physiologically, subjectively, and cognitively. Some behavioural responses may be interpreted in different ways; for example, erratic swimming behaviours may be interpreted as anticipation before feeding or anxiety in the presence of a potential predator. Physiological reactions are also subject to misinterpretation; an increase in opercular beat rate may be seen as a negative indicator of fear or a positive indicator of excitement. This is not to say that behavioural or physiological measures are without use, but rather that these measures should not be used as a standalone approach to

study affect – they require study designs that help avoid such ambiguity as described in the previous examples. The subjective representation of emotion is impossible to measure directly (in either humans and in non-human animals), but there has been some recent work in approaching the study of emotion through a cognitive level of analysis in fish (Gerlai, 2011; Oliveira, 2013). For example, a recent study demonstrated learned helplessness in larval zebrafish when they were exposed to an uncontrollable electric shock (Lee *et al.*, 2010). I recommend future studies using cognitive tests to assess fish welfare, including the effects of exposure to different anaesthetic agents. For example, in a similar design as the one used in the study mentioned above (Lee *et al.*, 2010), differences in avoidance behaviour may be monitored in fish that have or have not been pre-exposed to inescapable anaesthetic exposure. It was found that when fish were pre-exposed to a strong stressor, they failed to display avoidance behaviours preventing contact with aversive stimuli (Overmier and Seligman, 1967; Weiss and Glazer, 1975). If TMS is indeed strongly aversive to zebrafish, I predict that exposure to this agent will also lead to learned helplessness.

3.6 Conclusions

The results of my thesis are consistent with the findings recently published describing a behavioural study showing aversion to anaesthetics agents used for euthanasia of zebrafish (Readman *et al.*, 2013). However, this previous study was limited in that it was only able to show that zebrafish choose to avoid certain agents. In contrast, the results of my experiment show that a single exposure to these anaesthetics is sufficient to cause conditioned place avoidance, and that the motivation to avoid the conditioned chamber is greater than the motivation to approach a food reward. Collectively my work and that of Readman *et al.* (2013) provide strong evidence that zebrafish show a strong aversion to TMS. Readman *et al.* (2013)

found that zebrafish also avoided exposure to etomidate. Although this agent was not tested in my study, I found that aversion to metomidate (an etomidate analogue) was weaker than that to TMS. Readman *et al.* (2013) were unable to test aversion to clove oil, but the results of my study show that this agent is also less aversive to zebrafish than is TMS.

The CCAC (2005) recommends the use of TMS as the preferred method of euthanasia, as does the AVMA (2013) for finfish euthanasia. The findings summarized in this thesis suggest that these recommendations should be changed. The results of the current study are consistent with those from earlier research reporting negative physiological and behavioural responses to TMS in fish. Moreover, zebrafish showed less aversion to metomidate and clove oil; therefore I suggest that both of these latter agents be considered humane alternatives to TMS for euthanasia. From a practical standpoint, I recommend the use of clove oil over metomidate as it is considerably less expensive to use and veterinary approval is not required. Although concerns have been raised about a component of clove oil being a suspected carcinogen (FDA, 2002), this conclusion was based upon test concentrations 30 – 60 times higher than dosages required for zebrafish anaesthesia; lethal doses will likely be reached well before any risk of to human handlers (Matthew and Varga, 2012).

This thesis presents the first evidence of conditioned place avoidance in fish to assess the aversive effects of euthanasia agents. Unlike other methods used to assess aversion, such as approach-avoidance or preference tests, this study allows animals to make decisions without being under the influence of anaesthetic effects. In studies where response is measured during exposure, a lack of response may be due to immobility from anaesthesia while an increase in response may be attributed to excitatory effects of anaesthesia. In this study, where responses are measured after anaesthetic exposure, the results are distinctly representative of the experience of

anaesthetic exposure. I conclude that this approach is highly valuable in assessment of aversion, and that it should be considered in euthanasia studies for other species of laboratory animals, including rodents.

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