Anesthesia and Euthanasia in Zebrafish

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Abstract

Because of the relative ease of embryonic manipulation and observation, the ability to produce a great number of genetic mutations, efficient screening methods, and the continued advance of molecular genetic tools, such as the progress in sequencing and mapping of the zebrafish genome, the use of zebrafish (*Danio rerio*) as a biomedical model organism continues to expand. However, studies involving zebrafish husbandry and veterinary care struggle to keep pace with scientific progress. This article outlines some of the current, acceptable methods for providing anesthesia and euthanasia and provides some examples of how performance-based approaches can be used to advance the relatively limited number of anesthetic and euthanizing techniques available for zebrafish.

Introduction

Many of the institutions using zebrafish (*Danio rerio*) for research, testing, or teaching are funded by the Public Health Service (PHS) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Therefore, these institutions use the *Guide for the Care and Use of Laboratory Animals* (the *Guide*; NRC 2011) as a basis for designing, implementing, and evaluating the program for zebrafish care and use, including anesthesia and euthanasia. All institutions that receive PHS funds or support must have a defined policy or PHS Assurance that describes the institution's compliance with the PHS *Policy on the Humane Care and Use of Laboratory Animals* (PHS 2002) and the *Guide*.

PHS-funded and Association for Assessment and Accreditation of Laboratory Animal Care International–accredited zebrafish facilities must also have an institutional animal care and use committee (IACUC) to oversee the animal program, facilities, and animal procedures and to ensure that the institution's program is based on the *Guide* and PHS Policy. The *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* (IRAC 1985) form the basis of the *Guide* and can be used by IACUCs to evaluate their program and individual animal use protocols. Consistent with the difficulty of distinguishing between nociception and pain in fish, principle IV of the *US Government Principles*, which addresses the minimization of discomfort, distress, and pain, states: "Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals" (IRAC 1985, 1). In addition, principle V states: "Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on unanesthetized animals paralyzed by chemical agents" (IRAC 1985, 1). Because little is known about zebrafish pain, distress, and discomfort, this principle should obviously also be applied when evaluating potentially painful or distressing procedures in zebrafish.

The 2011 *Guide* provides general guidance on determining the appropriate method of anesthesia and euthanasia. According to the *Guide*: "The selection of appropriate analgesics and anesthetics should reflect professional veterinary judgment as to which best meets clinical and humane requirements as well as the needs of the research protocol. The selection depends on many factors, such as the species, age, and strain or stock of the animal, the type and degree of pain, the likely effects of particular agents on specific organ systems, the nature and length of the surgical or pain-inducing procedure, and the safety of the agent" (NRC 2011, 121). For evaluating appropriate euthanasia methods, some of the criteria that should be considered are "the ability to induce loss of consciousness and death with no or only momentary pain, distress, or anxiety; reliability; irreversibility; time required to induce unconsciousness; appropriateness for the species and age of the animal; compatibility with research objectives; and the safety of and emotional effect on personnel" (NRC 2011, 123). These criteria outlined in the *Guide* are based on those described in the American Veterinary Medical Association (AVMA) (2007) *Guidelines on Euthanasia* and are specified in the next paragraph. The specific agents and methods chosen for zebrafish euthanasia will depend upon age and the scientific objectives described in the IACUC animal care and use protocol (NRC 2011).

Both the PHS Policy and the *Guide* require the methods of euthanasia to be consistent with the AVMA *Guidelines on Euthanasia*. These guidelines were extensively revised in

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2011, approved by the AVMA Executive Board in September 2011, and are undergoing final edits (Nolen and AVMA Executive Board 2011). In evaluating various methods of euthanasia, the 2007 AVMA guidelines use the following criteria: "(1) ability to induce loss of consciousness and death without causing pain, distress, anxiety, or apprehension; (2) time required to induce loss of consciousness; (3) reliability; (4) safety of personnel; (5) irreversibility; (6) compatibility with requirement and purpose; (7) emotional effect on observers or operators; (8) compatibility with subsequent evaluation, examination, or use of tissue; (9) drug availability and human abuse potential; (10) compatibility with species, age, and health status; (11) ability to maintain equipment in proper working order; and (12) safety for predators/scavengers should the carcass be consumed" (AVMA 2007, 3). Whatever method is chosen for zebrafish euthanasia, "death must be confirmed by examining the animal for cessation of vital signs" (AVMA 2007, 4).

Indicators of Discomfort, Distress, and Pain in Fish

Humans are able to communicate feelings, subjective states of (dis)comfort, distress, or pain, including the level of intensity of these states. In contrast, animals cannot communicate these states directly and subjectively, so it is necessary to rely on interpretation and knowledge of the animals' species-specific behavior to assess their condition. Specifically, techniques for "remote biosensation" of a fish's state of well-being are limited at best and depend on an interpretation of behavior or a measuring of its physiological responses (Ross and Ross 1999). Thus, understanding of stress or pain in aquatic animals is complicated and indirect. More direct pain and/or stress assessments include netting or other handling of fish to measure, for example, plasma or whole-body cortisol levels (Ramsay et al. 2009a; Schreck 2000). This, however, introduces additional stressors and variables and complicates the differentiation between the specific source of discomfort or pain and the direct netting and/or handling. In spite of these difficulties, there are several physiological and behavioral assays available to establish a normal state versus a state of distress or pain in fish (Ross and Ross 1999; Sneddon 2009).

A stressful event can be defined as an abnormal environmental stimulus or threat that jeopardizes survival or negatively interferes with normal activities or physiological balance (homeostasis). For reasons of simplicity, we distinguish short-term (acute) and long-term (chronic) stressors even though stress in fish is a far more complex phenomenon and the term stress is neither well defined nor consistently used (Harper and Wolf 2009; Ramsay et al. 2006, 2009a).

The stress response in fish can be generally categorized into three physiological stages: primary, secondary, and tertiary (Casebolt et al. 1998; Iwama et al. 2004; Wedemeyer 1996; Wedemeyer and McLeay 1981; Wedemeyer et al. 1990). In addition, a fish's behavioral response can sometimes be immediately observed when a stressor is introduced. The behavioral response may be to avoid or mitigate the stressor (Schreck et al. 1997). Both behavioral and physiological responses are intimately related (Iwama et al. 2004).

The primary physiological response involves the release of "stress" hormones into the blood stream. Activation of the hypothalamic-pituitary-interrenal axis involves release of cortisol from interrenal cells located in the head kidney. These cells are activated by adrenocorticotropic hormone, which is released from the pituitary gland. The pituitary gland is stimulated by corticotrophin-releasing factor from the hypothalamus (Donaldson 1981; Wedemeyer et al. 1990). Activation of the sympathetico-chromaffin system (Casebolt et al. 1998) involves release of catecholamines (e.g., adrenaline, noradrenaline) from chromaffin tissue of the head kidney after direct stimulation by the sympathetic nervous system (Mazeaud and Mazeaud 1981; Wedemeyer et al. 1990).

The secondary response is composed of various changes to blood and tissue, such as elevated blood sugar levels, changes in electrolyte concentrations, such as hypochloremia, changes in hematology, such as reduced clotting times, and changes in the differential leucocyte count (Wedemeyer et al. 1990, 453). In addition, there may be changes in the tissues, "including depletion of liver glycogen and interrenal vitamin C, hemorrhage of the thymus, and hypertrophy of the interrenal body" (Wedemeyer et al. 1990, 453). Tertiary responses, for individuals and populations, include reduction in growth, reproduction, resistance to disease, and survival (Casebolt et al. 1998; Iwama et al. 2004; Wedemeyer et al. 1990).

Physiological indicators of stress resulting from stressful stimuli are, for example, the skipping of heartbeats and hematological effects such as changes in blood plasma ion composition (osmoregulation), swelling of erythrocytes, and dilution or concentration of heme (Ross and Ross 1999). Cortisol is a useful readout of the overall hormonal response of the hypothalamic-pituitary-interrenal axis. The increase of whole-body cortisol in response to acute and chronic stressors has been shown to impact the zebrafish immune system, leading to increased susceptibility to mycobacteria infections (Ramsay et al. 2006; Ramsay et al. 2009a,b). In larger species, blood plasma cortisol levels can be used instead. However, the analysis of such indicators depends on the establishment of a consistent and reproducible baseline that characterizes the normal state of an indicator. This "normal" state can fluctuate based on species, circadian rhythm, season, water quality, and other environmental factors (Barton et al. 2002; Iwama et al. 2004) and a stressed state may therefore exist—or not—depending on whether or not the stress indicator readout represents a true departure from the "baseline" at the time of testing.

A previous special issue of the *ILAR Journal* (2009, vol. 50, issue 4) was devoted to the practical, philosophical, and scientific aspects of whether or not fish can sense or feel pain, as well as their use as biomedical research organisms (Posner 2009). Pain, in addition to being accompanied by stress, can be defined as a noxious stimulus associated with

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an evasive behavioral response and potential or actual injury and tissue damage (Harper and Wolf 2009; Sneddon 2009). Several "internal" or physiological criteria support the fact that fish are capable of perceiving noxious stimuli or pain; for example, the existence of opiate neurotransmitters and neural connectivity in fish is comparable to the neurocircuitry that mediates pain in mammals (Gonzalez-Nunez and Rodriguez 2009). However, because it is unclear whether analogous or homologous evolutionary mechanisms could have given rise to similar structures, the debate about pain perception in fish continues to be unresolved, even though analogous evolution is less likely based on molecular data (Gonzalez-Nunez and Rodriguez 2009).

Several external behavioral signs can be used to differentiate between a normal and distressed state, including nociception and pain: excessive movement of the opercula (tachyventilation, hyperventilation), cough rate (reversal of water flow direction over the gills), color (pigmentation) changes, and increased movement (hypertaxia) or cessation of movement (ataxia) (Ross and Ross 1999) are useful indicators of stress in some species. However, species-specific differences do exist, making a generalized interpretation of several stress indicators difficult. Interestingly, when comparing opercular movements, use of cover, and swim rates, zebrafish share fewer stress indicators with the closely related carp (*Cyprinus carpio*) and more with the distantly related salmonid, trout (*Oncorhynchus mykiss*). Trout and zebrafish respond to a noxious stressor with increased ventilation, increased use of cover, and a decreased swim rate, whereas, based on these indicators, carp appear to be rather indifferent to the same stressor. Carp, however, respond with several anomalous (or, perhaps, species-specific) stress indicators such as off-balance swimming, loss of equilibrium, or rubbing of lips against the aquarium wall (Reilly et al. 2008).

Methods of Zebrafish Analgesia, Sedation, and Anesthesia

In fish, species-specific modes of respiration play a key role for the route of anesthetic administration. For example, injection of anesthetics is more efficient for species that are capable of piping, or performing surface aerial respiration under hypoxic conditions (Bruecker 1993). Zebrafish are cyprinids, and as such, they can resort to surface respiration under hypoxic conditions (University of Oregon 2007; Varga et al., unpublished study). Under normal conditions, however, they are obligatory gill breathers. Therefore, the most widely used method to anesthetize (Harper and Lawrence 2011; Westerfield 2007) or sedate (Trevarrow 2007, 2001) zebrafish is immersion, which, in fish, is analogous to inhalant anesthesia in terrestrials (Neiffer and Stamper 2009).

As with the assessment of discomfort and pain by behavioral observations, levels of sedation or anesthesia can also be characterized by several behavioral criteria. For fish specifically, six levels of anesthesia have been described, and of these, opercular movement is probably the most significant indicator to assess levels of anesthesia (McFarland and Klontz 1969). During light sedation (level 1), reaction to some external stimuli (visual and tactile) is slightly reduced, whereas under deep sedation (level 2), fish have slightly reduced opercular movement rates and do not react to any external stimuli except pressure. This is followed by partial loss of equilibrium (level 3), during which muscle tone is decreased, swimming is erratic, and the opercular rate is increased. At this level, fish still continue to react to strong tactile and vibrational stimuli. At the next level (level 4), however, equilibrium and muscle tone are completely lost, the opercular movement rate decreases again, and fish respond only to strong pressure as an external stimulus. After more prolonged anesthetic exposure (level 5), opercular movements become shallow and the heart rate is decreased. There is no reaction to external stimuli and all reflex reactivity is lost. Finally, the last stage (stage 6) is described as medullary collapse: opercular movement completely stops and the heartbeat is shallow and almost completely absent. If the anesthetic is not removed and attempts are not made to revive the fish, death follows due to cardiac arrest and hypoxia (McFarland and Klontz 1969).

These levels of fish anesthesia are, to some degree, similar to the stages observed in mammals. However, deviations may exist from these criteria. For example, even after opercular movements have ceased in tricaine methanesulfonate–anesthetized zebrafish, a hard knock on the work surface can still elicit an occasional, single-jolt flight reflex (Varga et al., unpublished study), indicating that some reflexes, and hence some neural activity, persist.

Ethyl 3-Aminobenzoate Methanesulfonic Acid

Tricaine methanesulfonate, also known as TMS, MS-222, Tricaine mesylate, Finquel® (Argent Laboratories, Redmond, Washington), and Tricaine-S® (Western Chemical, Inc., Ferndale, Washington), is currently the only anesthetic approved for some aquatic species by the US Food and Drug Administration (FDA) and the most widely used sedative and anesthetic for zebrafish. MS-222 is a muscle relaxant that blocks sodium and to a lesser degree potassium currents in nerve membranes. Action potentials as well as spontaneous contractions of muscles are eliminated by MS-222, including sensory input and reflexes (Frazier and Narahashi 1975).

MS-222 is available as a water-soluble powder; however, it is acidic and lowers the pH of unbuffered or weakly buffered solutions to as low as 5. Some studies suggested that the anesthetic effect of MS-222 is reduced under acidic conditions in several fish species (Gilderhus et al. 1973) but not in channel catfish (Welker et al. 2007). Welker and colleagues (2007) also showed that following buffered or unbuffered MS-222 exposure, the plasma cortisol levels remained essentially unaffected in catfish. In these studies, plasma cortisol and glucose levels also increased with higher MS-222 concentrations, suggesting that the stress responses resulted

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from the anesthetic rather than the pH of exposure water. In general, however, the lowering of water pH by MS-222 increases the risk of plasma acidosis in fish (Harper and Lawrence 2011) and should be avoided.

Zebrafish immersed in buffered or unbuffered MS-222 typically respond with hypertaxia and tachyventilation before righting reflex, body, and opercular movements cease (University of Oregon 2007; Wilson et al. 2009; Varga et al., unpublished study). Occasionally, gill bleeding occurs, and some fish do not recover from anesthesia. Presumably this is due to a general, anesthetic-induced stress response that includes acid stress and/or acidosis (in the unbuffered solution), changes in plasma osmolality, and a blood pressure increase that could damage the gills. MS-222 can be neutralized with sodium bicarbonate or sodium hydroxide (Harper and Lawrence 2011) or with Tris buffer (Westerfield 2007).

Another side effect of the muscle relaxant is a reduced heart rate, which increases the risk of accidental death during anesthesia. Simultaneous administration of isoflurane and MS-222 in the zebrafish reduces negative physiological effects, such as reduced heart rate, and effectively helps extend anesthesia duration and shorten recovery time (Huang et al. 2010).

In zebrafish, and presumably other fish, MS-222 dose response depends on age, size, and metabolic state. An increase in sensitivity to MS-222 (Rombough 2007) was observed in 3- to 9-day larvae, presumably due to changes in liver detoxification activity. Median lethal concentrations for 4- to 7-day zebrafish larvae suggested that key developmental changes, consistent with the maturation of gills (Shadrin and Ozernyuk 2002) and liver (Field et al. 2003; Sakaguchi et al. 2008), occur at this time.

The renowned toxicologist and zebrafish scholar von Hohenheim stated: "Poison is in everything, and nothing is without poison. The dosage makes it either a poison or a remedy" (Borzelleca 2000). Consistent with this observation, MS-222 has been used for zebrafish sedation [0.01 mg/ml (Trevarrow 2007, 2011)], anesthesia [0.168 mg/ml (Westerfield 2007)], and euthanasia [0.2-0.3 mg/ml (University of Oregon 2007; Wilson et al. 2009; Varga et al., unpublished study)]. In addition, studies in *Tilapia* (*Oreochromis niloticus, O. aureus,* and *O. mossambicus*) suggested that repeated anesthesia with MS-222 causes prolonged induction and recovery times, indicating that previous exposure should be taken into account when determining dose and exposure times for zebrafish (Smith et al. 1999).

Eugenol

Clove oil, which contains 70-95% eugenol (4-allyl-2-methoxyphenol), isoeugenol, and 5-15% methyleugenol, has been used for topical analgesia and local anesthesia in dentistry and has gained popularity among hobby aquarists for fish anesthesia and euthanasia. However, clove oil is currently not approved by the FDA as an anesthetic, mainly because methyleugenol is a suspected carcinogen (FDA 2002). However, the test concentrations currently used to test DNA adduct formation and tissue neoplasia induction in mice and rats are up to 30- to 60-fold above the dose typically used for zebrafish anesthesia or sedation (dosages are discussed in the last paragraph of this section). Thus, it is likely these higher dosages would be lethal for zebrafish before causing cancer to fish or humans, assuming similar species effects. However, eugenol is also thought to cause liver problems and could therefore negatively affect specific research results (FDA 2002; NTP 2000).

In direct comparison with MS-222, eugenol appears to combine several advantages for fish and humans, such as availability, low cost, and lower dosages needed to induce anesthesia in zebrafish. Specific stages or full anesthesia are induced more rapidly at comparably lower doses of eugenol, but recovery takes longer than with MS-222 (Grush and Noakes 2004). In contrast, our own observations suggest that, in addition to slower recovery, it takes longer to induce anesthesia with eugenol (J. Matthews and C. Carmichael, personal communication). Nevertheless, anesthesia-related mortalities with eugenol are low in zebrafish (Grush and Noakes 2004), and eugenol may have a wider safety margin than MS-222.

Eugenol is not readily water soluble and a 10% stock solution in ethanol needs to be prepared. To avoid light degradation, the stock solution can be stored at room temperature in brown glass or metal containers. A 2- to 5-µg/ml dosage concentration is recommended to sedate adult zebrafish, and 60 to 100 µg/ml is used for immersion anesthesia (Grush and Noakes 2004). In addition to a wider safety margin, eugenol also appears to mitigate crowding and handling stress in fathead minnows (*Pimephales promelas*) based on plasma cortisol level analysis and neutrophil function, whereas MS-222 did not appear to prevent that stress response (Palic et al. 2006; Welker et al. 2007). Further research with eugenol is needed to examine more fully its advantages and disadvantages as an anesthetic and euthanizing agent.

Anesthesia by Cooling

Cooling has been traditionally applied as a sedative and anesthetic in fisheries (Hovda and Linley 2000) but not in zebrafish. Gradual chilling of water will slow movements and reduce the general metabolic activity of fish, including excretion of ammonia and feces. Hence, oxygen consumption is reduced while, at the same time, the capacity for holding dissolved oxygen in water is increased. However, nerve conduction is, depending on the species, reduced but not completely blocked, as for example with the use of MS-222. Therefore, cooling anesthesia is not recommended for invasive procedures, and although it is a convenient method to immobilize fish, it is almost certainly not an effective anesthetic per se. In contrast, rapid chilling induces a lethal shock presumably due to disruption of osmoregulatory functions (Ross and Ross 1999).

Methods of Zebrafish Euthanasia

In the context of the most recent AVMA guidelines, euthanasia is determined to be "the act of inducing humane death in

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an animal" (AVMA 2007), one that involves minimal pain, distress, and discomfort. Euthanasia occurs through "three basic mechanisms: (1) hypoxia, direct or indirect; (2) direct depression of neurons necessary for life function; and (3) physical disruption of brain activity and destruction of neurons necessary for life" (AVMA 2007, 5).

Table 1 lists combined information from Appendix 1 and Appendix 2 of the 2007 AVMA *Guidelines on Euthanasia* and the following, currently acceptable methods for fish, which includes, but does not specifically mention, zebrafish. Consequently, several methods and agents listed, such as barbiturates, benzocaine hydrochloride, carbon dioxide, inhalant anesthetics, and 2-phenoxyethanol, have not been extensively tested in zebrafish.

MS-222 is by far the most common agent used to euthanize zebrafish. Overdose of MS-222 (200-300 mg/l) is accomplished by prolonged immersion and subsequent death by hypoxia. Adult fish should be left in the solution for at least 10 minutes following cessation of opercular movement (Harper and Lawrence 2011; Westerfield 2007).

Another euthanasia method uses MS-222 as an anesthetic (0.168 mg/ml), before euthanasia by immersion of the animal into liquid nitrogen (Westerfield 2007). This method is necessary, for example, for RNA isolation and preparation or other methods where it is important to preserve the status quo at time of death by preventing rapid degradation of susceptible biological material.

It is interesting to note that although the use of carbon dioxide is an AVMA-approved method of euthanasia for fish, it is slow acting and stressful (Marking and Meyer 1985). In fact, the Canadian Council on Animal Care guidelines on the care and use of fish in research, teaching, and testing state that the use of carbon dioxide is not an acceptable method of euthanasia (CCAC 2005).

Performance-Based Approaches for Euthanasia

The *Guide for the Care and Use of Laboratory Animals* (NRC 2011) emphasizes and even encourages performance-based standards, which allows institutions to develop and define their own goals, the methods for achieving those goals, and the means for evaluating them. This approach is particularly useful for zebrafish users, who have few engineering standards to follow.

The use of professional judgment is also critical in the development and assessment of performance-based methods. The AVMA panel states: "The panel is aware that circumstances may arise that are not clearly covered by this report. Whenever such situations arise, a veterinarian experienced with the species should use professional judgment and knowledge of clinically acceptable techniques in selecting an appropriate euthanasia technique. Professional judgment in these circumstances will take into consideration the animal's size and its species-specific physiologic and behavioral characteristics. In all circumstances, the euthanasia method should be selected and used with the highest ethical standards and social conscience" (AVMA 2007, 4).

For euthanasia of zebrafish, the performance goals are similar to the AVMA criteria described above, such as rapid loss of consciousness and death, minimization of pain and distress, reliability, safety of personnel, and so on. We will discuss three different methods of zebrafish euthanasia demonstrating this performance-based approach (Blessing et al. 2010; NIH 2009; University of Oregon 2007; Wilson et al. 2009; Varga et al., unpublished study). In all cases, the means for evaluating these different methods are death as evidenced by cessation of opercular movement and heartbeat, gross body movements, righting equilibrium, and responsiveness to touch or startling stimuli.

It is important to note that for all of the following performance-based approaches for euthanizing zebrafish by submersion in ice water (2-4ºC), the fish do not come into direct contact with the ice (please refer to protocol on fish species euthanasia at the end of this article).

Euthanasia of Adults: Immobilization by Cooling Followed by Maceration

The immobilization of zebrafish adults by submersion in ice water (2-4ºC) immediately followed by cranial maceration via an in-sink garbage disposal causes immediate destruction of all brain function and activity (Matthews et al. 2002; University of Oregon 2007; Varga et al., unpublished study). Immediate analysis of the tissue from adult zebrafish that was placed in the garbage disposal showed that none of the adult zebrafish survived and that the largest piece of ground up tissue found was about 0.5 in in diameter, with the majority of tissue substantially smaller. However, this method was not effective for zebrafish embryos or early stage larvae (University of Oregon 2007; Varga et al., unpublished study). This method meets several of the criteria specified in the AVMA guidelines. Specifically, these guidelines state: "Physical disruption of brain activity, caused by concussion, direct destruction of the brain, or electrical depolarization of neurons, induces rapid loss of consciousness" (AVMA 2007, 6). Although this method is not listed as one of the physical methods of cranial concussion, because death is almost instantaneous, it is consistent with the definition of euthanasia. This physical method is also consistent with the new physical method of maceration for chicks recently listed in the AVMA guidelines.

Public perception of this method was discussed during IACUC meetings (University of Oregon 2007; Varga et al., unpublished study). We recognize, as do the AVMA guidelines, that "[s]ome consider physical methods of euthanasia aesthetically displeasing. There are occasions, however, when what is perceived as aesthetic and what is most humane are in conflict" (AVMA 2007, 13). Because this physical method produces rapid loss of consciousness and subsequent death, thereby minimizing pain, distress, or discomfort to the animal, we believe it to be an acceptable euthanasia technique for adult zebrafish.

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Table 1 Summary of AVMA *Guidelines on Euthanasia*

Agent	Classification	Mode of action	Rapidity	Ease of performance	Safety for personnel	Efficacy and comments
Barbiturates	Hypoxia attributable to depression of vital centers	Direct depression of cerebral cortex, subcortical structures, and vital centers; dire depression of heart muscle	Rapid onset of anesthesia	Animal must be restrained; personnel must be skilled to perform intravenous injection	Safe except human abuse potential; Drug Enforcement Agency–controlled substance	Highly effective when appropriately administered; acceptable intraperitoneal in small animals and intravenous
Benzocaine hydrochloride	Hypoxia attributable to depression of vital centers	Depression of central nervous system	Very rapid depending on dose	Easily used	Safe	Effective but expensive
Carbon dioxide	Hypoxia attributable to depression of vital centers	Direct depression of cerebral cortex, subcortical structures, and vital centers; dire depression of heart muscle	Moderately rapid	Used in closed container	Minimal hazard	Effective, but time required may be prolonged in immature and neonatal animals
Inhalant anesthetics	Hypoxia attributable to depression of vital centers	Direct depression of cerebral cortex, subcortical structures, and vital centers	Moderately rapid onset of anesthesia, excitation may develop during induction	Easily performed with closed container	Must be properly scavenged or vented to minimize exposure to personnel	Highly effective provided that subject is sufficiently exposed; either is conditionally acceptable
2-Phenoxyethanol	Hypoxia attributable to depression of vital centers	Depression of central nervous system	Very rapid depending on dose	Easily used	Safe	Effective but expensive
Tricaine methanesulfonate (TMS, MS-222)	Hypoxia attributable to depression of vital centers	Depression of central nervous system	Very rapid depending on dose	Easily used	Safe	Effective but expensive
Decapitation[a]	Hypoxia due to disruption of vital centers	Direct depression of brain	Rapid	Requires training and skill	Guillotine poses potential employee injury hazard	Irreversible; violent muscle contraction can occur after decapitation

Composite of 2007 AVMA *Guidelines on Euthanasia* Appendix 1 and Appendix 2: Characteristics and Modes of Action. Some of these descriptions appear to be more applicable to warm-blooded species but can be generalized to cold-blooded species such as fish.
[a]Conditionally acceptable based on 2007 AVMA guidelines.

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Cooling as an Initial Method for Immobilization

Cooling as a method for anesthesia for fish has been studied and used for decades. The reference used to support the AVMA panel's statement that "there is no evidence that whole body cooling reduces pain or is clinically efficacious" is from the article "Evaluation of Hypothermia for Anesthesia in Reptiles and Amphibians" (Martin 1995, 187). In his review, Martin lists several references that characterize the "anesthetic aspects of hypothermia in mammals very well" and states that "[s]ome aspects of hypothermia in ectotherms have been described"; he goes on to list several studies involving reptiles and amphibians but not a single reference is given for fish. Martin (1995, 188) further states, "The critical question whether hypothermia is able to render ectotherms unconscious prior to inducing peripheral neuromuscular blockade has neither been established nor adequately addressed in the literature." He subsequently describes EEG studies involving tortoises and lizards, but he does not describe a single fish study. The statement that the AVMA panel uses comes from the last statement Martin makes: "Amphibians and reptiles include thousands of very diverse species. Although the available articles related to the subject are inadequate for such a large and diverse group, they generally do not support hypothermia as a clinically efficacious method of anesthesia." This comment does not pertain to fish, yet the AVMA panel broadened its application by applying it to all ectothermic species.

The American Fisheries Society, the American Institute of Fishery Research Biologists, and the American Society of Ichthyologists and Herpetologists (Nickum et al. 2004) have published *Guidelines for the Use of Fishes in Research*. These guidelines "were developed to provide a structure that ensures appropriate attention to valid experimental design and procedures while also ensuring humane treatment of the experimental subjects." In addition, "Policies, regulations, and recommendations developed for research on mammals, birds, reptiles, or even amphibians are frequently inappropriate for research on fishes. These guidelines provide recommendations that address the ethical concerns that underlie guidelines for other vertebrates while recognizing the unique nature of fishes" (Nickum et al. 2004, 6). And finally, the Uses of Fish in Research Committee (Nickum et al. 2004, 6) "suggests that these Guidelines should be endorsed and adopted (adapted, where necessary) by those state and federal agencies with regulatory responsibilities for fishes as well as by universities and research institutions."

The American Fisheries Society and colleagues (Nickum et al. 2004, 40) guidelines state: "Hypothermia and exposure to sublethal levels of carbon dioxide (a Low Regulatory Priority drug, see below) have been used in situations where anesthetics were contraindicated." The use of cold shock as a method of immobilization, immediately followed by cranial maceration via an in-sink garbage disposal, is also supported by the following paragraph from the American Fisheries Society and colleagues (Nickum et al. 2004, 40) guidelines: "Pithing, spinal cord dislocation, or decapitation generally are acceptable methods, provided the procedure is performed quickly and accurately. Small fish, less than 10 cm in length, may be euthanized instantly by immersion in liquid nitrogen. Depending on the size of the fish and experimental needs, some form of physical anesthesia, such as hypothermia, may be indicated prior to euthanasia. Cold shock and electrical shock are used commonly by fish processors preparing large numbers of animals for slaughter."

In addition, there are many other studies that support cold anesthesia for fish and support its efficaciousness for other activities such as handling and transportation (Chung 1980; Hovda and Linley 2000; Mittal and Whitear 1978; Yoshikawa et al. 1989). Although we are not proposing the use of hypothermia as a method of anesthesia for zebrafish, we are pointing out that cooling or hypothermia has been used as a simple method of short-term immobilization and anesthesia and that these studies are contrary to the statement from the 2007 AVMA guidelines.

Hypothermal Shock or Rapid Cooling for Adults, Larvae, and Embryos

Because immobilization by cooling followed by cranial concussion did not euthanize embryos or larvae effectively, we investigated using hypothermal shock alone as a means for euthanasia and compared it with MS-222 (University of Oregon 2007; Varga et al., unpublished study). This comparison was also performed by Wilson and colleagues (2009) using zebrafish adults only.

Zebrafish Adults

For zebrafish adults, hypothermal shock or rapid cooling is a more effective euthanasia method than an overdose of MS-222. Hypothermal shock is less stressful, produces death more rapidly as determined by the cessation of vital signs (opercular movement, righting equilibrium, and heartbeat), is more consistent, is easier to perform, and is safer for personnel than the use of MS-222 (University of Oregon 2007; Varga et al., unpublished study; Wilson et al. 2009). Histopathological analysis of adult zebrafish tissue showed no evidence of ice crystal formation (University of Oregon 2007; Wilson et al. 2009; Varga et al., unpublished study). These results are consistent for other warm water–adapted species (Blessing et al. 2010).

Zebrafish Larvae and Embryos

For 5- to 6-day-old (early stage) larvae and 1- to 2-day-old embryos, the use of MS-222, even at much higher concentrations (up to 1000 mg/L) is not an effective euthanasia method (University of Oregon 2007; Varga et al., unpublished study). Presumably this is because the normal cause of death for adult fish when using MS-222 is by asphyxiation

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due to the drug's ability to block gill ventilation, which leads to hypoxemia and eventually death. Zebrafish and other fish larvae use cutaneous gas exchange for their oxygen demands while their gills are still developing (Rombough 2007).

Hypothermal shock for these early-stage larvae is still an effective method of euthanasia as long as the larvae are exposed for at least 20 minutes. However, for 1- to 2-day-old embryos, rapid chilling is not an effective euthanasia method, taking at least 5 to 14 hours of exposure to ensure death (University of Oregon 2007; Varga et al., unpublished study). Because zebrafish 1- to 2-day-old embryos are not rapidly and effectively euthanized by hypothermal shock, we add chemicals, such as isopropyl alcohol or bleach, after rapid immersion in ice water to hasten their death.

Evaluation of Hypothermal Shock as a Performance-Based Euthanasia Method

The concern of whether or not fish possess the capacity to feel or experience pain is a current, hot topic (Braithwaite and Boulcott 2007; Chandroo et al. 2004a,b; Rose 2002; Sneddon 2009). Consistent with the *Guide* and US government principle IV, we should give the benefit of the doubt to fish and assume, at least for adults, that they possess the ability to perceive pain, distress, and discomfort.

To better understand whether or not hypothermal shock alone meets the criteria of a suitable euthanizing method, the first two criteria of the AVMA *Guidelines on Euthanasia* (2007) need to be considered: (1) ability to induce loss of consciousness and death without causing pain, distress, anxiety, or apprehension; and (2) time required to induce loss of consciousness. Because consciousness, pain, distress, anxiety, and apprehension all depend on a functioning nervous system, it is necessary to understand the relationship between thermal stress and its effect on the nervous system.

Elliott (1981, 209) defined thermal stress as "any temperature change that produces a significant disturbance in the normal functions of a freshwater teleost and thus decreases the probability of survival." Obligate poikilotherms (ectotherms), zebrafish, and most fish (except for some tunas and lamnid sharks) do not possess the ability to maintain body temperature independently of their environment. When the environmental temperature changes, thermal stress is affected by many variables, but the most important ones are the temperature of exposure, the length of time, and the previous acclimation temperature (Elliott 1981). The time it takes to reach equilibrium between body temperature and the environment is dependent on many factors, such as water movement and the shape, size, and activity of the fish. Approximately 70-90% of the heat transfer occurs through the body wall. Nevertheless, the rate of gill ventilation and blood flow do play some role and should be also considered (Elliott 1981).

There is a plethora of data that demonstrate the decrease in nerve activity with decreasing temperature for mammals (Martin 1995), invertebrates (Westerfield 1978), and fish (Harper et al. 1990; Prosser 1965). Prosser (1965) studied the peripheral nerves of catfish and showed that nerves from catfish (*Ictalurus melas*) that were acclimated at 24ºC were blocked at 3ºC, whereas nerves from those fish that were acclimated at 10ºC were blocked at 1ºC.

Several studies demonstrated a linear relationship between the critical thermal methodology (CTM), which can be defined as the endpoint at which the fish loses its equilibrium and locomotor abilities or even dies and the previous acclimation temperature. Currie and colleagues (1998) studied largemouth bass (*Micropterus salmoides*), channel catfish (*Ictalurus melas*), and rainbow trout (*Oncorhynchus mykiss*) acclimated at three different temperatures (20ºC, 25ºC, and 30ºC for bass and catfish; and 10ºC, 15ºC, and 20ºC for trout) and found that all three species of fish showed a positive linear relationship between acclimation temperature and critical thermal methodology minima and maxima—increasing critical thermal methodologies for increasing acclimation temperatures (Currie et al. 1998). Bennett and Judd (1992) studied the low temperature tolerance of Texas pinfish (*Lagodon rhomboids;* 2.9-9.3 cm) using death as the critical thermal methodology endpoint and found a positive linear relationship between critical thermal methodology and acclimation temperature.

Other studies with goldfish (*Carassius auratus auratus*) and bluegills (*Lepomis macrochirus*) showed that blocking temperatures for conditioned and learned responses are higher than blocking temperatures for simple reflexes (Roots and Prosser 1962). Previous studies also demonstrated the effect of two different acclimation temperatures (8ºC and 28ºC) on vagus nerve conduction, membrane fluidity, and lipid composition of brain tissue and found that conduction velocities of the vagus nerve were actually faster in cold water–acclimated fish (Harper et al. 1990). This suggests that specific adaptive mechanisms are activated in fish in response to prolonged temperature changes—for example, due to the season—and these mechanisms help the fish to cope with the changed environment.

Stauffer and colleagues (1988) acclimated blue tilapias (*O. aureus*, 12-20 cm) to 15ºC and 20ºC for at least 5 days and then subjected them to three patterns of temperature reduction: (1) rapid cold shock; (2) an intermediate temperature drop of −3ºC per hour; and (3) a slow temperature drop of −1ºC per hour. They found that cold shock susceptibility is dependent on both acclimation temperature and rate of cooling.

These studies all support our hypothesis that warm water–acclimated (28.5ºC) zebrafish, with their small body size and weight and, hence, large surface-to-volume ratio, loose heat rapidly when transferred to ice water (2-4ºC) and do not have the physiological tolerance limits or adaptation mechanisms to adjust to rapid exposure to ice water (2-4ºC). This is directly supported by the results of hypothermal shock experiments with adults (University of Oregon 2007; Wilson et al. 2009; Varga et al., unpublished study) and larvae (University of Oregon 2007; Varga et al., unpublished study). All vital signs and their responsiveness to

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touch, nociceptive stimuli, or startling stimuli ceased within 20 seconds. Hence, we infer that their ability to sense or perceive pain, discomfort, or distress also ceased within the same time after exposure to ice water.

A final question is whether or not the brief period of exposure to ice water prior to death from hypothermal shock—those 20 to 30 seconds—is potentially stressful or painful. As mentioned earlier, the stress response in fish can be generally categorized into three physiological stages: primary, secondary, and tertiary (Casebolt et al. 1998; Iwama et al. 2004; Wedemeyer 1996; Wedemeyer and McLeay 1981; Wedemeyer et al. 1990). The time course for the primary physiological responses (increase in cortisol and catecholamines in the blood stream) to an acute stressor happens rapidly (within the first minute) and is, of course, dependent on the type, intensity, and duration of the stressor as well as the species of fish, genetics, rearing history, and nutritional status (Iwama et al. 2004; Wedemeyer et al. 1990).

To date, no one has measured the acute primary physiological stress response for zebrafish euthanized by hypothermal shock. Others, however, have begun to utilize newer technologies to study the stress response of fish using cold shock as a stressor. Note, however, that these studies involve changes in temperature within the animal's thermal limits.

Tanck and colleagues (2000) studied the stress response of cold shock in carp by measuring plasma cortisol, glucose, and lactate after single or multiple temperature drops. Plasma cortisol levels increased with all temperature changes and peaked 20 minutes after cold shock. Stress-related metabolic changes were observed, and plasma glucose levels remained unchanged (Peeters et al. 2001; Tanck et al. 2000; van den Burg et al. 2005, 2006). To study the effects of sublethal cold stress, others have begun to use functional magnetic resonance imaging technology in addition to radioimmunoassays (Peeters et al. 2001; van den Burg et al. 2005, 2006). van den Burg found that a rapid 10ºC temperature drop caused a general decrease in cerebral blood volume throughout the brain and activated the primary secondary stress response and brain centers that induce motor activity (van den Burg et al. 2005, 2006). A more extreme temperature drop, such as the hypothermal shock we use for euthanasia, presumably means that zebrafish lose consciousness rapidly because of even more dramatically decreased blood supply to the brain. This rapid change presumably also prevents the primary stress response from eliciting any secondary responses.

Consistent with this idea and the above studies, we observed a brief reaction in adult zebrafish in the first 5 seconds when immersed into ice water (2-4ºC). Most become instantly immobilized; if any movement exists at all, it stops abruptly, and the fish lose all vital signs within 20 seconds after immersion. In comparison, netting as an acute stress stimulus leads to increased whole body cortisol levels over the time course of a few minutes (Ramsay et al. 2009b). It is possible that some fish are immersed into the ice water in a stressed state due to netting or transportation stress. In a few cases, we observed that fish struggle or attempt to escape, and this response can be attributed to a heightened state of alert. However, these fish also stop all movement within seconds. Overall, unless the fish enters the ice water (2-4ºC) in an acutely stressed state, the time in the ice water is too brief and the primary and secondary response in zebrafish too slow to account for an acute stress response. Cold-shocked zebrafish lose all vital signs, including reflexes (neural activity), in less than 20 seconds, whereas cortisol levels have been shown to peak in the range of minutes (Ramsay et al. 2009a). However, this emphasizes the importance of transferring fish swiftly into the ice water and keeping them stress-free immediately before doing so.

Conclusions

For the forseeable future, the use of zebrafish in biomedical research will continue to expand. Concomitantly, research on their husbandry, management, and veterinary care is also needed to provide more humane and consistent care and minimize variability, which could affect research results. Methods of anesthesia and methods of euthanasia are two critical areas that require the attention and care of animal care technicians, researchers, and veterinarians alike to ensure compliance and humane treatment. In addition, the *PHS Policy on the Humane Care and Use of Laboratory Animals,* the *Guide for the Care and Use of Laboratory Animals*, guidance from the NIH Office of Laboratory Animal Welfare, and the *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* allow for, and even encourage, institutions to develop their own performance-based standards. These standards should support the scientific objectives, support the health and welfare of animals, and include a justified performance-based index and the associated outcome criteria (OLAW 2012). Professional judgment and expertise, input from all stakeholders, and oversight from the IACUC are all critical for the success of performance-based standards.

We have summarized some of the currently accepted methods of anesthesia and euthanasia for zebrafish and provided some performance-based approaches whose criteria are clearly defined and based on acceptable guidelines and principles, such as those prescribed by AVMA (2007), and centered around the ability to induce loss of consciousness and death without causing pain, distress, anxiety, or apprehension.

The specific agents and methods used for anesthesia and euthanasia will also depend on the scientific objectives of the research, the developmental stage of the zebrafish, and the effects of the agents or methods on specific organ systems under study.

Protocols: Fish Species Euthanasia at the University of Oregon

Purpose: To describe the methods of fish euthanasia approved by the University of Oregon Institutional Animal Care and Use Committee.

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Responsibilities: All University of Oregon faculty, staff, researchers, and students who use or care for laboratory fish.

Procedures

Background: Small fish species (2-6 cm in length) typically used in laboratory research include zebrafish, spotted *Danio*, medaka, Mexican tetra, fathead minnow, goldfish, swordtail, and platyfish, among others. Large fish species (>6 cm in length) typically used include three-spined stickleback, salmon, trout, tilapia, catfish, hybrid-striped bass, bass, bluegill, sturgeon, and others.

Policy: Tricaine methanesulfonate (MS-222) and hypothermic shock should be used as the methods of choice for the euthanasia of all fish species. The MS-222 dosage required for euthanasia is higher than the anesthetic dose but varies greatly with species, size, and water temperature. In addition, a longer exposure time (relative to anesthesia) to the chemical agent or ice cold water is required to ensure that death occurs. Fish should be removed only after 10 min have passed since their last observed opercular movements (respiration).

Approved Methods

Euthanasia methods for fish include:

1. Overdose using MS-222
Fish should be removed only after 10 min have passed since their last observed opercular movements (respiration) occurred.
Note: Fish euthanized with MS-222 cannot be used for human consumption.

2. Hypothermal shock for tropical species
Because warm water–adapted, tropical fish species (e.g., zebrafish, medaka, and platyfish) have minimal to no physiologic adaptation mechanism for adjusting to cold (4°C) water, cooling to 4°C should be considered an acceptable method of euthanasia because the rapid decrease in temperature from 26°C (or higher) to 4°C induces rapid loss of consciousness and is lethal to these species. Fish euthanized by this method should not come in direct contact with the ice because this may cause thermal burns and induce pain, but rather the ice should be added to a lowered amount of water for a contact time of 20 min.

3. Anesthesia induced using MS-222 followed by rapid immersion in liquid nitrogen
Acknowledging that rapid freezing of tissue in liquid nitrogen will inhibit RNA degradation and noting that the Amphibians, Fish, and Reptiles section, Two-Stage Euthanasia Procedures subsection of the AVMA *Guidelines on Euthanasia* (2007) states: "Quick freezing of deeply anesthetized animals is acceptable," deeply anesthetizing fish using MS-222 and then immersing them into liquid nitrogen should be considered an acceptable method of euthanasia.

Procedure Details

1. Euthanasia by Overdose of MS-222

Protocol

I. Make MS-222 euthanizing solution (300 mg/L concentration).
   1. Wear approved gloves.
   2. Make MS-222 stock solution by combining the following items in a dark glass bottle with a screw cap:
      - 400 mg MS-222 powder (Finquel/MS-222, Argent Laboratories)
      - 97.9 ml double distilled water
      - ~2.1 ml 1 M Tris (pH 9)
      (Source: Westerfield 2007)

      Adjust solution pH to 7.0.
      - Use 1N sodium hydroxide to raise solution pH.
      - Use 1N hydrogen chloride to lower solution pH.
   3. Add 7.5 ml of MS-222 stock solution to 100 ml fish water.
      If more volume is required to accommodate size of fish or number of fish, ensure concentration of MS-222 euthanizing solution remains the same.

II. Euthanize fish.
   4. Place fish to be euthanized into beaker of euthanizing solution.
      - Use clean net to remove fish from tank.
   5. Wait at least 10 min following cessation of opercular movement before removing fish from euthanizing solution.

III. Clean up.
   6. Pour euthanizing solution down sink drain. Follow with lots of water; allow tap to run for 2 min or more to dilute anesthetic thoroughly. For small fish, place dead fish down sink drain. Follow with lots of water; allow tap to run for 2 min or more. If in-sink garbage disposal is present, use disposal to macerate dead fish. For large fish, place dead fish in plastic bag and freeze. Contact the Office of Veterinary Services and Animal Care for proper disposal.

2. Euthanasia by Hypothermal Shock for Tropical Species

Note that experiments conducted at the University of Oregon have shown that although adult and larval (5 to 6 days post-fertilization) fish are effectively euthanized within 20 min of immersion in ice slush, embryos less than 2 days old require immersion in ice slush for 5 to 14 hours before death occurs. The chilling times in the protocol have been extended so that

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adults, larvae, and embryos are effectively euthanized with the same procedure. In all cases, the AVMA *Guidelines on Euthanasia* should be followed, and fish should remain in the euthanizing solution at least 10 min past cessation of opercular movement.

Protocol

1. Set up a temperature-insulated container with ice slush every morning (5 parts ice to 1 part fish water).
2. Form a depression in the ice to expose water.
3. Throughout the day, rapidly transfer embryos, larvae, or adult fish into the ice-chilled water.
4. Replenish ice if necessary to keep water chilled to 2-4ºC.
5. Add bleach (5.75% sodium hypochlorite) to the container (at least 500 mg/L final concentration; pH 7.0) and leave overnight.
6. Collect dead fish in net/sieve and disposes of dead animals according to local regulations. These may include sink garbage disposal or storage in freezer for incineration.
7. Set up container with fresh ice slush.

Additional Notes

To ensure optimal hypothermal shock:

1. Ensure very rapid transition from room temperature to 2-4ºC! For example, pour embryos and/or larvae into ice slush using only minimal volumes of water. Use a net to drop adults into chilled water. A gradual transfer to cold temperatures does not work and causes unnecessary distress for the fish!
2. Form a depression in the ice slush to expose chilled water. This ensures optimal exposure of the entire body to cold temperatures and will rapidly chill the fish. There is no morphological or microscopical evidence that fish develop "hypothermal burns" when they come in close contact with ice. However, being in full contact with ice-cold water ensures optimal and rapid hypothermal shock of the fish.
3. Ensure that the water temperature does not exceed 4ºC. A temperature probe or constant monitoring is not necessary as long as ice is present in the slush. If this is the case, the water temperature will be constant at 0-4ºC (latent heat stored in ice crystals). To ensure low temperatures throughout the day, add more ice as needed.

3. Euthanasia by Anesthesia Induced Using MS-222 Then Rapid Immersion in Liquid Nitrogen

Protocol

MS-222 Anesthesia Procedure

I. Make MS-222 anesthetizing solution (168 mg/L concentration).

1. Wear approved gloves.
2. Make MS-222 stock solution by combining the following items in a dark glass bottle with a screw cap:
   - 400 mg MS-222 powder (Finquel/MS-222, Argent Laboratories)
   - 97.9 ml double distilled water
   - ~2.1 ml 1 M Tris (pH 9)

   (source: Westerfield 2007)

   Adjust solution pH to approximately 7.0.
   - Use 1N sodium hydroxide to raise solution pH.
   - Use 1N hydrogen chloride to lower solution pH.
3. Add 4.2 ml of MS-222 stock solution to 100 ml fish water.

   If more volume is required to accommodate size of fish or number of fish, ensure concentration of MS-222 anesthetizing solution remains the same.

II. Anesthetize fish.

4. Place fish to be anesthetized into beaker of anesthetizing solution.
5. Wait for cessation of opercular movement before removing fish from anesthetizing solution. For example, normal wait time for zebrafish is 3 to 8 min after immersion in anesthetizing solution.

Note: Ensure fish is deeply anesthetized before continuing to immersion step.

III. Immerse fish in liquid nitrogen.

6. Place fish into cryo-safe tube then place tube into liquid nitrogen.
7. Wait 5 min or more.
8. Remove tube from liquid nitrogen and process tissue for RNA extraction or store tissue in −80ºC freezer.

IV. Clean up.

9. Pour anesthetizing solution down sink drain. Follow with lots of water; allow tap to run for 2 min or more to dilute the anesthetic.

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