Report of Workshop on Euthanasia for Zebrafish—A Matter of Welfare and Science

Almut Köhler,¹ Chereen Collymore,² Karin Finger-Baier,³ Robert Geisler,⁴ Larissa Kaufmann,⁵
Kieran C. Pounder,⁶ Stefan Schulte-Merker,⁷ Ana Valentim,^{8,9} Zoltan M. Varga,¹⁰
Jürgen Weiss,¹¹ and Uwe Strähle^{5,11}

Abstract

The increasing importance of zebrafish as a biomedical model organism is reflected by the steadily growing number of publications and laboratories working with this species. Regulatory recommendations for euthanasia as issued in Directive 2010/63/EU are, however, based on experience with fish species used for food production and do not take the small size and specific physiology of zebrafish into account. Consequently, the currently recommended methods of euthanasia in the Directive 2010/63/EU are either not applicable or may interfere with research goals. An international workshop was held in Karlsruhe, Germany, March 9, 2017, to discuss and propose alternative methods for euthanasia of zebrafish. The aim was to identify methods that adequately address the physiology of zebrafish and its use as a biomedical research model, follow the principles of the 3Rs (Replacement, Reduction, and Refinement) in animal experimentation and consider animal welfare during anesthesia and euthanasia. The results of the workshop are summarized here in the form of a white paper.

Keywords: zebrafish, euthanasia, rapid cooling, anesthetics, Directive 2010/63/EU, hypothermic shock

Introduction

IN RECENT YEARS, THE ZEBRAFISH (*Danio rerio*) has become increasingly important in biomedical research. This is reflected by the steadily growing number of laboratories and PubMed-listed publications using zebrafish as a model organism for human conditions and disease.¹ In the United Kingdom, zebrafish have become the most frequently used laboratory animal after the mouse in biomedical research laboratories.² The specific properties of zebrafish make it a particularly well-suited research organism for human diseases, including kidney diseases, cancer, diabetes, myopathies, blood disorders, heart diseases, addictions,

neural and retinal degenerative disorders, infectious diseases, and many others.

Standardization of experimental procedures is the key to the reproducibility and validity of scientific results. Given its popularity in biomedical research, it is essential to develop the best possible standardization of experimental procedures for zebrafish, just as it has been achieved previously for rodent laboratory species. One of the most pressing ethical and scientific questions regarding the 3Rs³ is which method to apply for the humane killing of zebrafish. According to Appendix IV of the “Directive 2010/63/EU for the protection of animals used for scientific purposes”⁴ implemented by the European Council, only “anaesthetic overdose,” “concussion/

¹Safety and Environment, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany.

²Division of Comparative Medicine, University of Toronto, Toronto, Canada.

³Department of Genes–Circuits–Behavior, Max Planck Institute of Neurobiology, Martinsried, Germany.

⁴European Zebrafish Resource Center, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany.

⁵European Society for Fish Models in Biology and Medicine, Institute of Toxicology and Genetics, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany.

⁶Institute of Integrative Biology, University of Liverpool, Liverpool, United Kingdom.

⁷Faculty of Medicine, Institute for Cardiovascular Organogenesis and Regeneration, University of Münster, Münster, Germany.

⁸IS—Instituto de Investigação e Inovação em Saúde and IBMC—Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal.

⁹CITAB—Centro de Investigação e Tecnologias Agroambientais e Biológicas, Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal.

¹⁰Zebrafish International Resource Center, Institute of Neuroscience, University of Oregon, Eugene, Oregon.

¹¹Institute of Toxicology and Genetics, Karlsruhe Institute of Technology (KIT), Karlsruhe, Germany.

percussive blow to the head,” or “electrical stunning” are considered legal procedures to humanely kill zebrafish. The latter two are unsuitable: Adult zebrafish only attain a total length of up to 3 cm, which does not permit accurate placement of a blow to the skull that would achieve unconsciousness. Owing to their small size, there is also no adequately researched and approved mechanism or equipment for the electrical stunning of zebrafish. As a last resort, anesthetic overdose raises serious questions regarding the potential interference of anesthetic agents with experimental conditions, potentially affecting postmortem analysis of central and peripheral nervous systems and their target tissues (depending on the type, concentration, and administration modalities of the anesthetic agent). Hypothermic shock, however, that is, killing by rapid cooling, is not mentioned in Annex IV and, therefore, many European authorities will not authorize this method at all or only in conjunction with various restrictions, even though this method has been approved by the American Association for Laboratory Animal Science and is recommended by the American Veterinary Medical Association for zebrafish in their 2013 edition “Guidelines for the Euthanasia of Animals.”⁵

In 2016, the European Society for Fish Models in Biology and Medicine (EUFishBioMed) carried out a survey among its members, indicating that killing of adult zebrafish is practiced in European laboratories ($n = 53$) in different ways (Fig. 1). Most institutions (77%) performed euthanasia by overdose of anesthetics. Even in cases wherein overdose interfered with the experimental outcome, many authorities did not permit the use of methods other than those mentioned in Annex IV of the Directive 2010/63/EU.⁴ The most frequently used anesthetic was MS-222/Tricaine/ethyl 3-aminobenzoate methanesulfonic acid, a sodium channel blocker. Rapid cooling in ice water was often only permitted in conjunction with prior application of anesthetics (29%), which was more humane but scientifically problematic. Only in relatively few cases (17%) authorities allow rapid cooling without prior application of anesthetics. These findings were similar to another international survey that was performed in early 2017.⁶

The Institute for Toxicology and Genetics and the European Zebrafish Resource Center at the Karlsruhe Institute of Technology (KIT) organized a workshop on the humane killing of zebrafish on March 9, 2017, to discuss zebrafish welfare and to evaluate best practices for anesthesia and euthanasia. The workshop was sponsored by the European Society for Fish Models in Biology and Medicine (EUFishBioMed). The overall aim was to discuss the various methods available and to propose methods of killing zebrafish that are humane and compatible with scientific investigation. The participation of 78 specialists, 24 of which came from European countries other than Germany, highlighted the timeliness and importance of this topic.

Workshop

During the first part of the workshop, speakers from the United States, Canada, United Kingdom, and Germany shed light on various aspects of zebrafish nociception, physiology, anesthesia, and euthanasia, and evaluated both the scientific merit of our state of knowledge in these areas and the impact of the information on the humane treatment of aquatic animals.

Almut Köhler, KIT’s officer for animal welfare and a veterinarian, addressed the physiological diversity of fish. Karin Finger-Baier, a scientist at the Max Planck Institute for Neurobiology, presented the current literature on the topic of hypothermia and rapid cooling, that is, the shock-like cooling in cold water of 2–4°C with focus on zebrafish (see Supplementary Appendix 1 for standard protocol; Supplementary Data are available online at www.liebertpub.com/zeb). She presented evidence about type IV antifreeze proteins, which are expressed in small tropical fish such as zebrafish and whose only currently known function is in development.⁷ Other data demonstrated that hypothermia also reduces oxygen transport to the central nervous system (CNS) due to vasoconstriction and resulting reduction of blood flow.⁸ She also compared the acceptance of rapid cooling as a method of zebrafish euthanasia in other countries, showing that it is accepted in the United

FIG. 1. Results from a survey carried out among 53 European institutions between July and August of 2016.

States and currently under consideration in Canada. In Europe, some countries such as the Netherlands have already moved ahead and accepted rapid cooling as a permissible alternative to the methods listed in Annex IV of the EU-Directive.

Kieran C. Pounder, a researcher in Lynne Sneddon's group at the University of Liverpool, gave a lecture on the current knowledge on nociception in fish.^{9–12} He showed that different types of nociceptors are present in fish, which respond to mechanical, thermal, and chemical stimuli. Importantly, no cold-sensitive receptors have been identified in any fish species to date.¹³

Zoltan M. Varga, Director of the Zebrafish International Resource Center in Eugene, Oregon, presented test results about the efficiency and reliability of rapid cooling in embryonic, larval, and adult zebrafish. He showed that zebrafish rapid cooling induced a 20 times faster cessation of vital signs in adults (10.6 ± 3.28 s) compared with MS-222 overdose (216.3 ± 61 s). Previous research showed that the efficacy of rapid cooling for euthanasia depends on the normal environmental temperature, which correlates linearly with the critical thermal minima and maxima of fish.¹⁴ In addition to the rapid transition into ice-chilled water, which rapidly induces unconsciousness, an extended chilling period was required to ensure death by hypoxia. For adults, 10 min exposure after cessation of opercular movements induced

euthanasia effectively. In comparison, larvae required a chilling period of at least 20 min in ice-cold water to ensure death.¹⁵ However, neither prolonged MS-222 anesthesia (overdose) nor hypothermic shock was effective euthanasia methods for embryos. Embryos and larvae that had been exposed to adult doses (168 mg/L) of MS-222 or higher (250 and 500 mg/L) for several hours were not euthanized effectively and continued to develop during exposure to the anesthetic. Similarly, hypothermal shock and extended chilling periods also did not induce euthanasia in embryos effectively, necessitating the use of additional euthanizing agents.

Chereen Collymore, a veterinarian at the University of Toronto who is trained in laboratory animal science with extensive experience in anesthetic procedures, described the advantages and disadvantages of substances available for zebrafish anesthesia and euthanasia.^{16–18} She summarized that essentially all substances applied to fish for anesthesia and euthanasia elicit behavior indicative of aversion and/or stress, leading to increased opercular movement rate, aversive behaviors (such as erratic swimming), and/or blood plasma cortisol levels.

Bettina Bert, a veterinarian specialist in pharmacology and toxicology at the German Federal Institute of Risk Assessment, explained the legal framework as well as the

TABLE 1. ADVANTAGES AND DISADVANTAGES OF DIFFERENT EUTHANASIA METHODS FOR ZEBRAFISH

	<i>Advantages</i>	<i>Disadvantages</i>
Concussion/percussive blow to the head	Fast No residues Simple device	Uncertain performance Infeasible to study the brain Not appropriate for zebrafish due to their small size Not applicable in embryos or larvae
Electrical stunning	Rapid and reliable	No adequate methods and devices available for zebrafish Muscle tissue potentially damaged by contortion Not applicable in embryos or larvae
Anesthetic overdose	Extensive experience available Many substances to choose from	Aversive reaction of animals (stress reactions) to virtually all tested substances Chemical contamination (potential chronic exposure) Environmental protection Impact on experiments Personnel safety Necessitates increased cleaning due to chemical residues (oily properties) increasing costs Effect/results slower than with other methods Difficult procurement and disposal for controlled substances Low effectiveness in embryos and larvae
Rapid cooling	Fastest procedure (seconds) Brief stress period Reliable (no recovery of vital functions) No chemical contamination (environment, personnel, animal, experimental safety) Safe to personnel Simple procurement and disposal	Not suitable for surgical interventions Might not be effective with all fish species, in particular larger species or nontropical species Not reliable as singular method in embryos

process of decision-making in the context of German and European legislation.

The individual presentations were followed by a panel discussion during which the speakers discussed the different aspects of the methods with the audience and answered questions. The time required for zebrafish to lose consciousness was as much discussed as the fundamental question what fish unconsciousness entails and how it could be determined. It was emphasized that the fish should not be put on ice or into crushed ice without water because of air pockets. Water is a better conductor of temperature than air, and complete exposure of the body surface to ice-cold water ensures the most effective transfer of cold to superficial and deeper tissues in the animal. The formation of ice crystals within the body does not occur with this method of rapid cooling.¹⁹ Concerning the painful sensation of cold burns, it should be considered that cold temperatures inhibit signal propagation and synaptic transmission in the CNS and painful sensation occurs when frozen tissue is thawed, however, not during the cooling process itself. Indeed, sports injuries are often treated with cold to diminish pain sensation and swelling of injured tissues. It was also discussed that immersing animals in anesthetic agents can cause various levels of distress and that different anesthetic methods can exert various levels of cortisol and behavioral stress responses in fish before inducing unconsciousness or death. It was stressed that application of anesthetics also needs to be safe for the animal care personnel and the researchers handling the substances daily, the over extended periods of which have been suggested to be harmful to humans. Lastly, several anesthetics (e.g., ketamine) are controlled substances, or have different levels of legal restrictions in different countries. Thus, standardization, availability, and procurement of appropriate, scientifically indicated, or humane substances can be problematic and differ between countries. The advantages and disadvantages of the different methods are summarized in Table 1.

Conclusion

The workshop led to the conclusion that although various methods of killing zebrafish humanely are possible, it is only the expert use of rapid cooling that eliminates the unwanted potential of pharmacological effects on experimental results, and minimizes distress due to the short time leading to unconsciousness. Compared with all other euthanasia methods,²⁰ rapid cooling allows the fastest possible death in adults and juvenile stages (from 14 dpf¹⁹) and in larvae (120 hpf–14 dpf).^{20,21} For embryos, the fastest and most efficient procedure is transfer into liquid nitrogen. For immature stages, up to 5–6 dpf, additional tissue dissociation with sodium hypochlorite, proteinase K, or similar substances is necessary to complete euthanasia.

An *ad hoc* survey among workshop participants suggested that the overwhelming majority were in support of rapid cooling as a humane and scientifically appropriate method of killing adult and larval zebrafish. The participants urge authorities to reconsider decisions and to add the hypothermal shock euthanasia method for zebrafish and other small tropical fish in a revision to Annex IV of the Directive 2010/63/EU.

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Disclosure Statement

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Address correspondence to:

Almut Köhler, PhD

Safety and Environment

Karlsruhe Institute of Technology (KIT)

Karlsruhe 76344

Germany

E-mail: almut.koehler@kit.edu

Prof. Dr. Uwe Strähle

European Society for Fish Models in Biology and Medicine

Institute of Toxicology and Genetics

Karlsruhe Institute of Technology (KIT)

Karlsruhe 76344

Germany

E-mail: uwe.straehle@kit.edu