Euthanizing zebrafish legally in Europe

Are the approved methods of euthanizing zebrafish appropriate to research reality and animal welfare?

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Zebrafish have become an increasingly popular model organism in basic biological research. According to the European Commission's latest report on the number of animals used in research in the EU, the number of fish increased by 28.5% from 2008 to 2011 (310,307 more fish) [1]. The UK Home Office reported that fish were the second most used group of animals in research in 2015 in the UK (14%, 294,000 procedures), with zebrafish representing 50% of all fish species [2]. The growing use of fish in research is often regarded as a major achievement to replace mammalian model organisms, notably rodents [3]. However, the increasing relevance of zebrafish as an animal model creates an urgent need for techniques and methods that guarantee that any research with zebrafish can be carried out according to the same scientific and animal welfare standards as the laboratory rodents they are often replacing.

Legal methods to euthanize fish

From the moment zebrafish become free-feeding larvae, any intervention on them is regulated by EU legislation. This means that any procedure done in these animals from about 5 days after fertilization must comply with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes [4]. This includes euthanizing animals, longer needed, whose suffering needs to be ended or whose organs are collected for further analysis. The European Directive lists only three methods for humanely killing fish: anesthetic overdose, concussion, or electrical stunning. Other methods can be used in unconscious animals.

Each of these methods has clear limitations for research and for animal welfare. The zebrafish's small size makes efficient and controlled concussion or electrical stunning difficult to perform. Concussion severely damages the brain, which precludes it for most neuroscience research. This method also has a high margin for error, aggravated by the fact that it is perceived as very unpleasant for the operator. Electrical stunning requires a current high enough to induce unconsciousness and death, which

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causes strong muscular contractions that can damage the fish body, break bones, and cause hemorrhages. It may therefore not be suitable for morphological and histological studies. In addition, if an electrical current that does not induce stunning is used and is applied for a long time, it will cause total muscle exhaustion and the fish will be incorrectly considered unconscious. This leaves anesthetic overdose, which does not induce gross anatomical modifications and is apparently less stressful. However, it interferes with biochemical analysis of the nervous system and metabolizing organs.

All techniques approved by the Directive therefore limit postmortem analysis of fish tissue. In contrast, there is a much wider choice of approved methods for euthanizing rodents. Six different chemical (anesthetic overdose and other gases) and physical (cervical dislocation, concussion, and decapitation) methods are listed. This allows researchers to select the most adequate method for scientific and animal welfare purposes, compared to the limited options accepted by law for fish.

Does this discrepancy result from an overall different level of protection for rodents than for fish? This is a tempting interpretation based on the stronger emotional bond that we usually have with mammals compared with fish. But in reality, whereas European legislation definitely gives a higher level of protection to some species (notably non-human primates), no such distinction is made between fish and rodents: They are both equally protected by the EU Directive. Instead, the difference seems to stem from the process to establish acceptable methods of killing. While rodents have been considered a laboratory animal for a long time, fish traditionally belong to the domain of the food industry. This would explain why legislation regulating how to treat fish in research seems to be primarily based on knowledge from European aquaculture and fishery that involves many species very different from zebrafish. Given how highly heterogeneous fish are from cold to temperate waters, freshwater to saline water, different sizes, living in different depths of water, and so on it is obvious that one size will not fit all. Procedures that may be adequate for the small tropical zebrafish are not suitable for larger animals living in cold waters, such as salmon, and vice versa.

Rapid Cooling

The previous directive, Council Directive 86/609/FEEC, only required that all humane methods of killing animals should be performed by a trained person/expert. In the past, researchers often used rapid cooling to kill fish, which is easy to perform, inexpensive, and does not cause gross biochemical or physiological alterations that would impede postmortem analysis. This method requires the decrease in the water temperature to 2-4°C by adding ice and by preventing that the fish touch the ice before they become unconscious.

Under the present legislation, this method requires approval by the competent authority of the country where the research is performed (Directive 2010/63/EU, article 6, point 4). Any application must acknowledge that the use of the method requested is considered to be at least as humane as the accepted ones or that the purposes of the experiment cannot be achieved by using any of the approved methods. The competent authorities of various countries have actually approved a large number of requests to use rapid cooling. For example, in Germany, a special permit to use the rapid cooling method was requested and accepted at regional level. The rationale for it lies in the practicability of the method, the lack of biochemical and histological postmortem alterations, and the absence of evidence that it is problematic in terms of animal welfare. Indeed, the few scientific studies available advocate rapid cooling as a better method than MS222 overdose. The

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histopathological alterations were similar in both methods with no formation of ice crystals, while rapid cooling induced a quicker and irreversible death with less observable signs of distress in adults [5].

In regard to fish larvae, studies show that 14 days postfertilization larvae exposed to MS222, eugenol, or rapid cooling for 75 min had a 0% recovery rate with the cooling method only [6]. Actually, anesthetic overdose by immersion works by hypoxia, but since larvae breathe mainly through a high surface compared with the gills in adults, it makes them highly resistant to hypoxia. Thus, rapid cooling also seems to be more effective to kill young larval forms of zebrafish than anesthetics.

Why then is rapid cooling not included in the Directive's list of approved killing methods for fish? Most likely, any misgivings about rapid cooling as a humane euthanasia method stem from generalizations from species used in aquaculture, where the method has been less effective in killing large and/or cold-adapted species [7]. In addition, the developmental stage and differences in morphology and physiology will affect how fish of different species react to various methods for euthanasia.

Expanding methods list

With increasing requirements to detail all procedures performed in animal experiments, the use of rapid cooling to kill fish will be mentioned in scientific publications. This will look like European researchers do not comply with legislation, and, perhaps even worse, it will raise wrong ideas that methods that are not considered humane by law are being used to kill fish. This comes at a time when highly vociferous critics of animal research challenge European legislation and ask for a complete ban on the use of animals in research [8]. It would therefore be more prudent for all involved in animal research- competent authorities, researchers, and zebrafish- to update Annex IV on the approved methods for killing animals and add rapid cooling as an acceptable method for zebrafish; US recommendations have already changed in this regard [9].

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REFERENCES

1. European Commission (2013) Seventh Report on the Statistics on the Number of Animals Used for Experimental and Other Scientific Purposes in the Member States of the European Union. Brussels

2. Home Office (2015) Annual Statistics of Scientific Procedures on Living Animals, Great Britain, London, UK: The Stationery Office

3. Stewart AM, Braubach O, Spitsbergen J, Gerlai R, Kalueff AV (2014) Zebrafish models for translational neuroscience research: from tank to bedside. Trends Neurosci 37: 264278

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4. Strahle U, Scholz S, Geisler R, Greiner P, Hollert H, Rastegar S, Schumacher A, Selderslaghs |, Weiss C, Witters H et al (2012) Zebra- fish embryos as an alternative to animal experiments-a commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod Toxicol 33: 128-132

5. Wilson JM, Bunte RM, Carty AJ (2009) Evaluation of Rapid Cooling and Tricaine Methanesulfonate (MS222) as Methods of Euthanasia in Zebrafish (Danio rerio). / Am Assoc Lab Anim 48: 785-789

6. Strykowski JL, Schech JM (2015) Effectiveness of recommended euthanasia methods in larval zebrafish (Danio rerio).) Am Assoc Lab Anim 54:81-84

7. Roth B, Imsland AK, Foss A (2009) Live chilling of turbot and subsequent effect on behaviour, muscle stiffness, muscle quality, blood gases and chemistry. Anim Welfare 18: 33-41

8. European Commission (2015) Communication from the Commission on the European Citizen's Initiative "Stop Vivisection".

9. Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre CB, Gwaltney- Bran S, McCrackin MA, Meyer R (2013) AVMA guidelines for the euthanasia of animals: 2013 edition

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