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EUTHANASIA IN ZEBRAFISH: AN ISSUE TO BE DISCUSSED

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Abstract: *Danio rerio*, known as zebrafish, has become a widely used model in scientific research, with more than 5 million units used annually in 2017. Their popularity is due to their ease of handling, low cost and rapid reproduction, as well as the transparency of the embryos, which allows internal organs to be observed. However, the use of these fish raises ethical questions, especially in relation to animal welfare and pain during euthanasia and anesthesia procedures. The literature points to a growing concern about pain in zebrafish, since practices such as euthanasia, with methods approved by Directive 2010/63/EU, are still debated. MS-222 is widely used, but raises doubts about its efficacy and possible side effects. Alternative methods, such as rapid cooling and new anesthetics, are also discussed, revealing the need for more research into pain perception and the ethical management of zebrafish. The lack of consensus on the definition of pain in fish and the application of more humane practices are still crucial issues to be addressed in the scientific community.
Keywords: *Danio rerio;* zebrafish; pain; euthanasia

INTRODUCTION

Danio rerio (Hamilton-Buchanan, 1822), or commonly known as zebrafish, is one of the most used models in scientific research in recent years, in 2017 the annual number exceeded 5 million (Leyden et al., 2022; Lidster et al., 2017). Their intensive use is due to the fact that they are easier to manage than other models such as rats, as they have a short generation time, low housing costs, smaller size than others, are able to reproduce easily without human intervention, a pair of adult *Danio rerio* can have hundreds of embryos in one day, each with a complete cardiovascular system produced in two days, another attraction of embryos is their transparency, making it possible to observe the internal organs, development of tumors in adults (Bowley et al., 2021; Ohnesorge et al., 2021; da Silva et al., 2021; Ivanisevic; Want, 2019).

It's not hard to understand the expansive use of these animals and the ethical concerns within laboratories, which have been a recent topic of debate. In 2019, Message and Greenhough argued that in the UK fish were not viewed with the same empathy as other animals. Over time, this thinking has changed and interest in the cognitive responses and welfare of fish has gradually increased. At the time, the 3Rs (replace, reduce, refine) did not include them and there was limited consensus on which practices were applicable to zebrafish, while authors such as Safina (2018) argued that pain and fear were basic concepts for fish that had optimal social skills, such as interspecies cooperation, tool use.

Thinking about pain, there is a general definition of what it means for the scientific community, however, the interpretations of the definition are the most extensive, Sneddon et al. (2024) put that the severity of pain is based on duration, but intensity must also be considered and that in zebrafish, the measure is seen through their behavior (Hawkins et al., 2011). Ohnesorge et al. (2021) put forward the view of the International Association for the Study of Pain (IASP), which summarizes that pain is "an unpleasant sensory and emotional experience", but its debate is still under study, since it is not known which neurological structures in zebrafish process pain (Reilly et al., 2008).

Rose et al. (2012) went further, arguing that pain is an individual and subjective experience and cannot be measured or observed. The fact that fish have nociception is not a reliable indicator that they may have conscious or painful feelings, since they argue that for pain to exist there must be consciousness. The authors even gave an example of a surgery performed with the patient awake, with only

local anesthesia, where the nociceptors are activated, but the patient does not feel any pain, saying that it may be the same as what happens to zebrafish during pain experiments. Despite this view, it is necessary to understand what nociceptors are, because even though the debate and interpretation passes from author to author, these receptors are of great importance within fish management and it is through their activation that most research on pain in these animals is carried out (Ohnesorge et al., 2021). The process of capturing harmful stimuli is called nociception, these nociceptors are neurons with nerve endings capable of capturing harmful activities, an important reception channel is the TRP. Some of the members of the TRP are homologous to humans (Prober et al., 2008; Pan et al., 2012; Ohnesorge et al., 2021), in addition to also being normal in mammals, we can cite dopamine and serotonin as examples of neurotransmitters identical to these that in humans are associated with pain, hunger, thirst and fear (Rose et al., 2012; Sneddon, 2015; Safina, 2018; Ohnesorge et al., 2021).

Wahltinez et al. (2021) makes another point that a method that does not lead to rapid death or that causes trauma before unconsciousness cannot be considered euthanasia, so those that are considered are those that unconsciousness comes quickly, are capable of being reproduced in various laboratories, are not stressful and can be reversed (Close et al., 1997).

Thus, in this scientific review article, our main goal was to research the literature and analyze whether adult fish feel any pain during the euthanasia process, so that there is a more humane and ethical procedure in the context of zebrafish research, which is growing more and more every day.

MATERIAL AND METHODS

For a consistent qualitative analysis, we searched the Pubmed platform of the NIH (National Library of Medicine) using the words "zebrafish AND pain AND "euthanasia" and found only 2 articles from 2010 to 2025, so we removed the word euthanasia and the results in Pubmed were 265 articles, within this base we analyzed the abstracts, only about 100 articles made sense with the theme worked on. In Google Scholar, we searched for the words "adult zebrafish, pain, euthanasia". In order of relevance, we found 1,460 articles with a publication date from 2010 onwards, although within this database there were more than 1,000 references, we analyzed and used those that made the most sense with the research, excluding those that had already been read through the other platform.

With more than 100 articles in hand, we filtered out those that had new information compared to the others, new stunning methods, research on nocireceptors and that had experiments exclusively with *Danio rerio.* We also found some references from before 2010 cited in the articles we read, which we considered to be highly relevant.

RESULTS AND DISCUSSION

Methanosufonate tricaine, or just MS-222, is an anesthetic used exponentially for both anesthesia and euthanasia in *Danio rerio*. This is an ester-type agent, which acts when it is absorbed through the gills or skin, being distributed through the blood and thus acting on the muscle, because it is highly soluble, it reaches the muscle in such a way that the transmission of the action potential does not occur (Katz et al., 2020; Carter et al., 2011; Matthews and Varga, 2012; Spears et al., 2014; Topic Popovic et al, 2012) and, despite being considered safe and more suitable for such activities, the preference for its use is expressed by the ease of controlling anesthetic depth

and mortality through premedication, however, there are reports and studies regarding its side effects, such as the risk of mortality after repeated exposures and the need for analgesic drugs after painful procedures, also questioning whether the method is really painless to the model (Schroeder et al., 2021; Rácz et al., 2021; Martins et al., 2016; Lidster et al., 2017).

The same type of questioning occurs with another method widely used to euthanize fish: rapid cooling, known for not needing expensive equipment, but simply immersing the model at <4°C, causing them to die in a few minutes, although they are uncomfortable and the method is not valid for larvae (Schroeder et al., 2021; Wallace et al., 2018; Blessing et al., 2010; Wilson et al., 2009). It even divides opinion in legislation, not being allowed in Europe, but accepted by the AVMA (American Veterinary Medical Association) (da Silva et al., 2021).

There are no great doubts about environmental enrichment, feeding and stocking, but one topic that still causes debate and extensive study is euthanasia and anesthesia in this species, especially if the model feels pain during either of them, given the points of disagreement mentioned above. Nowadays, the discussion on euthanasia is based on better arguments. The rule according to Annex IV of Directive 2010/63/EU is that for fish the accepted methods are 'concussion/percussive blow to the head', 'electrical stunning' and 'anesthetic overdose', physical methods are alternatives to chemicals (Wallace et al., 2018; Wilson et al., 2009; Schroeder et al., 2021).

These physical methods, concussion and electrical stunning, both have their qualities, but always fall into the same concern of pain while unconsciousness is not achieved, in addition to stunning being approved by the Directive, but not commonly used in zebrafish laboratories (Mocho et al., 2022; Mocho and von Krogh, 2022; Lidster et al., 2017).

For chemical substances, the same Directive states that euthanasia requires an additional 10 minutes after stopping the opercular beat of the fish in the overdose solution and that physical means should be used to confirm death. von Krogh et al. (2021) state that, according to the literature, the lack of response occurs before the beat stops, this point is crucial and requires analysis (Ramos et al., 2021, Strykowski and Schech, 2015; Collymore et al., 2016, Close et al., 1997; Martins et al., 2016).

Even with so many counterpoints, the concern for animal welfare prevails in any study, it is worth mentioning that despite the numerous definitions of the term, the NFACC (National Farm Animal Care Council) (2021) puts it as "consideration of affective states, as well as health and biological functioning, and the display of normal and important behaviors", such characterization is common in other literature, such as Fraser's "three cycles of welfare" (2008) or the OIE's Five Freedoms concept of non-maleficence (2021) or more recently the Five Domains of beneficence for animals (Guirro, 2022), all of which converge on the point that bad feelings, be they pain, suffering or agony, do not classify welfare and show the capacity for sentience (Gaffney and Lavery, 2022).

In research published in the journal Nature, Saarinen et al. (2025) analyzed euthanasia by electric stunning, according to the authors, for the first time in a rearing tank, which will reduce the stress caused by the use of nets to transfer aquariums, handling and the condition of the water environment. They used 650 adult zebrafish for the study, varying the density of the fish and the volume of the aquariums, but in a single condition of electrical conductivity of the water, requiring further study , although for the larvae this type of euthanasia is effective after 30 minutes, guaranteeing brain death through calcium levels (Burkhardt et al., 2025).

Some other anesthetics are on the agenda as a replacement for MS-222, including isoflurane, isoeugenol and metomidate hydrochloride, which have been shown to be effective in sedating zebrafish during painful procedures (Weaver et al., 2024; Collymore et al., 2014). Ferreira et al. (2022a) carried out tests using propofol +lidocaine, clove oil, MS 222 and etomidate, with the latter, the animals acted differently before and after anesthesia, which makes room for the conclusion of an aversion and stress for this substance. Confirming this result, Ferreira et al. (2022b), in an experiment with the same substances studied above and including rapid cooling, observed that etomidate was the compound that took the longest to stop opercular movement and cooling the fastest, emphasizing that chemical methods can interfere in necropsy analyses. However, Schwartz et al. (2024) in an experiment with zebrafish concluded that etomidate caused minimal aversion, ease and safety, in addition to its high availability.

For all types of methods, we need to ask ourselves various questions: does the model feel pain before he loses his movements, and even when he has lost his movements? How sure can we be that it is in fact unconscious and no longer feels pain? Is it possible to consider only nociceptors as an indication of fish feeling this type of stimulus? Another viable question that opens up further research is whether the known methods cover both the biochemical profile of males and females, which differ in gender, size, lipid content and also in the aforementioned biochemical profile (da Silva et al., 2021; Ong et al., 2009; Saad et al., 2017).

As previously shown here, rapid cooling is accepted as a physical method, although not in all countries and is not recommended for zebrafish embryos under 3 dpf (American Veterinary Medical Association, 2020), as well as being uncomfortable for researchers, as there is a brief period of erratic hyper locomotion (Davis et al., 2022; Matthews and Varga, 2012). The discomfort occurs in the fish internally, with profound eye movements, heart rate and increased calcium levels in the foregut and hindbrain, indicating programmed cell death in the larvae, and may be more stressful and damaging than MS-222 (Leyden et al., 2022; Xu et al., 2017).

All the methods mentioned and analyzed here use their behavior as the main basis for understanding the zebrafish's pain, as well as the aversion test. Both techniques are the best indicators of pain that we currently have in fact, although improvement is necessary and growing, because yes, if a fish avoids a part of the tank or its swimming frequency changes, it means that it is under stress, but we can't say for sure if it is intrinsically linked to nociception, since everything is just a reproducible theory based on hypothesis, and can change depending on the population and strain of zebrafish (Taylor et al., 2017; Soares et al., 2019; Ohnesorge et al., 2021; Sneddon et al., 2024).

The use of nociceptor analysis as an indication of pain is also important, but if behavioral analysis divides opinion and is based on hypothesis, this type of analysis is even worse. Burgess and Burton (2023) sum it up by saying that pain requires processing in the forebrain, which nociception does not. The authors give a self-explanatory example of a decerebrate rat that responds normally to noxious stimuli, while showing no signs of fear, pain or distress (Woolf, 1984).

The experimental model of the zebrafish (*Danio rerio*) has established itself as a valuable tool in scientific research. Its expansive use in recent decades is justified by factors such as its rapid reproductive cycle, the transparency of its embryos and its ability to generate hundreds of embryos a day, as well as the similarity of its biological systems to those of humans, which makes it an efficient model for investigations related to physiology, development

and diseases (Bowley et al., 2021; Ohnesorge et al., 2021; da Silva et al., 2021). However, ethical concerns are a limiting factor in research, especially with regard to their pain and well-being in euthanasia.

The choice of euthanasia method in zebrafish is a topic of debate focusing on efficacy, safety and the possibility of causing suffering. Directive 2010/63/EU recommends methods such as concussion or percussive blows, electric stunning and overdose of anesthetics such as tricaine methanesufonate (MS-222) (Wallace et al., 2018).

Although MS-222 is widely used and considered effective, concerns about its long-term safety, such as the risk of mortality after repeated exposure and the need for additional analgesia after painful procedures are valid, so the research surrounding this topic is not complete and still generates insecurity about the anesthetic and its properties (Schroeder et al., 2021). Chu et al. (2020) carried out an experiment to replace MS-222 with propofol, even though it was not a drug permitted by the FDA, it was growing in scientific circles for the euthanasia of zebrafish because it has the ability to cause unconsciousness prior to cardiorespiratory arrest (Gholipourkanani and Ahadizadeh, 2013; Oda et al., 2014; Balko et al., 2017), the result was positive with immersion in 40mg/L for 20 minutes or 120 mg/L for 10 minutes. Davis et al. (2022), however, states that an exposure of zebrafish for 20 minutes has a failure rate of 18.75%, while the 30-minute exposure did not have the same rate and effectively euthanized all the fish tested without manifestation of aversion or acute pain.

Lidocaine has also been used for euthanasia in zebrafish, as it is safer, faster and less aversive (Davis et al., 2022; Collymore et al., 2014; Collymore et al., 2016), 2016), von Krogh et al. (2021) tested the same and found that 1 g/L of lidocaine with 2 g/L of NaHCO3 induces a loss of reflexes in 2 minutes, with lidocaine hydrochloride and ethanol being more humane methods for overdosing fish, but stressed that even though hydrochloride causes a loss of reflexes in a relatively short time, it is still an issue to be treated and studied carefully, since in the seven drugs tested the zebrafish appeared responsive even after breathing stopped, where the absence of a beat can be a clinical sign of deceptive death.

Uyttebroek et al. (2020) during their study recorded that the use of TNBS, also known as trinitrobenzenesulfonic acid, at 480 mM and 640 mM suffered from severe pain.

Schwartz et al. (2024), used etomidate to euthanize zebrafish, this is a mammalian analgesic, easily available for use in humans. In their research, they used two different doses in adult fish, and the result was positive in both concentrations: within 30 minutes there was no more movement. However, the authors make it clear that there is a need for further study, especially into brain death.

Meanwhile, Day et al. (2024) carried out tests using clove oil, where fish produced lower cortisol levels than those immersed in MS-222. Despite this, the authors argue that cortisol is a non-specific stress marker, demonstrating yet another irregularity and insecurity in the results of research into various means of euthanizing zebrafish.

Other techniques, whether widely used or not, such as rapid cooling, are also the target of criticism and questioning. In the case of cooling, the reservations are that it causes discomfort to the animals and the professional, as well as not being applicable to zebrafish embryos less than 3 days post fertilization (dpf) or not being accepted in all countries (da Silva et al., 2021). Methods such as concussion and electrical stunning, although approved in some guidelines, have been little used due to concerns about the pain experienced before loss of consciousness (Mocho et al., 2022; Mocho and von Krogh, 2022) as well as alternative

substances, propofol, isoflurane and etomidate being examples that have been explored as possible substitutes for MS-222, even though their efficacy results do not compare to MS-222, require more than one method for proven euthanasia (Ayala-Soldado et al., 2024; Schwartz et al., 2024; Weaver et al., 2024) or show zebrafish aversion to these substances.

Chereen Collymore, a veterinarian at the University of Toronto, reports in a nutshell that all substances used for euthanasia or anesthesia cause some kind of aversion in fish, either behaviorally or internally (Köhler et al., 2017). Thus, MS-222 continues to be the most suitable anesthetic for euthanizing *Danio rerio* due to ethical and management reasons (Leyden et al., 2022; Weaver et al., 2024). However, a recent literature review by Lavalle et al. (2025) concluded that many scientific articles do not provide a sufficient description of anesthesia or euthanasia methods to contribute to the progress of laboratory animal science, but that in short, euthanasia procedures should have at least two methods to ensure the safety and efficacy of the models.

In short, these neurological and behavioral results indicate that there is still much to be investigated regarding the best way to euthanize these animals ethically and effectively.

CONCLUSION

Differences of opinion on euthanasia within the scientific community not only affect research and laboratories, whether specialized or not, but also the well-being of the model, which according to Zhang et al (2024) constitutes "a transgression of ethical principles" and affects the reliability of research.

Thus, more research and a consensus in the scientific community is needed on what characterizes pain in zebrafish, because even after years of this fish being inserted as a good model in research, this type of discussion still doesn't have a final conclusion in which most researchers, guidelines and legislation agree, thus hindering the process of painless euthanasia of *Danio rerio.*

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