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# Anaesthesia and analgesia in laboratory adult zebrafish: a question of refinement

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## ABSTRACT

Anaesthesia is daily used in fish experimental procedures, however, the use of an inadequate anaesthetic protocol can compromise not only the animal's welfare but also results' reliability. The use of zebrafish (*Danio rerio*) in biomedical research increased in the last decades, highlighting the importance of appropriate anaesthetic regimes for this species. This article reviews the main anaesthetic agents and protocols used in laboratory adult zebrafish, and some analgesic methods that still need more research to be used in this species. In addition, it is proposed a systematized observation of signs to evaluate adult zebrafish welfare to reduce pain and distress.

## INTRODUCTION

The use of zebrafish (*Danio rerio*) in biomedical research has increased. Indeed, the percentage of publications using zebrafish almost tripled in the last decade <sup>1, 2</sup> in several research areas. In biomedical research, the behavioural or physiological changes that occur when an animal is exposed to a stressful or painful event can lead to unreliable results. The use of anaesthetic, sedative, or analgesic drugs is essential for reducing stress and/or pain in fish since they are sentient animals capable of pain perception<sup>3-5</sup>. Studies have demonstrated that fish show changes in normal behaviour and in physiological responses after being subjected to noxious stimuli, which may be ameliorated by the use of analgesics <sup>6-8</sup>. Despite these evidences, the discussion about pain perception in fish is still ongoing among researchers<sup>9</sup>. Nevertheless, there is an agreement regarding the importance of fish welfare and that efforts should be made to minimize painful and stressful conditions <sup>9, 10</sup>.

Although the use of anaesthetics is important to assure zebrafish welfare, these drugs can also have side-effects <sup>11</sup>, being essential to establish an anaesthetic regime (doses, combinations) that suits each research procedure in order to minimise collateral effects. This review summarizes the anaesthetic and analgesic drugs that are used in laboratory adult zebrafish, anaesthesia protocols, anaesthesia depth, and recovery. Also, during all the experiment is of major importance to monitor zebrafish welfare, and for that, we propose a score sheet to monitor distress and pain.



## ANAESTHESIA

Anaesthesia and sedation (Table 1) are routinely used in husbandry, clinical procedures and research. Thus, it is important to understand the side-effects of these drugs and how it may affect project outcomes in research. Anaesthesia has been described to negatively affect cognitive functions or to cause neurotoxicity, however, these effects are dependent on individuals' age, regime of administration, dose, duration and type of anaesthesia<sup>12</sup>. These factors combined with a yet scarce knowledge regarding anaesthesia in fish, make it difficult to choose an appropriate anaesthetic protocol suitable for each procedure.

Anaesthesia in fish may be achieved by diluting the anaesthetics in the water (inhalation anaesthesia), which induces a general anaesthesia with the drug being absorbed mainly through the gills but also through the skin in few species<sup>3, 13, 14</sup>. In fish, anaesthesia effects may vary depending on the administration route, pH, temperature, salinity, oxygenation, nitrogenous compounds and other water conditions<sup>3</sup>. Furthermore, anaesthesia depth and recovery depends on its duration, anaesthetic concentration, animals' body weight and metabolism, gill surface, fish health status, strain, age, and on the different particularities of fish species<sup>3, 13, 15</sup>. Thus, anaesthesia trials with small numbers of fish, i.e. pilot studies, must be performed to determine the optimal dosage and exposure time prior to the establishment of protocols. In addition, proper training and supervision of fish anaesthesia are essential to avoid complications that can lead to death. Not only anaesthesia should be carefully monitored but also complete fish recovery<sup>2, 16</sup>, which has been disregarded in the literature.

Table 1. Anaesthesia stages in fish			
Stage of anaesthesia	Description	Physiological and behavioural signs	Clinical interest
0	Normal	Total equilibrium. Normal muscle tone. Normal reaction to visual and tactile stimuli. Normal respiratory rate.	
I	Light sedation	Slight loss of reaction to visual and tactile stimuli.	Can reduce stress and physical trauma during transport
II	Deep sedation	Slight decrease in muscle tone. No reaction to visual and light tactile stimuli. Small decrease in respiratory rate.	Appropriate stage for close visual observation and for minimal manipulation, weighing and measuring.
III	Light narcosis / excitement phase	Partial loss of equilibrium/Weak responses to postural changes. Decrease in muscle tone. Increased reaction to visual and tactile stimuli. Respiratory rate increased and/or irregular.	Higher risk of physical injury or escape / jump from container or aquarium
IV	Deep narcosis	Total loss of equilibrium/Lack of responses to postural changes. No reaction to minor visual and tactile stimuli. Respiratory rate decreasing to almost normal.	Good plane for external sampling and blood sampling. Avoid painful procedures / analgesia may not be present. Suitable for imaging techniques
V	Light anaesthesia	Complete loss of muscle tone. No reaction to painful stimuli. Decrease in respiratory rate. Decrease in heart rate.	Minor surgical procedures: fin biopsies and gill biopsies
VI	Surgical anaesthesia	Absence of reaction to massive stimulation. Respiratory rate very low. Slow heart rate.	Major surgical procedures
VII	Medullary collapse/ Overdose	Flaccid muscle tone. Apnea – absence of respiratory rate, which can be followed in several minutes by cardiac arrest if anaesthesia depth is not decreased. Eventual death.	Appropriate for euthanasia
Adapted from Murray, 200217 and Pereira, 201618			

## ANAESTHETIC AGENTS USED IN FISH

The ideal anaesthetic agent should (i) be easy to administer and effective at low dose or exposure; (ii) be able to induce sedation or anaesthesia in less than 3 min with a minimum of stress; (iii) provide immobilisation and effective analgesia during all the procedure; (iv) induce a quick recovery from the anaesthetic stage, within 5 min, and (v) induce no or minimal changes in physiology and behaviour during or after anaesthesia<sup>15</sup>. Ideally, the anaesthetic should be affordable, easily available, practical to use, and safe to the operator.

A recent international survey showed that around 93% of the surveyed use MS-222 for zebrafish anaesthesia. The use of 2-phenoxyethanol, benzocaine, clove oil, isoeugenol, etomidate, and lidocaine was also referred<sup>19</sup>. Following, several anaesthetic agents are discussed.

### *MS-222*

Tricaine methanesulfonate or MS-222, classified as a local anaesthetic, is the most used inhalant anaesthetic in fish. MS-222 is highly absorbed through the gills and is administered by bath, inducing general anaesthesia. Overall it is a safe anaesthetic to fish<sup>20</sup>, although there are some concerns regarding risks of overdose in deeper stages of anaesthesia and long duration procedures, mainly in small animals as zebrafish<sup>2, 14, 20, 21</sup>. The anaesthetic solution of MS-222 should be buffered before use due to its acidic nature, which may cause aversion, epidermal and corneal lesions, and physiological alterations in the fish<sup>14, 22, 23</sup>. Also, MS-222 can be toxic to humans<sup>14, 24</sup>.

### *Clove oil*

Eugenol is the major constituent (70-90% by weight) of clove oil extracted from the plant *Syzygium aromaticum*. Clove oil and eugenol are used as inhalant anaesthetic, and must be mixed with ethanol to be soluble in the water bath. They showed rapid induction times and consistent anaesthesia, however, fish recovery takes longer than with MS-222<sup>3</sup>. Clove oil is efficient at a range of temperatures, easily available, and relatively inexpensive<sup>3</sup>. Aqui-S is a similar product available in the market constituted by isoeugenol, another compound of clove oil, which is soluble in water. Both have been suitable for harvesting and fish transportation<sup>3, 15</sup>. In general, there are equivocal evidence of carcinogenic activity of eugenol and isoeugenol, while methyleugenol, other clove oil constituent, is carcinogenic to rodents<sup>25</sup>.

### *Metomidate and Etomidate*

Metomidate and etomidate are nonbarbiturate hypnotic drugs, and both are used as inhalant anaesthetics in a water bath in fish. These agents induce quick anaesthesia induction and recovery but they should only be used for minor procedures, as they do not induce a surgical anaesthetic stage or analgesia<sup>3, 15</sup>. Also, they alter fish physiology by suppressing cortisol production<sup>11, 15</sup>.

## Lidocaine

Lidocaine hydrochloride, a local anaesthetic and analgesic<sup>15</sup>, is used as an immersion anaesthetic in fish. It induces anaesthesia within 1 min and the recovery is also rapid, taking about three to four times the induction period<sup>15</sup>. A high dose of lidocaine seems promising as an anaesthetic agent for surgical procedures but has a low margin of safety in zebrafish<sup>20</sup>. Thus, the addition of another agent, as propofol, may potentiate this effect and reduce the dosage<sup>2</sup>. Moreover, perioperative analgesia with lidocaine seemed to improve zebrafish welfare<sup>26</sup>.

## Propofol

Propofol is a sedative-hypnotic anaesthetic drug used for the induction and maintenance of general anaesthesia<sup>27</sup>. Propofol can be injected or used in an anaesthetic bath. It is rapidly metabolized, thus, lacking cumulative effects. Although propofol is highly lipophilic, it can induce anaesthesia in a rapid and smooth way. Moreover, the recovery from anaesthesia is also quick and complete<sup>2, 28</sup>. Although the preliminary results seem promising<sup>2</sup> it may not be fully soluble in the water and more research is needed.

## Ketamine

Ketamine is an injectable agent often used in mammals and induces a dissociative anaesthesia with some analgesia<sup>29, 30</sup>. In fish, it can be injected or used in an anaesthetic bath. The use of ketamine in fish depends largely on the species, since it could cause incomplete anaesthesia, apnoea, prolonged recovery and excitement in Salmonid species<sup>3</sup>. Ketamine revealed to be neurotoxic to zebrafish larvae<sup>31, 32</sup>, and to interfere with the development of embryos<sup>33</sup>.

## Isoflurane

Isoflurane is a hypnotic volatile drug routinely used in mammals anaesthesia. Studies evaluating volatiles in fishes are scarce, since the anaesthetic depth is difficult to control, causing overdose<sup>3, 20</sup>. Furthermore, anaesthetic preparation and anaesthesia should be conducted in a chemical fume hood for scavenging waste gas, reducing the risk to the operator. These characteristics and the observed clinical effects turn volatile anaesthetics less practicable in fish anaesthesia<sup>3, 20</sup>.

## ANAESTHESIA IN LABORATORY ADULT ZEBRAFISH

Laboratory zebrafish has emerged as a powerful vertebrate model system in research<sup>3, 34</sup>. Despite this interest, the research on welfare and refinement of procedures in this species are scarce. Pain and unexpected mortality due to incorrect anaesthesia in zebrafish can constitute a serious animal welfare issue, which increases data variability, carrying an important scientific and economical cost in daily research.

Table 2 provides a summary of the main anaesthetic agents used in adult zebrafish, and their effects. Immersion is the most common method used, especially in small fish as zebrafish, where other



invasive routes are impractical. Protocols for fish anaesthesia usually include only one anaesthetic agent instead of a combination, however, some studies have demonstrated that the mixture of two types of anaesthetics can result in a safer and more effective anaesthesia <sup>2, 21</sup>



Table 2. Anaesthetic and analgesic agents tested in laboratory adult zebrafish					
Species	Anaesthetic or analgesic agent	Anaesthetic stage/Analgesia	Dose	Observations reported/Comments	Reference
Zebrafish ( <i>Danio rerio</i> )	MS-222	Stage V	100, 120, 140, 160, 180 and 200 mg/L Immersion	The achievement of light anaesthesia, induced by all doses, ranged from a mean time of 1 min to 2.75 min in an inverse relationship response to dose. The achievement of surgical anaesthesia, induced by all doses, ranged from a mean time of 3 min to 14 min in an inverse relationship to dose. Inter-individual variability to achieve anaesthesia present at all doses. The total recovery of equilibrium, and reaction to external stimuli in the recovery phase of anaesthesia occurred at approximately 3 min regardless the dose.	Grush et al., 2004 <sup>35</sup>
		Stage III – IV	100 and 120 mg/L Immersion	Doses were unable to induce light anaesthesia in all subjects.	Huang et al., 2010 <sup>21</sup>
		Stage V	140, 160, 180 and 200 mg/L Immersion	Doses induced light anaesthesia within 2 min.	
			All doses (100 – 200 mg/L) Immersion	Time required to achieve respiratory arrest ranged from a mean time of 3 min to 12 min in an inverse relationship response to dose. Subjects took over 2.2 min to recover after showing sign of respiratory arrest.	
		N/A	100 mg/L Immersion	Induction of behavioural aversion.	Readman et al., 2013 <sup>22</sup>





Stage VI	150 mg/L Immersion	Surgical anaesthesia was achieved within a mean time of 4.2 min and recovery 2.3 min post-anaesthesia. No mortality occurred during anaesthesia or postanesthetic period. No distressful behaviours were observed during induction or recovery.	Collymore et al., 2014 <sup>20</sup>
N/A	168 mg/L Immersion	One exposure had no effect on several behavioural parameters of anxiety and activity immediately, 5 min, 30 min and 1h after recovery.	Nordgreen et al., 2014 <sup>16</sup>
N/A	150 mg/L Immersion	One exposure caused conditioned place aversion and 53% of the subjects completely rejected the previous preferred side of the tank.	Wong et al., 2014 <sup>36</sup>
Stage V	75 mg/L Immersion	Light anaesthesia was achieved in less than 2 min. Time to recover was less than 1 min.	Chambel et al., 2015 <sup>37</sup>
Stage V	100 mg/L Immersion	Light anaesthesia was achieved in less than 2 min. Time to recover was less than 1 min.	
Stage V	125 mg/L Immersion	Light anaesthesia was achieved within 1 min. Recovery occurred within 1 min.	
Stage V	150 mg/L Immersion	Light anaesthesia was achieved within 1 min. Recovery occurred within 1 min. Exposure for 30 min at this dose induced 50% of mortality.	
Stage V	200 mg/L Immersion	Light anaesthesia was achieved in less than 1 min. Recovery occurred within 1 min. Exposure for 30 min at this dose induced 100% of mortality.	



		Stage V	250 mg/L Immersion	Light anaesthesia was achieved in less than 1 min. Recovery occurred within 1 min. Exposure for 30 min at this dose induced 100% of mortality.	
		Stage VI	100 mg/L Immersion	No mortality occurred during anaesthesia or postanesthetic period.	Valentim et al., 2016 <sup>2</sup>
Zebrafish ( <i>Danio rerio</i> )	Clove oil	N/A	55 mg/L Immersion	One exposure caused some conditioned place aversion and only 19% of the subjects completely rejected the previous preferred side.	Wong et al., 2014 <sup>36</sup>
		Stage V	60, 80, 100, 120 and 140 mg/L Immersion	The achievement of light anaesthesia, induced by all doses, ranged from a mean time of 0.5 min to 1 min in an inverse relationship response to dose. The achievement of surgical anaesthesia, induced by all doses, ranged from a mean time of 2.5 min to 8.5 min in a negative exponential response to dose. The total recovery of equilibrium after anaesthesia occurred at approximately 5 min regardless of eugenol dose. The reaction to external stimuli in the recovery phase of anaesthesia occurred at approximately 8 min, regardless of eugenol dose.	Grush et al., 2004 <sup>35</sup>
Zebrafish ( <i>Danio rerio</i> )	Metomidate hydrochloride	Stage I	2 and 4 mg/L Immersion	The time to recover from anaesthesia ranged from 2.8 min to 5.2 min.	Collymore et al., 2014 <sup>20</sup>
		Stage IV	6, 8 and 10 mg/L Immersion	Light anaesthesia occurred between 2.3 min and 4.3 min. No surgical anaesthetic plane was achieved during the 10 min of exposure. The time to recovery from anaesthesia ranged from 5.6 min to 10.4 min regardless of the dose. No distressful behaviours observed during anaesthesia. No mortality during or after anaesthesia.	



		N/A	13.5 mg/L Immersion	One exposure caused some conditioned place aversion and only 11% of the subjects completely rejected the previous preferred side.	Wong et al., 2014 <sup>36</sup>
Zebrafish ( <i>Danio rerio</i> )	Etomidate	Stage IV	4 to 10 mg/L Immersion	Induction of light anaesthesia was achieved for doses of 4 mg/L and higher, and occurred in less than 1 min. The time to recovery varied from around 25 min for 4 mg/L to 60 min for the highest dose.	Amend et al., 1982 <sup>38</sup>
		Stage IV	8 to 10 mg/L Immersion	Doses higher than 8 mg/L induced mortality.	
		N/A	3 mg/L Immersion	Exposure for more than 10 min and 20 min showed 5% and 20% of mortality, respectively.	
		N/A	9 mg/L Immersion	Exposure for 120 s on three consecutive days induced 30% of mortality.	
		N/A	15 mg/L Immersion	Exposure for 120 s on three consecutive days induced 95% of mortality.	
		N/A	2 mg/L Immersion	No behavioural evidence of aversion was observed in the subjects exposed.	Readman et al., 2013 <sup>22</sup>
Zebrafish ( <i>Danio rerio</i> )	Lidocaine hydrochloride	N/A	100 mg/L Immersion	Induction of behavioural aversion.	Readman et al., 2013 <sup>22</sup>
		Stage I	300 mg/L Immersion	Induction of light sedation that occurred within a mean time of 5.4 min with a great variability. The recovery from anaesthesia occurred at a mean time of 3.4 min with a great variability. No mortality occurred during anaesthesia or postanesthetic period.	Collymore et al., 2014 <sup>20</sup>



		Stage VI	325 mg/L Immersion	Induction of surgical anaesthesia at a mean time of 0.85 min with a great variability. The recovery from anaesthesia occurred at a mean time of 5.8 min with a great variability. No mortality occurred during anaesthesia or postanesthetic period.	
		Stage VI/ VII	350 mg/L Immersion	Induction of surgical anaesthesia at a mean time of 1.7 min, and some subjects experienced Stage VII - Medullary Collapse/Overdose. The recovery from anaesthesia occurred at a mean time of 1.9 min with a great variability. 30% mortality observed during anaesthesia.	
		Analgesic	0.1-2 mg/kg (IM)	Analgesic drug. No side effects observed. Very efficient at 1 mg/kg.	Sneddon, 2012 <sup>11</sup>
		Analgesic	2-5 mg/L* Immersion	Pre and post-surgical administration for tail fin clipping. Reduction of pain-related behaviours.	Schroeder 2016 <sup>26</sup>
Zebrafish ( <i>Danio rerio</i> )	Propofol	Stage V	2.5 mg/L Immersion	The dose induced light anaesthesia in 93.4% of the subjects but was unable to induce analgesia in 40% of the subjects. This dose showed an occurrence of 33% mortality.	Valentim et al., 2016 <sup>2</sup>
		Stage V	5 and 7.5 mg/L Immersion	Both doses induced light anaesthesia but were unable to induce analgesia in 36% and 18% of the subjects, respectively. Both doses showed an occurrence of 9% mortality.	
Zebrafish ( <i>Danio rerio</i> )	Propofol (P) combined with Lidocaine (L)	Stage VI	2.5 mg/L(P)+50 mg/L(L); Immersion	No mortality occurred during anaesthesia or postanesthetic period.	Valentim et al., 2016 <sup>2</sup>



		Stage VI	2.5 mg/L(P)+100 mg/L(L); Immersion	Occurrence of 23% mortality.	
		Stage VI	2.5 mg/L(P)+150 mg/L(L) Immersion	Occurrence of 9% mortality.	
Zebrafish ( <i>Danio rerio</i> )	Ketamine	N/A	2000 mg/L (acute exposure during 5 min and chronic exposure for 5 consecutive days) Immersion	Sub-anaesthetic dose produced behavioural abnormalities such as increased circling behaviour, decreased gill movement (ventilatory response to hypoxia), and decreased stress response to hypoxia (body pulses). The repeated administration of ketamine did not cause tolerance or sensitization to specific drug effects.	Zakhary et al., 2011 <sup>39</sup>
		Stage IV (at least)	8000 mg/L Immersion	Physiological anaesthetic dose inducing a deep level of unconsciousness.	
Zebrafish ( <i>Danio rerio</i> )	Isoflurane	Stage III	0.5 mL/L Immersion	Distressful behaviours were observed (twitching, erratic swimming, and piping). Signs of disorientation, difficulty maintaining buoyancy, rolling, and swimming upside down also occurred. Recovery from anaesthesia within a mean time of 4.2 min. 30% mortality during anaesthesia and 1 day post-anaesthesia.	Collymore et al., 2014 <sup>20</sup>
Zebrafish ( <i>Danio rerio</i> )	Isoflurane (I) combined with MS-222 (M)	Stage III-IV; Stage V (10-20%)	50 mg/L(I)+50 mg/L(M); Immersion	Light anaesthesia was induced by this dose in only 10% - 20% of the subjects.	Huang et al., 2010 <sup>21</sup>



		Stage IV	60 mg/L(I)+60 mg/L(M); 65 mg/L(I)+65 mg/L(M); 70 mg/L(I)+70 mg/L(M); 80 mg/L(I)+80 mg/L(M) Immersion	The combination doses between 60+60 mg/L and 80+80 mg/L induced light anaesthesia within 1.5 min.
			All doses	Regarding all doses, time required to achieve respiratory arrest ranged from a mean time of 6 min to 55 min in a negative exponential response to dose. For all the combined doses the subjects recover within 2 min after showing sign of respiratory arrest.
<p>N/A – not applicable/not evaluated;          All anaesthetic agents were administered by immersion, with exceptions: IM – intramuscular injection.          *(Paul Schroeder, personal communication, July 19, 2016)</p>				



## MONITORING ZEBRAFISH WELFARE

In addition to the importance of a good anaesthetic protocol to ensure fish welfare during experimental procedures, animals should also be monitored for distress, discomfort and pain during all the experimental protocol and maintenance. Fish have demonstrated to react consistently to noxious chemical stimuli and present reliable phenotypes of stress, fear, and anxiety<sup>40, 41</sup>. Thus, the use of analgesic drugs in fish during and after painful experimental procedures should be considered, especially due to the fact that not all anaesthetic agents available for fish have proved to have adequate analgesic properties<sup>3</sup>. The information concerning the use of analgesics in zebrafish is extremely scarce (Table 2). Furthermore, the available analgesics tested in fish are administered intramuscularly and/or by the intraperitoneal route<sup>11</sup>, which make it difficult to perform in small fish, such as zebrafish, or in a large number of animals<sup>26</sup>. Lidocaine has recently been described as a promising analgesic for zebrafish<sup>26</sup>, which is an important step in the development of analgesic protocols to be administered in the water bath.

In Table 3 we suggest a score sheet to monitor the signs of distress and pain in adult zebrafish. This table is an initial proposal of a score sheet to monitor zebrafish welfare, and, as such, it should be further investigated for each individual protocol and genetic background.

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Table 3. Proposal of a pain and distress score sheet for laboratory adult zebrafish			
	Score		
<b>Physical appearance*</b>			
Normal	0		
Missing operculum, and missing fins repeatedly, indicating possible antagonist behaviours; darkening /inflammation of fin	1		
Mild scoliosis/ lordosis, tegument lesions, mucus production, over or under conditioned (obese or thin), abrupt colour change, especially blanching	2		
General emaciation (low body to head ratio), general body deformities, missing or protuberant scales	3		
<b>Food consumption</b>			
Normal	0		
Unresponsive to food during 1 day	1		
Unresponsive to food during 2 days, not even live food	2		
Unresponsive to food more than 3 days not even live food as artemia or rotifers (**)	3		
<b>Respiratory pattern</b>			
Normal	0		
Piping or extremely low rate, almost no opercular movement	3		
<b>Swimming behaviour (**)</b>			
Swimming through the water column	0		
Difficulties to control buoyancy and/or to maintain equilibrium	2		
Systematic swimming on the surface or in the bottom of the tank	3		
<b>Activity (**)</b>			
Normal	0		
Hyperactive (erratic swimming) or hypoactive	2		
Letargic, no reaction to external stimuli	3		
<b>Social behaviour (**)</b>			
Normal. Shoaling.	0		
Individual often isolated when group-housed	1		
Individual always chasing or being chased by conspecifics	2		
Individual does not respond to conspecific behaviours towards him	3		
<b>TOTAL</b>			
<p><b>Judgement:</b> 0-1 Normal; 2-8 Monitor carefully. Consider veterinary treatment including analgesics and consider also to analyse water quality; 9-12 Suffering, provide relief, consult the specialized veterinarian, consider euthanasia. 13-18 severe status, euthanasia, rethink experimental procedure. Euthanasia may be considered if a score of 3 is observed in any of the categories, except for behaviour-related categories (**), in which scores of 3 suggests repeated and close observation for a final decision regarding euthanasia. *Take in consideration animals' age.</p>			



## CONCLUDING REMARKS

The use of a suitable anaesthetic protocol able to produce anaesthesia with effective analgesia is an important refinement for painful procedures. Studies addressing the effects of anaesthetics in zebrafish are variable and lack important information such as the time during which the anaesthetic stage can be maintained without secondary effects, a clear description of the anaesthesia stage achieved with certain dose, and data about recovery. This lack of information can have a negative impact on zebrafish welfare when investigators are not familiarized with zebrafish anaesthesia. In addition, analgesia in zebrafish is another topic that needs further investigation to refine analgesic protocols during experimental procedures and for postoperative pain management, thus ensuring zebrafish welfare and reliable data collection.

In general, MS222 is a good anaesthetic, justifying its wide use in zebrafish<sup>19</sup>, but further refinement could be valuable when long duration procedures are required<sup>21, 21</sup>. Furthermore, two studies described zebrafish aversion towards this anaesthetic<sup>22,36</sup>. Thus, finding other anaesthetic protocols are advisable, for example, the use of anaesthetic combinations to decrease individual concentrations and the risk of unwanted effects. Also, lidocaine seems to be the more promising analgesic to be used in zebrafish, however, its efficacy needs to be tested in different painful procedures and experimental situations.

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## REFERENCES

1. Kalueff AV, Echevarria DJ and Stewart AM. Gaining translational momentum: more zebrafish models for neuroscience research. *Prog Neuropsychopharmacol Biol Psychiatry*. 2014; 55: 1-6.
2. Valentim AM, Felix LM, Carvalho L, Diniz E and Antunes LM. A New Anaesthetic Protocol for Adult Zebrafish (*Danio rerio*): Propofol Combined with Lidocaine. *PLoS One*. 2016; 11: e0147747.



3. Neiffer DL and Stamper MA. Fish sedation, analgesia, anesthesia, and euthanasia: considerations, methods, and types of drugs. *ILAR J.* 2009; 50: 343-60.
4. Sneddon LU. Pain in aquatic animals. *J Exp Biol.* 2015; 218: 967-76.
5. Jones RC. Fish sentience and the precautionary principle. *Animal Sentience: An Interdisciplinary Journal on Animal Feeling* 2016; 1.3: 10.
6. Sneddon LU, Braithwaite VA and Gentle MJ. Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. *Proc Biol Sci.* 2003; 270: 1115-21.
7. Sneddon LU, Braithwaite VA and Gentle MJ. Novel object test: examining nociception and fear in the rainbow trout. *J Pain.* 2003; 4: 431-40.
8. Mettam JJ, Oulton LJ, McCrohan CR and Sneddon LU. The efficacy of three types of analgesic drugs in reducing pain in the rainbow trout, *Oncorhynchus mykiss*. *Appl Anim Behav Sci.* 2011; 133: 265-74.
9. Rose JD, Arlinghaus R, Cooke SJ, et al. Can fish really feel pain? *Fish Fisheries* 2014; 15: 97-133.
10. Iwama GK. The welfare of fish. *Dis Aquat Organ.* 2007; 75: 155-8.
11. Sneddon LU. Clinical Anesthesia and Analgesia in Fish. *Journal of Exotic Pet Medicine* 2012; 21: 32-43.
12. Jevtovic-Todorovic V, Absalom AR, Blomgren K, et al. Anaesthetic neurotoxicity and neuroplasticity: an expert group report and statement based on the BJA Salzburg Seminar. *Br J Anaesth.* 2013; 111: 143-51.
13. Ferreira JT, Schoonbee HJ and Smit GL. The uptake of the anaesthetic benzocaine hydrochloride by the gills and the skin of three freshwater fish species. *Journal of Fish Biology.* 1984; 25: 35-41.
14. Carter KM, Woodley CM and Brown RS. A review of tricaine methanesulfonate for anesthesia of fish. *Reviews in Fish Biology and Fisheries.* 2011; 21: 51-9.
15. Ross LG and Ross B. *Anaesthetic and Sedative Techniques for Aquatic Animals.* Oxford, UK: Blackwell Publishing, 2008.
16. Nordgreen J, Tahamtani FM, Janczak AM and Horsberg TE. Behavioural effects of the commonly used fish anaesthetic tricaine methanesulfonate (MS-222) on zebrafish (*Danio rerio*) and its relevance for the acetic acid pain test. *PLoS One.* 2014; 9: e92116.
17. Murray MJ. Fish surgery. *Semin Avian Exot Pet Med.* 2002; 11: 246-57.
18. Pereira N. Introduction to Anaesthesia and Surgery in Fish. In: Oliveira M, Bernardo F and Robalo JI, (eds.). *Practical Notions on Fish Health and Production.* Sharjah: Bentham Science Publishers, 2016, p. 127-82.
19. Lidster K, Readman GD, Prescott MJ and Owen SF. International survey on the use of zebrafish in research. (Under submission).



20. Collymore C, Tolwani A, Lieggi C and Rasmussen S. Efficacy and safety of 5 anesthetics in adult zebrafish (*Danio rerio*). *J Am Assoc Lab Anim Sci*. 2014; 53: 198-203.
21. Huang WC, Hsieh YS, Chen IH, et al. Combined use of MS-222 (tricaine) and isoflurane extends anesthesia time and minimizes cardiac rhythm side effects in adult zebrafish. *Zebrafish*. 2010; 7: 297-304.
22. Readman GD, Owen SF, Murrell JC and Knowles TG. Do fish perceive anaesthetics as aversive? *PLoS One*. 2013; 8: e73773.
23. Davis MW, Stephenson J and Noga EJ. The effect of tricaine on use of the fluorescein test for detecting skin and corneal ulcers in fish. *J Aquat Anim Health*. 2008; 20: 86-95.
24. Berstein PS, Digre KB and Creel DJ. Retinal toxicity associated with occupational exposure to the fish anesthetic MS-222. *Amer J Ophthalmol* 1997; 124: 843-4.
25. Medicine CfV. Concerns Related to the use of Clove Oil as an Anesthetic for Fish. Rockville 2007.
26. Schroeder P. Exploring suitable analgesics in zebrafish – a combined approach. 13th Felasa Congress. Brussels, Belgium. 2016, p. 32.
27. Steinbacher DM. Propofol: a sedative-hypnotic anesthetic agent for use in ambulatory procedures. *Anesth Prog*. 2001; 48: 66-71.
28. Gholipourkanani H and Ahadzadeh S. Use of propofol as an anesthetic and its efficacy on some hematological values of ornamental fish *Carassius auratus*. *Springerplus*. 2013; 2: 76.
29. Karmarkar SW, Bottum KM and Tischkau SA. Considerations for the use of anesthetics in neurotoxicity studies. *Comp Med*. 2010; 60: 256-62.
30. Kohrs R and Durieux ME. Ketamine: teaching an old drug new tricks. *Anesth Analg*. 1998; 87: 1186-93.
31. Kanungo J, Cuevas E, Ali SF and Paule MG. Ketamine induces motor neuron toxicity and alters neurogenic and proneural gene expression in zebrafish. *J Appl Toxicol*. 2013; 33: 410-7.
32. Burgess HA and Granato M. Sensorimotor gating in larval zebrafish. *J Neurosci*. 2007; 27: 4984-94.
33. Felix LM, Antunes LM and Coimbra AM. Ketamine NMDA receptor-independent toxicity during zebrafish (*Danio rerio*) embryonic development. *Neurotoxicol Teratol*. 2014; 41: 27-34.
34. Oliveira RF. Mind the fish: zebrafish as a model in cognitive social neuroscience. *Front Neural Circuits*. 2013; 7: 131.
35. Grush J, Noakes DL and Moccia RD. The efficacy of clove oil as an anesthetic for the zebrafish, *Danio rerio* (Hamilton). *Zebrafish*. 2004; 1: 46-53.
36. Wong D, von Keyserlingk MA, Richards JG and Weary DM. Conditioned place avoidance of zebrafish (*Danio rerio*) to three chemicals used for euthanasia and anaesthesia. *PLoS One*. 2014; 9: e88030.



37. Chambel J, Pinho R, Sousa R, et al. The efficacy of MS-222 as anaesthetic agent in four freshwater aquarium fish species. *Aquaculture Research*. 2015; 46: 1582-9.
38. Amend DF, Goven BA and Elliot DG. Etomidate: effective dosages for a new fish anesthetic. *Trans Am Fish Soc*. 1982; 111: 337-41.
39. Zakhary SM, Ayubcha D, Ansari F, et al. A behavioral and molecular analysis of ketamine in zebrafish. *Synapse*. 2011; 65: 160-7.
40. Maximino C. Modulation of nociceptive-like behavior in zebrafish (*Danio rerio*) by environmental stressors. *Psychology & Neuroscience*. 2011; 4: 149-55.
41. Roques JA, Abbink W, Geurds F, van de Vis H and Flik G. Tailfin clipping, a painful procedure: Studies on Nile tilapia and common carp. *Physiol Behav*. 2010; 101: 533-40.