Anesthesia and Euthanasia of Laboratory Zebrafish

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7th Annual International Zebrafish Husbandry Course
Buguggiate, Italy
September 25-28, 2018

Overview

Preparation

Monitoring

Recovery

Methods of anesthesia

Special anesthetic considerations

Euthanasia

Anesthesia

Required for procedures that may cause pain and distress

General anesthesia suppresses central nervous system activity and results in unconsciousness and total lack of sensation

Sedation suppresses the central nervous system to a lesser degree, inhibiting both anxiety and creation of long-term memories without resulting in unconsciousness

Goals

Induce unconsciousness quickly

Minimize pain and distress

Reliable and reversible (full recovery)

Compatible with research objectives

Ideal Anesthetic Agent

Water soluble
Easy to prepare
Stable for long periods of time
Biodegradable
No long-lasting effects on fish health or behaviour
Induces anesthesia in less than 3 min
Recovery in less than 5 min
Wide margin of safety
Predictable levels of anesthesia
Ensure immobility and analgesia
Affordable
Non-toxic to fish or people

Preparation

Healthy Fish

Acclimated to their life support system (LSS)

No health problems

- Monitor for the presence of potential pathogens using the facility health surveillance data
- Pay attention to zoonotic agents

Fast fish for 12-24h before anesthesia

- May be shorter depending on the procedure

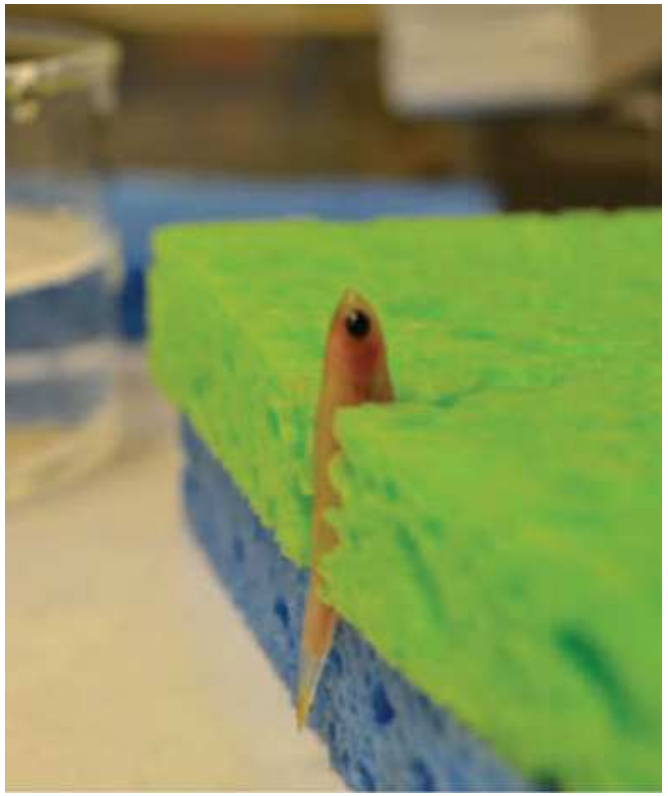
Materials & Equipment

Non-powdered gloves for personnel

Soft nets

All other materials for the procedure should be prepared in advance of the procedure

- Sponge without chemicals
- Air diffuser
- Water heater (if needed)
- Instruments



Dang et al. 2016

Water

Immersion in an anesthetic bath is most common method of anesthesia

Anesthetic water should come from the LSS the fish are acclimated to

- Minimizes changes in water quality parameters

Monitor water quality parameters if many fish will be anesthetized

Monitoring

Stages of Anesthesia

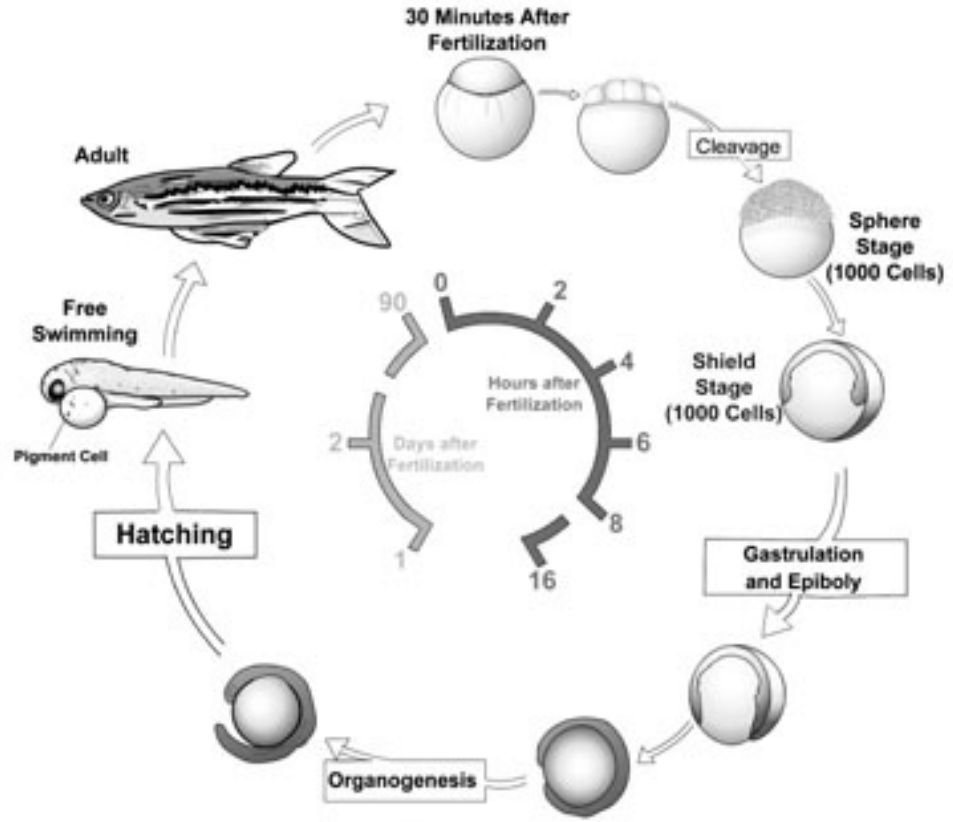
Table 1. Stages of anesthesia

Stage	Plane	Level of Anesthesia	General Behavior	Voluntary Locomotor Activity	Equilibrium	Opercular Movement Rate	Reflex Response	Heart Rate	Muscle Tone	Examples of Procedures
0		None	Normal	Normal	Normal	Normal	Normal	Normal	Normal	
I		Light sedation	Disorientation	Decreased	Difficult to maintain	Normal	Reduced	Normal	Normal	ENU mutagenesis, imaging
II		Excitation	Agitation	Increased	Lost	Increased	Increased	Increased	Normal	
III	1	Light anesthesia	Anesthetized	None	Lost	Decreased	Reduced	Regular	Decreased	Weighing, gill scrape, skin scrape
	2	Surgical anesthesia	Anesthetized	None	Lost	Shallow	None and no response to deep vibration	Reduced	Decreased	Gill biopsy, tail fin clipping, recovery surgery
	3	Deep	Anesthetized	None	Lost	Rare movements	None	Reduced	Relaxed	Non-recovery surgery
IV		Overdose	Apparently dead	None	Lost	None	None	Cardiac failure	None	Euthanasia

Modified from Collymore, 2014

Efficacy of Anesthesia

- Animal size
- Stage of development
- Body condition
- Presence of illness/pathogens
- Stress
- Water quality



D'Costa et al. 2009

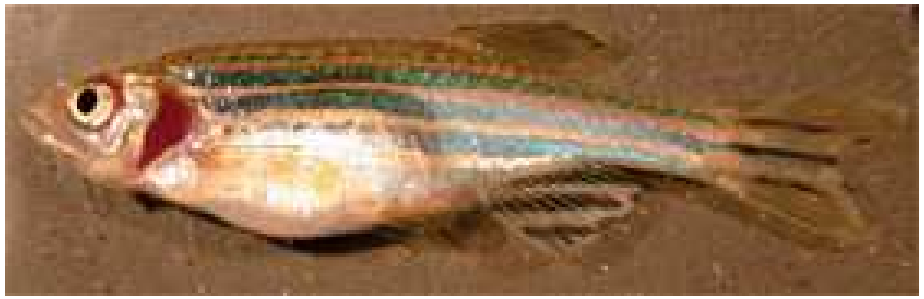
Opecular Beat Rate

Slows anesthesia deepens

Monitor continuously

‘Flaring’ is a warning sign

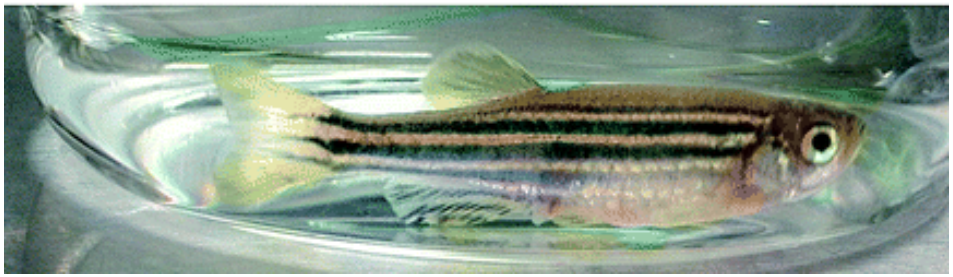
If opercular movement stops, fish should be removed from the anesthetic immediately

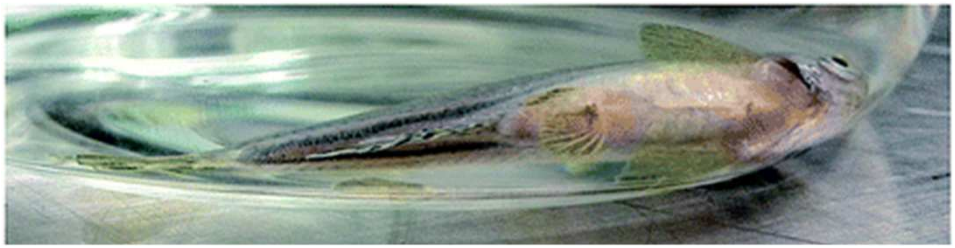


https://zebrafish.org/documents/faq.php

Immobility

- Reduction in swimming speed
- Loss of righting reflex
- Forward progress stops and eventually all movement stops



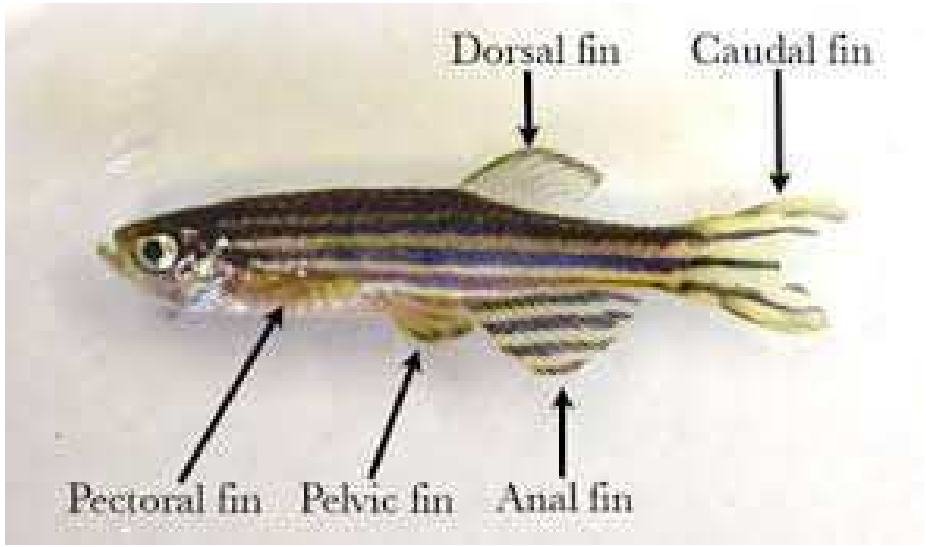


Chen et al. 2015

Response to Tail Fin Pinch

Perform before a painful procedure

Once the animal is immobilized, use your fingers or forceps to ensure the animal no longer responds to stimuli

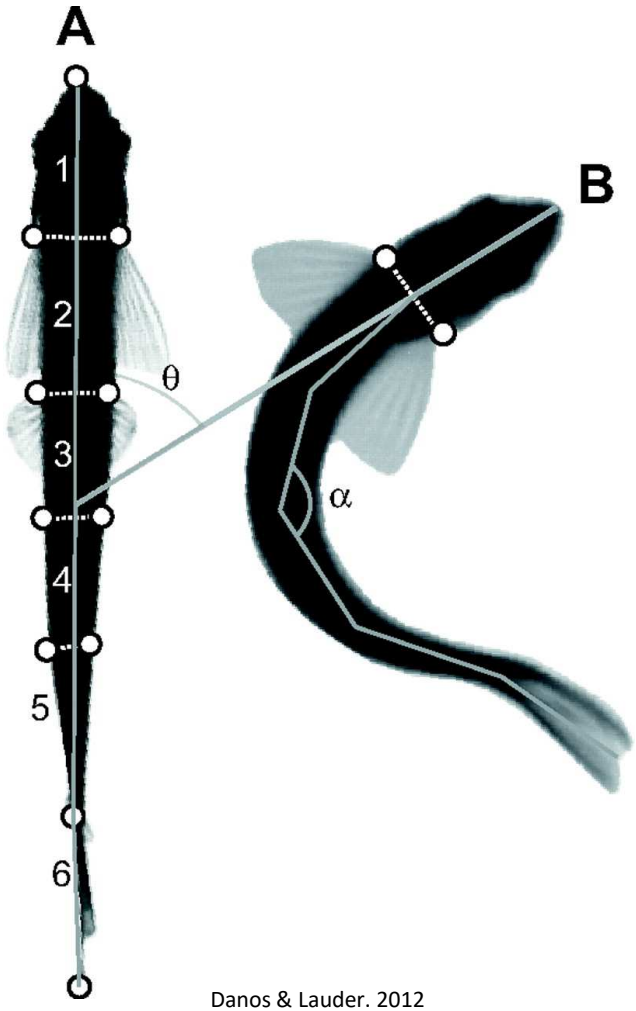


Gupta & Mullins. 2010

Response to a Deep Vibration

Fish not sufficiently anesthetized may respond to a knock on the tank walls

This reflex disappears with deeper anesthesia



Danos & Lauder. 2012

Coughing

Can be observed during induction

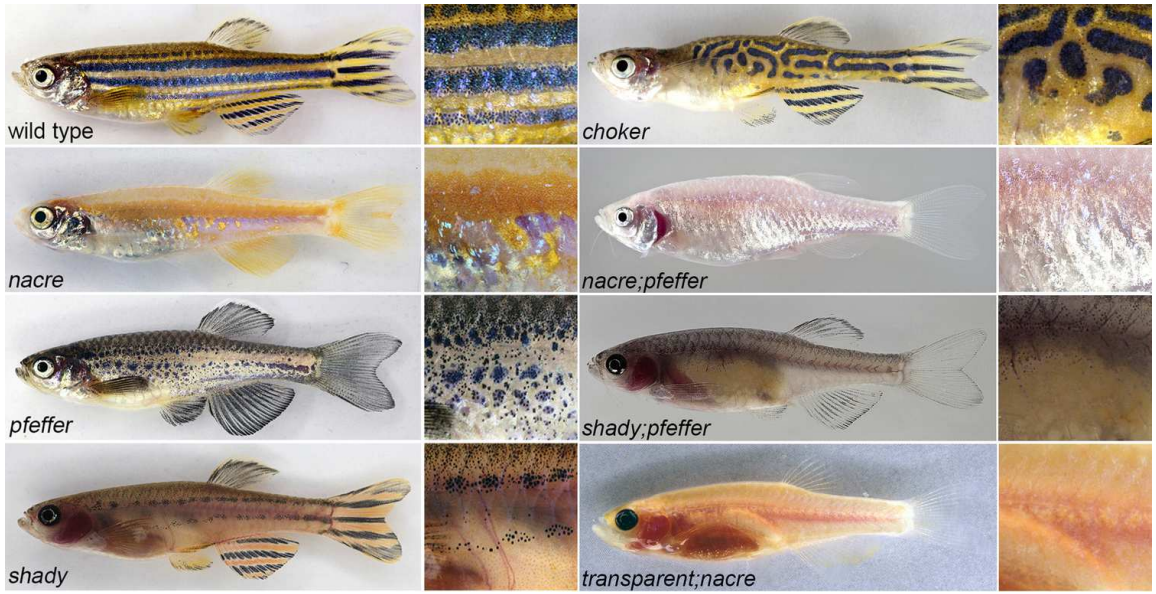
If the fish was not fasted, some food stuffs may enter the gills and prevent oxygen exchange

Colour

Fish may become darker under anesthesia

- Stress response

Return to normal colour when anesthetic agent is removed



http://www.eb.tuebingen.mpg.de/research/emeriti/research-group-colour-pattern-formation.html

Aversive Behaviours

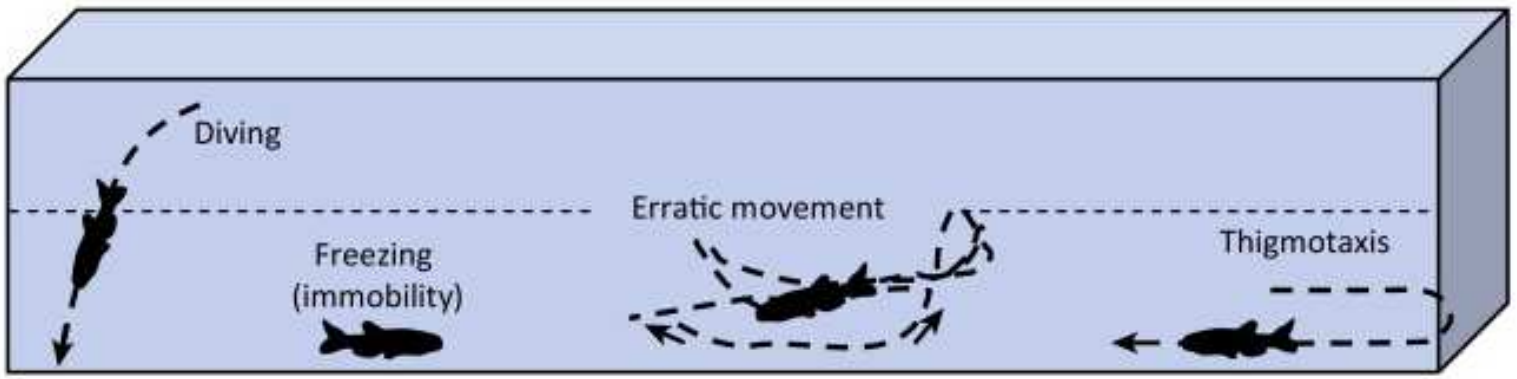
Piping

Erratic swimming

Twitching

Observed with aversive anesthetics

Stops with deeper anesthesia



Stewart et al. 2015.

Recovery

Recovery

Place fish in an anesthetic-free tank

Use water should from home LSS

Provide air diffusion and heat if needed

Re-check the animals for 2-3 days after anesthesia

Personnel Safety

Some anesthetic agents are toxic to people

Personal protective equipment should be worn when preparing stock solutions

- Especially gloves

Use respirators or fumehoods where necessary

Methods of Anesthesia

Anesthetic agents

Tricaine methanesulfonate

Clove oil/eugenol/isoeugenol

Lidocaine

Propofol

Others

Tricaine

Rapid induction of a surgical level of anesthesia

Acts by inhibiting sodium and potassium currents in nervous system cells

In adults absorbed by the gills; in larvae absorbed by the skin

Fast recovery

Wide margin of safety

Prolonged or repeated exposure causes high mortality

- Often due to hypoxia and cardiac effects

Tricaine

Aversive and induces distressful behaviour (Wong et al 2014; Readman et al 2013)

Affects hypothalamic-pituitary-adrenal axis

Needs to be protected from light

Toxic for personnel

- Powder form is a mucosal irritant in humans
- Retinotoxic in humans

Clove Oil/eugenol/isoeugenol

Natural anesthetic derived from flowers, flower stalks and leaves of the clove tree, *Syzgium aromaticum* (*Eugenia aromatica*)

Rapid loss of consciousness

Can recover high volume of serum after euthanasia

Must be dissolved in ethanol and protected from light

Aversive, but less than MS-222

Methyeugenol is considered equivocal carcinogen

Irritant to eyes, skin, respiratory tract

”Sticky”, tricky to handle → Equipment is easily contaminated

Grush et al. 2004; Davis et al. 2015

Lidocaine hydrochloride

Local anesthetic

Rapid loss of consciousness

Analgesic potential

Can provide surgical level anesthesia

Small margin of safety

Toxic for handler if injected in high doses

Mildly aversive

Unknown effects on scientific outcomes

Collymore et al. 2014

Propofol

Sedative-hypnotic

Rapid loss of consciousness

Cannot be stored for long periods of ti

May not dissolve evenly in solution

Must be used within 30 min of preparation

Toxic for handler if injected in high doses

Mildly aversive

Unknown effects on scientific outcomes

Can be combined with lidocaine for complete anesthesia and analgesia (Valentim et al 2016)

Valentim et al. 2016

Other Anesthetic Agents

Benzocaine

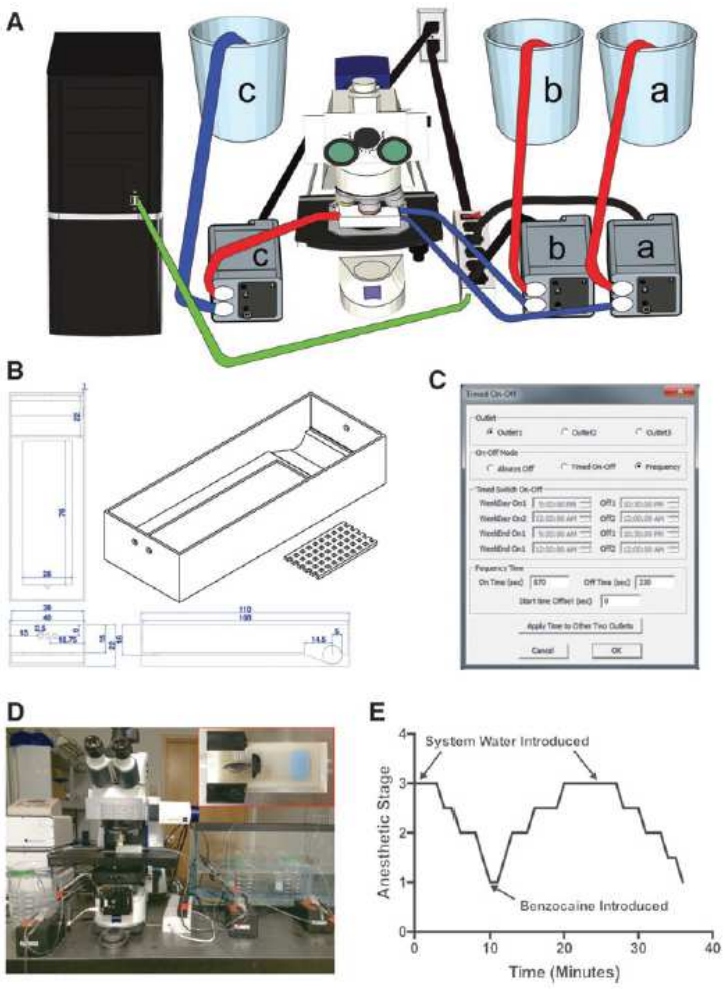
Metomidate and etomidate

2-Phenoxyethanol

Menthol

Benzocaine

- Parent compound of tricaine
- Less water-soluble
- Rapid induction but prolonged recovery
- Small margin of safety
- Aversive



Wynd et al. 2016

Metomidate and etomidate

Sedation

Reduces transport stress

Wide margin of safety

Rapid induction but prolonged recovery

Good for adults but not larvae

Reduces response to cortisol

Less aversive than tricaine

Collymore et al. 2014

2-Phenoxyethanol

Not as much published information

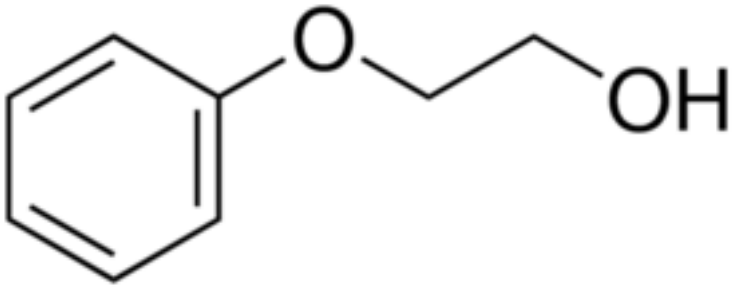
Popular in EU

Aversive

No advantages over other drugs

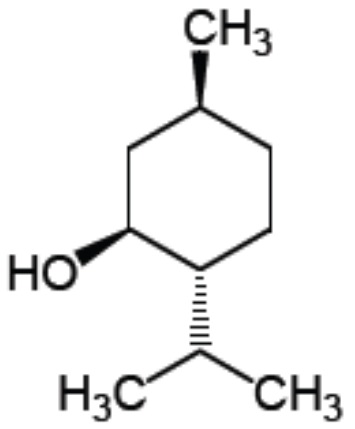
In other fish

- Maintains muscle reflexes
- No analgesia
- Hypoventilation

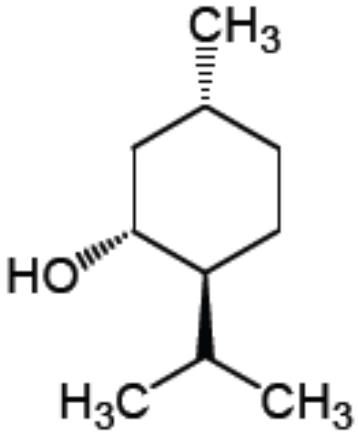


Menthol

0.5 mM induces surgical anesthesia in less than 3 min and recovery in less than 8 min



d-menthol



l-menthol

Kasai et al. 2014

Slow Chilling

Adults are immobilized at 10°C

- Good for injections, short transport
- Should not be used for painful procedures
- Near immediate recovery

For larvae less than 14 days

- Tolerate exposure to 0°C water for less than 10 min
- Rapid recovery

Chen et al. 2014

Anesthetic Doses

Table 2. Anesthetic agents used for zebrafish

Drug	Dosage	Anesthetic Stage	Observations & Comments
Tricaine methansulfonate	50 mg/L 50-100 mg/L 100-200 mg/L	Sedation Light anesthesia Surgical anesthesia	Also used to anesthetize larval zebrafish
Benzocaine	25-100 mg/L 35 mg/L	Light anesthesia Light anesthesia	After induction with MS222, intermittent dosing with benzocaine maintains anesthesia for median time of 7.5h
2-Phenoxyethanol	200-300 uL/L	Light anesthesia	
Clove oil/eugenol/isoeugenol	2-5 mg/L 60-100 mg/L	Sedation Surgical anesthesia	
Metomidate hydrochloride	2-4 mg/L 6-10 mg/L	Sedation Light anesthesia	
Lidocaine hydrochloride	300 mg/L 325 mg/L	Light anesthesia Surgical anesthesia	
Tricaine methanesulfonate and isoflurane	65 ppm + 65 ppm 175 ppm + 175 ppm	Light anesthesia Deep anesthesia	Maintains opercular movement for 20-60 min in adults For imaging for approximately 10 min duration
Hypothermia	12°C 10°C 0-4°C	Sedation Light anesthesia Anesthesia	Larval zebrafish can be exposed for up to 10 min

From Ackerman, 2006; Chen, 2014; Collymore, 2014; Grush, 2004; Huang 2010; Lockwood, 2017; Matthews, 2012;

*To be included in new ACLAM The Zebrafish in Biomedical Research textbook

Special Anesthetic Considerations

Special anesthetic considerations

ANESTHESIA OF EMBRYOS

1-3 dpf

Need very high concentrations of drugs for anesthesia

Rapid chilling may be used for short procedures

ANESTHESIA OF LARVAE

4-14 dpf

Need very high concentrations of drugs for anesthesia

Rapid chilling, and high doses of tricaine and eugenol may be used

Special anesthetic considerations

PROLONGED ANESTHESIA

- High mortality for most anesthetics
- Tricaine with isoflurane can provide 60 min anesthesia
- Intermittent dosing of benzocaine or tricaine

REPEATED ANESTHESIA

- 3 or more consecutive days of exposure to anesthesia
- High mortality rates
- No options currently

Tricaine and Isoflurane

Prolonged anesthesia without loss of heart beat or slowing of opercular beat rate

Must be used in a fumehood

Isoflurane alone should never be used

- Highly aversive
- Does not anesthetize the fish

TABLE 1. INDUCTION TIME REQUIRED TO REACH STAGE 4 ANESTHESIA

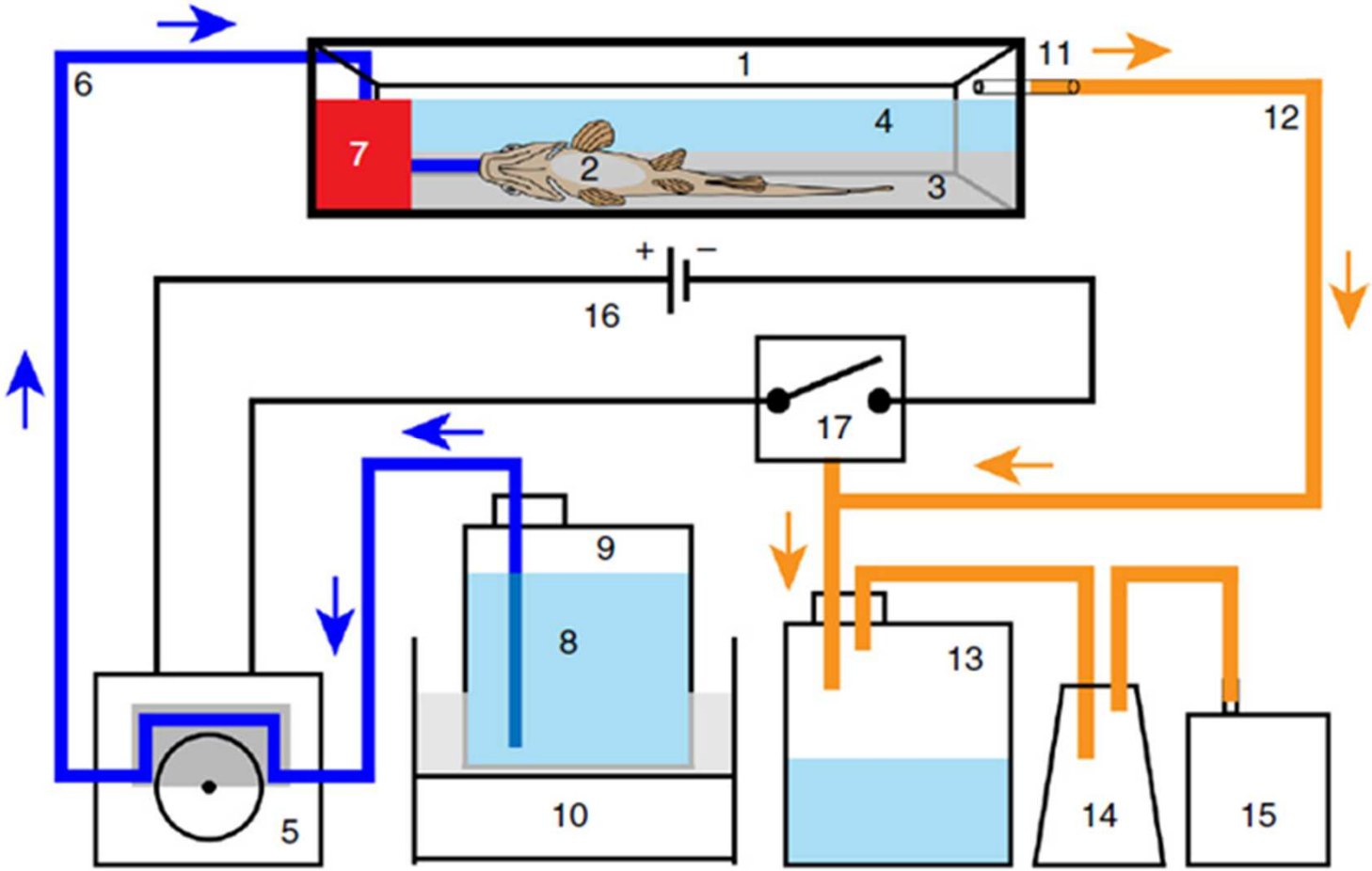
Anesthetic types	*Concentration (ppm)*	*Induction time (min)*
MS-222	200	0.59 ± 0.04
	180	0.79 ± 0.06
	160	1.48 ± 0.09
	140	2.01 ± 0.15
	120[a]	2.55 ± 0.17
	100[a]	2.48 ± 0.17
MS-222 + isoflurane	$80 + 80$	1.00 ± 0.06
	$70 + 70$	1.43 ± 0.14
	$65 + 65$	1.28 ± 0.10
	$60 + 60$	1.50 ± 0.09
	$50 + 50$[a]	3.68 ± 0.59

Data presented as mean ± standard error of the mean, $n = 30$ for each concentration.

[a]Indicates incomplete anesthesia, subjects occasionally flipped and briefly swam during anesthesia.

Huang et al. 2010

Intubation and Intermittent Dosing



Euthanasia

Euthanasia

Euthanasia (from the Greek word εὐθανασία; "good death": εὖ, *eu*; "well" or "good" – θάνατος, *thanatos*; "death") is the practice of intentionally ending a life to relieve pain and suffering

Motivations for euthanizing zebrafish

- Prevention of pain, suffering or injury
- Blood, tissue sampling for experiments
- Elimination due to breeding purposes
- Termination of an experiment
- Preventing escape of genetically modified animals

Ideal Euthanasia Method

Induces unconsciousness quickly

Leads to respiratory and cardiac arrest

Loss of brain function

Minimizes pain and distress

Easy to prepare and administer

Reliable and **irreversible**

Appropriate for species and age of the animal

Compatible with research objectives

Safe for personnel

Legislation

EU

European Directive 2010/63/EU, Annex IV

Methods allowed for fish:

- Anesthetic overdose
- Concussion/percussive blow to the head
- Electrical stunning

Methods other than those listed may be used on unconscious animals, providing the animal does not regain consciousness before death

USA

AVMA Guidelines for the Euthanasia of Animals: 2013 Edition

Methods allowed for fish

- Overdose of anesthetic agents
- Concussion/percussive blow to the head
- Electrical stunning
- Rapid chilling

"It [rapid chilling (hypothermic shock)] is appropriate for zebrafish and other small-bodied (3.8-cm-long or smaller) tropical and subtropical stenothermic finfish, for which the lower lethal temperature range is above 4°C."

Canada

Canadian Council on Animal Care Guidelines on Euthanasia Animals Used in Science and *CCAC Guidelines on the Care and Use of Fish in Research, Teaching and Testing*

Methods allowed for fish

- Overdose of anesthetic agents (must be followed by chemical or physical method to cause brain death)
- Concussion (conditionally acceptable)

But in "*FAQ: CCAC guidelines on: euthanasia of animals used in science*":

What about methods not mentioned in the guidelines as acceptable or conditionally acceptable - are they considered unacceptable?

"For practical reasons, not all possible methods of euthanasia and situations have been described [...]. Use of any methods that have not been specifically mentioned [...] should be discussed with a veterinarian knowledgeable about the species in question"

Methods of Euthanasia

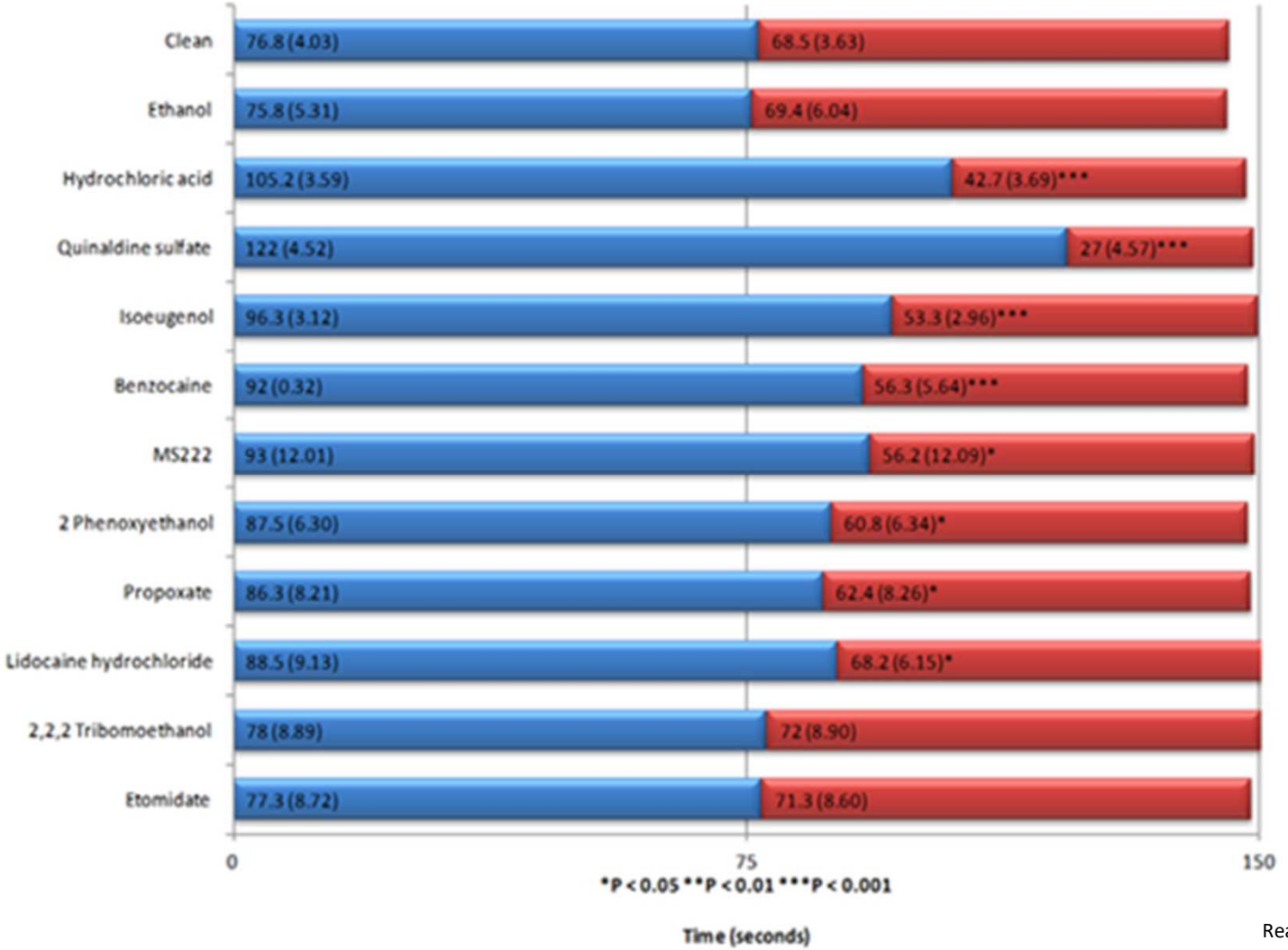
Common Methods

Chemical Methods: Overdose of Anesthetic through inhalation/immersion (mostly MS-222, Eugenol, Benzocaine)

Problems with MS-222

- Signs of stress even with strong evidence for anesthesia (Carter et al., 2011; Matthews and Varga, 2012)
- Cardiac arrest sometimes occurs after time periods > 3 h (Veterinary Office, University of California, Riverside)
- Some fish wake even after movement of operculum has stopped (4/23 fish) (Wilson et al., 2009)

Zebrafish Aversion to Anesthetics



Readman et al. 2013

Hypothermia (Hypothermal Shock, Rapid Chilling)

Hypothermia

The condition of having an abnormally (typically dangerously) low body temperature

Hypothermic Shock

Extreme and potentially lethal form of hypothermia, caused by rapid heat loss

Rapid chilling (in fish)

Quickly lowering the body temperature, usually through immersion in ice slush (water temp 1°C - 3°C).

Rapid freezing

Quickly lowering the body temperature to or below the freezing point, usually done with liquid nitrogen (LN2)

Rapid Chilling (courtesy of Almut Köhler)

Larval Zebrafish < 14dpf

Tricaine methanesulfonate

300, 600, 900 mg/L

At 900 mg/L hearts stopped at 10 min but all recovered within 30 min

Eugenol

500, 1000, 1500 uL/L

At 1500 uL/L hearts stopped in 45-50 min and only 3 returned during 30 min recovery

Larval Zebrafish < 14dpf

Lidocaine hydrochloride

- 400, 500, 600, 700, 800, 900, 1000 mg/L
- None lost heartbeat

Hypothermal shock

- Hearts stopped within 40 min and none recovered
- Younger fish require longer times (up to 12h exposure)

Embryos (0-3 dpf)

Anesthetize with previously discussed agents

- High concentrations may be required
- Longer exposure time

Hypothermal shock for 20 minutes (minimum)

Immersion in dilute bleach solution to assure complete destruction

NIH Guidelines 2013, AVMA 2014

Euthanasia Methods

Table 3. Euthanasia doses for zebrafish

Drug	Dosage	Observation & Comments
Adult zebrafish		
Tricaine methanesulfonate	> 200 mg/L	For 10 min after cessation of OM
Eugenol/Clove oil	> 100 mg/L	For 10 min after cessation of OM
Lidocaine hydrochloride	> 400 mg/L	For 10 min after cessation of OM
2-Phenoxyethanol	≥ 800 mg/L	For 5 min
Hypothermia	0-4°C	For 5 min
Larval fish		
Tricaine methanesulfonate	> 1800 mg/L > 1000 mg/L	For larvae 3-8 dpf with exposure for over 1h For larvae 3-8dpf followed by an adjunctive method of euthanasia
Isoeugenol	> 1500 uL/L	For larvae 14 dpf with exposure for at least 20 min after cessation of heartbeat
Hypothermia	0-4°C	Followed by an adjunctive method (bleach/decapitation/maceration) after exposure for at least 20 min after cessation of heart beat for larvae < 14 dpf Maintained in cold solution for at least 12h for larvae ≤ 14 dpf if no secondary adjunctive method used

From Collymore, 2014; Davis, 2015; Matthews, 2012; NIH Guidelines, 2013; Rombough, 2007; Schroeder, 2016; Strykowski, 2015, Wallace, *in press*

*To be included in new ACLAM The Zebrafish in Biomedical Research textbook

Acknowledgements

Karin Finger-Beier

Almut Kohler

References

American Veterinary Medical Association. 2013. AVMA Guidelines for the Euthanasia of Animals: 2013 Ed. Available at: https://www.avma.org/KB/Policies/Documents/euthanasia.pdf

Collymore C, Tolwani A, Lieggi C, Rasmussen S. 2014. Efficacy and Safety of 5 Anesthetics in Adult Zebrafish (*Danio rerio*). *JAALAS* 53(2):198-203.

Davis JD, Klug J, et al. 2015. Effects of Clove Oil as a Euthanasia Agent on Blood Collection Efficiency and Serum Cortisol Levels in *Danio rerio*. *JAALAS* 54(5): 564-567.

Martins T, Valentim AM, et al. 2016. Anesthesia and Analgesia in Laboratory Adult Zebrafish: A Question of Refinement. Lab Anim 50(6): 476-488.

Matthews M, Varga ZM. 2012. Anesthesia and Euthanasia in Zebrafish. *ILAR Journal* 53(2): 192-204.

National Institutes of Health. 2013. Guidelines for Use of Zebrafish in the NIH Intramural Research Program. Available at: http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf

References

Readman GD, Owen SF, Murrell JC, Knowles TG. 2013. Do fish Perceive Anaesthetics as Aversive? *PLoS ONE* 8(9):e73773.

Rombough PJ. 2007. Ontogenetic Changes in the Toxicity and Efficacy of the Anaesthetic MS222 (tricaine methanesulfonate) in zebrafish (*Danio rerio*) larvae. *Comp Biochem and Phys*, Part A 148:463-469.

Ross LG, Ross B. 2008. Anaesthetic and Sedative Techniques for Aquatic Animals, 3rd Ed. Ames (IO): Blackwell Publishing.

Schroeder PG, Sneddon LU. 2017. Exploring the Efficacy of Immersion Analgesics in Zebrafish Using an Integrative Approach. *Appl Anim Behav Sci* 187: 93-102.

Strykowski JL, Schech JM. 2015. Effectiveness of Recommended Euthanasia Methods in Larval Zebrafish (*Danio rerio*). *JAALAS* 54(1):81-84.

Valentim AM, Felix LM, et al. 2016. A new Anaesthetic Protocol for Adult Zebrafish (Danio rerio): Propofol combined with Lidocaine. PLoS ONE 11 (1): e0147747

Wong D, von Kerserlingk MAG, Richards JG, Weary DM. 2014. Conditioned Place Avoidance of Zebrafish (*Danio rerio*) to Three Chemicals Used for Euthanasia and Anaesthesia. *PLoS ONE* 9(2):e88030.