The Importance of Using Anaesthesia and Analgesia in Improving Fish Welfare

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Anaesthesia and Analgesia in fish

- 3Rs - Refinement
- Pain in fish?
- Assessing and alleviating pain in zebrafish
- Analgesia
- Anaesthesia – recovery and euthanasia
- Recommendations

PREPARE Guidelines

Original Article

Laboratory Animals
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PREPARE: guidelines for planning animal research and testing

Adrian J Smith[1], R Eddie Clutton[2], Elliot Lilley[3], Kristine E Aa Hansen[4] and Trond Brattelid[5]

Abstract

There is widespread concern about the quality, reproducibility and translatability of studies involving research animals. Although there are a number of reporting guidelines available, there is very little overarching guidance on how to *plan* animal experiments, despite the fact that this is the logical place to start ensuring quality. In this paper we present the PREPARE guidelines: Planning Research and Experimental Procedures on Animals: Recommendations for Excellence. PREPARE covers the three broad areas which determine the quality of the preparation for animal studies: formulation, dialogue between scientists and the animal facility, and quality control of the various components in the study. Some topics overlap and the PREPARE checklist should be adapted to suit specific needs, for example in field research. Advice on use of the checklist is available on the Norecopa website, with links to guidelines for animal research and testing, at https://norecopa.no/PREPARE.

Are you PREPAREd?

PREPARE:
Planning **R**esearch and **E**xperimental **P**rocedures on **A**nimals: **R**ecommendations for **E**xcellence

PREPARE covers 15 topics:

Formulation of the study
1. Literature searches
2. Legal issues
3. Ethical issues, harm-benefit assessment and humane endpoints
4. Experimental design and statistical analysis

Dialogue between scientists and the animal facility
5. Objectives and timescale, funding and division of labour
6. Facility evaluation
7. Education and training
8. Health risks, waste disposal and decontamination

Methods
9. Test substances and procedures
10. Experimental animals
11. Quarantine and health monitoring
12. Housing and husbandry
13. Experimental procedures
14. Humane killing, release, reuse or rehoming
15. Necropsy

Use of Protected Animals

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COMMENTARY

Considering aspects of the 3Rs principles within experimental animal biology

Lynne U. Sneddon[1,*], Lewis G. Halsey[2] and Nic R. Bury[3]

ABSTRACT

The 3Rs – Replacement, Reduction and Refinement – are embedded into the legislation and guidelines governing the ethics of animal use in experiments. Here, we consider the advantages of adopting key aspects of the 3Rs into experimental biology, represented mainly by the fields of animal behaviour, neurobiology, physiology, toxicology and biomechanics. Replacing protected animals with less sentient forms or species, cells, tissues or computer modelling approaches has been broadly successful. However, many studies investigate specific models that exhibit a particular adaptation, or a species that is a target for conservation, such that their replacement is inappropriate. Regardless of the species used, refining procedures to ensure the health and well-being of animals prior to and during experiments is crucial for the integrity of the results and legitimacy of the science. Although the concepts of health and welfare are developed for model organisms, relatively little is known regarding non-traditional species

from the general public that the use of animals in research is moral and ethically justifiable. A recent poll in the USA demonstrated that 50% of the public were opposed to the use of animals in research (http://www.pewinternet.org/2015/01/29/public-and-scientists-views-on-science-and-society/). In 2015, nine European countries presented a petition to the European Commission (EC) to ban animal research. However, the EC opposed this movement, but responded by stating that ethical justification and adoption of the 3Rs (Replacement, Reduction and Refinement) is a must for experimental studies (European Commission, 2015). Of course, it is in scientists' interest to adopt an ethical and humane approach to husbandry and experimental design, as healthy animals produce robust, reliable results, underlying valid scientific outputs. For example, improved husbandry and handling of rodents reduces stress, and this leads to less-variable data and more meaningful results (Hurst and West, 2010; Singhal et al., 2014). Embedding the

PREPARE
3Rs

Ethical Animal Experimentation

JUSTIFY STUDY

Is the study beneficial to humans or animals? Benefits can be medical, veterinary, economic, biological or educational.

Will it generate novel knowledge or have applied relevance?

Has the design been logged prior to commencing the experiment?

Is the experimental design appropriate to address the research question e.g. blinding, randomisation?

REPLACEMENT

Necessity for using whole animals? Can an immature form or invertebrate* model be used? Human volunteers, human cells and tissues or animal cell and tissue preparations? Is the model Relevant?

Mathematical or computational modelling of existing data sets rather than a new study using animals?

REDUCTION

Is the sample size just large enough to give sufficiently informative results but avoiding the use of too many animals?

Will the outcomes be published and/or included in a future meta-analysis?

REFINEMENT

Husbandry and housing – are animals kept in good health?

Will health be appropriately monitored and action taken quickly to improve welfare before and during experiments?

Are the least invasive techniques being employed to promote good welfare during experiments?

Where procedures compromise welfare are protocols in place to improve this e.g. pain-relief?

ARRIVE

Publish

Sneddon et al. (2017) J. Exp. Biol. 220, 3007-3016

REFINEMENT

Husbandry and housing – are animals kept in good health?

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Pain in fish?

Animal Behaviour 97 (2014) 201–212

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Review

Defining and assessing animal pain

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The detection and assessment of pain in animals is crucial to improving their welfare in a variety of contexts in which humans are ethically or legally bound to do so. Thus clear standards to judge whether pain is likely to occur in any animal species is vital to inform whether to alleviate pain or to drive the refinement of procedures to reduce invasiveness, thereby minimizing pain. We define two key concepts that can be used to evaluate the potential for pain in both invertebrate and vertebrate taxa. First, responses to noxious, potentially painful events should affect neurobiology, physiology and behaviour in a

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REVIEW

Pain in aquatic animals

Lynne U. Sneddon*

ABSTRACT

Recent developments in the study of pain in animals have demonstrated the potential for pain perception in a variety of wholly aquatic species such as molluscs, crustaceans and fish. This allows us to gain insight into how the ecological pressures and differential life history of living in a watery medium can yield novel data that inform the comparative physiology and evolution of pain. Nociception is the simple detection of potentially painful stimuli usually accompanied by a reflex withdrawal response, and nociceptors have been found in aquatic invertebrates such as the sea slug *Aplysia*. It would seem

injury and this is termed pain. The concept of pain occurring in animals has been extensively debated, with some authors suggesting only primates and humans can experience the adverse affective component as they possess a human (or similar in primates) neocortex (Rose, 2002; Rose et al., 2014). Opposing this opinion, scientists suggest that the negative experience that accompanies tissue damage is crucial in altering an animal's subsequent behaviour to perform protective and guarding reactions, enabling the animal to avoid such stimuli in future, and for avoidance learning to occur (Sneddon et al., 2014). This implies that

Assessing pain in fish?

- Popular experimental model
- 28% increase in Europe likely to reflect global use
- Need to ensure good welfare = good science
- Currently no tools for fish
- Important refinement

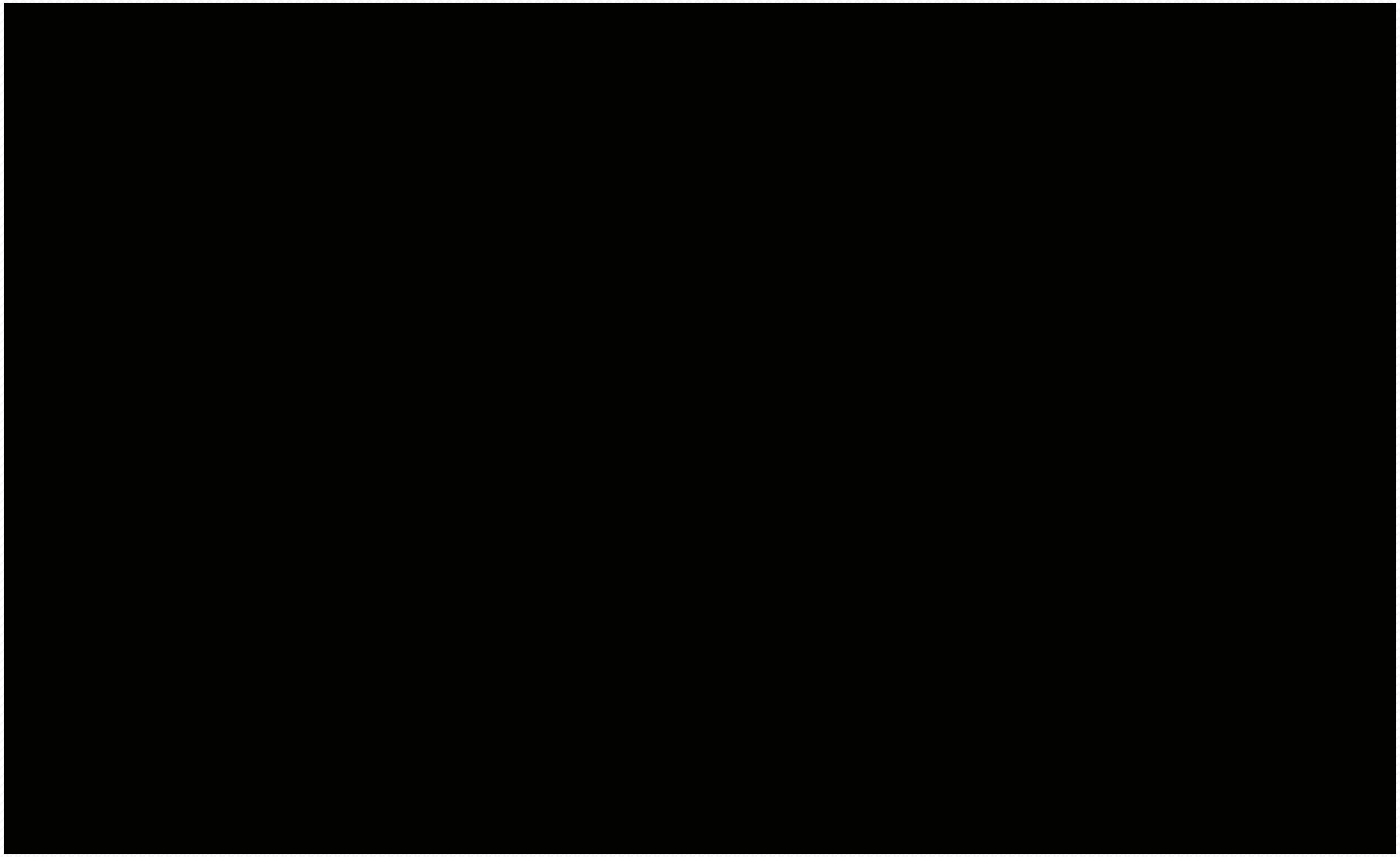
Using animal behaviour

- Many advantages
- Non-invasive
- Subtle changes may reflect initial attempts to cope
- Automated alert system would allow us to intervene

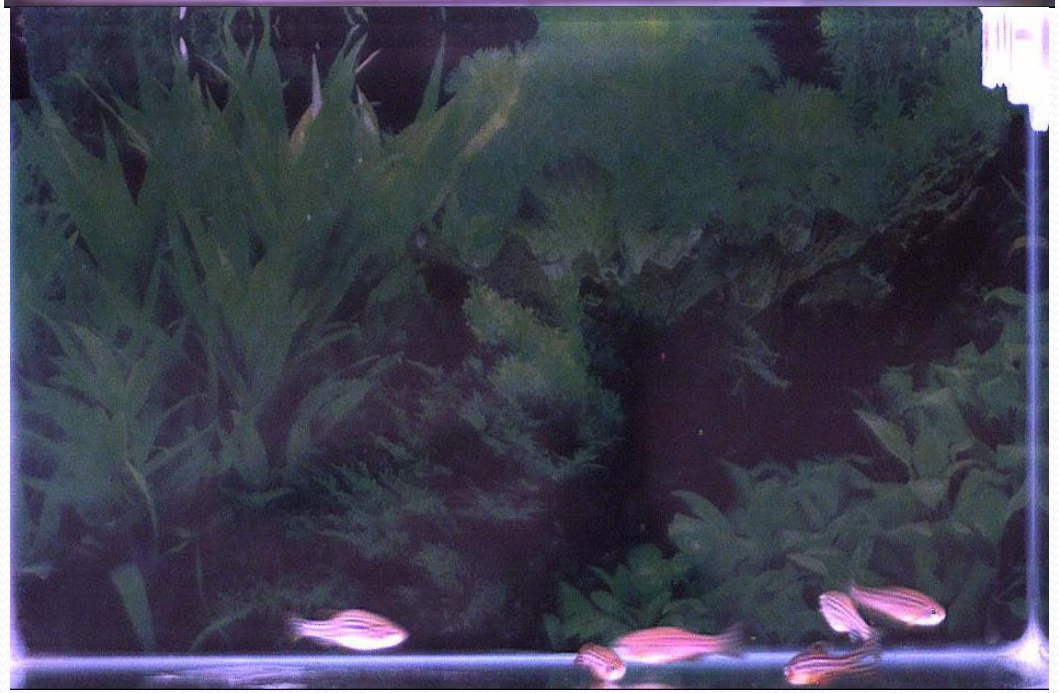
Objectives

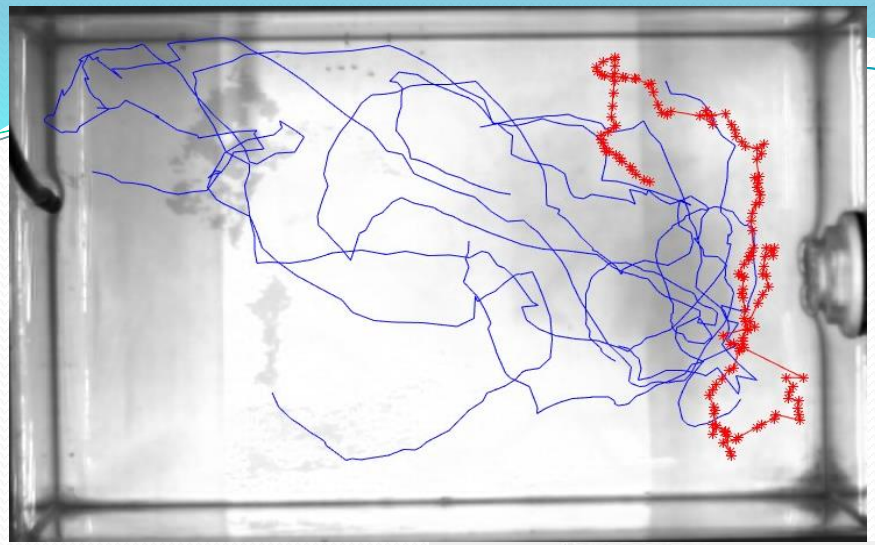
- Develop an automated software tool
- Test efficacy of immersion analgesia

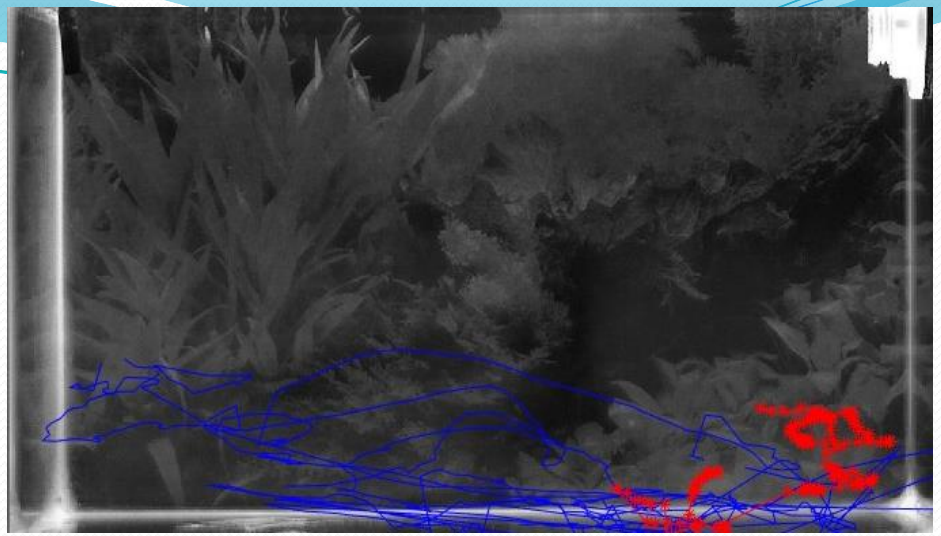
3D mapping of zebrafish behaviour

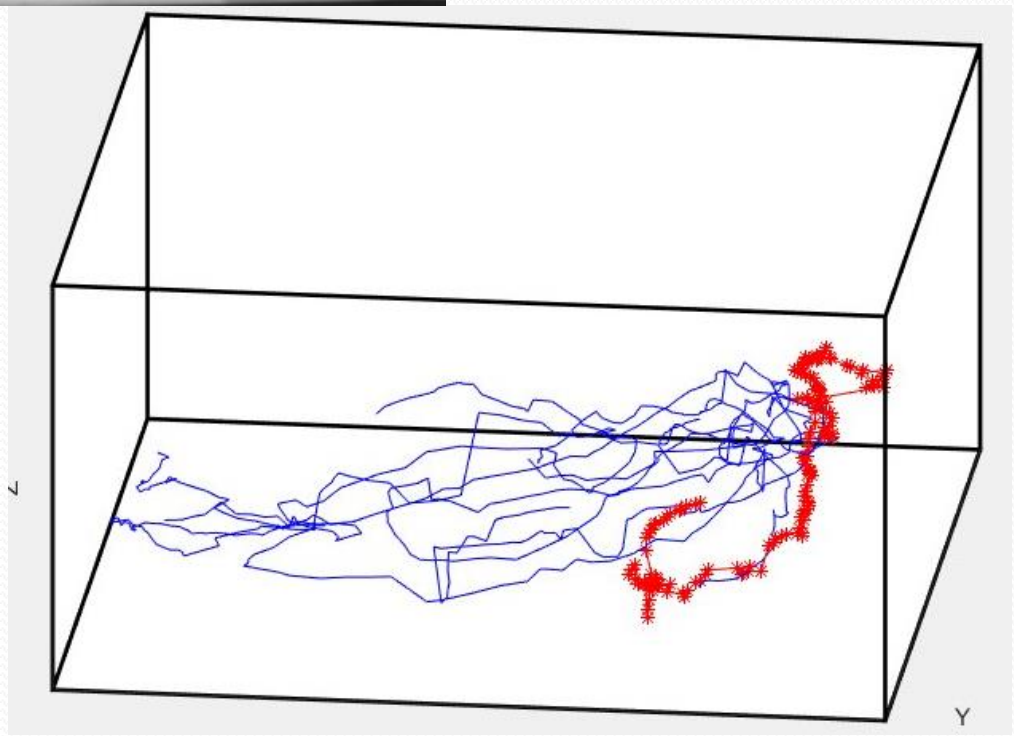


Hamza Alzu'bi and Waleed Al-Nuaimy, Electrical Engineering, unpublished data








Z
Y

Automatic analysis of behaviour

- Get real-time information
- Speed
- Distance travelled
- Acceleration
- Deceleration
- Time spent in specific areas
- Time spent active/motionless
- And many more

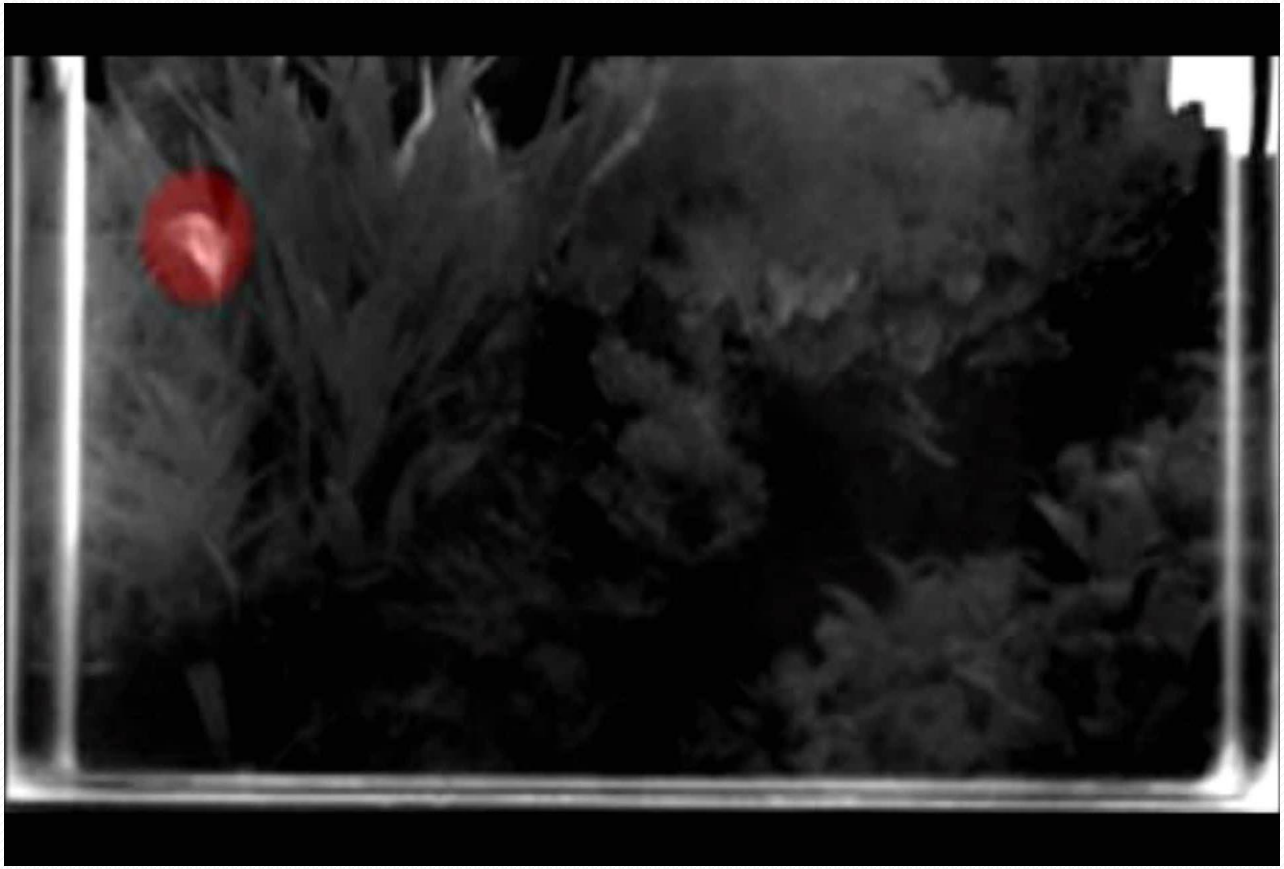
Recognising signs of pain in **individual** zebrafish

- **Healthy**
- Continuous swimming
- Swimming in mid water
- Calm swimming
- Gentle turns

Unhealthy

- Immobile
- Increased use of tank bottom
- Bursts of erratic swimming
- Unusual behaviours

Behaving normally / healthy? Fish A

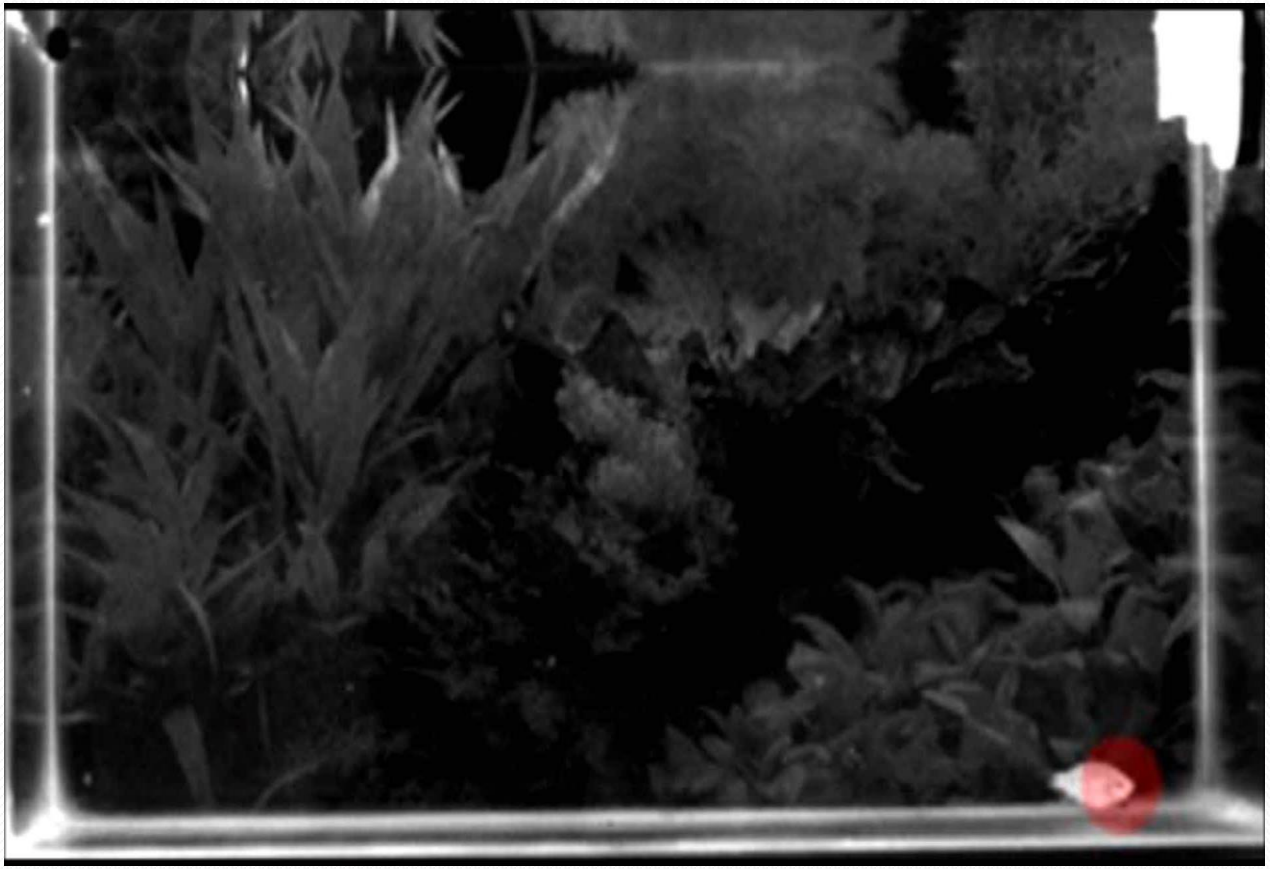


- Deakin et al. MS submitted

Behaving normally / healthy?

- Fish A – Normal, healthy

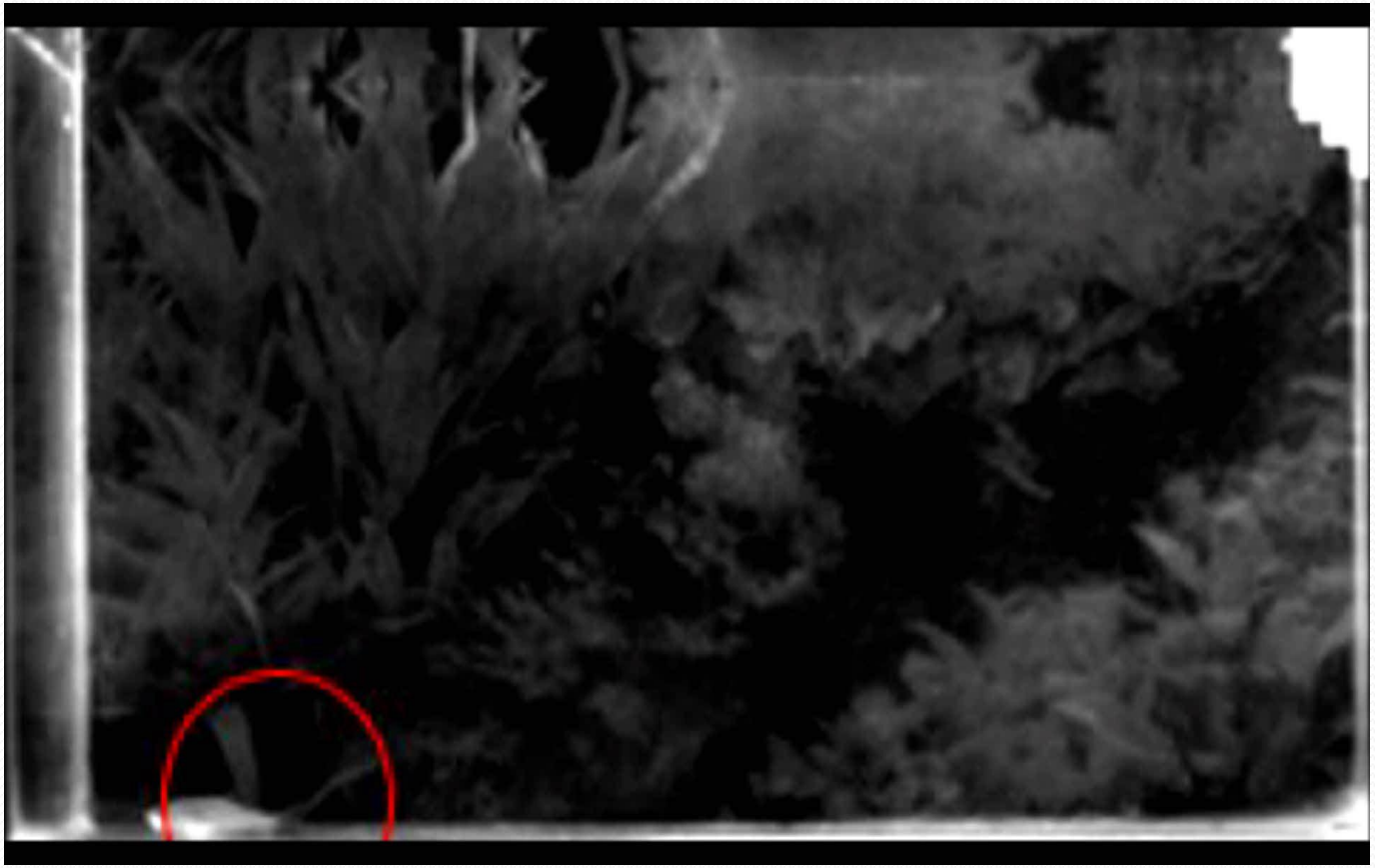
Behaving normally / healthy? Fish B



Behaving normally / healthy?

- Fish A – Normal, healthy
- Fish B – Unhealthy, fin clip

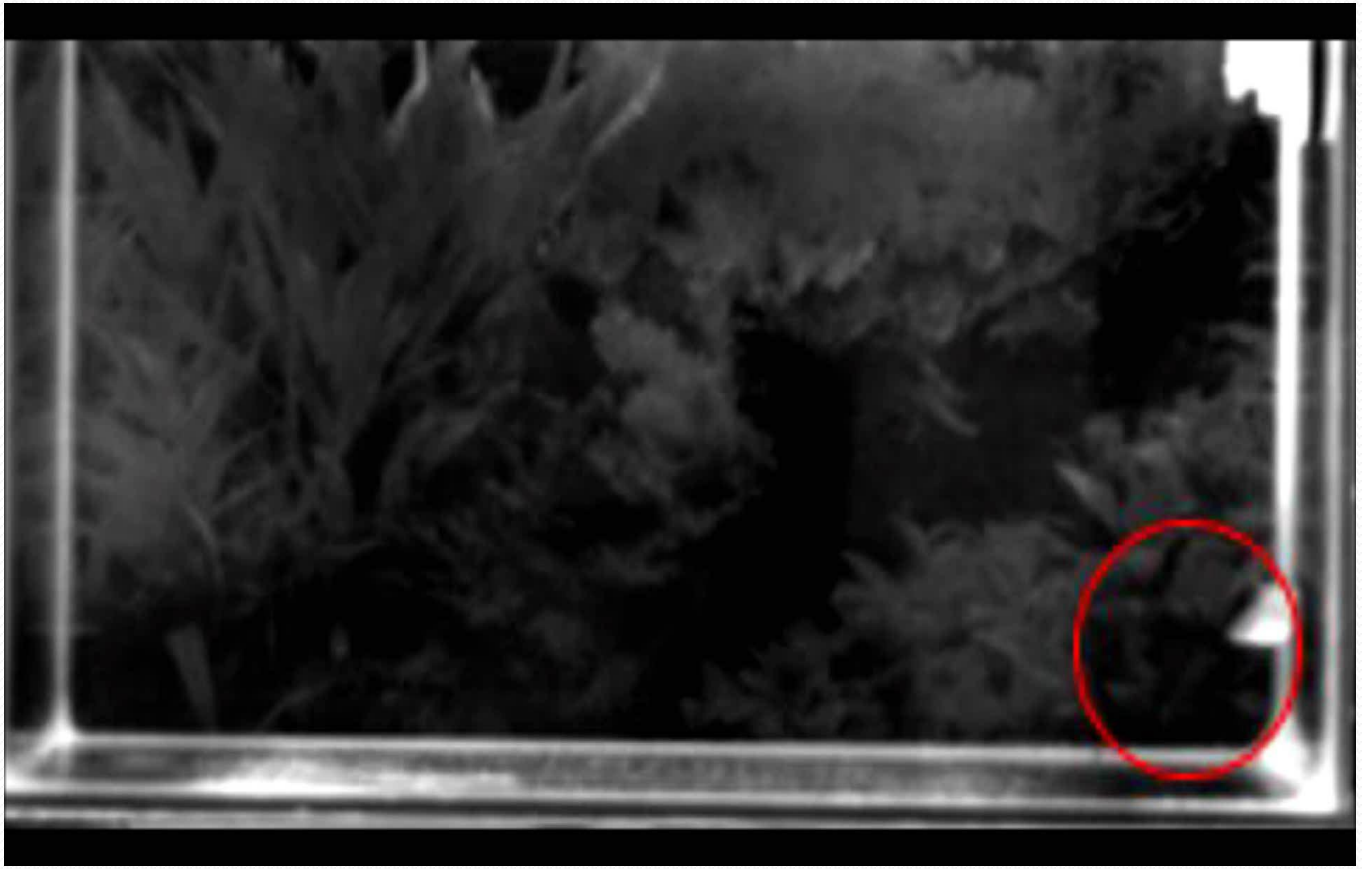
Behaving normally / healthy? Fish C



Behaving normally / healthy?

- Fish A – Normal, healthy
- Fish B – Unhealthy, fin clip
- Fish C – Unhealthy, PIT tag

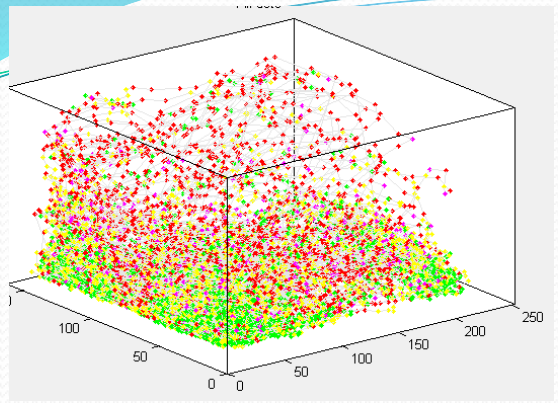
Behaving normally / healthy? Fish D



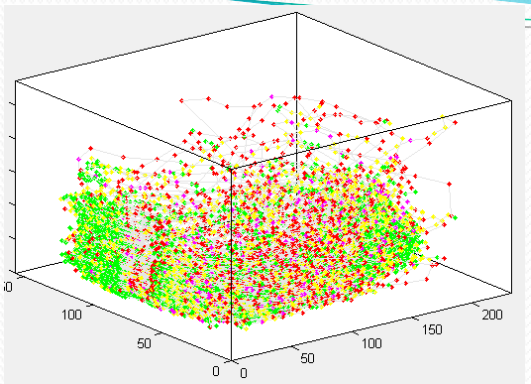
Behaving normally / healthy?

- Fish A – Normal, healthy
- Fish B – Unhealthy, fin clip
- Fish C – Unhealthy, PIT tag
- Fish D – Fin clip but given lidocaine at 5mg/L

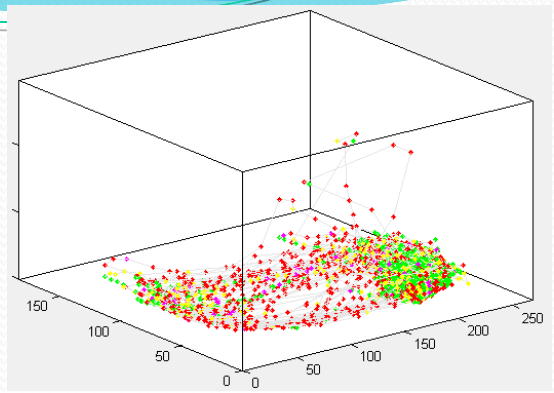
Control



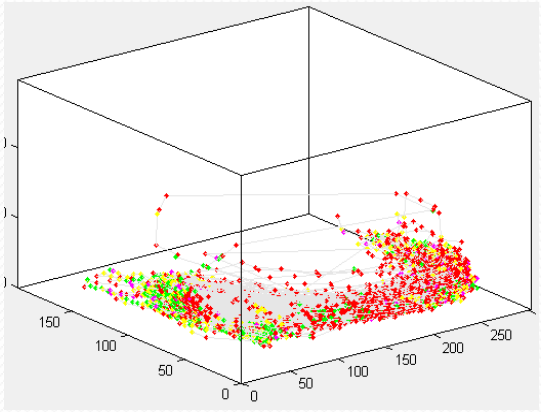
Sham handled



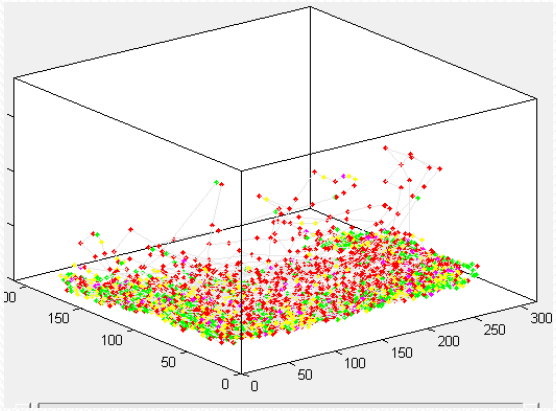
Fin clip



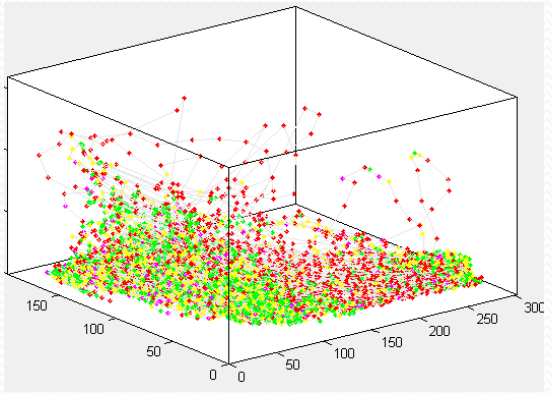
PIT tag insertion



Muscle

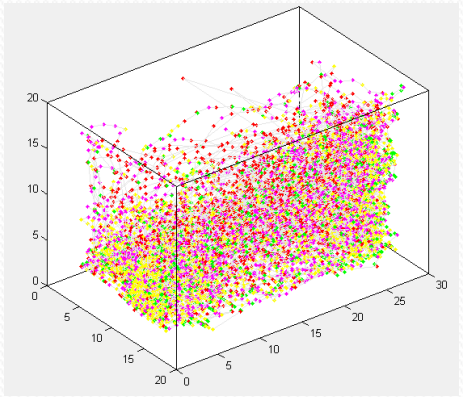


Chemical

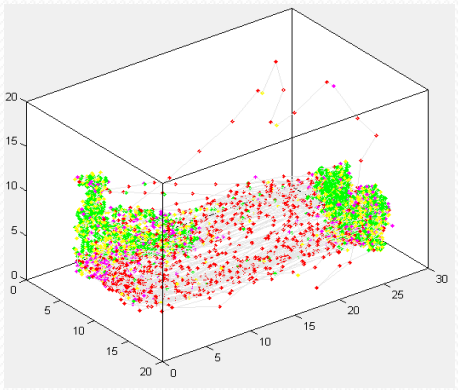


- Tracking behaviour at 2 hours after treatment; Deakin et al. MS submitted

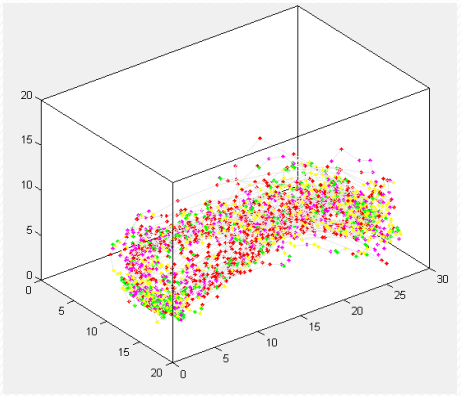
3D maps of analgesics



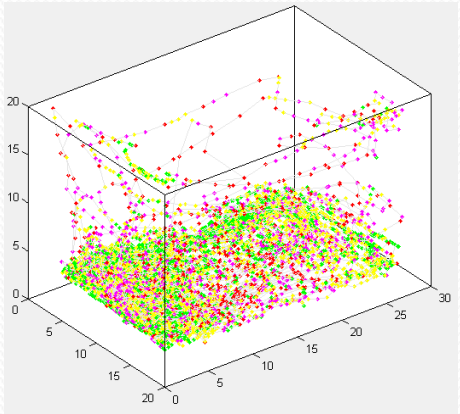
Lidocaine 5mg/L



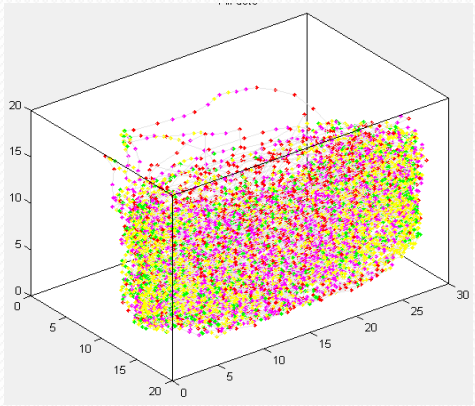
Flunixin 2mg/L



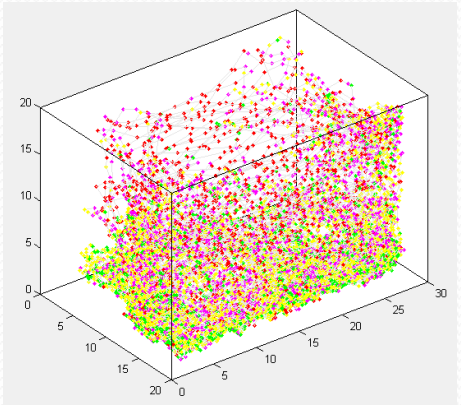
Morphine 3mg/L



Bupivacaine 1mg/L



Flunixin 8mg/L



Morphine 48mg/L

- Tracking behaviour at 2 hours after treatment; Deakin et al. MS submitted

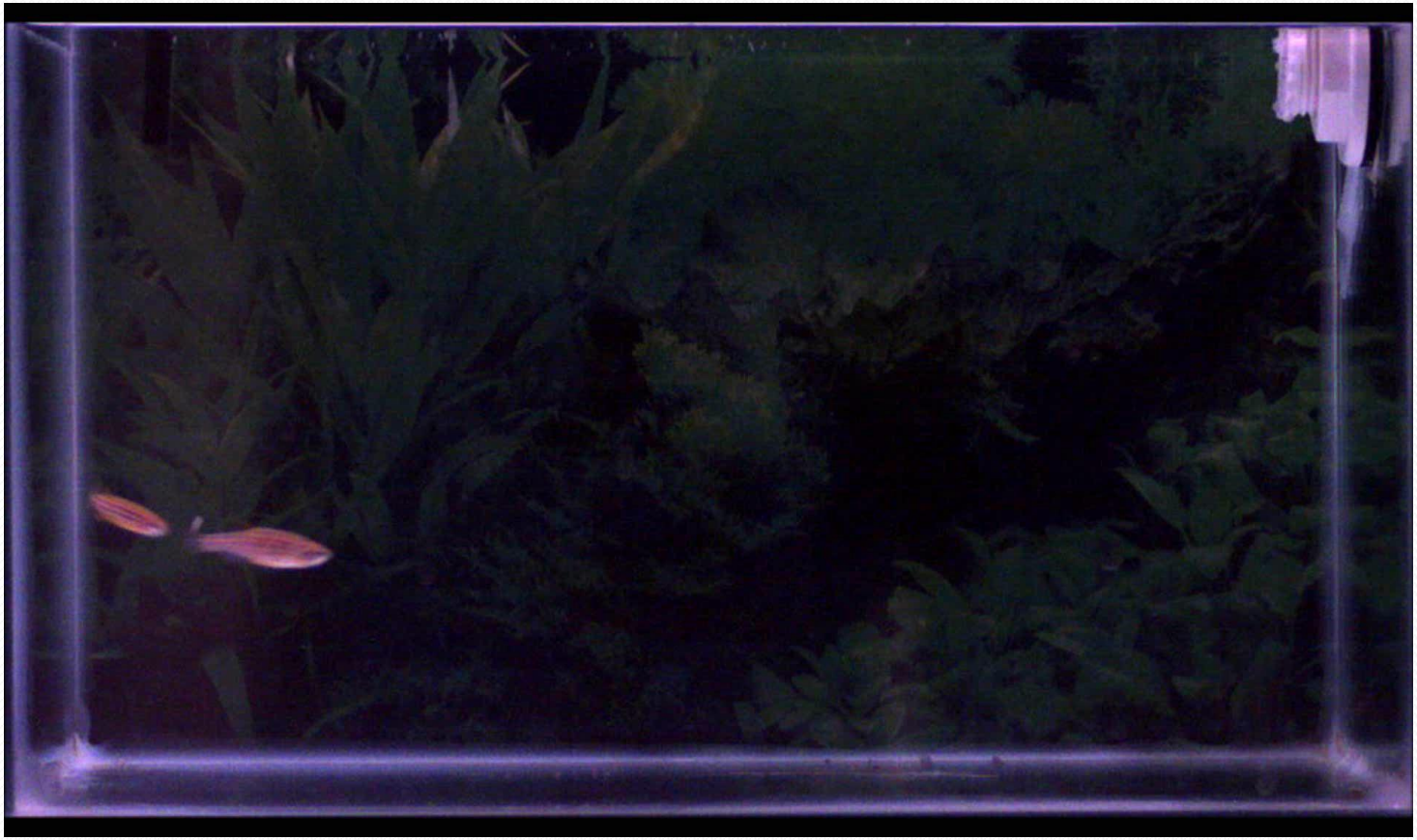
Recognising signs of pain in **pairs** of zebrafish

- **Healthy**
- Continuous swimming
- Swimming in mid water
- Calm swimming
- Gentle turns
- **Interacting continuously**

Unhealthy

- Immobile
- Increased use of tank bottom
- Bursts of erratic swimming
- Unusual behaviours

Pair - control



Pair – fin clip

Recognising signs of pain in **groups** of zebrafish

- **Healthy**
- Continuous swimming
- Swimming in mid water
- Calm swimming
- Gentle turns
- **Interacting continuously - dominance**

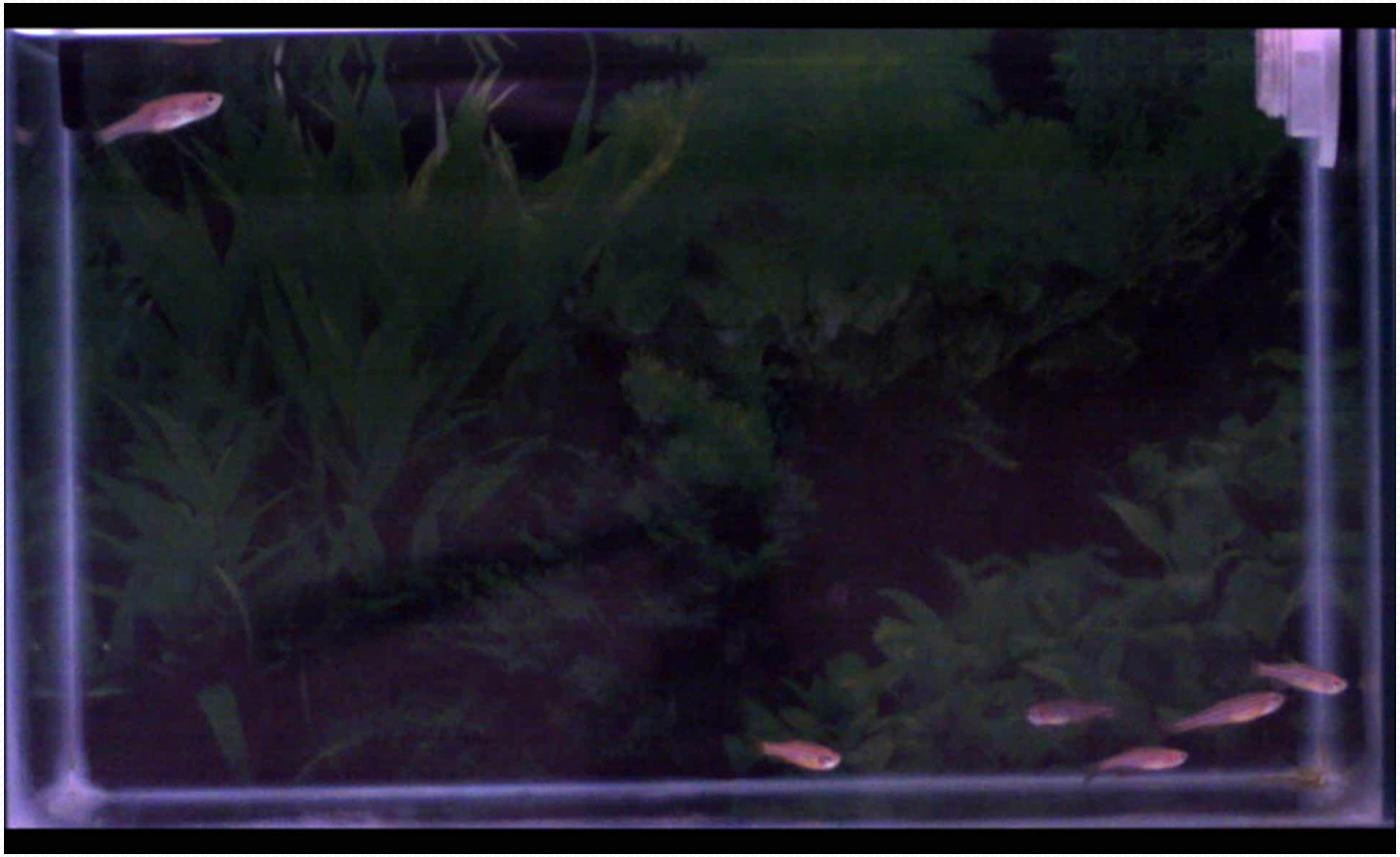
Unhealthy

- **Immobile (depends on pain type)**
- ~~Increased use of tank bottom~~
- Bursts of erratic swimming
- Unusual behaviours
- **Ostracised from the group if immobile**

Dominants do not display the same signs of pain when returned to a group; Ashley et al. (2009) Animal Behaviour 77, 403–410

Group - control

Group - fin clip



PCA – Key Behaviours

- Distance swum
- Location
- Activity
- Develop software - chromatic analysis
- Data extracted from videos in real time to provide health status
- Tested in 2D, blinded to treatment and on other species at Blue Planet Aquarium

Health Index

Normal
Ok
Unhealthy
Abnormal

based on scores for Activity, Location, Distance swum
30mins 20mins 10mins (1min)

ALERTS

	A	R	S	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
1	Count			1	1	1	0	0		0.00					
2	FPS			1	2	2	10			0.02		0			
3	Mins	0		1	3	3	186			0.39		-1	0	0	
4	Check fr	41.67		2	1	4	0			0.00					
5		83.33		2	2	5	0			0.00		Run			Z
6	Assume	125		2	3	6	982			2.05					Y
7	Max			3	1	7	0			0.00					X
8	Min			3	2	8	50			0.10		Stop			
9	VLC			3	3	9	3571	4799		7.44	10.0				
10	1		14400												
11	2	1	11964												
12	3														Z
13	4														Y
14	5														X
15	6														
16	7														
17	8														
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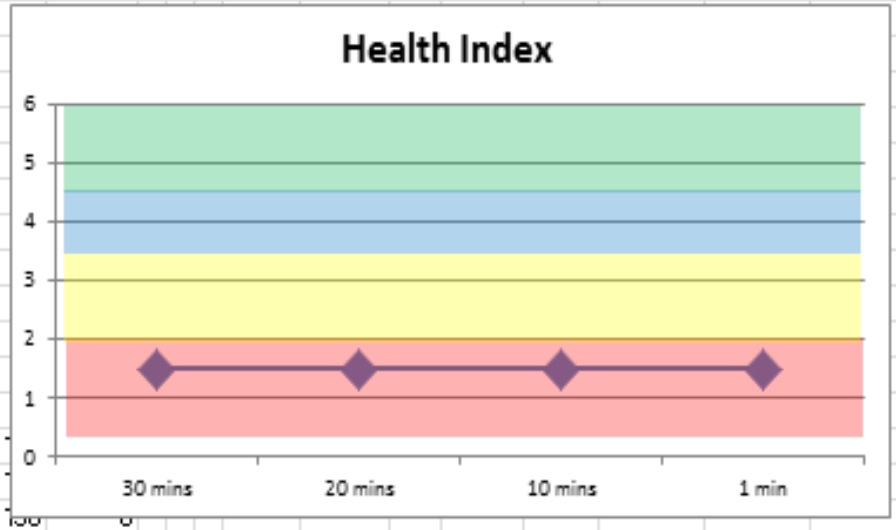
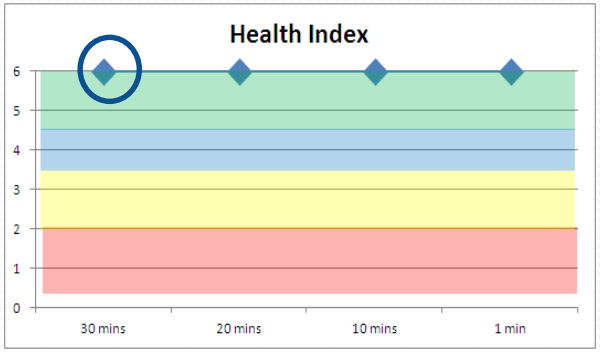


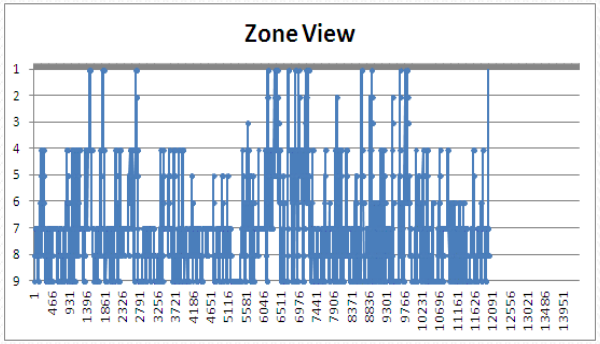
AF	AG	AH	AI	AJ
	Hrs	Mins	Secs	
TIME	0	10	0	
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	13,290	13,290	13,290	976
UTIL				
ACTIVITY	2.80	2.80	2.80	2.00
	Abnormal	Abnormal	Abnormal	Abnormal
Health Index	1.5	1.5	1.5	1.5
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	low	low	low	low
UTIL				
ACTIVITY	hypo	hypo	hypo	hypo
Time	10	10	10	1
Adj period dist	39,870	26,580	13,290	976
Avg. Distance/mi	1,329	1,329	1,329	976
Scores				
DISTANCE	1.5	1.5	1.5	1.5
UTIL				
ACTIVITY	0	0	0	0
Z	1	1	1	1

Sham 3

1 (U)

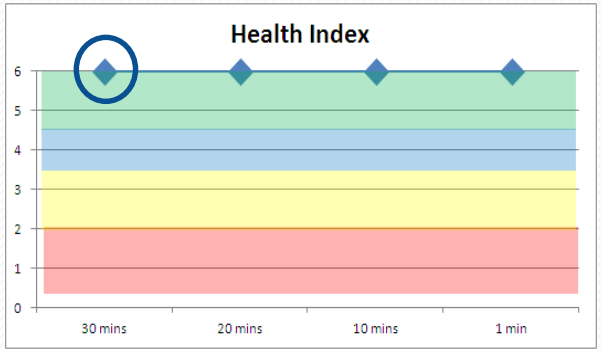
	Hrs	Mins	Secs	
TIME	0	25	0	
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	85,322	69,423	36,487	3,570
UTIL				
ACTIVITY	6.60	6.75	6.80	6.00
	Healthy	Healthy	Healthy	Healthy
Health Index	6	6	6	6
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	normal	normal	normal	normal
UTIL				
ACTIVITY	normal	normal	normal	normal

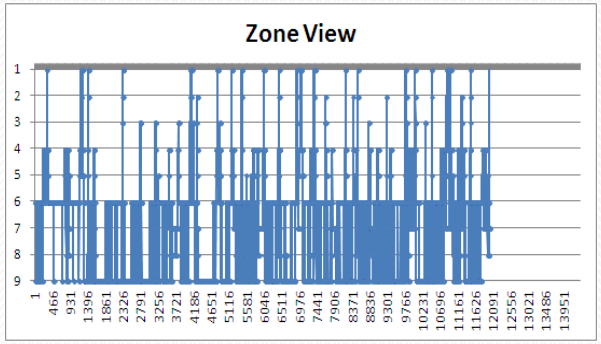




2 (T)

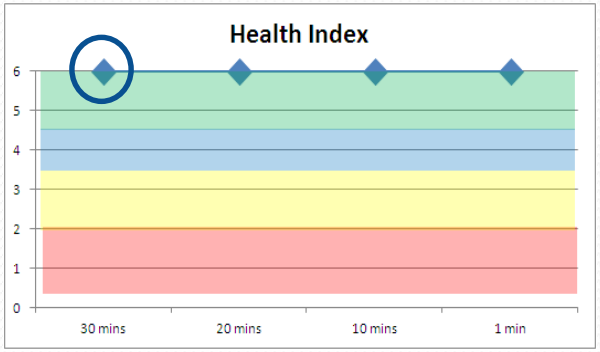
	Hrs	Mins	Secs	
TIME	0	25	0	
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	79,577	66,775	39,096	4,018
UTIL				
ACTIVITY	6.92	7.20	7.60	6.00
	Healthy	Healthy	Healthy	Healthy
Health Index	6	6	6	6
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	normal	normal	normal	normal
UTIL				
ACTIVITY	normal	normal	normal	normal

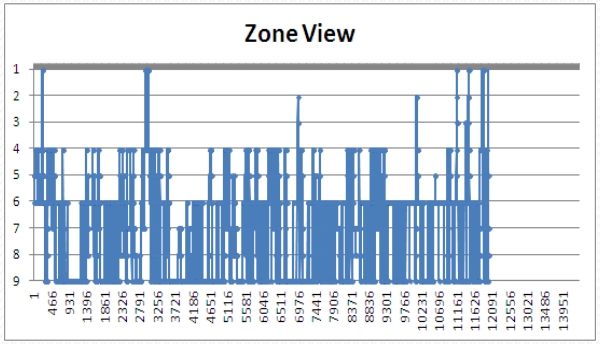




3 (T)

	Hrs	Mins	Secs	
TIME	0	25	0	
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	68,008	55,961	28,510	4,031
UTIL				
ACTIVITY	5.92	5.85	5.70	7.00
	Healthy	Healthy	Healthy	Healthy
Health Index	6	6	6	6
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	normal	normal	normal	normal
UTIL				
ACTIVITY	normal	normal	normal	normal





PIT d10N

1 (U)

	Hrs	Mins	Secs	
TIME	0	25	0	
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	98,143	81,390	45,805	5,446
UTIL				
ACTIVITY	5.76	5.90	6.50	8.00
	Healthy	Healthy	Healthy	Ok
Health Index	6	6	6	4
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	normal	normal	normal	hyper
UTIL				
ACTIVITY	normal	normal	normal	normal

2 (T)

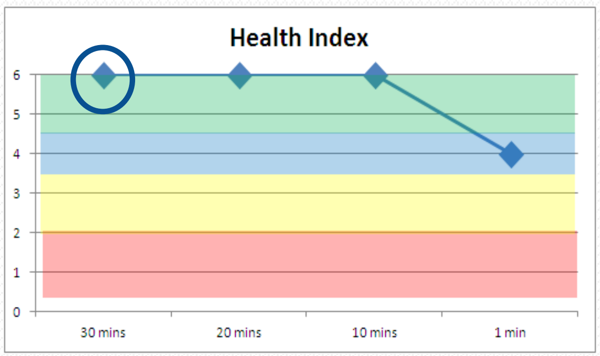
ALERTS

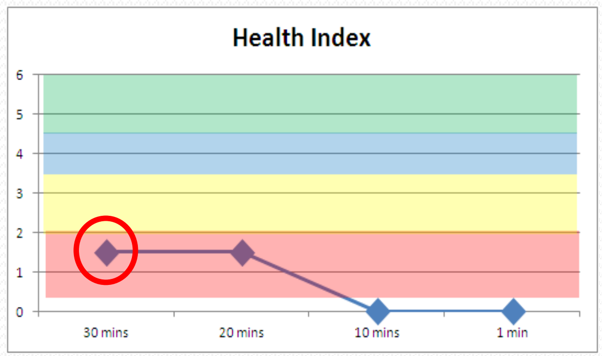
	Hrs	Mins	Secs	
TIME	0	25	0	
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	18,520	14,694	6,605	24
UTIL				
ACTIVITY	2.92	2.95	2.60	1.00
	Abnormal	Abnormal	Abnormal	Abnormal
Health Index	1.5	1.5	0	0
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	low	low	hypo	hypo
UTIL				
ACTIVITY	hypo	hypo	hypo	hypo

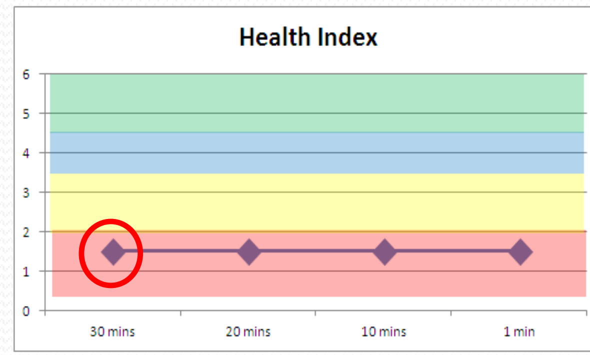
3 (T)

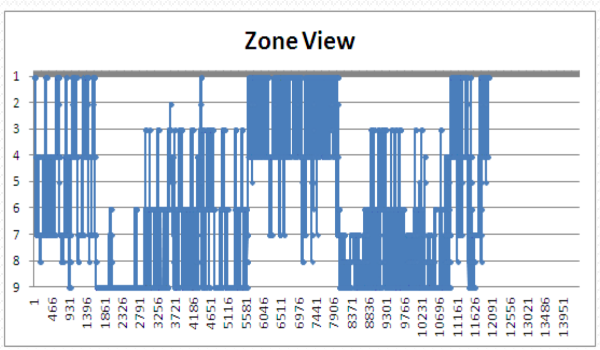
ALERTS

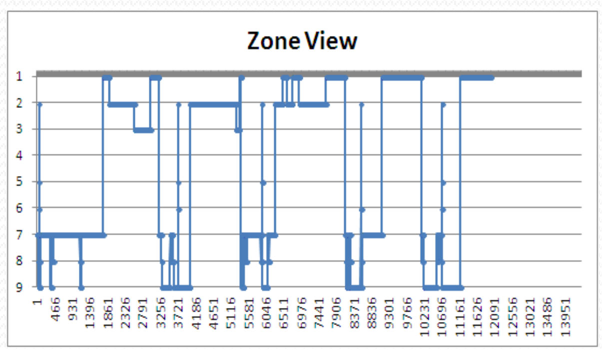
	Hrs	Mins	Secs	
TIME	0	25	0	
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	23,022	16,099	7,761	696
UTIL				
ACTIVITY	2.36	2.35	2.00	2.00
	Abnormal	Abnormal	Abnormal	Abnormal
Health Index	1.5	1.5	1.5	1.5
latest	30 mins	20 mins	10 mins	1 min
DISTANCE	low	low	low	low
UTIL				
ACTIVITY	hypo	hypo	hypo	hypo

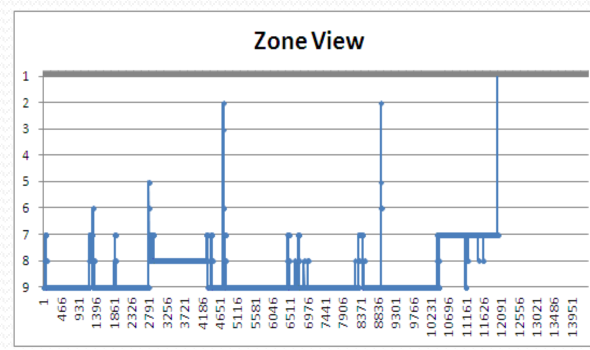




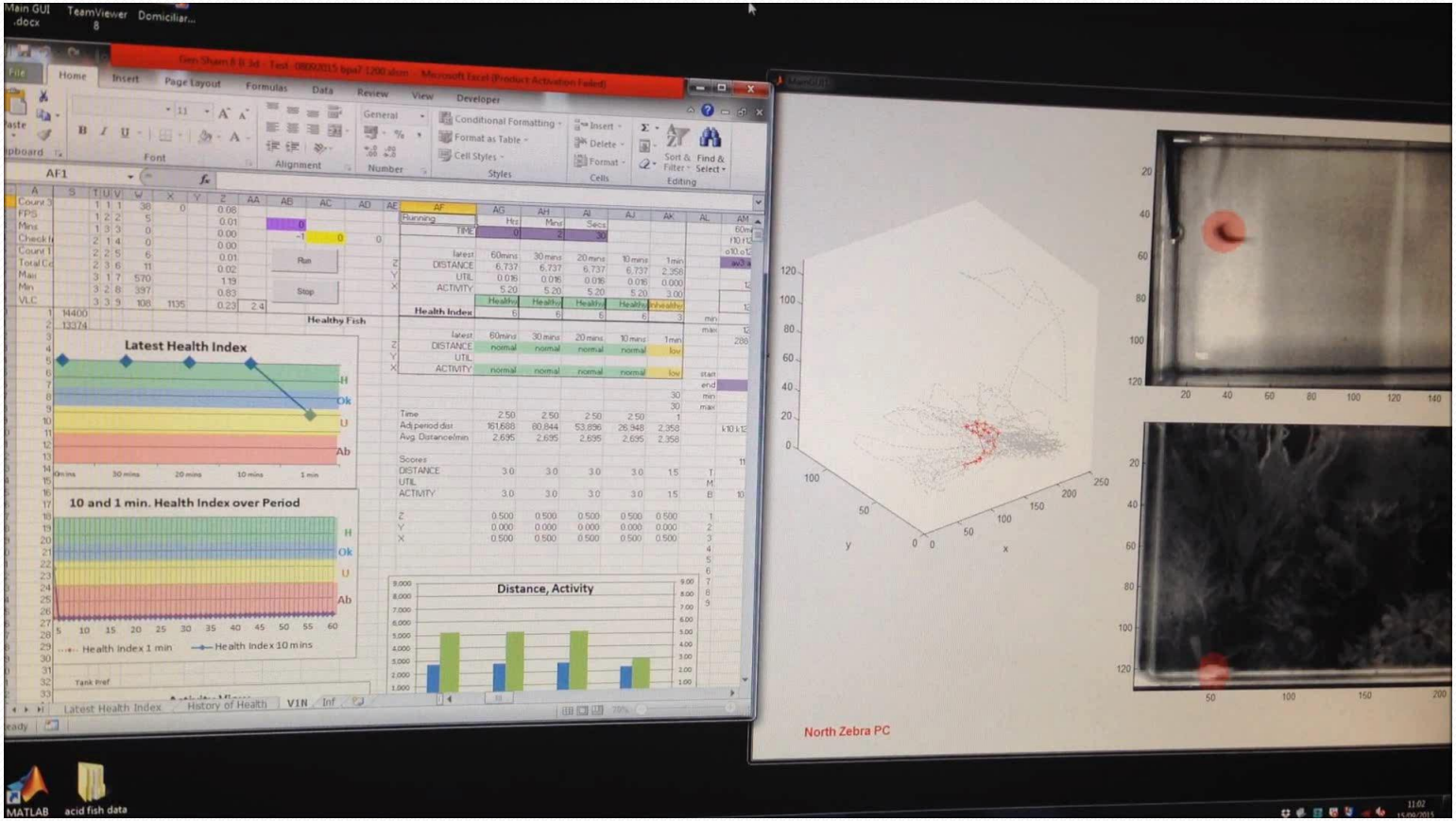




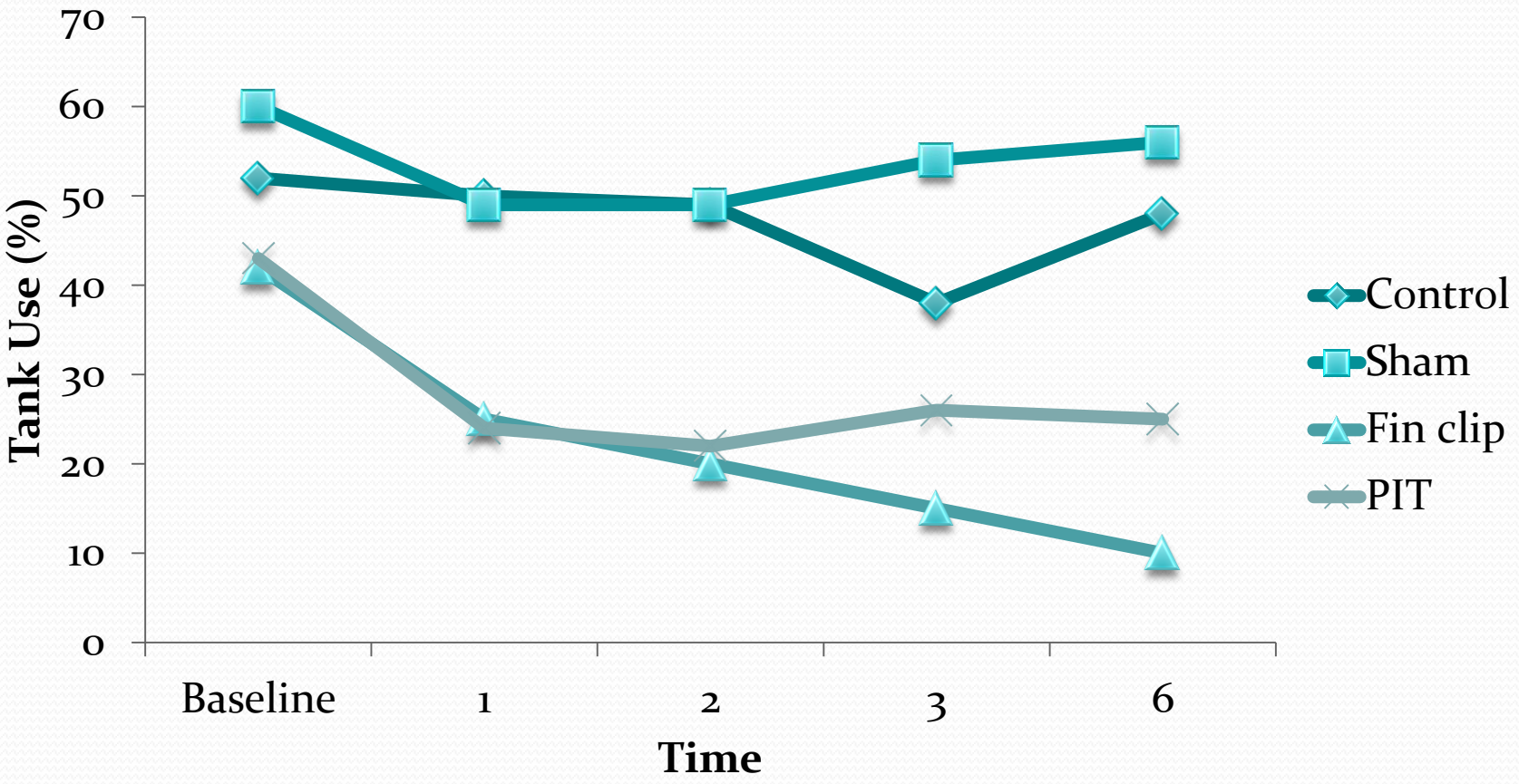




Normal – paused – fin clip behaviour



Recovery?


70
60
50
40
30
20
10
0
Tank Use (%)
Baseline
1
2
3
6
Time
Control
Sham
Fin clip
PIT

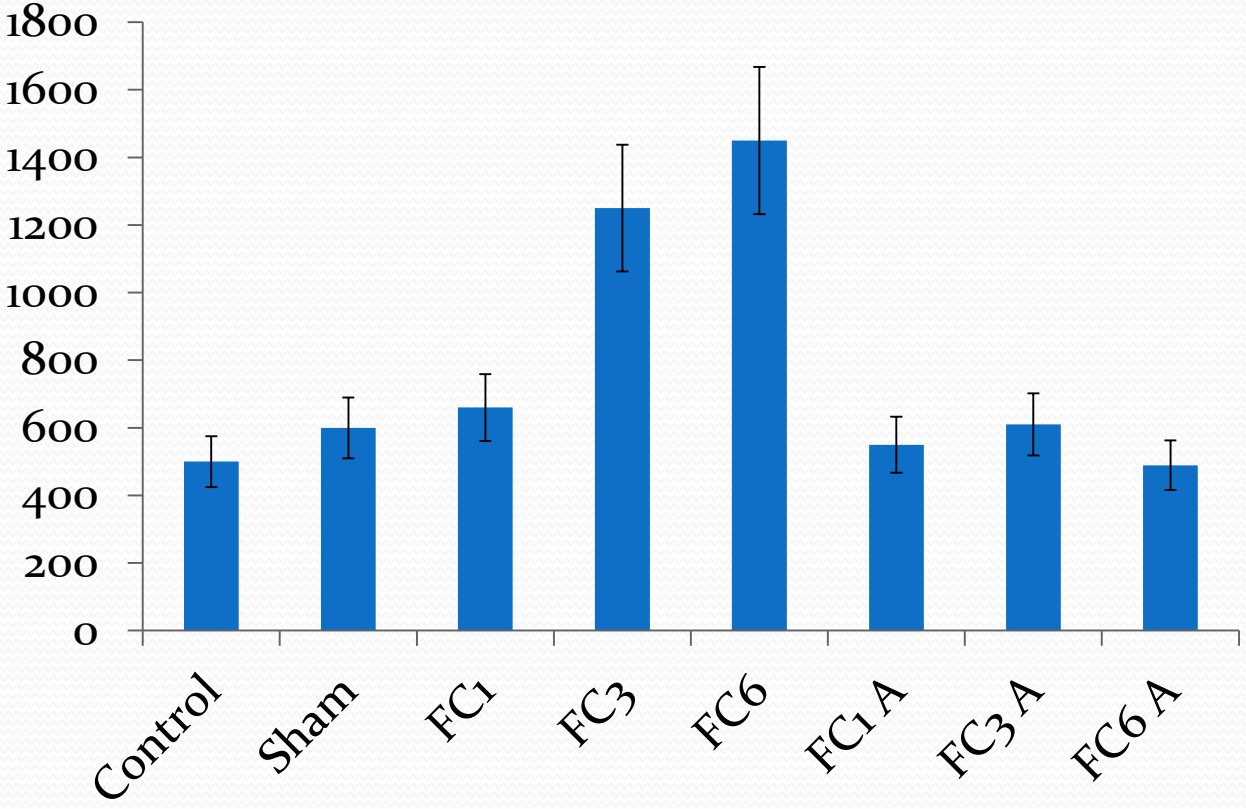
Fin clip in groups

Groups of 6 zebrafish (n = 8)

Fin clip 1, 3 or 6 fish (FC)

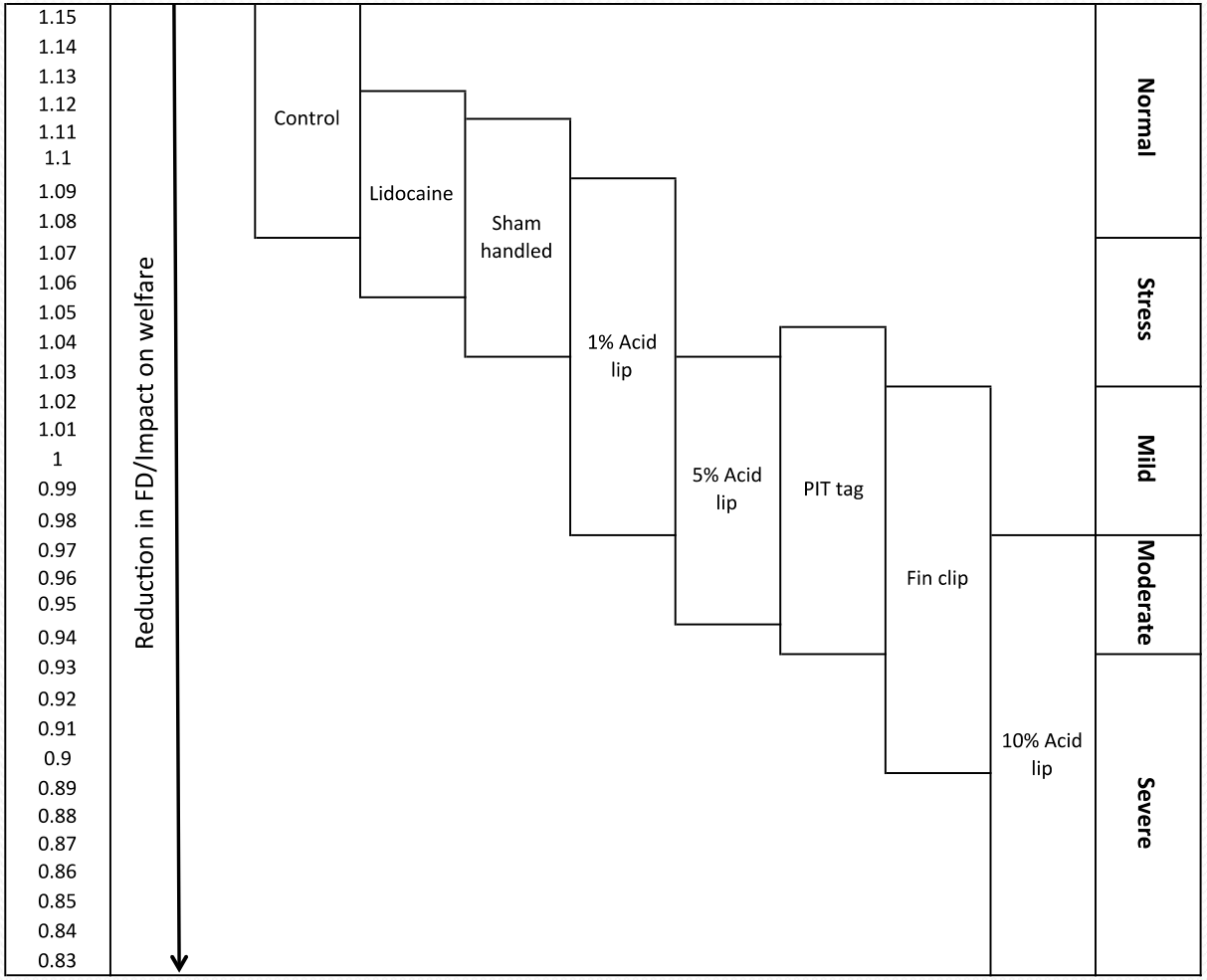
Fin clip plus 5mg/l lidocaine (A)

Measure saturation and hue in 2d = measure of activity



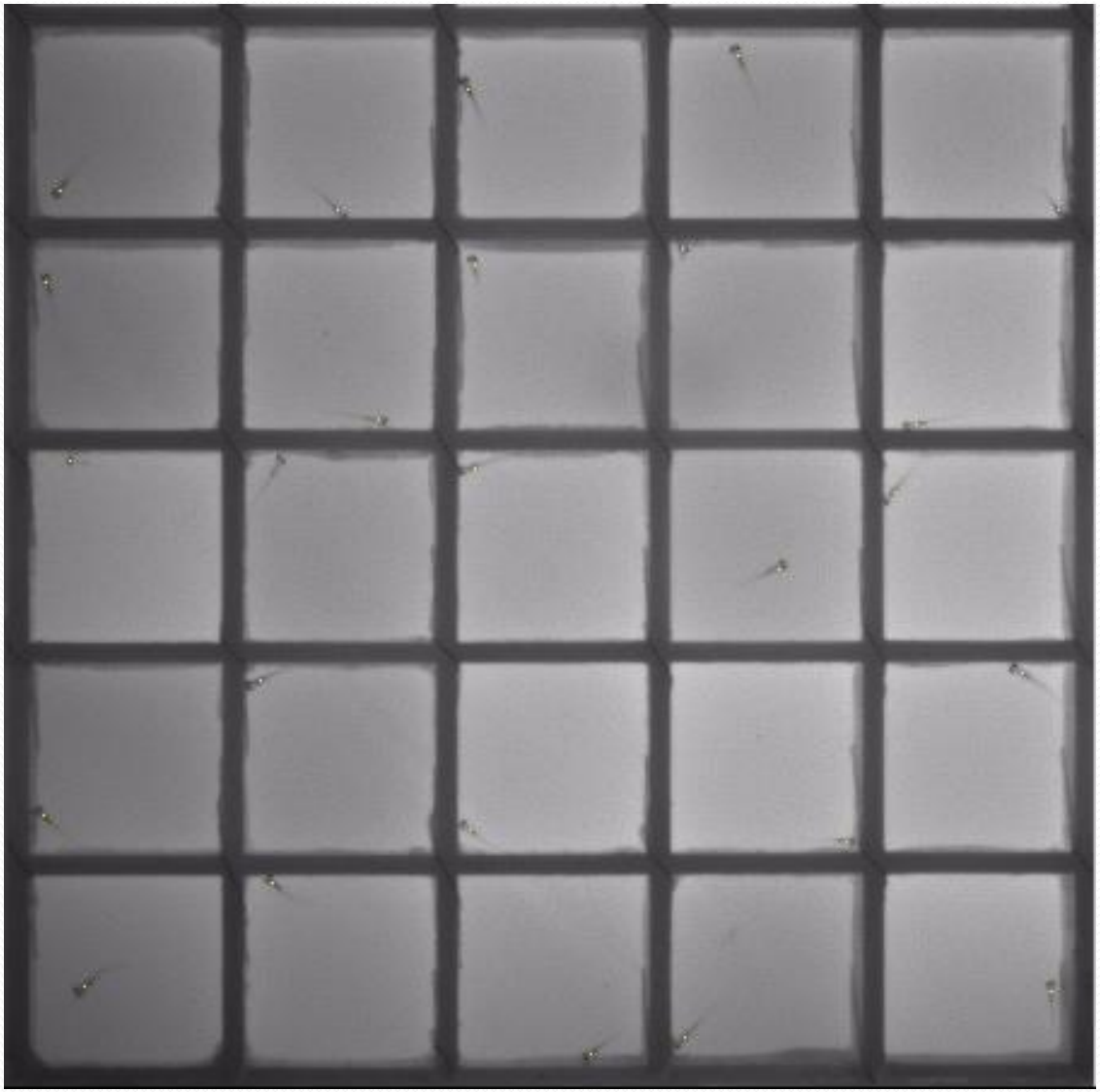
White et al. 2017. *Animal Behaviour* 132, 189-199

Actual Severity - Pain



Fish Species	Swimming	Ventilation Rate	Feeding	Plasma Cortisol	Light Preference	Changes in Gill Physiology	Anomalous Behaviours
Rainbow trout	↓	↑	↓	↑	NM	NM	√
Common carp	↔	↔	↓	NM	NM	NM	√
Zebrafish	↓	↑	↓	NM	NM	NM	√
Nile tilapia	↑	NM	NM	↔	↑	↑	NM

Behaviour of non-protected forms

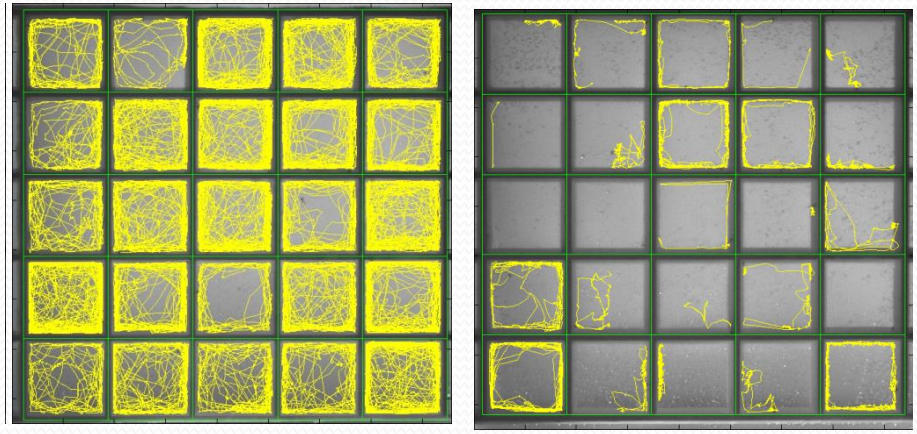


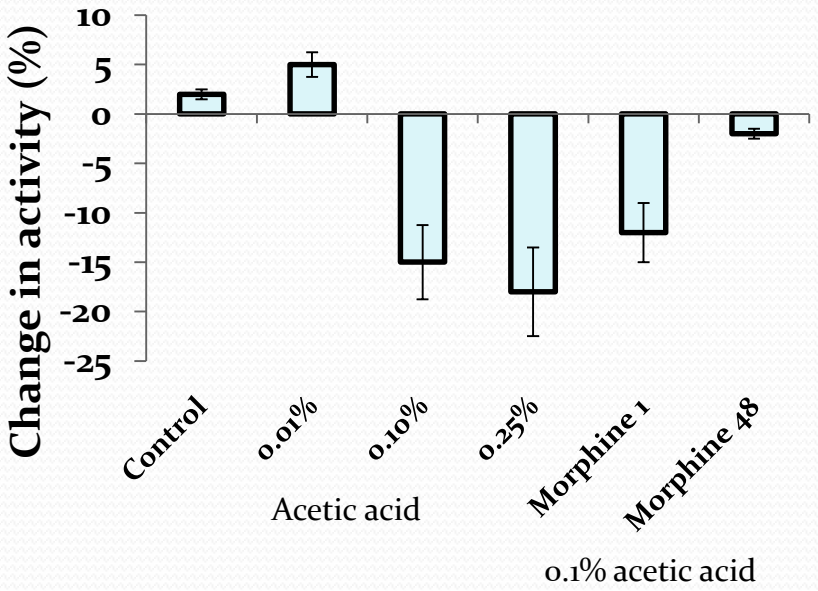
5dpf
zebrafish

Replacement for adults?

- Exposed to noxious stimuli
  - Chemicals - Lopez Luna et al. 2017 J Exp Biol 220, 1451-1458.
  - Thermal - Lopez Luna et al. 2017 App. Anim. Behav. Sci. 188, 97–105
  - CO_2 - Lopez Luna et al. 2017 App. Anim. Behav. Sci. in press.
  - Predator cue, stress, anxiolytics and anxiogenics – Lopez Luna et al. 2017 PLoS One, in press.
- Responses reduced by a range of analgesics

Replacement





Lopez Luna et al. 2017 J Exp Biol 220, 1451-1458.

Pain assessment

- Refinement in assessing fish welfare
- Behaviour invaluable
- Health status, humane endpoints, severity of procedures, response to other treatments?
- Replace adults with 5dpf zebrafish

NC 3Rs National Centre for the Replacement Refinement & Reduction of Animals in Research

MARIE CURIE ACTIONS

Analgesic use in fish

- When to administer?
- How to administer?
- What dose?
- Confounding effects?

Analgesia in zebrafish

Analgesic	Dose	Species	Side effects	Efficacy
Lidocaine	1-5mg/L	**Zebrafish (immersion)**	None observed	**Immersion at 2.5-5mg/L**
Morphine	2.5-50mg/kg 20µl of 0.2mg/ml 1 & 48 mg/L	**Zebrafish (i.m.)** **Zebrafish (i.p)** **Zebrafish (immersion)**	None observed	**i.m. at 5mg/kg** **Effective** **Immersion at 48mg/L**
Buprenorphine	0.01-0.3 mg/kg	**Zebrafish (immersion)**	None observed	**Immersion at 0.3mg/ml**
Aspirin	1-2.5mg/L	**Zebrafish (Immersion)**	None observed	**Immersion at 2.5mg/L**
Indomethacin	20µl of 0.2mg/ml	**Zebrafish (i.p.)**	None observed	**Effective**

Analgesic	Dose	Species	Side effects	Efficacy
Lidocaine	0.1-2mg/kg	Trout (i.m.)	None observed	Very efficient at 1mg injection site
Morphine	5-50mg/kg	Trout (i.m.) Flounder (i.p.) Goldfish (i.m.)	None observed	Very efficient at 5mg/kg
Buprenorphine	0.01-0.1 mg/kg	Trout (i.m.)	Reduced activity No impact on feeding or ventilation	Not efficient
Carprofen	1-5mg/kg	Trout (i.m.)	Depressed activity Increased ventilation	Reduced time to feed using 2.5mg/kg
Butorphanol	0.25-5mg/kg	Koi carp (0.4; i.m.) Dogfish (i.m.)		NS Koi – improved behaviour
Ketoprofen	1-4mg/kg	Koi carp (2; i.m.) Dogfish (i.m.)	No impact on behaviour in Koi	Not efficient
Flunixin	2-8 mg/l (adults) 2-20 mg/l (larvae)	**Zebrafish (Immersion)**	Decreased velocity in larvae	8 mg/l slight improvement of behaviour in larvae Effective in adults
Aspirin	1-2.5 mg/l	**Zebrafish (Immersion)**	Increased velocity of larvae Reduced activity	Improved behaviour at 2.5 mg/l
Bupivacaine	1 mg/l	**Zebrafish (Immersion)**	No impact on behaviour	Not efficient

Sneddon, 2012; Sneddon, 2003; Sneddon, unpub. data; Harms et al., 2005, Davis et al., 2006; Newby et al., 2006, Newby et al., 2009; Mettam et al., 2011, Nordgreen et al., 2009; Schroeder et al., 2017, Lopez-Luna et al., 2017; Lopez-Luna et al., 2017.

Recommendations

- Invasive procedures – use analgesia
- Untested species – lowest effective dose
- Consider possible side-effects
- Immersion – before to after procedure
- Injection (I.M. or I.P. or site) – during procedure

Anaesthetics

- Aids handling, weighing, tagging, surgery, humane killing
- Biological factors such as species, age, sex, body condition and weight, developmental stage, growth and physiological status, health, and reproductive condition
- Abiotic factors such as water quality, temperature, and oxygenation
- Recovery and euthanasia “good death”
- In UK Schedule 1 – overdose followed by brain destruction or exsanguination
- Evidence for avoidance in some species

Monitoring of anaesthesia

TABLE 1. Descriptions of the stages of anesthesia and the parameters used to monitor anesthesia in fish. A number of procedures are provided as examples of what can be done to the fish under these levels of anesthesia

Stage	Plane	Level of Anesthesia	General Demeanor	Activity	Equilibrium	Gill Ventilation Rate	Reactivity	Heart Rate	Muscle Tone	Examples of Procedures
0		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	
I		Lightly sedated	Disorientated	Reduced	Normal	Normal	Reduced	Normal	Normal	
II		Excitation	Agitated	Increased	Difficulty	Increased	Increased	Increased	Normal	
III	1	Light anesthesia	Anesthetized	None	Loss	Decreased	Reflex responses†	Regular	Decreased	Weight; close visual inspection; external noninvasive tags, gill scrape
	2	Surgical*	Anesthetized	None	Loss	Shallow	None	Reduced	Decreased	Invasive tags; tissue removal; injection; blood sampling; gill biopsy, lesion dressing, recovery surgery‡
	3	Deep	Anesthetized	None	Loss	Rare movements	None	Reduced	Relaxed	Nonrecovery surgery†
IV		Overdose	Apparently dead	None	Loss	None	None	Cardiac failure	None	

Adapted from Bell, 1987[92]; Burka et al, 1997[48]; McFarland, 1959[93]; McFarland and Klontz, 1969[94]; Summerfelt and Smith, 1990[19].

*Some authors suggest there is an intermediate stage between light and surgical termed medium plane anesthesia.

†An example of a reflex response is the fish swimming in response to a tail pinch.

‡Usually accompanied by the use of artificial ventilation where the gills are irrigated with fresh or anesthetic dosed water.

Sneddon 2012 J. Exotic. Pet Med. 21, 32-43

Anaesthetics

TABLE 2. Summary of selected anesthetic agents used in fish showing the range of doses, used in a variety of species and the resultant side effects (see Neiffer and Stamper, 2009[17] for species-specific information and also the citations in text)

Anesthetic Agent	Dose (mg/L^{-1})	Side Effects	
		Initial	Secondary
MS-222	50-400	Tachycardia Increased respiration Hyperglycemia	Decreased cardiovascular responses Hypoglycemia Increased lactate, hematocrit, and catecholamines Erythrocyte swelling
Benzocaine	25-150	Tachycardia Increased respiration Hyperglycemia	Decreased cardiovascular responses Hypoglycemia Increased lactate, hematocrit, and catecholamines Erythrocyte swelling Suppressed immune function
Clove 0il	4-150		Decreased ventilation and cardiovascular responses
Eugenol	20-200		Increased catecholamines and hematocrit
Isoeugenol	3.6-120		
Metomidate	0.06-10		Reduced adrenal steroid production leading to reduced cortisol Reduced respiration, circulation, and pH of blood Hypoxemia
2-Phenoxyethanol	0.25-600		Decreased ventilation rate, heart rate, blood pressure, and blood pH Increased adrenal hormones Hyperglycemia Reduced immune function
Quinaldine Quinaldine sulphate	10-50 5-100	Tachycardia	Decreased heart rate and respiratory function Increased cortisol and serum immunoglobulin M Hyperglycemia

These doses are not appropriate for all species or under all conditions (e.g., temperature, body size, and physiological state must be investigated before use). When working with unfamiliar species or agents, use the lowest doses and low numbers of fish to test anesthetic efficacy.

Humaneness of anaesthetics

- Tests measuring avoidance to low dose
- Conditioned place avoidance
- Recovery
- Physiological stress

Avoidance vs Aversion

Species	Water	HCl	Ethanol	Benzocaine	Etomidate	MS222
Carp	No	Yes	No	No	Yes	No
Fathead minnow	No	No	No	No	No	Yes
Medaka	No	Yes	No	Yes	No	Yes
Zebrafish*	No	Yes	No	Yes	No	Yes
Rainbow trout	No	Yes	No	No	No	No

*Readman et al. 2013 *PLoS ONE* 8(9): e73773; Readman et al. 2017 *Scientific Reports* 7, 7102.

Avoidance

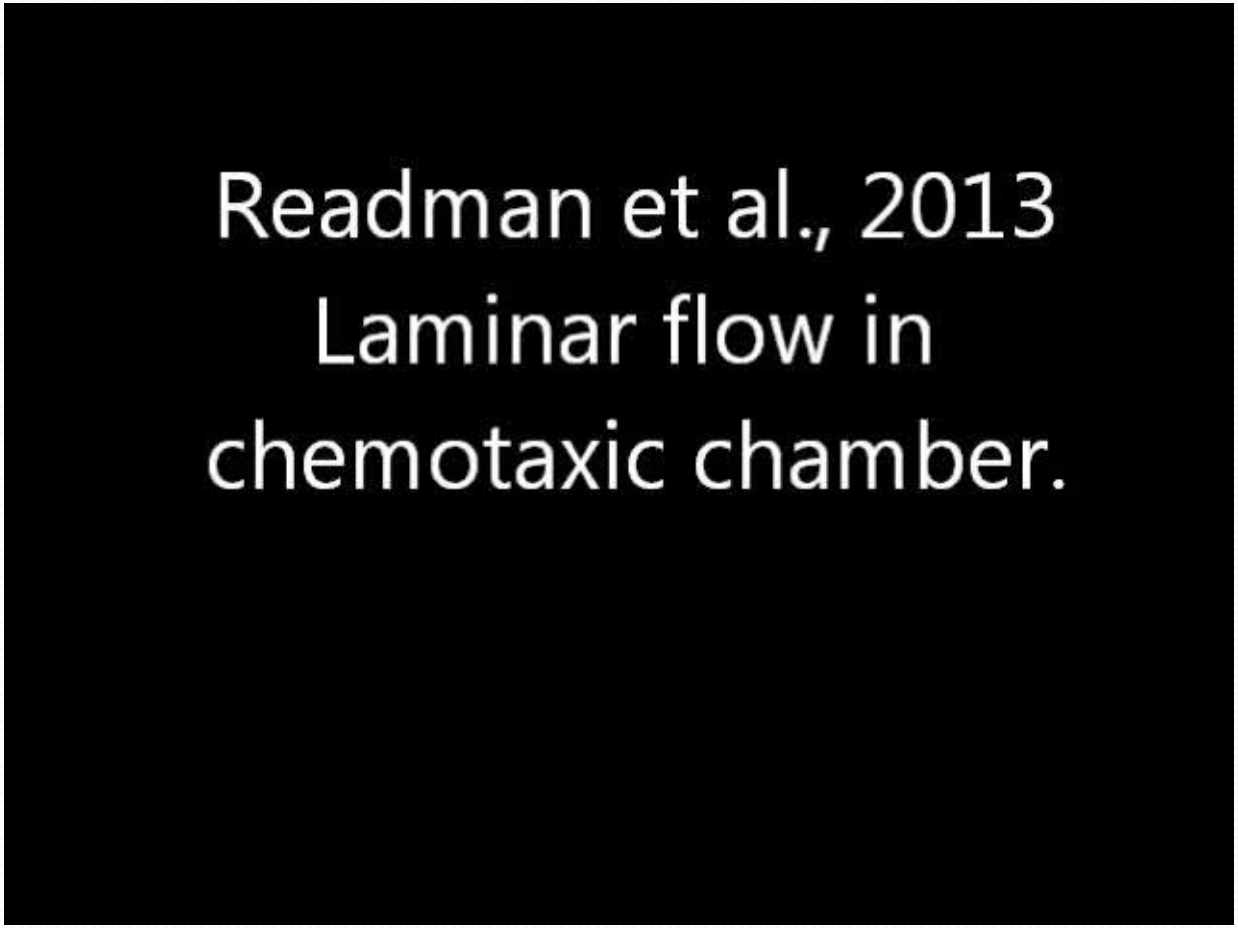
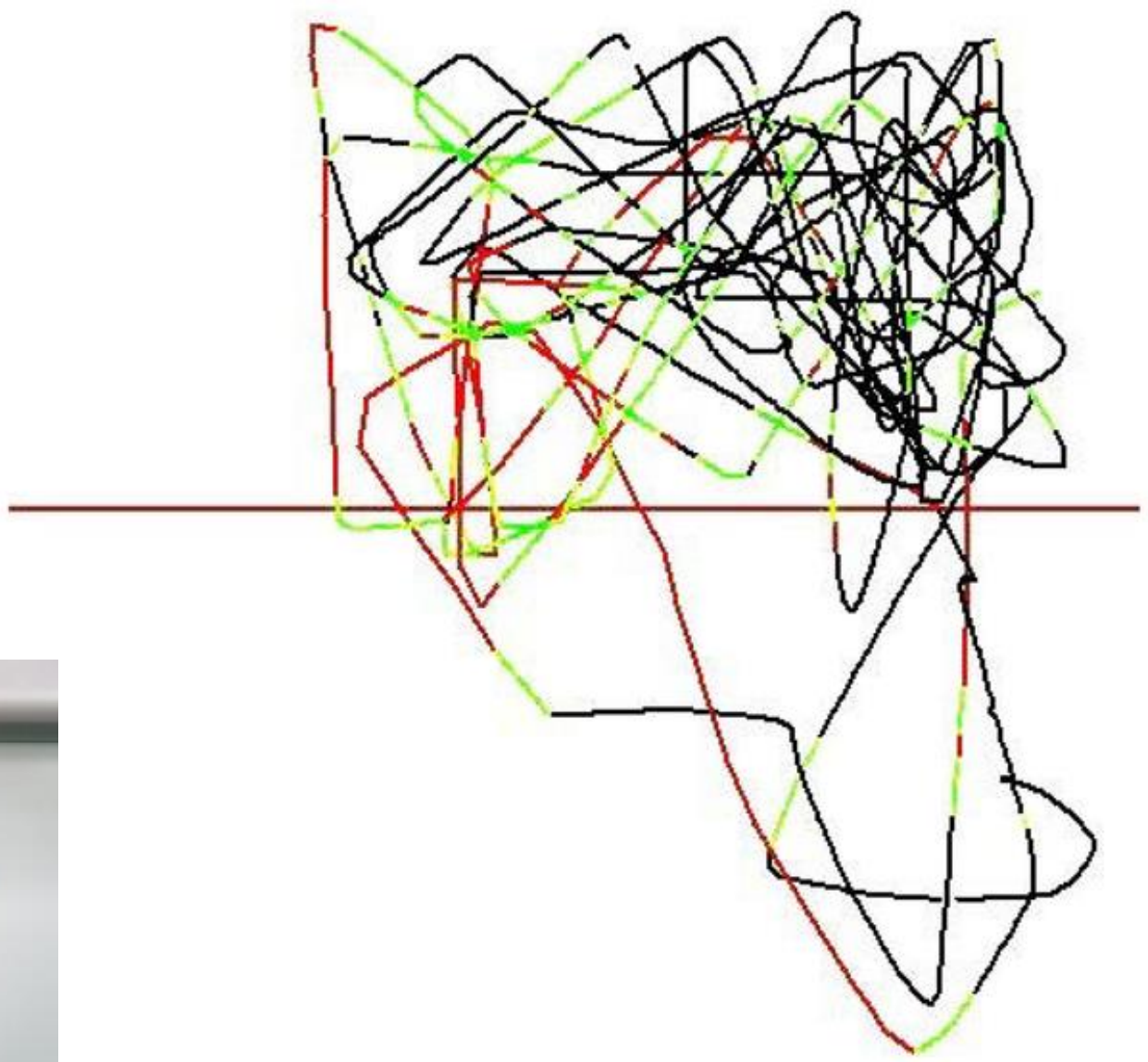


Figure 1. Image of the output from the ViewPoint software video tracked movement of a single adult zebrafish during exposure to hydrochloric acid (pH 3.0) viewed from above the choice chamber.
Readman et al. 2013 *PLoS ONE* 8(9): e73773



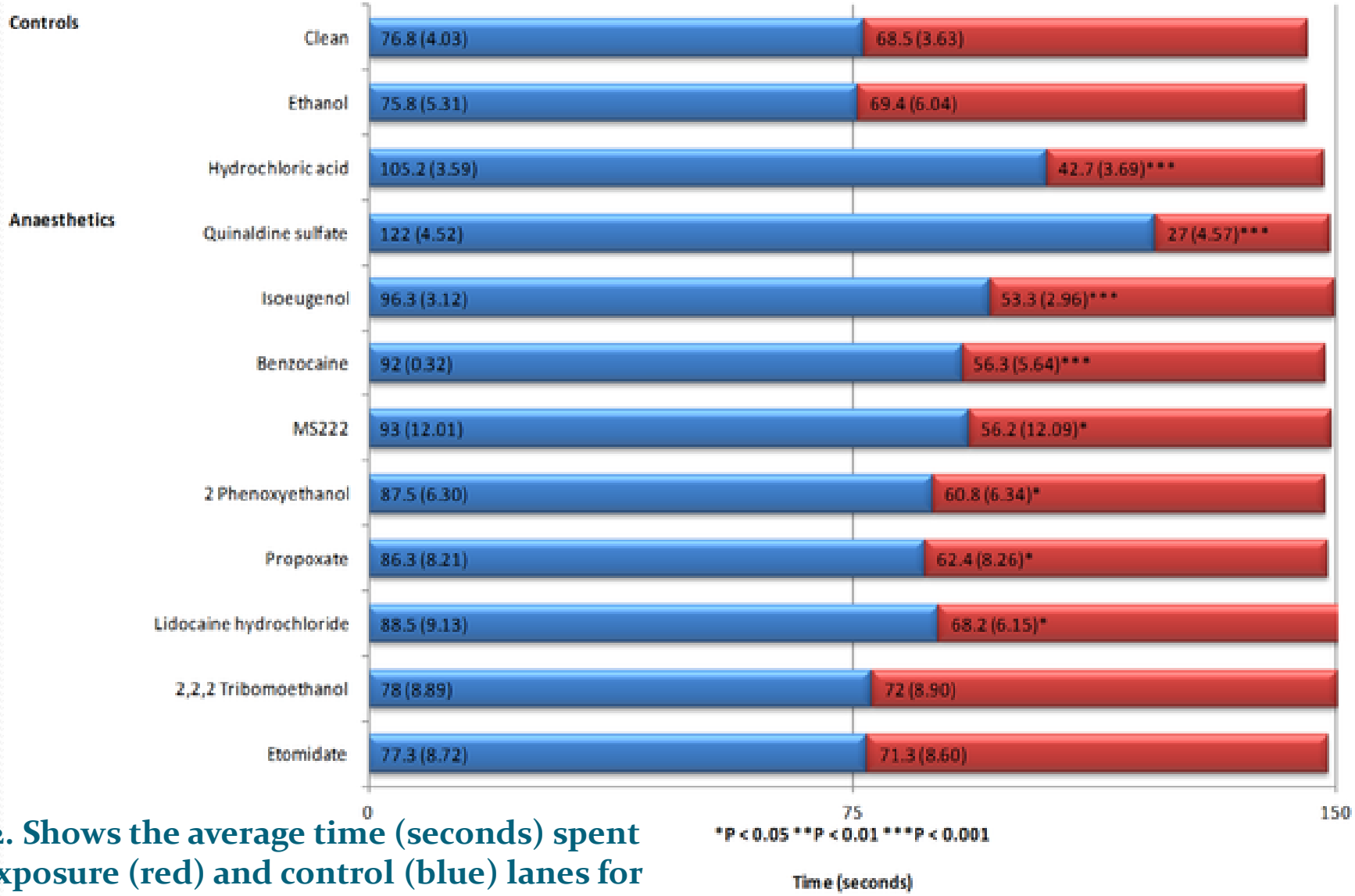
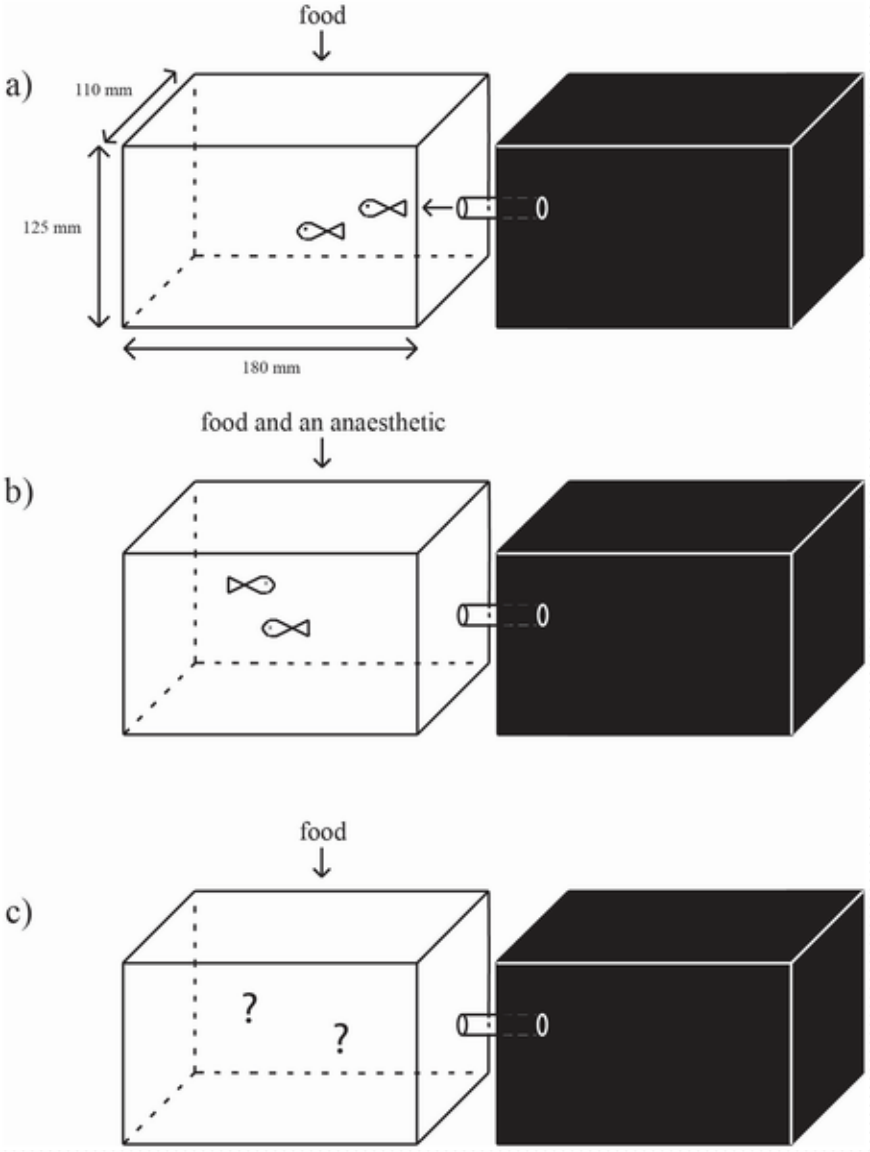
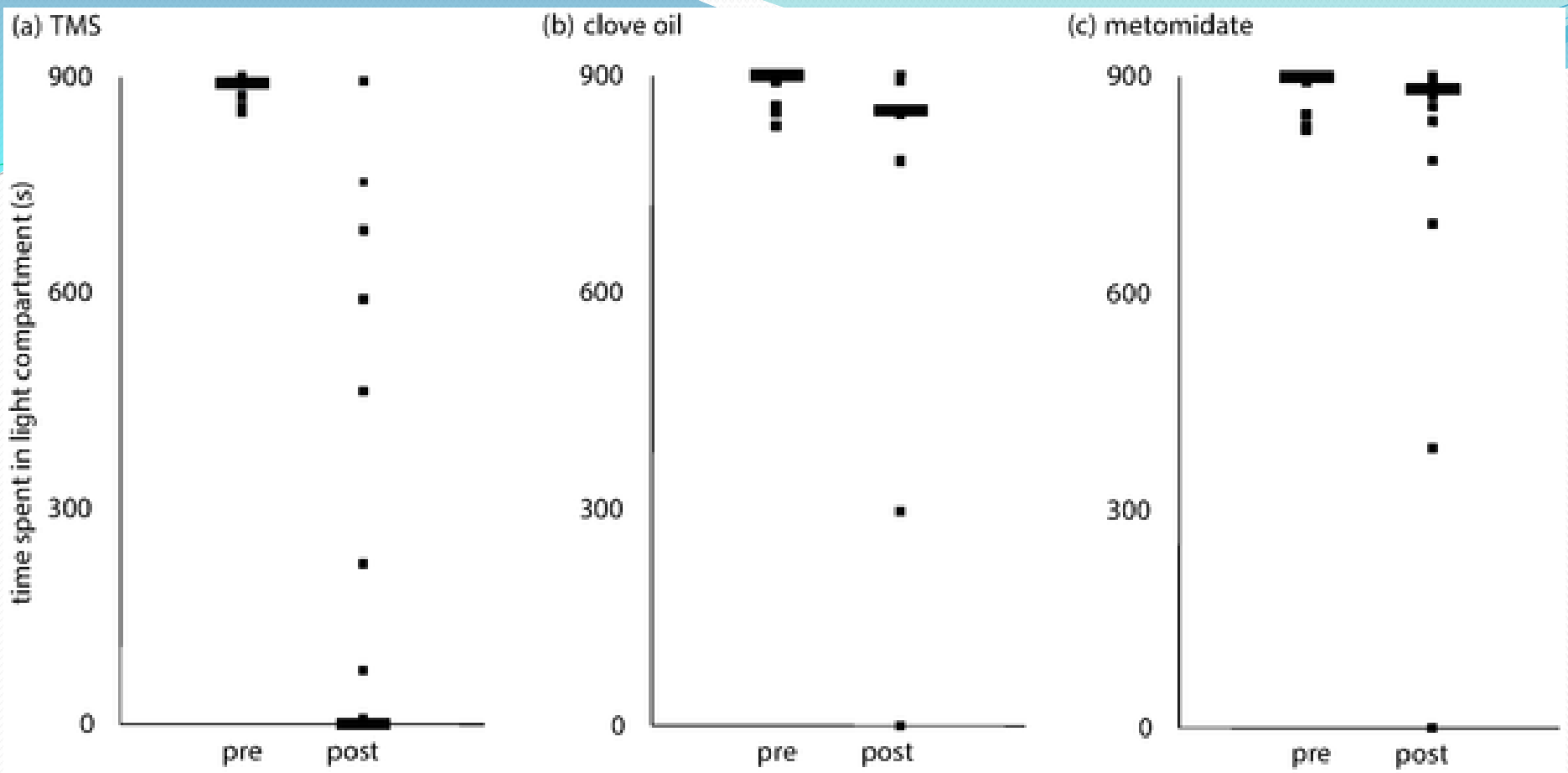


Figure 2. Shows the average time (seconds) spent in the exposure (red) and control (blue) lanes for each experiment (± SE, n = 10), ranked by aversion (highest to lowest) for the anaesthetics.

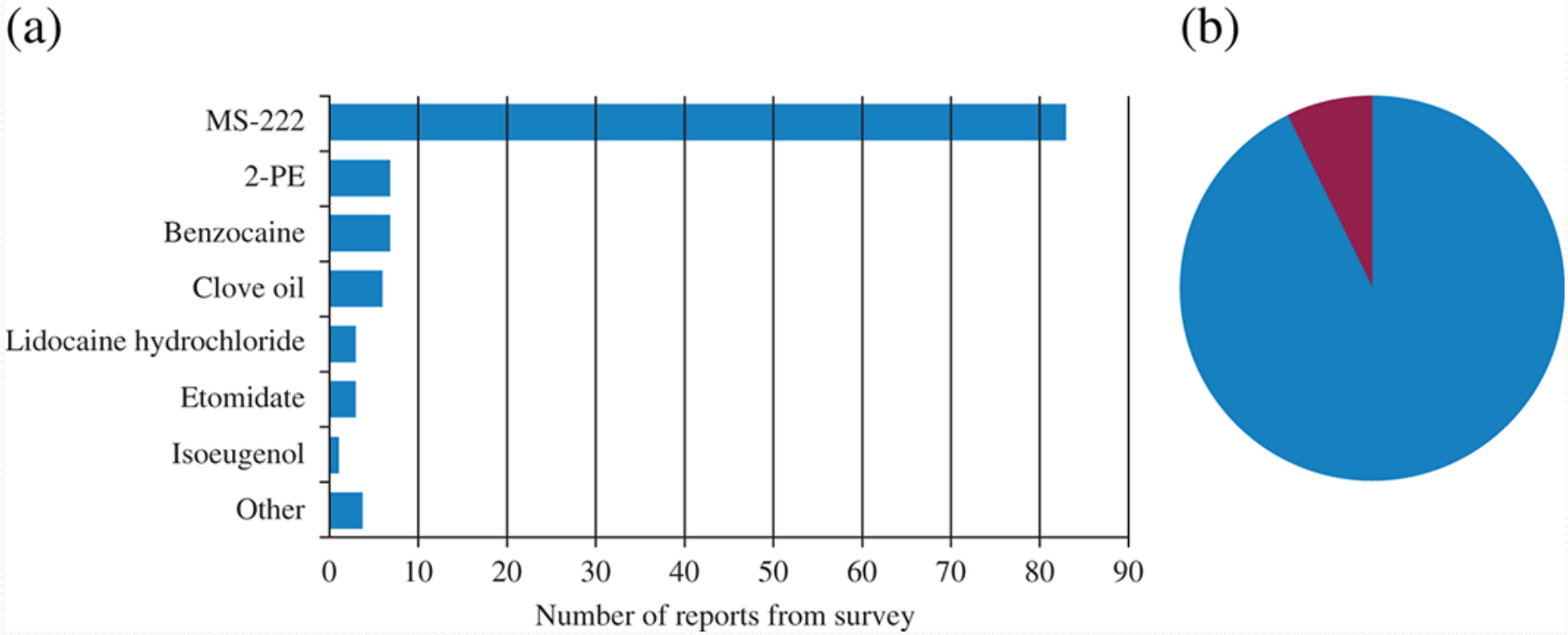


Wong et al. (2014)
Conditioned Place Avoidance of Zebrafish (*Danio rerio*) to Three Chemicals Used for Euthanasia and Anaesthesia.
PLOS ONE 9(2): e88030.



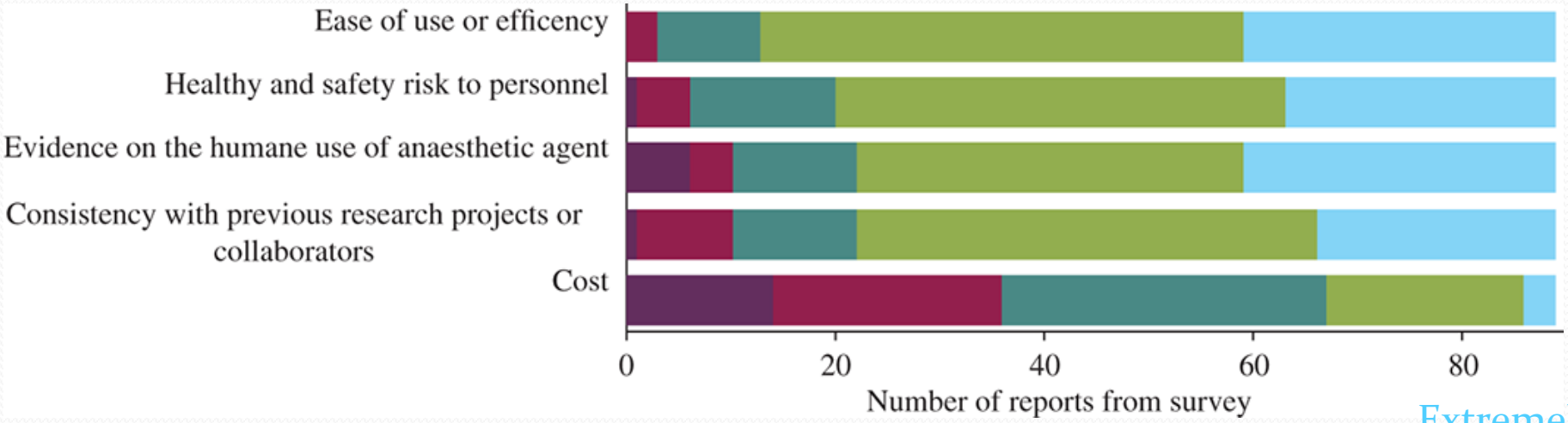
	Complete Rejection	Re-entered
TMS	9/17	8/17
Clove oil	3/16	13/16
Metomidate hydrochloride	2/18	16/18

International survey on the use and welfare of zebrafish *Danio rerio* in research



Lidster et al. 2017. Journal of Fish Biology, 90, 1891-1905

International survey on the use and welfare of zebrafish Danio rerio in research



Not important

Extremely important

Lidster et al. 2017. Journal of Fish Biology, 90, 1891-1905

After effects of MS222

Variable	Time	Tricaine (n = 8)	Control (n = 7)
Mean velocity (cm/s)	Baseline	4.0±0.9	3.3±1.5
	Immediate	3.0±0.6	3.7±1.2
	5-min	3.0±0.9	3.0±1.2
	30-min	3.0±1.0	3.5±2.1
	60-min	3.6±1.5	3.0±1.0
Time spent in lower third of the tank relative to duration of trial (percentage of duration of observation period)	Baseline	51.6 (26.4–68.0)	59.7 (26.1–80.7)
	Immediate	56.0 (41.9–71.2)	53.0 (43.9–95.9)
	5-min	60.3 (21.4–87.1)	60.0 (37.8–89.3)
	30-min	47.1 (26.4–92.2)	73.3 (11.1–89.9)
	60-min	66.0 (24.2–93.6)	80.1 (46.7–94.7)
Frequency of zone transitions between the lower third and the rest of the tank (per min)	Baseline	4.3±2.9	3.8±3.0
	Immediate	2.6±0.7	2.1±1.5
	5-min	2.3±1.5	2.1±1.6
	30-min	2.5±2.1	1.7±1.4
	60-min	2.6±2.9	1.2±0.6
Latency to leave the lower third of the tank (s)	Baseline	17.8 (10.3–29.0)	7.9 (4.8–59.0)
	Immediate	22.2 (6.1–68.4)	19.2 (9.3–215.8)
	5-min	12.1 (8.2–18.7)	8.6 (3.9–64.5)
	30-min	8.8 (1.7–36.7)	24.0 (14.9–36.8)
	60-min	5.2 (2.5–34.5)	16.8 (14.8–29.0)
Cumulative duration in the central part of the tank relative to the total duration of the trial (percentage of duration of observation period)	Baseline	44.2±23.6	36.1±24.7
	Immediate	46.3±15.9	30.2±23.1
	5-min	39.6±23.5	38.3±28.2
	30-min	32.7±18.0	23.0±18.3
	60-min	32.5±17.3	20.5±9.8

doi:10.1371/journal.pone.0092116.t003

Nordgreen et al.(2014) PLOS ONE 9(3): e92116.

Recovery, CPA and Euthanasia

Applied Animal Behaviour Science 199 (2018) 94–102

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journal homepage: www.elsevier.com/locate/applanim

Research Paper

Physiological and behavioural evaluation of common anaesthesia practices in the rainbow trout

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ARTICLE INFO

Keywords:
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Anaesthetic
2-phenoxyethanol
Aversion
Stress
Cortisol

ABSTRACT

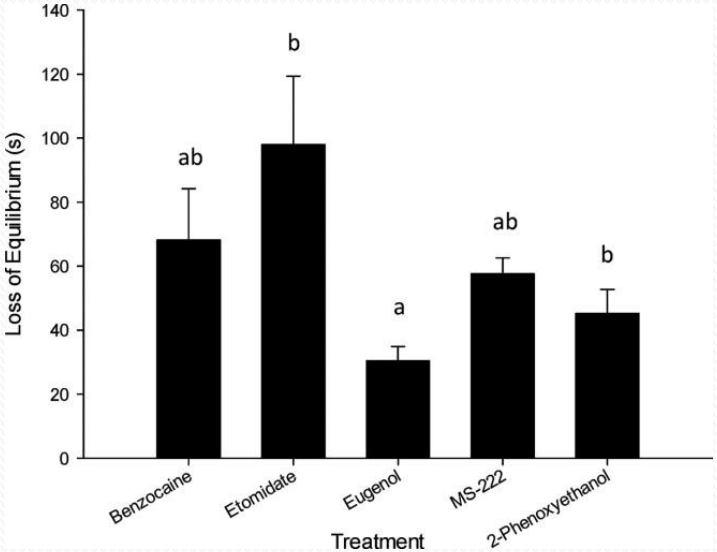
Anaesthetic drugs are commonly administered to fish in aquaculture, research and veterinary contexts. Anaesthesia causes temporary absence of consciousness and may reduce the stress and/or pain associated with handling and certain invasive procedures. The rainbow trout (*Oncorhynchus mykiss*) is a widely-used model species with relevance to both aquaculture and natural ecosystems. This study sought to establish the relative acute impact of commonly used anaesthetics on rainbow trout when used for anaesthesia or euthanasia by exploring their effects on aversion behaviour and stress physiology. Five widely used anaesthetics were investigated at two concentrations reflective of common laboratory practises: MS-222, benzocaine, 2-phenoxyethanol, etomidate and eugenol. The anaesthetics were administered via immersion and fish were: 1) euthanised with anaesthetic; or 2) allowed to recover from deep plane anaesthesia; or 3) subjected to a conditioned place avoidance paradigm. Behaviour, opercular beat rate and plasma cortisol concentrations and cortisol release rates to water were quantified to investigate the effects of the five drugs. Based upon longer induction to deep plane anaesthesia, and increased plasma cortisol levels post-anaesthesia the widely-used and recommended anaesthetic MS-222 may be relatively stressful for rainbow trout. Whereas 2-phenoxyethanol, due to a combi-

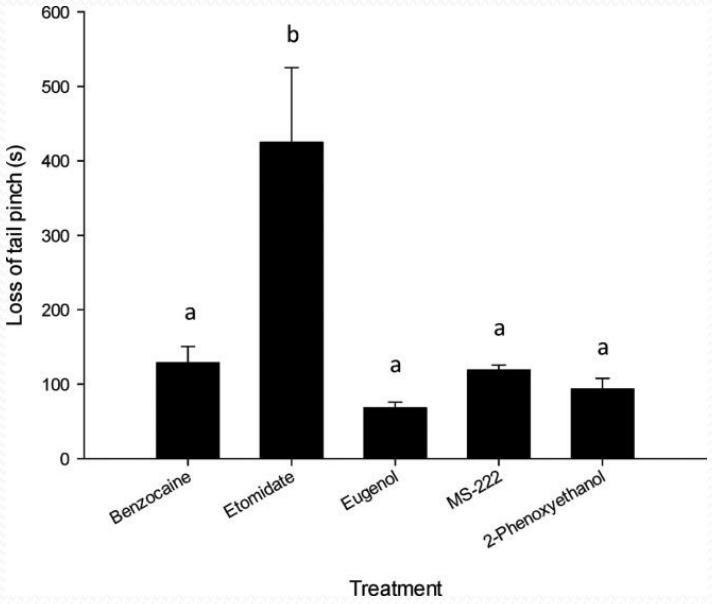
Recovery and Euthanasia

- Concussion (E)
- Benzocaine (100; 150 mg)
- MS222 (100; 200 mg)
- 2-Phenoxyethanol (Aquased 2; 6 ml)
- Etomidate (5; 10 mg)
- Eugenol (100; 200 ml)
- Low (R) and high concentrations (E)
- 25 l vessel with lid
- Water from system
- Same temperature
- Aerated
- Opercular beat rate
- Time to lose equilibrium, slow and no OBR, to death (E), loss of reflex response (tail pinch) and then cortisol immediately, 1 and 2 h afterwards

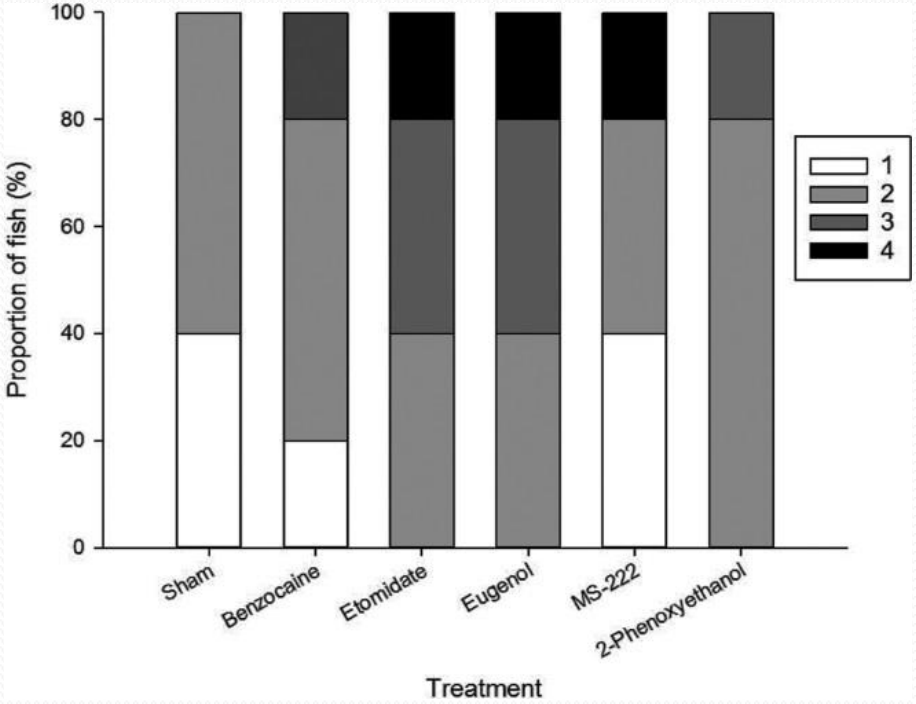
Euthanasia

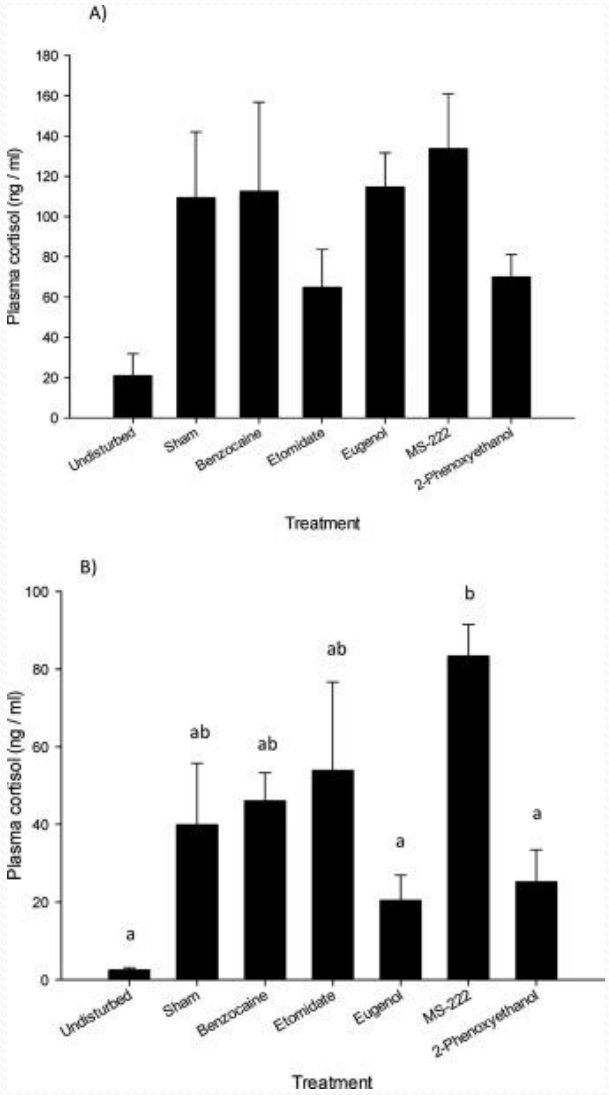
- No difference in plasma cortisol between concussion and overdose of any drugs
- Etomidate longer induction





Recovery





Pounder et al. 2018. Appl. Anim. Behav. Sci. 199, 94-102

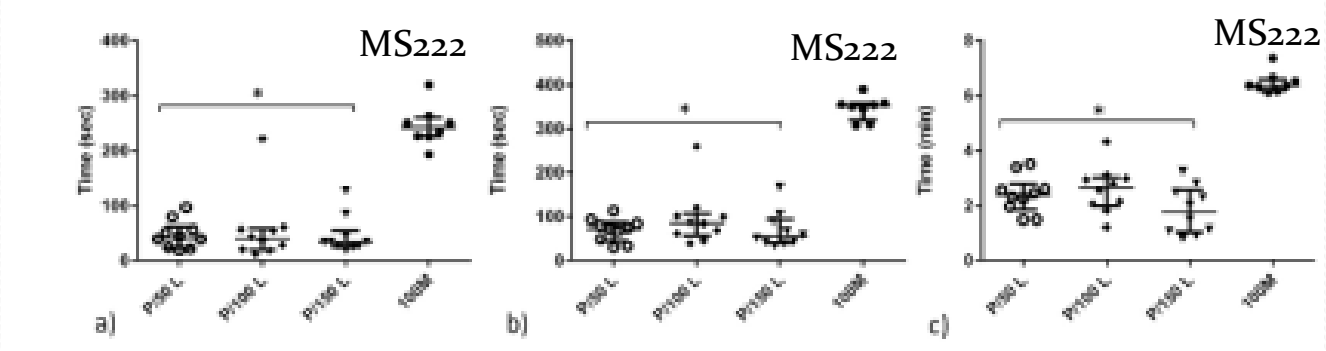
Conditioned Place Avoidance

- Home tank split into two by partition
- Doorway allowed swimming between
- Fed in one side left or right
- Developed CPP
- Anaesthetised in preferred side
- Held in vessel for 1h then returned and fed
- Latency to return and feed
- Water and plasma cortisol

- Trout very calmly went under anaesthesia
- No differences in the latency of return to the positive conditioned side ($H = 3.00$, $p = 0.558$) or in the time taken to resume feeding ($H = 2.48$, $p = 0.648$).
- MS222 higher plasma cortisol at 2h
- 2-phenoxyethanol lowest cortisol

Combination anaesthesia

- If drugs are avoided – can we use lower doses in combination?
- Routinely done in medical practice
- Propofol/lidocaine in zebrafish 2.5/50µg/ml P/ L
- Valentim et al.(2016) *PLoS ONE* 11(1): e0147747.



Equilibrium Tail pinch Painful stimulus

Novel Compounds

Essential oils and plant extracts in gilthead seabream

Comparison with 3 anaesthetic drugs

Survival and genotoxicity (DNA damage)

Cinnamon was evaluated as an equal competent anaesthetic agent satisfying the criteria of the ideal anaesthetic, reducing DNA strand breakage and indicating anti-stress, anti-genotoxic and geno-protective effect

Table 4. Mortality rate 6 days post-treatment.

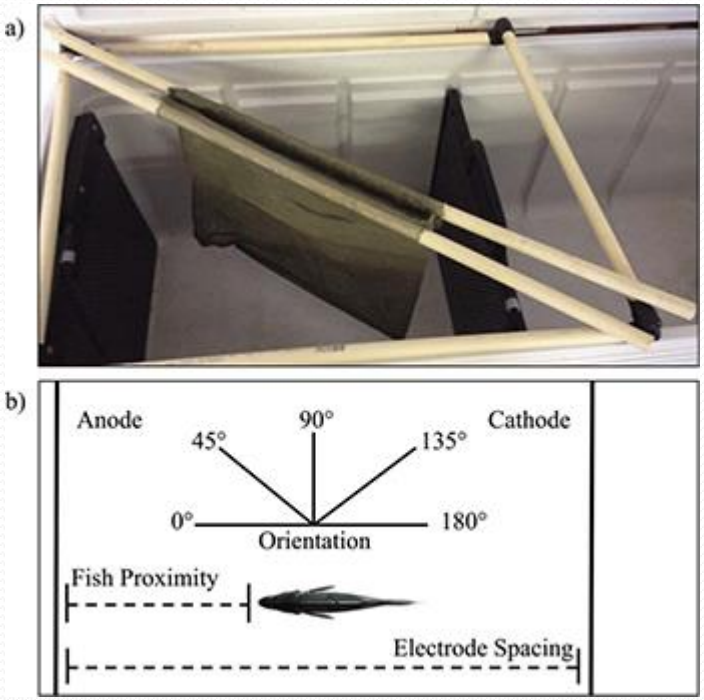
	Post-treatment mortality rate (%)							
Anaesthetic agent	0 h	24 h	48 h	72 h	96 h	120 h	144 h	Cumulative mortality (%)
Origanum vulgare	0	7	0	0	0	0	0	7
Eugenia aromatica	0	13	7	0	7	0	0	27
Aloysia triphylla	7	13	7	0	0	0	0	27
Melaleuca alternifolia	0	7	0	0	0	0	0	7
Juniperus communis	20	7	0	7	0	0	0	34
Cinnamomum zeylanicum	0	0	0	0	0	0	0	0
Tricaine (MS-222)	0	0	0	0	0	0	0	0
2-Phenoxyethanol	0	0	0	0	0	0	0	0
Benzocaine	0	0	0	0	0	0	0	0

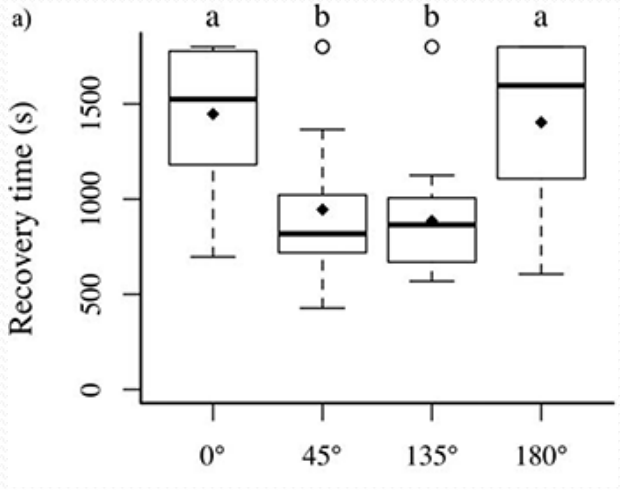
Golomazou et al. 2016. *Aquaculture* 464, 673-682.

Non-chemical method

- Electrosedation
- Largemouth bass – increased glucose and lactate; pallid sturgeon – higher plasma osmolality
- Grass carp, cobia, hybrid striped bass – acute stress response
- Orientation to electrodes affects recovery in bluegill pulsed DC with a standardized frequency (100 Hz), voltage (90 V), and shock duration (3 s)

Electrosedation





Rous et al. 2015. Transactions of the American Fisheries Society 144, 820-828,

Non-chemical method

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- Electrical stunning – cod, herring – followed by a secondary method

Recommendations

- Long term impact of anaesthetics?
- Repeated anaesthesia?
- Novel compounds and electrosedation?
- Most humane methods – avoidance versus analgesia
- Home tank preferable
- Euthanasia

Fish Euthanasia

Guidelines

- UK Schedule 1 – overdose
- Concussion followed by destruction of the brain before the return of consciousness
- AVMA 2013 –CO_2-saturated water, decapitation, cervical transection, concussion followed by pithing, rapid chilling

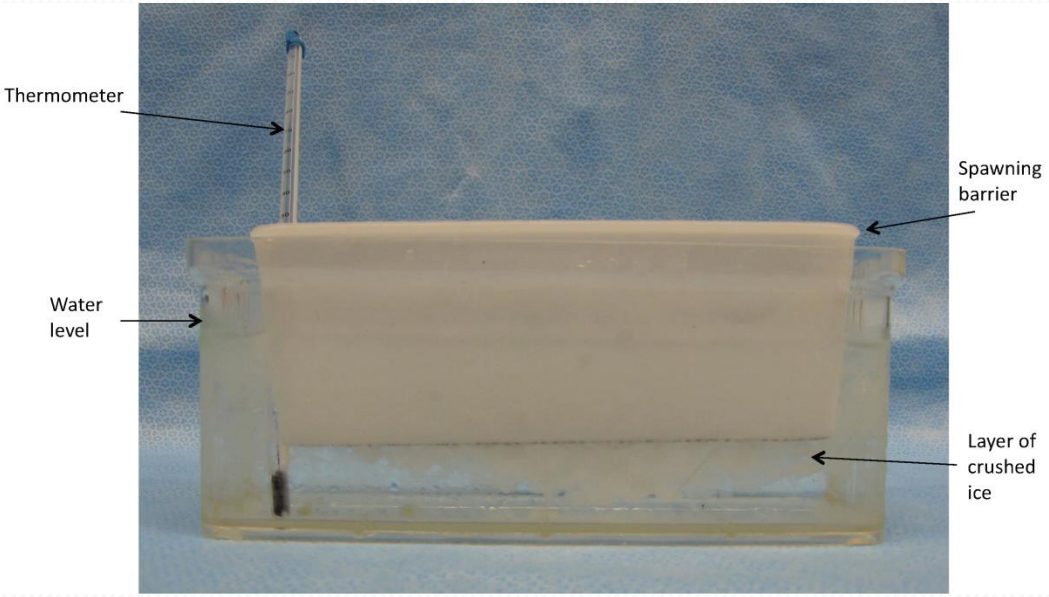
Research

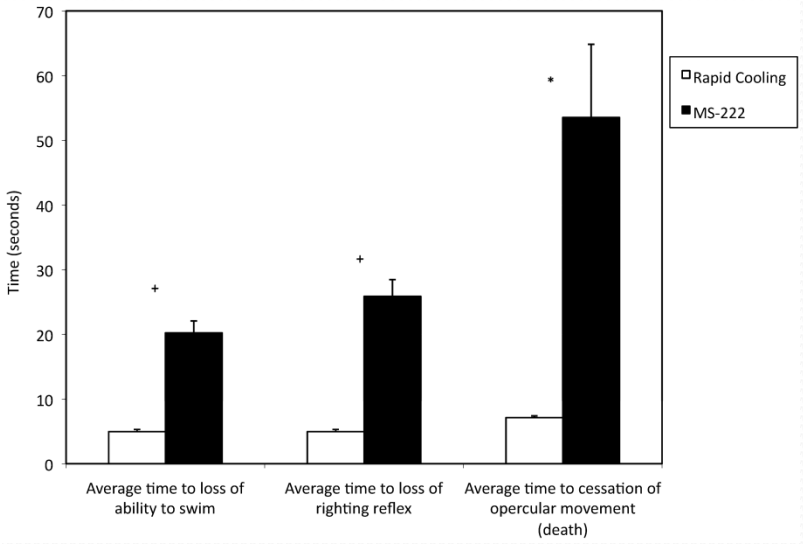
- CO_2 saturated water excites nociceptors in trout (Mettam et al. 2012 J. Exp. Biol. 215, 685-693.)

- Euthanasia in iced water less stressful and quicker than overdose in zebrafish (Wilson et al. 2009 JAALAS 48, 785-789). Only small tropical sp.

Euthanasia of zebrafish

- Small tropical species – overdose using MS222 or ice bath (Matthews & Varga 2012, ILAR J 53, 192-204)
- Comparison of MS222 and ice (<10 s; Wilson et al. 2009, JAALAS 48, 785-789)





Euthanasia in fish

- More research required e.g. species specific responses, humaneness of immersion anaesthetics, abiotic and biotic factors
- Public health

Acknowledgements

- www.nc3rs.org.uk; EU FP7
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