RED KITE
VETERINARY CONSULTANTS

PIL A/B Fish

Paul Schroeder BSc (Hons) Biol., MSc (Fish Biol.), DVM, PhD, MRCVS

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AGENDA

- Introduction and quiz
- Introduction to fish biology & welfare
- Husbandry, biosecurity & disease prevention
- Fish anaesthesia & Sch1
- Minor procedures & severity assessments
- Refinements
- Breakout session
- Written Assessment (20 minutes)

2

PIL A&K Fish

3

Introduction to fish biology and the most popular research species

4

Fishes

Suitable definition: "Craniate, gilled ectothermic aquatic animals with limbs in the shape of fins and lacking digits. These morphological traits can have evolved homologously or analogously."

Edward Branson, Fish Welfare (2008)

Not a systematic term!

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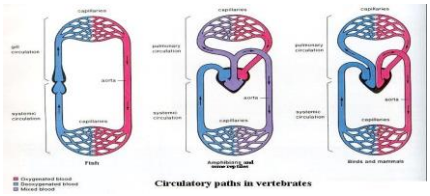
Overview of fish biology



Circulatory paths in vertebrates

www.Nature.com

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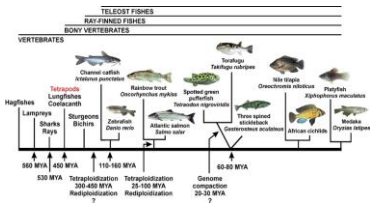
Overview of fish biology



www.Nature.com

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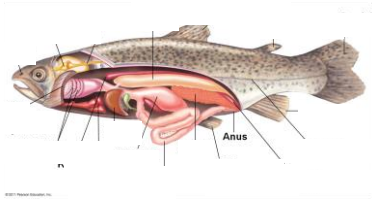
Fish Anatomy



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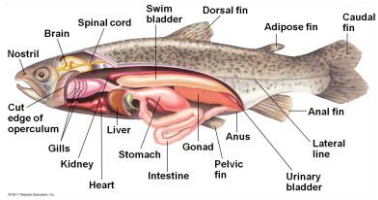
Fish Anatomy



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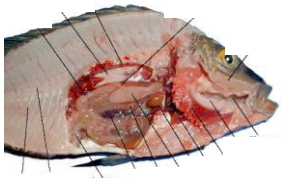
Fish Anatomy



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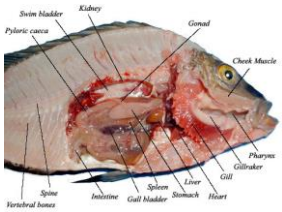
Fish Anatomy



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Fish Anatomy

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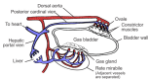
Some (more or less) unique anatomical features



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Some (more or less) unique morphological and physiological features

- No thoracic diaphragm
- No middle ear
- No limbs or limb girdles
- Swim bladder (teleosts)
- Bony fin rays without skeletal support
- Otoliths
- Heart consists of single chamber
- 95% of gas exchange through gills, 5% through skin

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How we should classify fishes in research facilities

- Small warm freshwater - modular re-circulation systems

- Freshwater (cold water aquaculture)

- Marine (cold water)

- Marine (warm water)

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How the Home Office classifies fishes in research facilities

- Zebrafish
- Freshwater fishes: typical species trout & carp
- Marine fishes: typical species seabass & salmon

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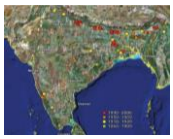
Zebra fish

- Zebrafish (*Danio rerio*) – small **shoaling** members of Cyprinidae family (carps & minnows)
- Max total length 50mm, weight up to 1.8g
- **Sexually mature 12 weeks after hatching**
- **Photoperiodic breeders – right after sunrise**
- **Females spawn every 2-3d, producing clutches of 100-200 eggs**
- **Larvae hatch 2-3d pf at 28.5°C**
- Live up to 5 years
- Natural geographic range: South and South East Asia (Pakistan to Myanmar)
- Habitat: well oxygenated (>5mg dissolved O_2 /l) freshwater - slow flowing streams, flooded plains, rice paddies; water temperature range 14-34°C (in captivity - ideally 26-29 °C)
- Diet: copepods & other small crustaceans, insects & insect larvae, zooplankton…



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Zebra fish use

- Zebrafish popular aquarium species before development of ZF research model
- Fast development (motile transparent larvae 48h post fertilization), rapid wound regeneration, cheap husbandry → ideal for toxicology, surgical models and genetic research
- 2nd most common (appr. 15% of total) animal used in scientific procedures
- As vertebrates, zebrafish are protected under ASPA from the time they can feed independently (day 5 p.f)
- some husbandry recommendations in Home Office CoP (max 5 fish/litre, unspecified structural enrichment)

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Freshwater fishes

20

Marine species

37

European seabass

- Protected under ASPA from 6dph (7-10 days pf)
- **Temperature** 15-25ºC
- **Diet:** rotifera, artemia & nauplii from d6ph;
- D40-50ph: transfer to nurseries & transition from live prey to granulates
- Commercial fish meal & fish oil pellets
- In the wild: slow growing species that takes several years to **reach full adulthood (4-7y)**
- Wild seabass can grow to **1m/12kg (females); 7kg (males)**
- AQ: 1.5-2 years to slaughter (350-500g)
- Seasonal migration from **summer coastal feeding to winter offshore spawning grounds**

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European seabass

- *Dicentrarchus ibrax*
- Salt- and brackish coastal waters; NW Atlantic incl. Mediterranean
- "Pioneer" species for marine aquaculture (sustainable seafood, aquaponics)

Regulated procedures UK:

- Nutritional research
- Physiological studies
- Marine ecotoxicology

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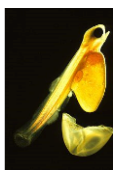
Atlantic Salmon

- **Atlantic Salmon** (*Salmo salar*)

- N Atlantic and tributaries;
- Anadromous (landlocked forms exist)
- "Classic" commercial aquaculture species (since early 1980s)
- Hatcheries & nurseries (FW)
- Grow out/fattening (SW) in sea pens/sea cages;
- Or combined egg to table aquaculture facilities



- Slaughter weight 3kg after 1.5-2 (organic: 3) years
- Perennial disease issues: sealice, ISA etc.

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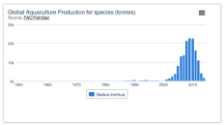
Wild caught species

- e.g. **spiny dogfish** (Squalus acanthias)
- Temperate coastal waters S & N hemisphere
- Sexual maturity >20yo
- Ovoviviparous
- Caught by anglers & trawl bycatch
- Optimal temperature 7-14ºC
- Poor survival in conventional recirc systems

- e.g. **Atlantic cod** (Gadus morua)
- Continental shelf Atlantic N of 40˚ latitude
- Mature at 3-5yo
- Usually young fish (<1.5yo) caught by coastal angling but heavy transfer losses
- Adults survive in conventional recirc systems (11-15ºC)

POLE needs to be specified in PPL application!



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Marine fish

- Can usually tolerate wide range of salinities as they are excellent osmoregulators
- Though in saltwater ammonia and nitrite has less toxicity relative to FW but still needs to be monitored; high stocking densities can produce extreme levels!!
- Sourcing?
  Not Sch2 - e.g. commercial AQ
  Wild caught – POLE
- Health issues – hygiene, C&D, screening to limit parasites & viral diseases
- Grading - Separating by size cohorts to minimize aggression and cannibalism
- Feeding - Give sufficient quantities to avert aggression and competition stress

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Between evidence base & speciesism – paradigms and debates on fish welfare

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Fishes as subject of animal welfare science

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11,000,000 – 80,000,000 fish/year

50

10,000,000-20,000,000 fish per/year

51

4,000,000,000,000 fish per year

52

Fish in laboratories

- 470 000 fish procedures in UK 2018, 82% of which ZF (HO returns, 2019)
- Worldwide estimate: >3,250 institutes in >100 countries work with zebrafish and >5 million are used per year *Lidster K, Readman GD, Prescott MJ, Owen SF (2017) International survey on the use and welfare of zebrafish in research. Journal of Fish Biology doi:10.1111/jfb.13278*

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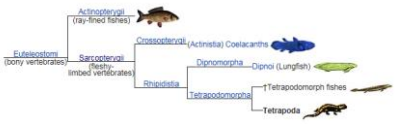
Humans are fish…

- (cladistically) as all tetrapods, including mammals, stem from lobe finned fishes emerging during the Devonian period



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First steps in recognising pain sensitivity in fish & the perennial debate on sentience

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General principles in fish welfare – pain sensitivity?

Sentience - "the capacity to feel, perceive, or experience subjectively"

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General principles in fish welfare –pain sensitivity?

Verheijen (1983):

- Hooked and released 30 juvenile carp:
- Reactions to hook – spitting, headshaking (even with slack line) – "pain" ?
- Buoyancy changes due to expulsion of spitgas when line was pulled taught

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General principles in fish welfare – pain sensitivity?

Verheijen (1983):

- Different behavioural pattern when line was kept slack
- ↑ time to feeding, erratic behaviours – fear, anxiety (?)

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General principles in fish welfare – pain sensitivity?

Verheijen (1983):

- Work was partially funded by the Dutch Angling Society who subsequently refused permission for international publication of results;
- First mentioned in New Scientist feature in 1987; now considered seminal work

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General principles in fish welfare – pain sensitivity?

Sneddon (2003):

- 3 nociceptor types on head of rainbow trout, A-δ and C fibres in trigeminal ganglion
- nociceptive events could change the motivational state of trout & post nociceptive behaviours after injection of acetic acid and bee venom:
- Lip rubbing, ↓↑OBR, ↓activity

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"Why fish do not feel pain"

Brian Key (2016)

"Accepting at face that fish may feel pain (…) comparable to believing causal association between MMR vaccination and the development of autism in children"

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"Why fish do not feel pain"

Brian Key (2016)

- Devastating consequences through legislative restrictions on fish-related activities with potentially serious negative implications for
- native subsistence fishing,
- human food supply
- economic development
- "Benefit of the doubt" argument can quickly lead to unsupported anthropomorphic conclusions

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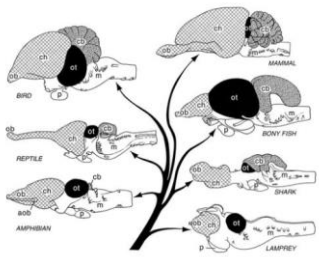

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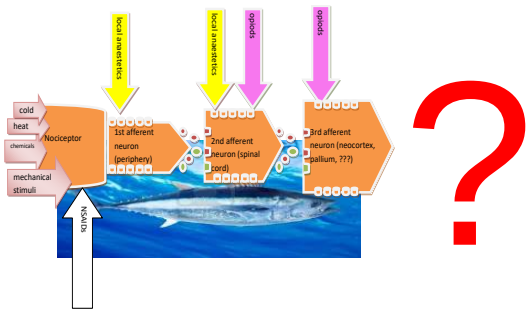

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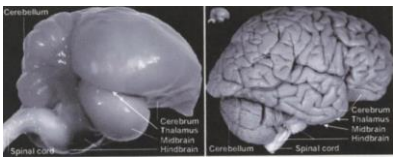
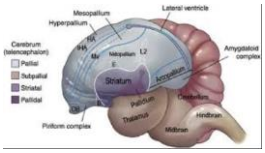


Jarvis et al. (2005)



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Speciesism

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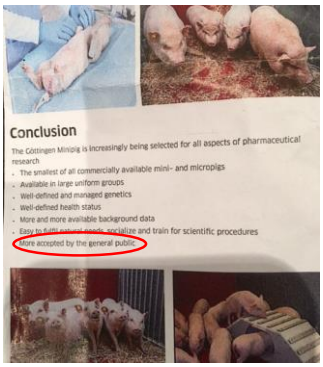

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Speciesism

- Assigning certain value on membership of a specific genetic clade/species/taxon
- For example – special protection for dogs, cats & horses under European laboratory animal legislation (but not pigs, psittacines..)

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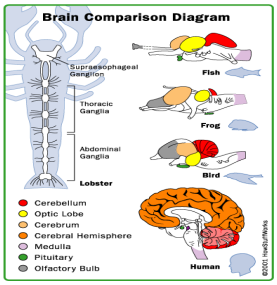
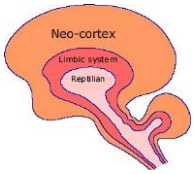
Fish – sentient species?





brainevolution.html

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Fish – sentient species?

- Birds and reptiles would be equally incapable of any "higher" response as they do not possess a neocortex
- Different species can use different neurological structures and systems to handle the same functions
- E.g. avian pallium => functional rather than structural homologies between avian and mammalian brains

Jarvis, Güntürkün et al. 2005

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The perennial debate…

Key, Brian (2016) Why fish do not feel pain
Animal Sentience 2016.003

Author Website
http://www.uq.edu.au/soms/staff/brian-key

Abstract

Abstract Only humans can report feeling pain. In contrast, pain in animals is typically inferred on the basis of nonverbal behaviour. Unfortunately, these behavioural data can be problematic when the reliability and validity of the behavioural tests are questionable. The thesis proposed here is based on the bioengineering principle that structure determines function. Basic functional homologies can be mapped to structural homologies across a broad spectrum of vertebrate species. For example, olfaction depends on olfactory glomeruli in the olfactory bulbs of the forebrain, visual orientation responses depend on the laminated optic tectum in the midbrain, and locomotion depends on pattern generators in the spinal cord from most vertebrate phylogeny, from fish to humans. Here I delineate the region of the human brain that is directly responsible for feeling painful stimuli. The principal structural features of this region are identified and then used as biomarkers to infer whether fish are, at least, anatomically capable of feeling pain. Using this strategy, I conclude that fish lack the necessary neurocytoarchitecture, microcircuitry, and structural connectivity for the neural processing required for feeling pain.

Fish do not feel pain and its implications for understanding phenomenal consciousness

Brian Key

Received: 14 April 2014 / Accepted: 8 December 2014 / Published online: 16 December 2014
© The Author(s) 2014. This article is published with open access at Springerlink.com

Abstract Phenomenal consciousness or the subjective experience of feeling sensory stimuli is fundamental to human existence. Because of the ubiquity of their subjective experiences, humans seem to readily accept the anthropomorphic extension of these mental states to other animals. Humans will typically extrapolate feelings of pain to animals if they respond physiologically and behaviourally to noxious stimuli. The alternative view that fish instead respond to noxious stimuli reflexly and with a limited behavioural repertoire is defended within the context of our current understanding of the neuroanatomy and neurophysiology of mental states. Consequently, a set of fundamental properties of neural tissue necessary for feeling pain or experiencing affective states in vertebrates is proposed. While mammals and birds possess the prerequisite neural architecture for phenomenal consciousness, it is concluded that fish lack these essential characteristics and hence do not feel pain.

…e human brain that is directly responsible

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Speciesism

- Assigning certain value on membership of a specific genetic clade/species/taxon

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State of (zebra)fish welfare today

"Two pillars"
- "Does the animal have what it wants?"
- "Is it physically healthy?"

(Stamp Dawkins 2012)

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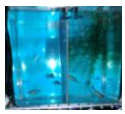
Does the animal have what it wants?

Paul Schroeder, Soffia Jones, Iain S Young and Lynne U Sneddon (2014): What do zebrafish want? Impact of social grouping, dominance and gender on preference for enrichment. Laboratory Animals ***48 (4)*** *328-327*

- Most enrichment cues significantly preferred over barren environment except airstones (significantly avoided
- Images of (gravel) substrate alone sufficient to elicit preference behaviour, attracting occupancy rates almost as high as the actual substrate



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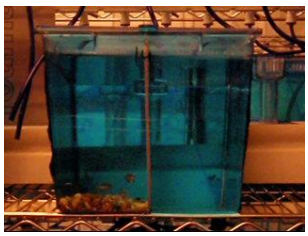

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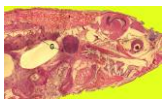
Is the animal healthy?

- Microsporidiosis
- *Pseudoloma neurophilia.* "skinny fish disease" or scoliosis
- 90-95% prevalence in European ZF facilities (estimate, QM Diagnostics, 2015) with 10-30% of healthy fish and 90% of emaciated fish infected (Matthews et al. 2001)
- **Diagnosis –** histology; PCR (water, eggs)
- **Control** - Pre-screening all brood fish by PCR → Pseudoloma free stock.



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State of (zebra)fish welfare today

"Two pillars"
- "Does the animal have what it wants?"
- "Is it physically healthy?"

(Stamp Dawkins 2012)

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Husbandry:
housing, breeding & production, nutrition, transport, GMOs

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General - Water quality

- Fish ectothermic, water passing **by** and passing **through**
- There is nowhere to hide if anything is wrong with tank water

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Water quality

- PH 6.8-7.6 (ZF); 6.8-8.0 (trout) optimal;
- Temperature 26-29° (ZF); 22-24° (seabass); 8-15° (salmon)
- Conductivity 3-500 ms
- Salinity 1-3 ppt

Buffering to resist changes in pH; 50-150mg/L $CaCO_3$

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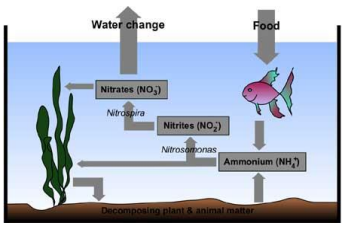
Water quality



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Ammonia, nitrite and nitrate

- Ammonia (NH3/NH4+): Either dissolved as ammonium ions or more dangerously as free ammonia.
- Levels of free ammonia increase with increased pH or temperature.
- Ideally<0.06ppm, toxic>0.3ppm.
- Nitrite less toxic than ammonia.
- Toxic from 0.5 mg/l; death at levels>10mg/l
- Nitrate depends on bacterial nitrification (final product in the nitrogen cycle)
- Recommended maximum 50mg/l

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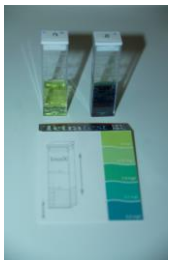
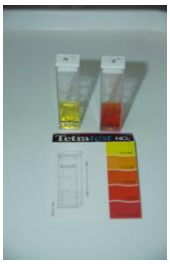
Ammonia, nitrite and nitrate





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Temperature

- Fish should be kept “as close to natural environmental temperature” (CoP) yet this is 12-34°C *(Zebrafish in the wild; Engeszer et al. 2004)*
- **Zebrafish**
  - Optimum temperature of 26-29°C
  - Above 31C and below 25C → decreased breeding performance.
  - Room temperature at 25°C as temperature buffer.
- **Rainbow trout**
  - 12-20°C
  - Room temperature 14°C
- **Common carp**
  - 14-20°C
  - Room temperature 14-17°C

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Temperature change

- Osmo-regulatory dysfunction.
- Suppression of immune system.
- Loss of equilibrium.
- High temp. - Increased activity.
- Low temp – Ataxia and coma.

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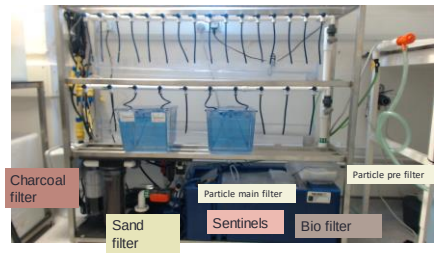
Filtration



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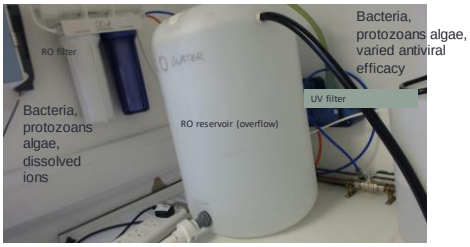
Filtration



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Housing - zebrafish

- For ZF - facility sizes vary from one tabletop rack to those with tens of thousands of tanks (ZF Resource Center Karlsruhe, Sanger Institute).

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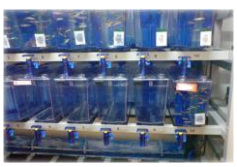
Housing - zebrafish



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Nutrition

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LO: 3.1.5 & 4.6 98

Feeding – presentation, frequency, hazards

- Controlled ration vs ad libitum
- Timed vs continuous
- Frequent (several times/day) vs infrequent (once/day or less)
- Pellet/commercial vs live/natural feeds
- Depends on species requirements: energy expenditure (breeding, activity levels & metabolic state as well as digestive morphology)
  - Example: ZF – controlled ration, timed, frequent, commercial & live
  - Example: Juvenile carp (cool water system) infrequent, commercial, timed ad libitum
  - Example: Juvenile carp (warm water system) frequent, natural & commercial, timed, controlled ration

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LO: 3.1.5 & 4.6 99

Feed related hazards

- Overfeed
  - Obesity
  - Accumulation & degradation in tank edges, overflows
  - Overload of physical & biological filtration process; displacement & overgrowth of denitrifiers by decomposition bacteria
  - Increased resistance for effluent leg of recirc system, slowing down water replacement
  - Can lead to increased circulating microorganisms/pathogens – as fine waste food particles & pigments may pass through physical & biofilters, reducing efficacy of UV filtration through shading

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LO: 3.1.5 & 4.6 100

Feed related hazards

- Live feed as vector for parasites

Paramecium, Rotifers & Artemia

- cultured in freshwater, veritable "bacterial soups," shown to support diverse microbial communities, including *Vibrio*.
- Commonly utilized zooplankton species indiscriminately feed on bacteria, it is entirely possible that they would then have infective potential if they ingested pathogens and then in turn were themselves ingested by fish.
- Documented cases not limited to *Vibrio*: *"Paramecium caudatum*, a commonly utilized prey item for first feeding zebrafish, experimentally enhanced transmission of both *Mycobacterium chelonae* and *M. marinum* to zebrafish larvae"

Watts et al. (2016)

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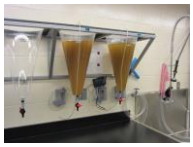
LO: 3.1.5 & 4.6 101

Zebrafish/medaka/guppy nutrition

- Minimum requirement:
- ZF & medaka < 10-12 days typically fed Paramecium
- ZF & medaka > 10-12 days dried flakes 000 with feed particle size increased progressively from then
- Guppies: fry formula or paramecium <3weeks, flakes for older fish
- Live feeds with artemia at least 2-3 times per week (ideally daily)

AVOID TUBIFEX & DAPHNIA!

AVOID OVERFEEDING!!



101

LO: 3.1.5 & 4.6 102

Zebrafish nutrition

- Survival and subsequent health status of juvenile ZF related to:

1. Frequency of feed

1. swift removal of uneaten food remainders

Not so much feed brand/type!

102

LO: 3.1.5 & 4.6 103

Trout/salmon/seabass nutrition

- Adult salmonids and bass feed on variety of invertebrates e.g., freshwater shrimp, aquatic and terrestrial insects, and small fishes.
- Fry can be weaned onto an artificial diet.
  - Starter feeds, starting from when approximately 50% have reached the 'swim-up' stage.
  - When most fish actively feeding, give 10% of their weight daily for 2–3weeks.
  - When fish reach 15–25 mm long feeding based on temperature and fish size.
  - Overfeeding must be avoided.
  - Fish moved to larger tanks to reduce density as they grow.
- Adults fed pelleted diet as a % of body weight (typically 1-2%) depending on how much growth you want!

103

LO: 4.7 & 7.1 104

Fish handling, capture & restraint

- Fish are invariably stressed through all forms of handling
- Struggle during capture in wild and captive environment
- Significant damage to eyes, gills, epidermis & scales can result from rough handling, followed by osmoregulatory & neurological pathology
- For larger ornamental or AC species, sedation may be necessary prior to netting

104

LO: 4.7 & 7.1 105

Capture & netting

- Some management activities e.g. cleaning of the tank, moving fish to another tank, require netting.
- Suitable non-abrasive/knotless net
- Attempt to reduce chasing with a net during capture.
- Ensure that fish are out of water for the shortest possible time.
- Well-established routines should be applied to the care to both minimize the frequency of cleaning and the moving of fish.
- Zebrafish, medaka, guppies and other small ornamental breeds are caught and transferred from tank to tank by the use of soft non-abrasive nets to minimise any stress and damage to scales and skin.
- Any skin damage should be avoided as this may lead to infection.

105

LO: 4.7 & 7.1 106

Handling & restraint

- Handle with clean, soap free, moist hands or gloves.
- Place animals onto plastic covered foam or soft moist cloth to avoid skin abrasions.
- Larger fish may require restraining device for quick out-of-water procedures

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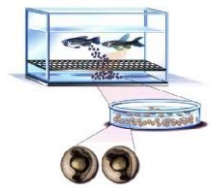
Breeding/production systems

107

LO: 4.10 108

Breeding systems - ZF

- "Low tech" breeding systems or fully automated nurseries
- Install screens to stop egg predation
- Install enrichment cues to encourage spawning
- Select and monitor brood stock
- Natural or in vitro fertilization



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Breeding systems - ZF

- Select fertilized eggs
- Keep in fry tanks or deep petri dishes
- Frequent water change & removal of dead eggs/larvae
- Feed Paramecium from day 5 pf

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Fish Transport

- ZF, medaka, sticklebacks, juvenile carp - adults or juveniles in bags (system water) in insulated boxes
- For medaka & ZF>3 months no more than 5 fish/l
- Air pocket must be locked into bag!
- Post transport quarantine
- Careful of any **netting damage- skin surface delicate!**

Large fish

- Barrelled in groups or bagged individually as above - lower temperature (within species appropriate range) for longer transport to reduce oxygen demand. Ideally no longer than 2h
- Consider alternative methods for short distances – pipes, chutes

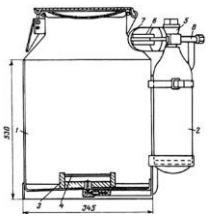
110

Transport

- Transport stressful to all fish species, resulting immunosuppression and ↓ oxygen can lead to high transport mortality
- Higher oxygen demand with higher temperatures
- Published loading rates in need of revision
- Allow for temperature acclimation pre and post transport

Weight (in kg) of channel catfish that can be transported per litre of 10°C water (Piper et al., 1982)

Number of fish (per kg)	Transit period (in h)		
	6	12	18
2	0.73	0.66	0.57
4	0.71	0.57	0.41
8	0.60	0.40	0.35
110	0.41	0.30	0.24
276	0.35	0.26	0.21
552	0.26	0.21	0.19
1 100	0.21	0.20	0.15
2 200	0.15	0.12	0.09
22 000	0.02	0.02	0.02



Piper et al. (1982)

111

Genetic modifications

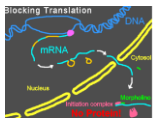
- Morpholinos (knockdowns)
- Transgenics
- Knockouts

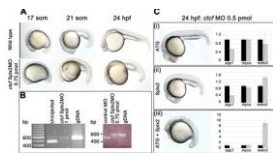
112

Genetic modifications

- Morpholinos (knockdowns)

used in molecular biology to modify gene expression. DNA bases attached to a backbone of methylenemorpholine rings. Morpholinos block access of small (~25 base) specific sequences of the base-pairing surfaces of ribonucleic acid (mRNA). Morpholinos are used as research tools for reverse genetics by knocking down gene function.





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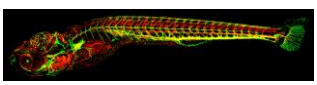
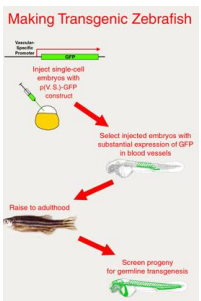
Genetic modifications

Transgenics





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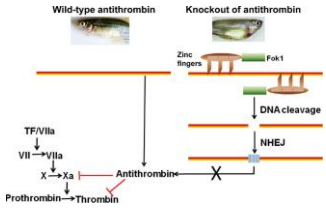
Genetic modifications

Knockouts

- ↑membrane permeability with electroporation before chemically induced DNA cleavage



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Husbandry – biosecurity & disease prevention

116

Diseases

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General

- Relatively little knowledge on ZF diseases
- No "known viral pathogens" in research facilities (but some suspicious cases)
- Several persistent infections with high morbidity but low mortality, affecting many facilities.
- As with other laboratory animals used in research, imperative to conduct studies with disease-free fish (Kent 2009).
- Unlike rodent models, where there are many certified specific pathogen free (SPF) strains, it is early days still for SPF stocks of zebrafish

118

Examination

- External - **Fish behavior**
- Flashing (rubbing on the sides of tanks), may indicate external parasites.
- Cessation of feeding, lethargy, loss of equilibrium, abnormal position in the aquarium (e.g., at surface or bottom).
- Abnormal respiratory pattern (gill damage, other stress).
- whirling or spiralling swimming often indicates neurological damage.

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Further examination

- **Necropsy** conducted on diseased fish that are collected while still alive
- Several affected fish should be examined whenever possible
- Include apparently normal, asymptomatic fish to detect early pathological changes
- Dead fish – CAVEAT post-mortem autolysis.

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Further examination

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Further examination

- **Close up examination** with stereo dissecting microscope.
- Surface abnormalities (e.g., frayed fins, cloudy eyes, ulcers, skin discolorations, parasites, and tumors).
- Prepare wet mounts of skin mucus/scales by scraping surface of fish with glass cover → put on slide; reduce light and lower condenser for higher contrast

122

Preservation for diagnosis (zebrafish.org)

Preservation methods of fish tissues and their uses in fish disease diagnostic examinations. +++ = optimal; ++ = satisfactory in most cases; + = suboptimal, can be used if no other tissue available; 0 = useless.

	Live	Iced	Frozen	Preserved *
Parasitology	+++	++	+	+
Bacteriology	+++	+	+	0
Virology	+++	++	+	0
Toxicology	+++	++	+++	0 to +++
Histology	+++	+	+	+++
Electron Microscopy	+++	+	0	+++
PCR	+++	++	+++	+++

* Preserved in formalin-based fixative (e.g., Dietrich's Fixative) for histology, glutaraldehyde-based fixative for electron microscopy, 95% ethanol for PCR testing.

123

Protozoan infections

- **1. Microsporidiosis**

- *Pseudoloma neurophilia* (Matthews et al. 2001)

- "Skinny disease"
- Scoliosis

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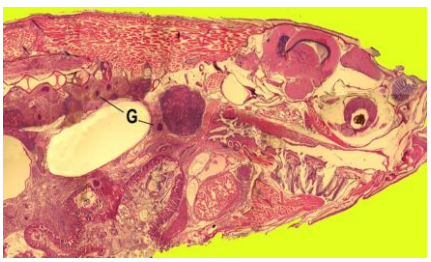

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Protozoan infections

- Most "skinny" fish and up to 1/3rd of healthy fish infected → infection routinely in normal, healthy appearing fish
- stress causes increased severity of the infection
- **Transmission:** obligate intracellular fungus-like parasites which produces infectious and resistant spore

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Protozoan infections

- Transmission via the ingestion of infective spore stage;
- Fry fish are very susceptible, and may show more acute form
- Transmitted by feeding on infected carcases, but also via water in recirc system!

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Protozoan infections

- **Diagnosis –** histology; spores ovoid to pyriform, with a prominent posterior vacuole, and average 5.4 x 2.7 um.
- Xenomas within the spinal cord and hindbrain.
- PCR tests (non-invasive test based on PCR of water and eggs in development).
- Pre-screening all brood fish by PCR → Pseudoloma free stock.

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Protozoan infections

- **Control and Treatment –** *UV* at 30-50,000 µWsec/cm^2 kills the parasite and thus prevents it transmission in a recirculating system.
- Fumagillin as oral treatment for ornamental fish and aquaculture species (eel, salmon; originally developed for honeybee microsporidiosis).
- Microsporidian spores resistant to disinfectants!
- chlorine levels routinely used by zebrafish facilities (i.e., 25 or 50 ppm for 10 min) not effective for killing spores, especially those persisting in eggs.

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Protozoan infections

- **Ichthyophthirius (white spot/Ich)**
  - Most common disease in FW ornamentals
  - Excessive mucus production, labored breathing, and lethargy
  - White, raised nodules on the skin ("white spot")

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Protozoan infections

- **Treatment/control** - Ich penetrates the epithelium hence difficult to eradicate with external baths or dips.
- Formaldehyde 1:5000; Methylene blue
- Multiple treatments!
- UV filters upstream & downstream, especially in recirc systems
- Quarantine racks with separate recirc/flow through system

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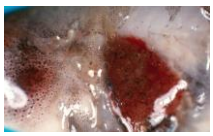
Bacterial infections

- **Mycobacteriosis (Fish TB)**
- Chronic, systemic bacterial infections by various *Mycobacterium* species
- *M. marinum*, *M. abscessus*, *M. chelonae*, *M. Fortuitum*, *M. peregrinum* and *M. haemophilum* associated with mycobacteriosis in zebrafish



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Bacterial infections

- usually chronic with low level mortality
- rarely acute or peracute with severe losses in ZF colonies.
- Factors responsible for the differences yet to be determined (strain, species, water quality, stress etc.).

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Bacterial infections

- **Zoonosis**! "Fish handlers disease"(usually extremities) which needs aggressive antibiotic treatments, can even be lethal to immune compromised individuals
- **Wear gloves!** Wash hands after coming in contact with water containing fish, and avoid exposing open lesions to aquarium water and fishes.
- Most cases of human mycobacteriosis associated with fishes caused by *M. marinum*.

→ check that *M. marinum* is covered by AFB scan (if applicable)

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Bacterial infections

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Aquatic health screening – rationale & epidemiology

- What do we screen

- (Epidemiological) Population, a group of conspecific individuals sharing a defined space or medium, so that they have an approximately equal chance of contact with a hypothetical pathogen

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Aquatic health screening – rationale & epidemiology

- Why do we screen?

Reactive
- Understanding cause of acute mortality event
- Morbidity
- Failure to thrive
- Known biosecurity breach

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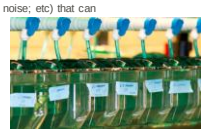
Aquatic health screening – rationale & epidemiology

- Why do we screen?

Preventive
- ID pathogens before clinical manifestation
- ID pathogens because clinical manifestations other than death are unlikely to be spotted by anyone
- ↓ Statistical noise (screening for infections which result in confounding subclinical conditions)
- Protect human health (screening for zoonotic pathogens such as *Mycobacterium* spp., *Anisakis* etc.)
- Quality assurance of genetic integrity, especially inbred strains bred and maintained in facility
- Environmental factors (quality of feed, water, and bedding; lighting; noise; etc) that can affect colony health.



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Aquatic health screening – rationale & epidemiology

- How do we screen?

Indirect (e.g., tank water)

Direct:
- non lethal, non invasive (e.g. faeces)
- Non lethal, minimally invasive (e.g. mucus swab from gills or skin)
- Non lethal, moderately invasive (e.g. scale extraction, peripheral blood sample, gill biopsy, fin clip, stripping)
- Non lethal, highly invasive (e.g. liver biopsy, surgical egg removal)
- Lethal (whole fish or tissue samples)

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Aquatic health screening – rationale & epidemiology

If samples are to be sent away, live fish usually preferred
e.g. CR Health Surveillance 360 Diagnostics™

Testing Type	Required Sample Type(s)
Signature Health Inspection	65 live fish selected randomly from the population
Diagnostic Investigation	10 live fish: ideally some, or all, of the fish submitted will be moribund, or displaying signs of disease. Please also health history of the animal/colony, being as detailed as possible.
Histopathology	Live fish or fish fixed in 10% neutral buffered formalin (euthanized immediately before fixing, with belly carefully slit to allow optimal fixation)
Aerobic Culture	Live fish
Molecular Testing	Live fish, frozen fish, or fish preserved in RNAlater or ethanol (euthanized immediately before preservation, with belly carefully slit to allow optimal preservation)
Virus Isolation	Live fish

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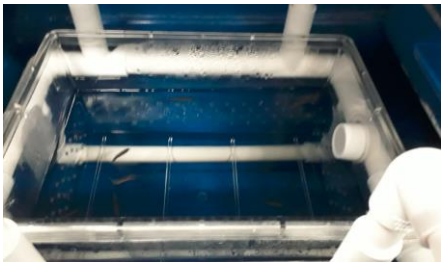

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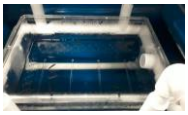
Aquatic health screening – rationale & epidemiology

- Preventive screening

Sentinel or randomly chosen tank?
- Depends on facility design
- How strongly are we convinced that our filtration system and gradual water replacement significantly reduces circulating pathogens?



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Required sample sizes

- Depends on sensitivity and specificity of test
  - Sensitivity = likelihood of truly infected animal to be detected by test
  - Specificity = likelihood of truly uninfected animal to be cleared by test
- Generally, tests offered by health screening services based on serology &/or PCR have ↑specificity ↓sensitivity
  - → few false positives, lots of false negatives
  - Sensitivity can be augmented by also adding histology & cultures (bacteria, fungi, viruses)
- Measure of certainty that test positive animal is truly infected:
  -Positive predictive value (PPV)

= true positives/(true positives/false positives)

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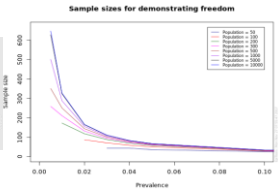
Required sample sizes

It is a numbers game and depends on:

- Prevalence
- Test sensitivity
- Desired confidence interval

Signature Health Inspection	The Signature Health Inspection provides a 95% confidence level in detection, assuming pathogen prevalence is at least 5%. This approach to health monitoring in aquatic systems is based on the recommendations of the Office International Des Epizooties (OIE) and the American Fisheries Society-Fish Health Section.



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Required sample sizes for demonstrating disease freedom

e.g. Pseudoloma PCR assay, 99.4% specificity, 85% sensitivity, CI95%

	Prevalence = 0.005	Prevalence = 0.01	Prevalence = 0.02	Prevalence = 0.03	Prevalence = 0.04	Prevalence = 0.05	Prevalence = 0.1	Prevalence = 0.2
Population = 50				46	46	38	27	16
Population = 100			92	75	63	54	31	17
Population = 200		183	125	93	74	61	33	17
Population = 300	275	223	139	100	78	64	34	18
Population = 500	372	266	153	107	82	67	35	18
Population = 1000	531	305	164	112	85	69	35	18
Population = 5000	665	343	174	117	88	71	36	18
Population = 10000	685	348	175	117	88	71	36	18
Population = 1e+05	703	352	177	118	89	71	36	18
Population = 1e+06	705	353	177	118	89	71	36	18

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Required sample sizes for demonstrating disease freedom

- The lower the prevalence, the higher the required sample size for any given test sensitivity
- The lower the test sensitivity, the higher the required sample size for any given prevalence
- The size of population under surveillance only affects sample sizes up to n=10,000

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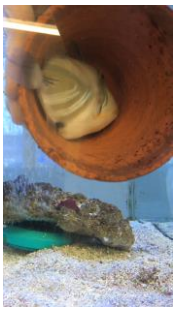
Lethargy & inappetence



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Adspection & wet mount

- Heavy sedation MS 222 75mg/l (10min)
- During inspection gills appeared greyish pale.
- Wet mount largely inconspicuous, except for copious amounts of mucous embedding the secondary lamellae.

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Anaesthesia & Sch1

160

Fish anaesthesia in the laboratory – principles

- Definitions, limitations and pre-anaesthetic considerations
- Refinement issues
- Recent developments – how do we interpret the evidence base?
- Analgesic considerations

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Definitions

- 96% of fish species gilled teleosts
- 50% of regulated fish procedures on ZF; rest variety of spp. incl. trout, stickleback, cave fish

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Definitions

Non-immersion fish anaesthesia:

- i.v. - restraint issues
- i.c. – visceral damage
- i.m. – (dorsal musculature) – leakage
- oral – precise dosing difficult, absorption?

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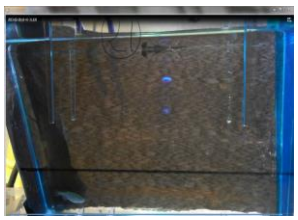
Main method of choice for fish

- anaesthesia: immersion (water medication)



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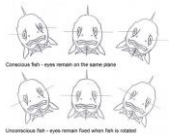
	Mammal	Fish (immersion route)
Premed	Anti-emetic Anticholinergic Sedatives Anaesthetic potentiators Analgesics	?
Short acting anaesthetic	Inhalant anaesthetic Propofol	MS222, benzocaine, 2POH, isoeugenol
Longer acting anaesthetic	α2, diss. agents, opioids etc..	
Adjusting	↓↑Inhalant conc. ↑Top-up with injectant	↑Top-up immersion bath with anaesthetic stock solution; ↓bags of anaesthetic-free water if using reservoir
Monitoring	Righting reflex Spinal reflexes Ocular reflexes Respiration Temperature Pulse O_2; CO_2	Righting reflex Spinal reflexes Ocular reflexes Respiration
Reversal	Opioids, α2	n/a; "gill flushing"



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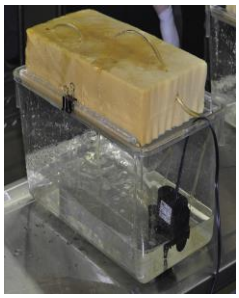

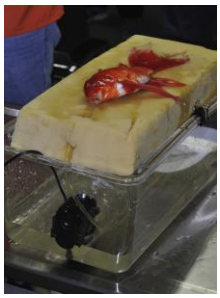

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Pre-anaesthetic considerations

Routes of administration and pharmacokinetic considerations:

- Immersion drug uptake mostly gills,
- Generally very little through olfactory tract and skin
- But species dependent with less scaled fish absorbing higher proportion through skin
- E.g. for electric eels more quinaldine may be absorbed through skin than gills (Neiffer & Stamper 2007)
- Permeability of skin to immersion drugs varies between animals and with age

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Pre-anaesthetic considerations

Routes of administration and pharmacokinetic considerations:

- Skin only permeable to water and small, nonpolar molecules limited by mucus and scales
- Gills with varying absorption - lipophilic compounds will diffuse across gills but not ions with mol weight > 100;
- even with ideal absorption 95% of immersion anaesthetic wasted!

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Pre anaesthetic considerations

- Fasting -12/24/48h (WF cost?)
- Health?
- Preparation of transport, anaesthetic and recovery tanks
- Oxygenation of water
- Out of water procedure?
- poikilo/endothermy? Could temperature become an issue?

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Pre anaesthetic considerations

Peri-procedural parameters to be monitored:

- Opercular beat rate
- Swimming behaviour: coordination, activity level
- Righting reflex (maintenance of equilibrium?)
- Responsiveness to tactile stimuli (blunt forceps)
- Eye roll
- Return-to-feeding time (post recovery)

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Criteria for suitability of anaesthetic (Brown, 1994/2010):

Induce stage III anaesthesia within 3 mins

- Offer a 90s window for out-of-water procedure
- be safe to fish when used for 30 mins
- allow full recovery within 5 mins
- Rapid metabolisation or secretion
- Zero or short withdrawal period*

*degree-days, calculated by multiplying mean daily water temperature by total number of days on which temperature was measured; for any off-label use 500 d/d is minimum withdrawal period established by European Economic Commission Directive No. 82/2001

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Criteria for suitability of anaesthetic (NC3Rs/CEFAS Weymouth Fish WF workshop proceedings May 2016):

- Induce surgical anaesthesia
- Offer a suitable window for out-of-water procedure
- Minimal aversive properties
- …

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Anaesthesia in fish – anaesthetic agents

Agent	Dose to induce surgical anaesthesia	Properties
Benzocaine	25-200mg/l	+ Rapid uptake - Hypoxia Efficacy affected by water temperature --> poor safety margins for warm water species; Poor safety margin for salmonids
Buffered tricain (MS 222)	100-300mg/l	+ Rapid uptake through gills due to high lipid solubility, inexpensive - Induction of hypoxia; Effects on CV system / vasoconstriction make long term anaesthesia hard to manage, Sensitive to light
Clove oil + polysorbate 80 (Aqui-S®)	15-25mg/l	+ No withdrawal period, very fast recovery for lower to medium doses - Small safety margin when using higher concentrations, interactions with local anaesthetics
2-Phenoxyethanol	200-800mg/l	+ Inexpensive, easy to dose, Fast onset - Questionable analgesic properties, oily consistency requires mixing with water
Buffered quinaldine sulphate	25-40mg/l	+ Very fast induction and recovery - Expensive, poor safety margin in some species

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Anaesthesia in Fish - stages

Stage	Plane	Description	Signs	Dose required (MS 222, mg/l, 3-5 mins)
I	1	Light sedation	Reduced motion, responsive to stimuli, ↓ventilation possible	20-30
	2	Deep sedation	Same as 1 but less responsive to stimuli, some analgesia	50-80
II	1	Light anaesthesia	Loss of equilibrium, still some response to tactile stimuli, good analgesia, ventilation ↓↓	100 (Leopard) 125 (AB)
	2	Deeper anaesthesia		125-150 (?)
III	n/a	Surgical anaesthesia (formerly "stage 4")	As in II but total loss of responsiveness to stimuli, ventilation ↓↓↓ and very shallow	125-200
IV	1	Anaesthetic overdose	Gasping, no other signs of ventilation	250-300
	2	Medullary collapse	Total absence of opercular movements, death (after 15-30mins)	>400

adapted for zebrafish from own data, 2011-14, Treves-Brown (2000) Applied Fish Pharmacology and Roberts (2010) Fundamentals of Ornamental Fish Health

176

Anaesthesia in fish – recommended species' doses

- MS 222 (buffered tricain)
  - ZF: 100-200mg/l
  - Trout: 80-180mg/l
  - Carp: 30-200mg/l
  - Medaka: 200-300mg/l
- Benzocaine
  - ZF: 50-100mg/l
  - Trout: 25-35mg/l
  - Carp: 50mg/l

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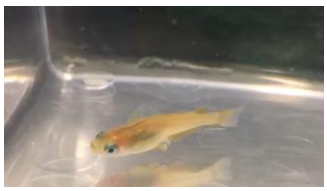
Why such differences?

- Mucus layer (gill, tegument) and scale cover vary from fish to fish
- MS 222 is fat soluble



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Euthanasia / Sch 1

Which of these methods is consistent with ASPA 1986 (2012 Amendment Regulation) Schedule: 1, listing humane killing methods for protected species?

5.2kg salmon: concussion with heavy metal rod then destruction of the brain

Yes – ANIMAL EXCEEDED WEIGHT LIMIT FOR THIS METHOD UNTIL LAST YEAR

179

Euthanasia / Sch 1

Which of these methods is consistent with ASPA 1986 (2012 Amendment Regulation) Schedule 1, listing humane killing methods for protected species?

1 yo Zebrafish: netting, concussion by "snipping" index finger onto cranium, followed by checking reflexes for vital signs

NO – CHECKING REFLEXES NOT AN ACCEPTABLE METHOD FOR CONFIRMING DEATH

180

Euthanasia / Sch 1

Which of these methods is consistent with ASPA 1986 (2012 Amendment Regulation) Schedule 1, listing humane killing methods for protected species?

4 wo Zebrafish: netting, injection of 2ml/kg pentobarbital with 35 gauge needle, followed by destruction of the brain

YES – CONSISTENT WITH SCH1

181

Euthanasia / Sch 1

Which of these methods is consistent with ASPA 1986 (2012 Amendment Regulation) Schedule 1, listing humane killing methods for protected species?

3mo rainbow trout: electrocution with 24V/14A, followed by destruction of the brain

NO – ELECTROCUTION NOT IN SCH1

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Euthanasia / Sch 1

- Physical methods – concussion, then destruction of the brain
- Non physical methods – Overdose of anaesthetic
- Injection of pentobarbital - 0.06mg/g body weight
- Immersion bath using range of anaesthetics:

183

Euthanasia / Sch 1

- MS 222 (>500mg/l, 30 min)
- 2PO (>1500mg/l, >15 -20 min)
- Benzocaine (>250mg/l, 30 min)

Euthanise individually!

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LO: 7.5 & 8.1 185

Minor procedures and aseptic technique

- Injection sites
- Fin clipping & other ID systems
- Aseptic technique
- Post op care

Harms (2005)

185

LO: 7.5 & 8.1 186

Injection sites

- IM – 85% of fish may be muscle so easy enough to find! E.g. dorsal musculature,
- IP – along midline between pectoral fin and anus, fish in dorsal recumbency
- IV – lateral caudal peduncle, tricky in smaller fish (blind technique)

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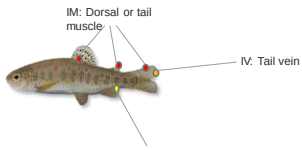
LO: 7.5 & 8.1 187

Injection techniques



BEVEL ALWAYS POINTS UPWARDS!!!

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LO: 4.8 188

Fin clip and other minor procedures (e.g., scale removal)

- For batch ID or for genetic material
- Size 10/15 curved scalpel – remove max 50% of tailfin distal of forking
- 2-6 weeks to full regeneration (ZF)
- Anaesthetize to allow at least 60s out-of-water procedure
-
- Aseptic technique similar to that in other species: sterile nets; sterile scalpel/surgical scissors; sterile drape or fine kitchen towel; sterile system water for squirting/drenching BUT DON'T DISINFECT SKIN; JUST SWAB EXCESS MUCUS
- Batch surgery – use bead sterilizer or autoclave
  - in between (or have several sets of sterile
  - instruments ready

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LO: 4.8 189

Tagging – Passive integrated transponder (PIT)

- First developed to monitor salmon movements in the 1980s
- 8-12mm long, not suitable for fish < 15cm TL
- Less invasive alternative preferable (VIE, even fin clip
  - for smaller fish)
- Can exceed limits of "minor procedure" e.g. surgical implantation of PIT tag in coelom

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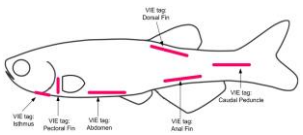
LO: 4.8 190

Tagging – Visible implant elastomer (VIE)

- Implant applied subcutaneously in several sites
- Batch tagging, no additional data stored electronically (unlike PIT tag)
- Smaller and thinner than PIT tag



190

LO: 20.12 191

Post surgery

- Check on full recovery/feeding
- Analgesia: lidocaine 2-5mg/l

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LO: 5.5 192

Severity considerations

- Sub – threshold
- Non-recovery
- Mild
- Moderate
- Severe

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LO: 5.5 193

Severity considerations – examples

1. Control animals in genetic study → Sch1?
2. ZF: 16 hours food retention effecting slight behavioural change?
3. ZF: Cardiac excision procedure with 10-15% mortality?
4. Carp: Fin clip under anaesthesia?
5. ZF: VIE implant under anaesthesia?
6. Medaka: superficial mucus swab without anaesthesia?
7. Rainbow trout: blood sample for health screening ?
8. Fish anaesthetised for surgical implantation of PIT tag in coelom

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Some final legal considerations

- Protection from free swimming stage – use minimum; guppies protected from day one!
- Prospective **&** reported severity – surgical procedures often "severe"
- Refinement obligation
- Sch1 with completion!!
- Sch2!!!

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Refinement matters

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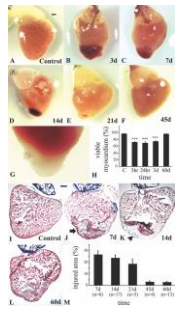
Refinement of fish anaesthesia -Systematic review on piscine models of myocardial infarction (Oct 2014)[1]

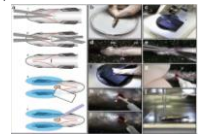


The ARRIVE guidelines (Kilkenny, Browne et al. 2010) were devised to improve transparency where studies reported results derived from animal research. Ideally, any publications adhering to these rules objectively describe the welfare cost to the animals involved. Three guidelines are particularly linked to this:

(1)Provide precise details of all procedures carried out, for example (…) anaesthesia and analgesia, (…) surgical procedure. (2) Provide details of (…) welfare related assessments and interventions that were carried out prior to, during, or after the experiment. (3) Specify the total number of animals used in each experiment.

[1]The Welfare of Laboratory Fishes. In Fish Welfare (ed. Peter Southgate) 2nd edition. Wiley-Blackwell.

Mercader et al. 2012



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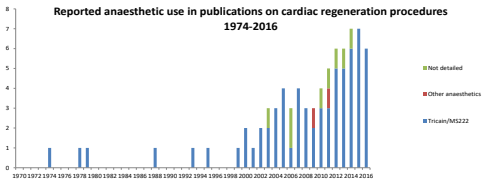

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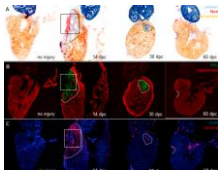
So, I shall use nothing but MS222...

- But how much for this fish?
- For example, cardiac excision & cardiac cryoinjury: publications 2012-2016; for ZF recovery anaesthesia **all** used tricain but with dose ranging from 0.090 to 0.032 (90-320mg/l)



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MS222 doses for cryoinjury and cardiac excision

Cryoinjury

Fish were anesthetized with 0.02% Tricaine (MS-222) and transferred to a moist sponge for surgery. After visually locating the posterior medial margin of the heart straight iridectomy scissors were used to puncture the skin and the silvery pericardial sac. Subsequently, an incision was made through both the skin and pericardium starting from the junction of the pericardium and peritoneum and reaching anteriorly for about 2/3 of the length of the heart. The incision was spread open laterally using fine forceps to expose the ventricle. Small pieces of dry ice were formed into a conical shape with a length of ~20 mm, one end with a diameter of ~2 mm and the other end with a pointed tip. The pointed tip of the dry ice cone was applied to the posterior apex of the ventricle for 10 seconds to cause the cryoinjury. After surgery the fish were returned to holding tanks. To revitalize the fish a pipette was used

Schnabel et al. 2016

Methods

Animal Procedures

The following zebrafish strains were used in this study: wild-type AB (Oregon) strain, Ekkwil (EK) strain, transgenic strain cmlc2::DsRed2-nuc [26] to visualize cardiomyocytes nuclei, and transgenic strain cmlc2::EGFP [27] to analyze the injured area on the whole heart. Fish aged 6-18 months were anesthetized in 0.1% tricaine

Chablais et al. 2011

Experimental design

Fish are anesthetized by immersion in 0.032% (wt/vol) Tricaine and immobilized by placing them with the ventral side facing up into a foam holder under a dissecting microscope. After removing some of the ventral scales with forceps, a small incision is made through the body wall and the pericardium. This is done using forceps and microdissection scissors, tearing the tissue rather than making a clean cut, in order to facilitate healing (see Fig. 1 for an illustration of the main steps in the procedure). Once the

Mercader et al. 2012

Cryoinjury

Cryoinjury was performed as described previously (González-Rosa and Mercader, 2012). For analysis of regeneration, animals were euthanized at different times post-injury by immersion in 0.16% tricaine (Sigma), and the hearts

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Refinement of aquatic anaesthesia

- Length of out-of-water procedure?
- Only specified in Mercarder et al. (2012) as 3-5 min

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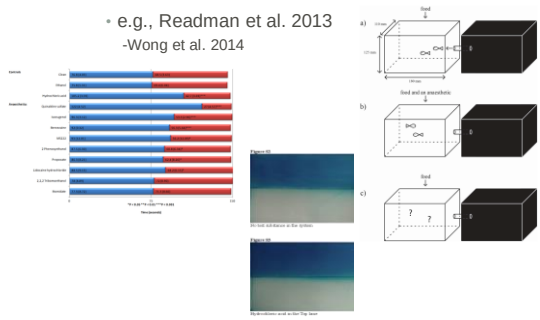
Refinement of anaesthetic procedures - recent evidence base?

- e.g., Readman et al. 2013
  -Wong et al. 2014



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Some historical context

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Some historical context

- **MS 222** used since 1960 (experimental); validated 1967 (Schoettger et al.)
- Observation at the time: huge variation in induction time and dose response within same species
- In 1960 **sodium thiopental** was the most widely used human general anaesthetic; neither etomidate nor fentanyl had been invented
- In 1960, for laboratory mice, the **ether jar** was the most popular anaesthetic delivery method

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Some historical context

- Attempts at balanced fish anaesthesia using mix of compounds is not that new either…

SYNERGIC MIXTURES OF MS-222 AND QUINALDINE AS ANESTHETICS FOR RAINBOW TROUT AND NORTHERN PIKE

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Fish Control Laboratory, La Crosse, Wisconsin 54601
and
Erwin W. Steucke, Jr.
Bureau of Sport Fisheries and Wildlife
Minneapolis, Minnesota 55111

ANESTHETICS are essential tools for the proper handling of sport and commercial fishes during artificial spawning, marking, weighing, measuring, surgery, photography, and research. For example, Bailey (1965) reported the use of MS-222 to anesthetize millions of lake trout for fin-clipping. Literature reviews by Kistler and Backhuis (1960), Kiuchi (1964), Bell (1967), Bov6 (1967), Schoettger (1967), and Schoettger and Julin (1968) have demonstrated the extent and variety of uses of MS-222 and quinaldine as fish anesthetics.

In general, either MS-222 or quinaldine is selected by fishery workers because its properties are suited to a specific need. MS-222 is water-soluble and odorless, and desired depths of anesthesia are relatively easy to control with concentrations (Schoettger and Julin, 1967), but concentrations that quickly render fish immobile

A better anesthetic would combine certain properties of both these compounds. Specifically, it would immobilize fish rapidly, completely, and maintain them safely in this condition for a reasonable period. Therefore, the objective of our study was to determine whether MS-222 and quinaldine could be used together to achieve safe and more effective anesthesia of fish.

Materials and Methods

Practical-grade (95 percent) quinaldine, 2-methylquinoline, was purchased from Eastman Kodak Company. Technical-grade (99.6 percent) MS-222, the methanesulfonate of m-aminobenzoic acid ethyl ester, was obtained from Sandoz Pharmaceuticals.[2]

Rainbow trout (Salmo gairdneri) used in this work were obtained in two lots from the National Fish Hatchery at Manchester,

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Perioperative analgesia

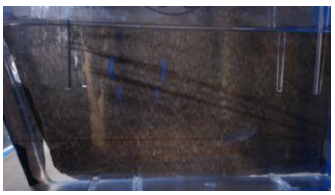
- Objective:
  - Can perioperative immersion analgesia protocols be beneficial to ZF welfare?

Paul Schroeder & Lynne Sneddon (2017): Exploring the efficacy of immersion analgesics in zebrafish – an integrated approach. Applied Animal Behaviour Science

205

Tail-wafting

- Duration (s) of high frequency tail-beat movements that do not lead to propulsion in the water
- First seen in study on ZF post-nociceptive behaviour, after acid injection near tail fin ("tail beating") Maximino 2011



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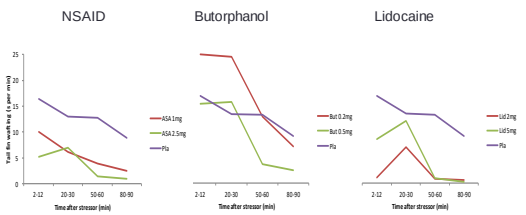
Tail-wafting



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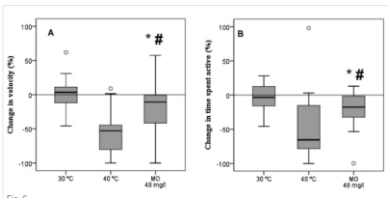
See also

- Lopez-Luna et al. (2017)
- Analgesic protocol for 5dpf ZF (morphine, lidocaine)



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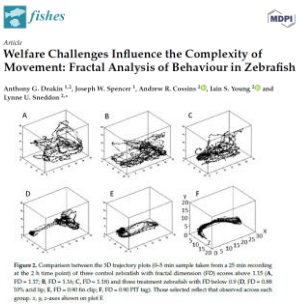
And



fishes

MDPI

Article

Welfare Challenges Influence the Complexity of Movement: Fractal Analysis of Behaviour in Zebrafish

Anthony G. Deakin [1,2], Joseph W. Spencer [1], Andrew R. Cossins [2], Iain S. Young [3] and Lynne U. Sneddon [2,*]

Figure 2. Comparison between the 3D trajectory plots (5–5 min sample taken from a 25 min recording at the 2 h time point) of three control zebrafish with fractal dimension (FD) scores above 1.55 (A, FD = 1.57; B, FD = 1.56; C, FD = 1.58) and three treatment zebrafish with FD below 0.9 (D, FD = 0.88 10% acid tip; E, FD = 0.90 flu clip; F, FD = 0.90 PIT tag). These selected reflect that observed across each group. x, y, z-axes shown on plot F.

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"The only animal species which we can justifiably use for invasive surgical research procedures is the dog"

Prof Eddie Clutton (2013)

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Break out session 2

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Questions

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