

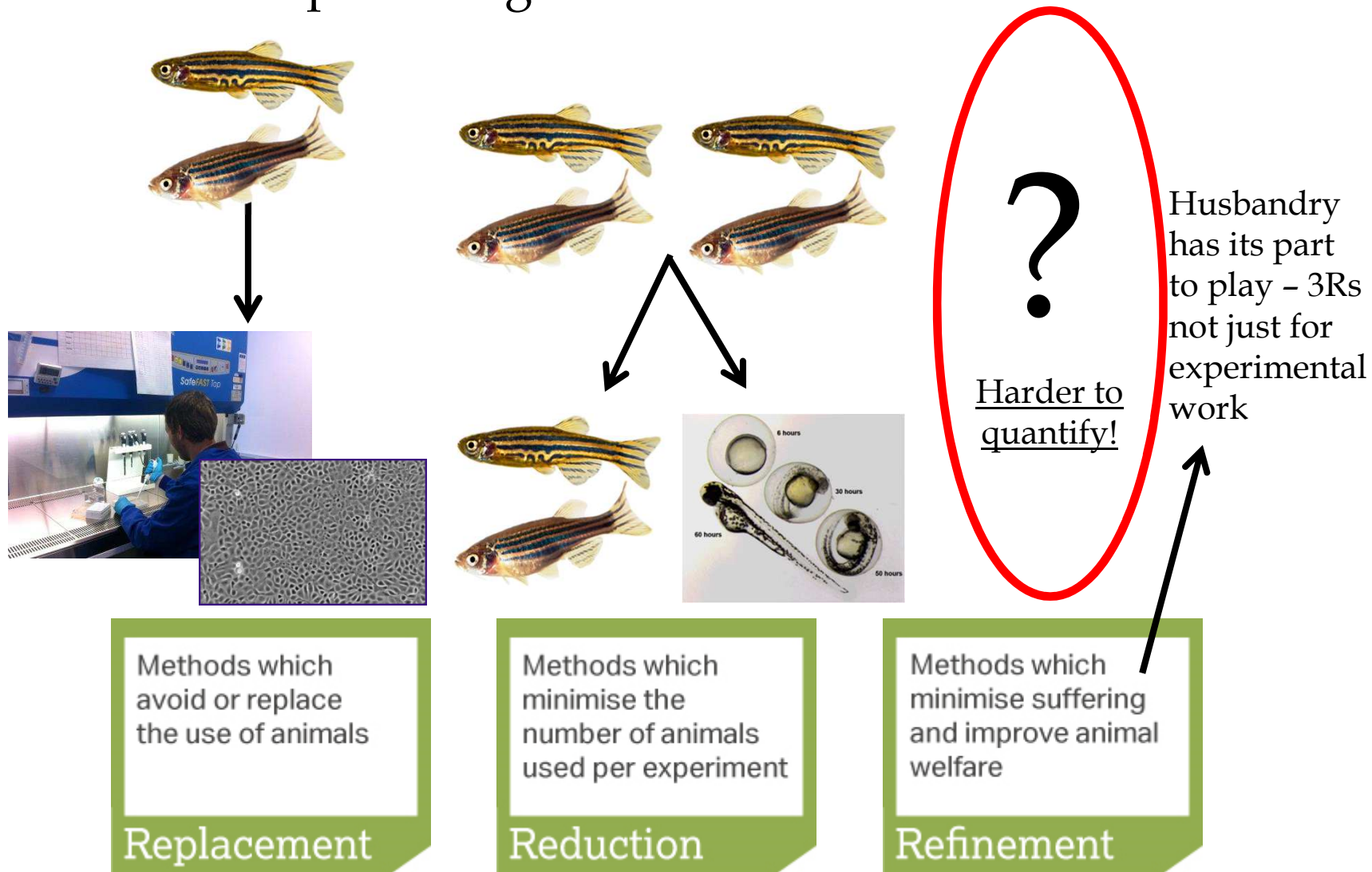
Zebrafish Refinement



Dr Gregory Paull

7th Annual International Zebrafish Husbandry
Meeting

The 3Rs – providing more humane animal research



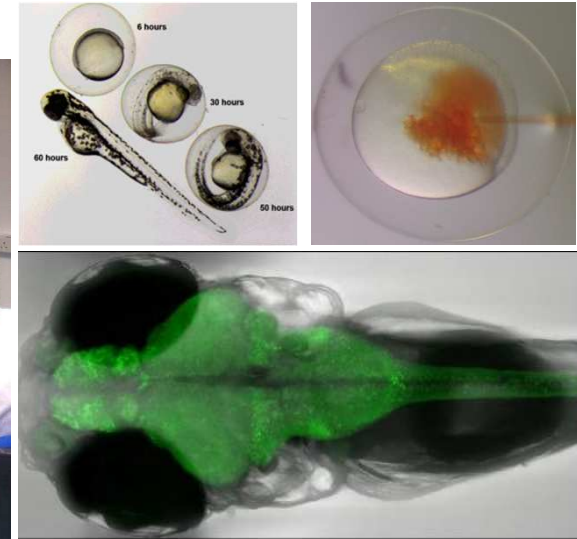
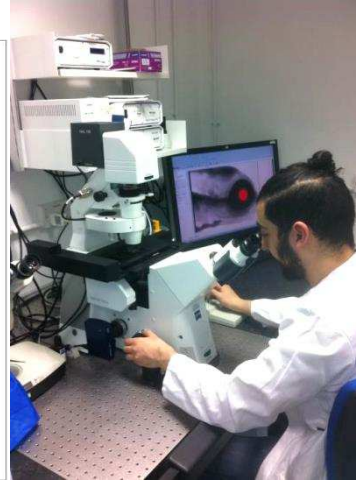
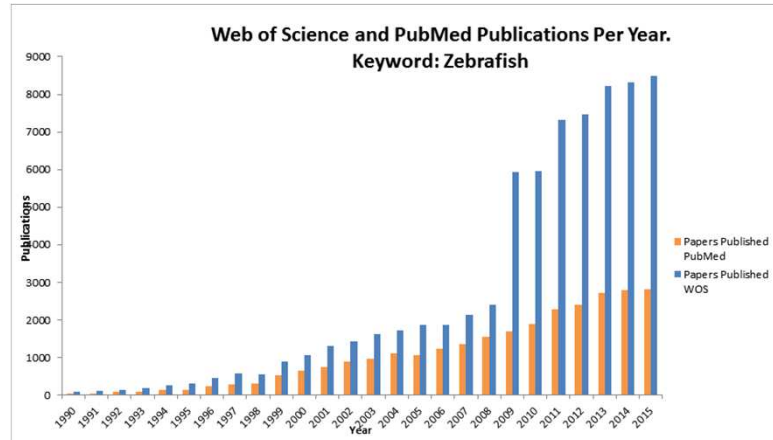
Talk Outline:



- Rapid expansion of the zebrafish ‘model’
- Enrichment – too much focus?
- What do the demands of modern facilities and today’s researchers mean for fish welfare?
- Identifying quick wins for improving the welfare of our animals!

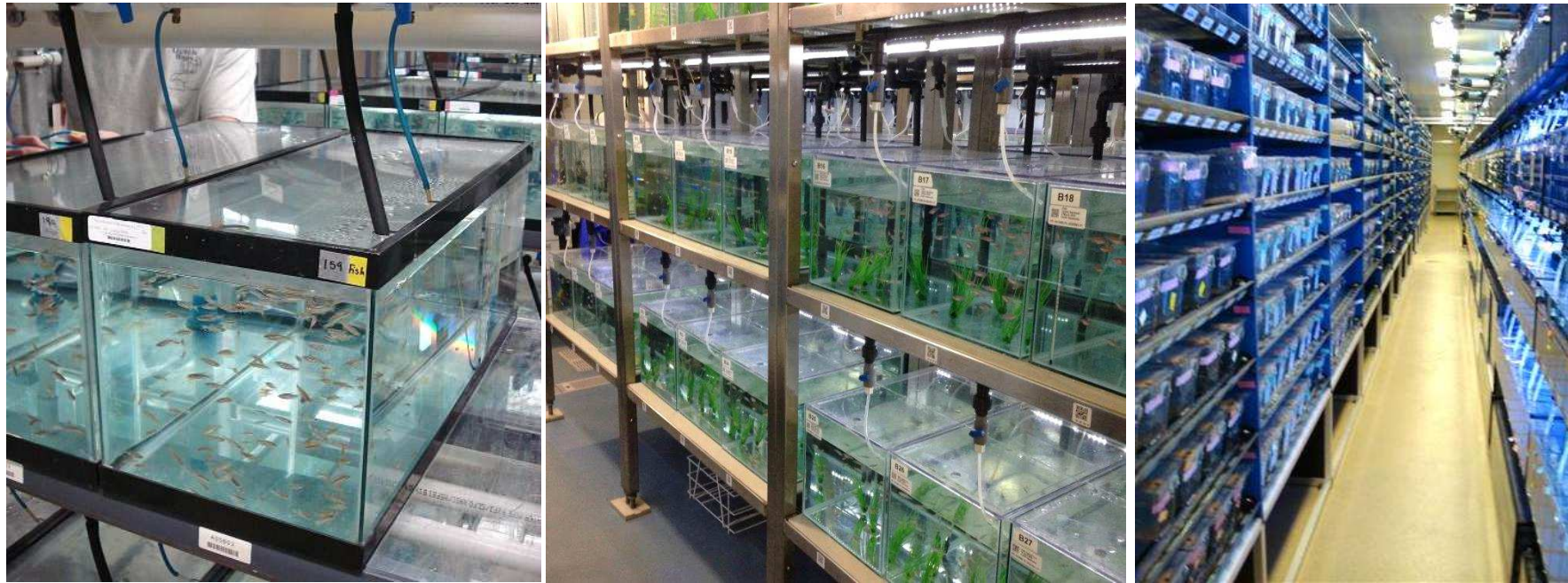
Rapid Expansion of the Zebrafish 'Model'

Rapid Expansion of the Zebrafish 'Model'



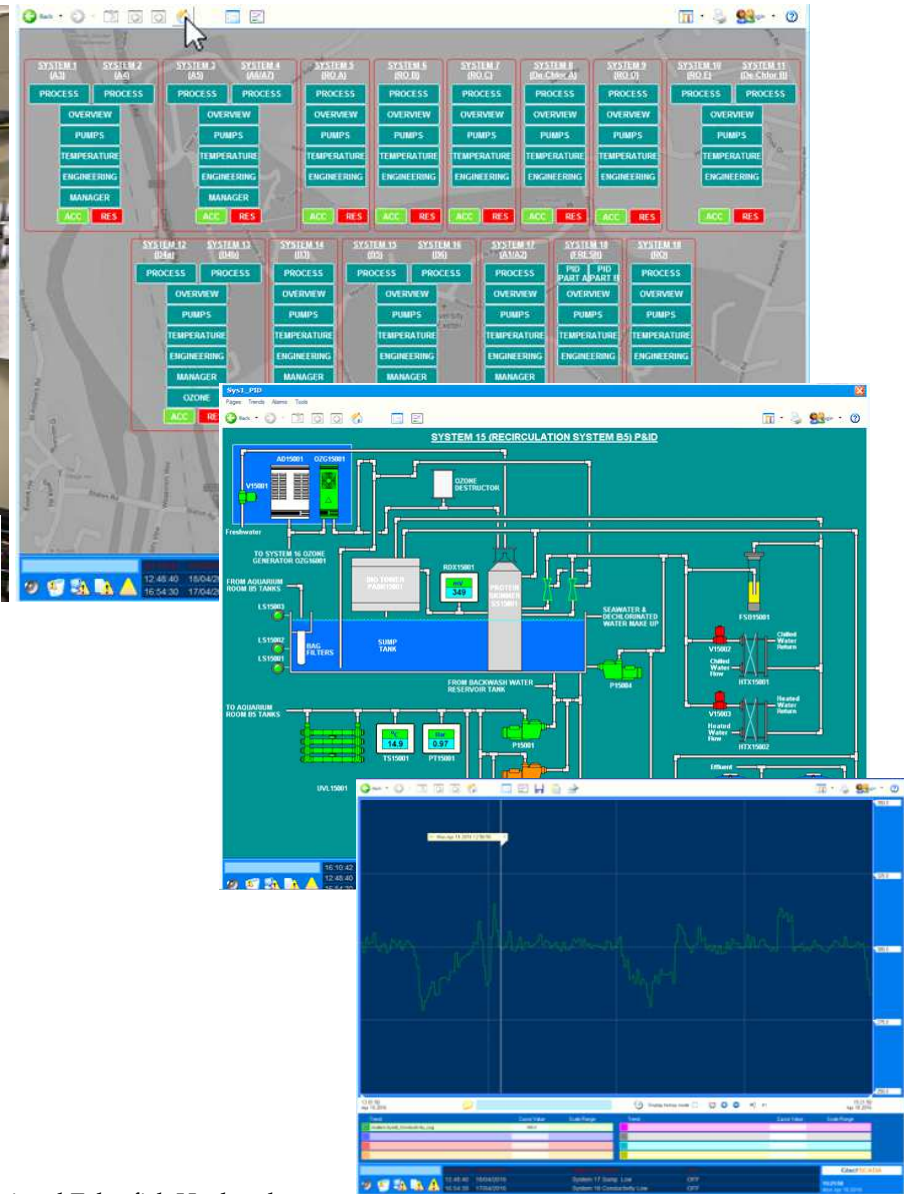
- 1981, Streisinger published his pioneering work on mutagenesis
- 1993, 'The Big Screen'. 4000 mutant phenotypes characterised. 37 papers in a special issue of *Development*, confirming the zebrafish as the foremost research model for development biology
- 2001, Wellcome Trust Sanger Institute began sequencing the entire zebrafish genome. 71% of human genes have a zebrafish orthologue and 82% of genes linked with human disease have a zebrafish equivalent. Genetic similarities made the zebrafish a valuable model for studying human development and disease and for discovering and screening new drugs
- 2013, implementation of advanced gene editing techniques such as CRISPR allowing us to better understand the function of affected genes and model human disease

Rapid Expansion of the Zebrafish 'Model'



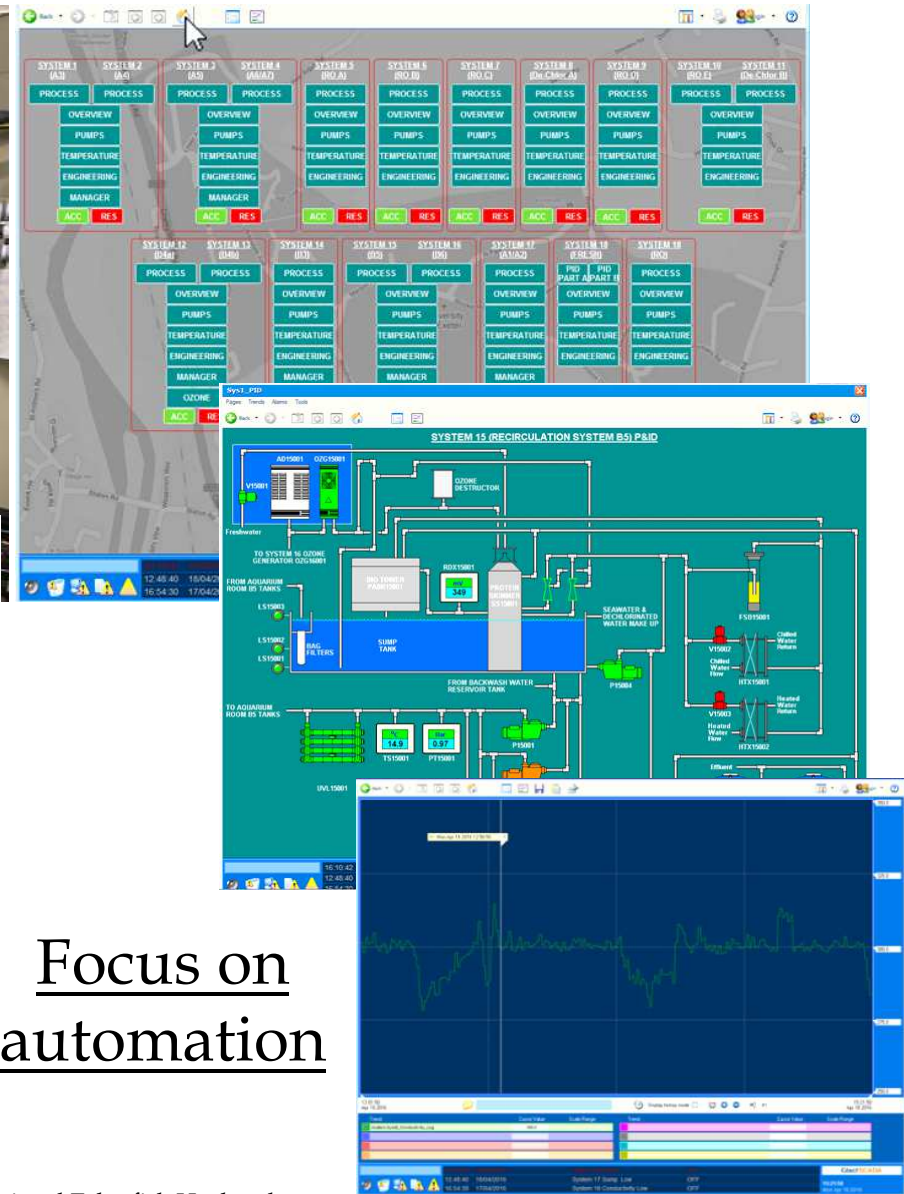
- Changing the way our fish facilities look
- Large increase in tank numbers to cope with the number of zebrafish lines but without the concurrent increase in facility size
- Tank size has decreased and fish density has increased
- Behavioural/interactive restriction: Provision of sufficient space - freedom of movement

Rapid Expansion of the Zebrafish 'Model'



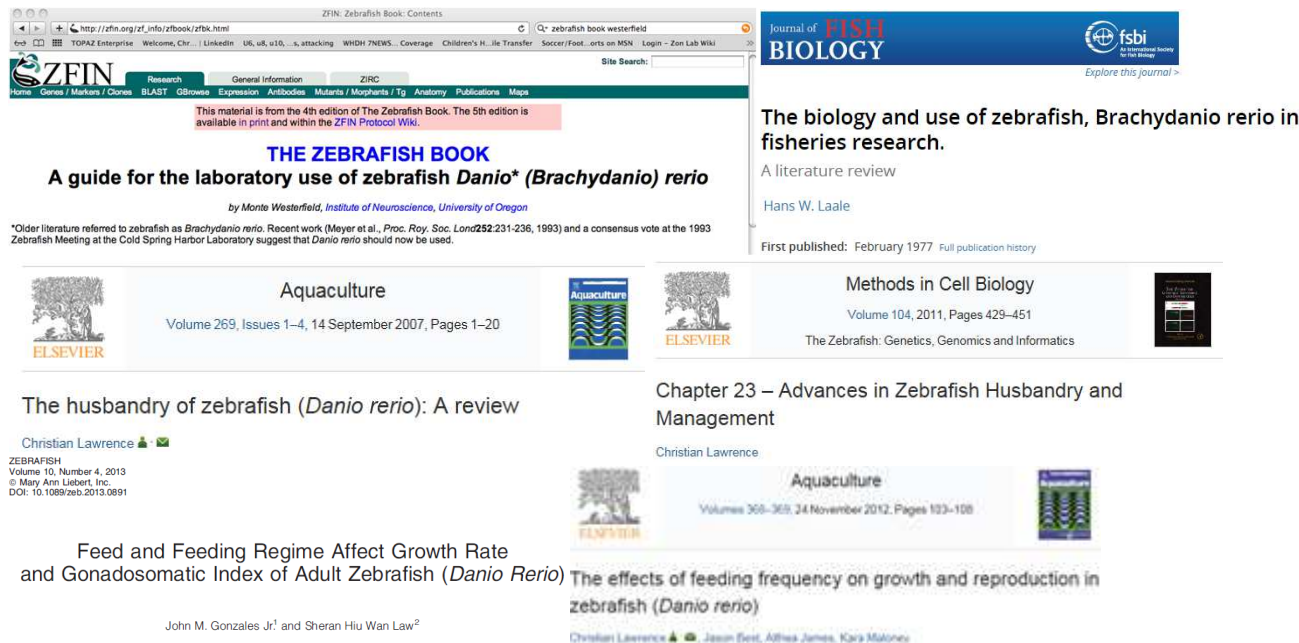
7th Annual International Zebrafish Husbandry Meeting

Rapid Expansion of the Zebrafish 'Model'



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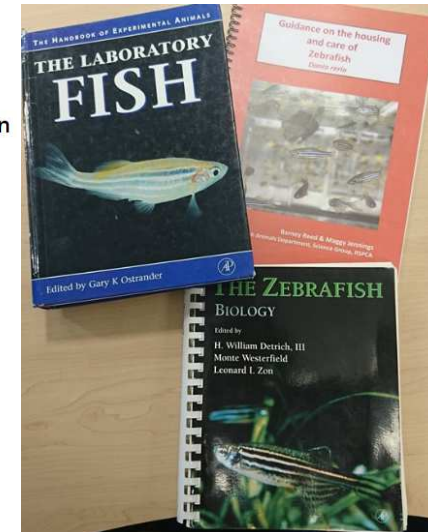
Rapid Expansion of the Zebrafish 'Model'



THE ZEBRAFISH BOOK
A guide for the laboratory use of zebrafish *Danio** (*Brachydanio*) *rerio*
by Monte Westerfield, *Institute of Neuroscience, University of Oregon*

*Older literature referred to zebrafish as *Brachydanio rerio*. Recent work (Meyer et al., *Proc. Roy. Soc. Lond.* 252:231-236, 1993) and a consensus vote at the 1993 Zebrafish Meeting at the Cold Spring Harbor Laboratory suggest that *Danio rerio* should now be used.

Aquaculture	Methods in Cell Biology
Volume 269, Issues 1-4, 14 September 2007, Pages 1-20	Volume 104, 2011, Pages 429-451
The husbandry of zebrafish (<i>Danio rerio</i>): A review Christian Lawrence	Chapter 23 – Advances in Zebrafish Husbandry and Management Christian Lawrence
Feed and Feeding Regime Affect Growth Rate and Gonadosomatic Index of Adult Zebrafish (<i>Danio Rerio</i>) John M. Gonzales Jr. ¹ and Sheran Hiu Wan Law ²	The effects of feeding frequency on growth and reproduction in zebrafish (<i>Danio rerio</i>) Christian Lawrence, Jason Best, Alfie James, Kara Maloney



Rapid growth and out-crossing promote female development in zebrafish (*Danio rerio*)

Christian Lawrence · John P. Ebersole · Richard V. Kesseli

Growth and Survival of Zebrafish (*Danio rerio*) Fed Different Commercial and Laboratory Diets

Anthony J. Siccardi III,¹ Heath W. Garriss,¹ Warren T. Jones,¹ Dorothy B. Moseley,¹ Louis R. D'Abramo,² and Stephen A. Watts¹

A New System for the Rapid Collection of Large Numbers of Developmentally Staged Zebrafish Embryos

Isaac Adatto^{1,2}, Christian Lawrence⁵, Michael Thompson⁶, Leonard I. Zon^{1,2,3,4,*}

The Effect of Stocking Densities on Reproductive Performance in Laboratory Zebrafish (*Danio rerio*)

Daniel Castranova,¹ Angela Lawton,^{2,a} Christian Lawrence,^{3,a} Diana P. Baumann,^{4,b} Jason Best,^{3,b} Jordi Coscolla,^{5,b} Amy Doherty,^{6,b} Juan Ramos,^{5,b} Jenna Hakkestee,^{7,b} Chongmin Wang,^{1,b} Carole Wilson,^{7,b} James Malley,⁸ and Brant M. Weinstein¹

[Zebrafish](#), 2007 Spring;4(1):21-40.

Zebrafish in the wild: a review of natural history and new notes from the field.

Enqeszer RE¹, Patterson LB, Rao AA, Parichy DM.

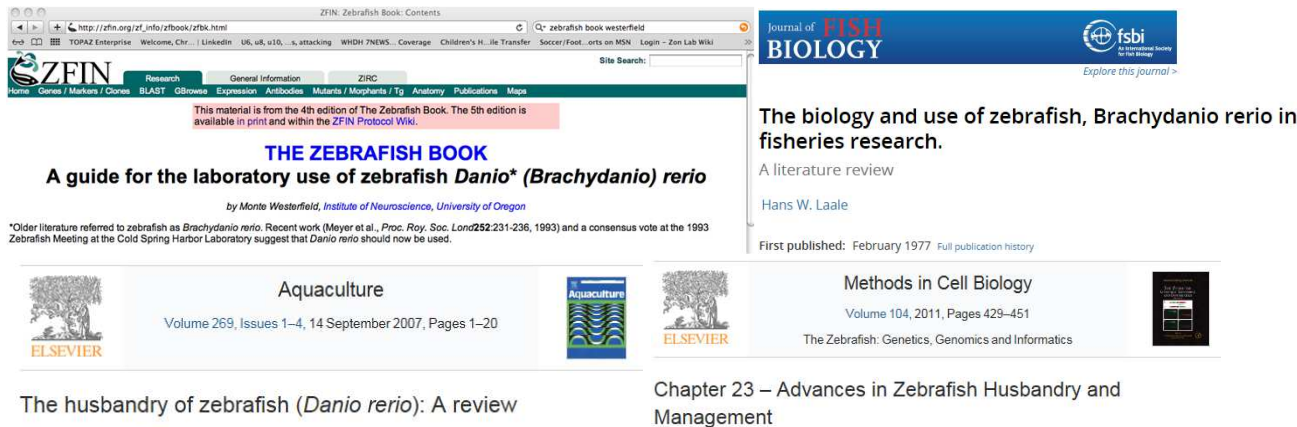
[Elife](#), 2015 Mar 25;4. doi: 10.7554/eLife.05635.

The behaviour and ecology of the zebrafish, *Danio rerio*

Rowena Spence^{1*}, Gabriele Gerlach², Christian Lawrence³ and Carl Smith¹

Advancing biology through a deeper understanding of zebrafish ecology and evolution.
Parichy DM¹.

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This material is from the 4th edition of The Zebrafish Book. The 5th edition is available in print and within the ZFIN Protocol Wiki.

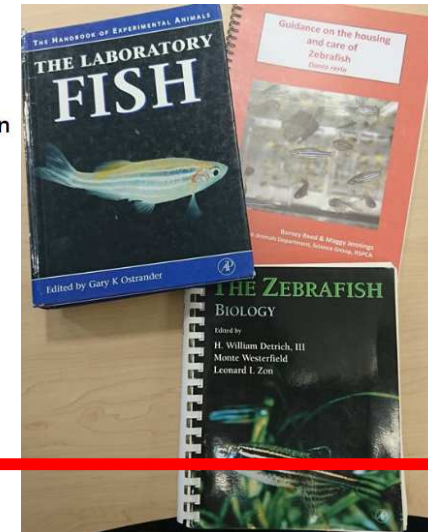
Journal of FISH BIOLOGY
The biology and use of zebrafish, *Brachydanio rerio* in fisheries research.
A literature review
Hans W. Laale
First published: February 1977 Full publication history

Aquaculture
Volume 269, Issues 1–4, 14 September 2007, Pages 1–20

Methods in Cell Biology
Volume 104, 2011, Pages 429–451
The Zebrafish: Genetics, Genomics and Informatics

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Focus on rapid growth and higher fecundity – research driven

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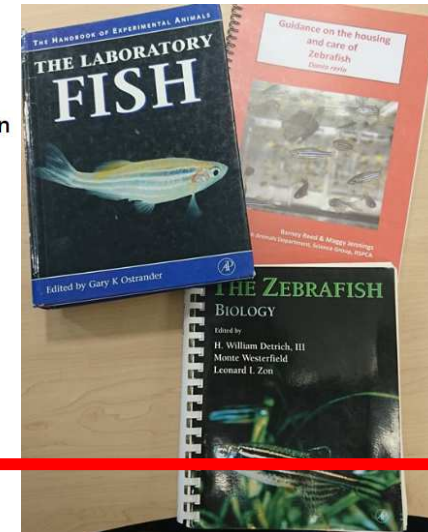
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Chapter 23 – Advances in Zebrafish Husbandry and Management

Aquaculture
Volumes 368–369, 24 November 2012, Pages 103–108

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Focus back on the life-experience of the fish in captivity

Enrichment – too much focus?



Enrichment – too much focus?

MIXED VIEWS

- Problem of how we measure the impact of enrichment on fish welfare?
- Practicality of employing enrichment
- Unknown impact on research?



Enrichment – too much focus?

Table 1
Summary of reports investigating effects of environmental enrichment on zebrafish

Study	Enrichment	Rearing environment prior to study	Social context		Age of fish at start of study	Measures	Results
			Before study	During study			
Schroeder et al. (2014)	Gravel, sand, image of gravel, image of sand, artificial plants (floating and submerged), air stone	Barren tanks	Mixed-sex groups of 10	Pairs and groups of 8	9 months	Preference for enrichments and combinations of enrichments	Pairs preferred substrate over barren tanks, no preference for submerged plants versus barren area Groups preferred substrates and plants over barren areas, strongest preference for gravel and images of gravel, males preferred floating plants to submerged plants, air stones not preferred
von Krogh et al. (2010)	Stones (4-9 mm) as substrate, plastic plants	Barren tank	Mixed-sex group of 100	Single males	Adult	Latency to feed, locomotor activity, cortisol levels, neurogenesis	Compared to control group, fish housed in enriched tanks showed no difference in latency to feed, lower locomotor activity, higher cortisol level, increased telencephalic proliferation
Langen, E.M.A. (2012)	Gravel, small rocks, real and plastic plants, piece of flowerpot	Similar to study environment	Mixed-sex groups	Groups of 20 females	Shoaling: 4 months; novel object: 9 months	Shoaling preferences response to novel object	Rearing environment did not affect shoaling preferences or response to a novel object
Basquill & Grant (1998)	Black plastic strips (25 x 2 cm) to simulate vegetation	Barren tanks	Groups of 25	Groups of 3 (1 large, 1 medium and 1 small)	Juvenile	Levels of aggression, monopolisation of resources	Levels of aggression and food monopolization by dominant fish were lower in the complex habitat than in the simple one
Maximino et al. (2010)	Hiding places, rocks, vegetation and natural substrate	Same as study environment	Not reported	Not reported	Not reported	Exploratory behaviour in dark/light preference test	Fish reared in enriched tanks were more exploratory and showed decreased anxiety-like behaviour (BCF transient swimming or freezing) compared to fish reared in plain tanks
Carfagnini et al. (2009)	Artificial plants anchored with aquarium gravel	Barren tanks	Single-sex groups of 4 females, 20 males	During mating sessions, groups of 2 f and 2 m were placed in breeding tanks; returned to single-sex	8 months	Levels of aggression and fecundity	Reduced aggression in females in complex aquaria; in complex aquaria, no relationship between aggression and fecundity; in bare tanks, females with highest aggression showed reduced fecundity

Delaney et al. (2002)	Artificial plants	Not reported	Not reported	groups after spawning Group of 4 males and 4 females	10 months	Choice of planted or non-planted areas of aquarium	Fish spent 99% of their time in areas containing plants
Hamilton & Dill (2002)	'Vegetation': black plastic strips (10 x 1 cm) 'Cover': overhead mesh cover	Bare tank	Group of 60	Groups of 3	Not reported	Choice of habitats, levels of aggression, monopolisation of resources	Covered habitats used more than bare ones; no difference in use of vegetated and bare habitats; levels of aggression did not differ between habitats; monopolisation of resources was greater in bare habitats than in covered, but not vegetated, habitats.
Kistler et al. (2011)	Sand substrate, plants, clay pots	Bare tanks	Not reported	Groups of 6–9, gender undefined	Not reported	Choice of structured or bare habitat; behaviour (exploration, foraging, social behaviour)	Structured habitat preferred over bare habitat. No difference in behavioural diversity between habitats
Spence et al. (2007)	Gravel, silt, gravel and plants, silt and plants	Not reported	Not reported	Group of 4 males and 4 females	Not reported	Choice of spawning sites, egg survival	Gravel substrate preferred by both males and females and vegetation preferred over non-vegetation; egg survival greatest in gravel
Spence et al. (2011)	Artificial plants	Same as study environment	Groups of 12	Groups of 3 familiar individuals	6 months	Adult learning rate (performance in maze task), body length	Fish raised in bare tank had slower rate of learning than those reared in complex environment; fish reared in complex environment were smaller than those reared in bare tanks
Wilkes et al. (2012)	Black opaque glass rods to simulate vertical stems of plants	Bare tanks	Not reported	Groups of 6	35 dpf	Levels of activity and aggression, shoaling density, whole-body cortisol	Activity and shoaling density showed no response to tank structures, aggression in control tanks declined faster than in structured tanks, cortisol levels did not vary between control and structured tanks
Wafer et al. (2016)	Plastic grass, plastic leaves	Bare tanks	Groups of 30	Pairs	90–180 dpf; 0–6 dpf	Fertility, fecundity, survivorship of larvae	More eggs spawned on 'grass' than on 'leaves'; no effect of enrichment on survivorship of fry to 6 dpf
Bhat et al. (2015)	Plastic plants, water flow	Bare tanks	Groups of 15–20	Groups of 6	Unreported; 2 wild populations, 1 lab reared	Latency to feed, aggression, shoal distance	Vegetation increased aggression; water flow decreased latency to feed

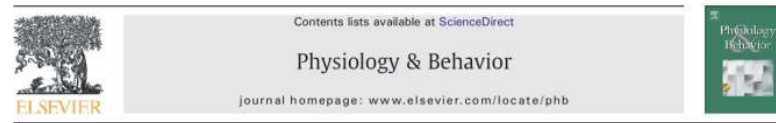
Enrichment – too much focus?

- Concerned with impact on research - ultimately drives change!

- forebrain cell proliferation > in enrichment environments

- measure of neural plasticity

- maintenance of adaptive cognitive and emotional processes in mammal - altered brain cell proliferation in zebrafish should be further explored

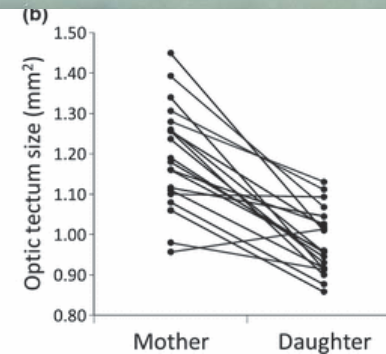
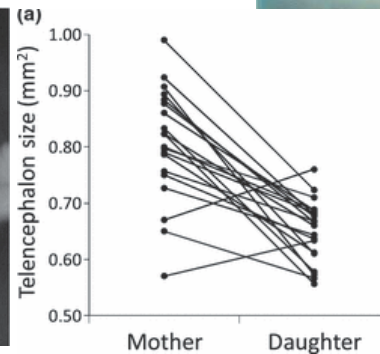
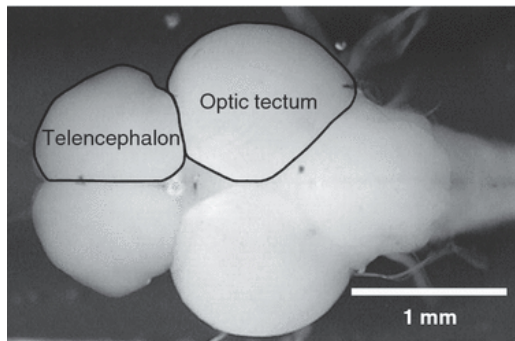


Forebrain cell proliferation, behavior, and physiology of zebrafish, *Danio rerio*, kept in enriched or barren environments

Kristine von Krogh^a, Christina Sørensen^b, Göran E. Nilsson^b, Øyvind Øverli^{c,*}

Rearing Environment Affects the Brain Size of Guppies: Lab-Reared Guppies have Smaller Brains than Wild-Caught Guppies

James G. Burns, Arthy Saravanan & F. Helen Rodd



Telencephalon
serves –
spatial
memory

Optic Tectum
serves – visual
processing

Enrichment – too much focus?

- Habitat complexity affected brain development in salmonids:

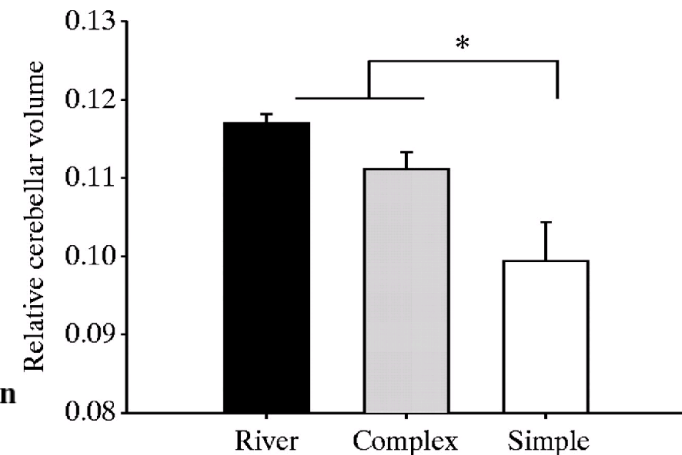
- Cerebellum size: wild>complex>simple
- Cerebellum involved in locomotion
- Bigger cerebellum = better maintenance tank position

The Journal of Experimental Biology 209, 504-509
Published by The Company of Biologists 2006
doi:10.1242/jeb.02019

Early rearing environment impacts cerebellar growth in juvenile salmon

Rebecca L. Kihlslinger* and Gabrielle A. Nevitt

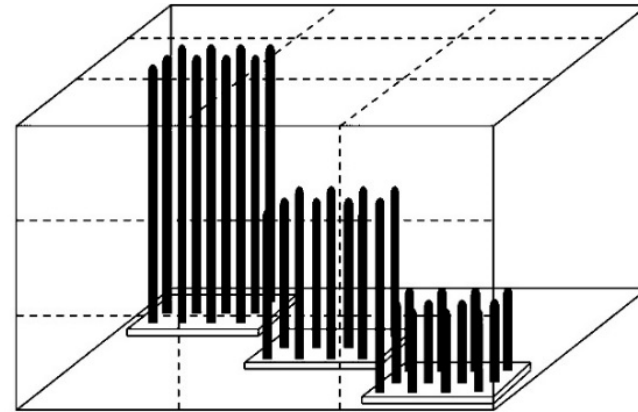
Section Neurobiology, Physiology and Behavior, UC Davis, Davis CA, USA



- Rearing environment can affect cognitive development & subsequent behaviour
- Potential impact on research using fish kept in bare/simple environments!

Enrichment – too much focus?

- This study found no impact but.....



- Problem 1 - Regulatory toxicology strict criteria regarding holding conditions and experimental design
- Problem 2 – What value do glass rods have to a zebrafish?
- No differences found in activity levels, shoaling density and levels of cortisol
- The addition of glass rod structures did not result in improvements in welfare
- Fit for purpose? - relevance, both, scientifically and to the life-history traits of the species in question!

Enrichment – too much focus?

- Literature suggests a welfare benefit of enrichment for other lab animals.

Journal of Alzheimer's Disease 11 (2007) 359–370
IOS Press

Environmental Enrichment Delays the Onset of Memory Deficits and Reduces Neuropathological Hallmarks in a Mouse Model of Alzheimer-Like Neurodegeneration

Nicoletta Berardi^{a,b,c,d}, Chiara Braschi^{a,d}, Simona Capsoni^{c,d}, Antonino Cattaneo^{c,d} and Lamberto Maffei^{a,c}

359

European Journal of Neuroscience, Vol. 20, pp. 1341–1347, 2004

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Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity

N. Benaroya-Milshtein,¹ N. Hollander,² A. Apter,³ T. Kukulansky,² N. Raz,² A. Wilf,¹ I. Yaniv⁴ and C.G. Pick¹

Available online at www.sciencedirect.com

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Applied Animal Behaviour Science 102 (2007) 163–188

APPLIED ANIMAL
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Why and how should we use environmental enrichment to tackle stereotypic behaviour?[☆]

G. Mason^{*}, R. Clubb, N. Latham, S. Vickery

- Environmental enrichment should be regarded as an essential component of an overall animal care program, equally as important as nutrition and veterinary care. It is also important to evaluate the impact of enrichment on scientific outcome. Baumans, V. (2005).
- Understanding the relationship between group size and cognition in animals may yield practical animal management benefits and improved animal welfare. Croney, C.C. and Newberry, R. C. (2007).
- As many behaviour problems are related to the way people house and handle domestic animals, possibly the most important aspect of clinical ethology is its focus on the human-animal interaction. Ladewig, J. (2005).

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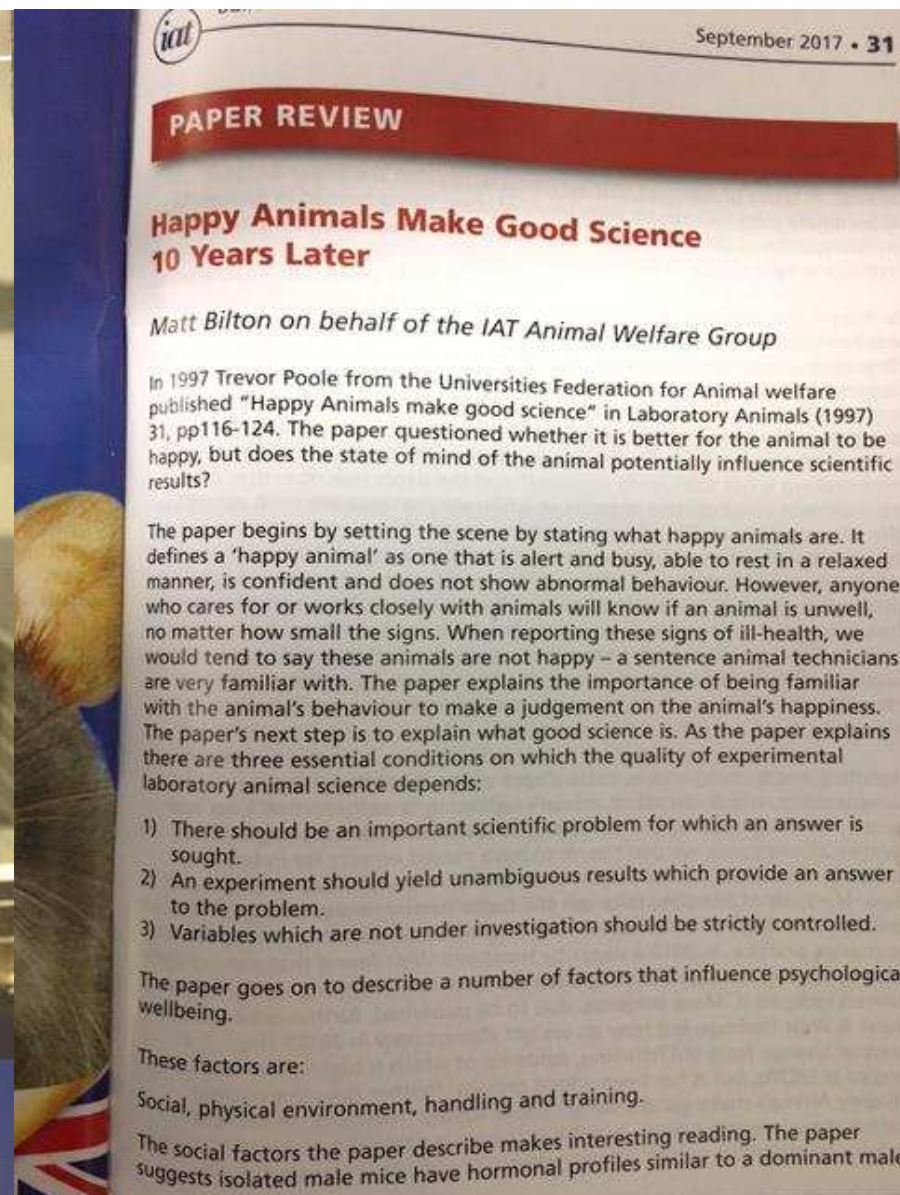
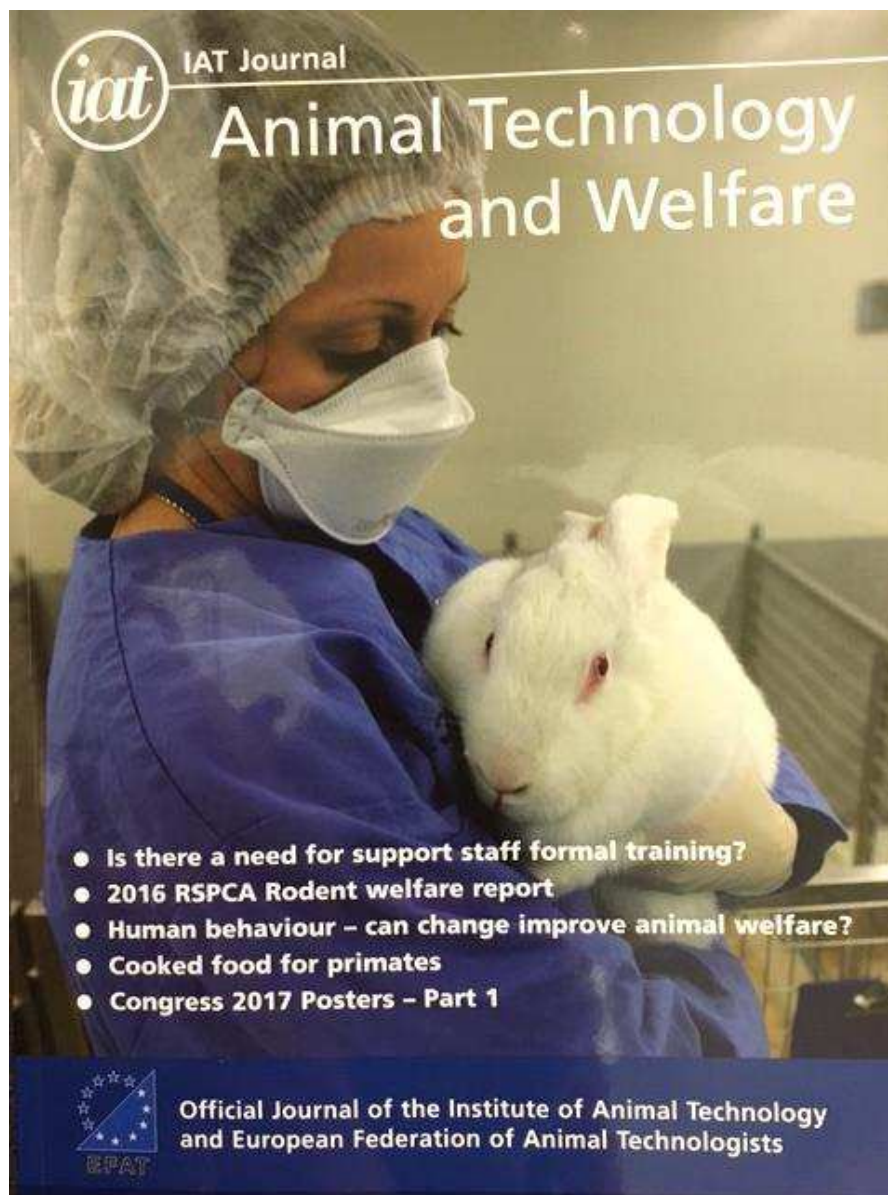
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Our focus?

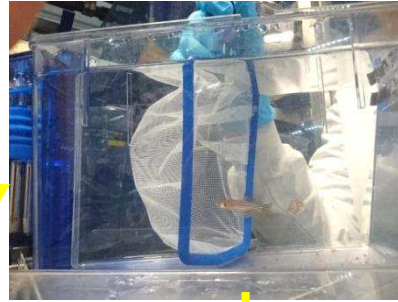


Take a step back and reflect on current practices



What do the demands of
modern facilities and today's
researchers mean for fish
welfare?

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Observation!



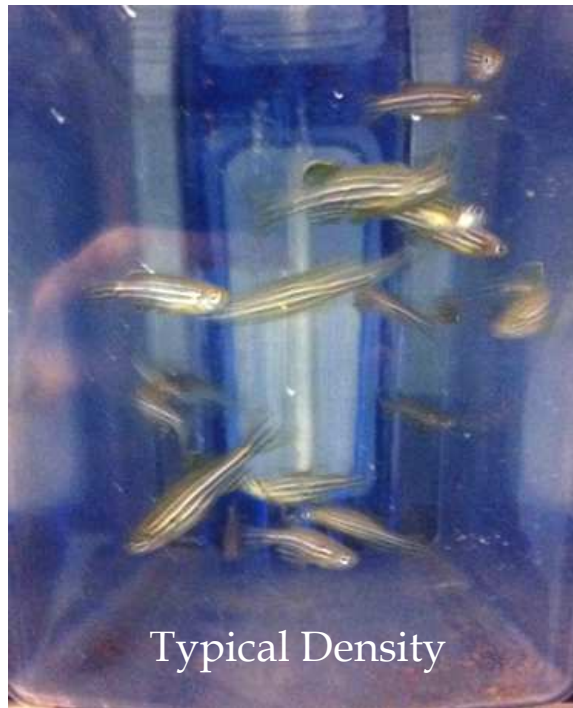
- Understand the life-experience of our fish throughout its time in our facilities
- Fish are routinely moved between differing housing types and social groups
- Understand the impact this has on their welfare
- Observe and collect data and publish it
- Develop new husbandry methods based on new data
- Work with technology companies to drive best practice

We actually know some of this stuff already – published work!

Density Effects

What do the demands of modern facilities and today's researchers mean for fish welfare?

- Take a walk around your facility!
- Recommended stocking densities (5fish/L) but....
- Variation exists between facilities and between housing type
- Continuous movement of animals - inevitable inappropriate groupings are left - welfare issue!



Low Density – elevated aggression: Activation of ‘stress axis’ in brain

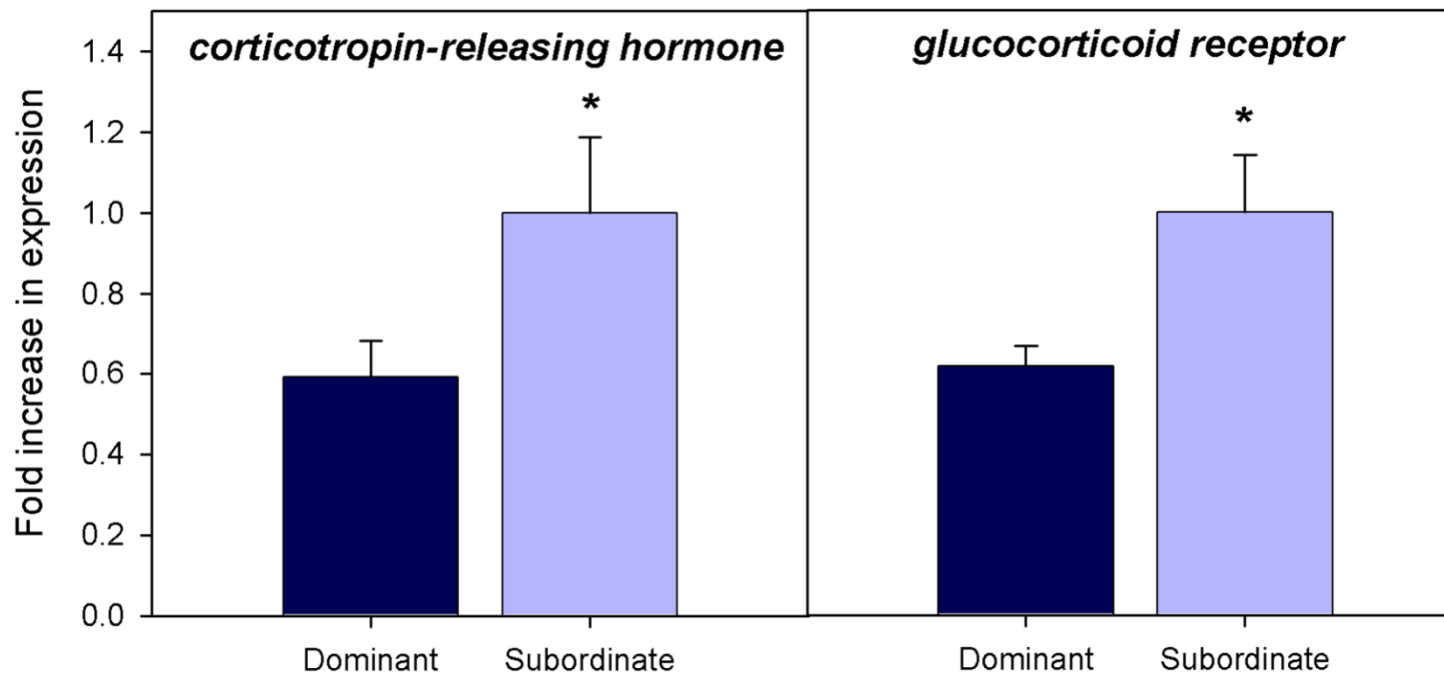
Physiological and health consequences of social status in zebrafish (*Danio rerio*)

Amy L. Filby^{a,*}, Gregory C. Paull^{a,1}, Emily J. Bartlett^a, Katrien J.W. Van Look^{b,2}, Charles R. Tyler^a

^a School of Biosciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter, Devon EX4 4PS, United Kingdom

^b Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, United Kingdom

Density Effects



Rapid - Day 1 – pre-cursor to cortisol production

Low Density – elevated aggression: Activation of ‘stress axis’ in brain

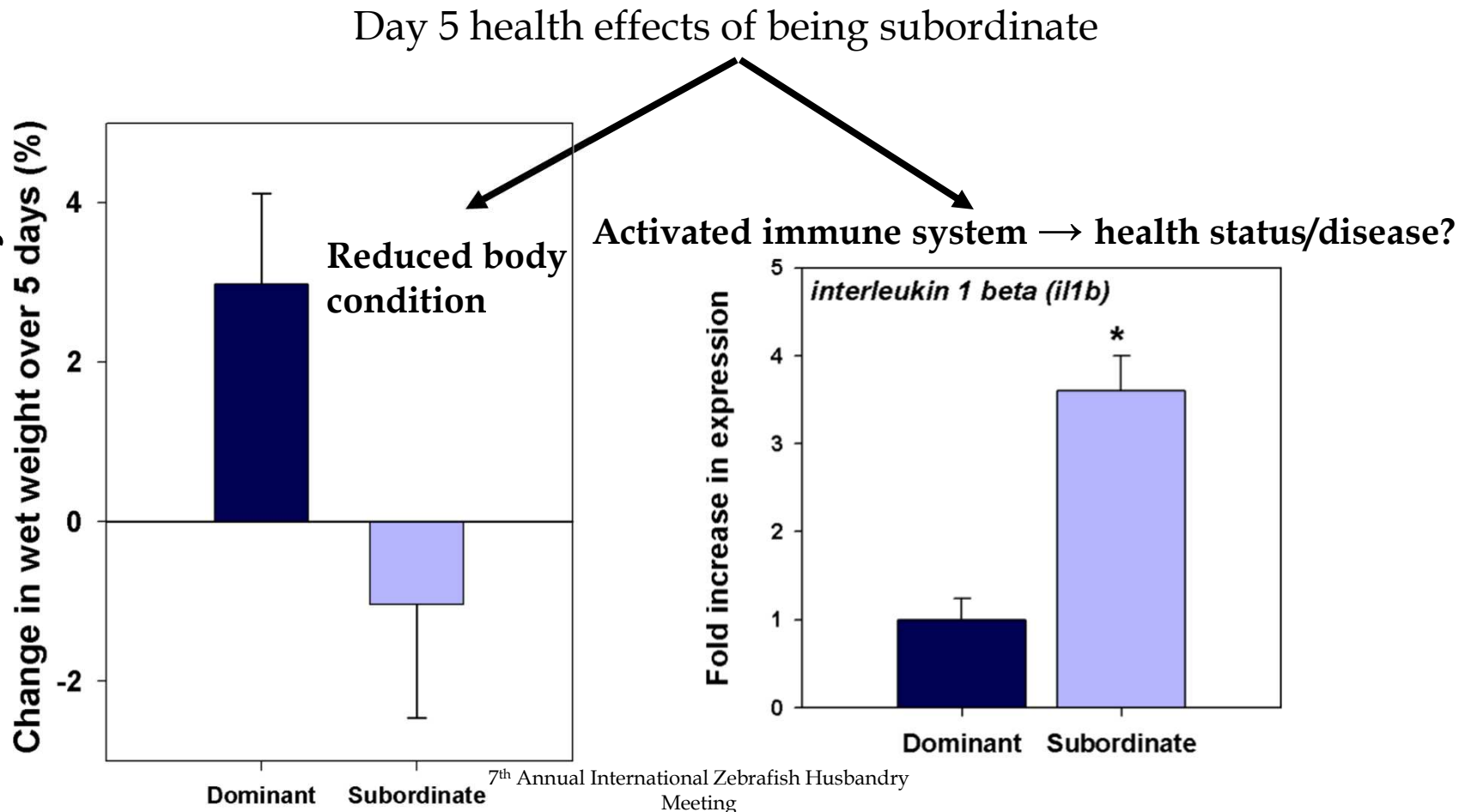
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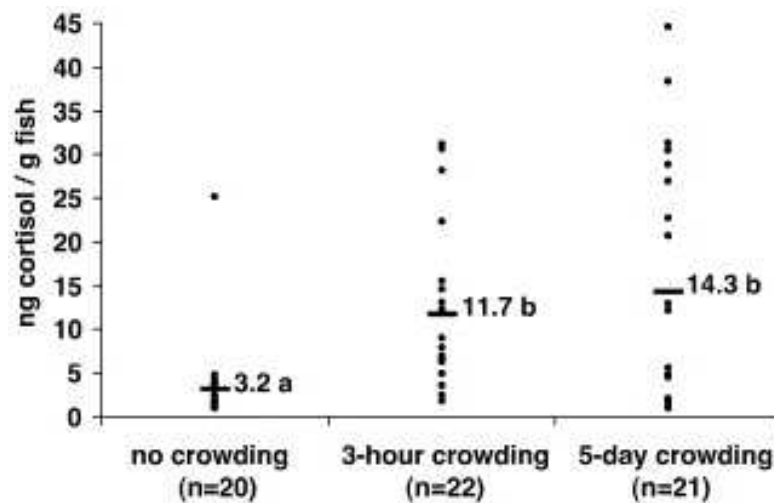
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Density Effects

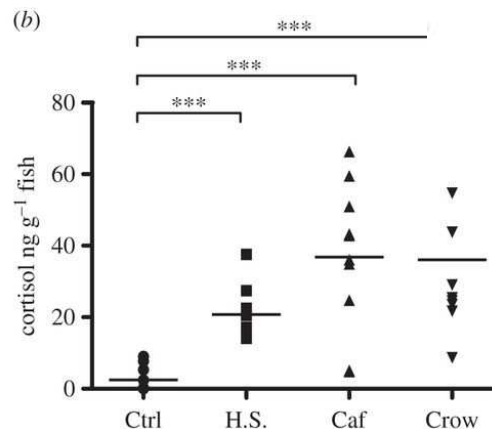
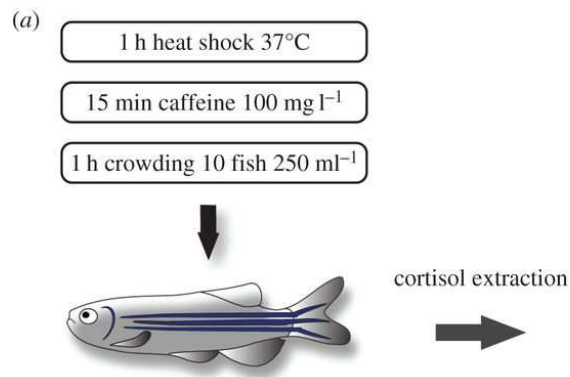


Overcrowding/high density – stressor?

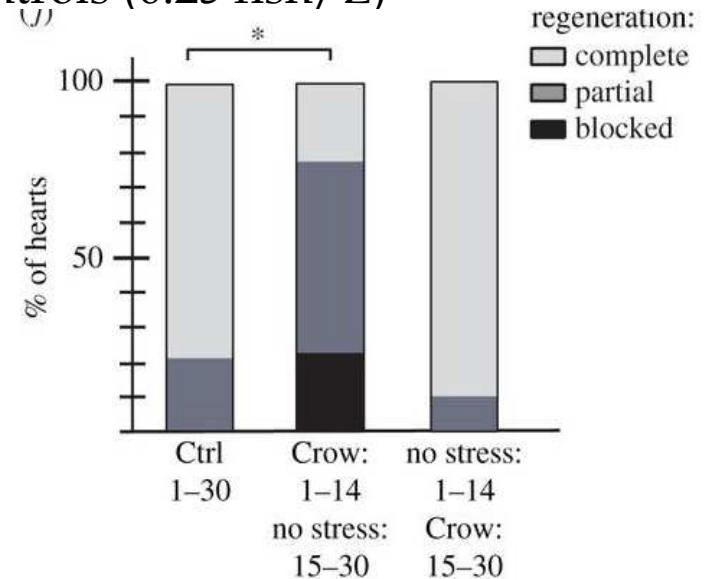
Density Effects



- Study by Sallin and Jazwinka, Acute stress is detrimental to heart regeneration in zebrafish



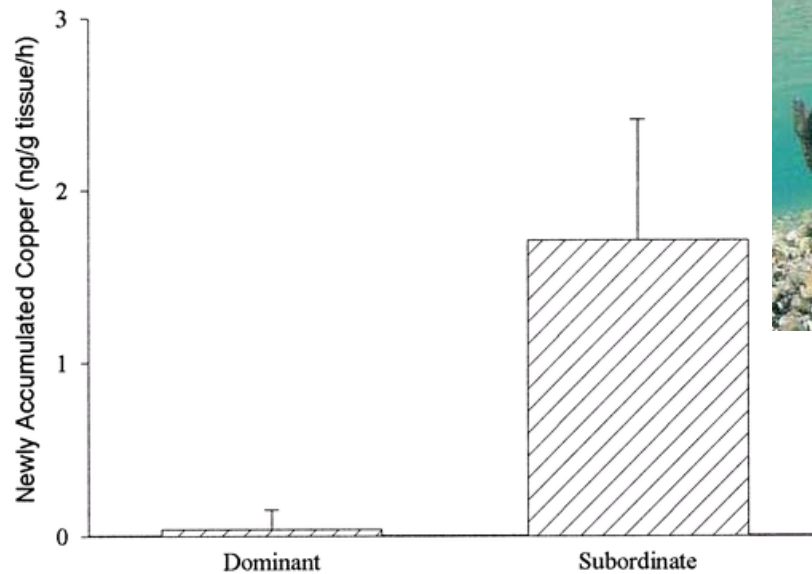
- Study by Ramsey et al, Whole-body cortisol is an indicator of crowding stress in adult zebrafish
- Crowding (40 fish/L) 4 fold increase in cortisol levels vs controls (0.25 fish/L)



- Daily exposure to crowding impaired heart regeneration in 78% of animals

Social interactions can affect physiological consequences of exposure to toxicants

Density Effects



Greater uptake of copper in subordinate vs. dominant fish

Sloman et al. (2002) – ET&C 21:1255-1263

Poor handling techniques: Activation of stress response

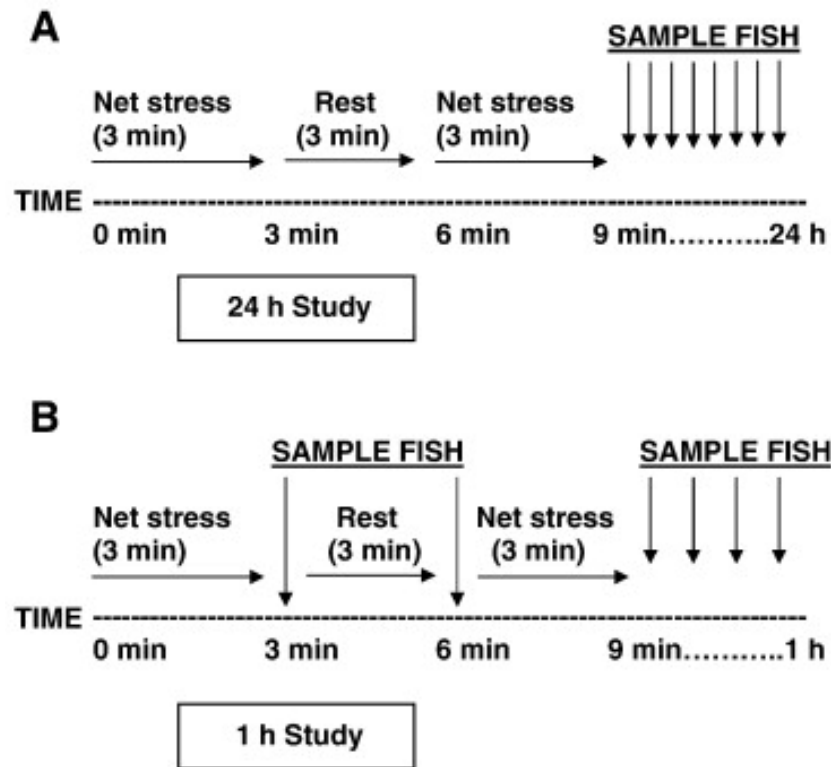
Handling Effects

- Users have widely different abilities/care
- Mitigate variable skill sets through provision of appropriate equipment
- Notably, provide appropriately sized nets and the correct number of nets for the different tanks in your facility
- Provide training!

One net versus two?

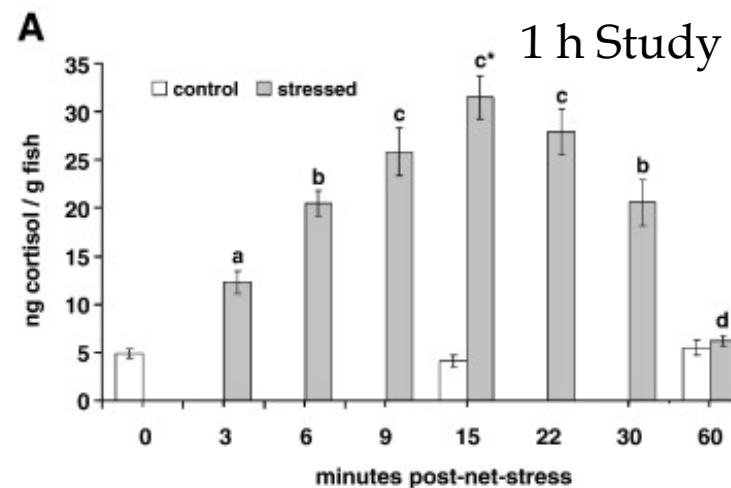
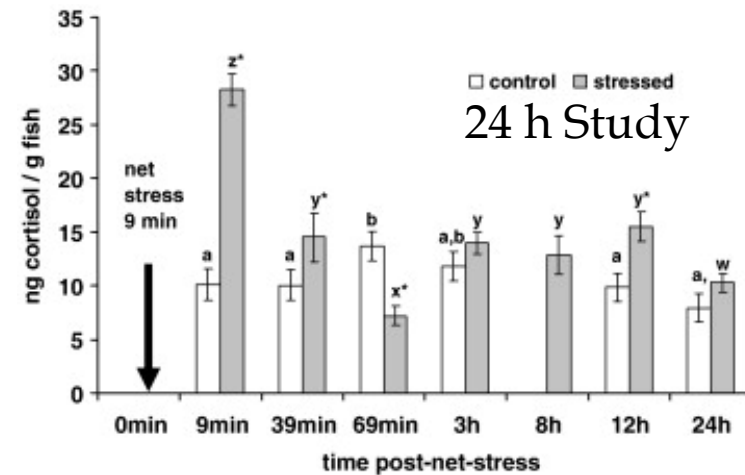


Handling Effects



Fish were netted from their tanks, suspended in the air for 3 min, returned to their tanks to rest for 3 min and subjected to a second 3 min net stressor. Ramsey et al, 2009. Whole-body cortisol

Better understanding how zebrafish respond to handling will allow researchers to create a rearing environment for optimal health and reproduction while minimizing husbandry-associated sources of variation (Kent et al., 2009).



Physiological consequences of housing practices

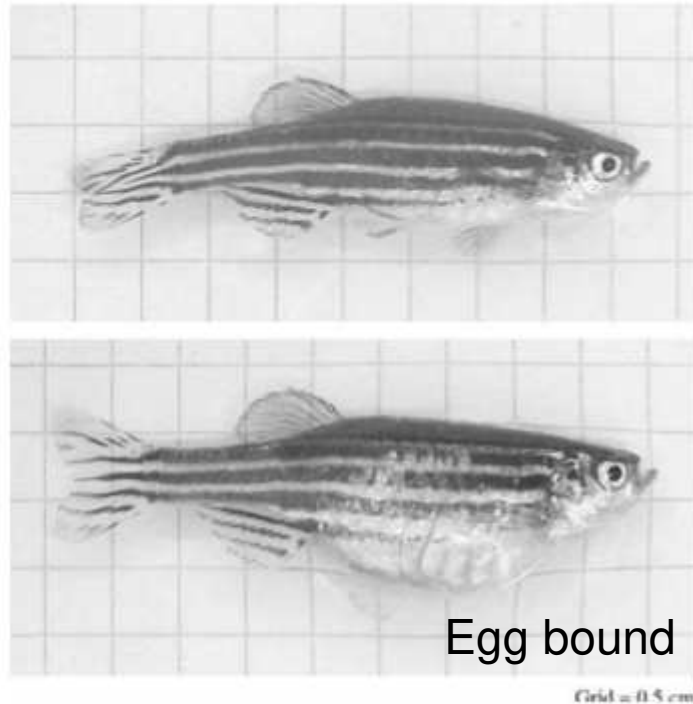


Fig. 2. Female zebrafish before (upper panel) and after being housed alone for three weeks (lower panel). The mean \pm SD belly height of the females increased, by $69 \pm 24\%$ ($N = 10$).

Spence et al., The behaviour and ecology of the zebrafish. 2008

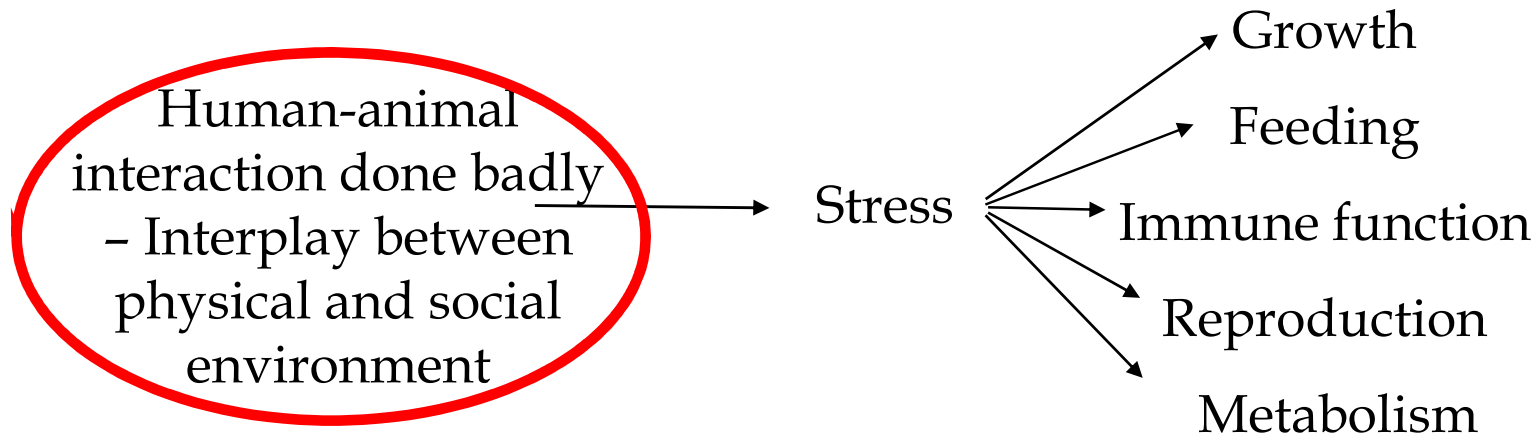
- Atretic oocytes; poor spawners (quality & quantity, frequency of spawning); health consequences
- Completely isolated females
- Isolated by dividers
- Mismatched pairs
- Female biased populations
- Not just a zebrafish problem

Physiological consequences of housing practices

- Stickleback *In Vitro* Fertilisation Protocol
- **Important:**
 - As well as for the purposes of IVE, stripping eggs from females is an important husbandry technique to prevent atresia and damage to the fish.
 - When kept in dense groups, female stickleback will not lay eggs. They must be removed. If developed eggs remain in the ovary for too long, they may become atretic inside the female. Females should be observed regularly to identify when they need to be stripped to prevent ill health. An indicator of this is a bubbly, raspberry-like texture on the abdomen of the female, and when removed the eggs will look yellow and may feel hard and crunchy.
 - If atretic eggs can be removed without excessive force and without causing damage to the female, the fish will usually recover well. However, if atresia is too advanced (usually when eggs have formed a hard mass inside the female) it may be too difficult to remove them without causing damage to the fish, and the fish should be euthanised by a schedule 1 method. Regular observation and stripping of females can prevent this occurring, and this is the responsibility of the user.



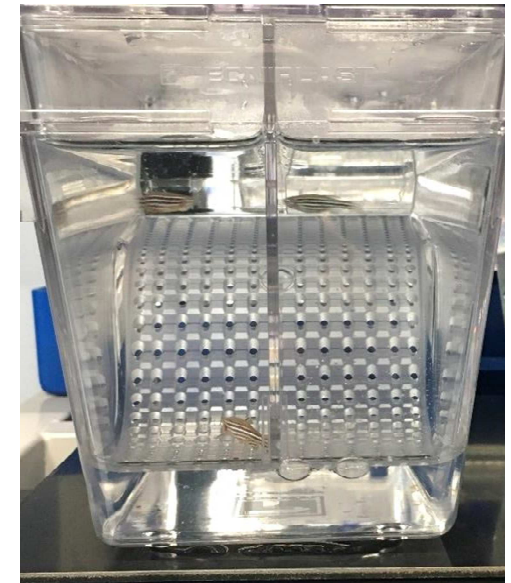
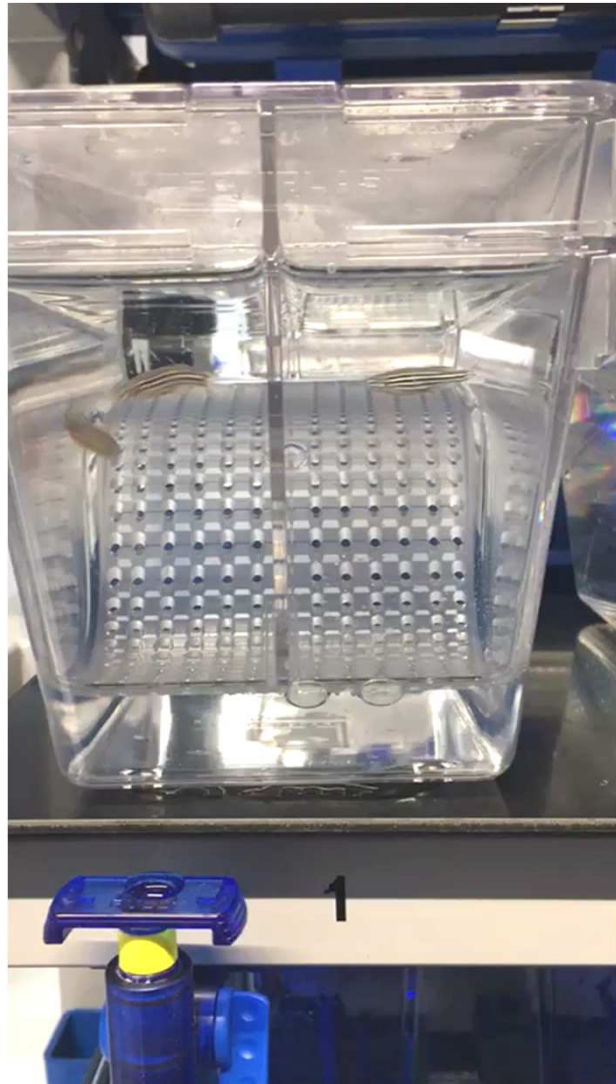
What do the demands of modern facilities and today's researchers mean for fish welfare?



- We know very little about the long-term/cumulative effects of repeated stressors from human-animal interactions & their impact on fish wellbeing

What do the demands of modern facilities and today's researchers mean for fish welfare?

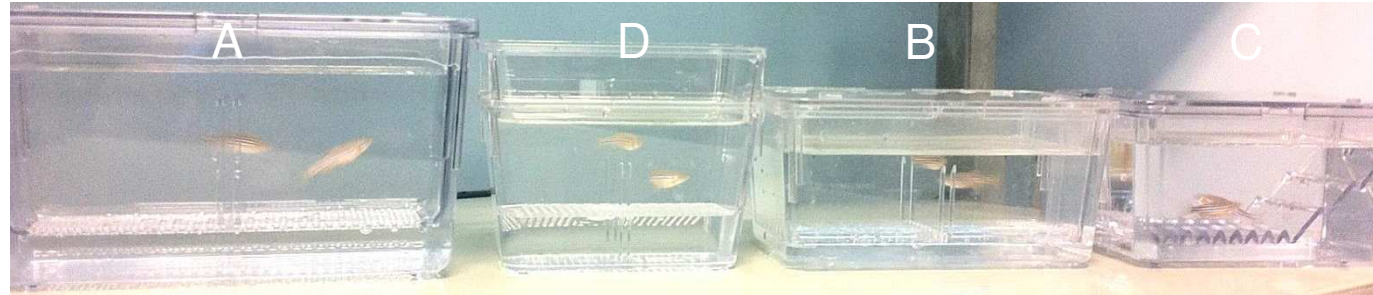
- Limited information on 'best practice' guidelines for pair-wise mating, genotyping, screening
 - 1mx1f; 2mx1f; 1mx2f; small groups of random sexes; size of spawning chamber; duration left for; volume of water; size and age of fish used
 - exposing fish to confinement - stressor!
 - exposing fish to aggression – stressor!
- Often no records kept of how often fish are used/handled



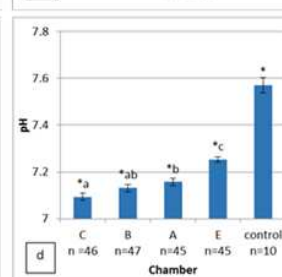
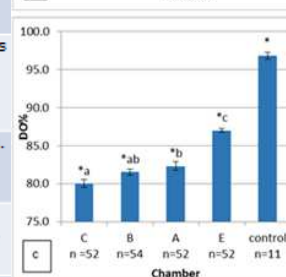
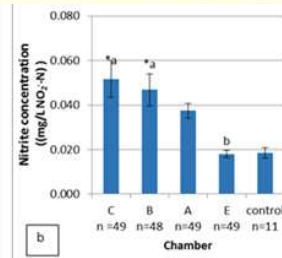
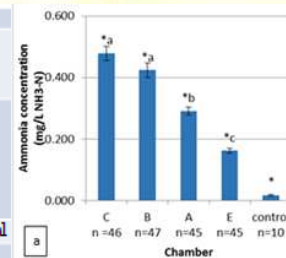
Identifying quick wins for
improving the welfare of our
animals!

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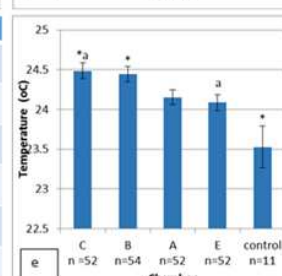
Goolish et al., @ 200ml
& 100ml only 48% &
26% of control egg
production (no effect >
300ml)



Questionnaire criteria	Zebrafish Research Facility		
	?	?	?
Chamber type used (see Table.1 for chamber identification letter)	A	E	A & C
Water volume (ml)	1000ml	2000ml	1000ml and 2000ml
Static or flow-through water	Static	Static	Static
How many chambers (on average) are set up a week?	~140 chambers	100-150 per room per day.	~350-400 chambers
Time left in chamber (chamber duration)	19 hours (15:00-10:00)	Max. 24 hours	Normally 24 hours. Sometimes 48 hours.
Gender ratio f:m	1:1	3:2	1:1 but occasionally 2:1 or 1:2
'Divider' between male and female in chamber?	Yes. Removed 9.30am	Occasionally	Occasionally
How often are the fish used?	Weekly	Once every few days	Max twice weekly
Enrichment added to chamber (e.g. marbles)	Yes. Plastic plant on male side of the chamber	Plastic plant	No
Monitored water parameters in breeding chambers?	No	No. (previous measurements taken)	No (previous measurements taken)

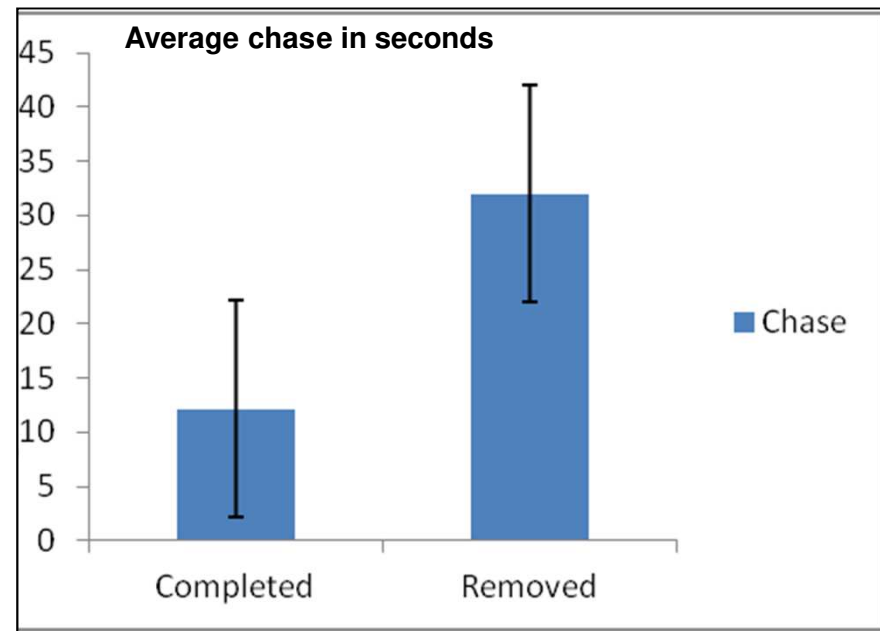
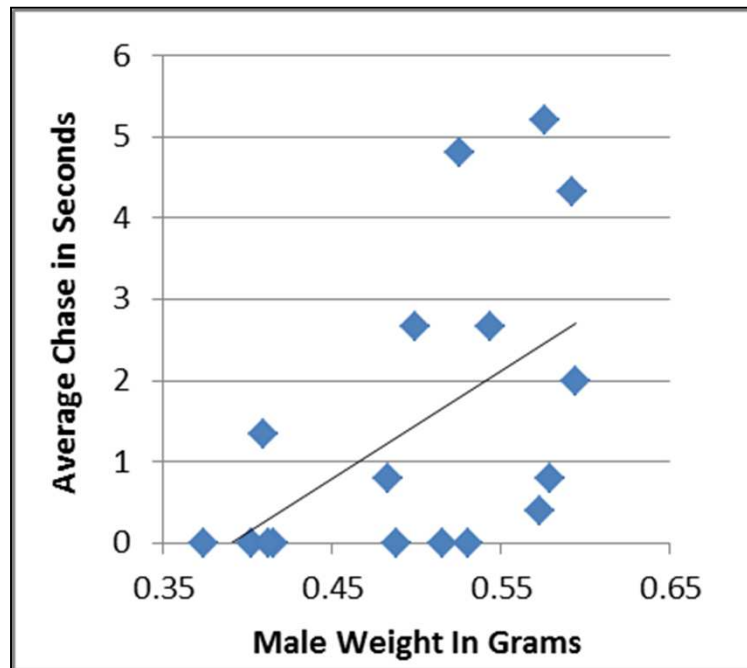


Mean water parameters values					
Chamber	Ammonia (mg/L)	Nitrite (mg/L)	DO (%)	pH	Temp (°C)
A	0.291	0.037	82.3	7.17	24.15
B	0.424	0.047	81.5	7.11	24.44
C	0.478	0.052	80	7.08	24.48
E	0.162	0.016	87	7.26	24.08
Control	0.017	0.010	96.0	7.50	23.53



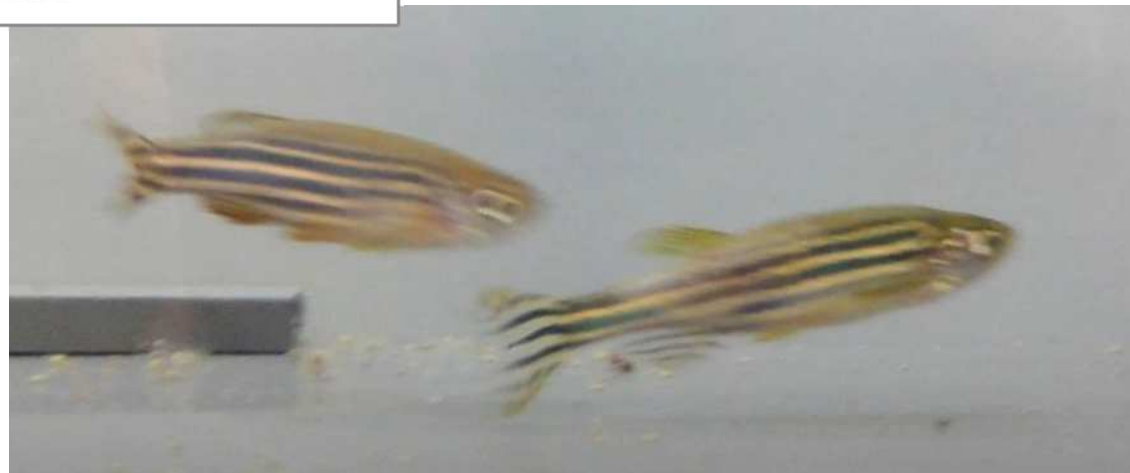
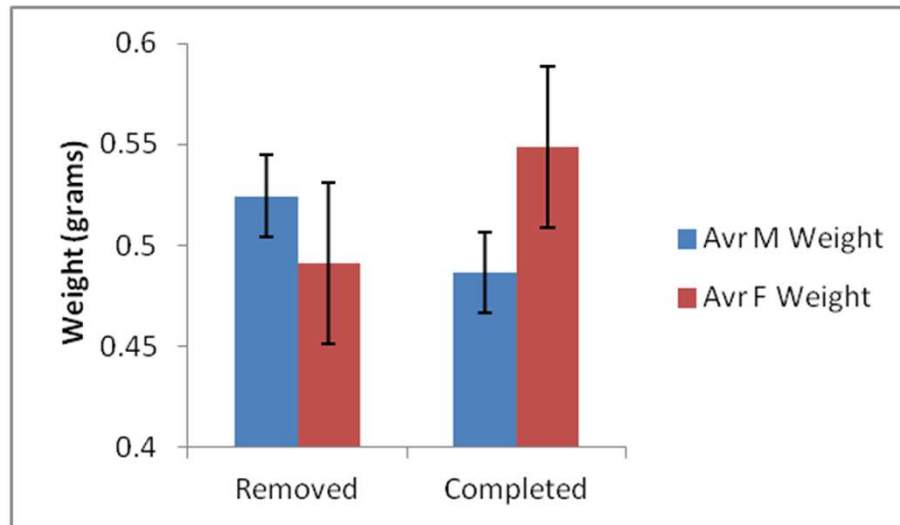
Identifying quick wins for improving the welfare of our animals!

- Pair-wise mating (or any occasion fish housed at low density) can invoke aggressive behaviour in zebrafish, invoked particularly by larger males.



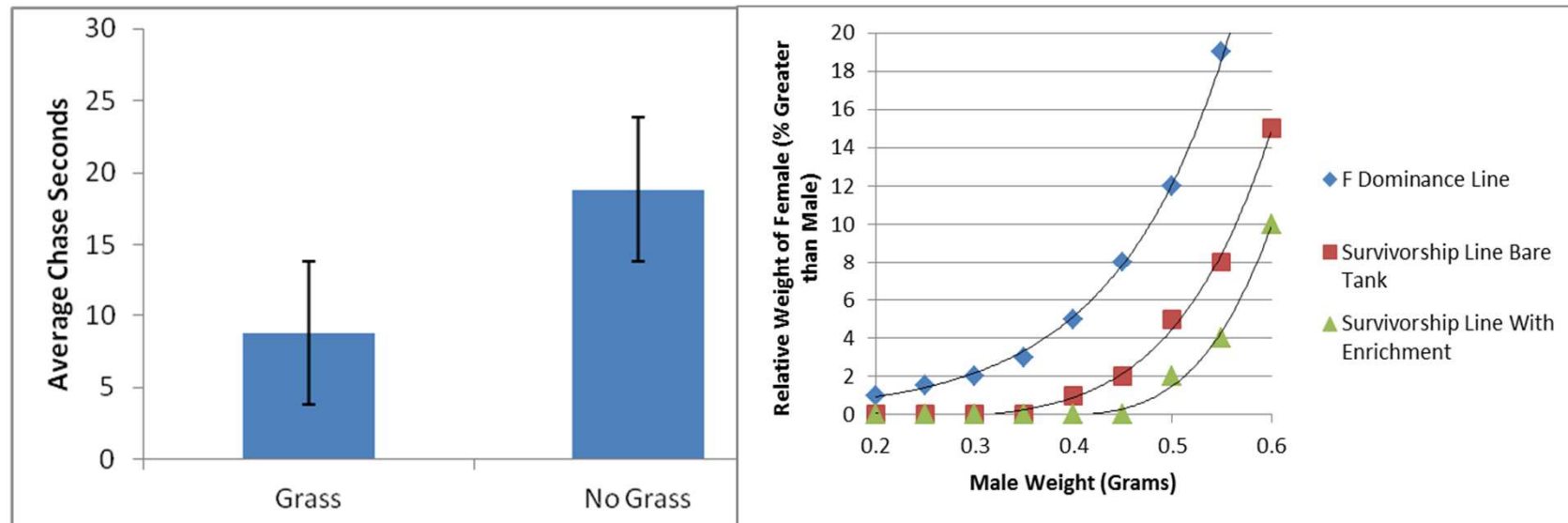
Identifying quick wins for improving the welfare of our animals!

- Unlike for females, subordinate males showed less signs of stress and importantly no physical damage even when females were dominant.

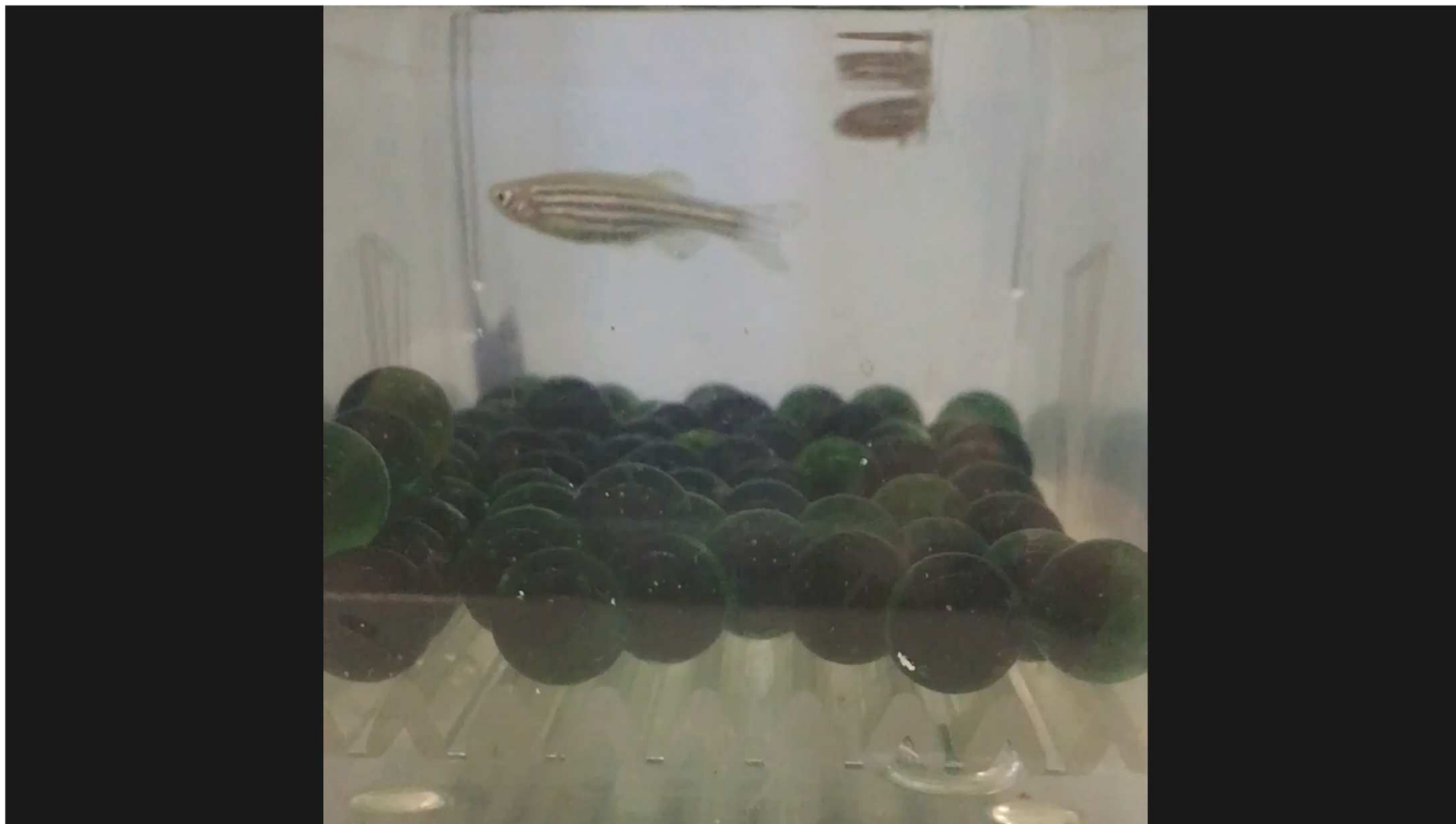


Identifying quick wins for improving the welfare of our animals!

- Simple tank enrichment and size matching mitigated levels of aggression



- Often the best principle is to avoid extremes....



7th Annual International Zebrafish
Husbandry Meeting

Identifying quick wins for improving the welfare of our animals!

Novel approaches to husbandry practices!

- Change matters?
 - The importance of Environmental change for wellbeing and life history success
 - Significant welfare benefits can be achieved through simple and practical husbandry practices
- Hypothesis: Enriched environments that don't change maybe less stimulating over time than bare tanks that do change?
- Fish will be housed in bare tanks and at differing time points moved to identical clean bare tanks
 - Fish Condition
 - Reproductive Performance
 - Behaviour, e.g. activity (foraging, occupation of tank space, aggression, dominance hierarchies)
 - Physiology, e.g. notably brain size and morphology
- Can we elicit the same levels of stimulation (behavioural and physiological) that have been reported in enriched environments?

Conclusion

▣ Refinement and Best Practice:

- Reflect on current practices and technology and refine animal usage through a better understanding of human-animal interactions and fish behaviour dependent on the social or housing environment kept
- Simple changes may offer immediate improvements to fish welfare: E.g. do we always need to spawn in pairs or 2:1's? Preference for small groups to reduce aggressive behaviour and the formation of extreme hierarchies. Preference for using larger spawning vessels that help maintain better water quality
- Look for alternate welfare solutions that are practical and more likely to be applied in large facilities
 - ▣ Do some of your own experiments?
 - ▣ The response 'That is how we have always done it' doesn't mean that it is best practice today!
 - ▣ Publish your findings!





▣ My happiest fish.....



Ethical Rationale

Can fish exposed to adverse conditions experience pain?

- Fish lack a neocortex, therefore they are believed incapable of consciousness and cannot appreciate pain nor suffer
- Homologous structures (involved in conscious experience) with mammalian neuroanatomy have been identified in fish
- Many studies show fish are capable of nociception –
noxious stimulus → electrical impulse → brain



Ethical Rationale

- Fish can experience fear-like states and avoid situations in which they have experienced adverse conditions
- Currently the precautionary principle should be that it is likely that fish can feel pain & studies designed accordingly

