



THE UNIVERSITY  
OF QUEENSLAND  
AUSTRALIA

# 5<sup>th</sup> Annual International Zebrafish Husbandry Course

## Spawning & Reproduction

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Aquatics Manager  
UQ Biological Resources*



 **TECNIPLAST**

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# Spawning Overview

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Reproductive biology

Nutritional Management

Genetic Management

Behavioural Management

Age/Health Management

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# Reproductive Biology

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Asynchronous batch spawners, capable of spawning on a daily basis

- 🧬 Egg scatterers, no parental care
- 🧬 Males necessary for oviposition

Clutch size variable

- 🧬 <100 – 1000+ eggs per event

*Castranova et al., 2011*

- 🧬 Correlated with female body size, interspawning interval

Olfaction critical in reproduction

- 🧬 pheromonal feedback loop controls spawning
- 🧬 Mate choice - non-kin over kin

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# Sexing Zebrafish

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## ♂ Male characteristics

- ♂ Fusiform bodies
- ♂ Red anal fin
- ♂ Breeding Tubercles

*McMillan et al., 2015*



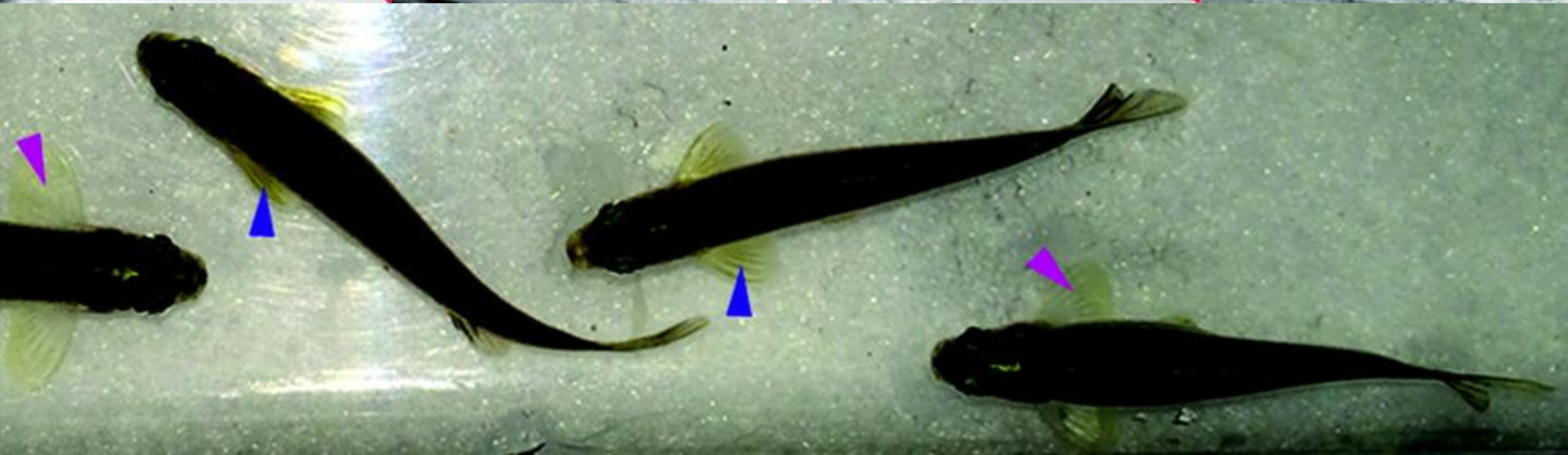
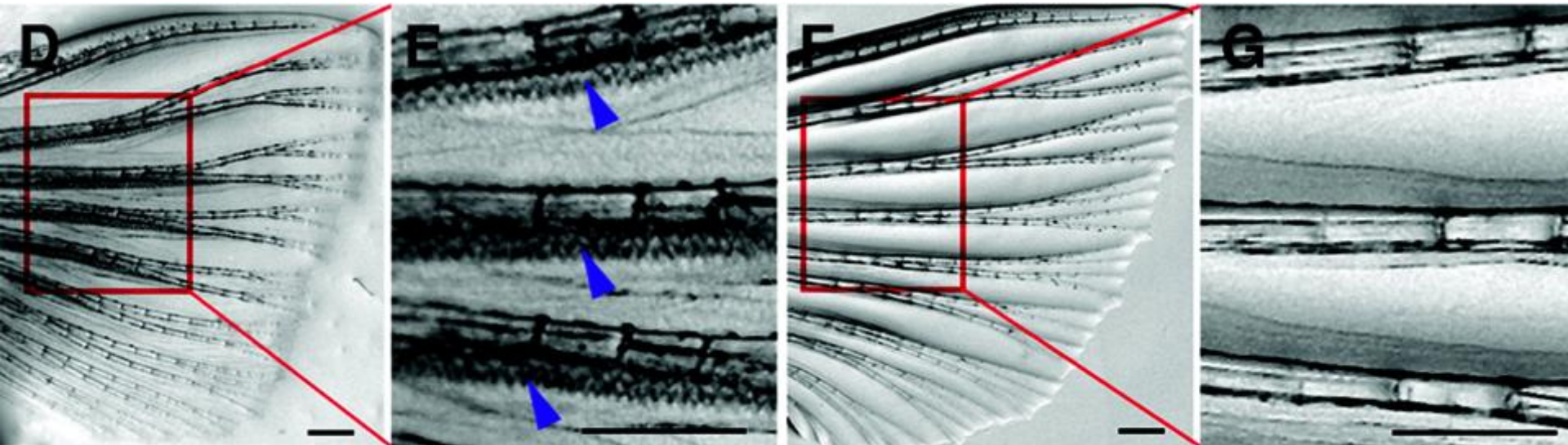
zebrafish, adult male, ~0.50 g,  
~40mm





# Breeding Tubercles

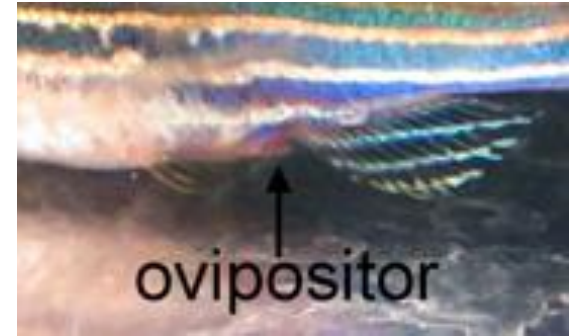
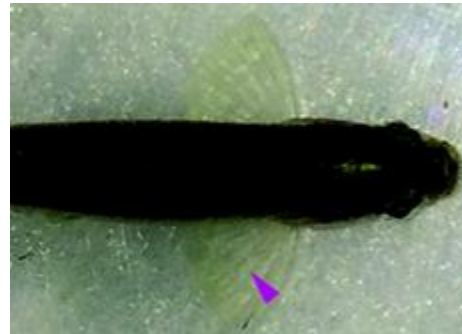
(McMillan et al., 2015)



# Sexing Zebrafish

## ♀ Female characteristics

- ♀ Rotund bodies
- ♀ (usually) clear anal fin
- ♀ No Breeding Tubercles
- ♀ Genital papilla



zebrafish, adult female, ~0.75 g,  
~40mm



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# Photoperiod

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Egg maturation and ovulation stimulated by onset of daylight

- 🧬 Breeding behavior typically commences at onset of daylight
- 🧬 Pattern observed in the wild and in the laboratory

Fish will spawn throughout day, although with less regularity

- 🧬 Many fish in laboratory populations will also spawn in the late evening
- 🧬 Fish observed in nature spawning at all times of day during heavy rains

Patterns in nature and captivity suggest that zebrafish are crepuscular spawners

- 🧬 Dawn and dusk
- 🧬 Dawn seems to be predominant time for spawning
- 🧬 Abrupt alterations in photoperiod can inhibit reproduction



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# Water Quality and Spawning

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Zebrafish display spawning patterns typical of many species adapted to monsoonal climate regimes

Spawning triggered by onset of rains

- 🧬 Changes in water chemistry
- 🧬 Dilution, drop in pH, °C, and salinity

In RAS and typical lab spawning, dilution may also flush metabolites in water

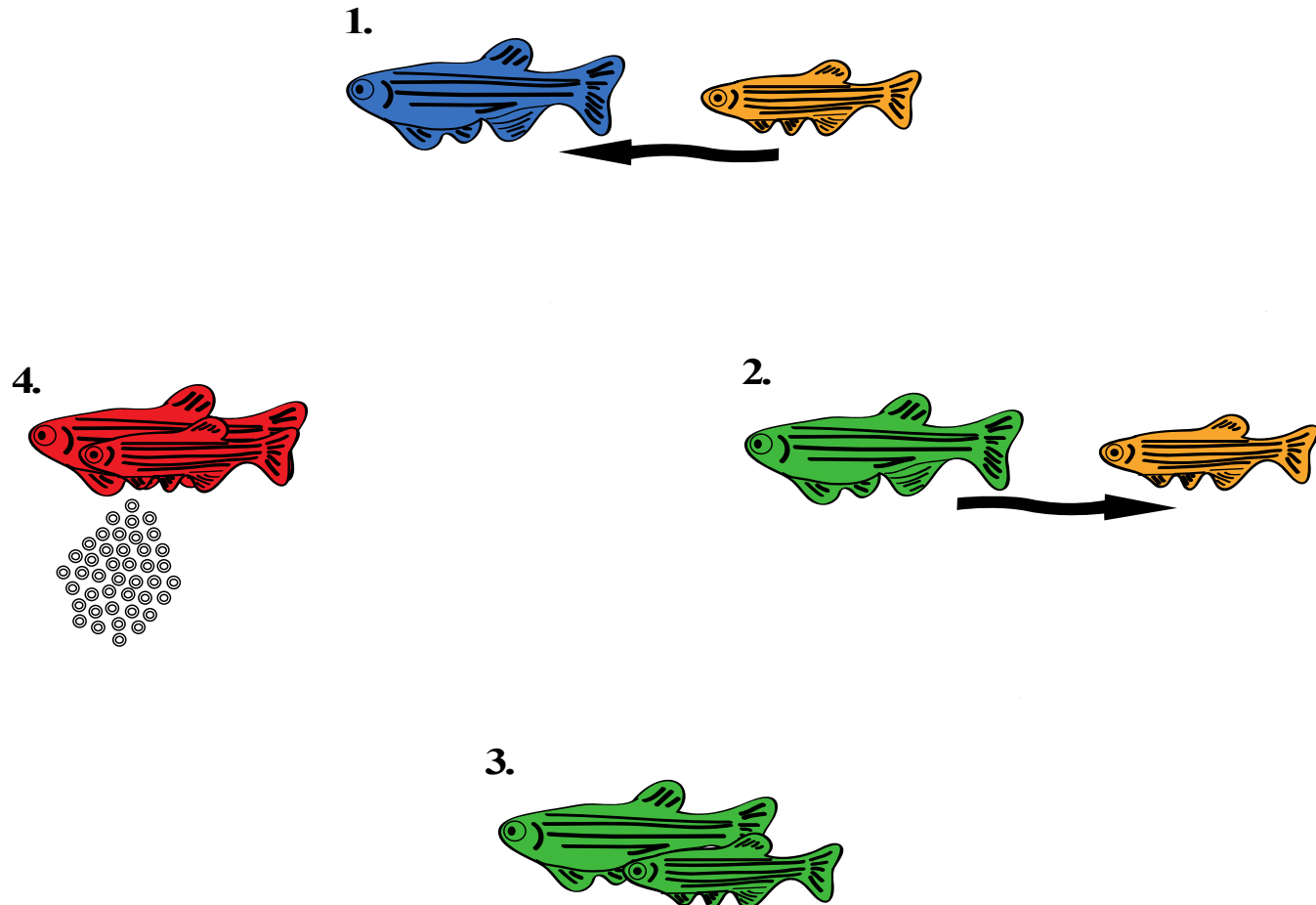
- 🧬 Ammonia (static)
- 🧬 Nitrates
- 🧬 Pheromones? (repressive)





# Olfactory control of zebrafish spawning

(Harper & Lawrence, 2010)



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# Normal Reproductive Behaviour

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Spawning activity typically involves males actively pursuing females up and down the water column before diving to the substratum to spawn.

## Non-receptive female behaviors

- 🐟 Fleeing to a safe zone (plant)
- 🐟 Retreat and repel
- 🐟 Chasing and biting

## Reasons

- 🐟 Not attracted to the male
- 🐟 Eggs not mature
- 🐟 Already “spawned out”

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# Rejection of Spawning Stimulus

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# Abnormal Mating Behaviour

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# Typical laboratory habitats

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Ponds, lakes, puddles, rivers, streams, rice paddies

- 🐟 Slow or no flow; backwaters, margins

High visibility, high turbidity

Barely vegetated

Stable seasonally

- 🐟 Temperature, chemistry,

- 🐟 Habitat size and connectivity



# Spawning Techniques

## Within holding tank

- 🐟 Fish are spawned in holding tanks
- 🐟 Fish spawn over traps inserted in tanks; eggs drop into traps

## External tank

- 🐟 Fish are taken out of holding tanks and spawned in separate tanks
- 🐟 Separate tanks have inserts
- 🐟 Fish spawn in inserts, eggs drop through floor/grate of insert
- 🐟 May be static or on flow



# Within Tank



Trap dropped in holding tanks when eggs are desired (lights on )

- 🧬 No issues with changes in water quality
- 🧬 No handling of animals required

Sex ratios

- 🧬 Female bias important
- 🧬 2 or 3:1 female to male ratios

Practical for group spawning

- 🧬 Gene knockdowns, chemical screens

Difficult/impractical for genetic studies



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# External Tank

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Males and females transferred from holding tanks to external tanks for spawning

- Typically done during the evening prior to day of spawning
- Issues with water quality can be important
- Breaking down of social hierarchies

Various types of tanks available

- Differ in size, maneuverability

Suitable for all applications

- Pairs
- Group spawning
- Dividers for timed spawning/staging embryos





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# Factors impacting performance

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## Nutrition

- 🧬 Diet must contain nutrients essential for reproductive function

## Behavior

- 🧬 Equipment must facilitate preferences
- 🧬 Behavioral management of fish in tanks

## Water quality

- 🧬 Some evidence of metabolite inhibition of spawning

## Genetics

- 🧬 The more disparately related the more the attraction



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# Nutrition and Reproduction

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Diet must contain nutrients essential for reproductive function

- 🧬 Essential fatty acids

- 🧬 Inclusion of both  $\omega 3$  and  $\omega 6$  HUFAs & PUFAs

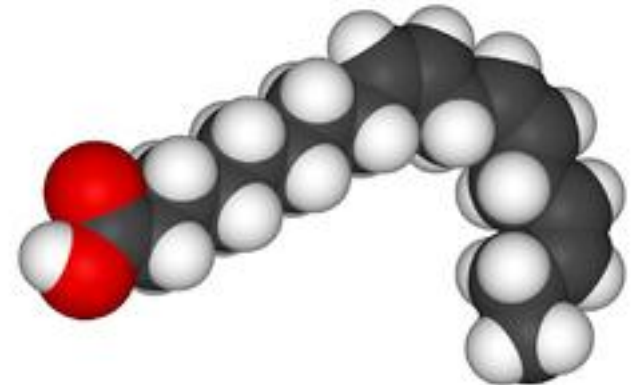
- 🧬 High  $\omega 6:\omega 3$  ratio

- 🧬 8:1 ratio of  $\omega 6:\omega 3$  results in larger, less obese fish Powel et al. (2015)

- 🧬 Vitamin E – 500mg/Kg Miller et al. 2012

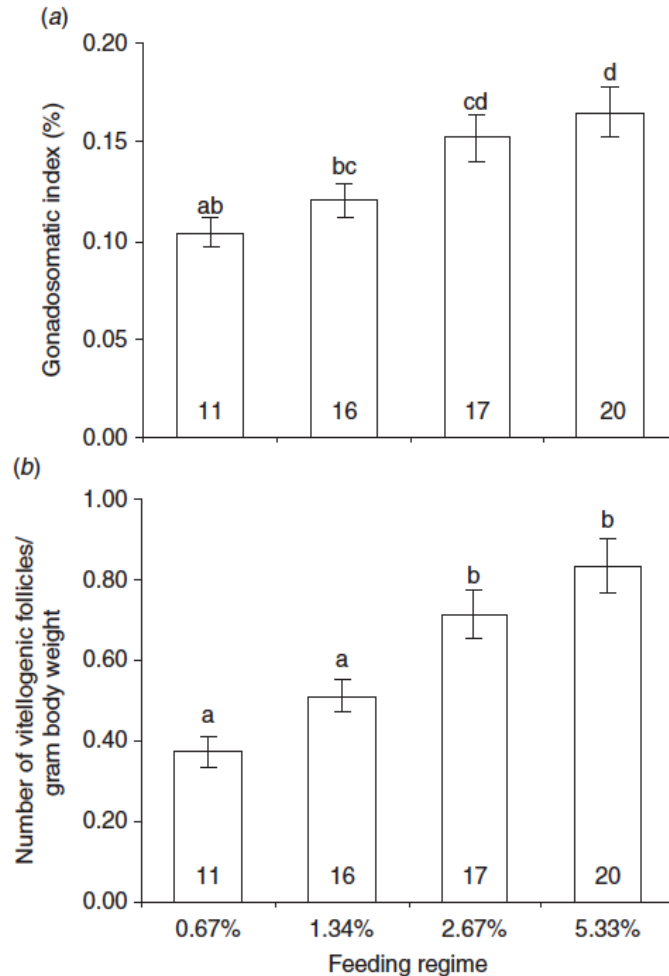
- 🧬 Vitamin C

- 🧬 Vitamin A - >50,000IU/Kg Alsop et al. 2008

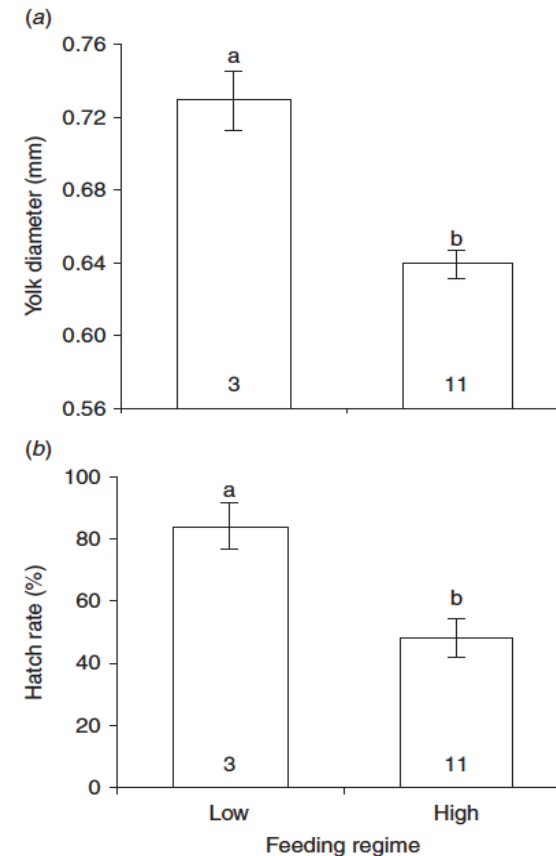


# Impact of feeding rates on fecundity

(Forbes et al., 2010)



**Fig. 1.** (a) Average gonadosomatic index (GSI) and (b) average relative fecundity of female zebrafish subjected to four different feeding regimens. The numbers in the bars indicate the number of females within each regimen. Data are the mean  $\pm$  s.e.m. Columns with different letters above them differ significantly ( $P < 0.05$ ).



**Fig. 5.** Relationship between ration size and (a) yolk diameter and (b) hatch rate of eggs spawned from zebrafish subjected to two feeding regimens. The average number of eggs per spawn was 71 for the low regimen and 203 for the high regimen. The numbers in the bars indicate the number of females within each group. Data are the mean  $\pm$  s.e.m. Columns with different letters above them differ significantly ( $P < 0.05$ ).

# Impact of fatty acids on reproduction

(Jaya-Ram et al., 2008)

**Table 2**

Proximate (% dry matter basis) and fatty acid composition (% of total fatty acids by weight) of experimental diets ( $n=3$ ) containing graded levels of squid/linseed oils

	SO	SLO	LO
<i>Proximate composition</i>			
Crude protein (%)	34.5	33.7	33.6
Crude lipid (%)	11.3	11.3	11.5
Ash (%)	11.6	11.4	11.1
Moisture (%)	3.8	6.1	3.4
Fibre (%)	13.2	13.8	12.2
Gross energy (MJ/kg) <sup>a</sup>	17.1	16.7	17.4
$n-3$ HUFA <sup>b</sup>	3.1	2.2	1.0
<i>Fatty acid composition</i>			
Total saturates	23.6	18.2	13.7
Total monoenes	38.8	21.2	16.6
18:2 $n-6$	1.4	7.1	12.1
20:4 $n-6$	1.5	0.9	0.3
Total $n-6$ PUFA	2.8	8.0	12.4
18:3 $n-3$	0.6	21.7	41.7
18:4 $n-3$	1.0	0.5	0.2
20:5 $n-3$	12.9	9.1	4.2
22:6 $n-3$	15.0	10.8	4.6
Total $n-3$ PUFA	31.4	44.1	51.3
Total $n-3$ HUFA	27.9	19.9	8.8
Total PUFA	36.0	58.4	67.8
$n-3/n-6$ PUFA	11.2	5.5	4.1
DHA/EPA	1.2	1.2	1.1
EPA/ARA	8.6	10.1	14.0

<sup>a</sup> Gross energy, calculated based on 0.17, 0.237, 0.398 MJ/g for carbohydrate, protein and lipid, respectively.

<sup>b</sup> Calculated from lipid content  $\times$  total  $n-3$  HUFA.

**Table 3**

Mean value  $\pm$  S.E. ( $n=30$ ) of various growth and reproductive parameters of female zebrafish fed different levels of dietary HUFA after 12 weeks

	Diet		
	SO	SLO	LO
Initial weight (g)	0.40 $\pm$ 0.02	0.41 $\pm$ 0.02	0.41 $\pm$ 0.02
Final weight (g)	0.53 $\pm$ 0.04	0.52 $\pm$ 0.02	0.56 $\pm$ 0.03
Weight gain (g)	0.13 $\pm$ 0.04	0.11 $\pm$ 0.02	0.15 $\pm$ 0.04
SGR (%)	0.27 $\pm$ 0.08	0.25 $\pm$ 0.04	0.31 $\pm$ 0.07
FCR	25.05 $\pm$ 8.59	22.89 $\pm$ 1.48	20.32 $\pm$ 2.95
Total egg production per female	3681 $\pm$ 170 <sup>a</sup>	5006 $\pm$ 325 <sup>b</sup>	4464 $\pm$ 384 <sup>ab</sup>
Relative fecundity	694.55 $\pm$ 32.04 <sup>a</sup>	962.77 $\pm$ 62.47 <sup>b</sup>	797.15 $\pm$ 68.61 <sup>ab</sup>
Hatching rate (%)	60.2 $\pm$ 3.1 <sup>a</sup>	75.4 $\pm$ 2.5 <sup>b</sup>	63.2 $\pm$ 3.1 <sup>a</sup>
Larval survival (%)	90 $\pm$ 2	93 $\pm$ 1	91 $\pm$ 5
GSI (%)	12.84 $\pm$ 0.94	11.43 $\pm$ 1.04	13.01 $\pm$ 1.15

Mean values in similar row with different superscript letters are significantly different (Tukey's HSD,  $P < 0.05$ ).



# Impact of omega fatty acids

(Meinelt et al., 1999)

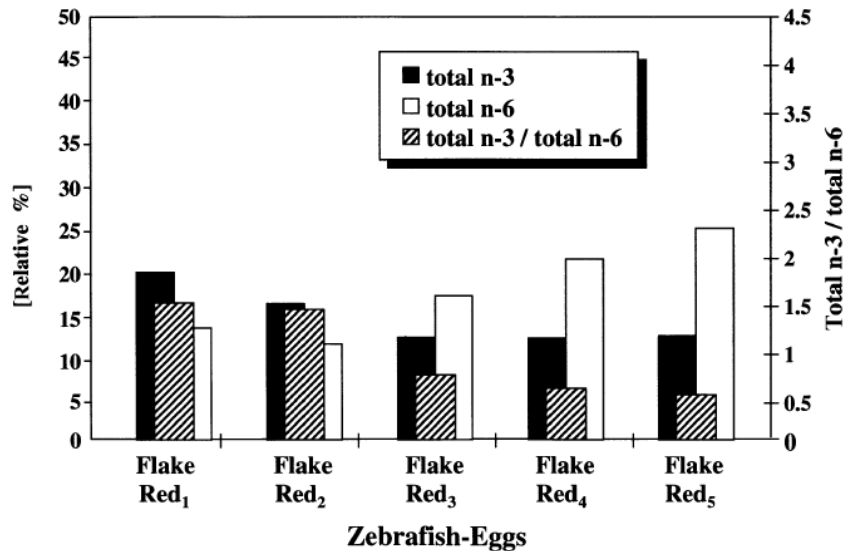


Fig. 1. Relative percentage of total n-3 and total n-6 FA and ratio of total n-3/total n-6 FA in the triglyceride fraction (TG) of zebrafish eggs following eight weeks of flake diets

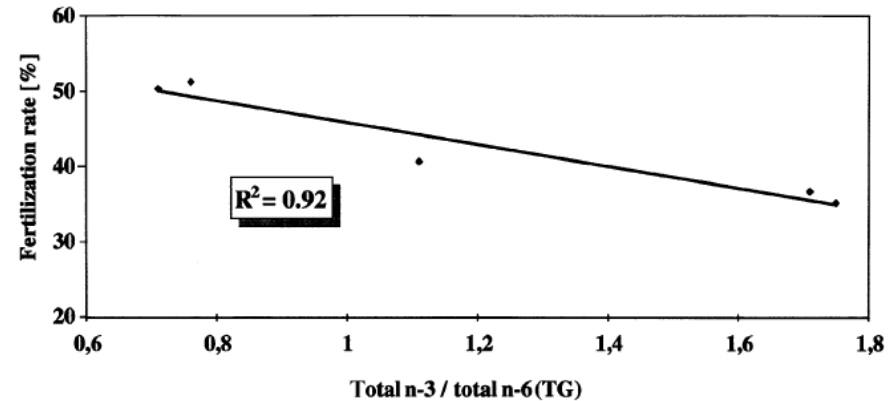
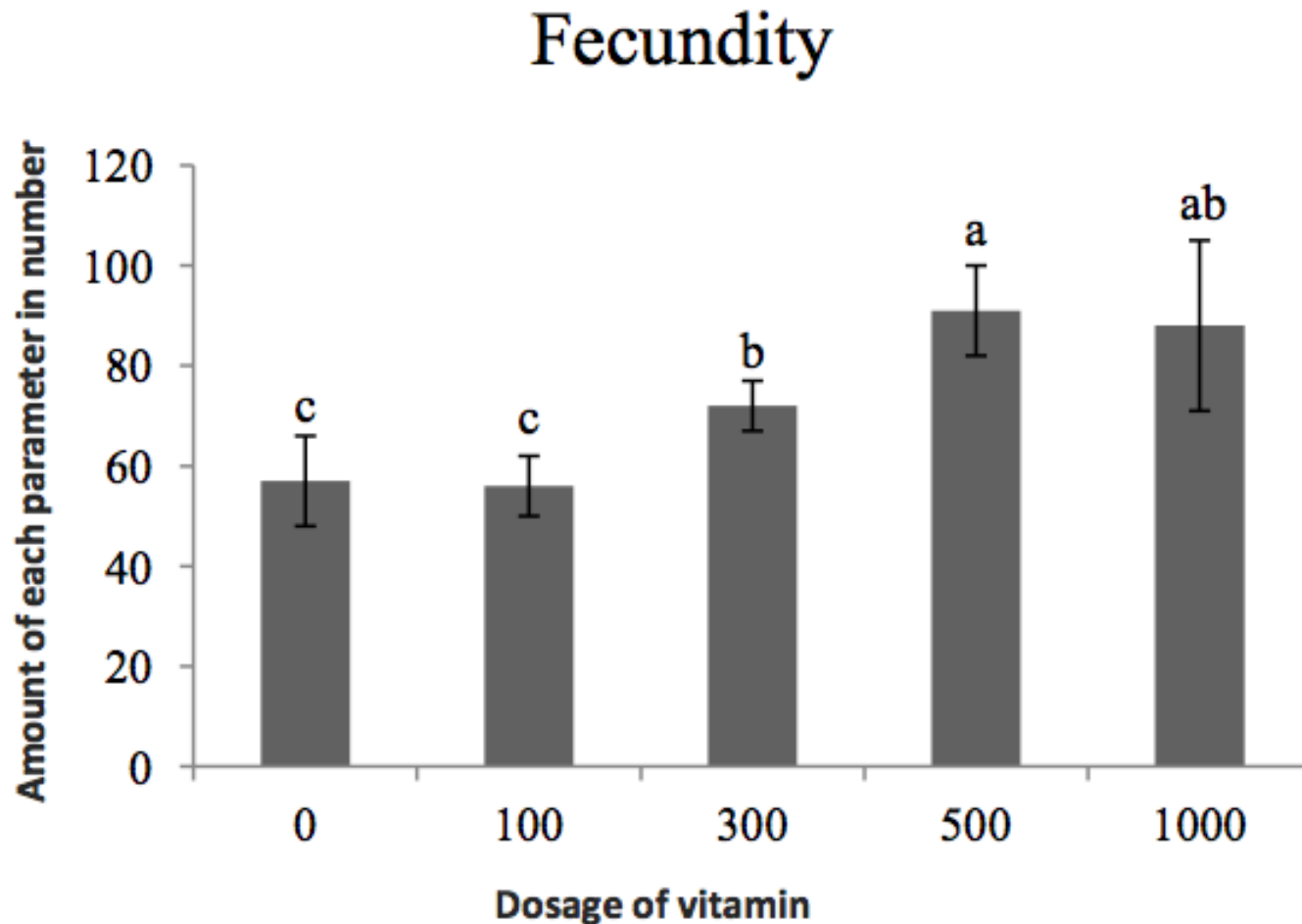


Fig. 6. Regression and correlation coefficient between composition of flake diets and fertilization rate

# Importance of Vitamin E for fecundity

(Mehrad et al., 2011)



**Figure 7.** Effects of vitamin E on fecundity of zebrafish.

# Vitamin E effects on embryo viability

(Miller et al., 2012)

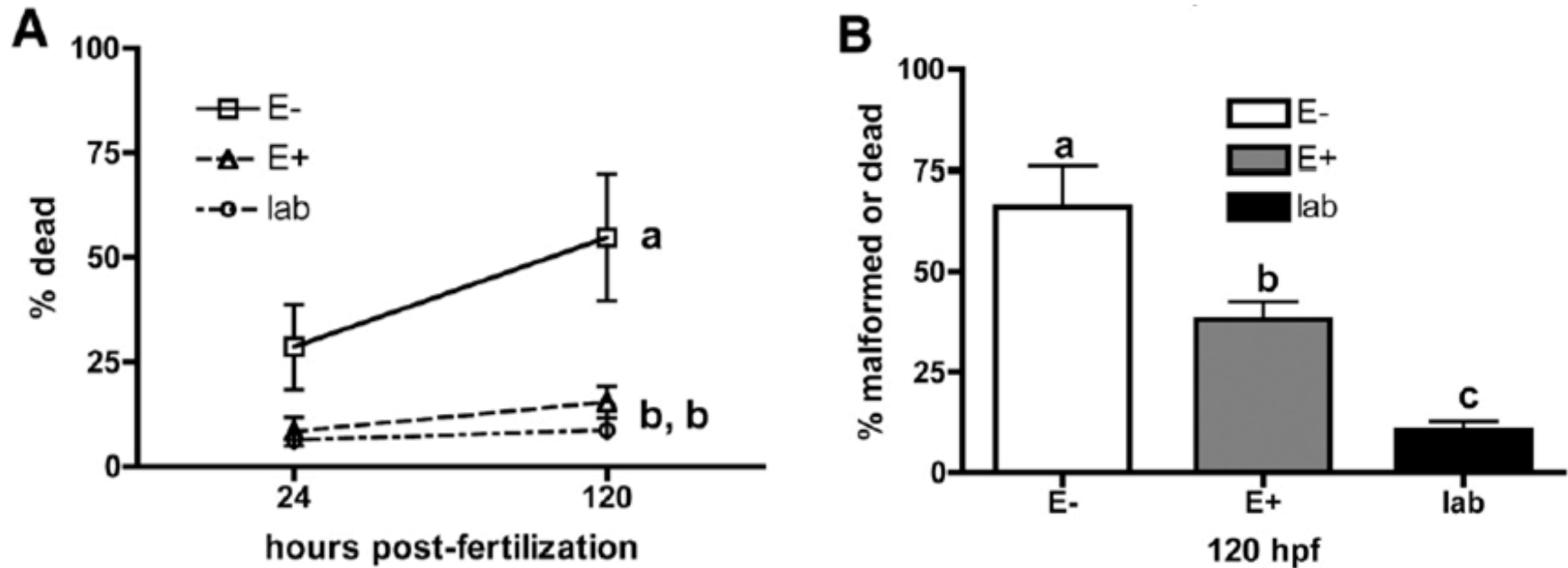


Fig. 5. Malformations and mortality of zebrafish embryos. (A) Increased mortality (mean±S.E.M.) was observed in the E-embryos at 24 and 120 hpf compared with the other diet groups (diet × time interaction,  $P=0.0001$ ), but at 24 hpf, the differences between diet groups did not reach statistical significance. Mortality increased from 24 to 120 hpf in the E- (squares;  $P=0.01$ ) and the E+ embryos (triangles;  $P=0.01$ ), but not in the lab diet embryos (circles). At 120 hpf, the E-embryos displayed significantly higher levels of mortality compared with the E+ and lab diet embryos (diet effect,  $P=0.005$ ; E- (a) N E+ (b) or lab (b);  $P=0.05$  for paired comparisons). (B) Higher levels of both malformations and mortality were observed at 120 hpf in the E- embryos compared with E+ ( $P=0.05$ ; a) or lab diet embryos ( $P=0.001$ ; b); E+ had greater malformations than did lab diet embryos ( $P=0.05$ ; c). Embryos were analyzed in 96-well plates, one embryo per well with 48 to 120 embryos per group per spawn. Results are expressed as percentages affected per total number of embryos ( $n=6$  spawns per group)

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# Genetics

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Genetic background important for mate choice

- 🧬 The more disparately related, the greater the attraction
- 🧬 Larval fish prefer odors of siblings (Gerlach and Lysiak, 2006)
- 🧬 Sexually mature fish prefer odors of non-sibs
- 🧬 Mechanism to avoid inbreeding

Preference can be overridden (sibs will mate) but this is not as easy

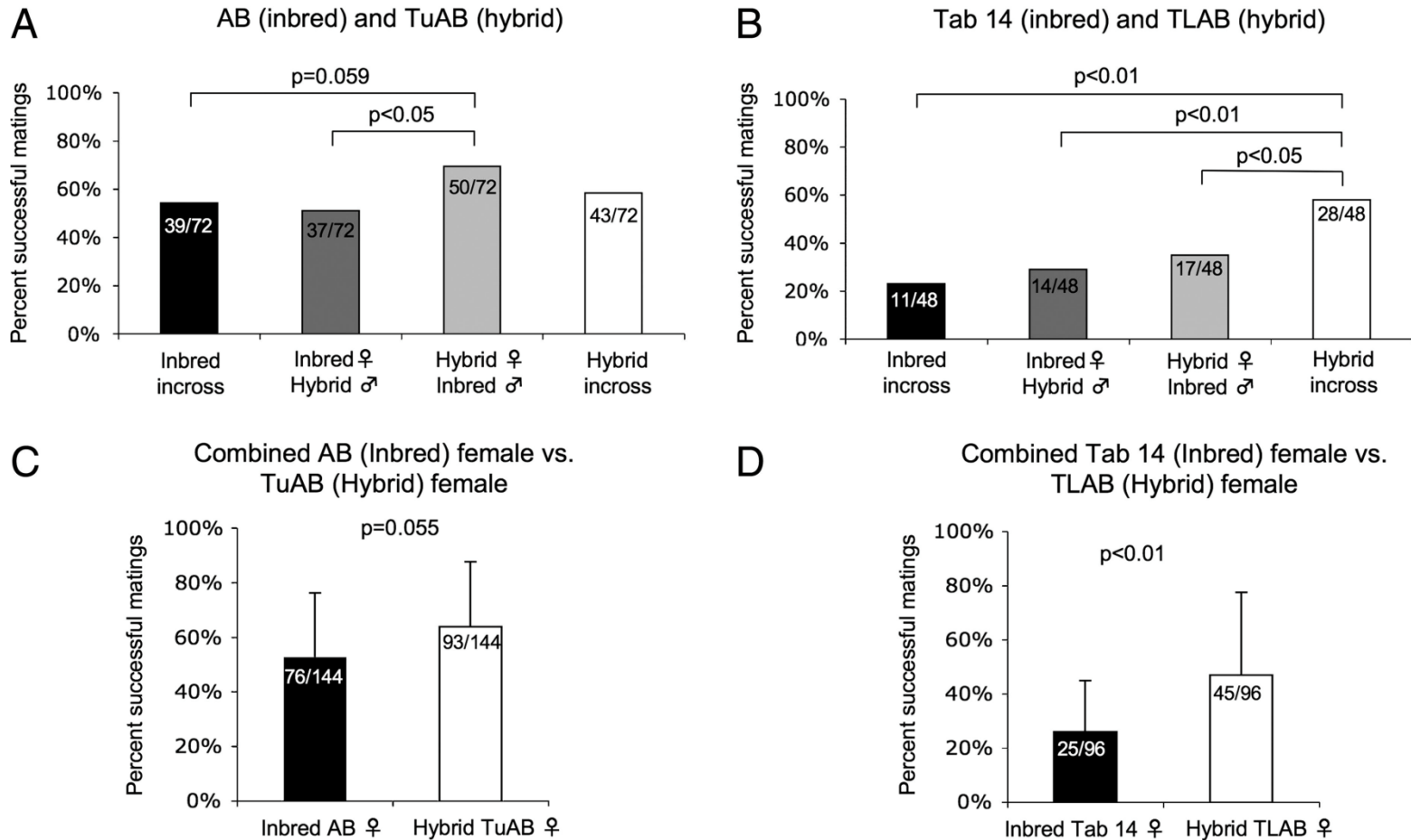
- 🧬 One reason for lack of inbred zebrafish

This dynamic is influential in a typical zebrafish research facility

# Hybridization improves reproduction

(Monson and Sadler, 2010)

The more disparately related the more the attraction





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# Genetic Management

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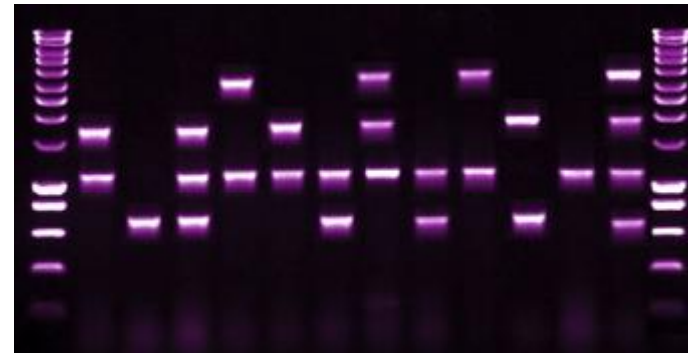
Genetic management of stocks HUGELY important, but often overlooked

The classic problem: How do you manage the genetics of small, closed populations?

Heterozygosity must be maintained in the face of inevitable loss of genetic diversity due to drift, inbreeding.

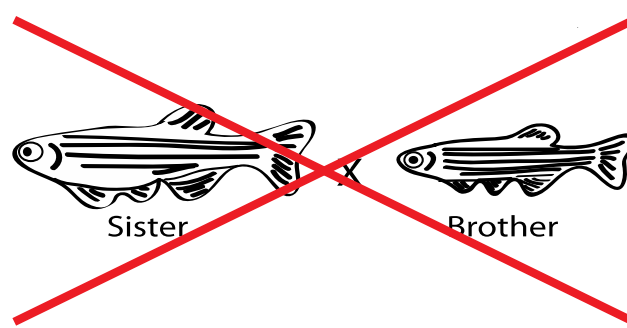
Drift and inbreeding depression

- 🧬 Long term health of lines
- 🧬 Breeding efficiency
- 🧬 Sex ratios
- 🧬 Reproducibility of experiments



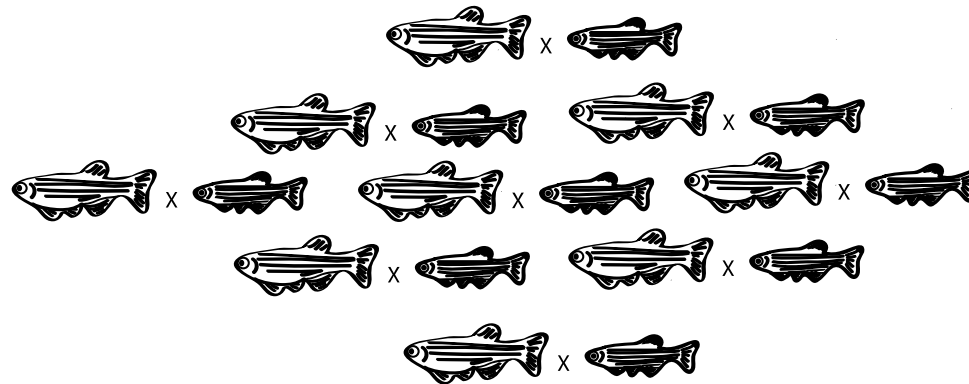
Inbreeding depression is a major source of problems in zebrafish facilities

1.



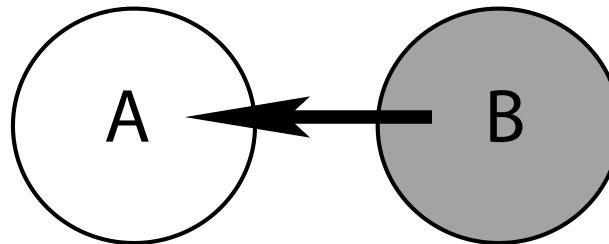
Eliminate sib matings

2.



Maximize effective population size

3.



Harper and Lawrence 2010

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# Genetic Management Strategies

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1. Short-term, easily implemented approaches for:
  - 🧬 Wild-type (fount from which everything else springs)
  - 🧬 Mutants, transgenics should be maintained by outcrossing large numbers of carriers!
  
2. Long-term (BIG PICTURE)
  - 🧬 Need to implement systematic, standardized approach
  - 🧬 Akin to what is done in rodents; e.g. Genetic Standardization Program employed by Charles River

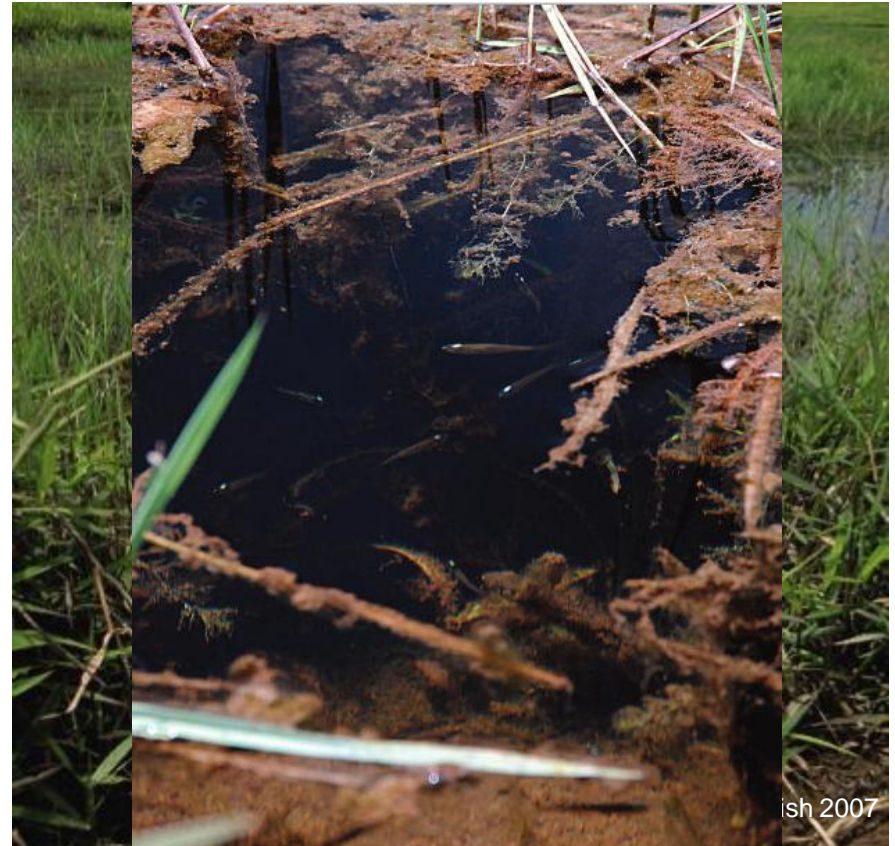
# Behavioral Preferences in Nature

It is thought that zebrafish spawn in shallow water, in and around margins of water bodies they inhabit.



Photo courtesy of Spence and Smith

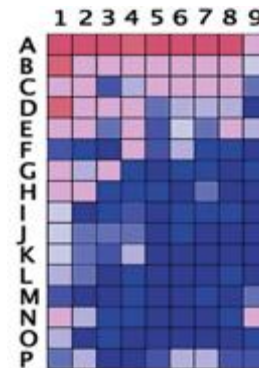
It has also been demonstrated that they prefer to spawn in vegetated sites.



ish 2007

# Behavioural preferences in the Lab

(Sessa *et al.* 2008)



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# Spawning behavior with no depth gradient

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(external tank)



courtesy of Lawrence, C



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# Spawning behavior with depth gradient

(external tank)

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courtesy of Lawrence, C

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# Spawning behavior with depth gradient

(external beach tank)

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# Shallow spawning behavior

(external tank – iSpawn)

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courtesy of Tecniplast



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# Shallow spawning behavior

(external tank – iSpawnS)

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# Behaviour Impacts

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Dominant individuals sire significantly more offspring

- ♂ Males – Paull et al., 2010

- ♀ Females – Gerlach, 2006

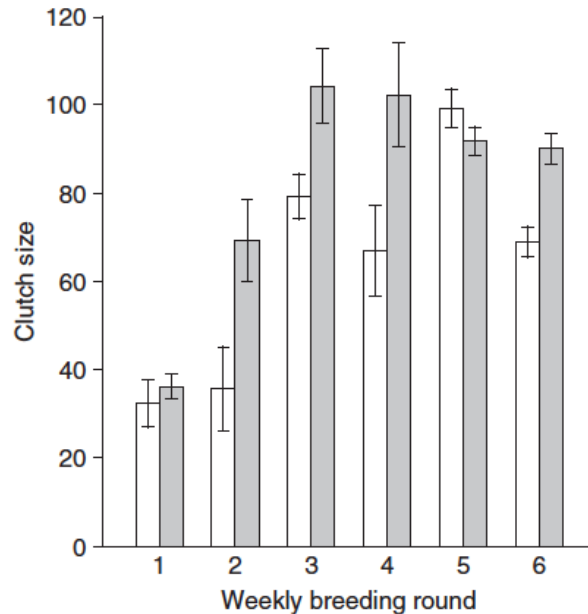
Reduces overall production in groups where hierarchies are intact

- ♂ Closed, static environments = housing tanks

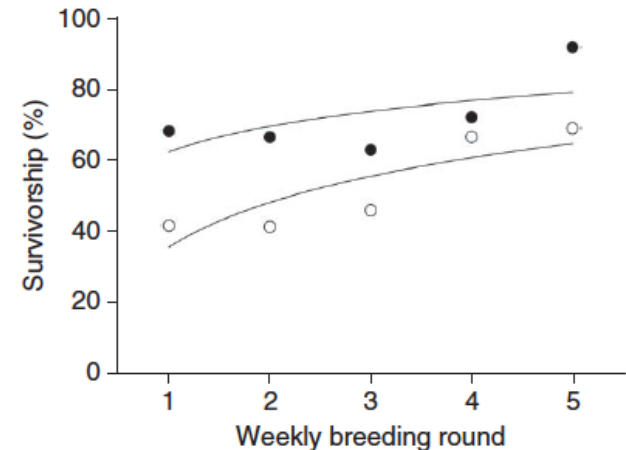
- ♂ Counteract by frequent mixing and matching

# Segregation effect on reproduction

(Kurtzman et al., 2010)



**FIGURE 1** | Clutch size (total eggs collected including unfertilized) from a total of 144 harem breedings (2 male 1 female) of fish housed together (empty bars) or separately by gender (shaded bars). Fecundity in both groups was significantly greater in the third breeding round than in first ( $P = 0.01$ ). In the second breeding round, clutch size was significantly greater for the sex-separated group than the mixed-gender group ( $P = 0.041$ ).



**FIGURE 2** | Survivorship of eggs at 30 hpf from fish housed together (open circles) or separately by gender (filled circles) from a total of 144 harem breedings. Nonlinear regression lines indicate the trend of increasing embryo survivorship (not statistically significant) in both experimental groups during successive pairings.

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# Behavioural management

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Holding densities very important

- 🧬 Simple way to impact welfare of fish

High Density

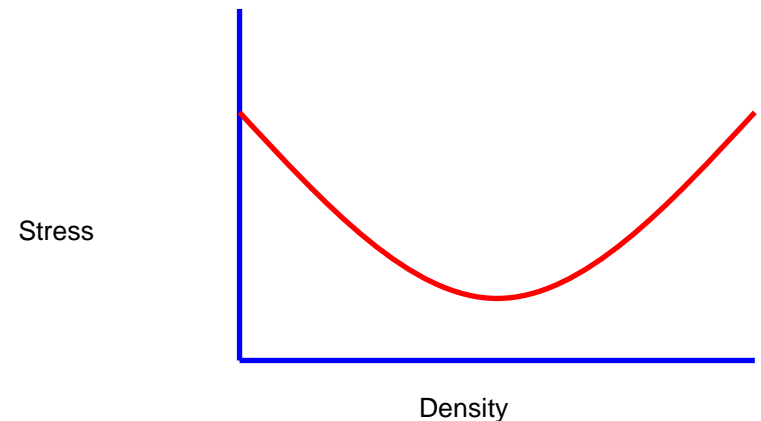
- 🧬 Fish display stress response at elevated densities

Low Density

- 🧬 Aggressive interactions highest

Optimal Density

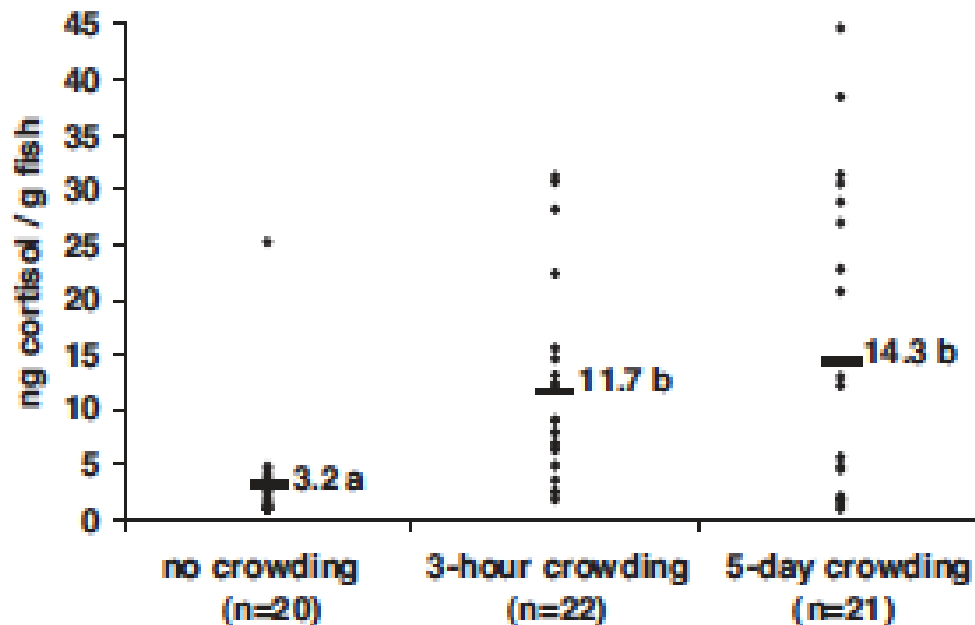
- 🧬 Performance is best



Harper and Lawrence 2010

# Crowding increases cortisol in zebrafish

(Ramsay, JM et al. 2006)



# Target Stocking Density

(Castranova et al. 2011)

ZEBRAFISH  
Volume 00, Number 00, 2011  
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DOI: 10.1089/zeb.2011.0688

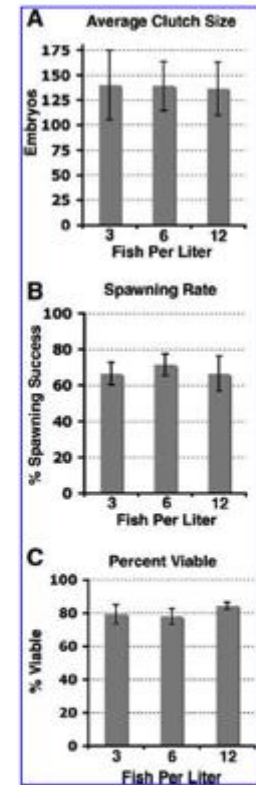
*The Fish Haus*

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

Daniel Castranova,<sup>1</sup> Angela Lawton,<sup>2,a</sup> Christian Lawrence,<sup>3,a</sup> Diana P. Baumann,<sup>4,b</sup> Jason Best,<sup>3,b</sup> Jordi Coscolla,<sup>5,b</sup> Amy Doherty,<sup>6,b</sup> Juan Ramos,<sup>5,b</sup> Jenna Hakkesteeg,<sup>7,b</sup> Chongmin Wang,<sup>1,b</sup> Carole Wilson,<sup>7,b</sup> James Malley,<sup>8</sup> and Brant M. Weinstein<sup>1</sup>

### Abstract

Despite the growing popularity of the zebrafish model system, the optimal husbandry conditions for this animal are not well defined. The aim of this study was to examine the effect of stocking density on reproductive performance in zebrafish. In this study, undertaken by eight different zebrafish facilities, clutches of at least 200 wild-type zebrafish embryos from a single pairwise mating were produced at each participating institution and subsequently reared according to “in-house protocols” until they were 14 weeks old. Fish were then randomly assigned into treatment groups with balanced sex ratios and densities of 3, 6, or 12 fish/L. After a 1-month acclimation period, fish were spawned in pair crosses every 2 weeks for 3 months, for a total of six spawning dates. The number of viable and nonviable embryos produced in each clutch were counted at 1 day post-fertilization. Although there was a great deal of variability in clutch size and percent spawning success among laboratories, there were no significant differences in average clutch size, spawning success, or percent viable among the treatment densities. These data suggest that using stocking densities as high as 12 fish/L does not have a negative impact on performance, when measured by reproductive performance.

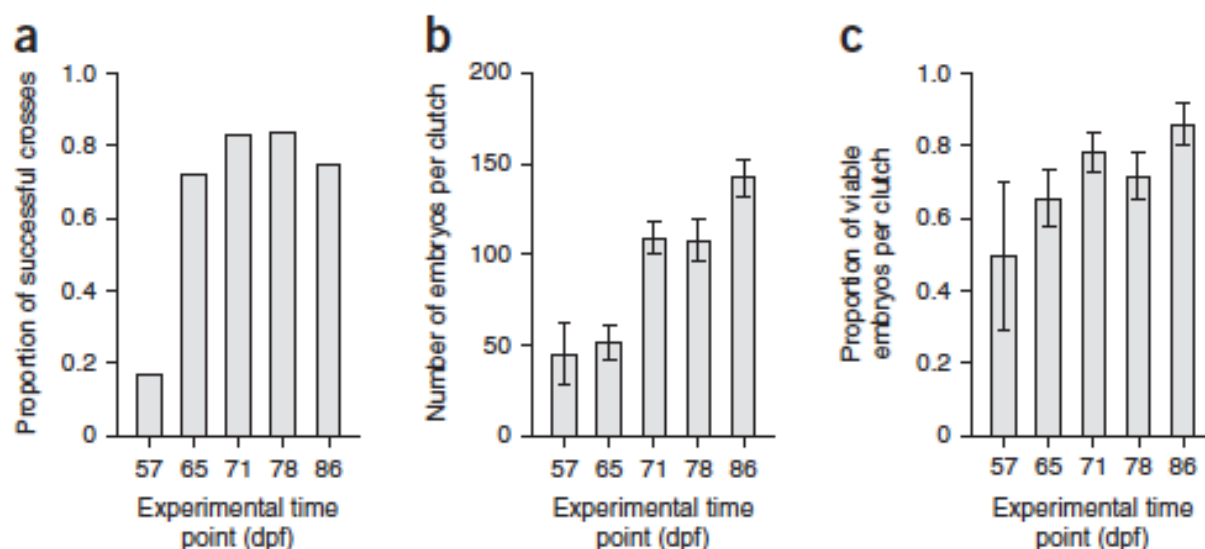


**FIG. 1.** Average clutch size (A), spawning rate (B), and percent viable at 1 day postfertilization (C) for individual pairwise crosses of zebrafish housed at three different stocking densities: 3, 6, and 12 fish/L. Data were averaged from eight zebrafish laboratories collected on six spawning dates. Vertical bars represent the standard error of the mean. No significant differences were detected among any of the three densities for any parameter using one-way analysis of variance.



## Generation time of zebrafish (*Danio rerio*) and medakas (*Oryzias latipes*) housed in the same aquaculture facility

Christian Lawrence, MS<sup>1</sup>, Isaac Adatto, MS<sup>2</sup>, Jason Best, BS<sup>1</sup>, Althea James<sup>1</sup> & Kara Maloney, BS<sup>1</sup>



**FIGURE 3 |** Reproductive performance of zebrafish. (a) Proportion of crosses that yielded embryos. (b) Mean number of embryos produced per clutch. (c) Mean proportion of viable embryos per clutch. Error bars represent s.e.m.

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# Stocking density policy

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Target, minimum, maximum densities defined

- 🧬 By life stage, application, and tank type

Exceptions on high side

- 🧬 must be approved by ethics committee (AEC / IACUC)

Low side require enrichment

- 🧬 Isolation cages
- 🧬 Social or Structural enrichment

# UQZF Stocking Policy



## STOCKING DENSITY POLICY FOR ADULT ZEBRAFISH (45<sup>+</sup>DPF)

TANK TYPE SIZE	MINIMUM FISH # <sup>1</sup>	FISH PER LITRE	RECOMMENDED FISH #	FISH PER LITRE	MAXIMUM FISH # <sup>2</sup>	FISH PER LITRE
ZebTec 0.7L (Static)	-	-	2	3	3	4
ZebTec 1.0L (Static)	-	-	2	2	6	6
AHAB 2.0L (Static)	-	-	2	1	12	6
ZebTec 0.7L (Flow)	1	1	1	1	2	3
ZebTec <sup>3</sup> 1.1L	1	1	11	10	20	18
AHAB 1.5L	2	1	15	10	22	15
AHAB 3L	15	5	30	10	45	15
ZebTec 3.5L	18	5	35	10	53	15
ZebTec 8.0L	20	2.5	80	10	120	15
AHAB 10L	20	2	100	10	150	15

DPF = Days post-fertilization

FPL = Fish per litre

**Purple Shading** – Target (optimal) densities per life stage. Fish maintained under these densities will exhibit optimal performance (growth, survival, fecundity, immune function) under our rearing and maintenance regime. Fish will be sexually mature within 60-90 dpf. We recommend fish densities (per litre) should be reduced from 15 to 10 fpl once fish reach the sub-adult stage (30dpf).

**Olive Shading** – Fish can be maintained at these densities their entire lifespan (from swim-up through death). Densities do not have to be reduced once fish reach the sub-adult stage (30dpf). Note that fish reared under these densities will be sexually mature in approximately 50-60 days.

<sup>1</sup> This represents the minimum density of animals allowed under the AEC approved policy. This is not strictly enforced; i.e. keeping fish below this threshold does not require special approval by the AEC. However, if densities need to be kept below this threshold, arrangements should be made with UQBR|Aquatics staff to provide environmental enrichment in tanks.

<sup>2</sup> This represents the maximum density of animals allowed under the AEC approved policy. Any exception requires prior approval from the AEC.

<sup>3</sup> When two fish are maintained in an isolation tank, environmental enrichment must be provided to negate dominant/submissive aggression.

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# What to do in low densities

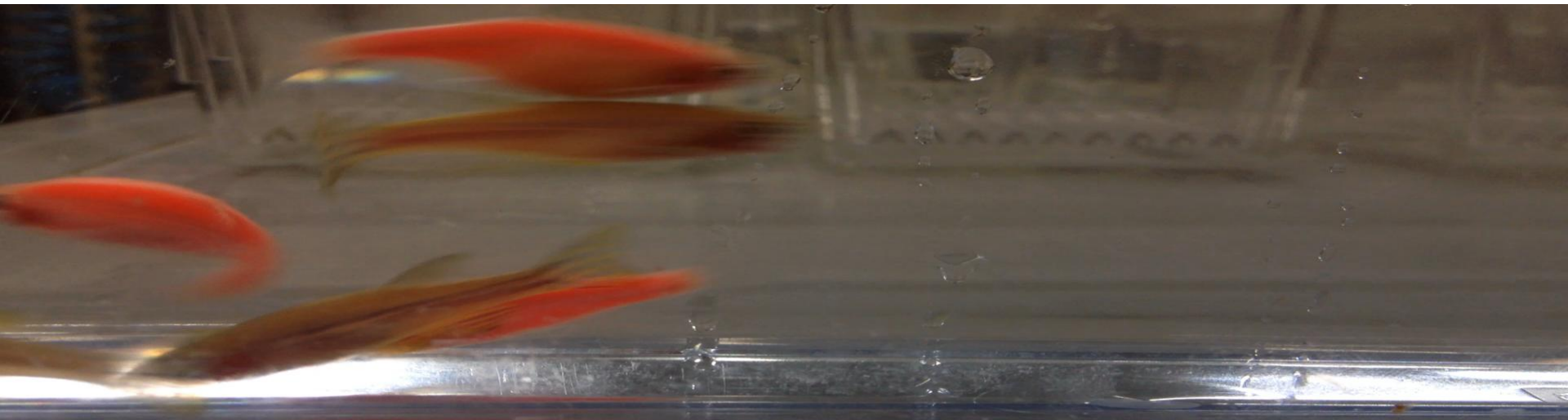
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Don't do it!

- 🧬 Keep important individuals in mixed sex groups with other fish that have phenotypic differences

If you must

- ♂ - okay by themselves
  - Watch for aggression during spawning
- ♀ - have to be managed
  - Set up regularly to avoid getting egg bound





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# Enrichment Options

(Social Enrichment – low density housing)

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# Enrichment Options

(Structural Enrichment – low density housing)

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courtesy of Lawrence, C



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# Enrichment Options

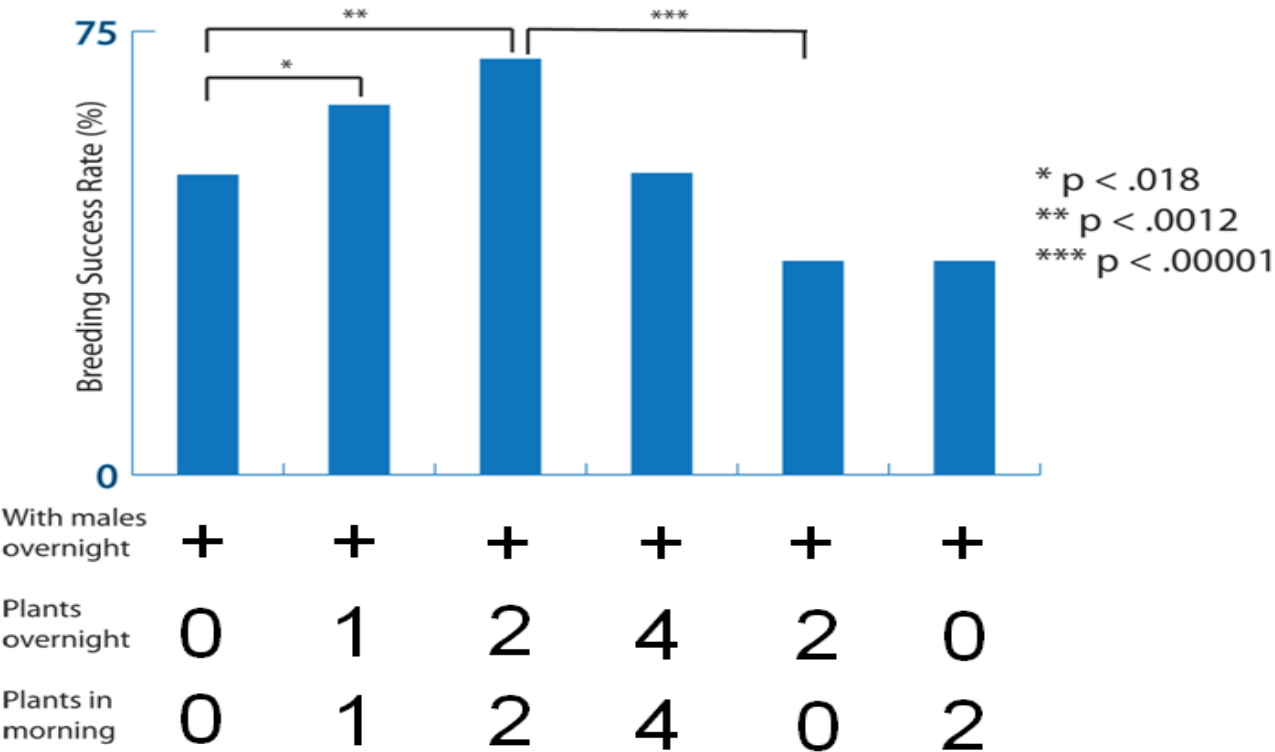
(Structural Enrichment - breeders)

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# Sex Toys for Zebrafish

courtesy of Sanders, E



Plants enhance breeding when included in breeding tank both overnight and in morning

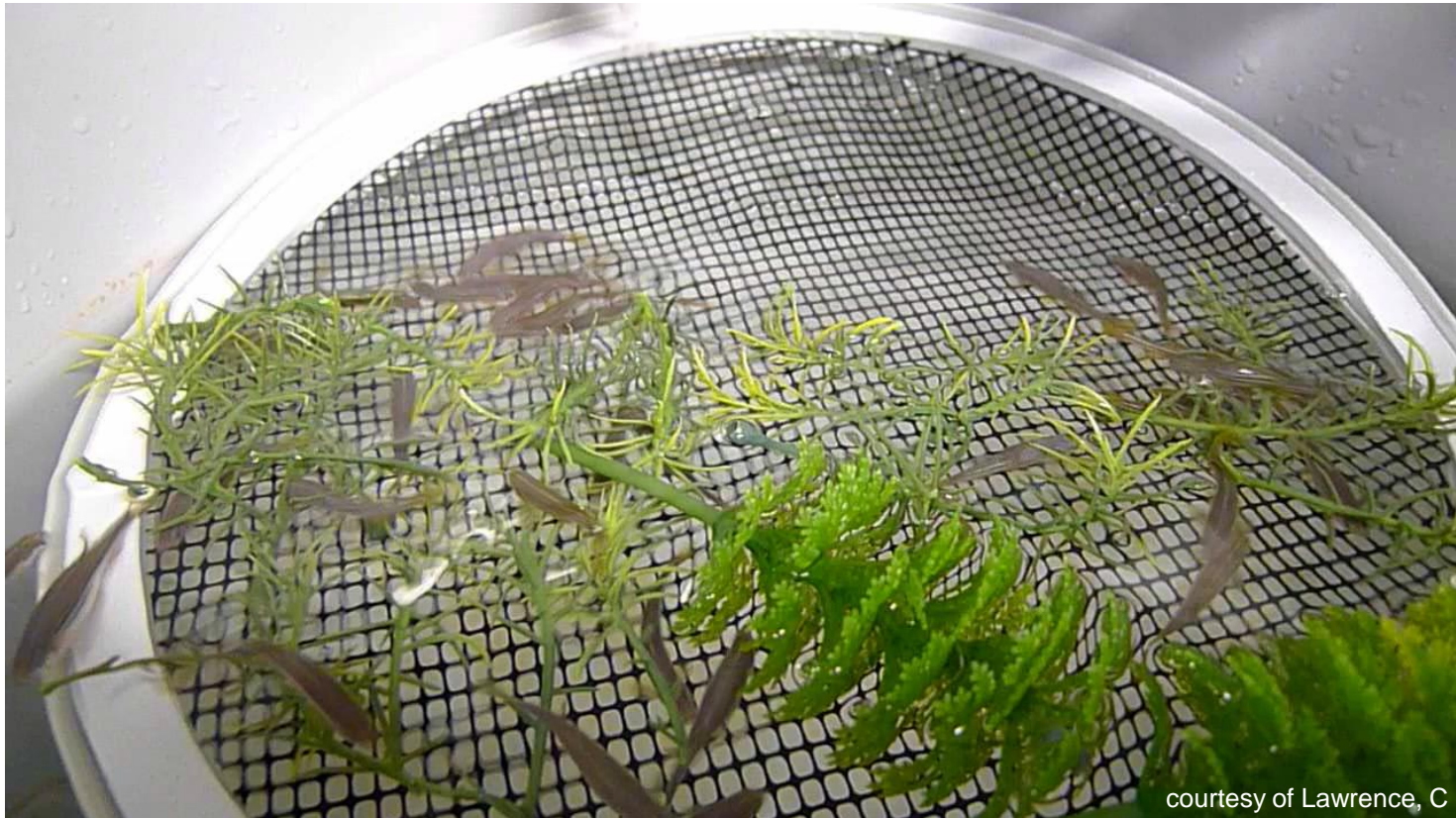
Plants ineffective when included in only one stage

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# Enrichment Options

(Structural Enrichment Within Holding Tank - MEPS)

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courtesy of Lawrence, C

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# Age turnover policy

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## Zebrafish lifespan

- 🧬 In the laboratory: 5+ years
- 🧬 In the wild: ~ 1 year (primarily an annual fish?)

Fish are most productive shortly after attaining sexual maturity

- 🧬 Peak production 6-10 months
- 🧬 Tails off after 1 year
- 🧬 Susceptibility to pathogens concomitantly increases

The rate at which they age depends on usage and management

- 🧬 Age “quickly” if handled frequently, used for egg production, kept at high temperatures

Setting facility age limits important

- 🧬 18 months with exceptions



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# Husbandry Refinement

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Establish policies on housing densities and turnover frequency

Train caregivers and users on normal and maladaptive behaviours

Controlled photoperiod

- 🐟 Dawn/dusk simulation

Breeding, low density housing

- 🐟 Socially enriched housing

- 🐟 Consider plastic plants, or other environmental structures

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# Acknowledgements

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Christian Lawrence (Children's Hospital Boston)





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# Questions?

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*Photo by  
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