Zebrafish Larviculture
embryo and larval care

a detailed examination

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Basic Larval Biology

1) Zebrafish larviculture begins at fertilization of the egg
2) At 26-28C hatch at 2.5-3 dpf
3) Inflate gas bladder by 5 dpf
4) Yolk sac gone at 7 dpf (will die at 10-dpf if not eating)
5) Nearly obligate water column feeders
6) Must feed nearly continuously upon hatching to meet nutrient demands
7) Management challenge is to meet this demand without compromising physico-chemical environment
8) Stability more important than absolute water quality

Presentation Overview

1) Embryo collection - Proper rinsing and media
2) The first 24-hrs - handling, sorting and storage
3) 48-hpf to 72-hpf - clean-up and hatching
4) 4-dpf to 5-dpf - Swim-up, transfer to holding cage and first feed
5) First Feeding to metamorphosis

Collect in a sieve or strainer, and **rinse thoroughly with embryo medium** to remove feces, scales, and other debris.

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What is Embryo Medium?
(a.k.a egg water, embryo water, E2, E3, etc.)

- It's essential qualities are:
  - Water
    - adequate purity (RO, DI, distilled, filter sterilized, etc)
    - Chemically defined (no chlorine, chloramines, nitrogen, phosphorus, etc.)
    - Biologically inert- not from the fish system!
  - Salts (ionic compound) that impart:
    - Adequate pH (~7.5)
      - Typically from a salt (buffer) such as Soduim Bicarbonate ($NaHCO_3$) a salt composed of sodium ions and bicarbonate ions
    - Adequate conductivity (500-1000uS)
      - Typically from a Sodium Chloride (NaCl)

Step 2:

Sorting the eggs to remove the embryos from the non-fertilized eggs

choose your tool wisely!

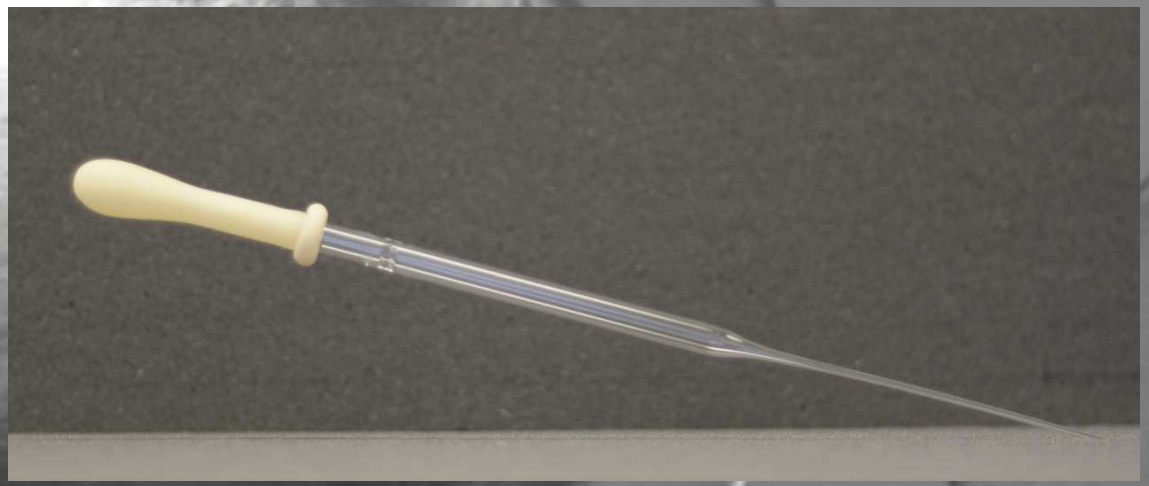
Pipette Pump Bel-Art F37898-0000

Transfer pipette 3mL

The first 24-hrs

choose your tool wisely!

Pipette Pump
Bel-Art F37898-0000



Pasteur transfer pipette

(Fisher Scientific Item #NC9993639)

Pasteur transfer pipette

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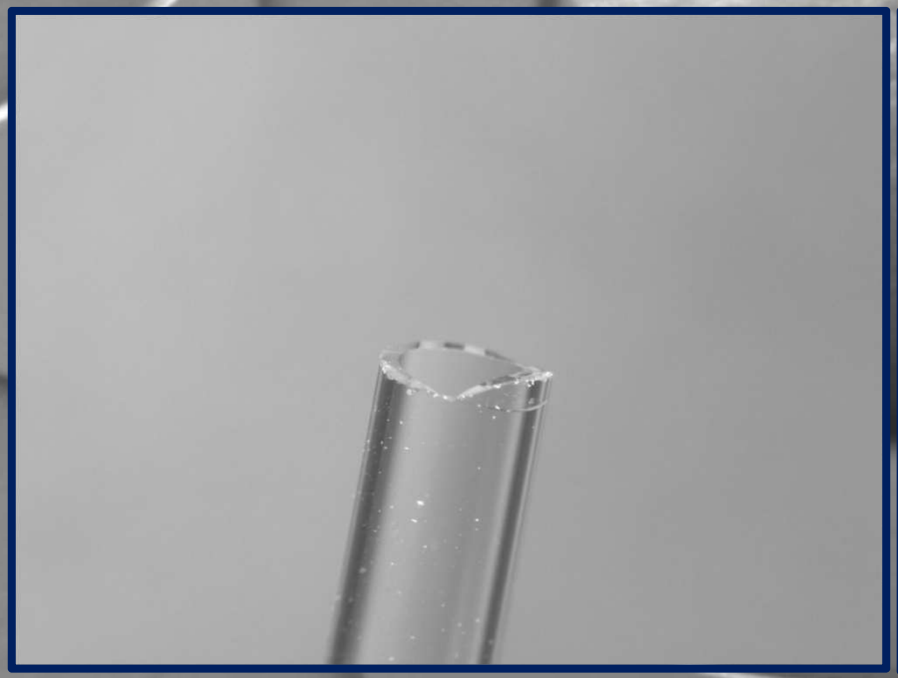
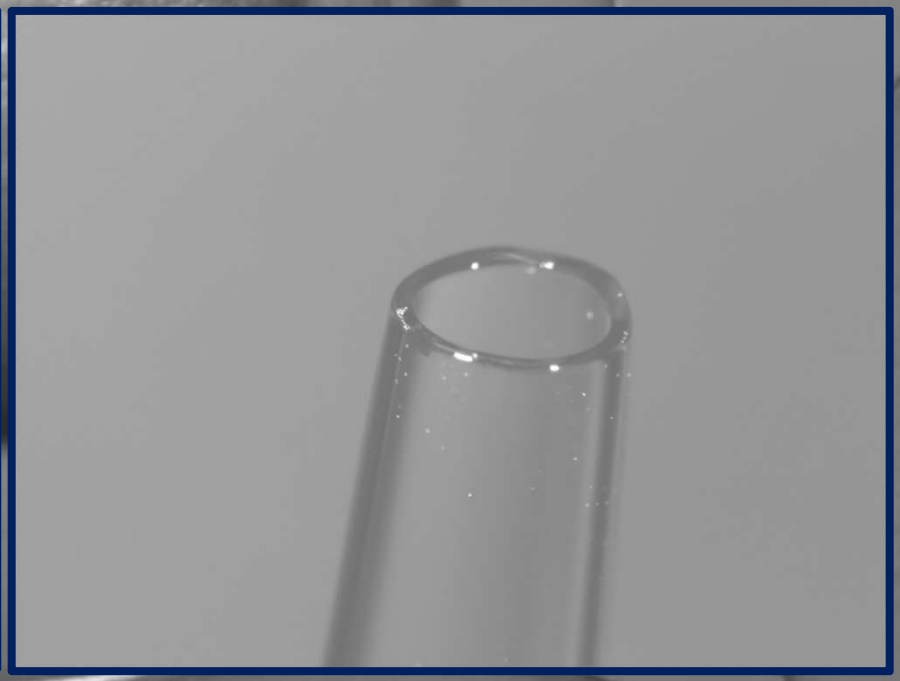
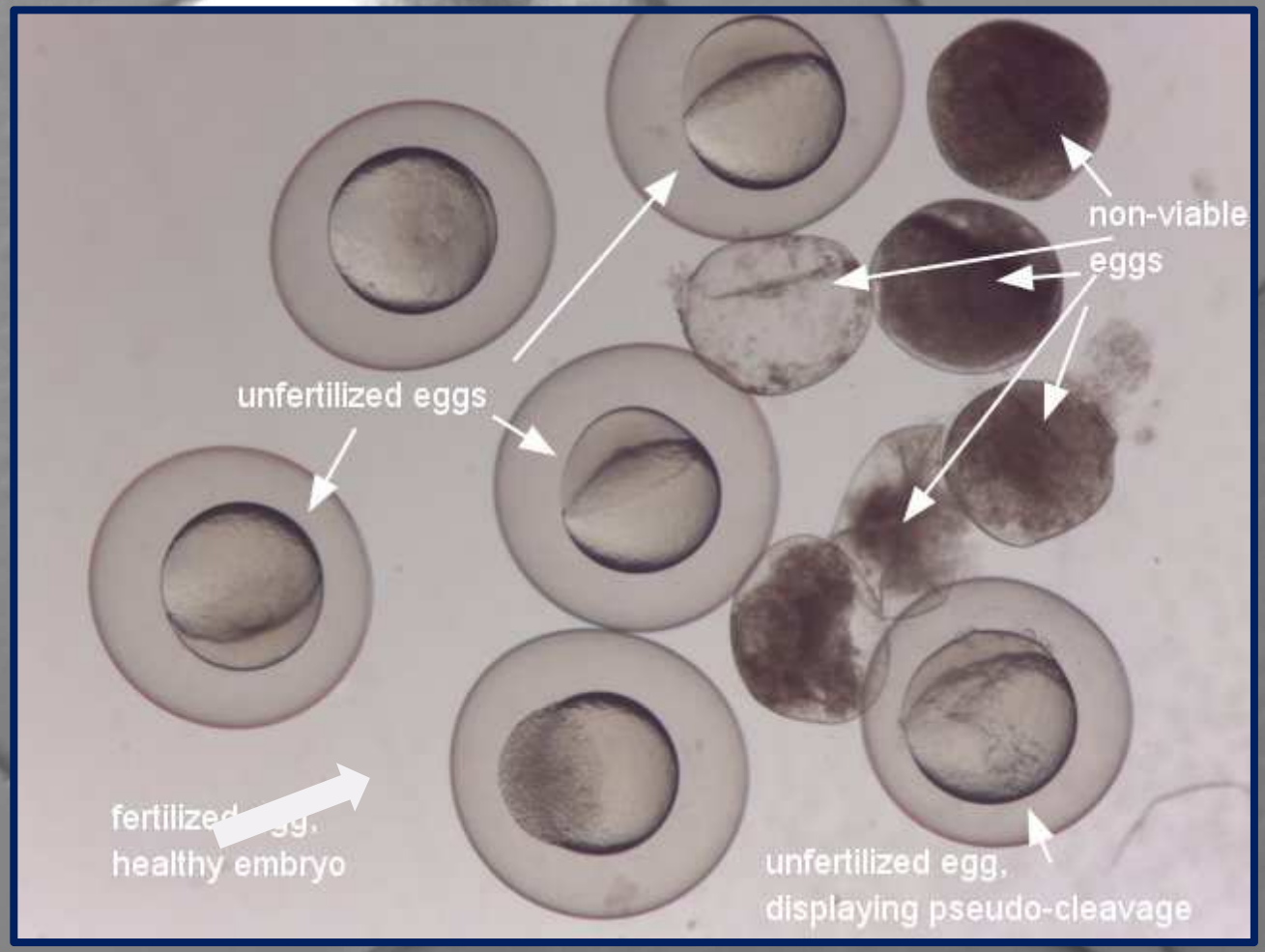




out of the box vs. fire-polished

Clean-up of embryos is critical to success

0-dpf cleanup of embryos



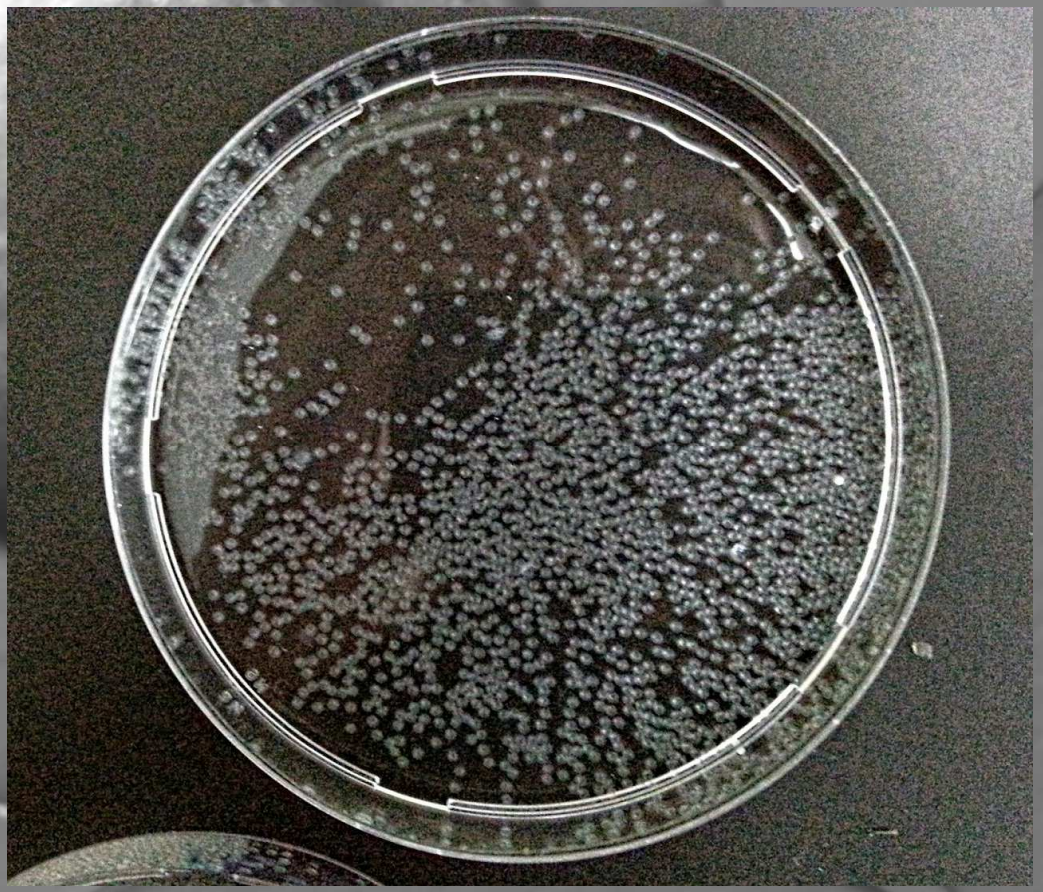
The first 24-hrs

What actually happens to those non-fertilized embryos and those that die of normal attrition?

21-hrs of development in 24-seconds
Time-lapse in 5-min intervals.
Start: 11:42AM End: 08:22AM

Recommended storage density of embryos:

- 50-embryos per Petri dish (~50ml)

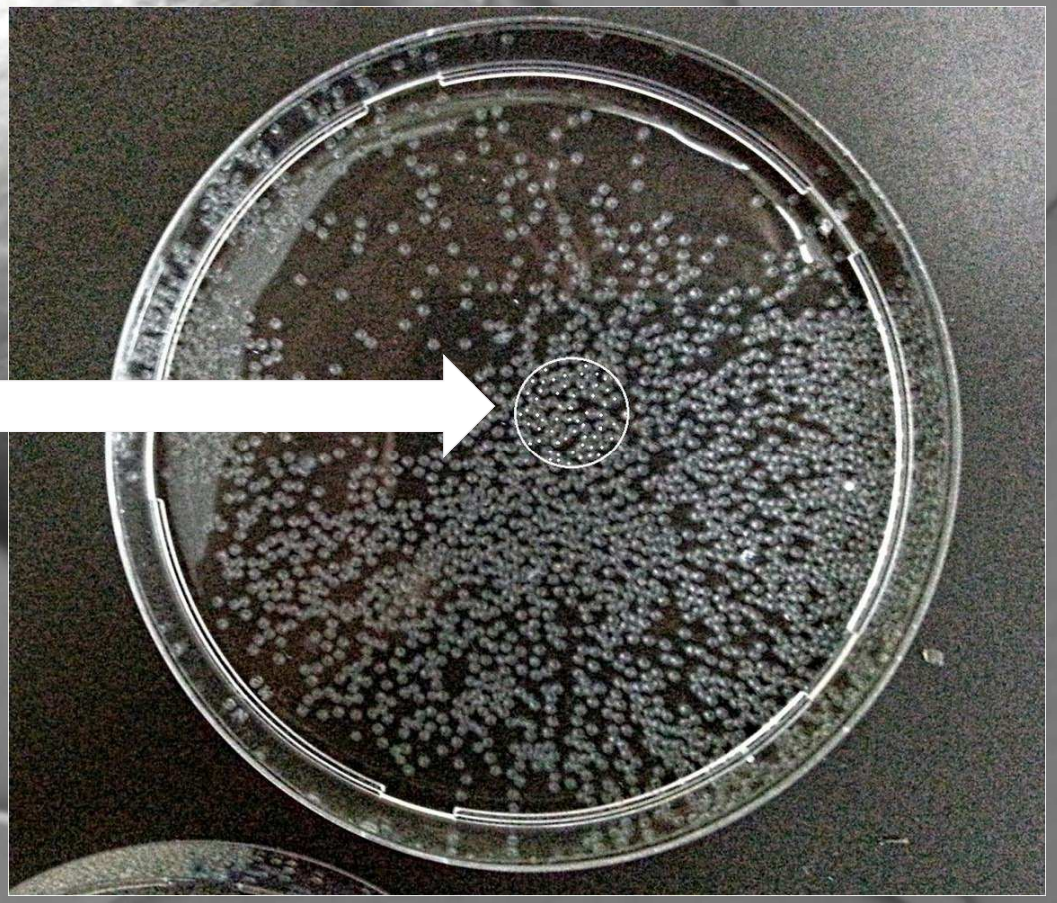


The first 24-hrs

Recommended storage density of embryos:

- 50-embryos per Petri dish (~50ml)

THIS is what 50-embryos looks like



good embryo care is critical for success

- Best practice is to move healthy embryos to a new dish with fresh media
- Use of Methylene blue is most common anti-fungal used in zebrafish culture
  - Very little is required
  - Too much can stain tissues and interfere with imaging and in-situ results
  - Not needed after chorion detritus is removed
  - Is detrimental to live feeds if transferred into container for first-feeding

The first 24-hrs through day 3

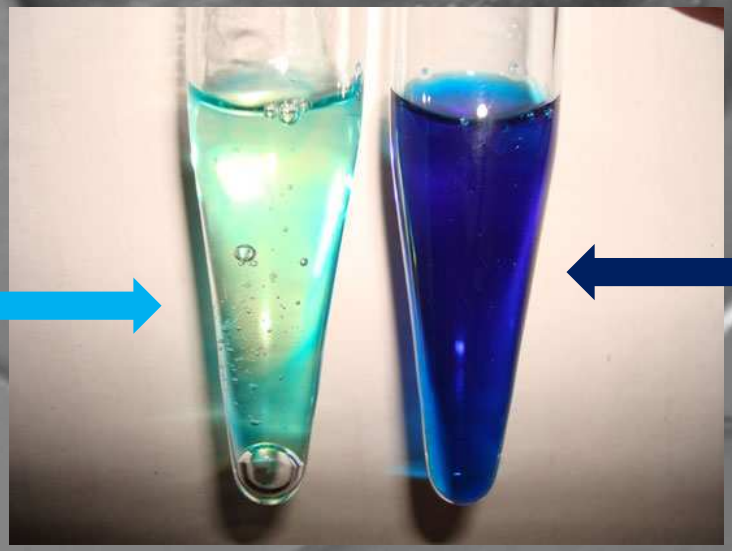
Methylene Blue Stock solution

- 1 g methylene blue powder ***(M9140 SIGMA-ALDRICH)***
- 1 L Reverse Osmosis or suitably pure water

Store at Room temperature

Only 1 to 2 drops of the stock solution is needed for each Petri dish of embryos!

Less is more!



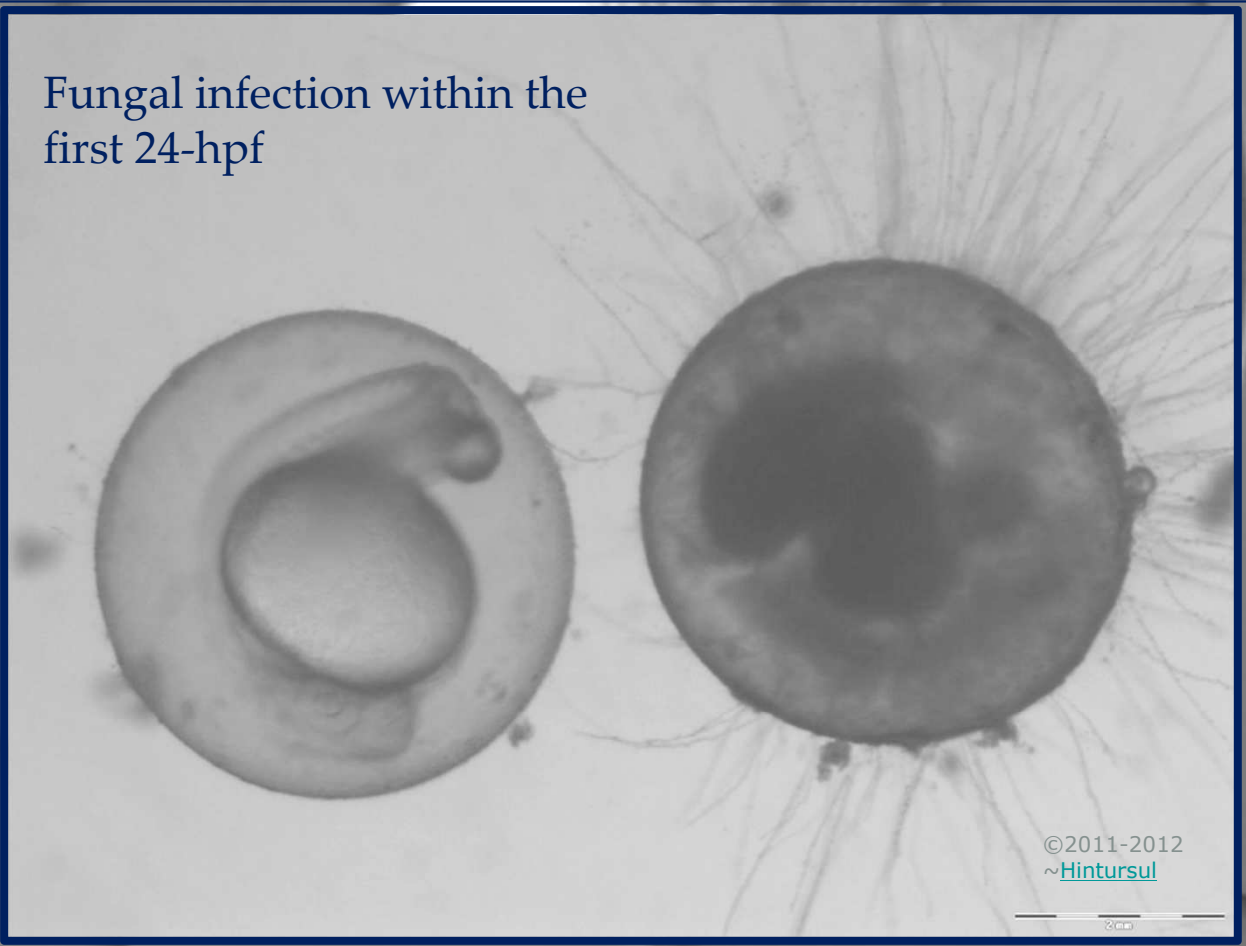
Much too much!

Consequences of failing to do a good job?

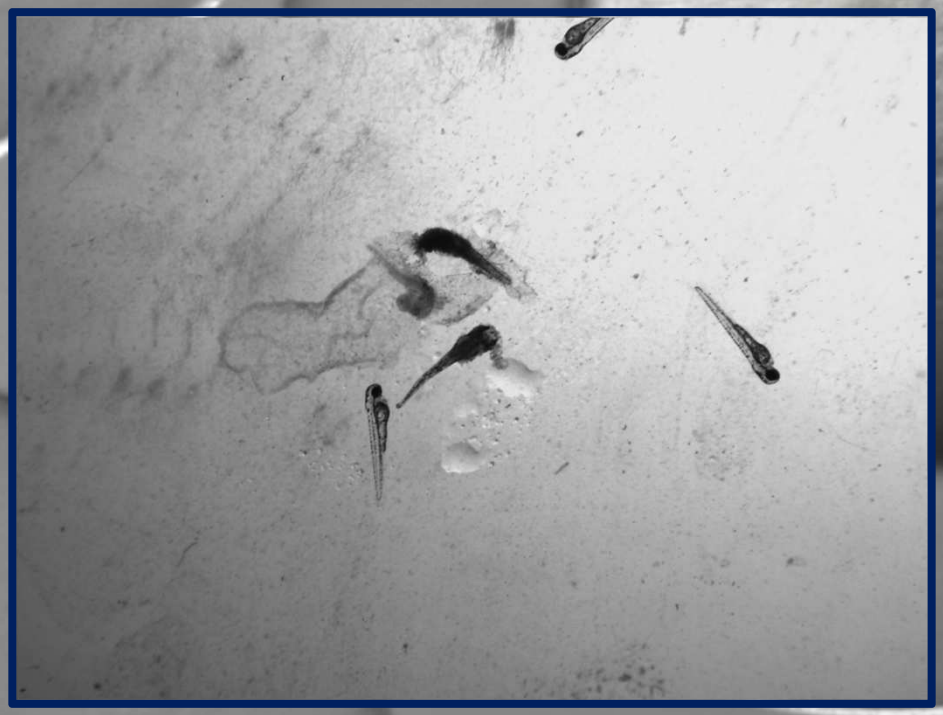
- Fungi – serious losses of embryos
- Fouling of water in dish resulting in serious losses- can extend beyond a single dish!
- Protozoan blooms
- hypoxia

What happens if fungi is not prevented (you don't use methylene blue)?

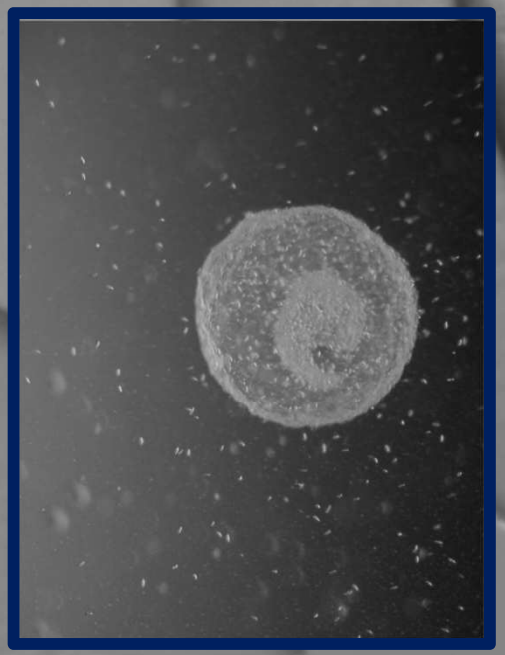
Fungal infection within the first 24-hpf



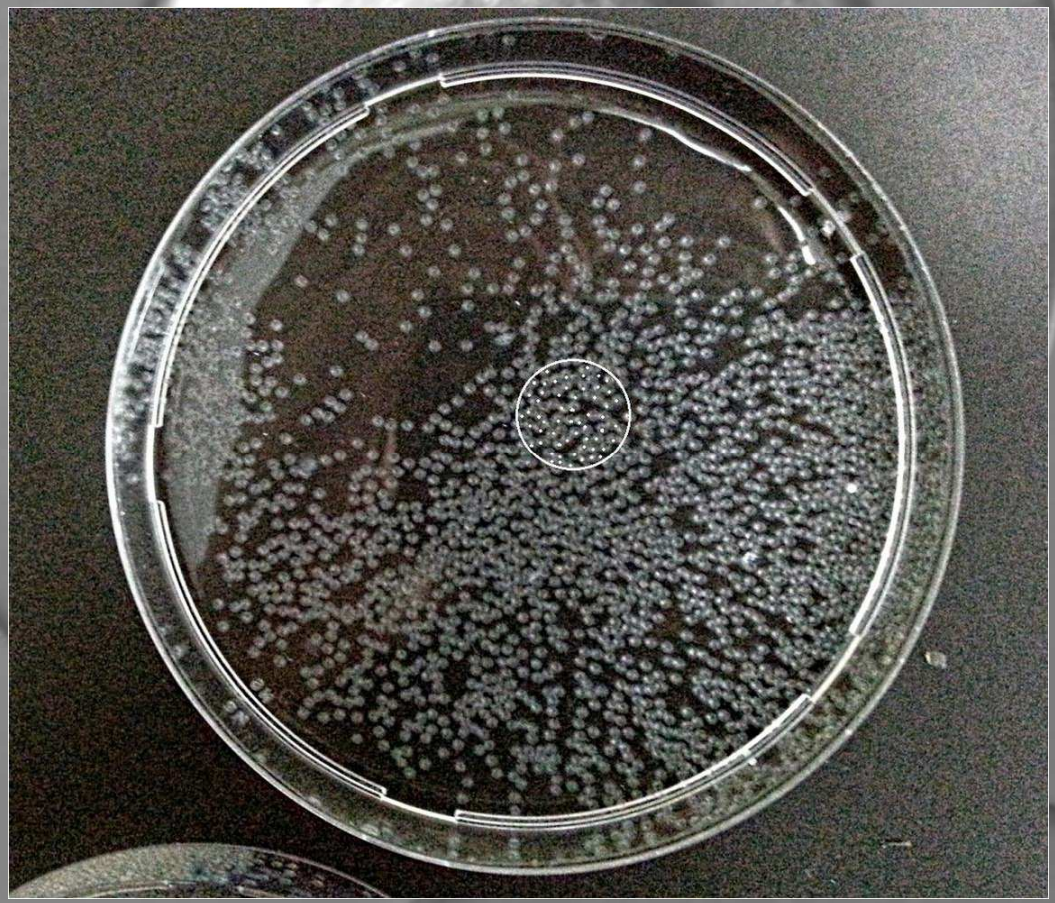
Look familiar?

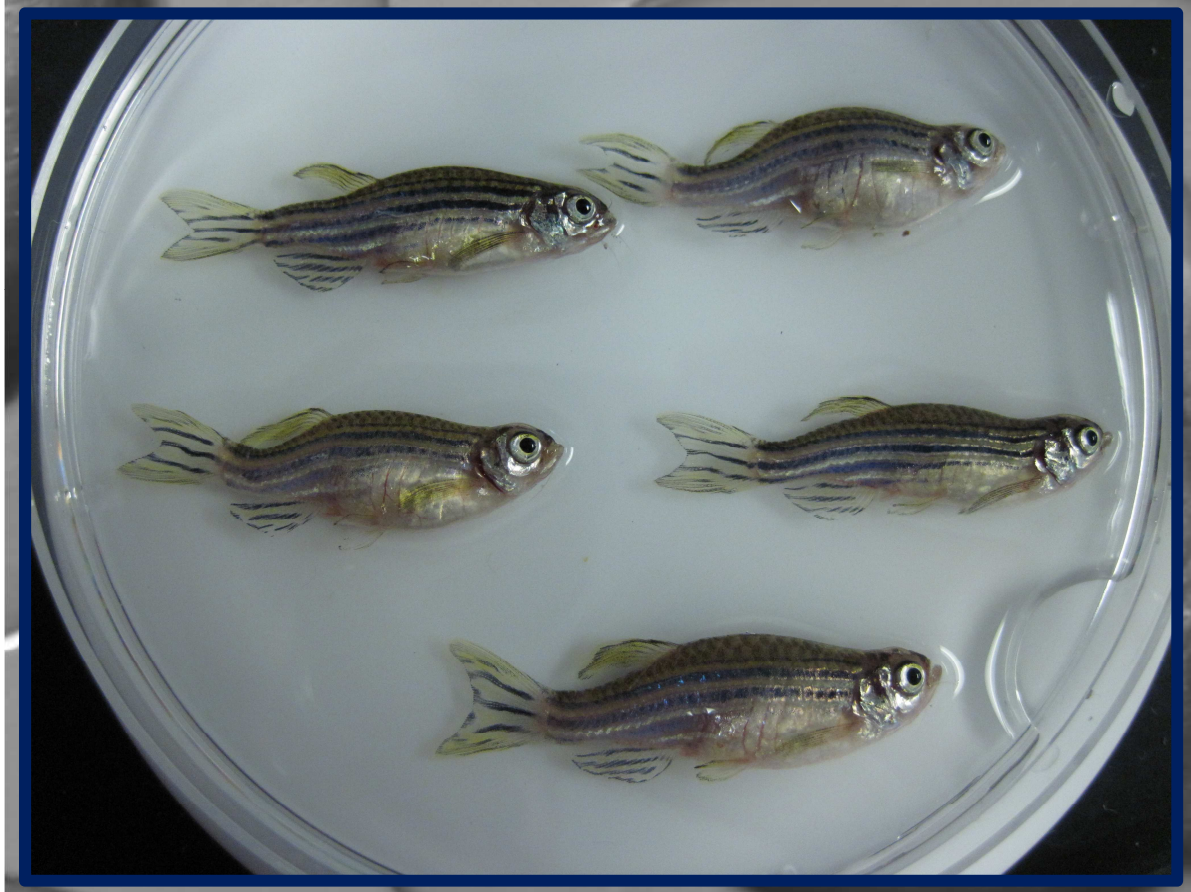


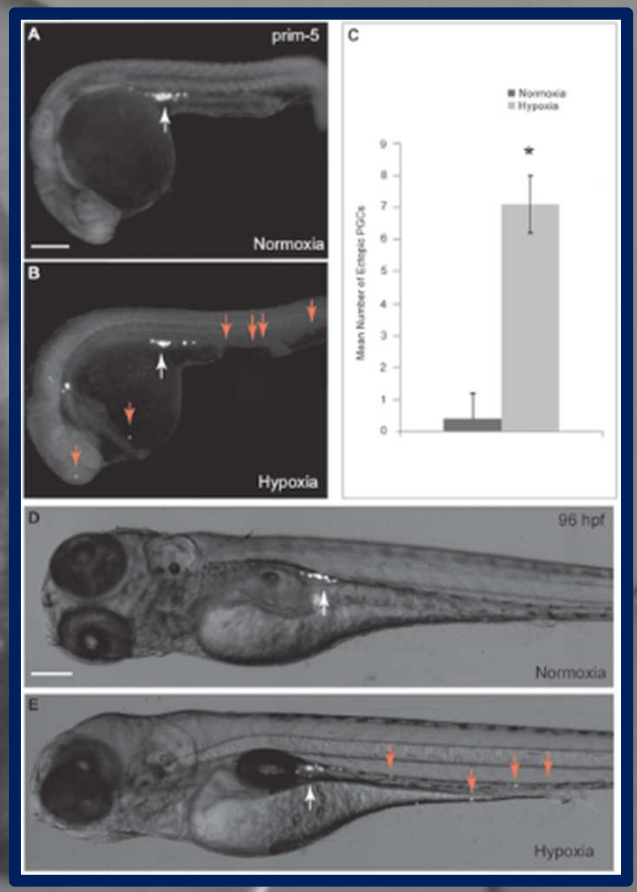
- ***Coleps***
  - feeds on bacteria, algae, flagellates, living and dead ciliates, animal and plant tissues.
  - *Coleps* uses toxicysts, poison it carries to capture its prey from its oral area.
  - extrudes tube-like structures to force toxicysts into its prey and wait until its prey becomes paralyzed.
  - These toxicysts take 5–10 minutes to be effective on the prey of the *Coleps* and it separates itself from the prey during this time.
  - If there are numerous *Coleps* hunting for the same prey, some *Coleps* will cling to its prey until the toxicysts become effective and fragment the prey, consuming only few parts



What can happen if you crowd the embryos?







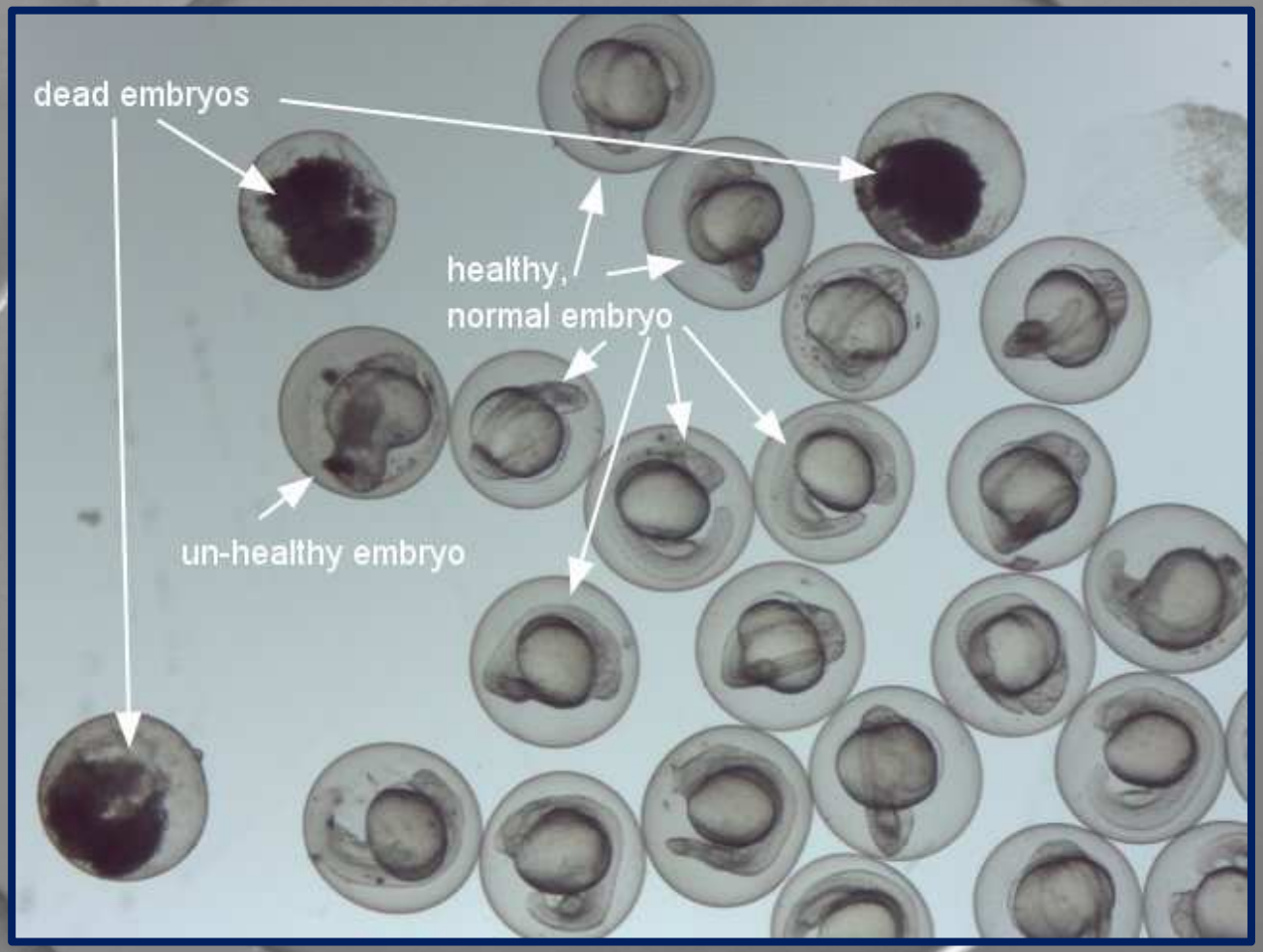
good embryo care is critical

hypoxia

Effects of hypoxia are wide-ranging. From developmental retardation and abnormalities to primordial germ cell migration defects, and disruption of pathfinding of forebrain neurons. more than +25.7K hits on Google scholar “zebrafish hypoxia”

Day 2

Approx
. 24-hpf



Continued, diligent cleanup
Move embryos to new dishes

48-hpf to 72-hpf

Continued, diligent cleanup of embryos

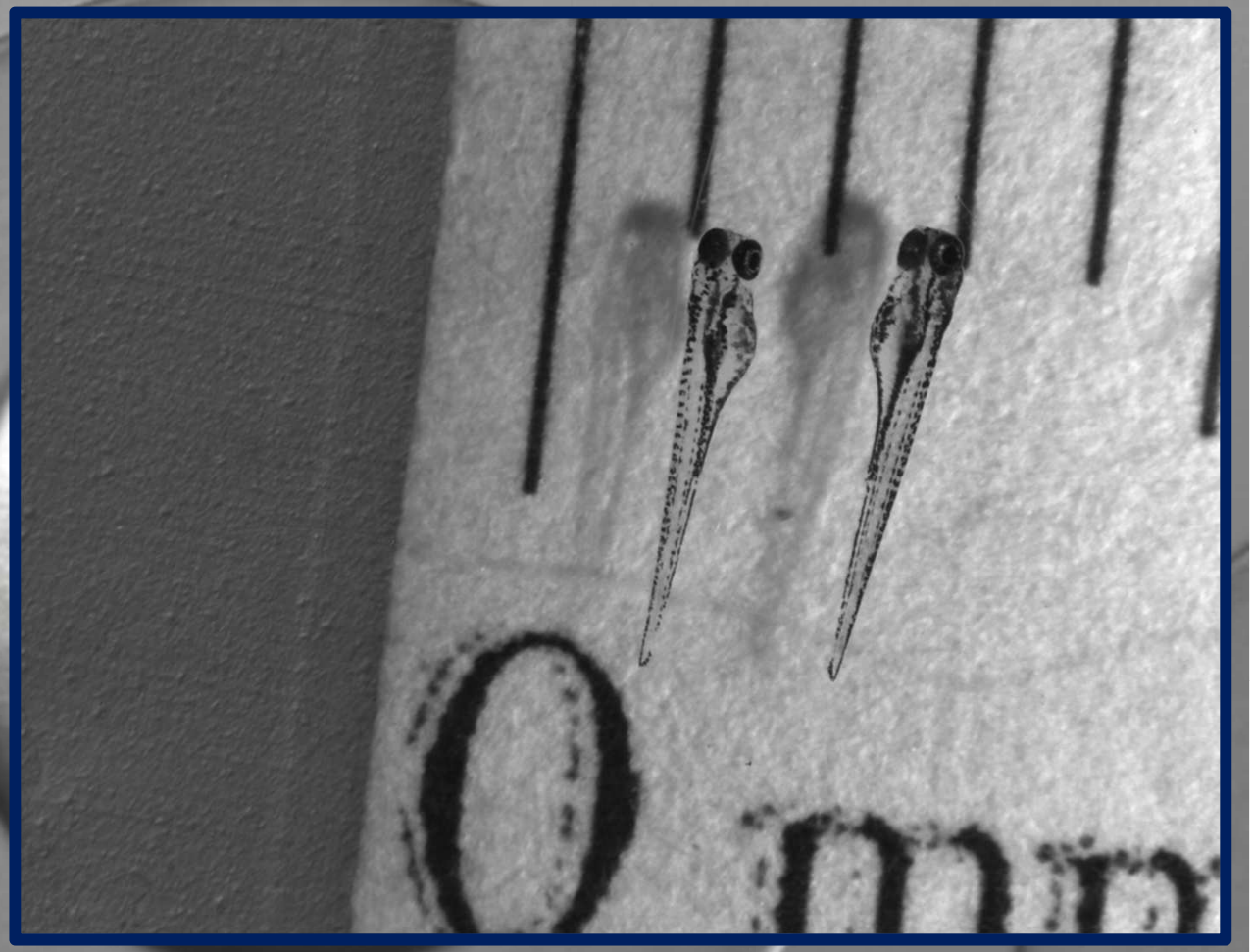
Approx 48-hpf

Continued, diligent cleanup

Move embryos to new dishes

Approx. 72-hpf

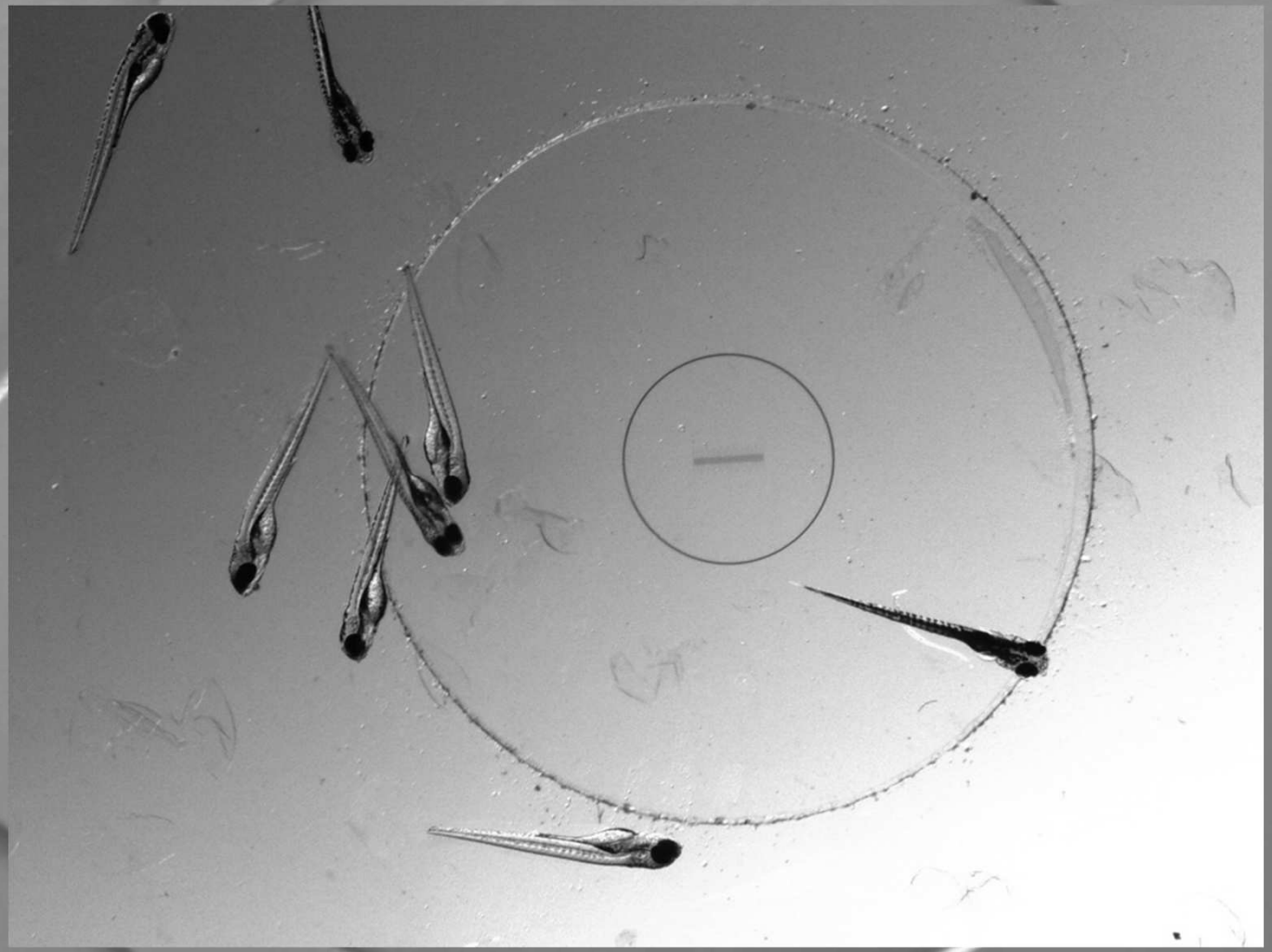
Continued, diligent cleanup
Move embryos to new dishes



clear definitions

embryo & larvae

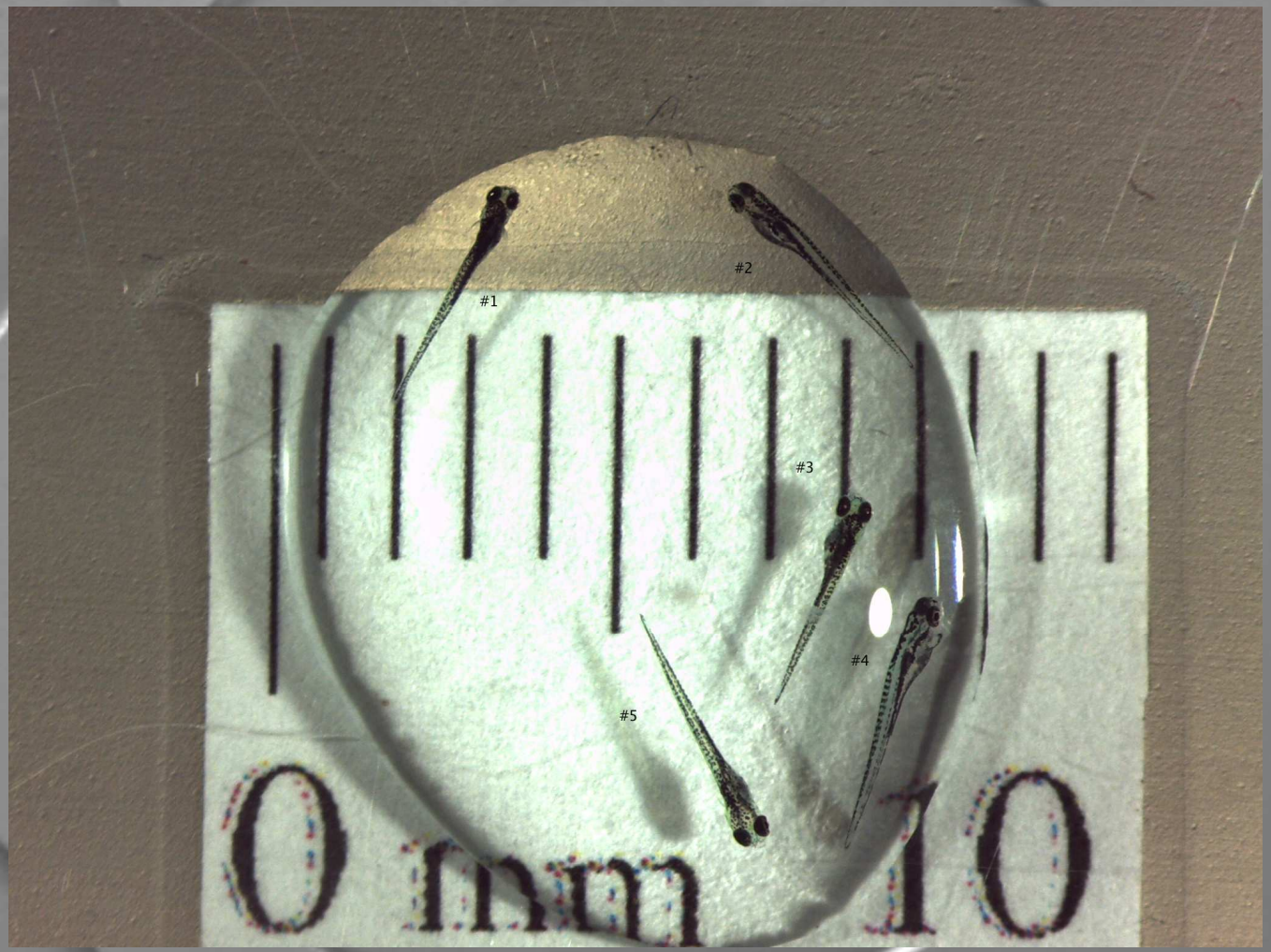
Approx. 4-dpf



Continued, diligent cleanup of embryos

Move larvae to new dishes!

Approx. 5-dpf



Continued, diligent cleanup of embryos. Move larvae to new dishes!

Days 4 and 5

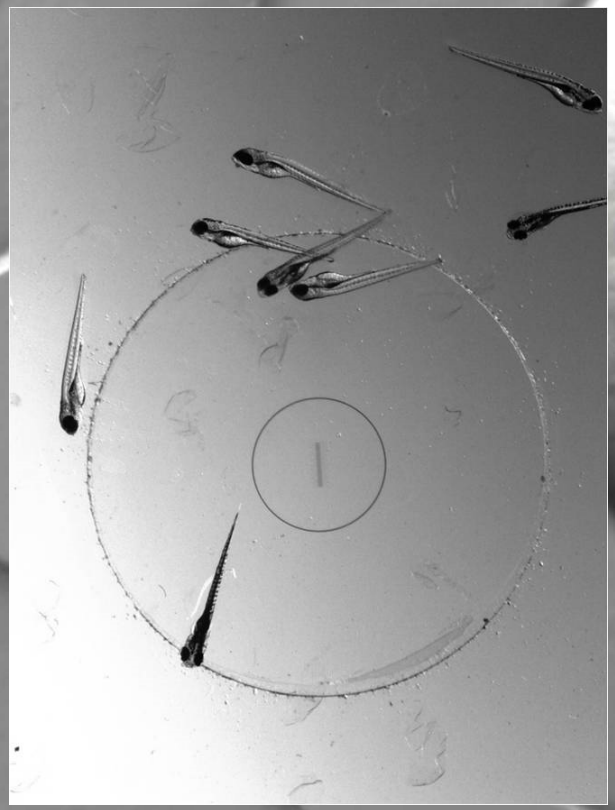
When to start feeding??

time is not the best or absolute answer

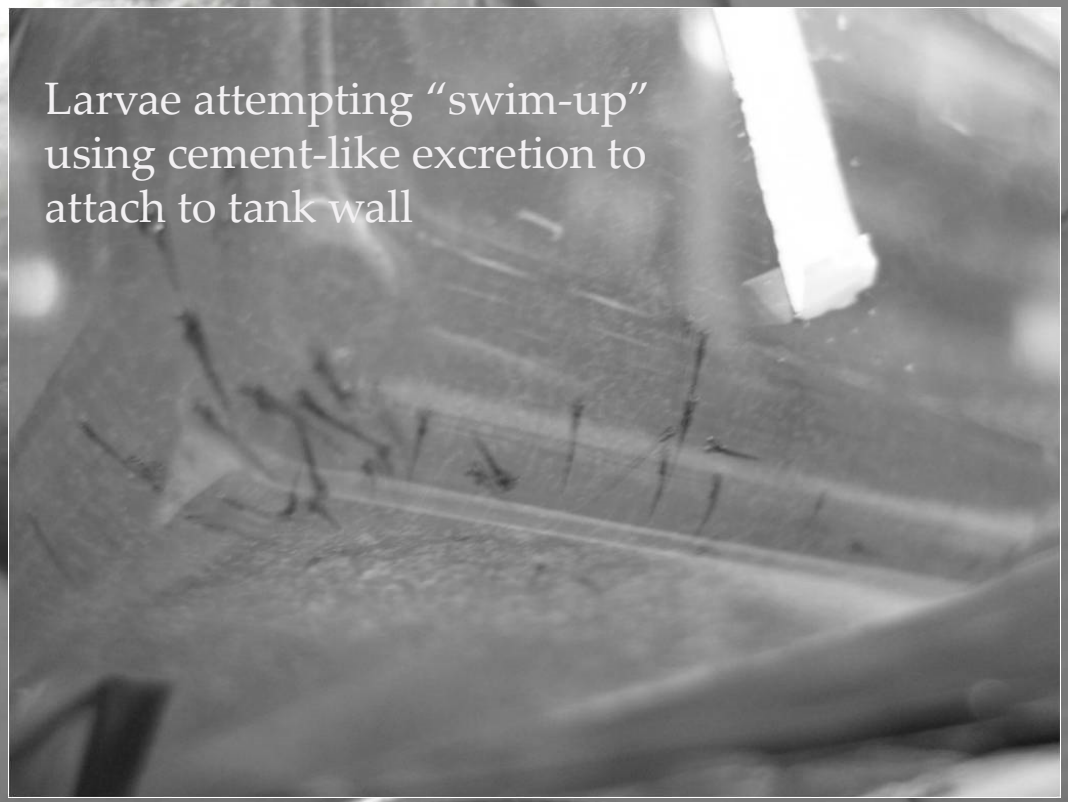
- *Some fish lines exhibit delayed gas-bladder inflation (examples: casper, albino)*
- *+75% of larvae should be swimming in water column and have inflated gas bladders before offering food*

Days 4 and 5

When to start feeding??



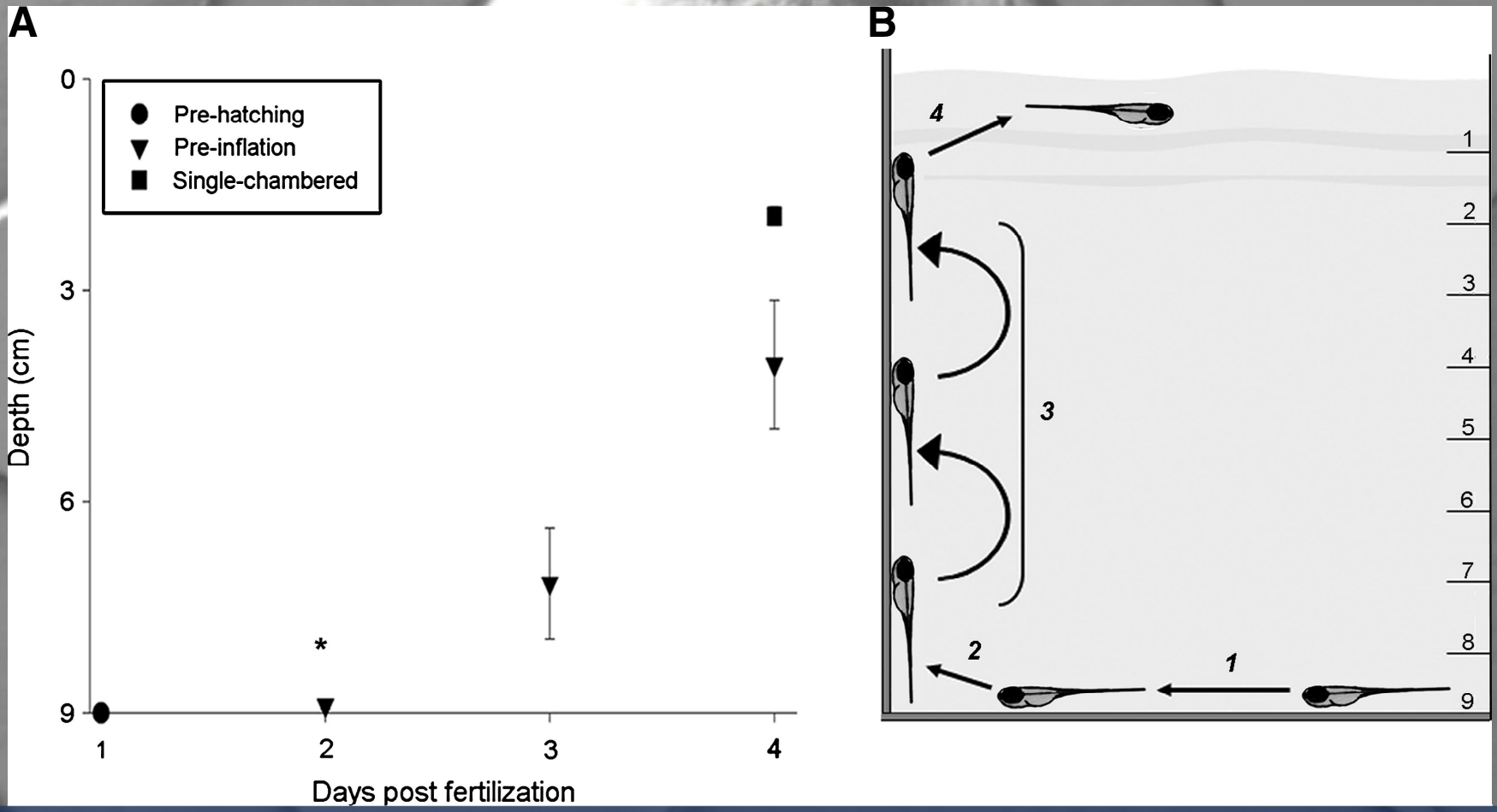
Too early

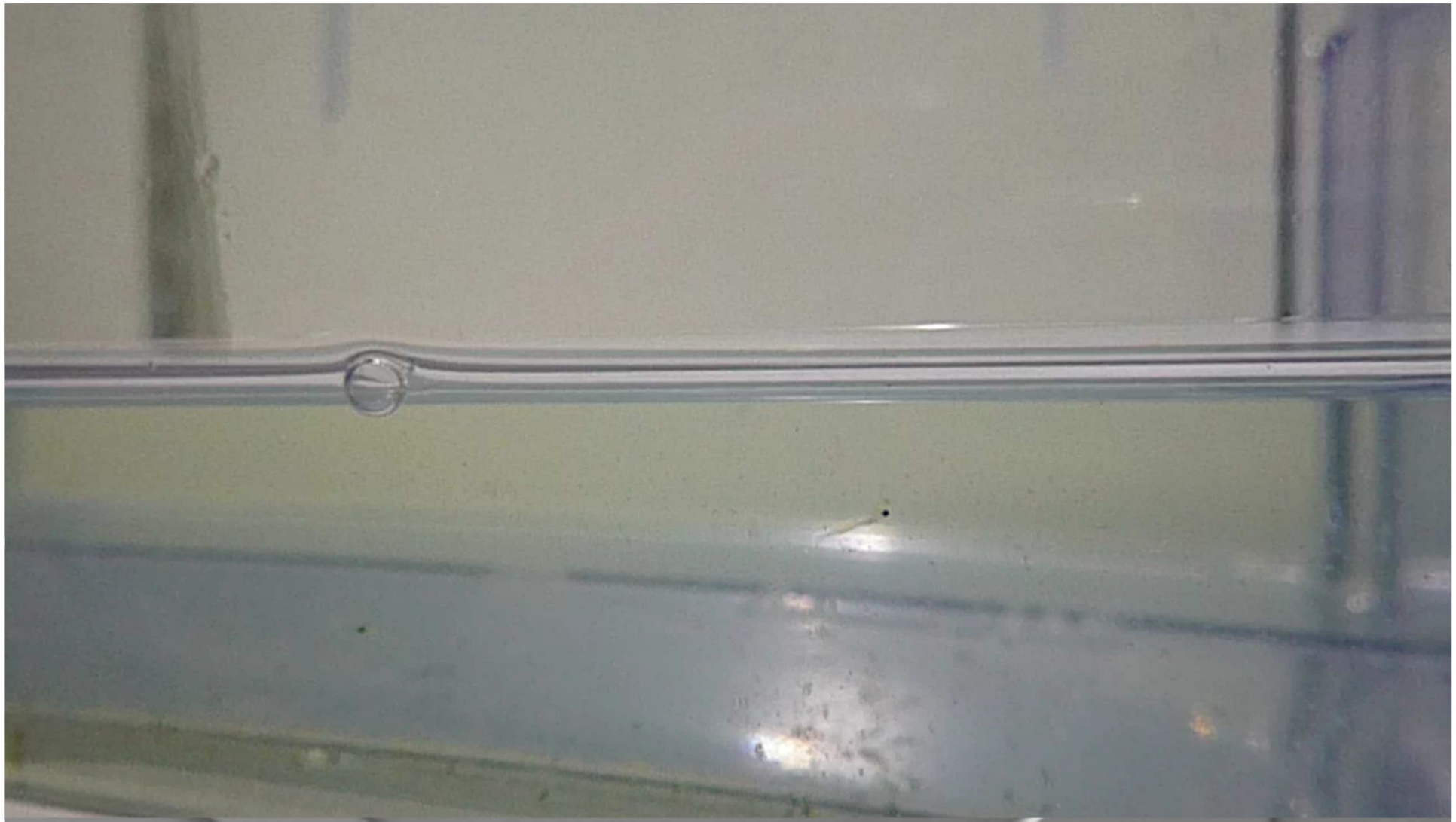


and too much water

Days 4 and 5

Understanding swim-up

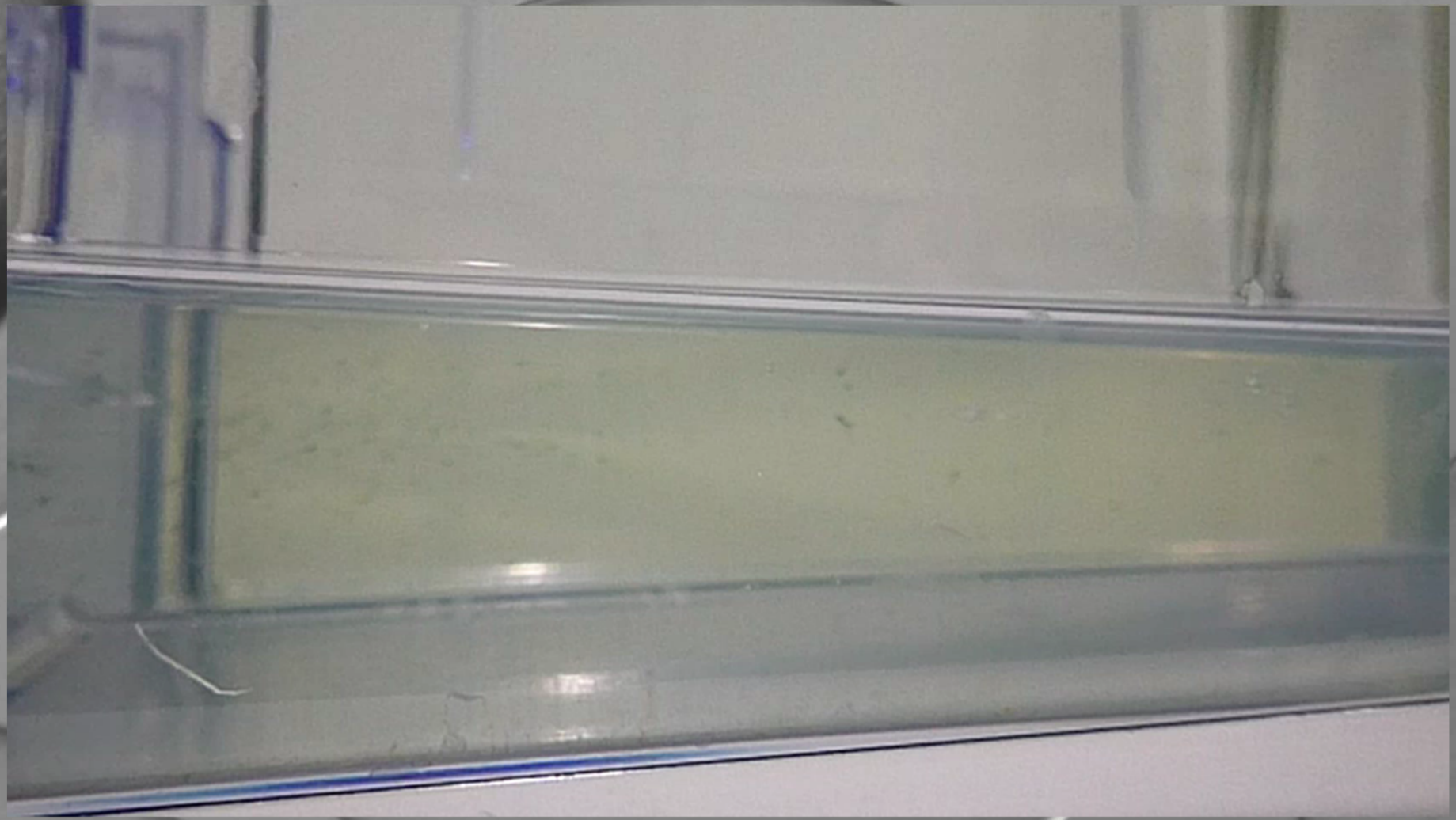




Too early

Without inflated gas-bladders, the fish will fail to feed and survive

4-dpf



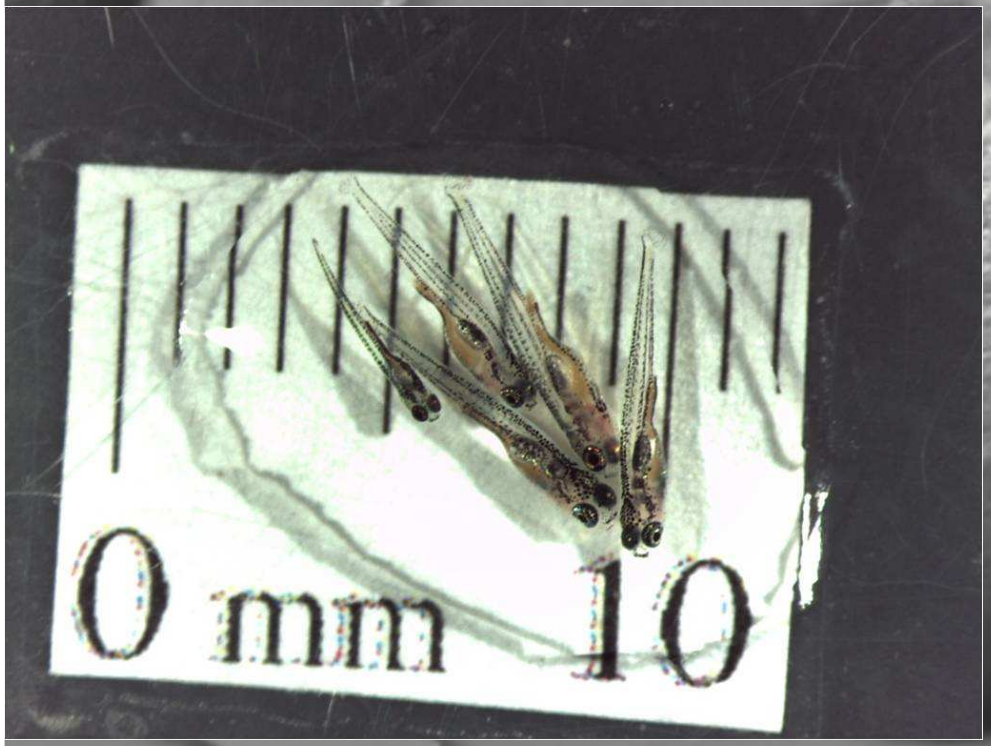
Just right

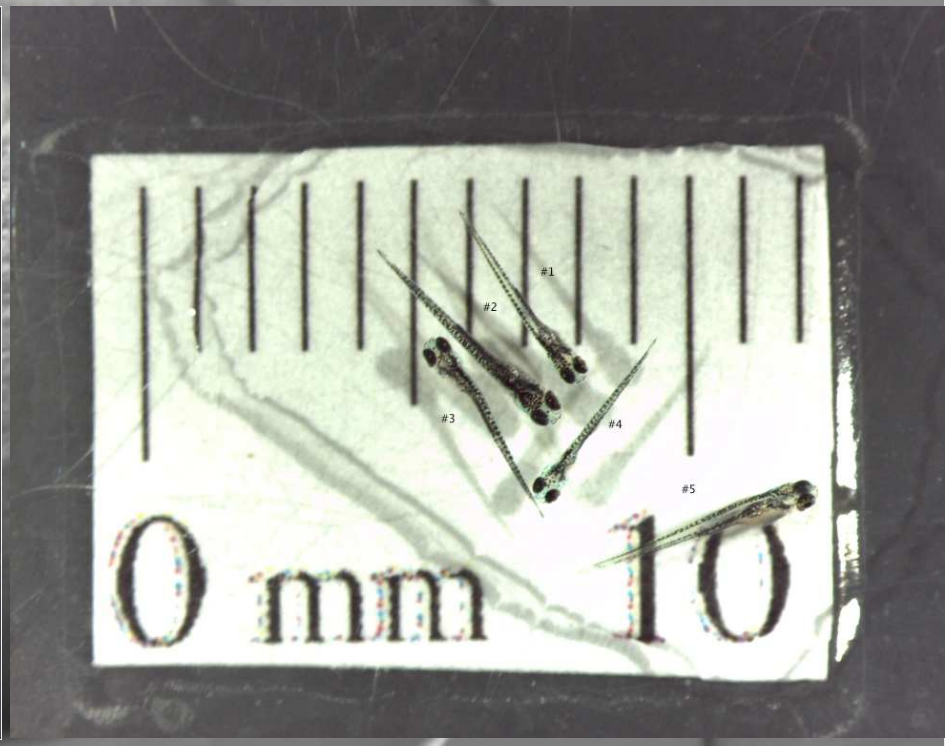
these fish have inflated gas-bladders and are capable of swimming in all levels of the water column.

5-dpf

these fish have inflated gas-bladders and are capable of swimming in all levels of the water column.

When to start feeding??

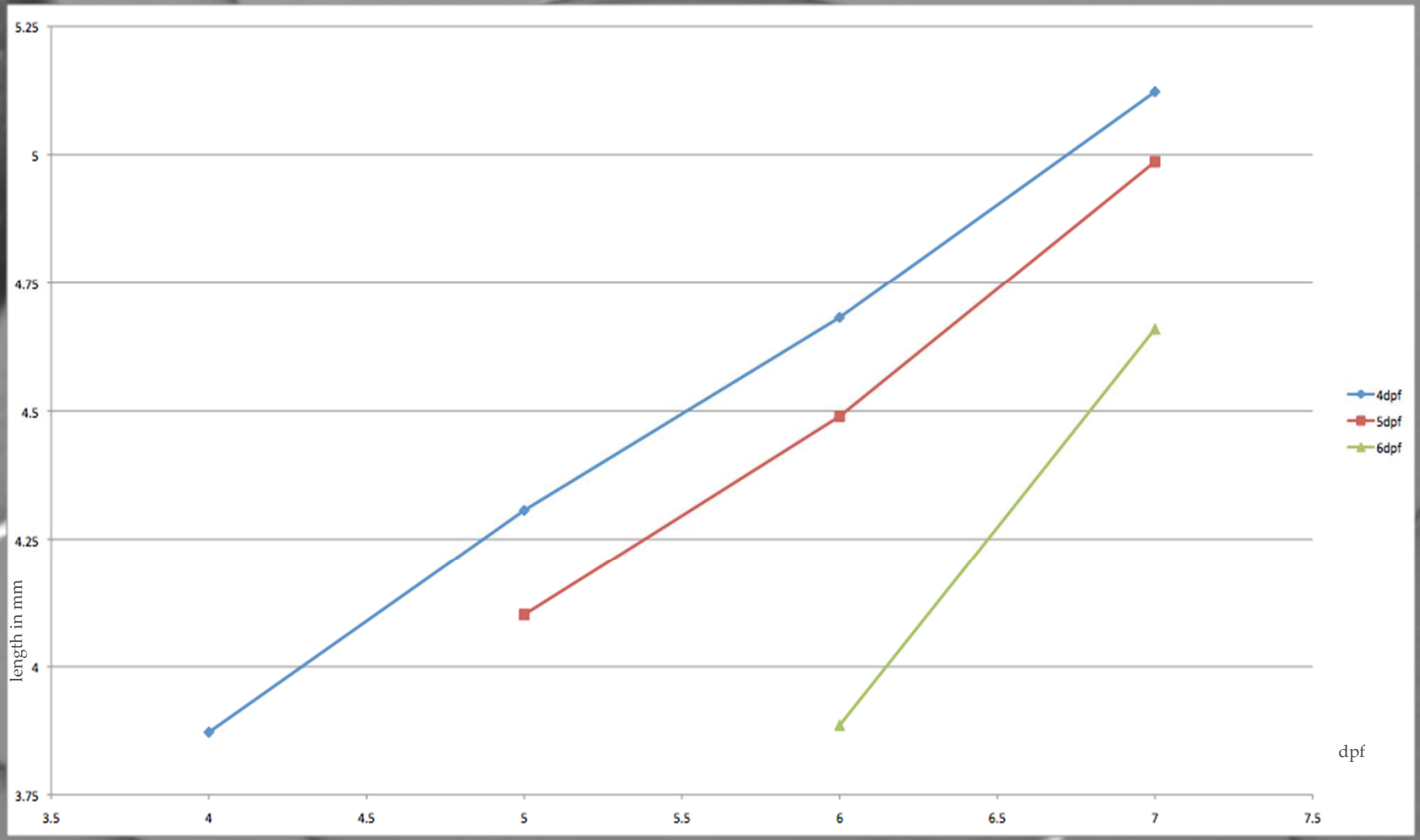




Waiting too long can be disasterous

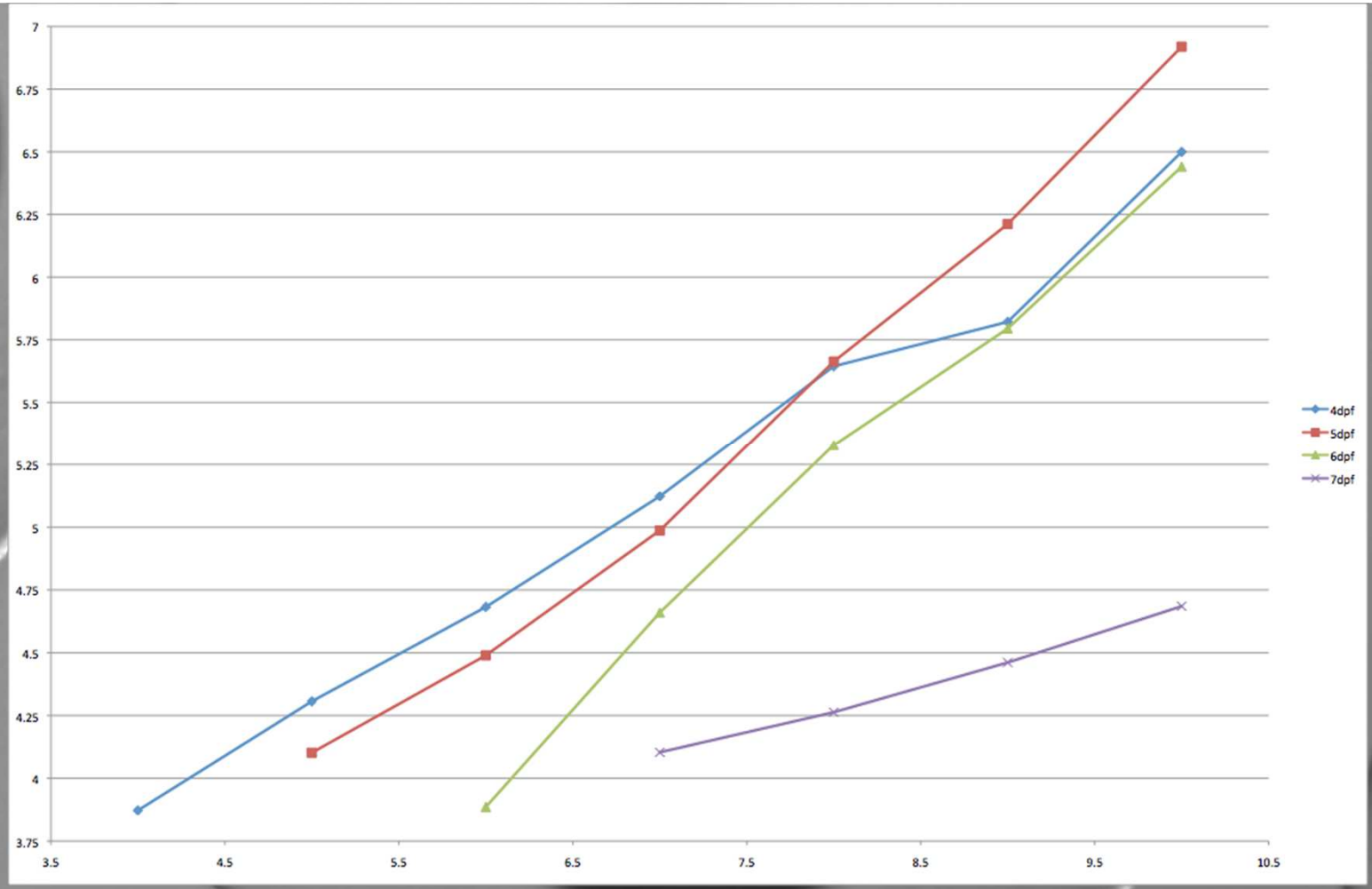
both are: 10dpf , same cluteh

the left, offered food (rotifers) at 4pdf, on the right offered food at 7dpf



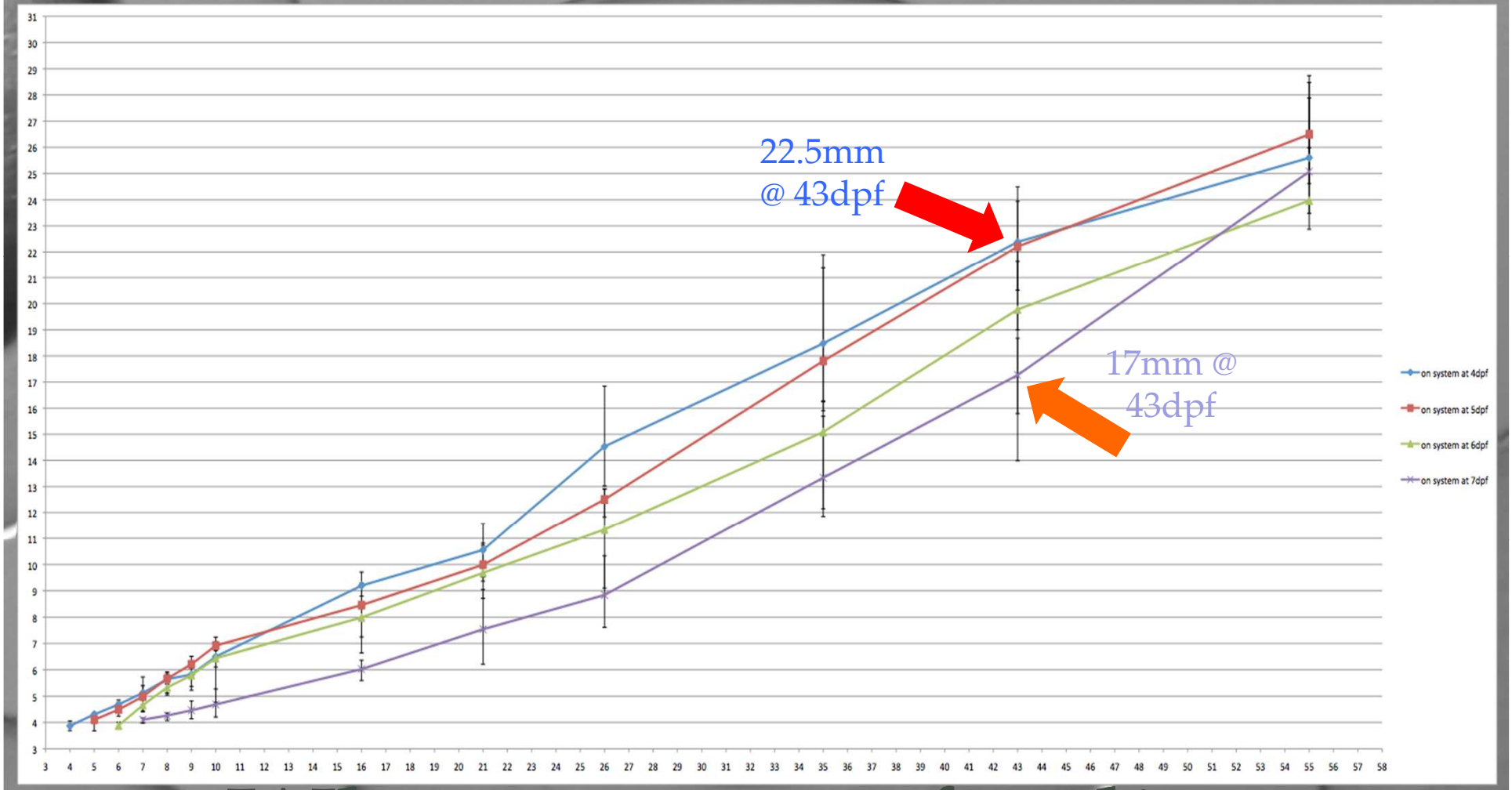
When to start feeding

time isn't the best or absolute answer



When to start feeding

time isn't the best or absolute answer



When to start feeding

time isn't the best or absolute answer

feed choices *live diets*

Artemia

- inferior due to large size- most spp. are larger than gape/buccal cavity of larval zebrafish at 5-7dpf.
- "good" nutritional profile (high lipids, protein)
- good swimming (prey) behaviour
- expensive to procure, with costs ever-increasing
- amenable to production (from cysts), not easy or cost effective to culture in the lab
- wild-caught product. must be sanitized to ensure bio-security
- must be decapsulated to remove chitinous shell/capsule- more work and $$$

Paramecia

- adequate size for larval zebrafish mouth/buccal cavity
- inferior nutritional profile (compared to artemia and rotifers)
- less than ideal swimming (prey) behaviour
- inexpensive starter cultures available (unknown health status)
- amenable to lab culture, but require large footprint compared to harvest yield (STINKY!)
- known to enhance transmission of mycobacterium (Peterson et al)

Rotifers (branchionous spp.)

- ideal size for larval zebrafish mouth/buccal cavity
- excellent nutritional profile when enriched or "gut-loaded" with micro-algae such as nannochloropsis
- excellent swimming (prey") behavoiour
- inexpensive starter cultures available (known health status)
- amenable to lab culture and scalable, with relatively small footprint when compared to harvest yield
- perhaps the predominant first-feed live organism for zebrafish currently

feed choices *prepared diets*

aquaculture feeds vs. hobbyist feeds

- more digestible protein sources and bio-availability
- fatty acid profiles tuned to warm-water vs. cold-water fish
- carotenoids (antioxidants)
- differing buoyancies (dispersal differences) –highly engineered!
- traceability
- extruded micropellets/agglomerations > crumble/mash > flake
- extreme care must be excersied in the proper storage of prepared diets!

Leaching of water soluble vitamins from feeds upon hydration

TABLE 2

Effect of vitamin leaching from experimental diet 2 after a period of 30 s in water (n = 1)

Vitamin	Concentration before contact with water	Percentage vitamin loss in 30 s
	mg · kg dry diet^{-1}	
Pyridoxine	49	6.1
Pteroyl-monoglutamic acid	25	16.0
Choline	3700	27.0
Pantotenic acid	76	47.4
Ascorbic acid	470	66.0
Cyanocobalamin	0.6	90.0

From Pannevis and Earle, *J. Nutrition 1994*

Leaching of water soluble protein from feeds upon hydration

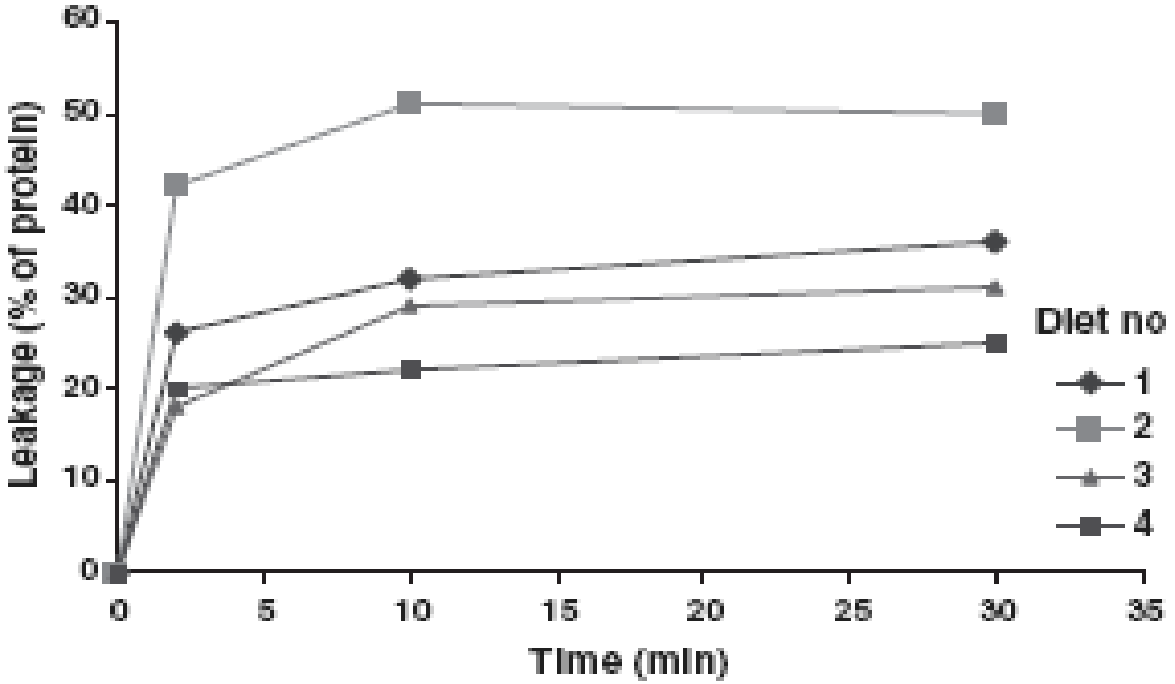


Figure 1 Leaching of crude protein (%) from formulated larval feeds. Diets 1 and 4 are experimental feeds, diet 2 and 3 are commercial feeds. All diets were micro-bound. Leaching was measured by incubating 1 g of diet in 100 mL seawater for variable time intervals. Protein leached to the water was measured as N × 6.25 after filtration and partly evaporation of the water phase (Hamre 2006).

From Kvale et al. , *Aquac. Nutrit.*, 2007

Feed application techniques

prepared diets

top fed (dry)-

- superior
- The way these feeds are designed to be used
- manual dispensers (metered-dose)
- automated feeders

Liquified- or mixed into water:

- Poorest choice
- water soluble vitamins are gone before you get it to the fish

Effect of temperature on lipids in feeds

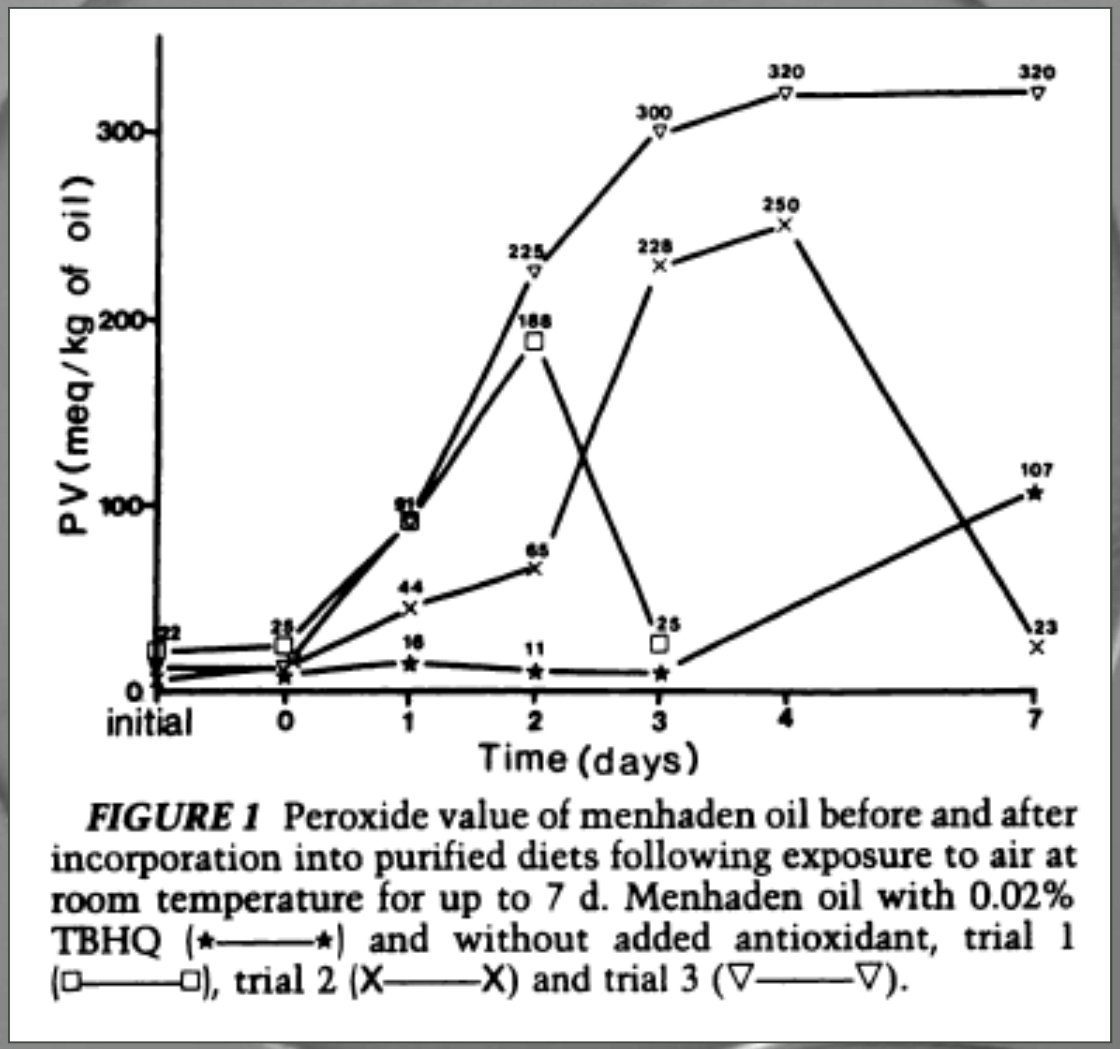
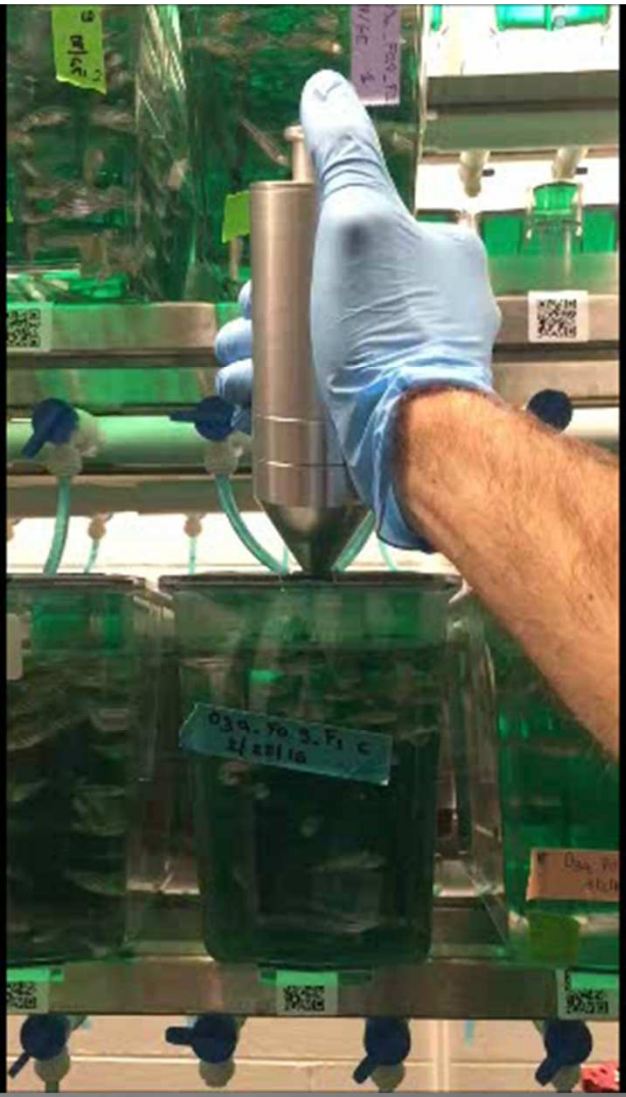


FIGURE 1 Peroxide value of menhaden oil before and after incorporation into purified diets following exposure to air at room temperature for up to 7 d. Menhaden oil with 0.02% TBHQ (★——★) and without added antioxidant, trial 1 (□——□), trial 2 (X——X) and trial 3 (▽——▽).

From Fritsche and Johnston, *J. Nutrit.* 1988



DanioLab manual feeder

Tecniplast Tritone Automatic Feeding System

Feed application techniques
live diets

Artemia

- must be 1st instar to be compatible with zebrafish mouth/buccal cavity size
- survival of nauplii not possible at salinities which larval zebrafish thrive
- must be offered several times per day to meet metabolic demands of larval zebrafish

Paramecium spp.

- Adequate
- Better size than most artemia spp. for first feed

Rotifers (branchionus spp.

- superior
- The way these feeds are designed to be used

How much (many) rotifers?

1) In aquaculture there is one unit of measure that is consistently used to communicate how much live feed is offered to (larval) fish- that being **prey density** (# of prey items/ml is very common when talking about microscopic organisms).

2) While related in some ways, the question I most often get from zebrafish labs is "how many rotifers should I feed my baby fish?"

How much (many) rotifers?

According to Nutrient Requirements of Fish and Shrimp, published by the National research Council of the National Academies (2011)

"Certain prey densities seem to be effective across a number of larval fish species (e.g. 10-20-rotifers per mL); however, optimum prey density may vary with the species. Ontogeny, size of prey, and culture system (Lee and Ostrowski, 2001)

How much (many) rotifers?

According to Nutrient Requirements of Fish and Shrimp, published by the National research Council of the National Academies (2011)

"The best way to determine optimal densities is to monitor both number of larval prey at intake and density of prey in the culture system to avoid under and over feeding (Palmer et al,. 2007). Underfeeding retards larval growth and development, whereas, overfeeding can result in reduced capture success and can also lower water quality…(Lee and Ostrowski, 2001)."

How much (many) rotifers?

# of tanks (3.5L) to feed rotifers to	# of L needed for feedout (30mL/tank)	# rotifers (M) needed for feedout
300	9	31.5
275	8.25	28.875
250	7.5	26.25
225	6.75	23.625
200	6	21
190	5.7	19.95
180	5.4	18.9
170	5.1	17.85
160	4.8	16.8
150	4.5	15.75
140	4.2	14.7
130	3.9	13.65
120	3.6	12.6
110	3.3	11.55
100	3	10.5
90	2.7	9.45
80	2.4	8.4
70	2.1	7.35
60	1.8	6.3
50	1.5	5.25
40	1.2	4.2
30	0.9	3.15
20	0.6	2.1
10	0.3	1.05

- ~1000-rotifers (L-type) per fish, ~30000/tank/day

- This is my "ideal" situation. Less will work fine.

- Rotifers may not need to be added to the tanks every day- sometimes the rotifers bloom in the tank, eliminating the need to add more.

If you have a method that is working for you, analyze what you have been doing and develop tables, charts, etc. to ensure consistency and accountability in reporting

Polyculture method

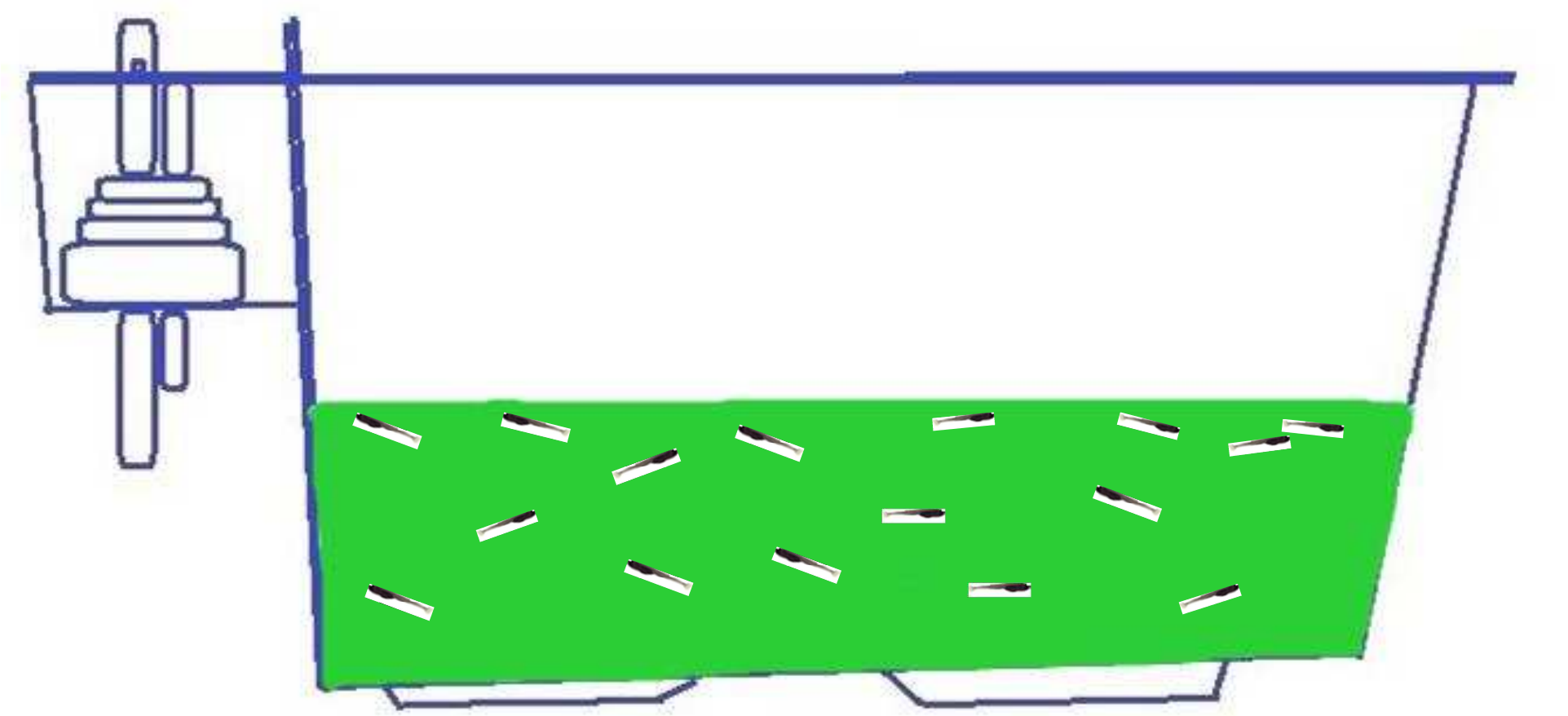
"set it & forget it"

Overview

- *Combining into one tank:*
  - *rotifers*
  - *algae (rotifer food)*
  - *Larvae*
- *Total water level changes little over time*

Polyculture method

- Larval fish 5-14dpf
  - Rotifers (1000 per larval fish/day*9days= 9000rotifers)
  - Add 2-3inches of water (at least 2ppt)



Polyculture method

- *Pros*
  - *Low level of labor and involvement*
  - *Excellent survival and growth expected*
- *Cons*
  - *may require daily addition of rotifers*
  - *may hinder view of fish until end of polyculture phase due to algae*
  - *HUGE differences between labs in terms of methods*

Polyculture method

- *HUGE differences between labs in terms of*

publication info			Rotifer Information		Statistics	
			rotifers/fish/day	Prey Density (rotifers/ml in larval rearing		
paper ref.	year published	journal	typical max	typical max	reported survival %	sample size (n=)
Lee Ostrowski,	2001	Aquaculture	1000	20	N/A	N/A
Markovich Brown	2005	WAS Abstract	3000	unknown	42	4
allen wallace sisson	2016	Zebrafish	321	6	82	2
best et al	2010	Zebrafish	2664	333	94	5
martins et al	2016	Zebrafish	2571	180	N/A	N/A
lawrence et al	2015	Zebrafish	10667	800	98	6
lawrence et al	2016	JOVE	18675	747	95	1
Hedge et al	2015	WAS Poster	2500	200	N/A	N/A
big lab	current	N/A	1000	unknown	95	N/A
Aoyama	2015	Zebrafish	153	22	91.1	9

Incremental method

- *Overview*
  - *Combining into one tank:*
    - *Rotifers (gut-loaded with algae)*
    - *Larval fish*

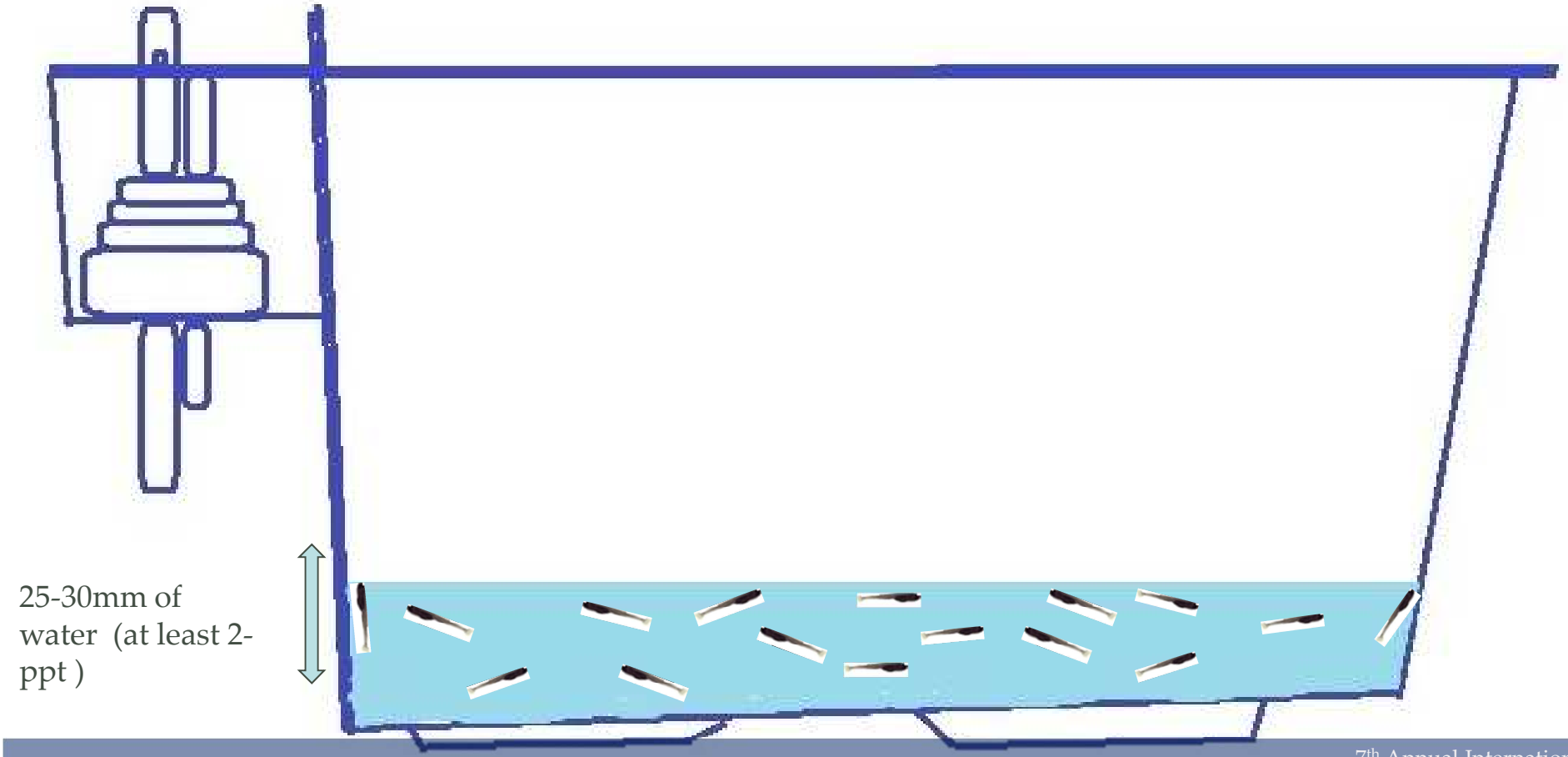
Incremental method

- *Pros*
  - *Excellent growth and survival expected*
  - *More consistent prey density*
  - *Fewer rotifers needed each day*
  - *Larval fish are visible at all times*
- *Cons*
  - *requires daily addition of rotifers*
  - *Additional level of labor and involvement*
  - *Requires regular addition of water to culture*

Incremental method

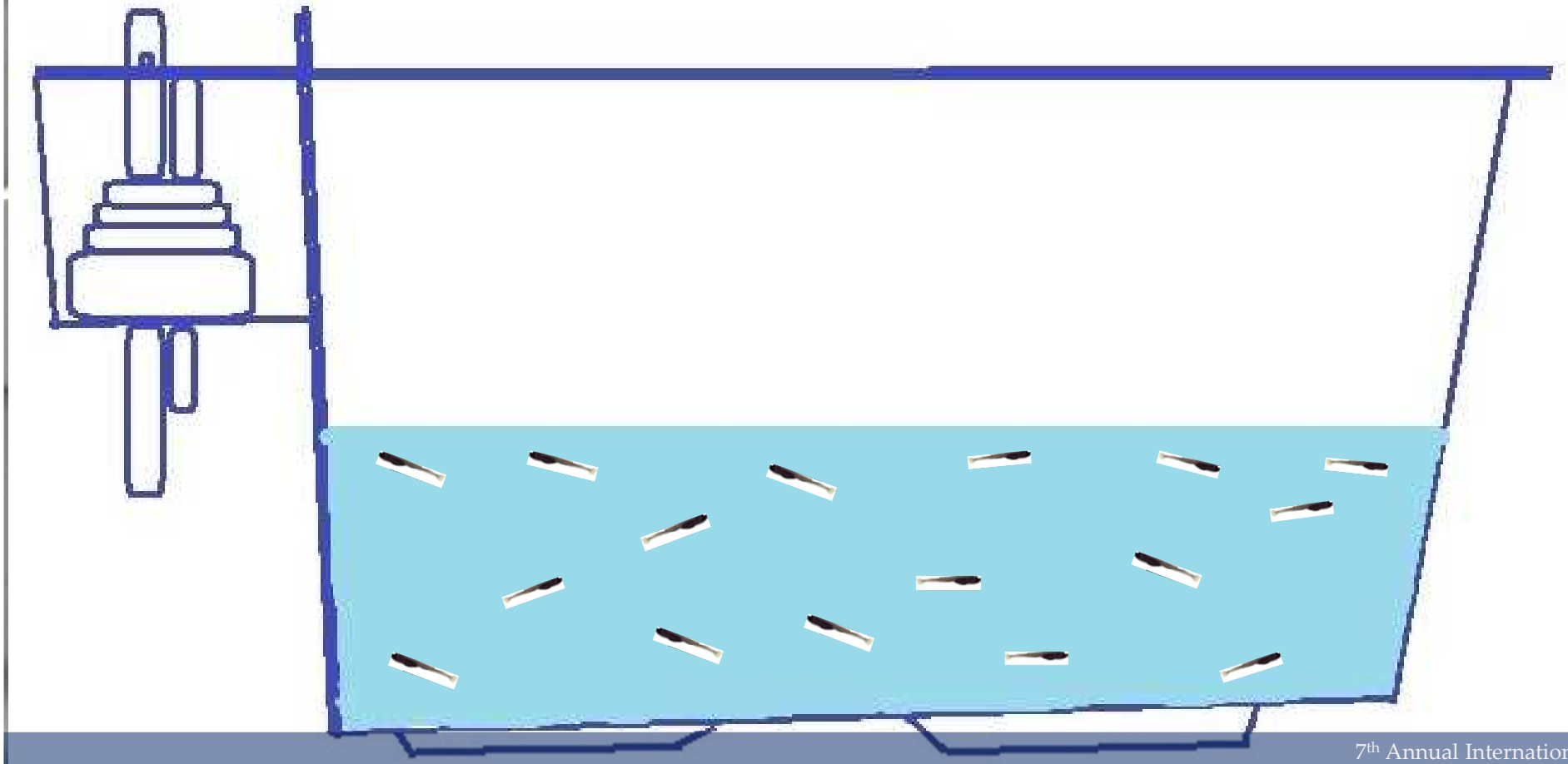
Larval fish 5-6dpf

Rotifers (1000 per larval fish/day)



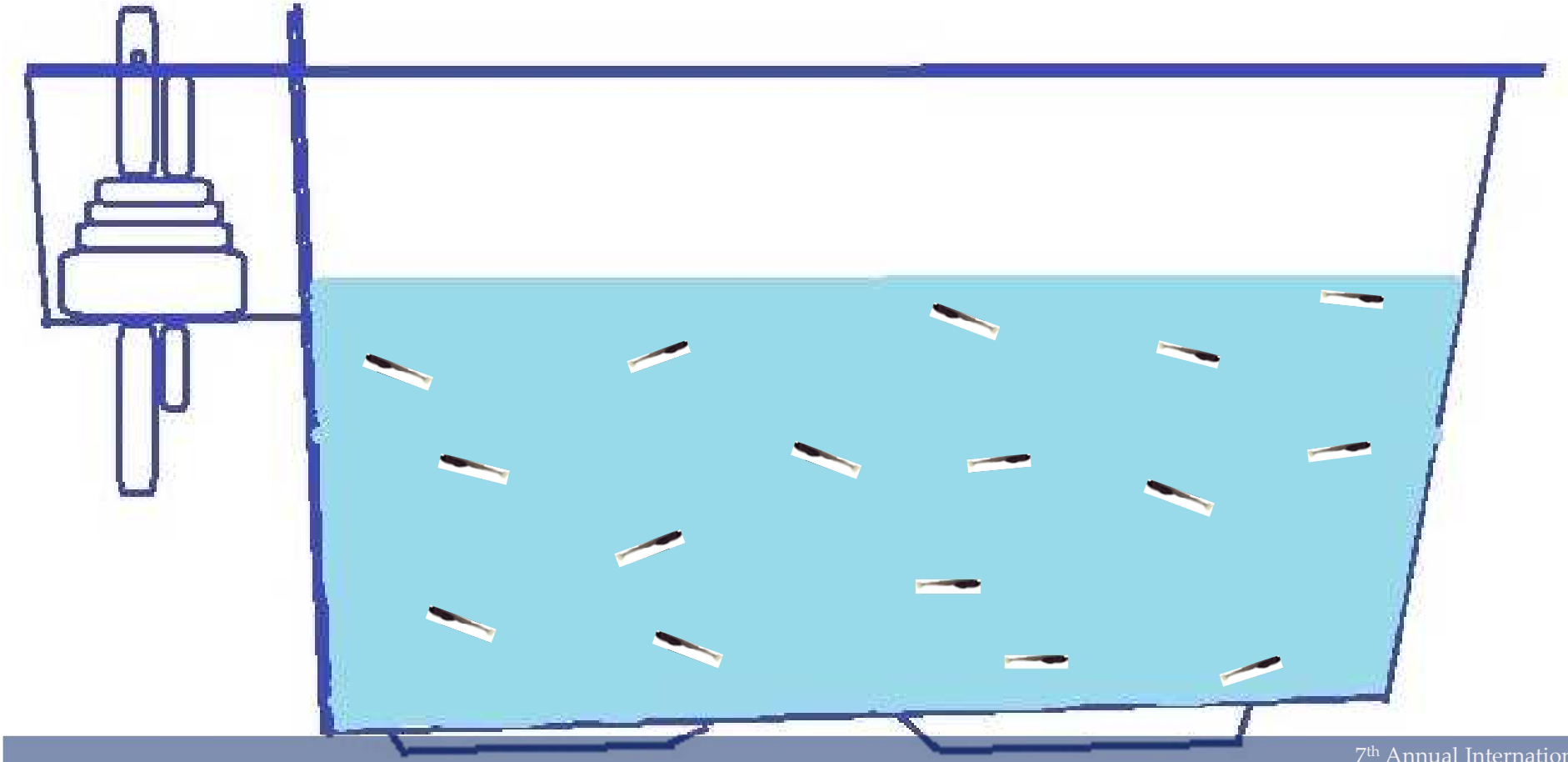
Incremental method

- Larval fish 7-8dpf
  - Rotifers (1000 per larval fish/day)
  - Add 25-30mm water (at least 2ppt)



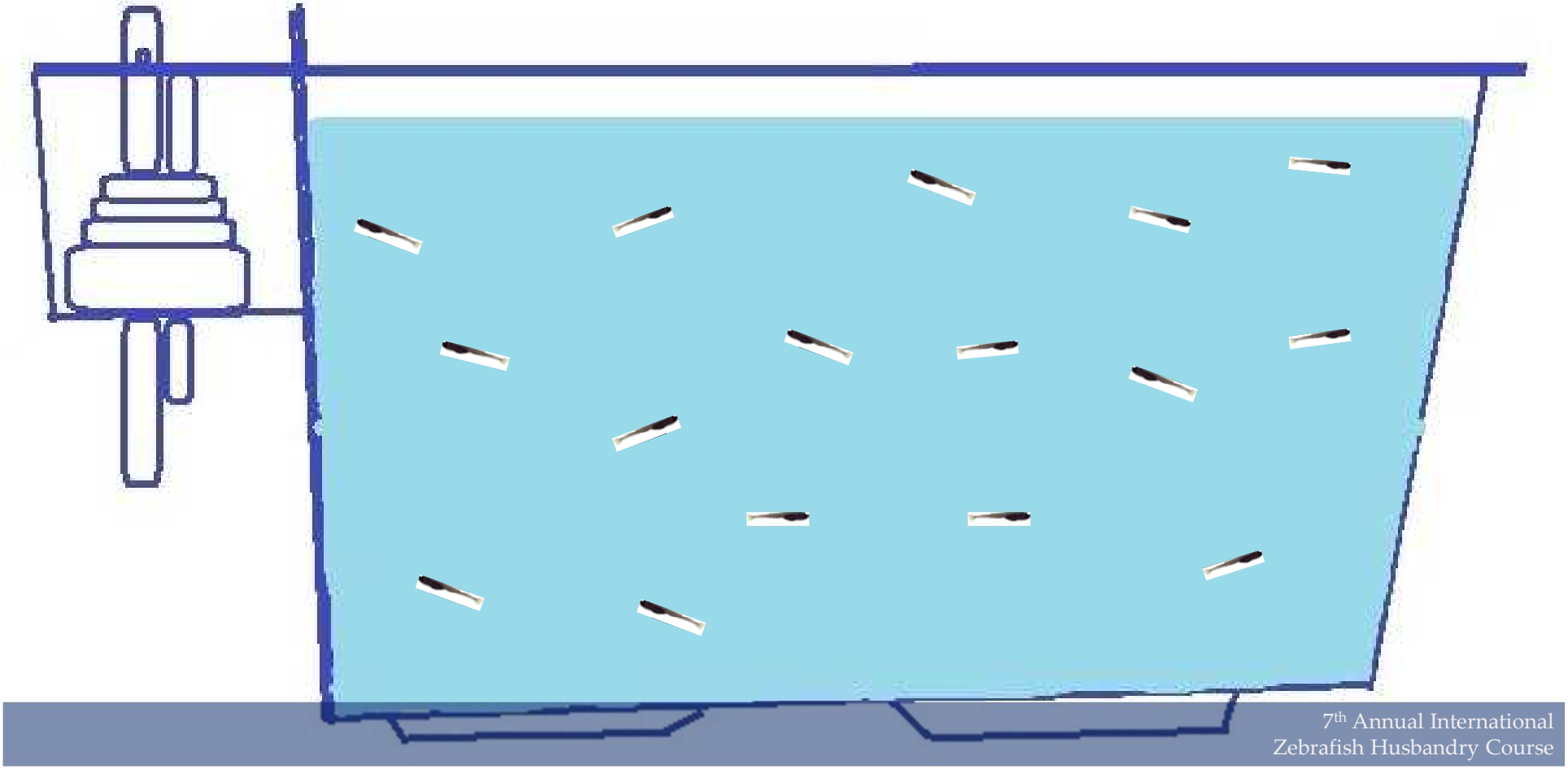
Incremental method

- Larval fish 9-10dpf
  - Rotifers (1000 per larval fish/day)
  - Add 25-30mm water (at least 2ppt)



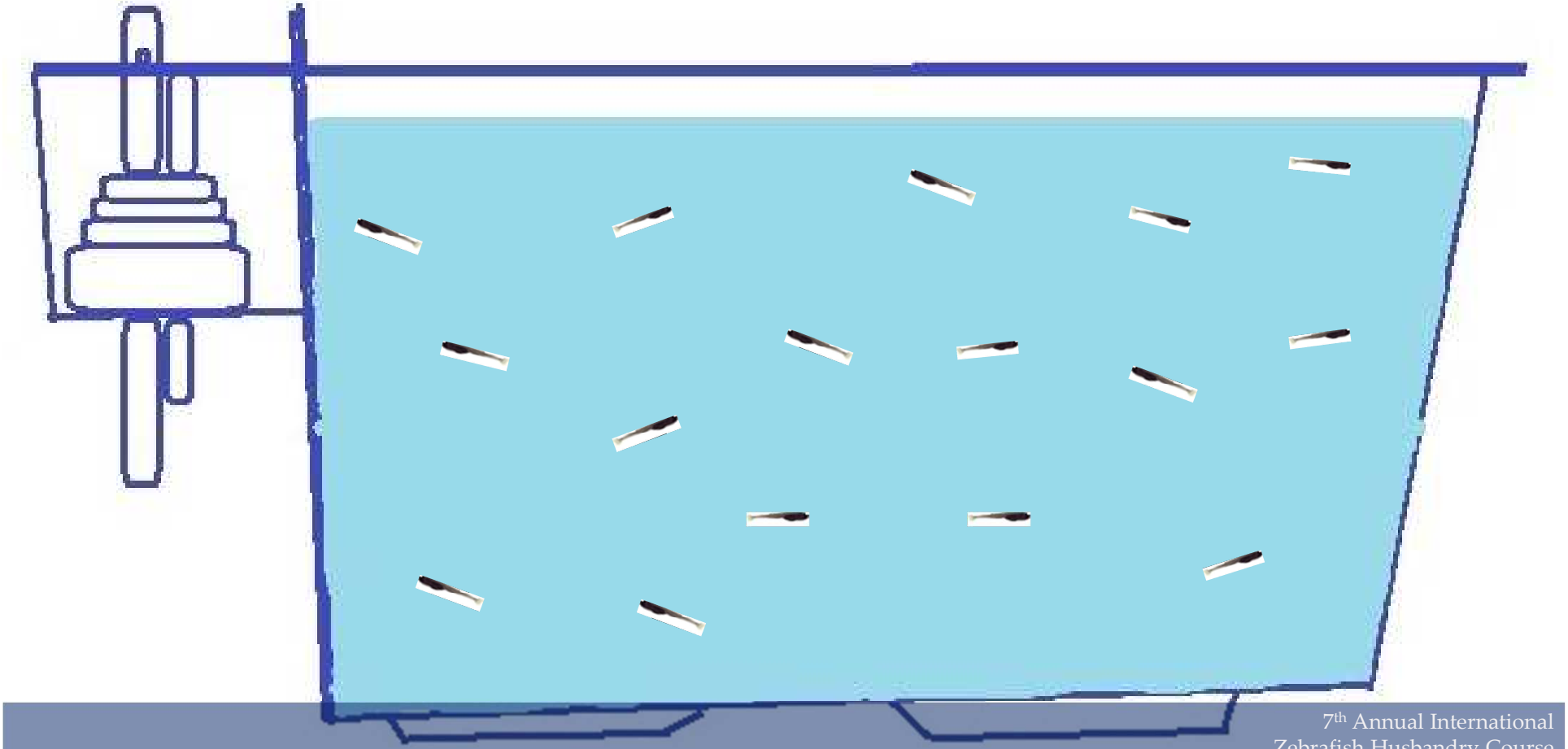
Incremental method

- Larval fish 9-10dpf
  - Rotifers (1000 per larval fish/day)
  - Add 25-30mm water (at least 2ppt)



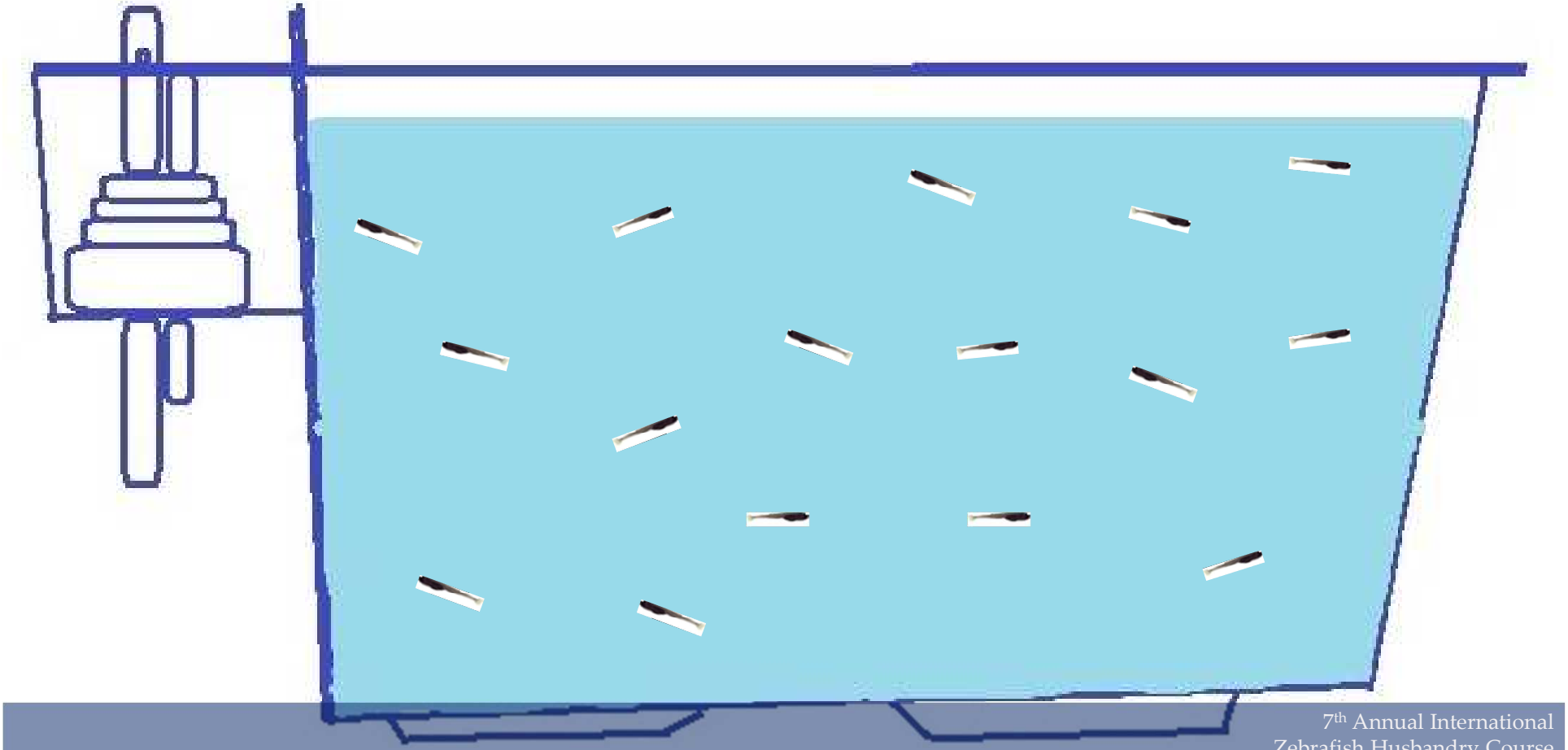
Incremental method

- Larval fish 11-12dpf
  - Rotifers (1000 per larval fish/day)
  - Add 25-30mm water (at least 2ppt) to flush waste



Incremental method

- Larval fish 13-14dpf
  - Rotifers (1000 per larval fish/day)
  - If fish readily eat prepared food (150-250micron), start low-flow and feed both dry-food and rotifers

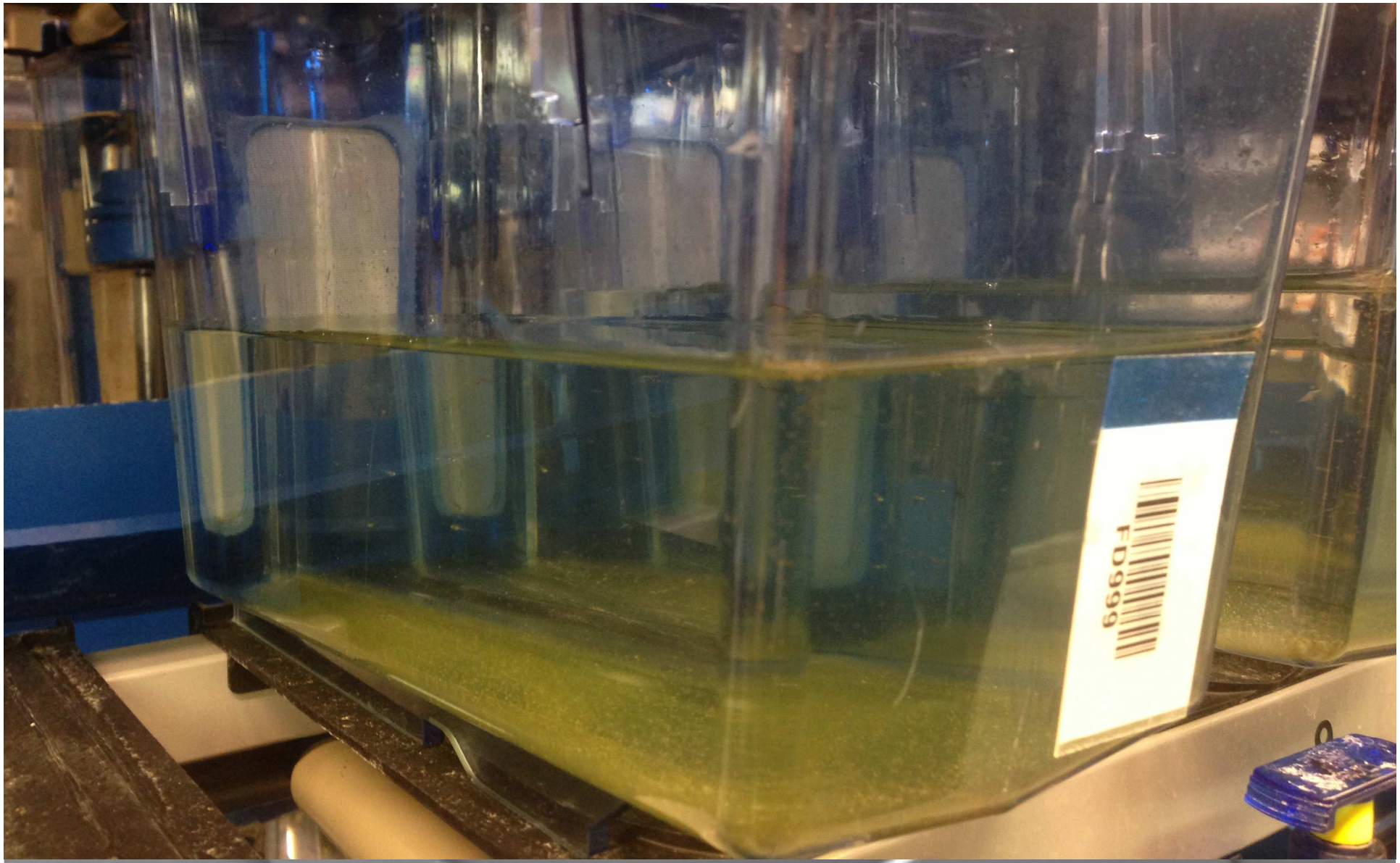


larval fish feeding on rotifers

Notice the high encounter-rate of the fish with the rotifers

larval fish feeding on rotifers

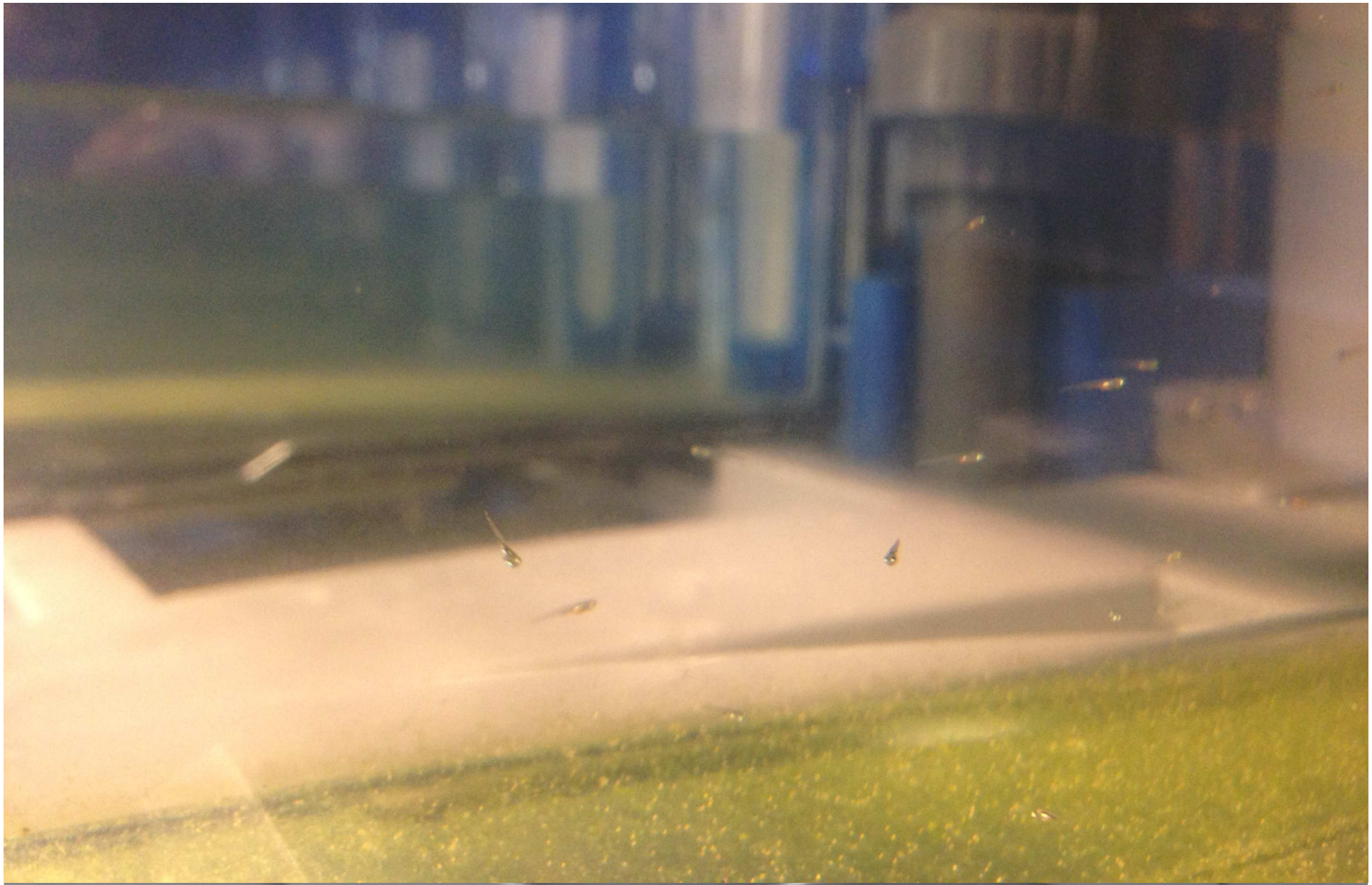
Notice the high encounter-rate of the fish with the rotifers



larval fish tanks with rotifers

7th Annual International
Zebrafish Husbandry Course

larval fish tanks with rotifers



larval fish tanks with rotifers

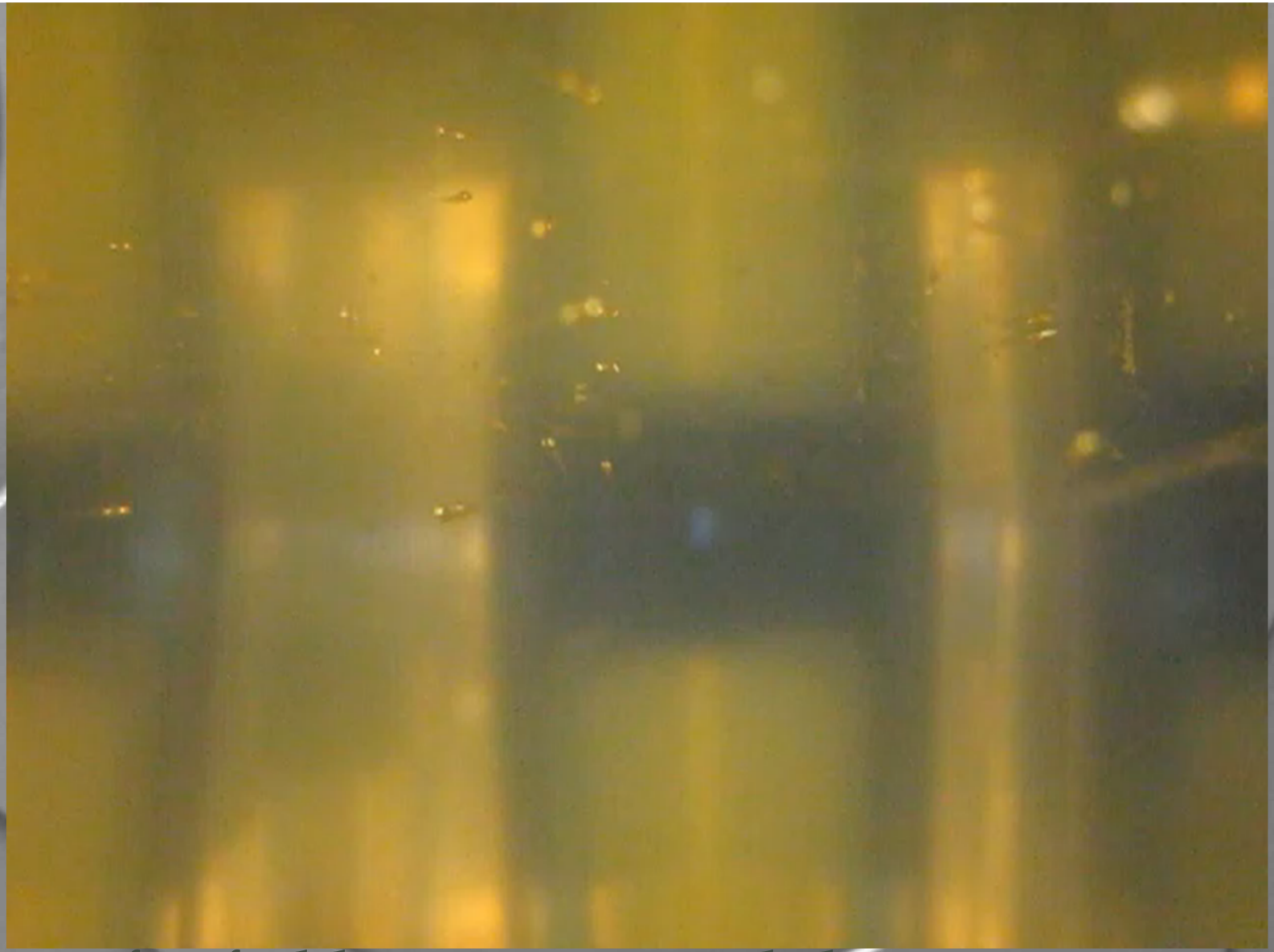
Common Problems in the early stages of raising zebrafish

Trust your senses and your intuition

use your nose and your eyes!

If it smells bad.....
If it looks milky/hazy/cloudy...
If there is a scum or a slick.....

These conditions require an **ACTION** from you
What actions do you take?



rotifer fed larvae stressed due to scum on water

What Can be Done?

To break up scum, flush out fouled water, and add oxygen you must initiate water exchange.

First, best option:

- Initiate water flow
  - Slow and easy is preferable, but not always possible
  - Finish by slowly pouring out water to return the tank to the original fill level

What Can be Done?

Alternatives to flushing with water:

- To remove scum only: use bristles of a paint brush to skim the offending material from the surface
  - A stack of folded paper towels can work as well
- Light aeration without air-stone can break up scum also

Please note difference in water surface between slow-stream to rapid drip

Let them eat...

your observations are the basis for diet changes

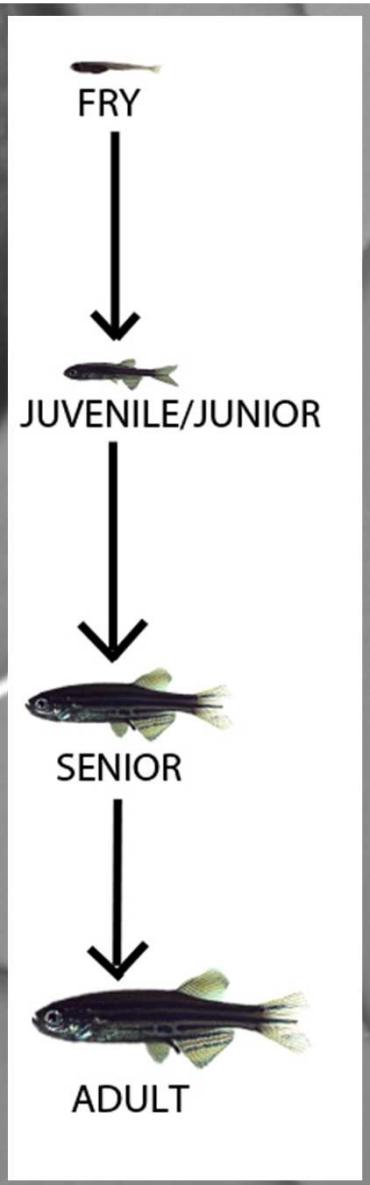
Can larger fish actually see and eat rotifers?

Young adults frenzying on rotifers

Proper Feeding Frequencies

- provide adequate nutrition
- exploit the rapid-growth potential of the model

- Larvae - constant
- Juvenile - high frequency
- sub-adult - high frequency



graphic tools

empower the staff and researchers to make decisions and avoid making mistakes

Images like these can reflect you feeding practices, and avoid problems with offering the wrong feed type or amount to your fish.

Thanks

Tecniplast and IWT, and the entire Bernardini, Brocca, Frangelli, Nisi, and Sala families

Kathleen, Lillian, Finley, and Robin Sanker-Sanders