EPA United States Environmental Protection Agency

Overview of Our Zebrafish Facility and Research With Larval Zebrafish (*Danio rerio)*

Joan M. Hedge

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Office of Research and Development
Center for Computational Toxicology and Exposure/ Biomolecular and Computational Toxicology Division/Advanced Experimental Toxicology Models Branch
Research Triangle Park, North Carolina

50 EPA at Progress for a Stronger Future

Agenda

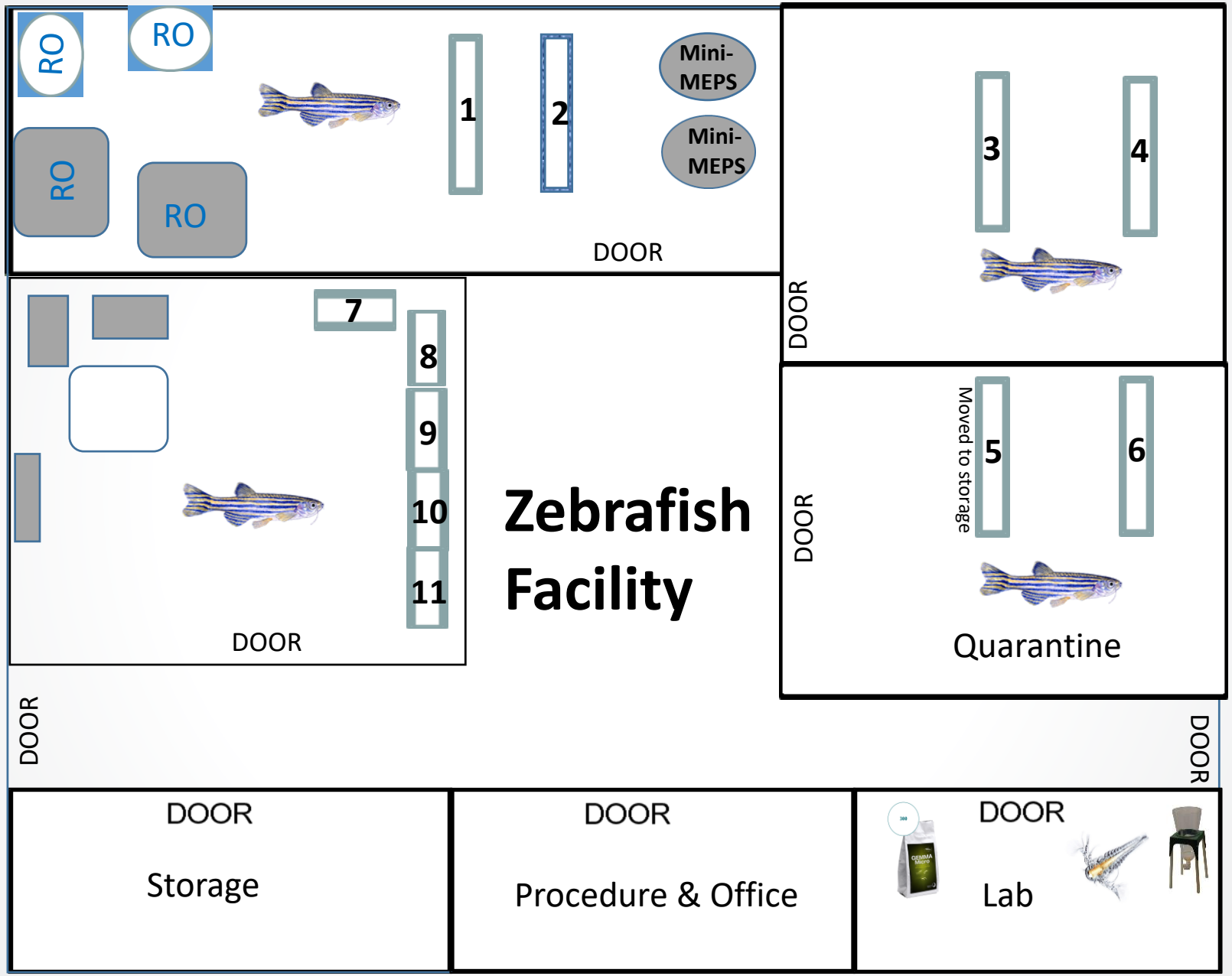
- Overview of our zebrafish facility & its procedures

- Larval zebrafish behavior
  - in response to time lights come on
  - in response to light intensity
  - in response to chemicals

- Dysmorphology and locomotor activity assessment

Our Zebrafish Facility

- Current Inventory ~ 5225 adult zebrafish
- Space for ~28,000 zebrafish if every tank on every rack were filled to capacity, but realistic capacity is ~ 15,000
- Our normal stocking density ~ 8 adults per liter, males and females housed together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Mini-MEPS
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Zebrafish Facility
Moved to storage
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Quarantine
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Storage
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Procedure & Office
DOOR
Lab

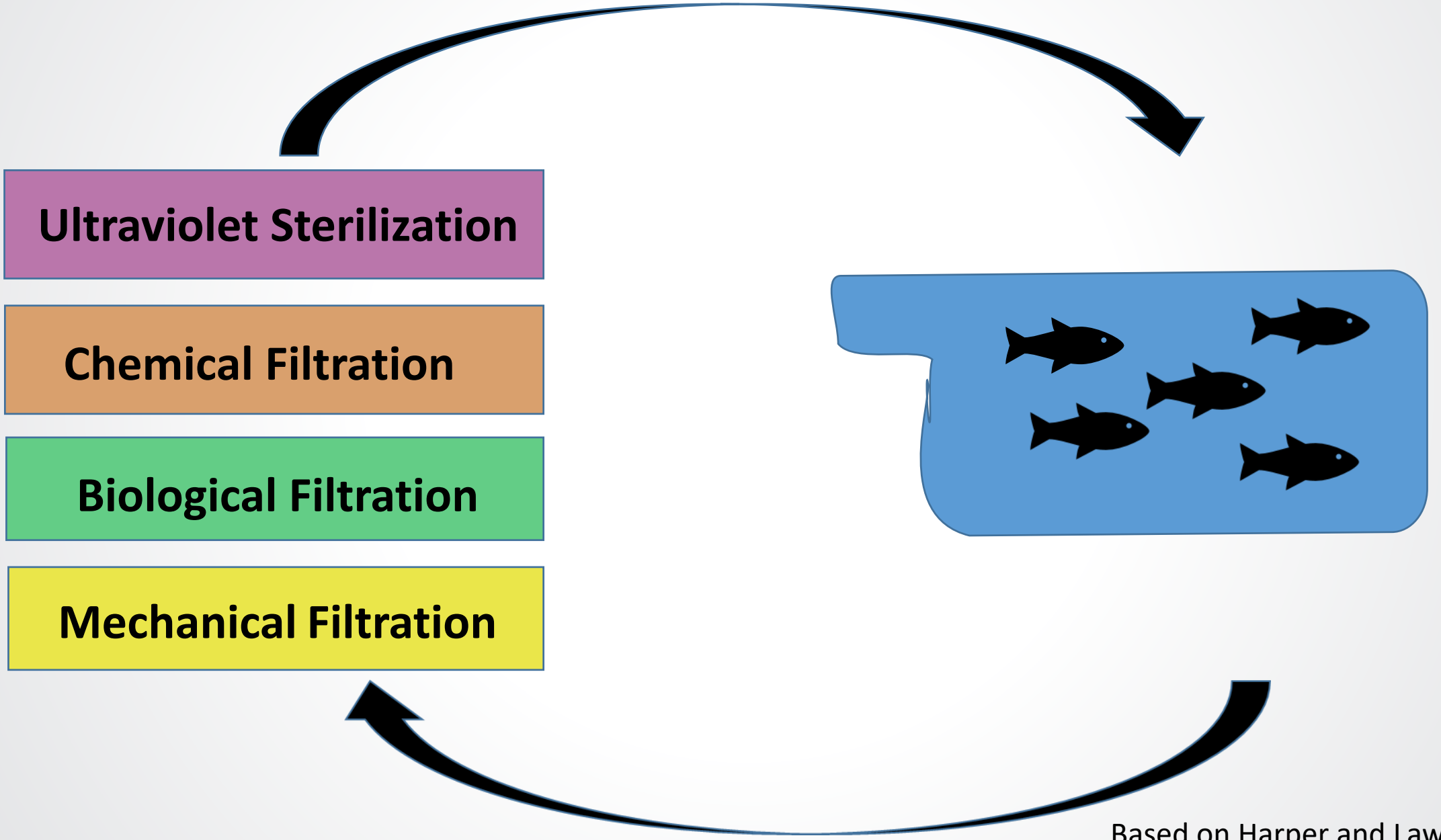
Water

- Incoming Durham, NC city tap water is subjected to reverse osmosis (RO).
- Two reverse osmosis systems and two water storage holding tanks ensure redundancy and adequate supply for all housing racks systems.
- RO water is then treated with sodium bicarbonate and instant ocean sea salt to buffer it before circulating into the zebrafish tanks.

FACILITY 2020 AVERAGE WATER PARAMETERS

Parameters	Tecniplast System	Aquaneering Racks	Target Range	Testing Frequency	Testing Methods
Tank Temperature °C	28 ± 0	26.76 ± 0.05	26 to 29	continuous, when warranted	continuous, handheld meter
pH	7.37 ± 0.002	7.47 ± 0.005	7.2 to 7.6	continuous, daily, when warranted	continuous, hand held meter
Conductivity µs	999.52 ± 0.28	976.08 ± 2.31	800 to 1200	continuous, when warranted	continuous, handheld meter
Ammonia (TAN) ppm	0	0	0	weekly, daily when warranted	colorimetric test
Nitrite ppm	0	0	0	weekly, daily when warranted	colorimetric test
Nitrate ppm	9.56 ± 0.24	6.97 ± 0.26	<20	weekly, daily when warranted	colorimetric test
Alkalinity ppm	not available	not available	50 - 150	weekly, when warranted	colorimetric test, test strips
Hardness ppm	not available	not available	> 75 - 200	weekly, when warranted	colorimetric test, test strips
Dissolved Oxygen %	not available	not available	6 -8	weekly, when warranted	handheld meter

Recirculating Zebrafish Housing Systems



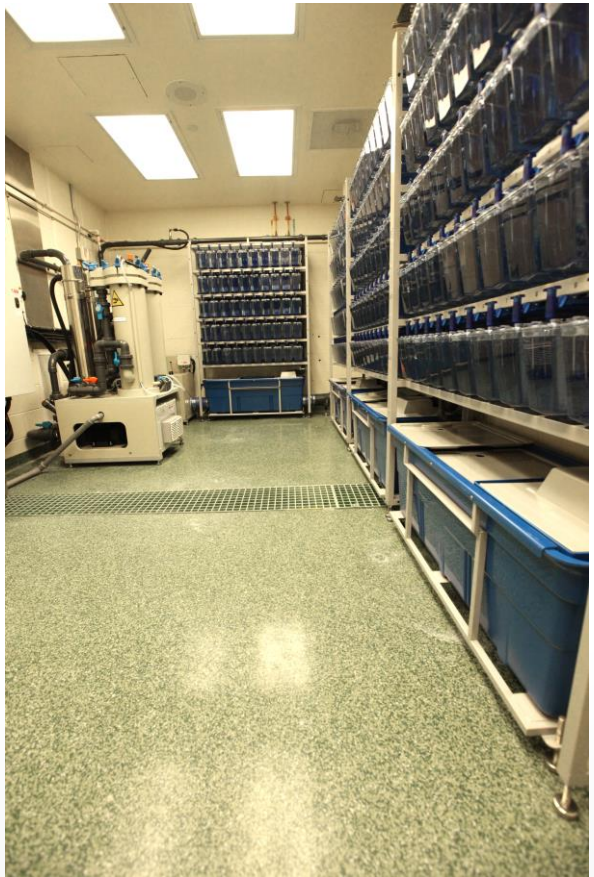
Based on Harper and Lawrence, 2010

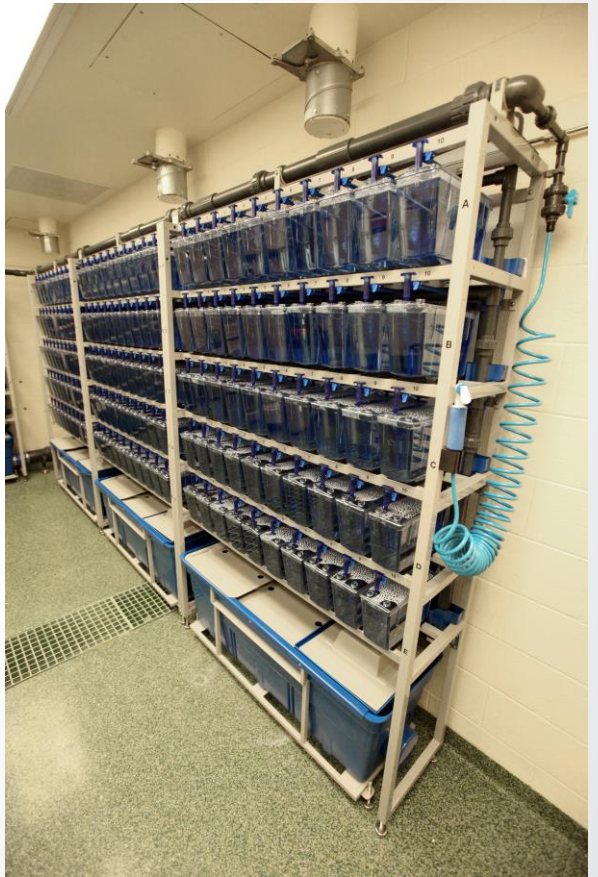
Aquaneering Recirculating Stand Alone Racks

- 5 double-sided housing racks (plus 1 currently in storage)
- Individual water treatment systems for each rack in the sump, no alarm system
- Fluidized bed biofilter
- Maximum 288 of the 6 liter tanks = 14,400 fish capacity (including quarantine)
- Realistically can house ~ 9,000 adult fish

Tecniplast Multi-linking Recirculating Rack System With Pallet Based Water Treatment (ZebTec)

- 5 single-sided housing racks; connected water treatment system can handle up to 12 racks, all racks = common water
- Some advanced capabilities and alarm system; Glax ring aerated bed biofilter in sump under each rack, one shared technical sump for water treatment
- Currently 250 of the 3.5 liter tanks or 6250 total fish capacity, realistically house ~5000 fish





Mass Embryo Production Units (mini-MEPS)

- 2 units used to house and spawn adult zebrafish
- Unit capacity = 80 liters of water and ~640 adult zebrafish/unit
- Realistically house about 1000 zebrafish between the 2 units
- Light timer and spawning platform with embryo collection funnel
- Must be connected to another housing rack for water filtration

Environmental Parameters

Water Temperature = 28°C

Photoperiod = 14 hour light: 10 hour dark

Lights on at 700 h Lights off at 2100 h

Water Turnover = 10 to 20%/day

Tank Exchange ~ every 30 – 60 days,
Sides scrapped, and baffle, siphon, spring & lid exchanged when spawned

Room Temperature = 24°C

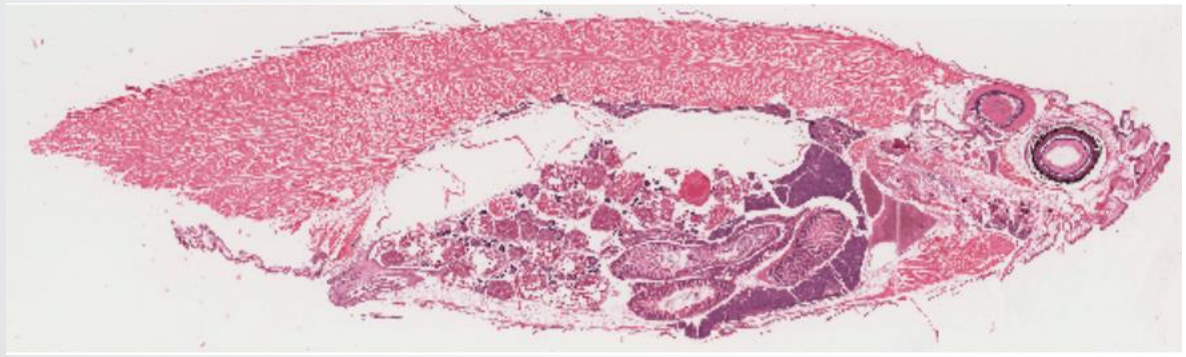
Room Humidity = 50%

All animal research procedures are approved by the United States Environmental Protection Agency Office of Research and Development's Health Institutional Animal Care and Use Committee (IACUC).

Our vivarium is also accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Health Monitoring

- Tank level checks of all zebrafish at least twice daily on week days, once on weekends and holidays
- Sumps checked frequently and any loose zebrafish removed
- Zebrafish sent for routine histological evaluation at least twice a year or when warranted
  - all tissues evaluated and with various stains
- Zebrafish sent for PCR evaluation at least once a year or when warranted
  - Zebrafish Comprehensive Panel or Mycobacterium Panel
- Environmental samples (biofilm, debris and feed samples, including dry feed, rotifers and artemia) sent for evaluation once a year



https://zebrafish.org/wiki/health/diagnostic_testing/start

Biosecurity – Personnel Procedures

- Limited number of staff allowed, access limited by key card
- Strict vivarium visitor policy – cannot have been in another vivarium, pet store or laboratory where animals are utilized within 24 hours of visit.
- Visitors must shower and wear clean clothes and shoes or shower in and wear our scrubs on day of visit.
- Disposable lab coats, shoe covers and gloves must be worn (masks and safety glasses in some instances).
- Gloves should be changed after working on quarantine rack or when wet between rooms.

Biosecurity – Zebrafish

- Never netted on rack, always moved to a cart. Before moving, carts must be disinfected.
- Any zebrafish that falls to the floor or sump must be euthanized.
- Dead, sick, or compromised fish should be separated as soon as possible. Any fish found in the sump should be euthanized as soon as possible.
- Fish are only set up to spawn with other fish of their same age group and only in their housing room.
- New zebrafish are never brought into the colony as adults, only as bleached embryos.
- Spermatozoa from our in house wild type line, “Z” are cryopreserved and stored off site.

Biosecurity – Equipment and Procedures

- Separate feeders are kept for each housing room, and the quarantine rack.
- Each rack must have it's own dedicated net and container of disinfecting net soak, changed per manufacturer's recommendations, and labelled accordingly with date.
- Any tank, baffle, lid, breeding tank, etc. that is used for zebrafish must be soaked in bleach solution for at least 20 minutes and then sent to cage wash, heated and rinsed throughly, rinsed with distilled water and allowed to air dry before returning to use in the facility.
- Housing room floors are sanitized daily.
- Approved Operating Procedures are maintained in the facility and personnel are familiar with them.

Emergency Procedures

- Vivarium Emergency / Disaster Plan is in place including special document pertaining only to zebrafish colony emergencies.
- Emergency back-up power supplied to all zebrafish housing rooms including housing racks.
- Back-up air supplies and extension cords available in case of system or room specific electricity problems to keep biological filter aerated.
- Other vivarium personnel are cross trained to fill in the zebrafish facility when needed.
- A new zebrafish housing suite is being planned on another floor of the vivarium as a back-up housing space.

Our Feeding Regimen

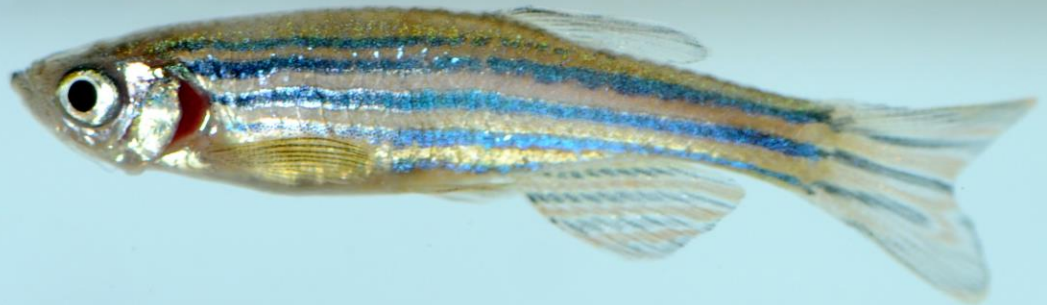
Age of zebrafish^	Food to use	Color code on Gemma food	How often to feed
0 to 2 weeks #	rotifers, artemia and Gemma 75 (GM75)	YELLOW (GM75)	2x a day (make food continuously available starting on PM of day 5)
2 weeks to 6 weeks	Gemma 75 (GM75) AND artemia	YELLOW (GM75)	GM75 (2x/day) AND artemia (2x/day)
7 weeks to 14 weeks	Gemma 300 (GM300) AND artemia	BLUE (GM300)	GM300 (2x/day) AND artemia (2x/day)
15 weeks +	Gemma 300 (GM300) AND artemia OR Gemma 500 (GM500) AND artemia	BLUE (GM300) / GREEN (GM500)	GM300 (2x/day) OR GM500 (2x/day) AND artemia (2x/day)

^ Age of zebrafish can be determined by looking at DOF (Date of Fertilization) on zebrafish tank labels and counting up to the present day.

For the first 2 weeks zebrafish are kept in the Padilla lab and fed by lab staff there. They are put on the racks at about 14 days old.

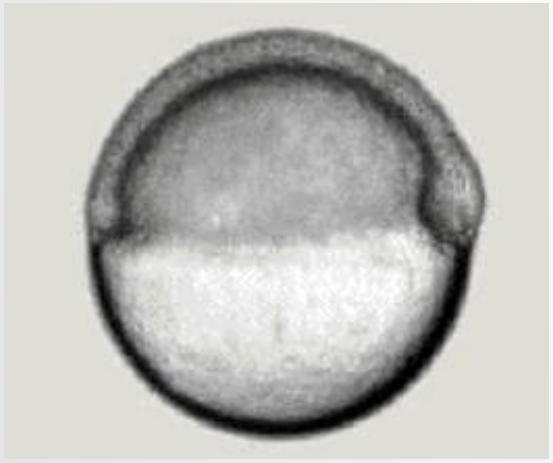
Zebrafish Colony Stock

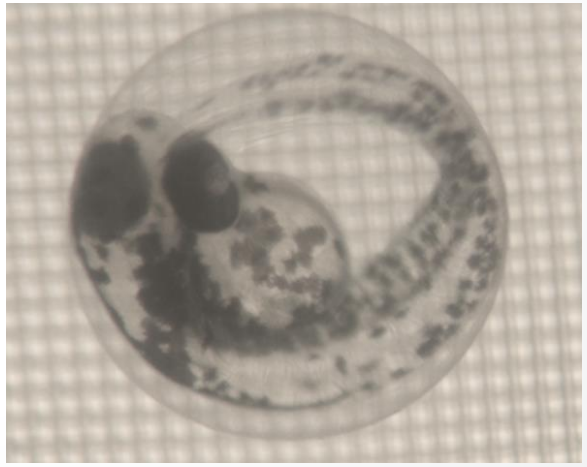
- We call our in house wild-type stock the “Z” strain
- Created by combining 2 sets of undefined, outbred stock of wild-type AB strain background zebrafish from 2 separate commercial fish suppliers ~15 years ago
  (Aquatic Research Organisms, Hampton, NH, and EkkWill Waterlife Resources, Ruskin, FL)



- Since then the line has been maintained and cleaned up through testing, exclusion, and breeding every year

- We currently do not conduct research with adult zebrafish.
- Adult zebrafish however are very important to our program and are used as breeding stock.
- All adult zebrafish are spawned at least every 2-3 weeks.
- All our research is conducted with zebrafish embryos.
- These zebrafish embryos are obtained by spawning in 3 ways.





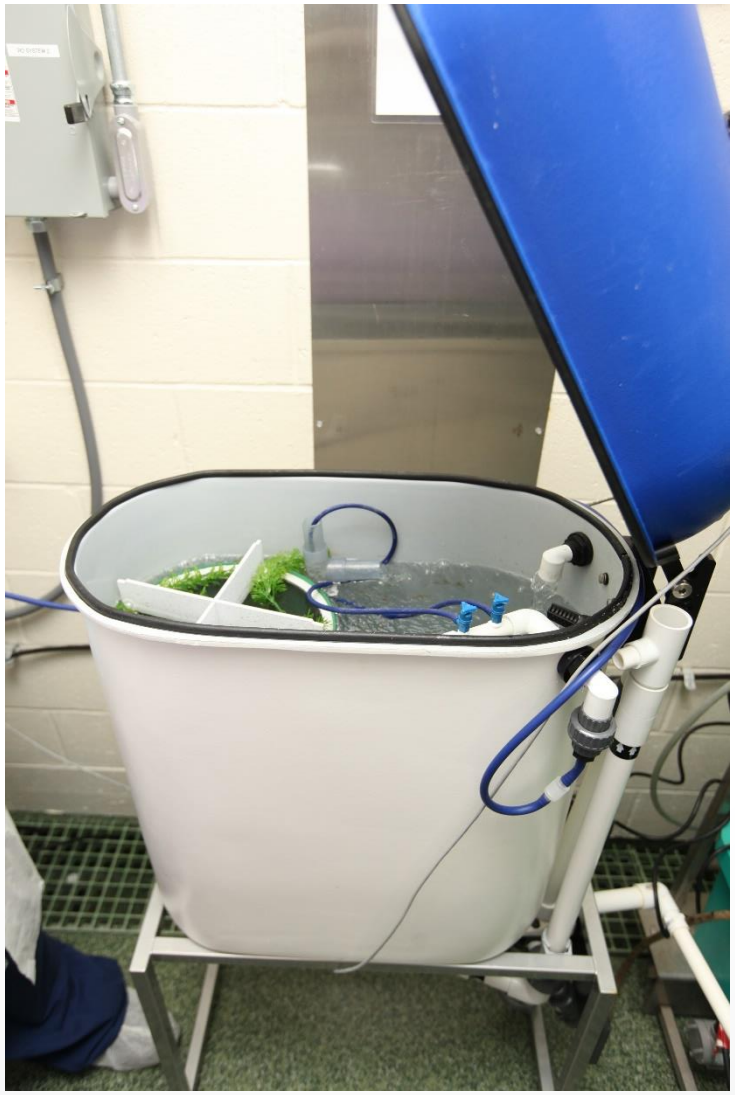
(Haffter *et al.*, 1996

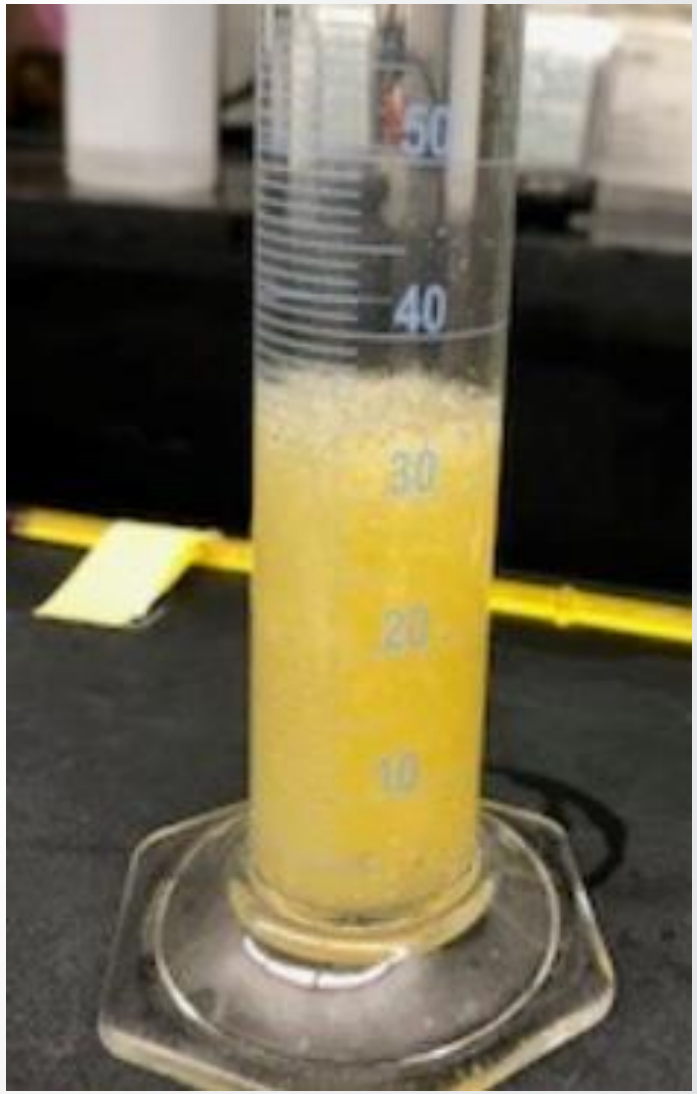
1. Spawning in modified Cambro food service pans:

- Two pan design, zebrafish held ~ 16 hours overnight
- Sloped mesh partition with shallow end
- Holds 75 to 100 adults from 2 to 3 home tanks

2. Spawning in mini-MEPS (Mass Embryo Production System):

- Zebrafish live in the unit so set up is minimal
- House about 500 zebrafish in each unit
- Spawning platform lowered & embryos collected once a week





3. Small group crossings:

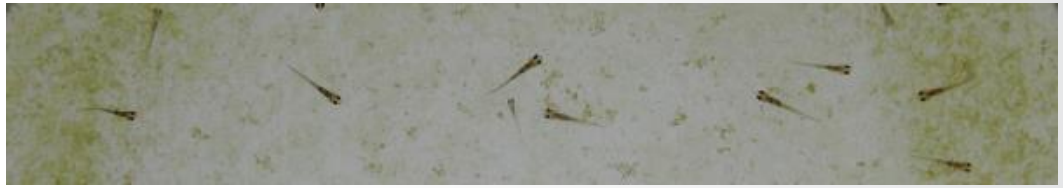
- Sexes can be segregated to synchronize matings
- Timed embryos good for injections / genetic manipulations

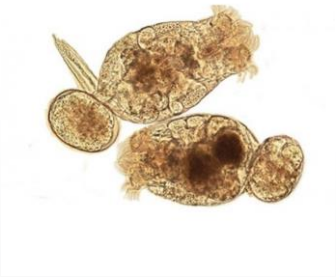
http://zebrafishfacility.wustl.edu/data1/images/ffpic12x.jpg

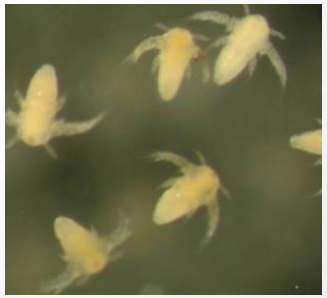
Rearing replacement breeders:

- Breeding stock replaced at 15 months old, larvae reared ~ every 3 months.
- Some embryos selected from all zebrafish spawned across entire week & washed for 2 five minute washes in 0.06% bleach solution.
- Embryos reared in glass dishes in incubator at 28°C on 14 hour light:10 hour dark light cycle. Placed on housing rack at 14 days post fertilization (dpf).

Solution	10% Hanks Balanced Salt Solution dpf 0-4	4 g/L Instant Ocean Sea Salt dpf 4-11	0.75 g/L Instant Ocean Sea Salt dpf 11-14
Feeding	L-Type Marine Rotifers + Algae dpf 5-14	Instar I Artemia + Gemma 75 dpf 12-14	
Cleaning	Daily dpf 6-14	Remove and replace 80% solution dpf 9-14	







The Personnel That Keep Our Zebrafish Colony Running…….

RESEARCH STAFF

VETERINARIAN

IACUC

ANIMAL CARE STAFF

ANIMAL PROGRAM STAFF

FACILITIES STAFF

EQUIPMENT MANUFACTURERS

Larval Zebrafish Behavior

- Animal behavior recognized as an informative endpoint for assessing chemical effects on developing nervous system

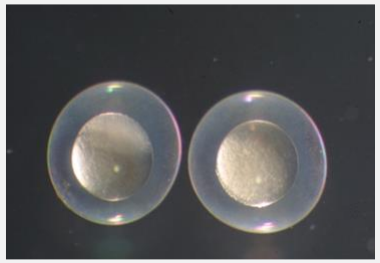
- Zebrafish increasingly being utilized as an alternative model for developmental neurotoxicology
  - smaller and easier to maintain
  - shorter generation time
  - high fecundity with external fertilization
  - transparent and rapid development
  - lower cost and higher throughput

- Behavioral alterations caused by neurotoxicants usually occur below those inducing mortality or malformations

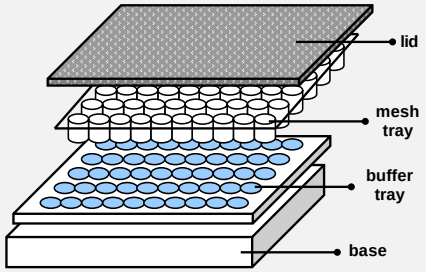
- Larval behavior assay examples: light : dark locomotor activity, circadian rhythm mapping, touch response, thigmotaxis, prey-capture, and rest/wake

- Our laboratory utilizes an early life stage zebrafish light : dark locomotor activity assay

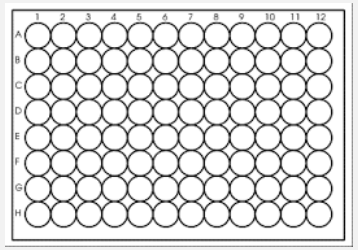
EXPERIMENTAL METHODOLOGY



Embryos Washed
- 1- 2 hours post fertilization
- 0.06% bleach

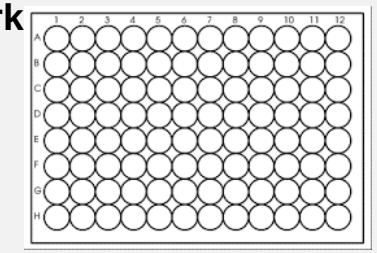


Embryos Plated
- 6 to 8 hours post fertilization
- 1 embryo / well
- 10% Hanks' buffer



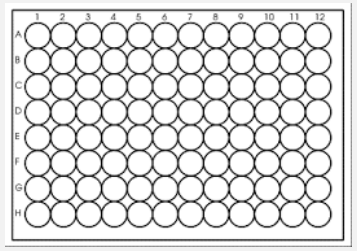
Embryos Treated
- days post fertilization 0 and 3 only
- Control (0.4 % dimethyl sulfoxide vehicle) or Chemical

14:10 light : dark 26ºC



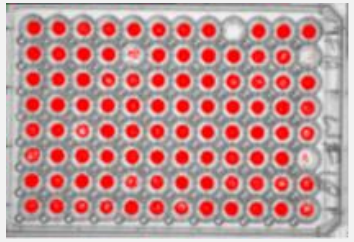
Depuration
- 5 days post fertilization
- Embryos changed to 10% Hanks' buffer

14:10 light : dark 26ºC



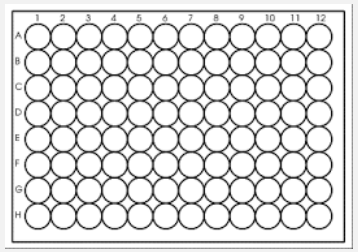
Solution Change
- 6 days post fertilization
- 10% Hanks' buffer

26ºC

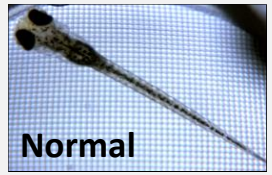


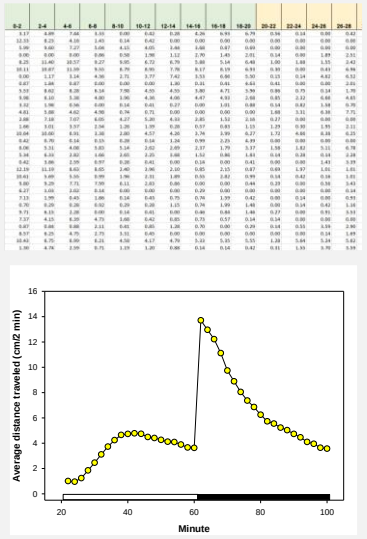
Behavioral Testing
- 6 days post fertilization
- 20 minute basal dark
- 40 minute light
- 40 minute dark

26ºC



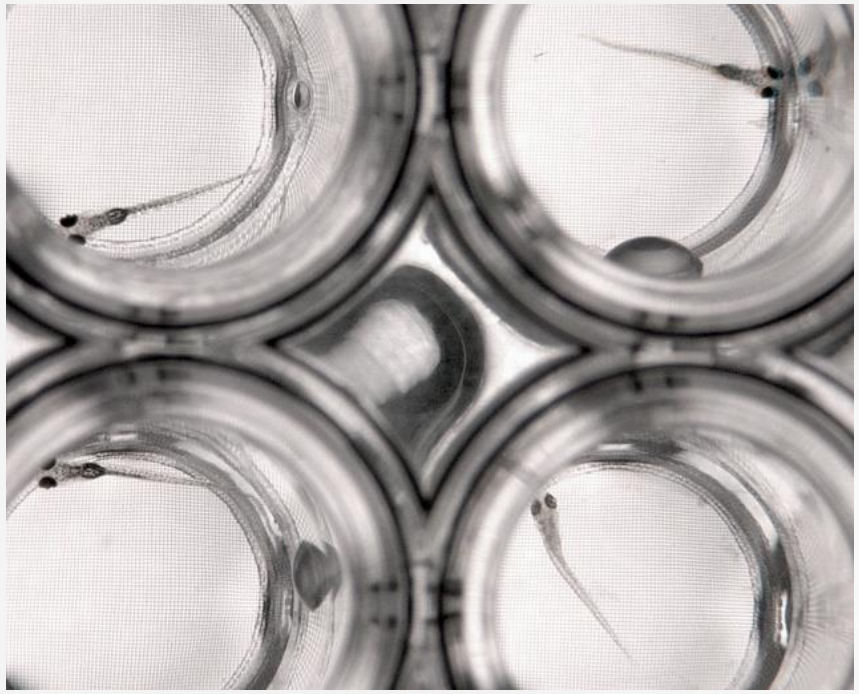
Assessment Post Test
- 6 days post fertilization
- Gross morphology and survival

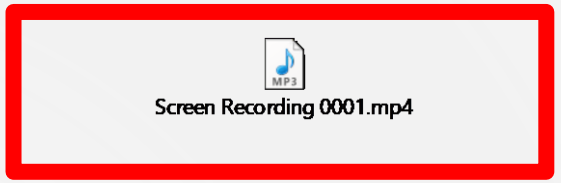




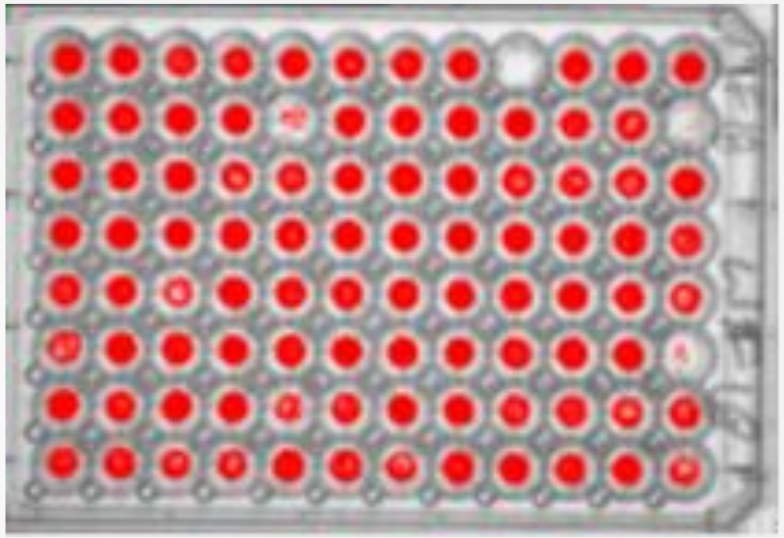
Tracking of behavior and data analysis

Locomotor Behavior Assessment Testing





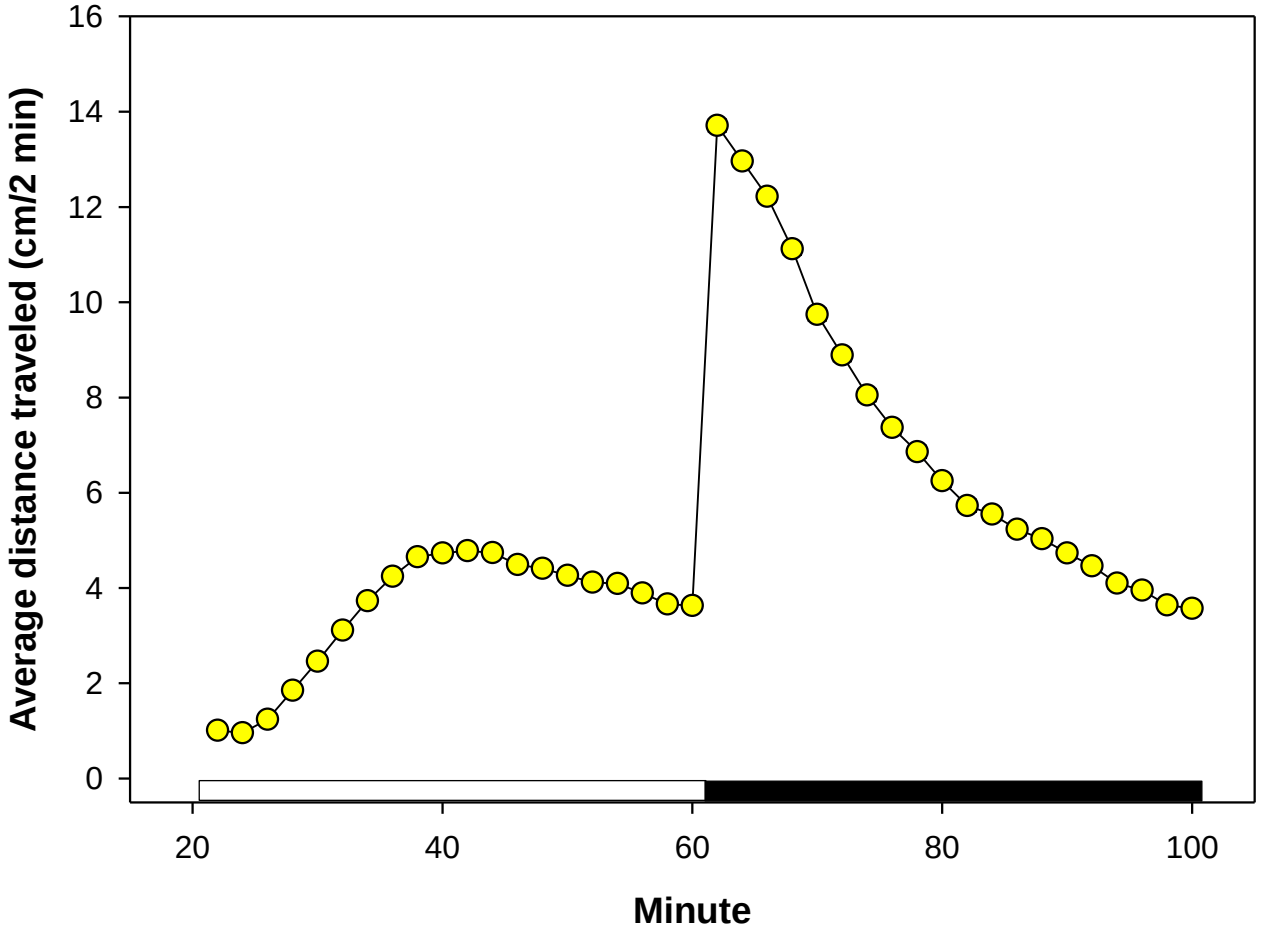
Tracking Video



- All testing is performed on 6 days post fertilization larvae in the same 96 well plates where they are dosed and reared
- All testing occurs between 1230 and 1630h

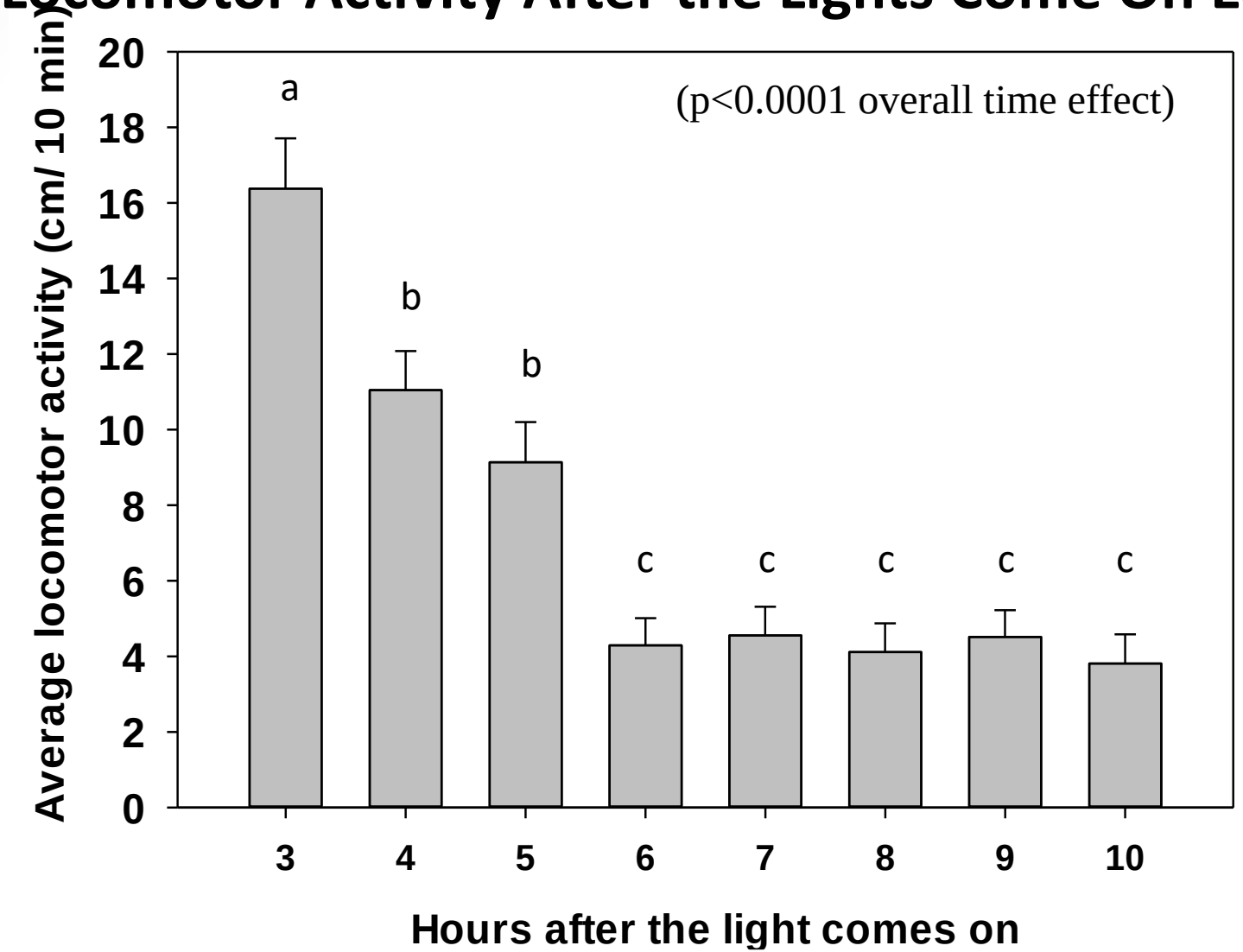
- Plates of embryos are tested using a Noldus Tower Tracking System (Noldus Information Technology, Leesburg, VA) with 40 minutes of 18 lux luminance visible light, followed by 40 minutes of dark
- Fish movement (locomotion) is tracked from videos using Ethovision Software Version 13 (Noldus)

Typical Pattern of Locomotor Behavior at 6 days post fertilization



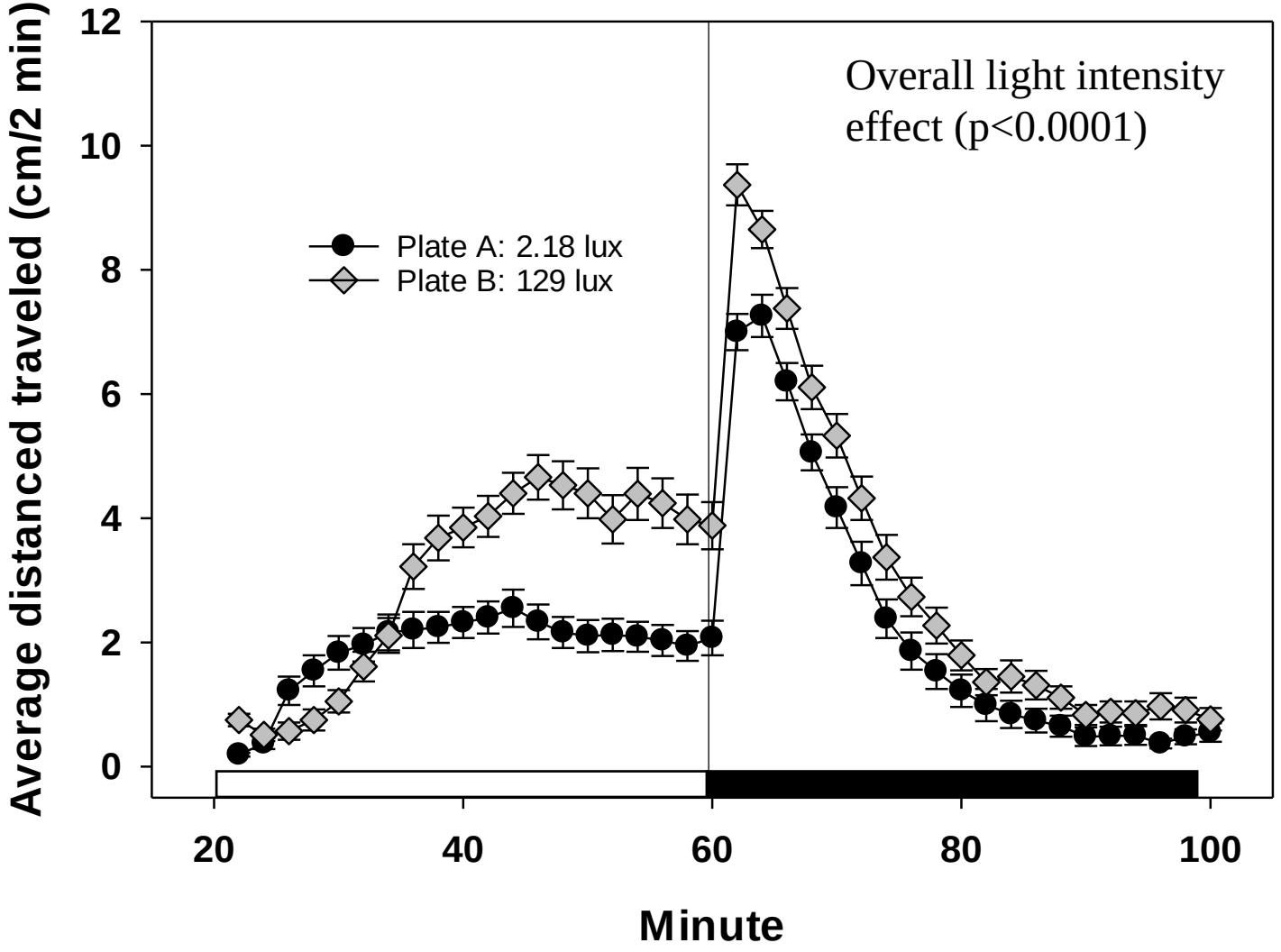
- 20 minute basal dark period for acclimation is not analyzed
- 40 minutes of 18 lux light is represented by the white bar and is analyzed
- 40 minutes of dark is represented by the black bar and is analyzed
- General light-to-dark pattern exists, startle activity when light goes out and then greatly increased movement in the dark initially which slowly decreases over time

Locomotor Activity After the Lights Come On Each Day



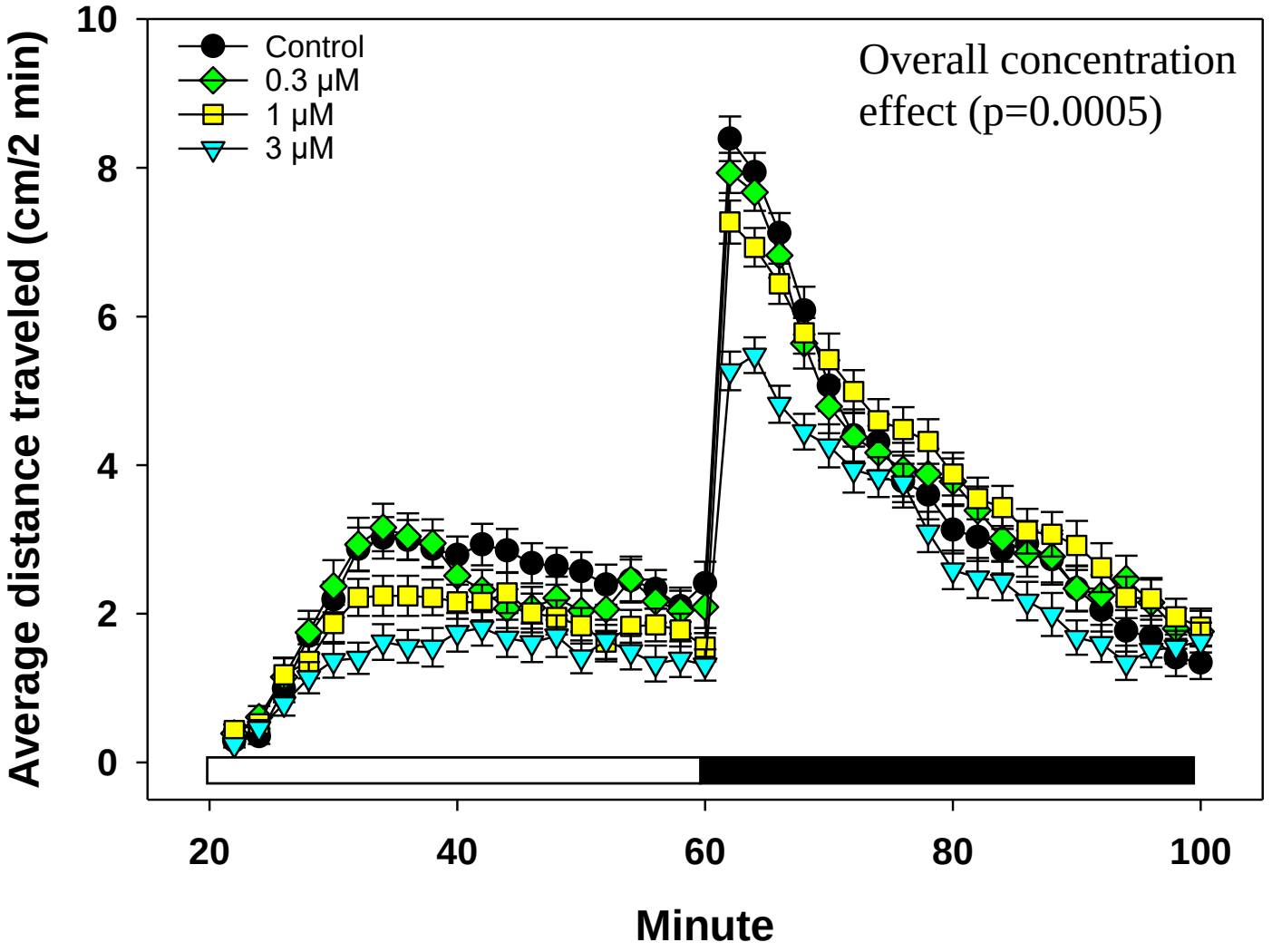
- Alternating light and dark photoperiods produce consistent patterns of locomotion.
- Zebrafish are very responsive to light and have a definite circadian rhythm that can influence your behavioral testing results.
- Studies in our laboratory have determined the least variable time after the lights come on each day to test locomotor behavior in the larval zebrafish, 5.5 to 10.5 hours.

Effect Of Intensity Of Light On Locomotor Behavior



- Locomotor activity is also affected by the intensity of the light photoperiod.
- Light intensity affects the activity levels when larvae are returned to total darkness.
- The brighter the light, the higher the activity level when abruptly returned to a dark photoperiod.

Effect of an Organophosphate Pesticide on Locomotor Behavior



- Developmental neurotoxicants may affect larvae in one or both photoperiods
- Common positive control organophosphate pesticide decreases larvae locomotor behavior in the light and dark photoperiods in a concentration dependent manner.

- Knowing how larval zebrafish respond to varying photoperiods, you can then look for variations in these patterns in response to chemical exposure during development.

- There is not one standard zebrafish locomotor activity protocol.

- Our usual schedule for assessing locomotor activity:
    - 20 minutes of dark (acclimation phase) to reduce problems associated with moving plate to the behavioral platform
    - 40 minutes of 18 lux light
    - 40 minutes of dark

Dysmorphology and Locomotor Activity Assessment In Larval Zebrafish (*Danio rerio)*

To screen and prioritize chemicals for developmental neurotoxicology, the United States Environmental Protection Agency is utilizing:

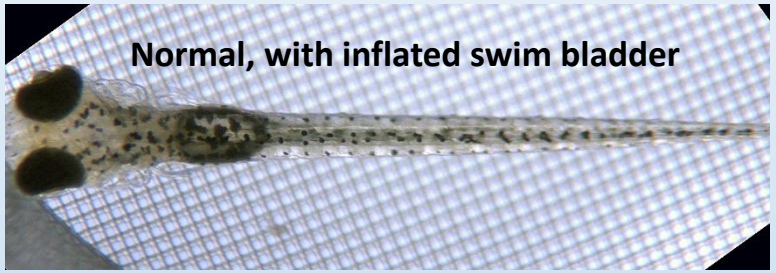
- non-mammalian models
- higher throughput testing approaches
- behavioral endpoints

Confounding factors must be carefully considered:

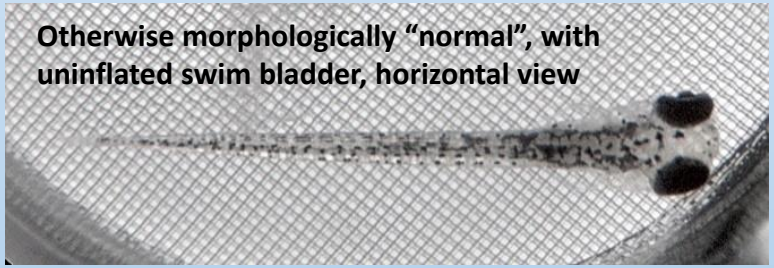
- rarely addressed in behavioral publications
- morphological assessment

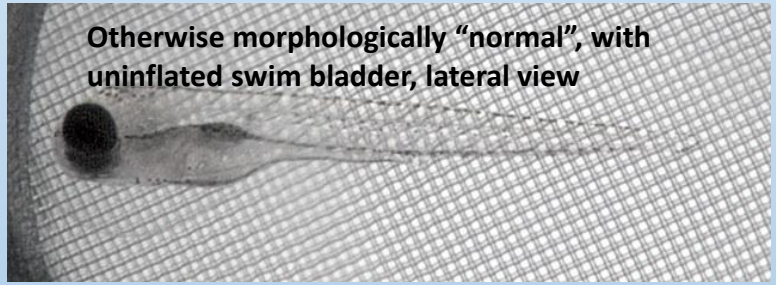
Morphological Assessment of Zebrafish Larvae at 6 Days Post Fertilization

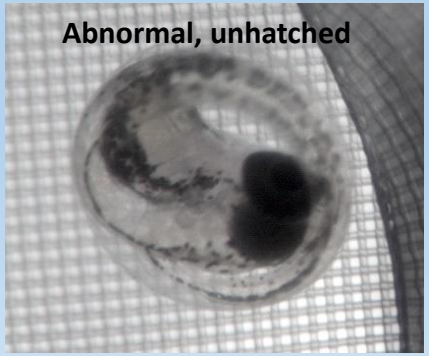
Normal larvae:

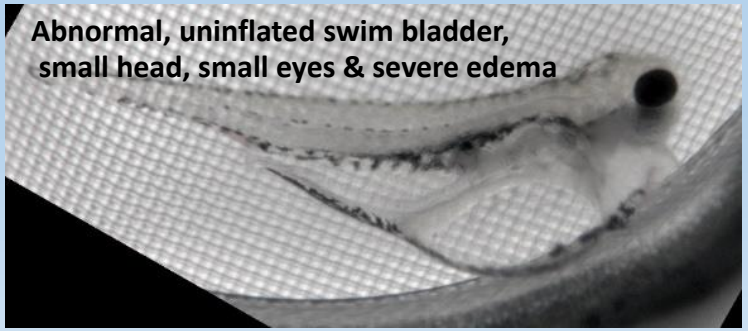


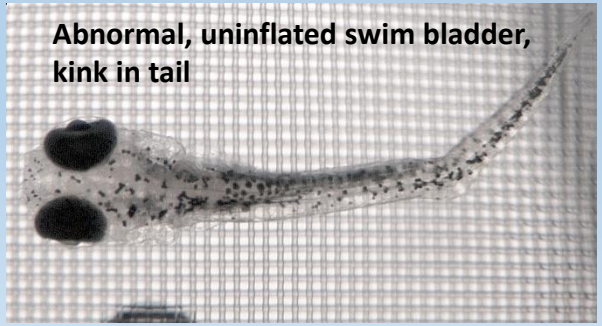
Common dysmorphologies:







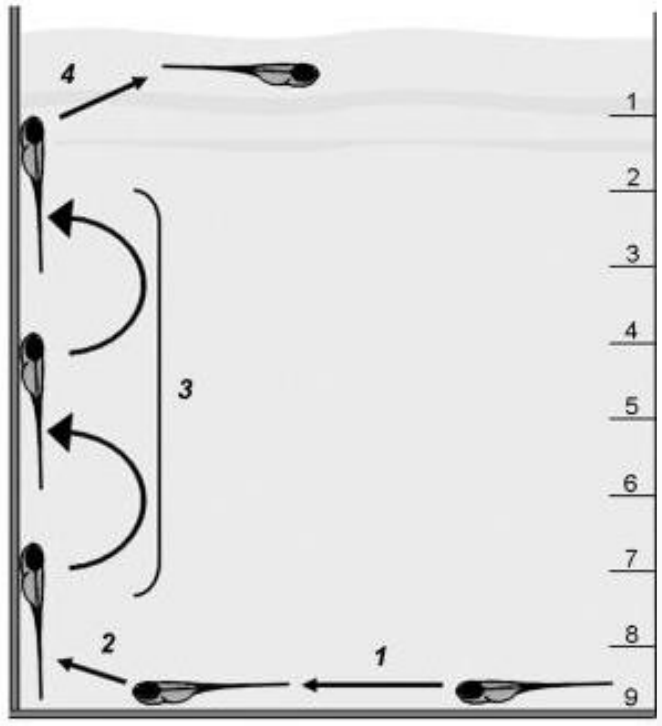




The swim bladder (posterior) maintains buoyancy and without it larvae will expend more energy to move through the water column, thus it is considered essential for survival.

- Develops as a two chamber organ. The first chamber, the posterior swim bladder, develops about 3 days post hatch.

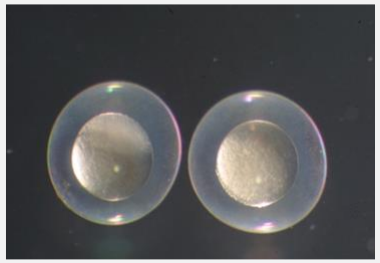
- Zebrafish larvae require a breath of air to inflate the first swim bladder chamber.



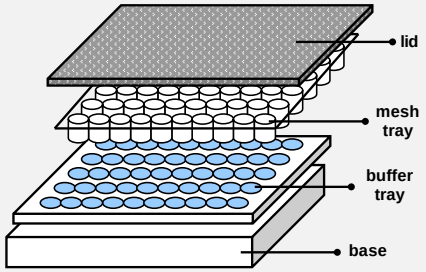
Zebrafish larvae, 6 days post fertilization with first chamber of swim bladder inflated

Lindsey et al. 2010. From inflation to Floatation: Contribution of the Swimbladder to Whole-Body Density and Swimming Depth During Development of the Zebrafish (*Danio rerio*) *Zebrafish* 7(1)

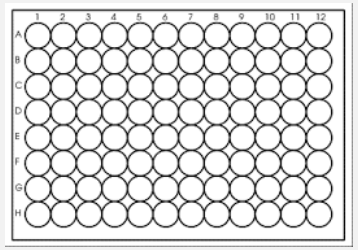
EXPERIMENTAL METHODOLOGY



Embryos Washed
- 1- 2 hours post fertilization
- 0.06% bleach

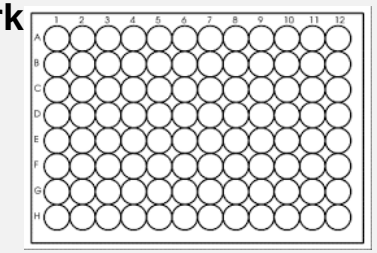


Embryos Plated
- 6 to 8 hours post fertilization
- 1 embryo / well
- 10% Hanks' buffer



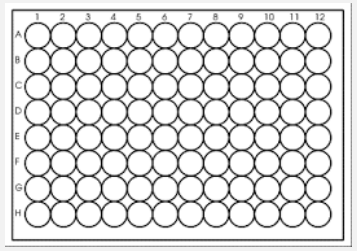
Embryos Treated
- days post fertilization 0 and 3 only
- Control (0.4 % dimethyl sulfoxide vehicle)

14:10 light : dark 26ºC



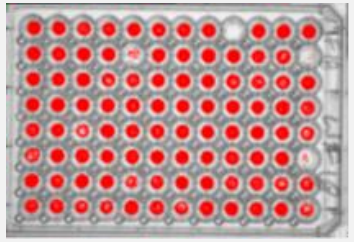
Depuration
- 5 days post fertilization
- Embryos changed to 10% Hanks' buffer

14:10 light : dark 26ºC



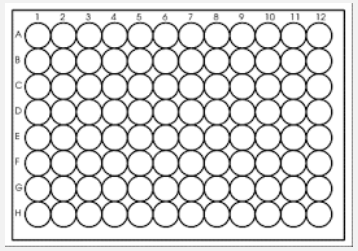
Solution Change
- 6 days post fertilization
- 10% Hanks' buffer

26ºC

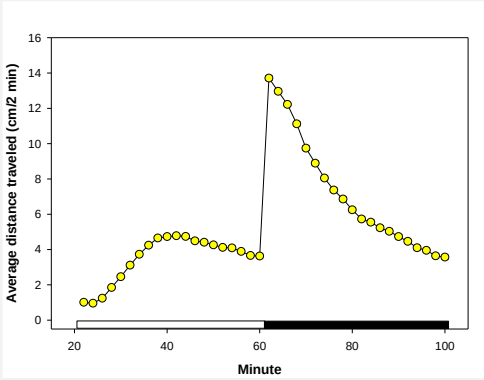


Behavioral Testing
- 6 days post fertilization
- 20 minute basal dark
- 40 minute light
- 40 minute dark

26ºC

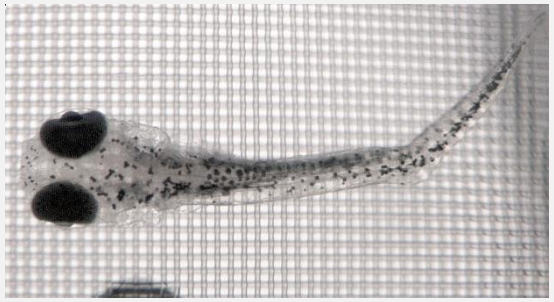
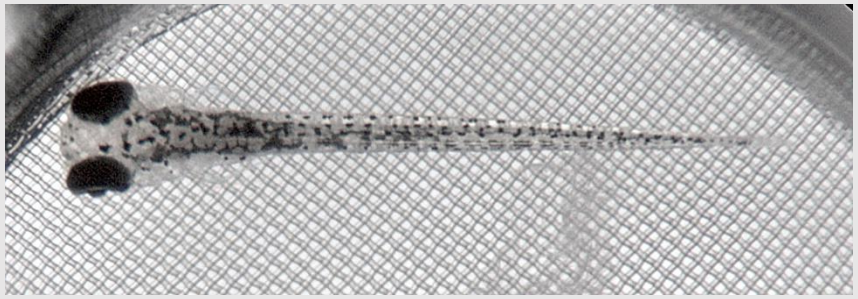


Assessment Post Test
- 6 days post fertilization
- Gross morphology and survival

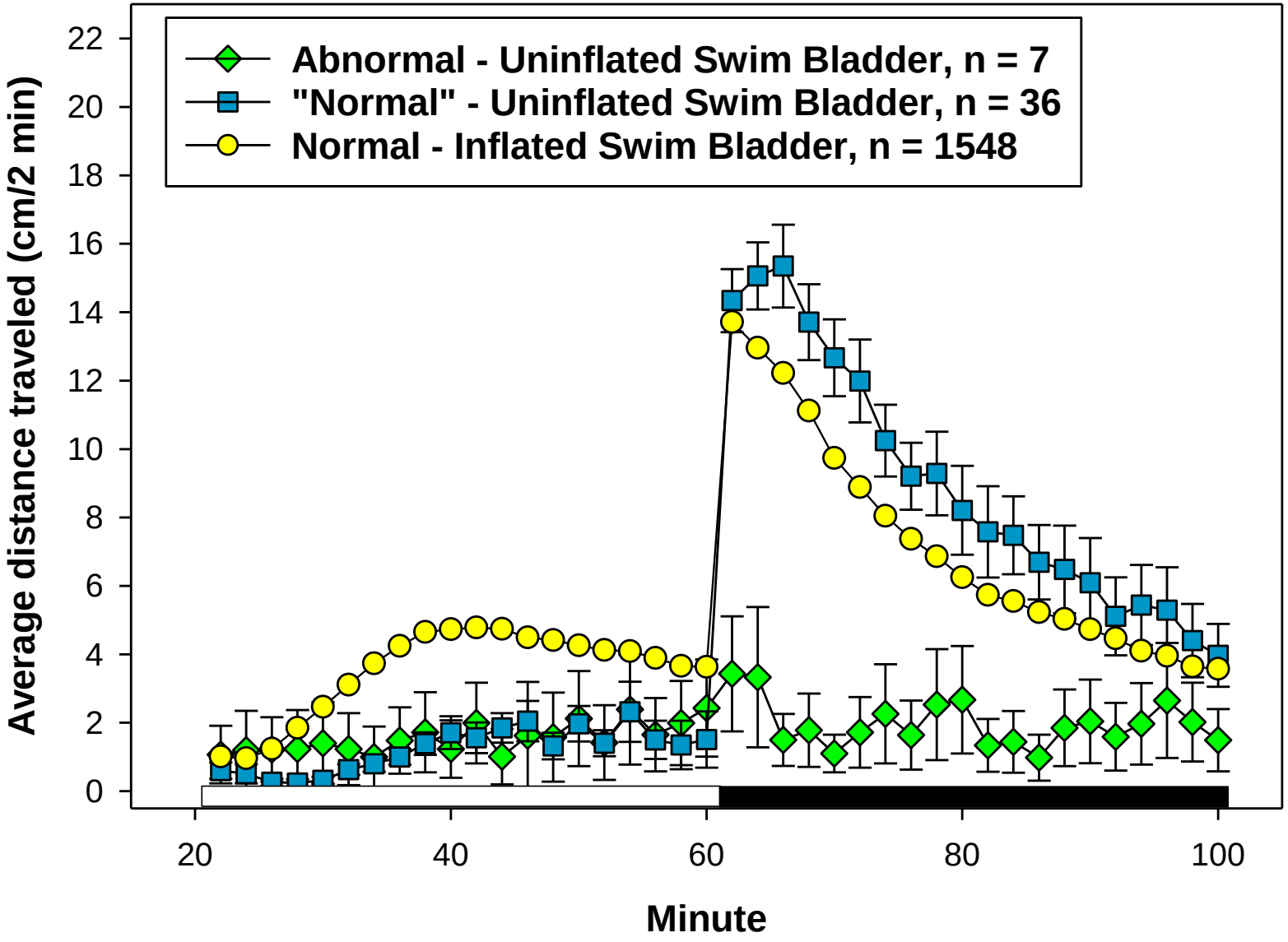


Tracking of behavior and data analysis

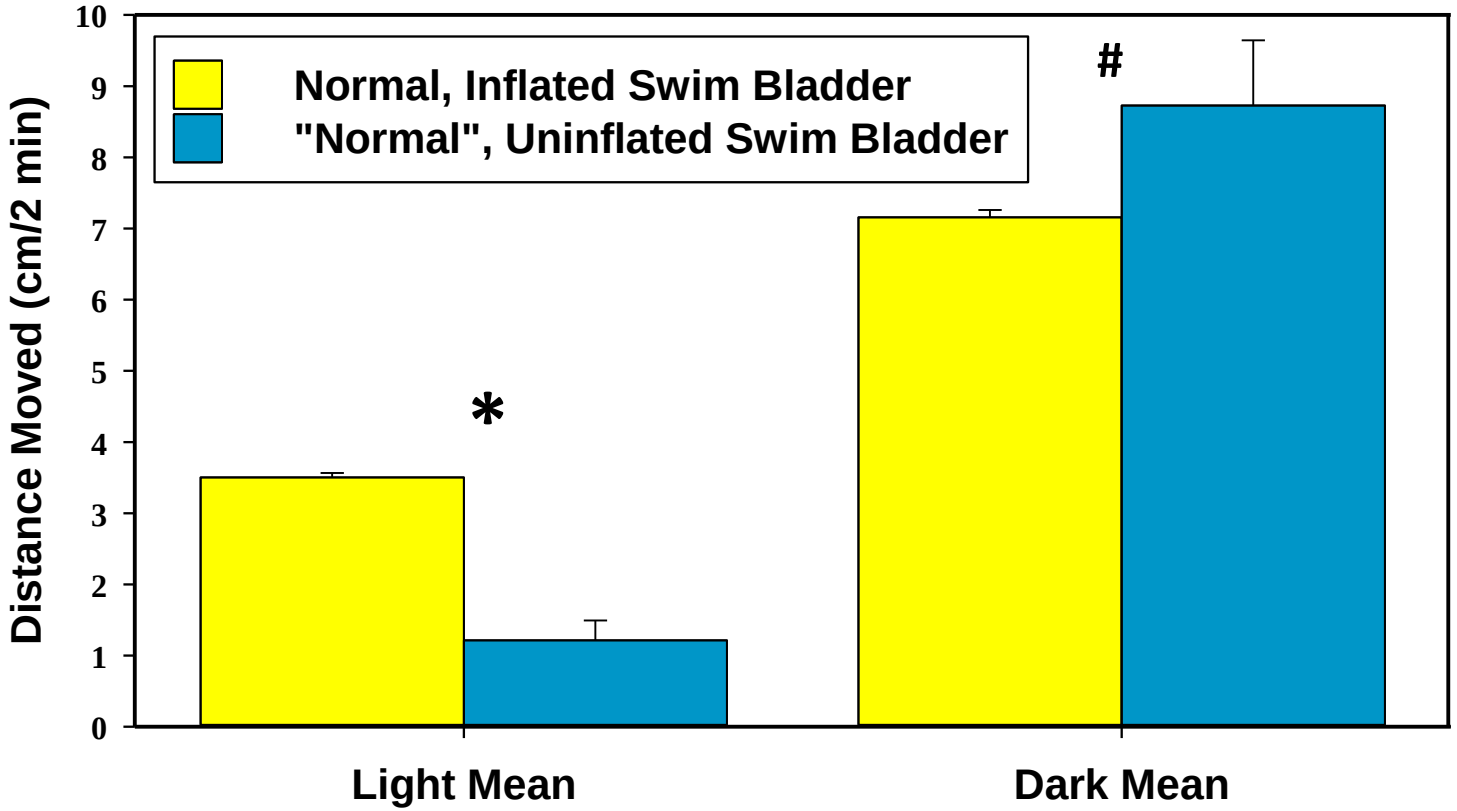
Data on 1591 CONTROL zebrafish larvae were examined:

Example	# of larvae	Condition of larvae at 6 days post fertilization
	7	Morphologically Abnormal with Uninflated Swim Bladder
	36	Otherwise Morphologically “Normal” with Uninflated Swim Bladder
	1548	Morphologically Normal with Inflated Swim Bladder

General Behavioral Patterns of Locomotion



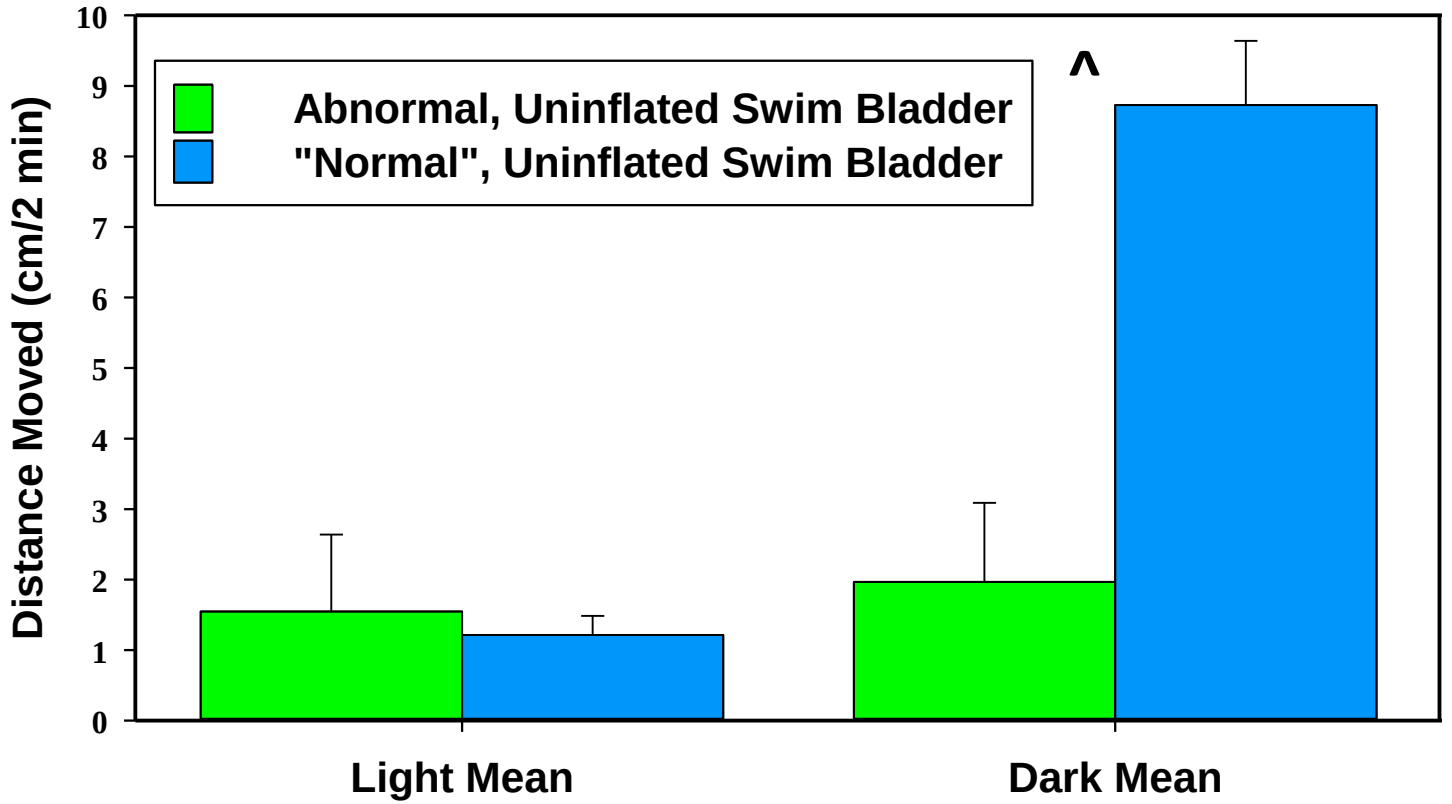
In otherwise morphologically “normal” larvae, swim bladder inflation status made a difference in locomotor activity level in both the light and the dark phases



- Otherwise morphologically “normal” larvae with uninflated swim bladders (blue bars) showed 3 times less activity in the light phase than normal larvae with inflated swim bladders (yellow bars).
- Conversely, in the dark phase, otherwise morphologically “normal” larvae with uninflated swim bladders (blue bars) had slightly more activity than normal larvae with inflated swim bladders (yellow bars).

[Note: Non-parametric statistical analysis with the Mann-Whitney U Test ; *= $p < 0.0001$; # = $p = 0.047$]

Morphology status of zebrafish larvae with uninflated swim bladders made a difference in locomotor activity in the dark phase but not in the light phase



- There was no difference in activity between abnormal zebrafish larvae with uninflated swim bladders (green bars) and otherwise morphologically “normal” zebrafish larvae with uninflated swim bladders (blue bars) in the light phase.
- However, in the dark phase, abnormal zebrafish larvae with uninflated swim bladders (green bars) had 4.5 times less activity than otherwise morphologically “normal” zebrafish larvae with uninflated swim bladders (blue bars).

[Note: Non-parametric statistical analysis with the Mann-Whitney U Test; ^= p = 0.001]

SUMMARY AND CONCLUSIONS

- **Both swim bladder inflation and dysmorphology profoundly affect behavior in zebrafish larvae and therefore are important confounding variables in the locomotor behavioral assay.**

- **These data illustrate the importance of morphological assessments and reporting in larval zebrafish behavior testing and the need to control for these variables.**

- **As human health and ecotoxicology rely more heavily on behavioral assessments in zebrafish and other non-mammalian species, it is essential that consideration of confounders be carefully addressed.**

THANK YOU!

All for your time and attention and especially to **Dr. George Sanders** for inviting me to speak today.

To the Animal Colony Care & Veterinary contract staff: Donald Holman, Guillermo Orozco, Femi Yerumo, Clark Kridler, Jenelle Dunn, Kimberly Wingate & Leslie Jarrell

To Keith Tarpley and Chuck Gaul for some of the photographs of the zebrafish and facility.

And most importantly to my ORISE & EPA collaborators: Matthew Waalkes, David Korest, Bridget Hill, Kimberly Jarema, Jeanene Olin, Deborah Hunter & Stephanie Padilla

QUESTIONS?