Methylene Blue and Developing Zebrafish – Effects on Mortality, Morphology and Behavior

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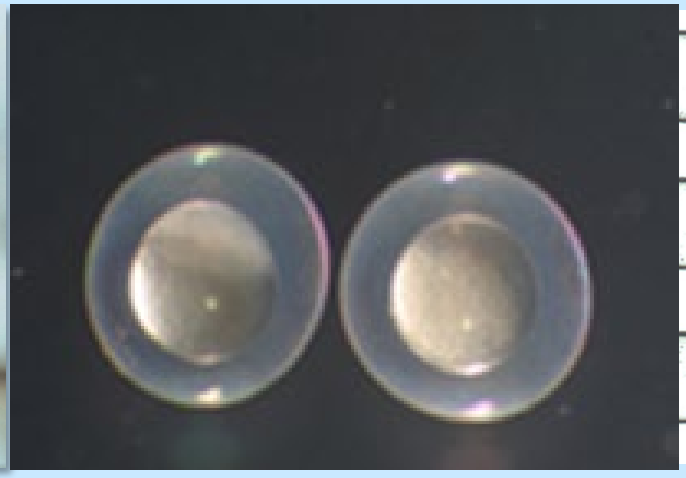

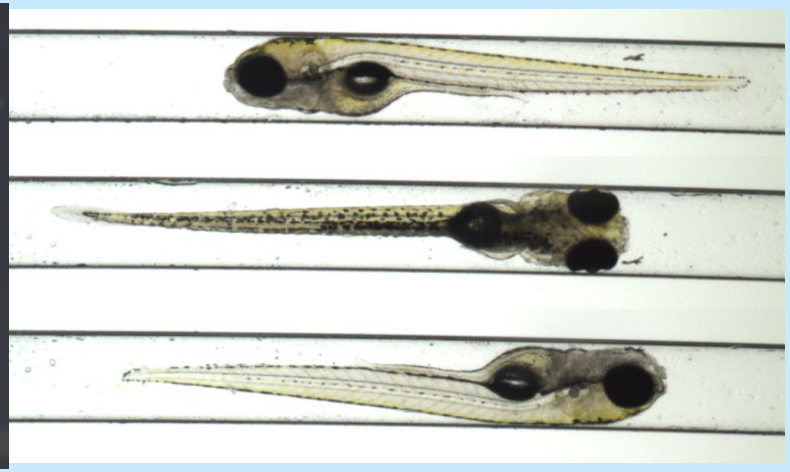

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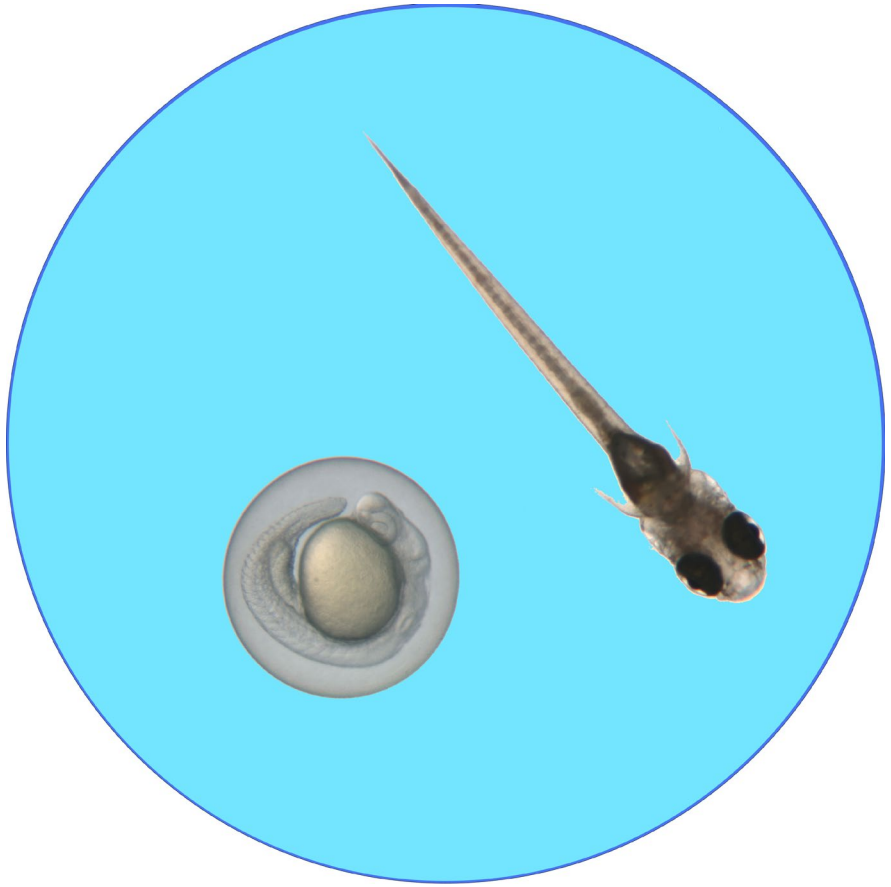
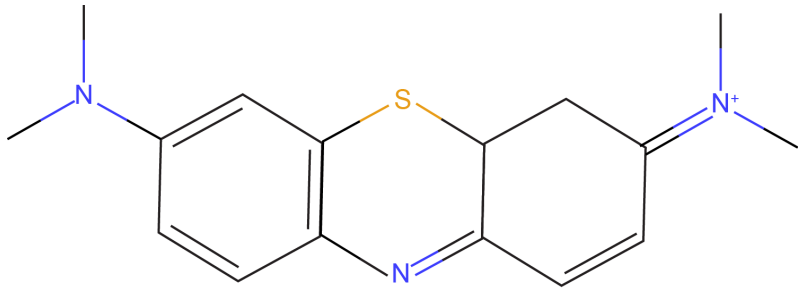
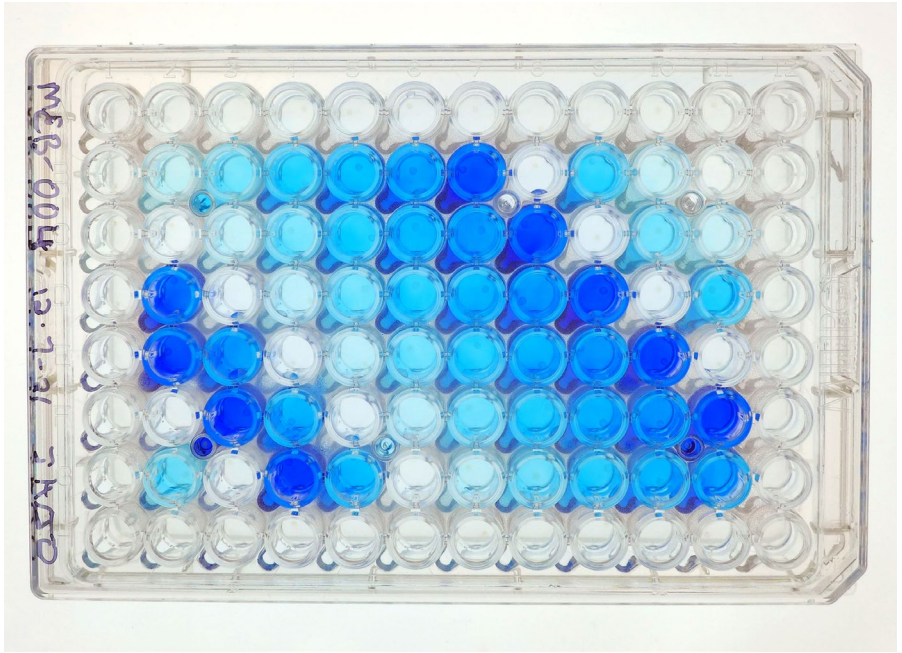
Does your embryo media with methylene blue added look like a darker blue than this?



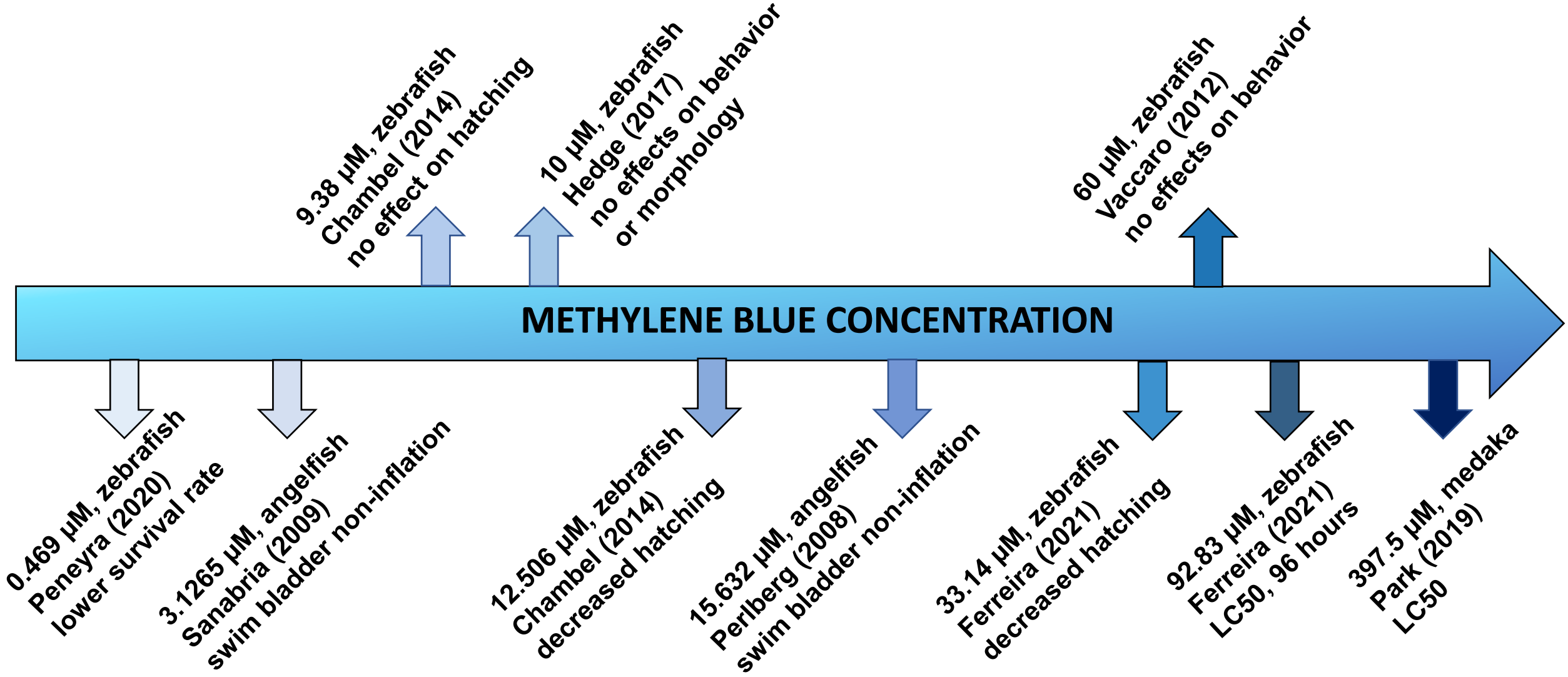
If so, your zebrafish may be in trouble!

Methylene Blue





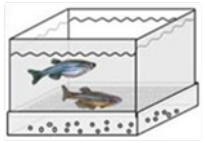
- “First fully synthetic drug used in medicine”
- Humans - treatment of methemoglobinemia, marking for polyps/tumor removal and to show lymphatic drainage
- Humans – previously used for cyanide poisoning, urinary tract infections, malaria, bipolar disorder, Alzheimer’s disease, RNA viruses, and marking the amniotic sac
- Mice - shown to cause both axial skeleton and neural tube defects
- Aquaculture/research and tropical fish hobbyists - treatment for fungal infections and to protect newly fertilized fish embryos from infection



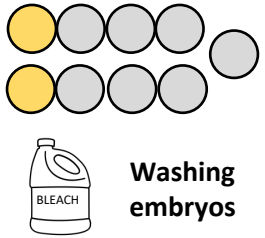
- Use on zebrafish embryos appears to be widespread
- Reported concentrations vary - 0.03 µM to over 3100 µM (husbandry manuals, experimental protocols & research papers)
- More commonly used concentrations on zebrafish embryos are 10 µM or less
- No systematic study of toxicological effects on developing zebrafish / no standardization for use

Experimental Protocol

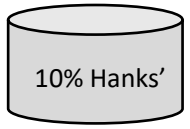
Pre-assay



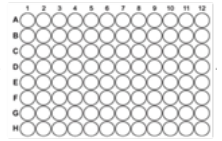
Zebrafish spawning



Washing embryos



Selecting optimal embryos



Plating 1 embryo per well at 6-8 hpf

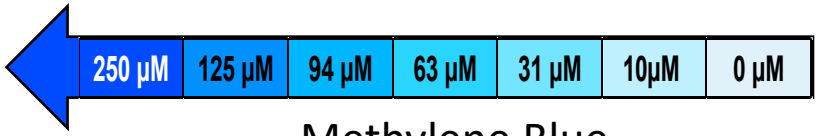
During assay

Locomotor testing and recording

10% Hanks'

6 dpf (transferred)

5 dpf (transferred)



4 dpf

3 dpf

2 dpf

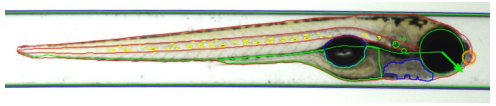
1 dpf

6-8 hpf (exposure begins)

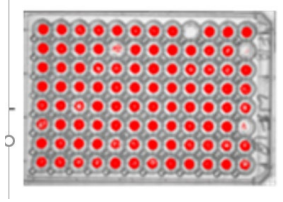
Post- assay

Assessing larvae for malformation and death

VAST images acquisition

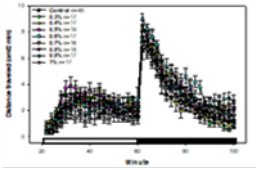


VAST image analysis

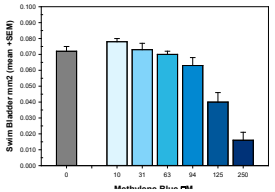


Locomotor activity tracking

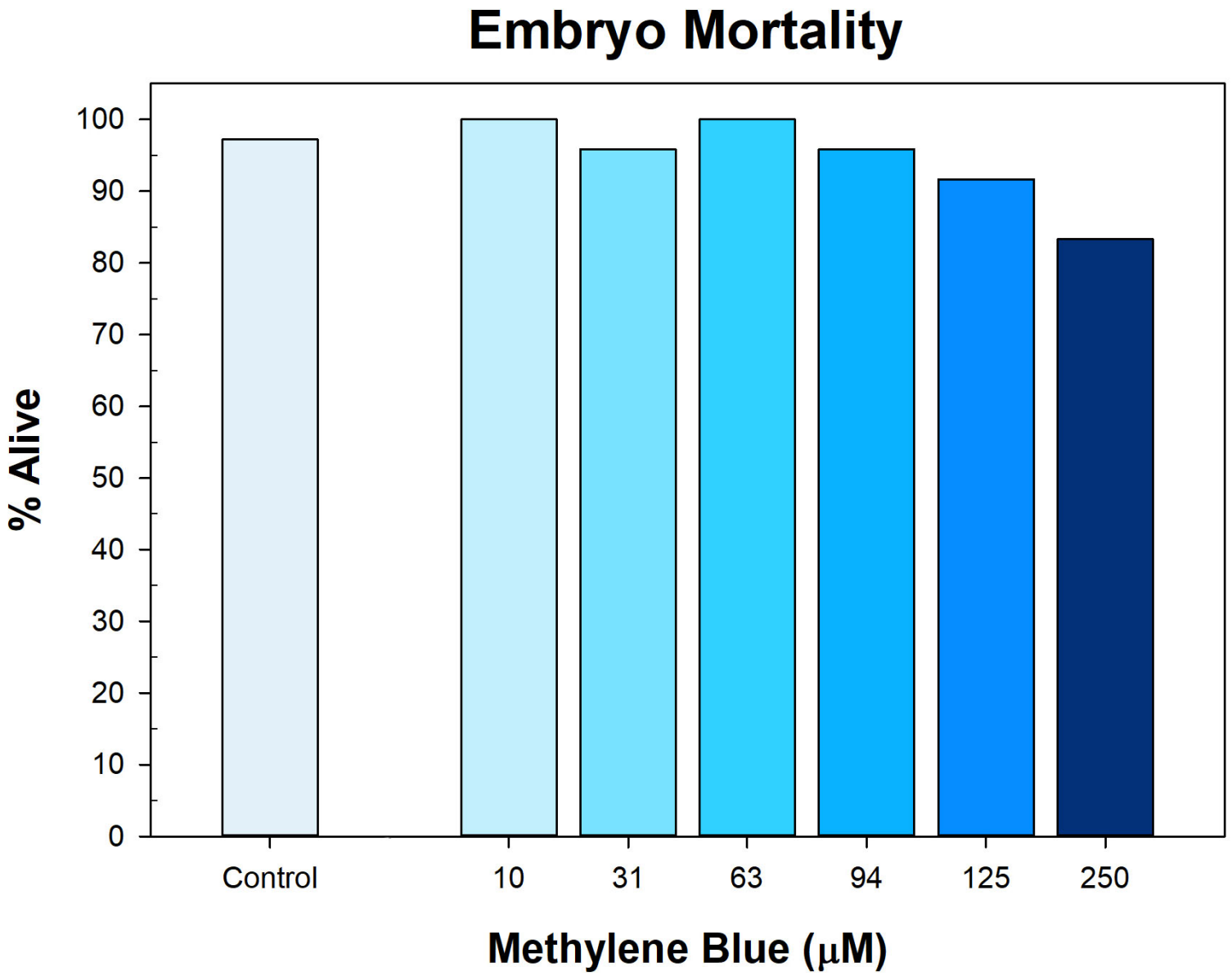
Compiling activity data and excluding malformations/death



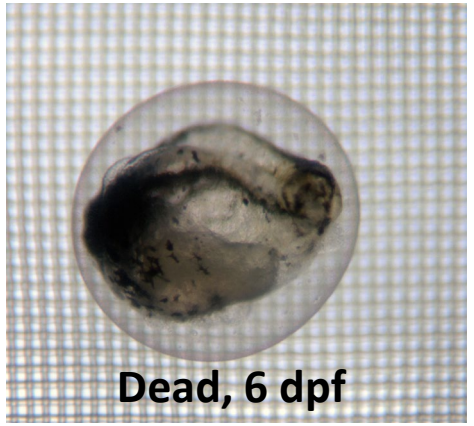
Generating activity graph and statistical testing

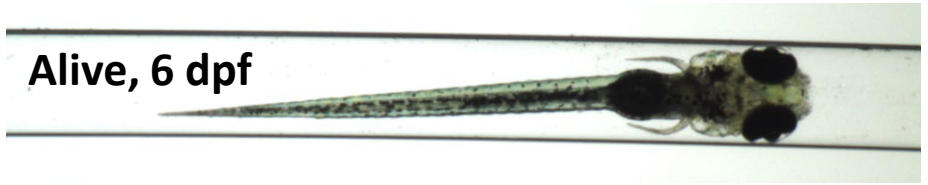


Compiling and graphing VAST data plus statistical testing



- **Embryo survival was not impacted by methylene blue in concentrations up to 250 µM**

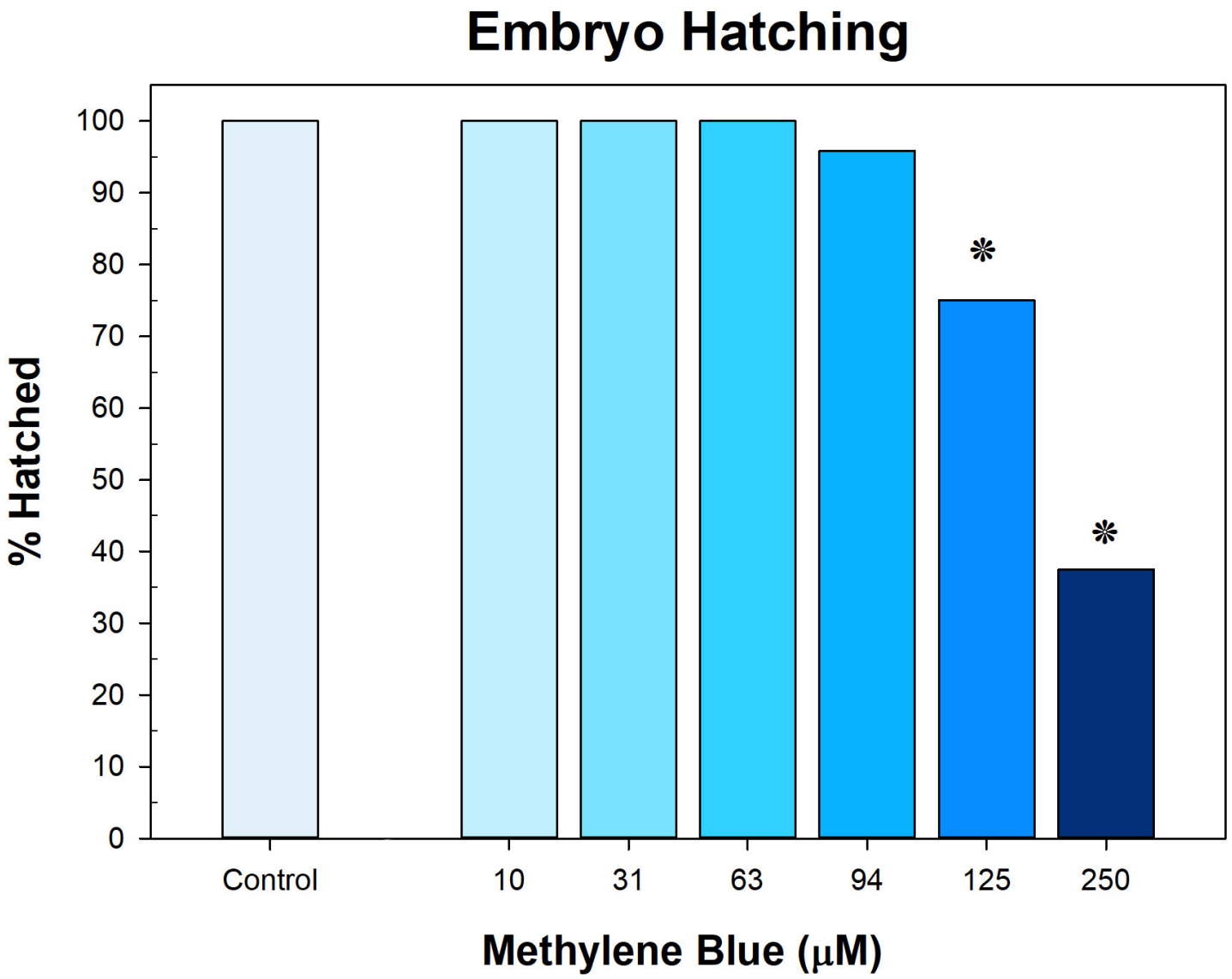




Control n=36

Doses 10-250 µM, n=24

Fisher's Exact Test on Incidence Data, P > 0.05



- **Reduction in percent of embryos hatched with concentrations of ≥ 125 µM methylene blue**

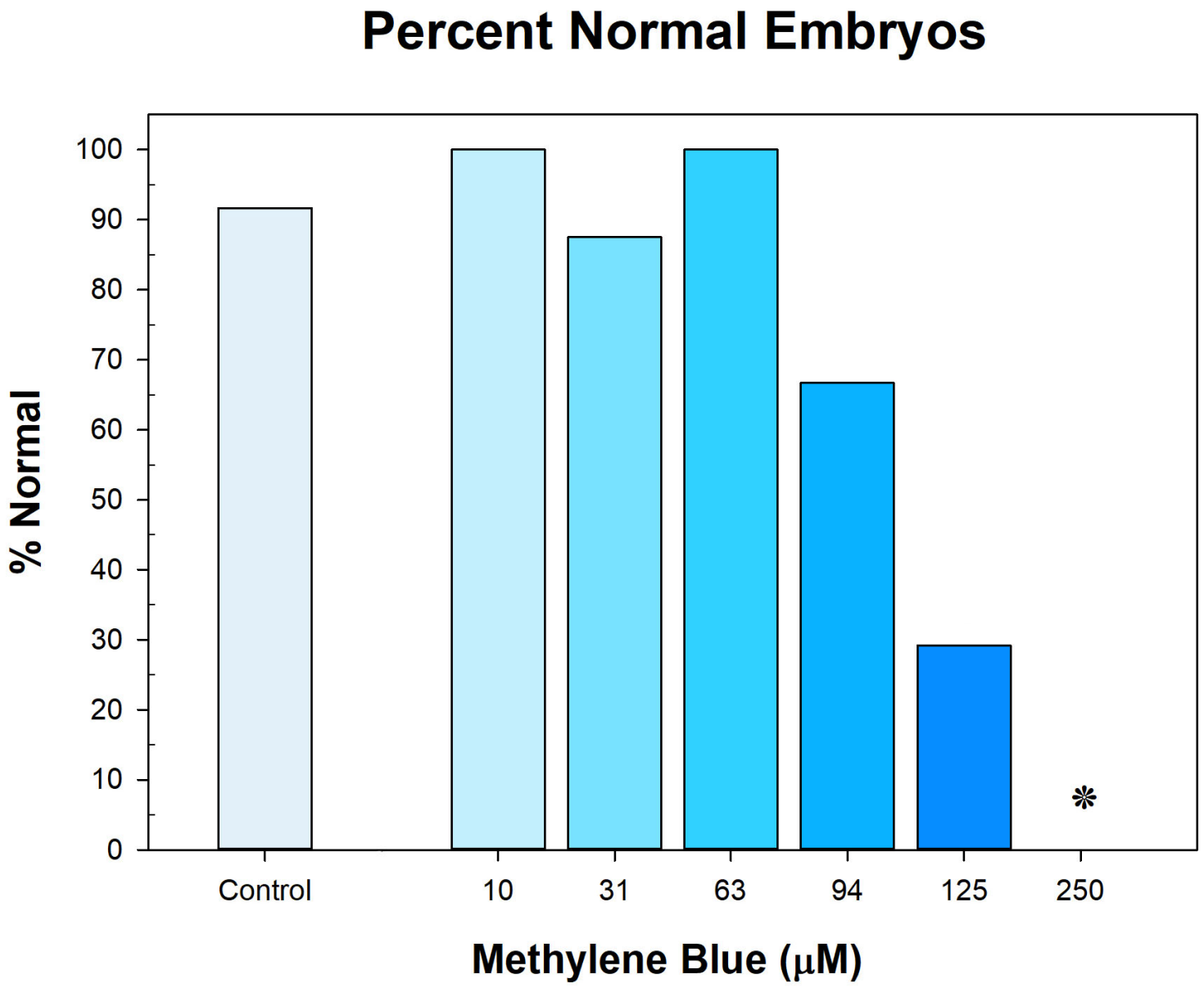
Unhatched, 6 dpf

Hatched, 6 dpf

Control n=36

Doses 10-250 µM, n=24

Fisher's Exact Test on Incidence Data, P < 0.0055 (125µM); P< 0.00001 (250 µM)

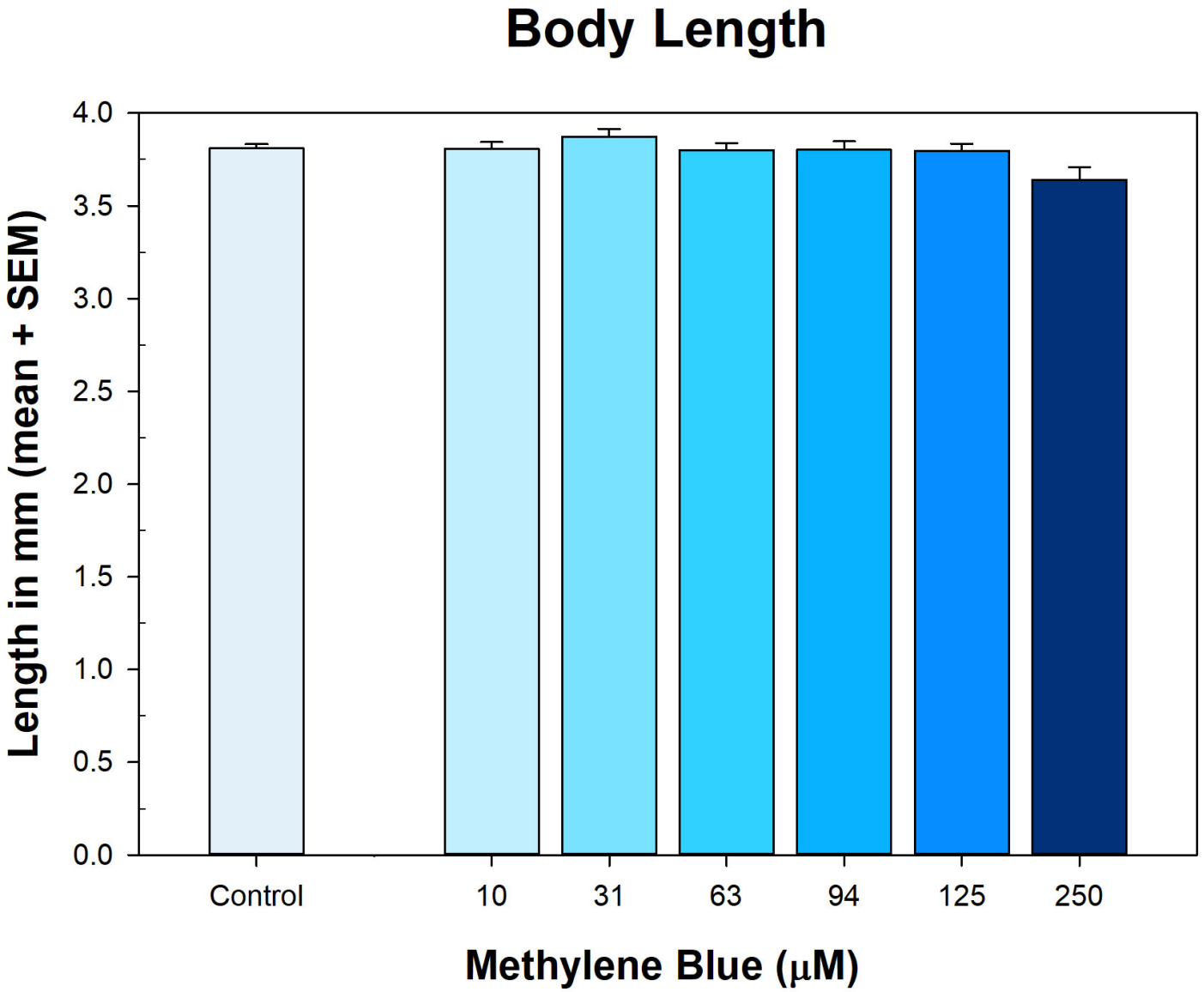


Control n=36 Doses 10-250 µM, n=24 Fisher's Exact Test on Incidence Data, P < 0.00001

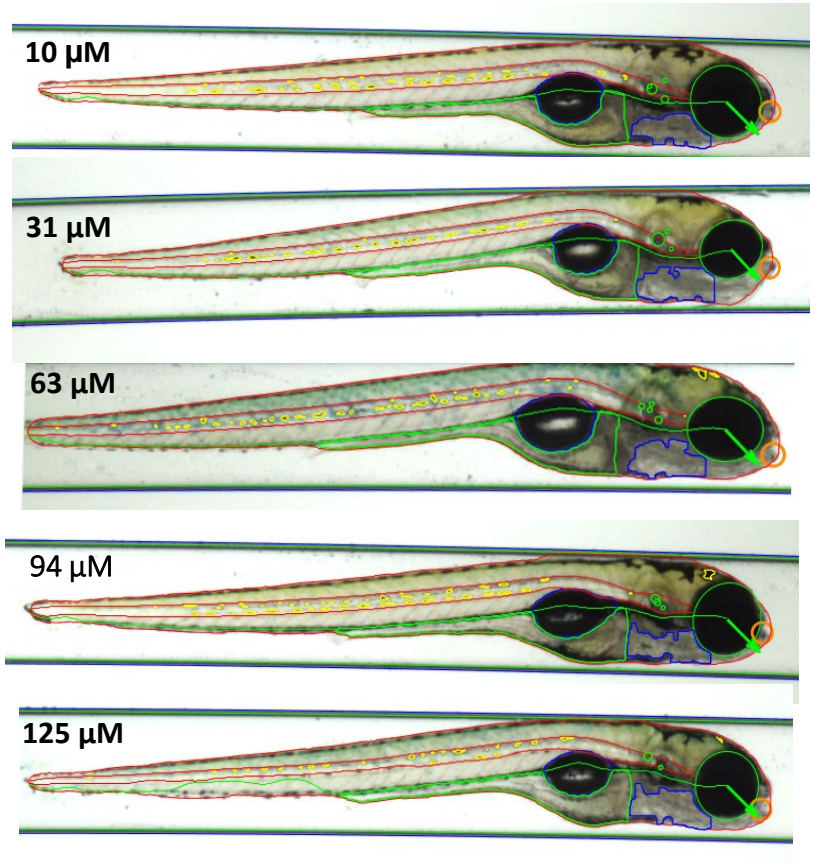
- **Removing dead, unhatched, abnormal (swim bladder uninflation, etc.) embryos, % "normal" was calculated**
- **Reduction in the percent of "normal" embryos with 250 µM methylene blue**
- **No "normal" embryos found at 250 µM methylene blue**

Uninflated swim bladder, 6 dpf

Normal, 6 dpf



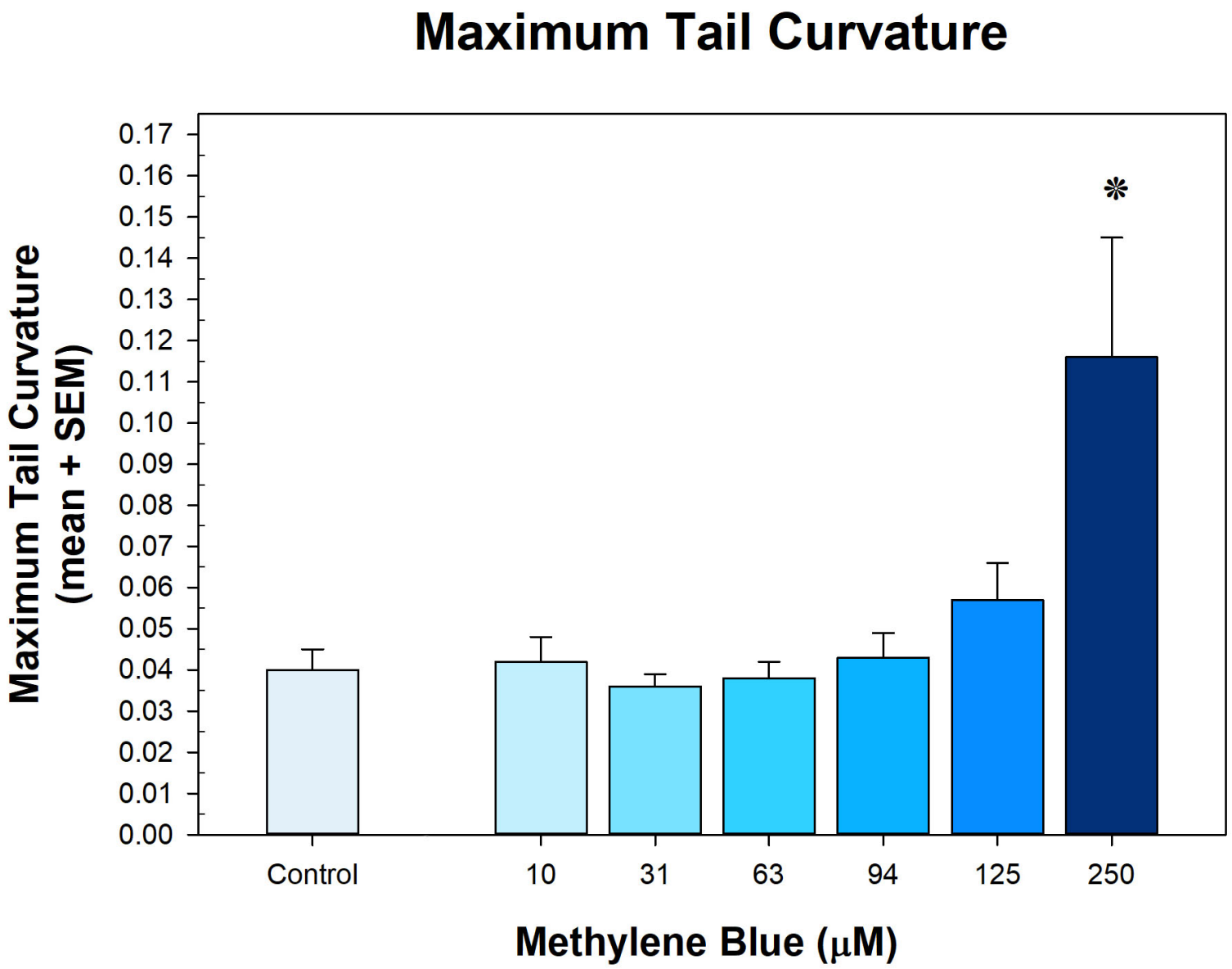
- **No dose response relationship between methylene blue and body length.**



Control n=36

Doses 10-250 µM, n=10-24

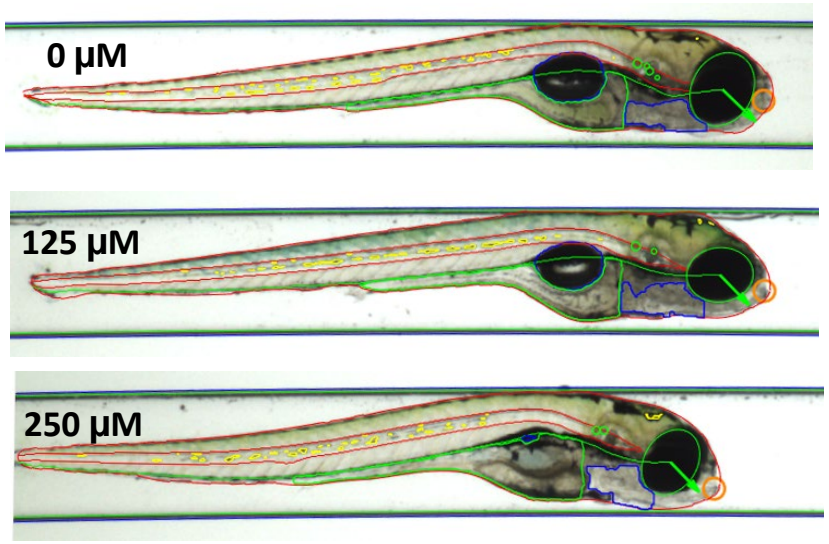
ANOVA, P-Value = 0.0571



Control n=36

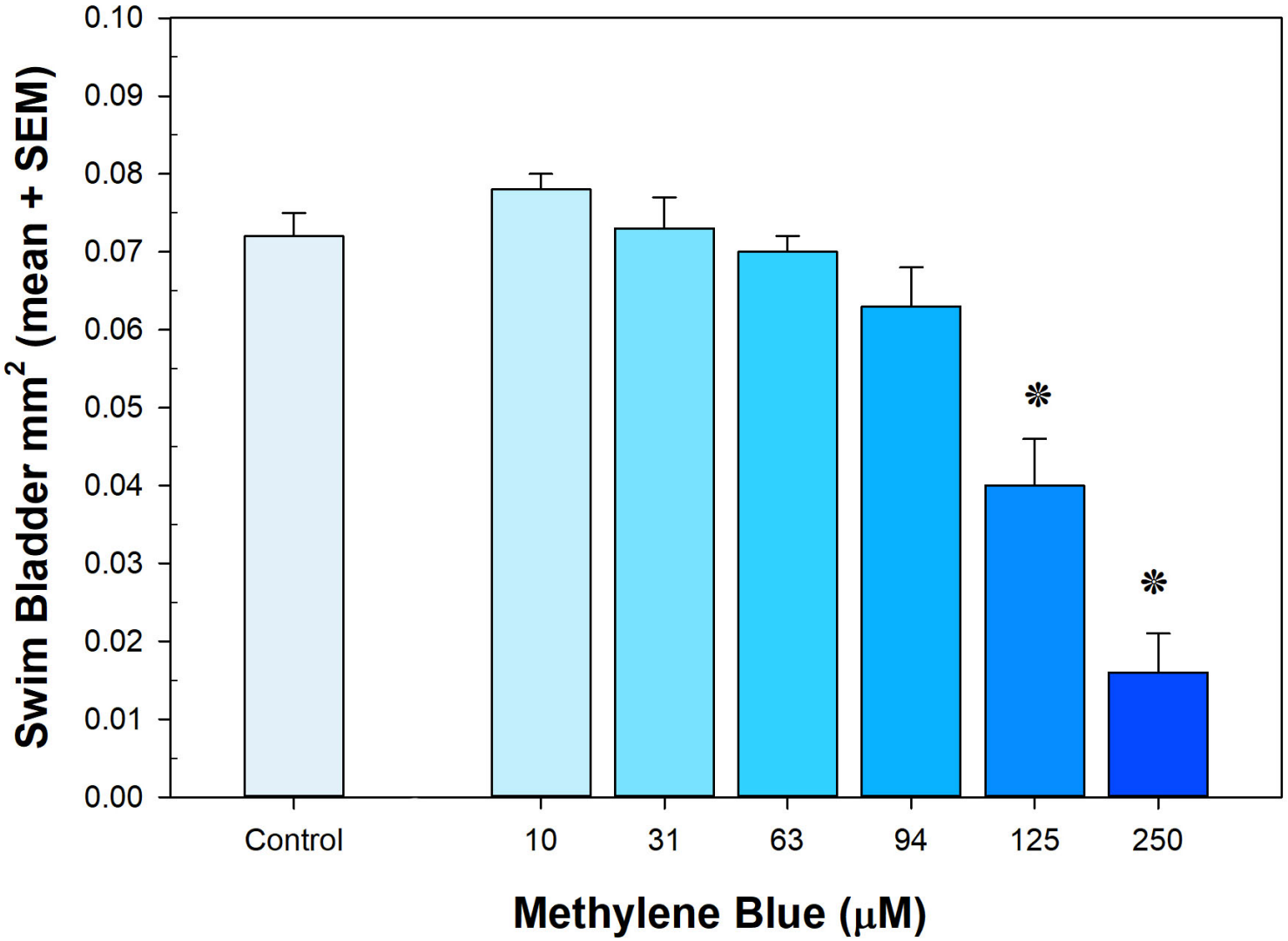
Doses 10-250 µM, n=10-24

- **Maximum tail curvature increased with 250 µM methylene blue**

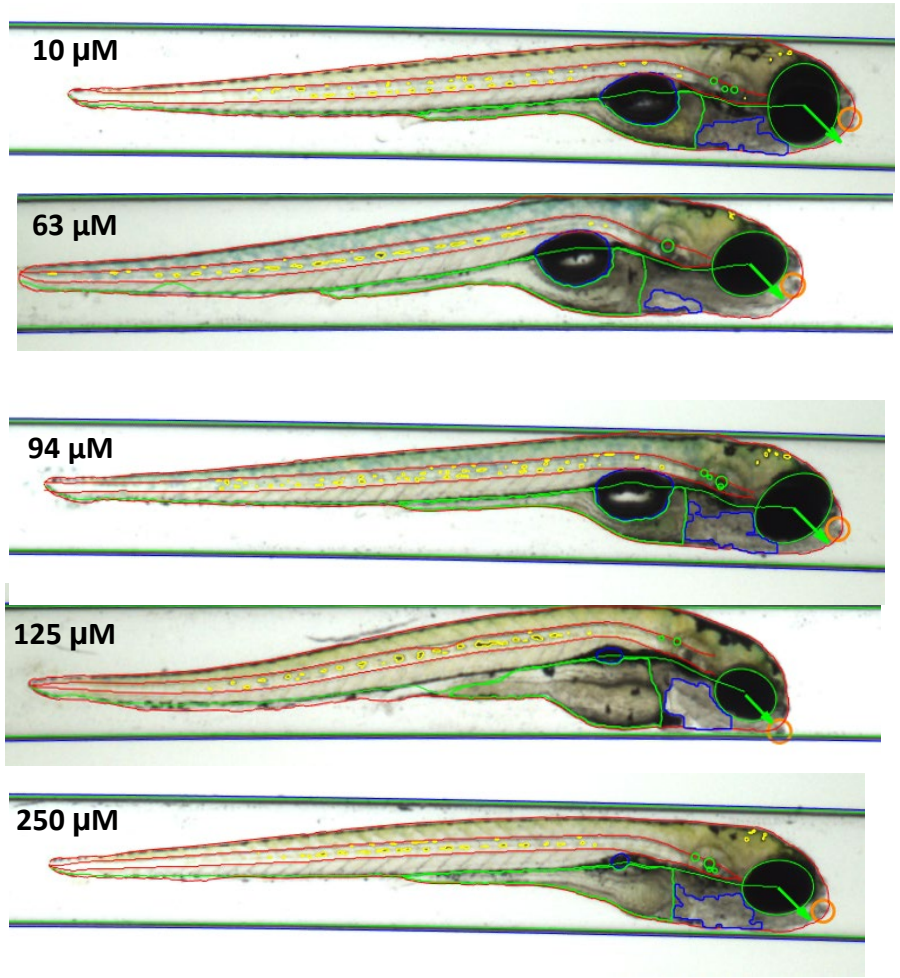


ANOVA, P-Value < 0.0001, followed by Fisher's PLSD

Swim Bladder Size



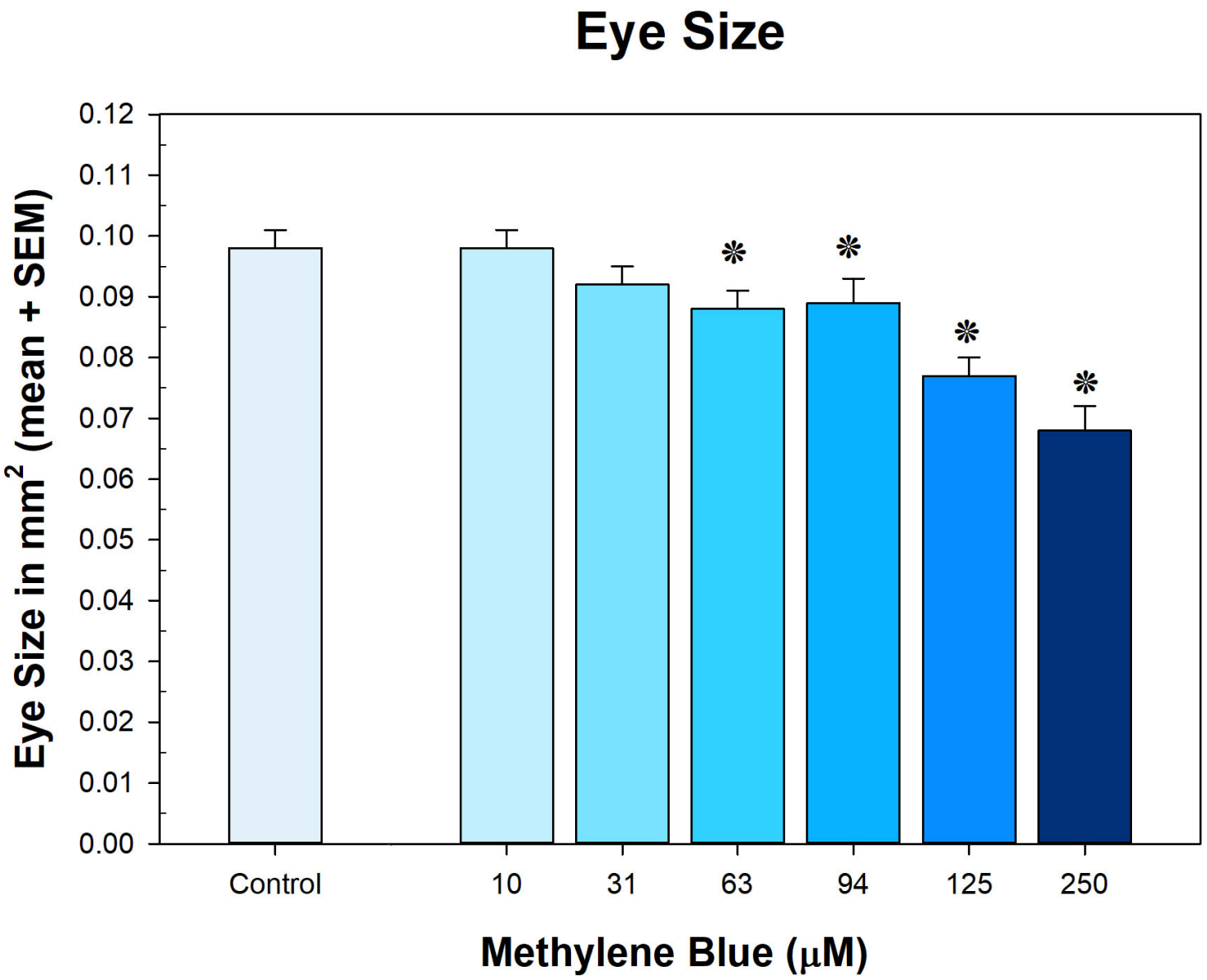
- **Swim bladder size reduced in larvae with 125 µM or > methylene blue**



Control n=36

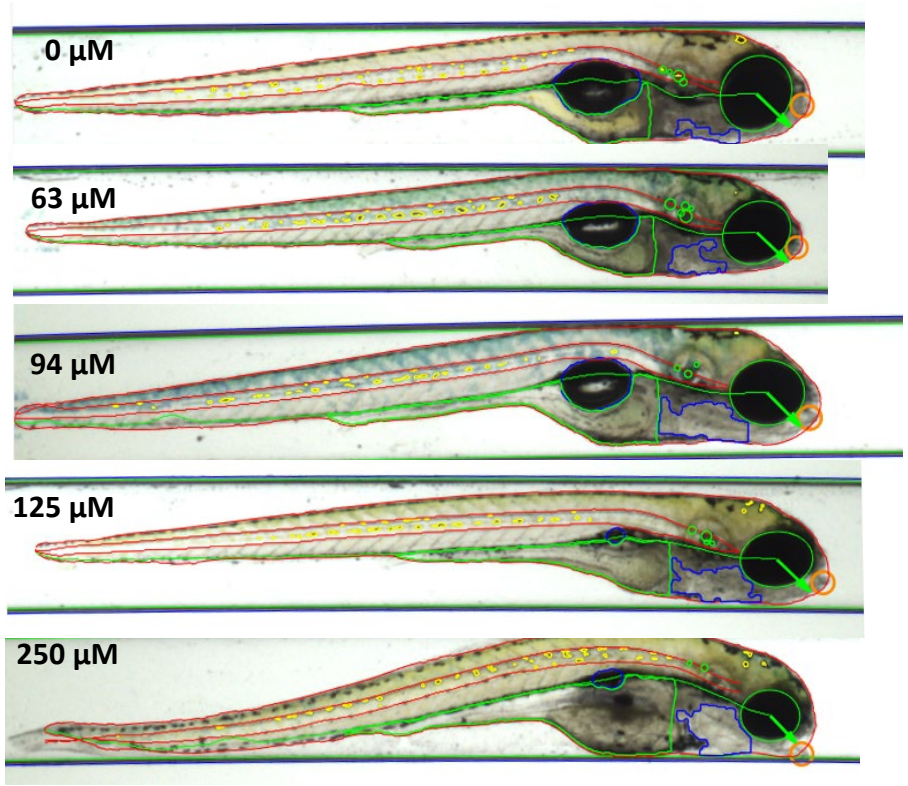
Doses 10-250 µM, n=10-24

ANOVA, P-Value < 0.0001, followed by Fisher's PLSD



Control n=36 Doses 10-250 µM, n=10-24 ANOVA, P-Value < 0.0001, followed by Fisher's PLSD

- **Eye size = most sensitive endpoint assessed**
- **63 µM or > methylene blue caused a significant reduction in eye size**



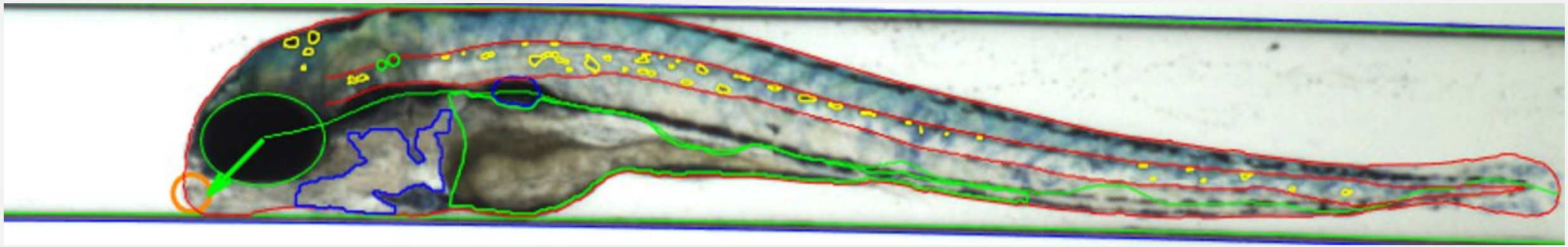
Effects of Methylene Blue

	Locomotor Behavior	Mortality	Hatching	% Normal	Body Length	Tail Curvature	Swim Bladder Size	Eye Size
less than 10 µM	-	-	-	-	ND	ND	ND	ND
10 µM	ND	-	-	-	-	-	-	-
31 µM	ND	-	-	-	-	-	-	-
63 µM	ND	-	-	-	-	-	-	+
94 µM	ND	-	-	-	-	-	-	+
125 µM	ND	-	+	-	-	-	+	+
250 µM	ND	-	+	+	-	+	+	+

0 µM	10µM	31 µM	63 µM	94 µM	125 µM	250 µM

Conclusions

- Including our previous work, methylene blue appears safe for use with zebrafish embryos up to **10 µM** which falls within the most commonly used concentrations.
- Concentrations above **31 µM** methylene blue are not recommended.
- Use of methylene blue when the exact concentration is not known is not safe for use with zebrafish embryos.



Contributors to this research

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- Morgan Lowery
- Kimberly Jarema
- Alan Tennant
- Susan Farmer

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