Zebrafish Models for Human Acute Organophosphorus Poisoning

Natàlia Garcia-Reyero
US Army Engineer R&D Center
USACE

ERDC
Engineer Research and Development Center

CSIC
idaea

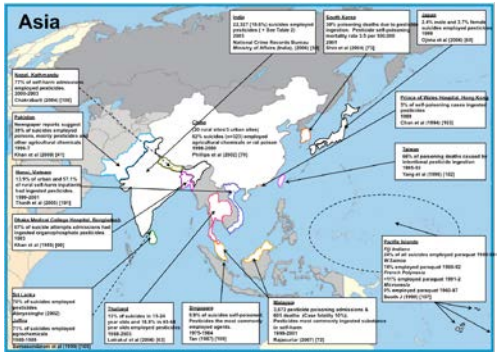
Acute organophosphorus poisoning, as a major public health concern some facts

Developing countries

Self-poisoning

around **3 million** cases

300,000 deaths



Developed countries

Intentionally (**terrorist attack**)/ chemicals released from transportation or storage facilities after an **accident** or a **natural disaster**

Eddleston, M., Buckley, N. A., Eyer, P., & Dawson, A. H. (2008). *The Lancet*, *371*, 597-607; Gunnell, D., Eddleston, M., Phillips, M. R., & Konradsen, F. (2007). *BMC public health*, *7*, 357.

Acute Organophosphorus Poisoning: looking for new therapeutic strategies

Although pathophysiology of OPP is complex....

- AChE inhibition
- ACh accumulation
- Nicotinic & muscarinic AChR Activation
- EAA release
- Seizures
- **Cholinergic neurones overstimulation**
- NMDA-receptor activation
- Ca^{2+} disregulation
- Inflammatory response
- Mitochondrial respiration
- Oxidative stress
- Immune response
- Cell death

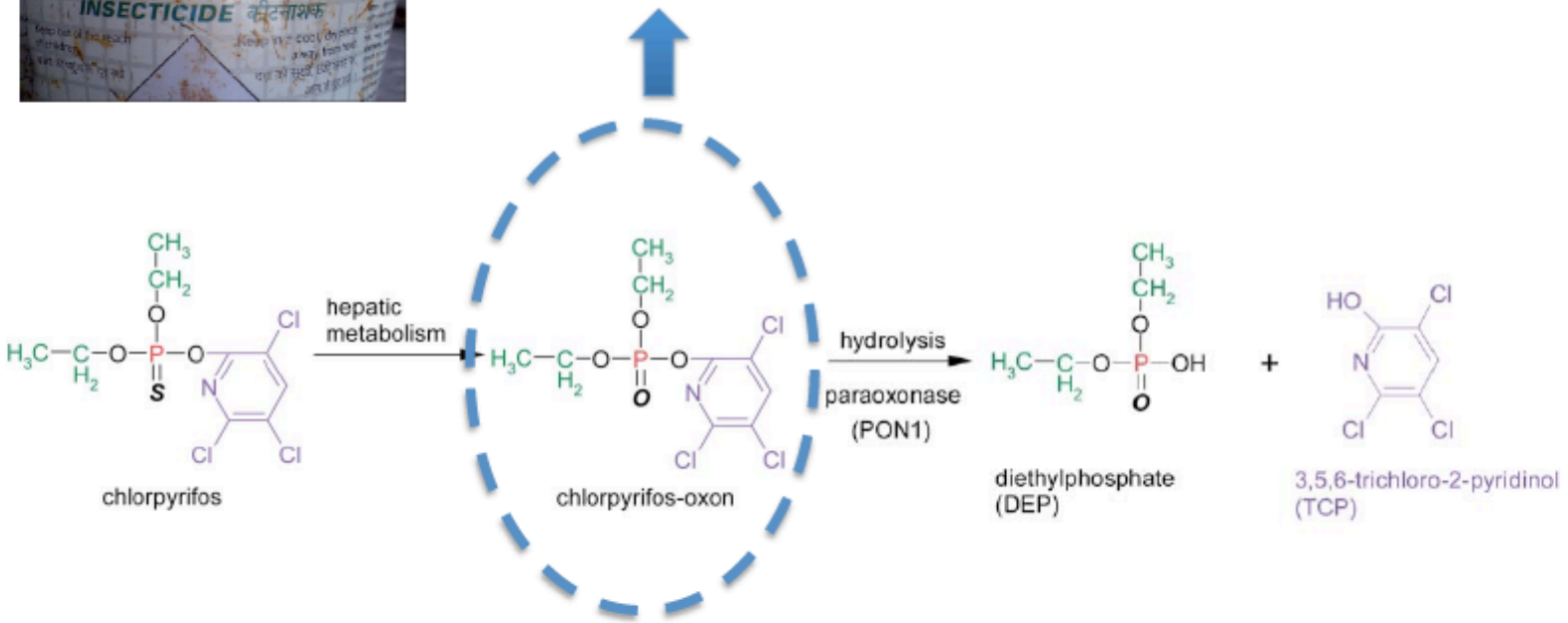
... standard therapy mainly targets:

- **AChE reactivators**
- **Muscarinic AChR antagonist**
- Anticonvulsants

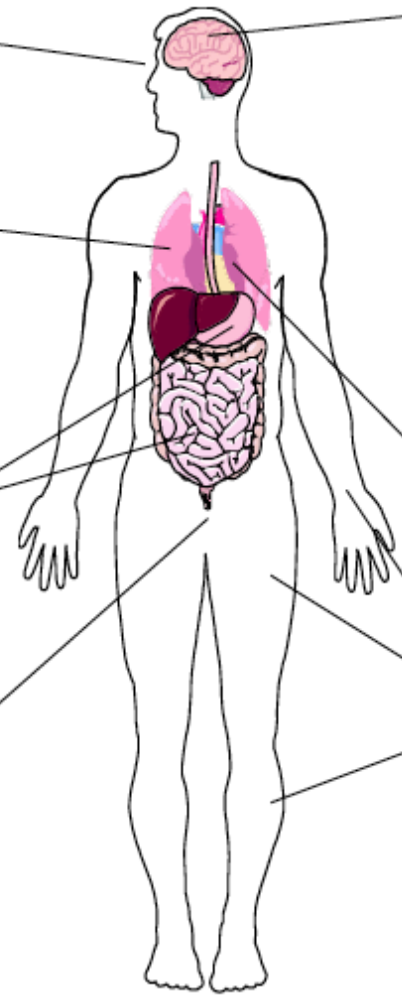
"Multifunctional drug therapies"

CHOLINERGIC TOXIDROME

ACETYLCHOLINESTERASE INHIBITION



Manifestations of Organophosphate Poisoning



Optic System
Pupil Constriction
Blurred Vision
Lacrimation

Respiratory System
Bronchospasm
Bronchial Secretion
Pulmonary Edema
Tightness of Chest
Wheezing
Cough
Difficulty Breathing

Gastrointestinal Tract
Salivation
Nausea
Cramps
Abdominal Pain
Vomiting
Diarrhea
Fecal Incontinence

Urinary - Genital
Urinary Incontinence
Impotence
Uterus Contraction

Brain
Headache
Dizziness
Vertigo
Anxiety
Apathy
Confusion
Anorexia
Insomnia
Lethargy
Fatigue
Inability to Concentrate
Memory Impairment
Convulsion
Coma

Cardiovascular Svstem
Bradycardia, Tachycardia
Increased Blood Pressure

Musculature
Weakness
Tremor
Fasciculations
Twitching
Cramps
Increased Sweating

ANTIDOTES against the cholinergic toxidrome

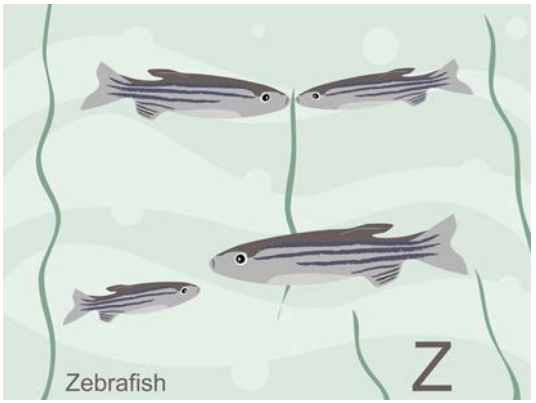
Military MARK I Kit containing atropine and 2-PAM autoinjectors.

1- Atropine: competitive antagonist of muscarinic cholinergic receptors in both the CNS and the PNS (improves respiratory function by decreasing secretions)

2.- Pralidoxime (2-PAM): prevents aging of AChE and reverse muscle paralysis with OP poisoning

3- Benzodiazepines: Depresses all levels of CNS (eg, limbic and reticular formation) by increasing activity of GABA. Used for treatment of seizures.

DEVELOPMENT OF A ZEBRAFISH MODEL OF CHOLINERGIC TOXIDROME, AS A TOOL FOR IDENTIFICATION OF ANTIDOTES



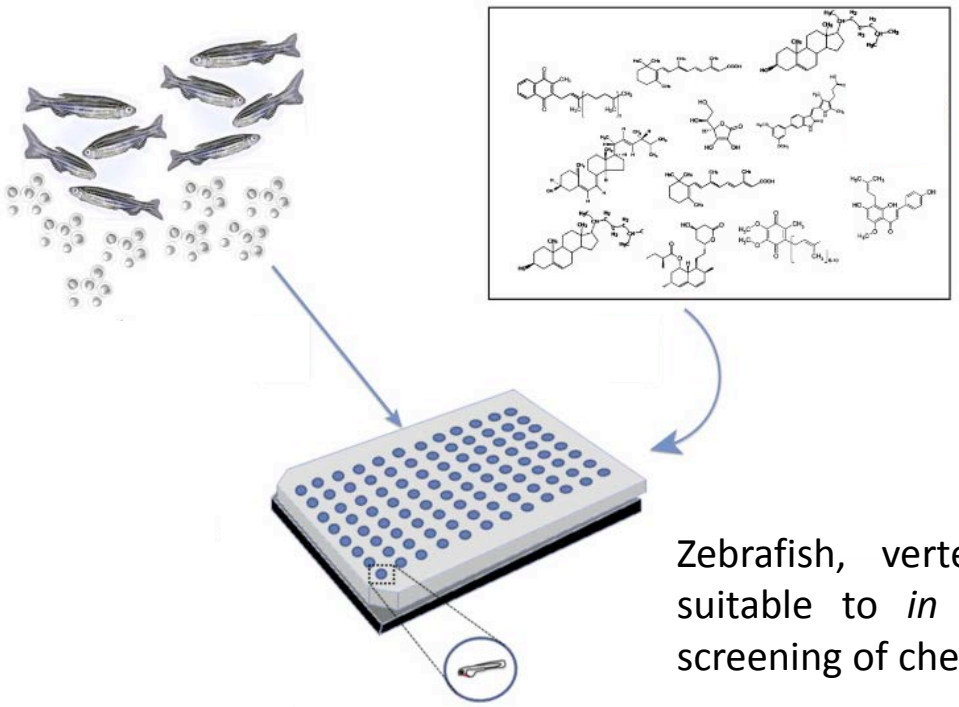
Muscarinic effects

Cholinergic Toxidrome Flashcard

Acute Organophosphorus Poisoning: some facts

Identification of new medical countermeasures against OPP by the **development** and **validation** of ***in vivo* animal models** for rapid **screening** of **molecular libraries**

NIH CounterACT program



Zebrafish, vertebrate model of human diseases suitable to *in vivo* medium and high-throughput screening of chemicals

Objectives

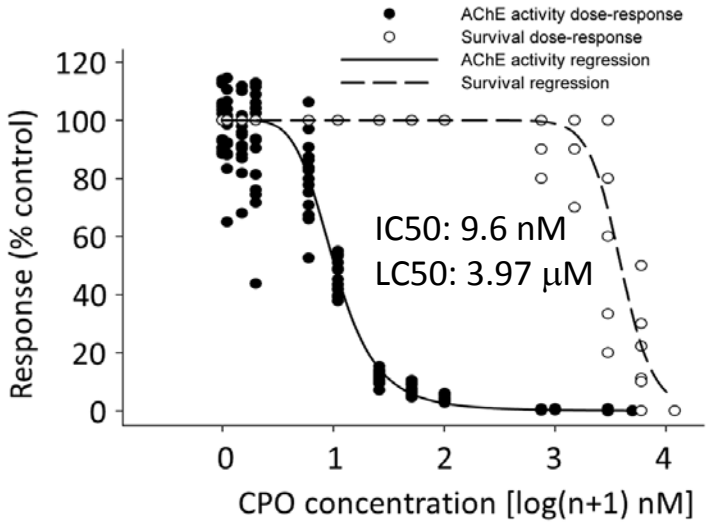
To **develop and validate** new OPP **mechanistic models** suitable for *in vivo* **medium and high throughput screening** with drugs of therapeutic value.

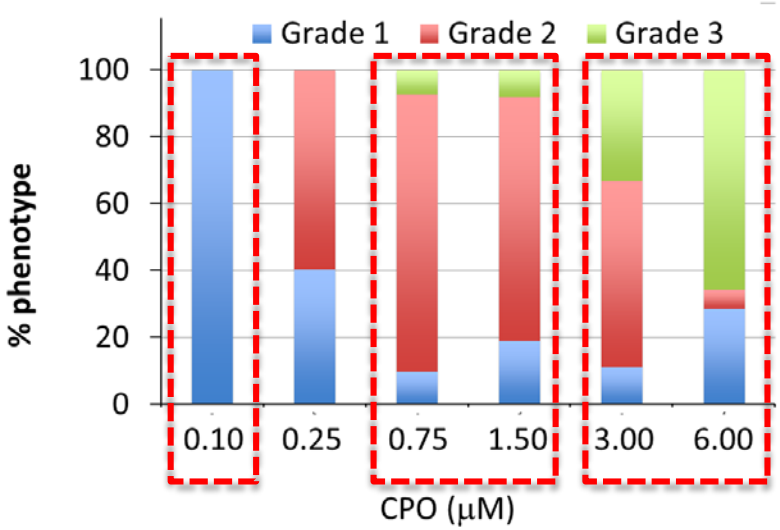
1. **Development of chemical model**s of OPP, with different grades of severity, in zebrafish larvae by using chlorpyrifos-oxon as a prototypic OP compound
2. **Characterization** of the models, by analysing the adverse effects at different levels of organization (transcriptional, biochemical, ultrastructural, cellular & tissular, organismal and behavioural)
3. Deciphering the **pathophysiological pathways** involved in OPP development in our models by using a pharmacological approach and the analysis of the perturbed KEGG pathways

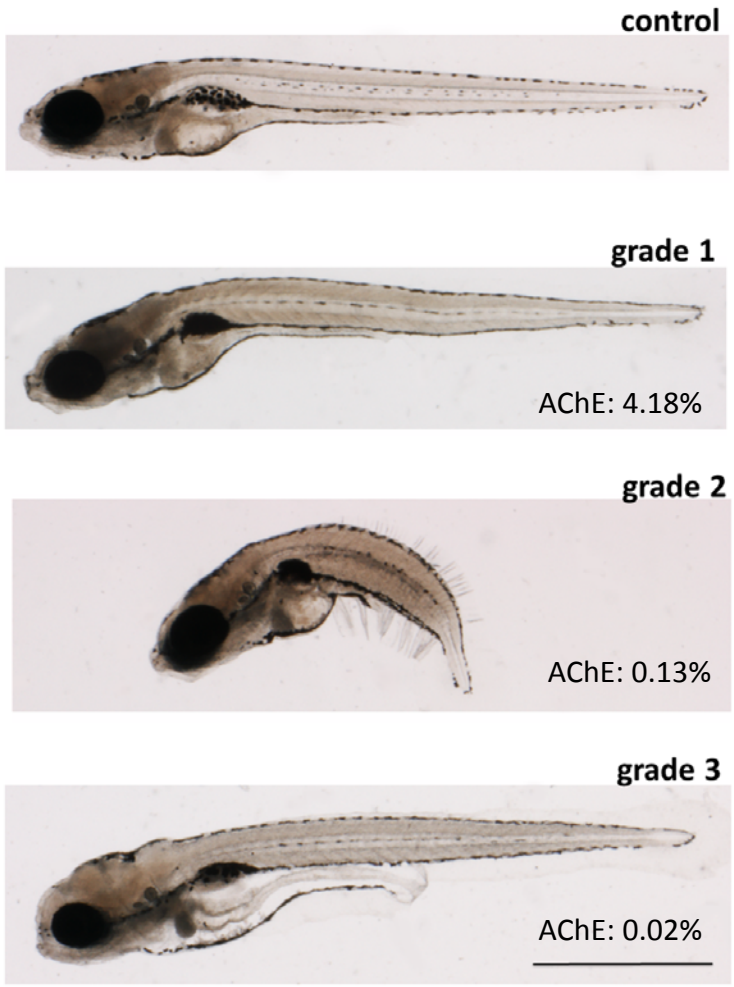
Methods

- Biochemical determination: AChE activity (individual fish), SOD, CAT, GSH (pools of 5 larvae)
- LPO determination
- In vivo detection of ROS generation
- Histopathology
- Behavior: basal locomotor activity, visual motor response, and touch-evoked escape response
- RNAseq
- Oxygen consumption
- Adenine nucleotide levels (AMP, ADT, ATP)

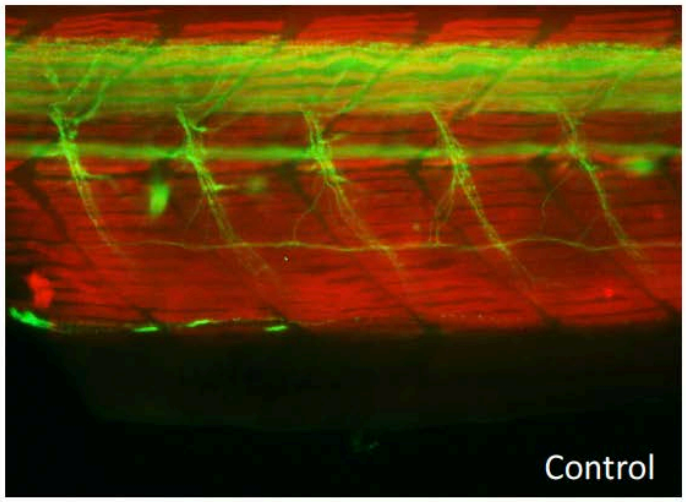
Grading OPP severity in zebrafish larvae

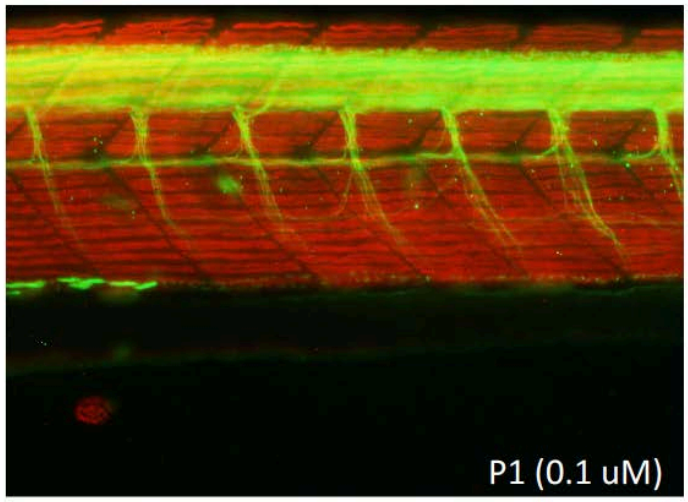


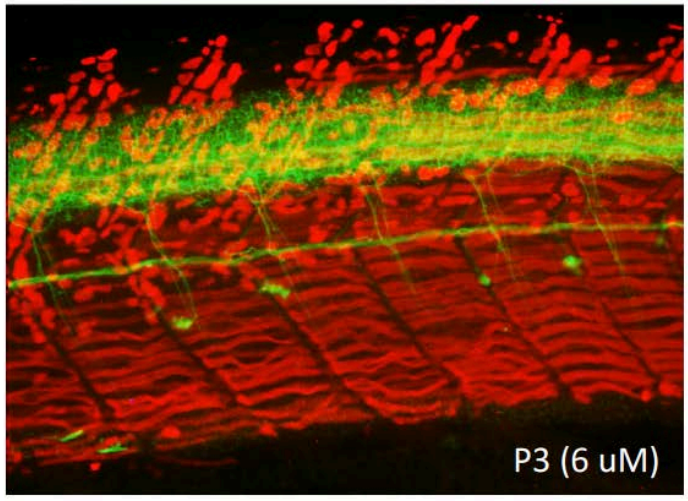


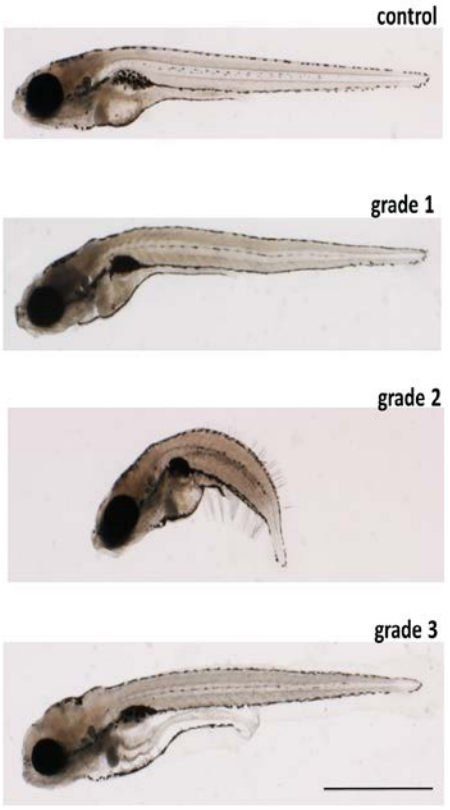


Grading OPP severity in zebrafish larvae

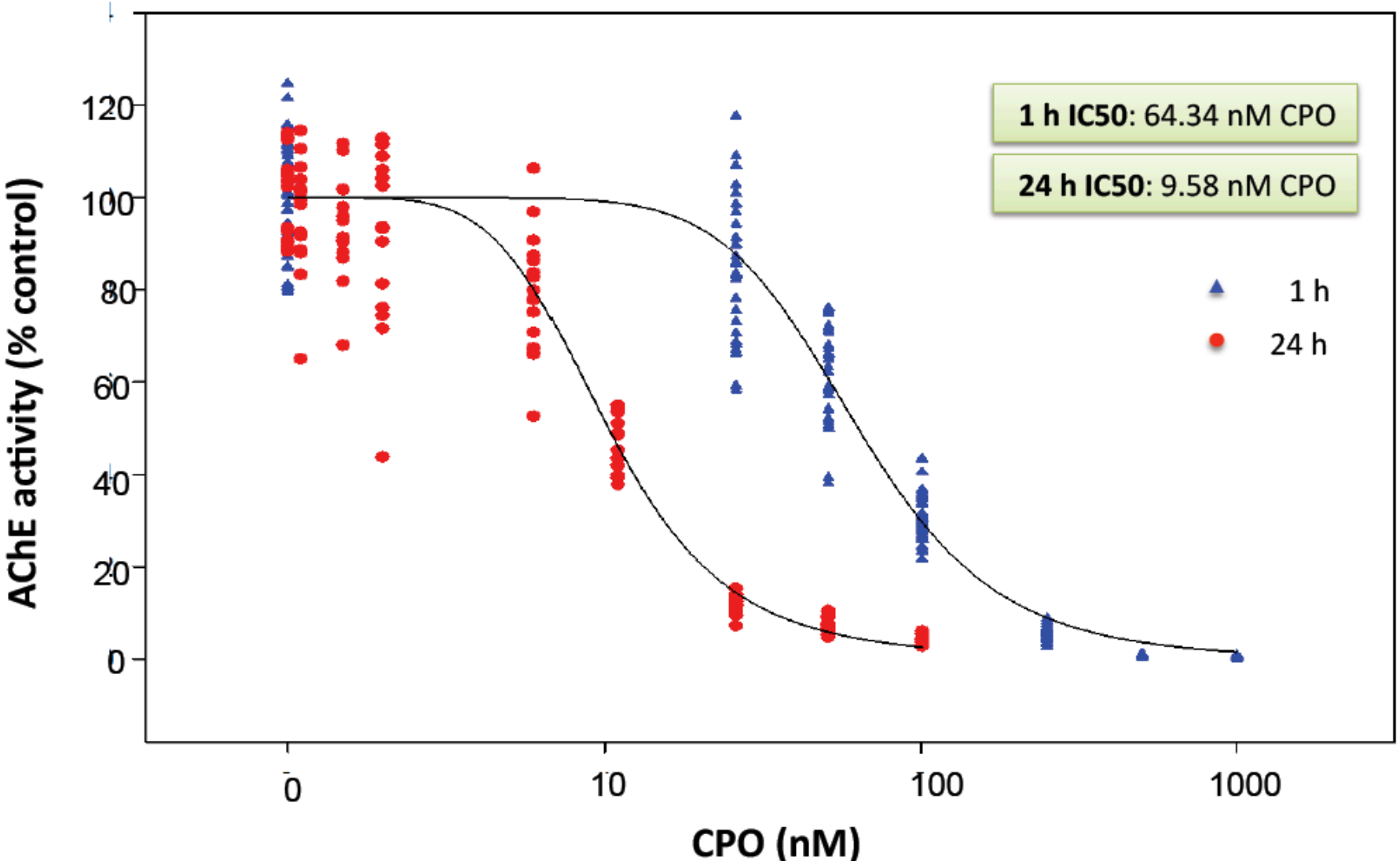




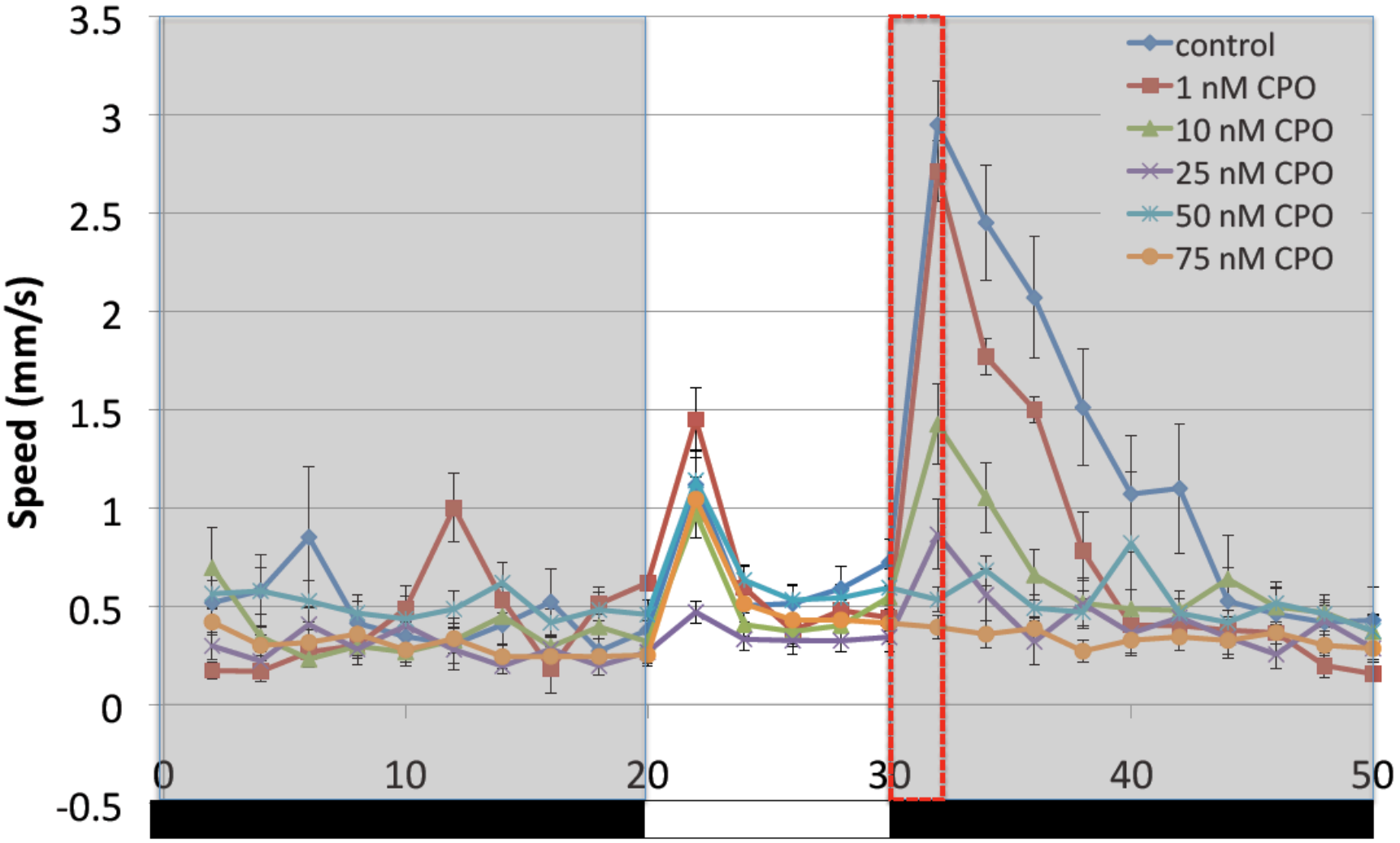




Concentration-response: in vivo inhibition of zebrafish AChE activity by CPO

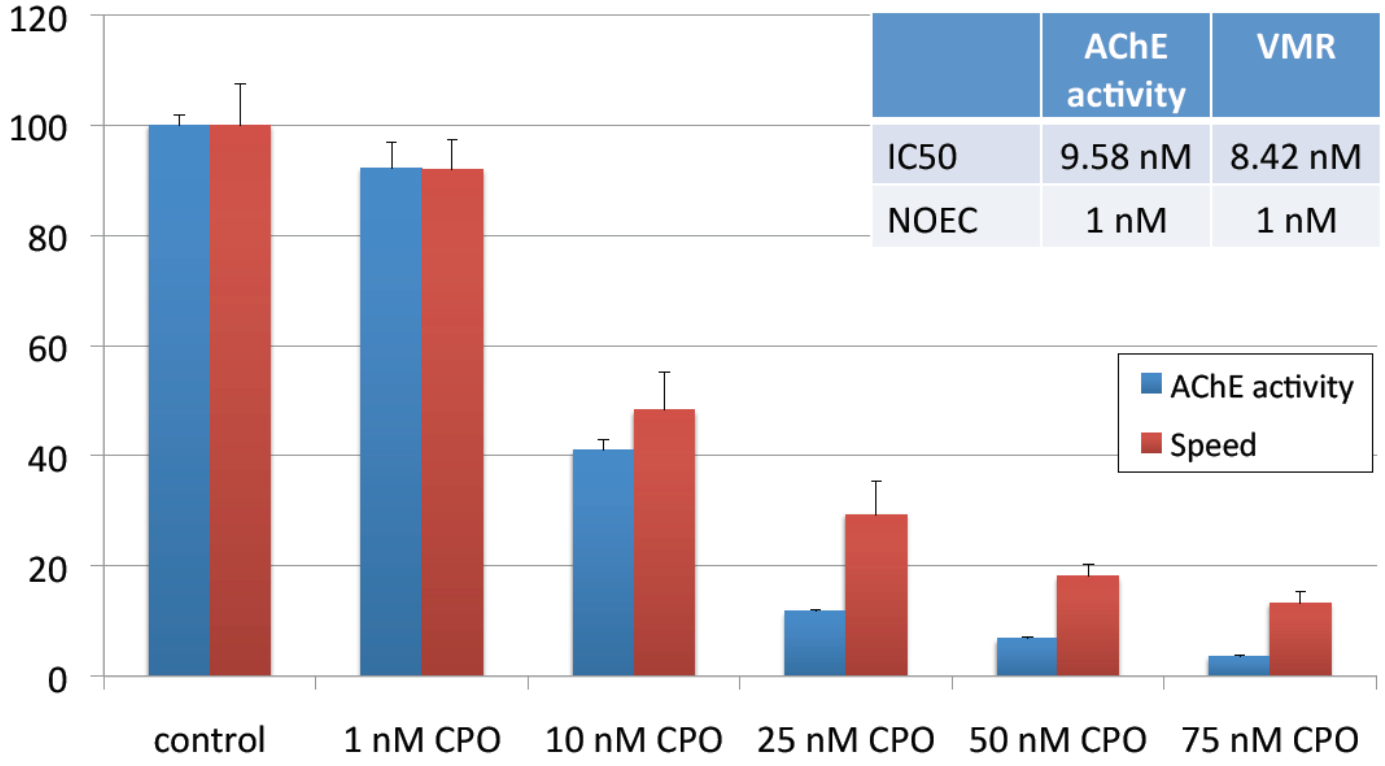


Visual motor response (VMR) is impaired in larvae exposed to low concentrations of CPO



VMR parallels AChE inhibition in larvae exposed to low concentrations of CPO

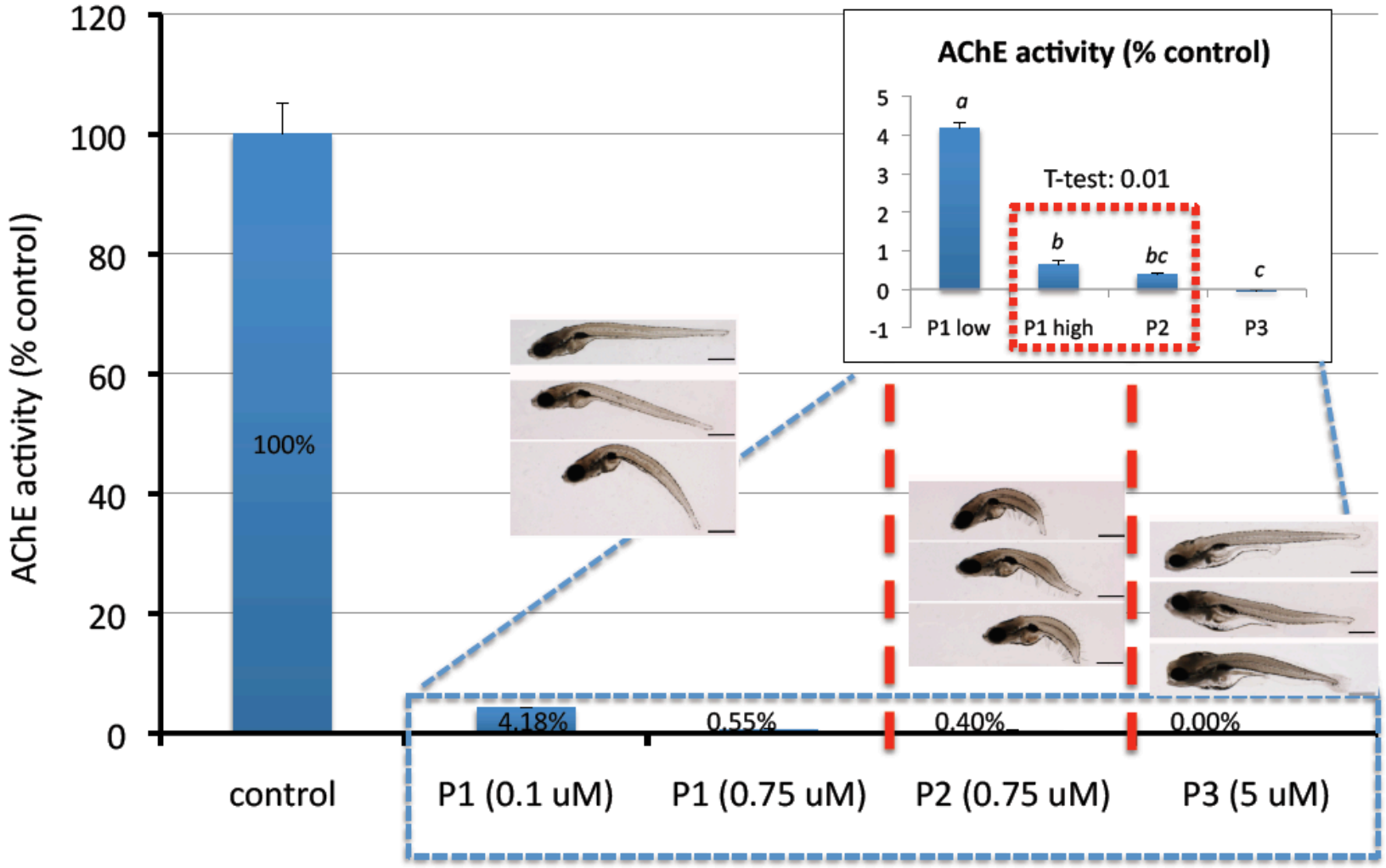
- Behavioral phenotype in larvae exposed to low concentrations of CPO (1-100 nM) is fully explained by the inhibition of AChE activity



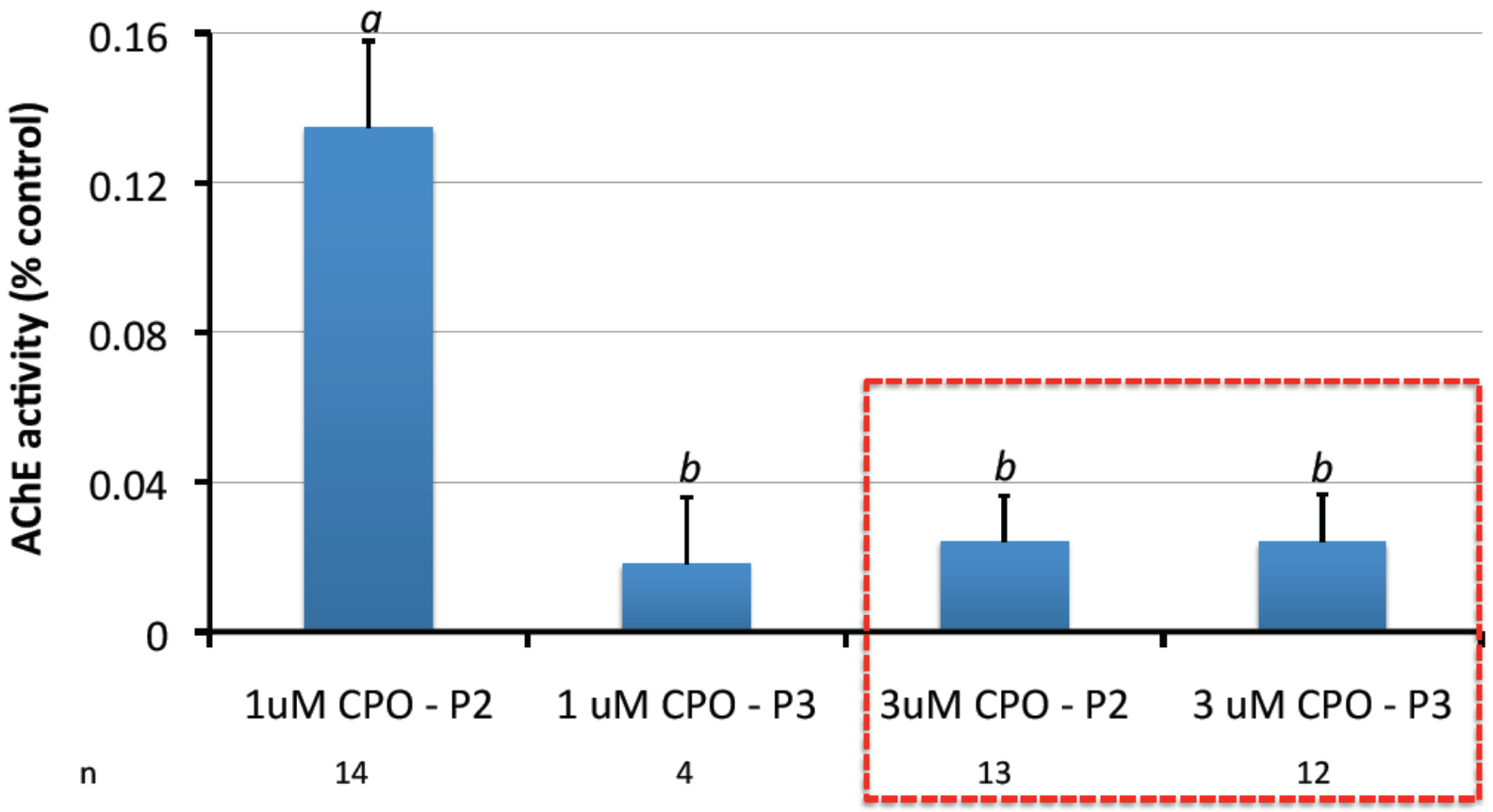
	AChE activity	VMR
IC50	9.58 nM	8.42 nM
NOEC	1 nM	1 nM

AChE activity and speed (% control values). Average speed was measured between 30-32 min of the assay

AChE activity in larvae exhibiting P2 and P3 is lower than 1% of the control values



Larvae exposed to the same concentration of CPO exhibiting a similar degree of AChE residual activity can exhibit different phenotypes



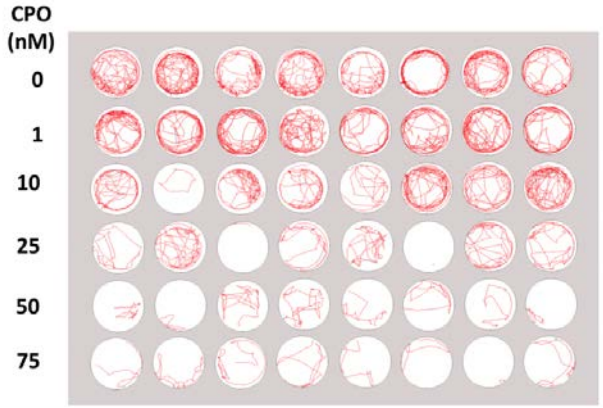
- At high concentrations of CPO, the phenotype presented by the larvae is not explained by the degree of AChE inhibition.

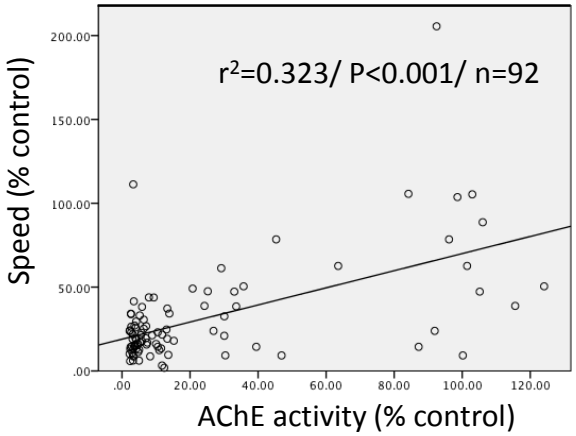
Mild OPP zebrafish model

- No morphological defects at CPO concentrations below 100 nM
- Mild but significant decrease in the length of the trunk
- Histopathological assessment: any effect at CNS/PNS, retina, axial muscle fibers
- No oxidative stress
- Large-scale transcriptomic analysis (RNAseq): 80 DEGs (FDR adjusted $p \leq 0.05$)

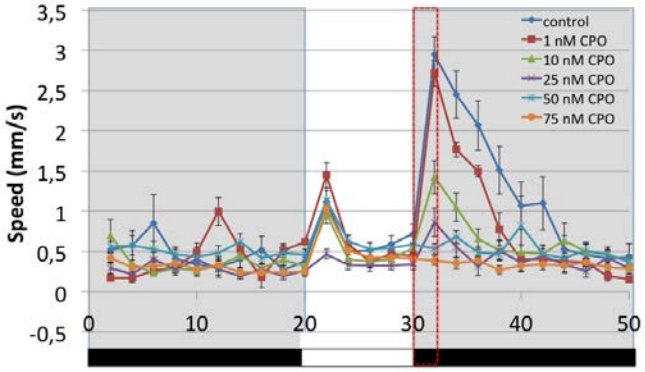
- 4 down-regulated KEGG pathways

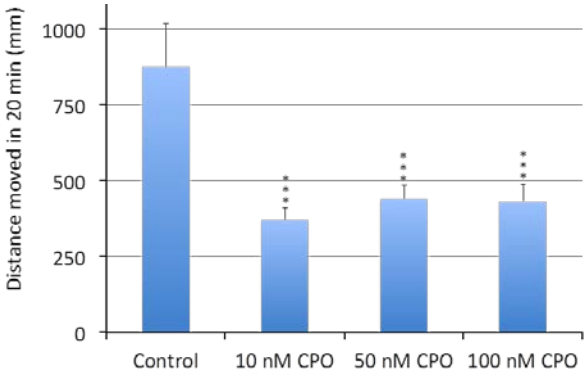
Visual Motor Response is strongly impaired in the mild OPP model



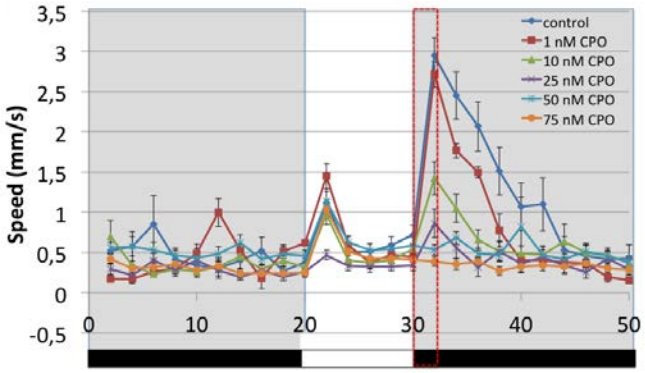


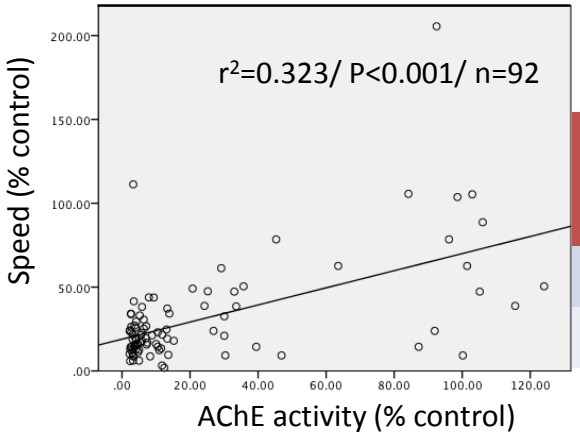
	IC50/ EC50 (nM)	NOEC (nM)
AChE	9.58	1
VMR	8.42	1



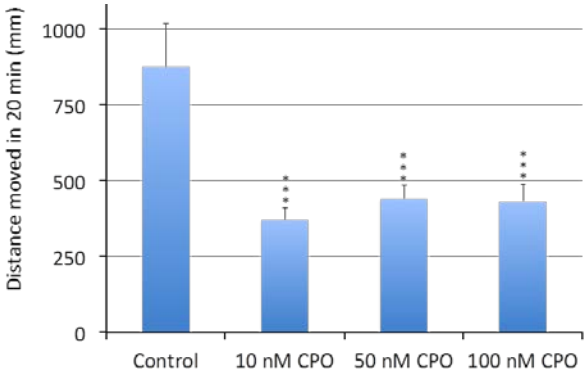


Visual Motor Response is strongly impaired in the mild OPP model



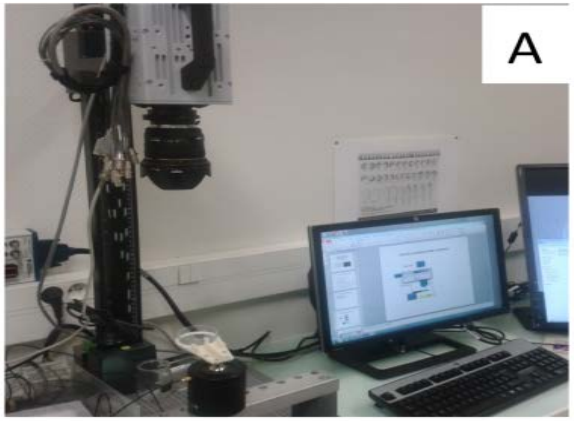


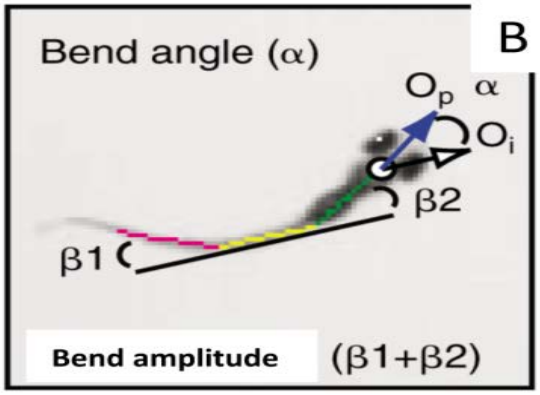
	IC50/ EC50 (nM)	NOEC (nM)
AChE	9.58	1
VMR	8.42	1

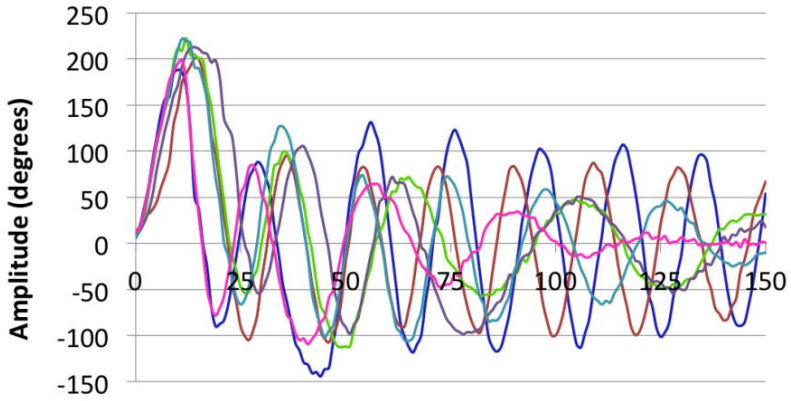


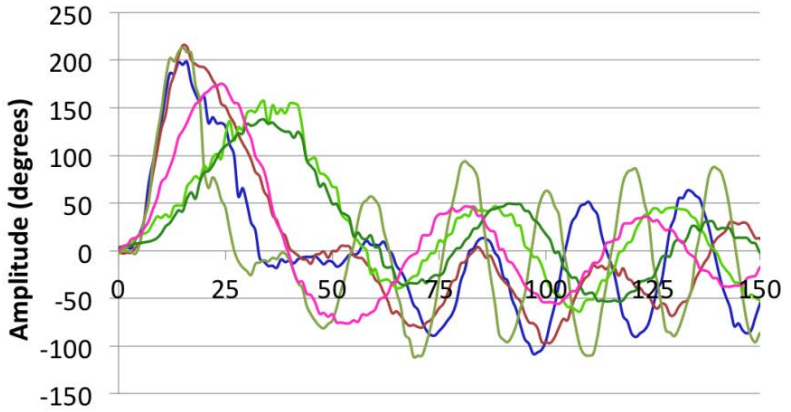
"**Phototransduction**" (dre04744)

Although kinematic of the touch-evoked escape response is altered, mild OPP model is responsive to the touch stimulus



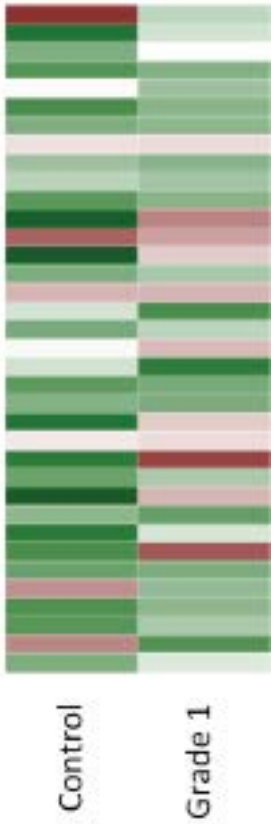




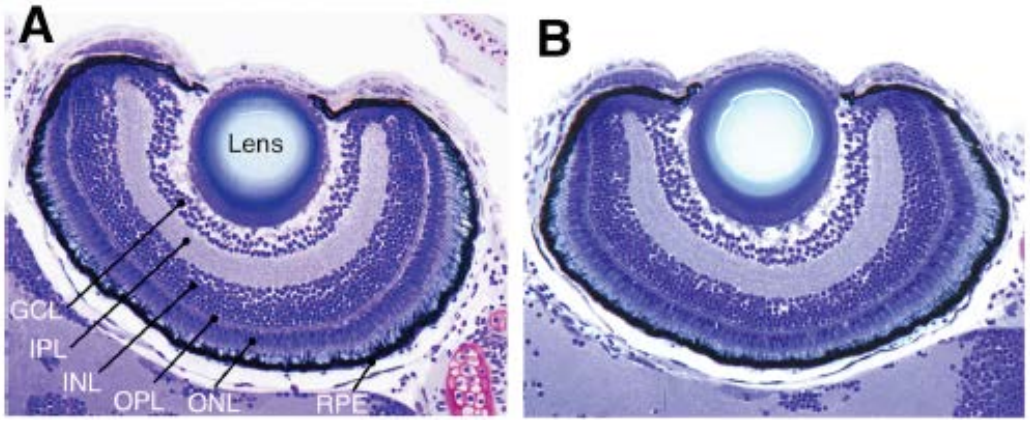


Visual Motor Response is strongly impaired in the mild OPP model

"**Phototransduction**" (dre04744)



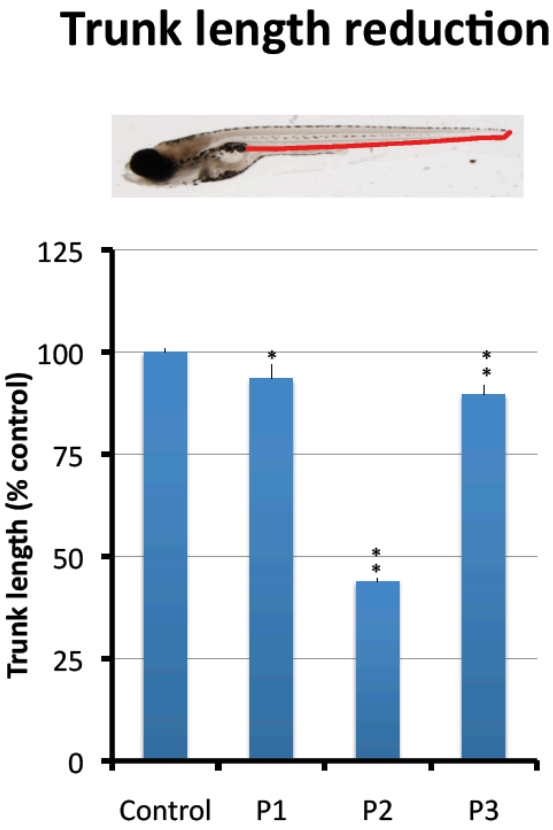
Retina architecture impairment

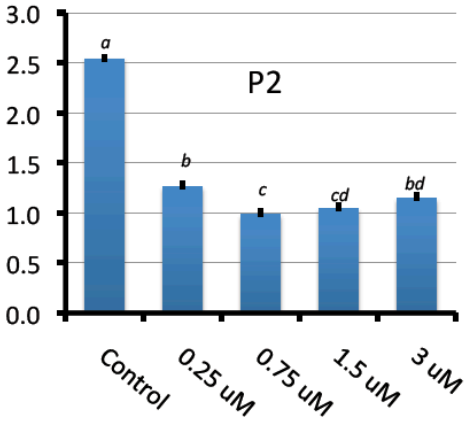


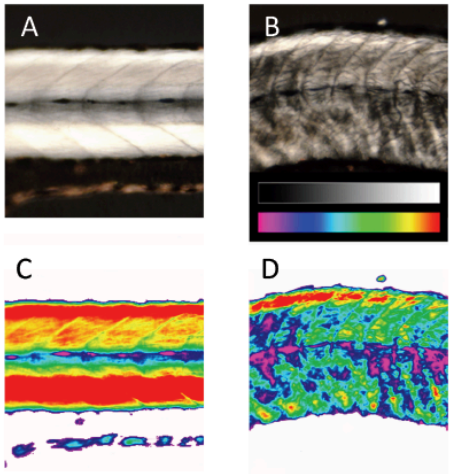
	IC50/ EC50 (nM)	NOEC (nM)
AChE	9.58	1
VMR	8.42	1

Moderate OPP zebrafish model

Severe decrease in the length of the trunk (around 50%!)

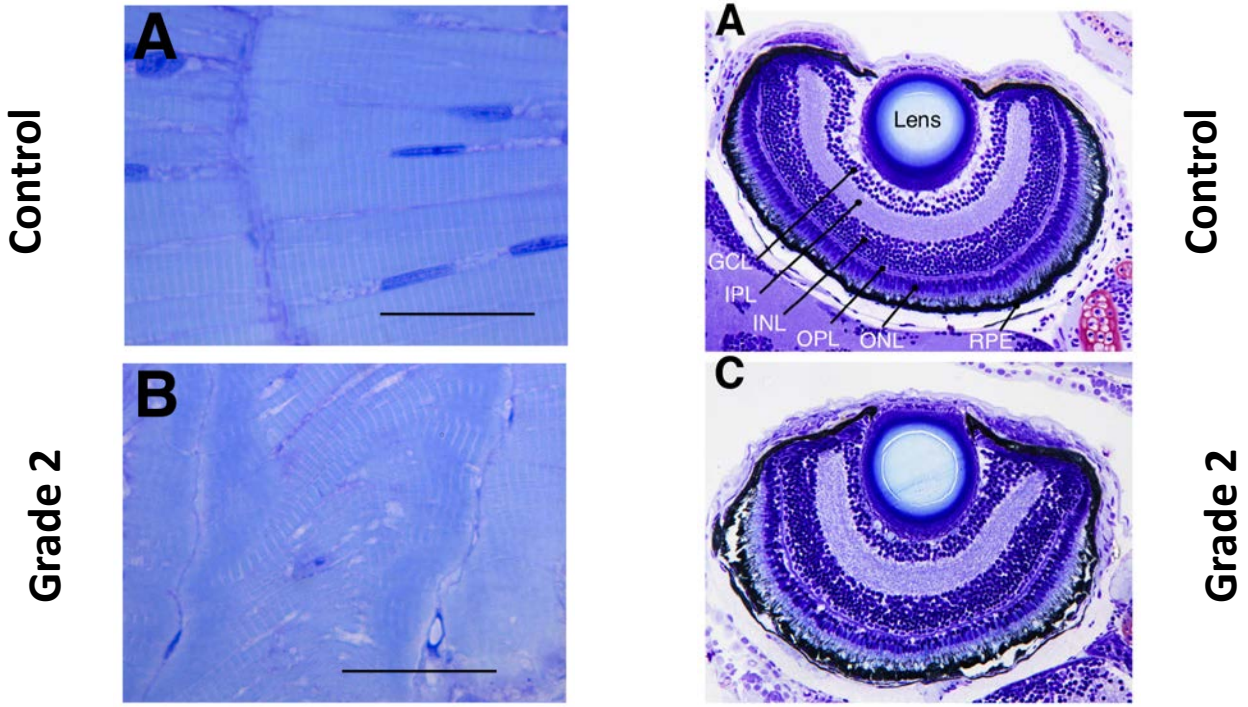






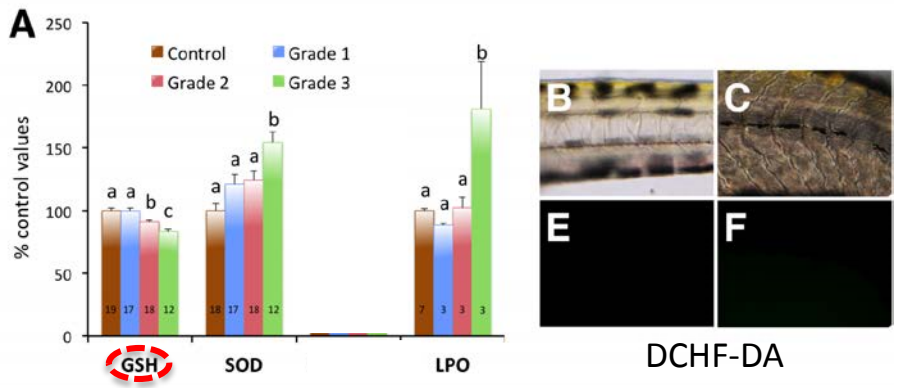
Moderate OPP zebrafish model

Severe decrease in the length of the trunk (around 50%!)
Histopathological assessment: **Altered axial muscle fibers and re**tina, but no CNS



Moderate OPP zebrafish model

No oxidative stress



Transcriptomic analysis (RNAseq): 4,568 DEGs (FDR adjusted p≤0.05)

30 down-regulated KEGG pathways

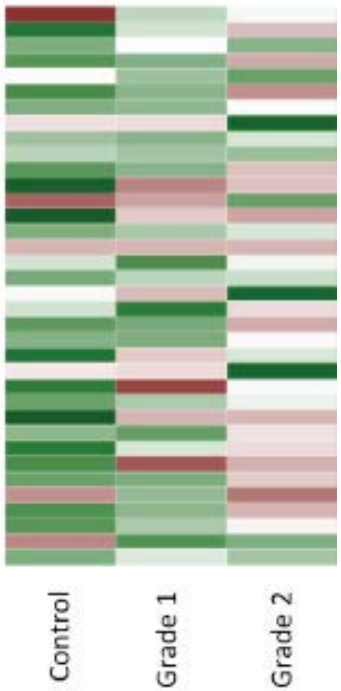
"**Phototransduction**" (dre04744)

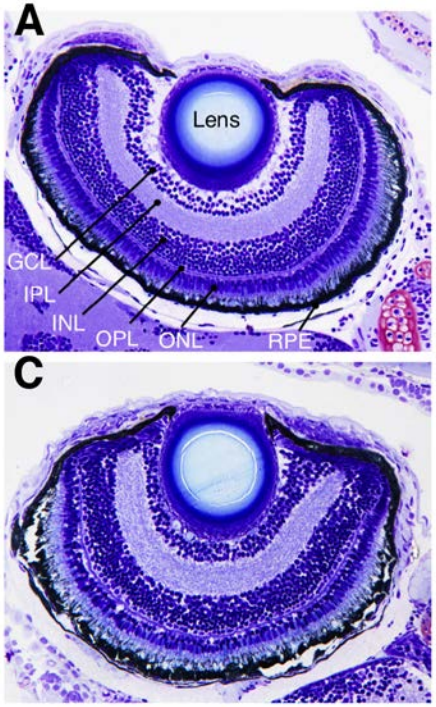
41 up-regulated KEGG pathways

Immune and inflammatory response

Proteosome (dre03050), toll-like receptor signaling pathway (dre04620), MAPK signaling pathway (dre04010), RIG-I like receptor (dre04622)

Moderate OPP zebrafish model





Control

Grade 2

Transcriptomic analysis (RNAseq): 4,568 DEGs (FDR adjusted p≤0.05)

30 down-regulated KEGG pathways

"**Phototransduction**" (dre04744)

41 up-regulated KEGG pathways

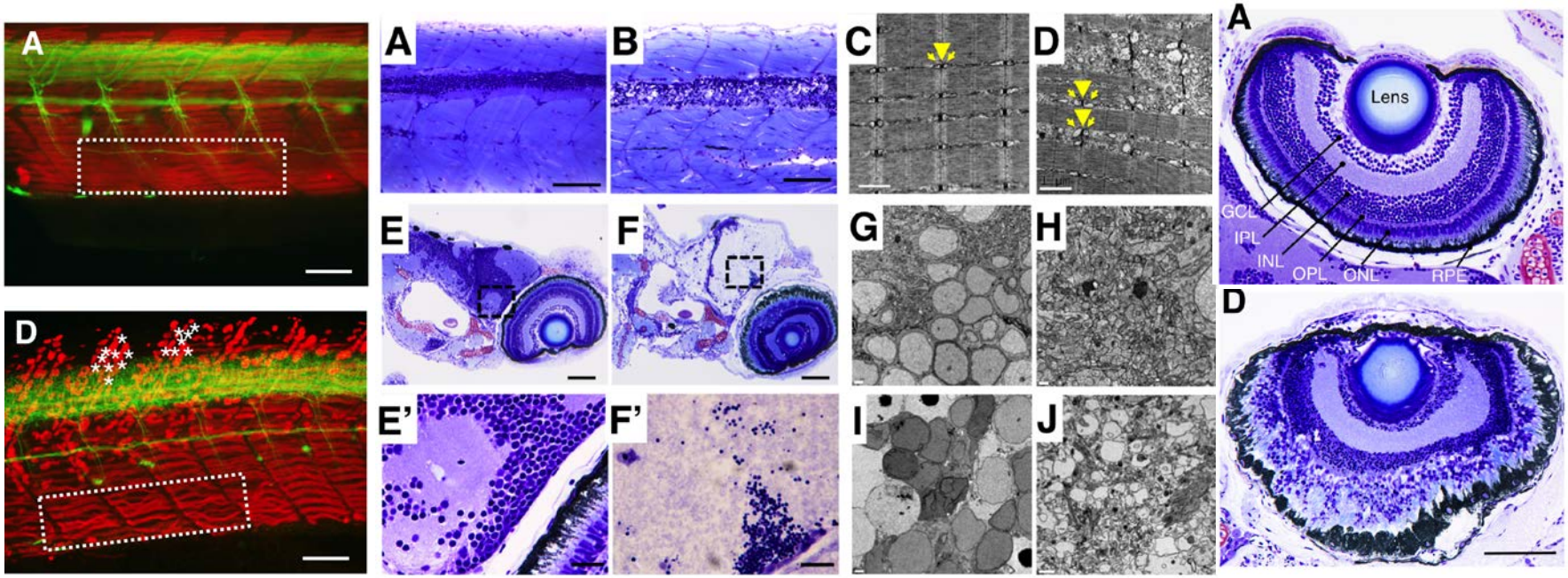
Immune and inflammatory response

Proteosome (dre03050), toll-like receptor signaling pathway (dre04620), MAPK signaling pathway (dre04010), RIG-I like receptor (dre04622)

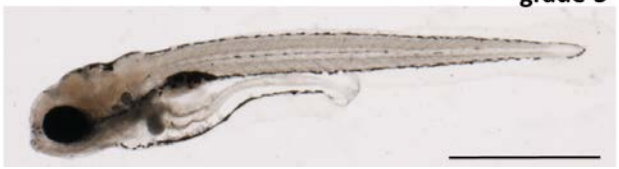
Severe OPP zebrafish model

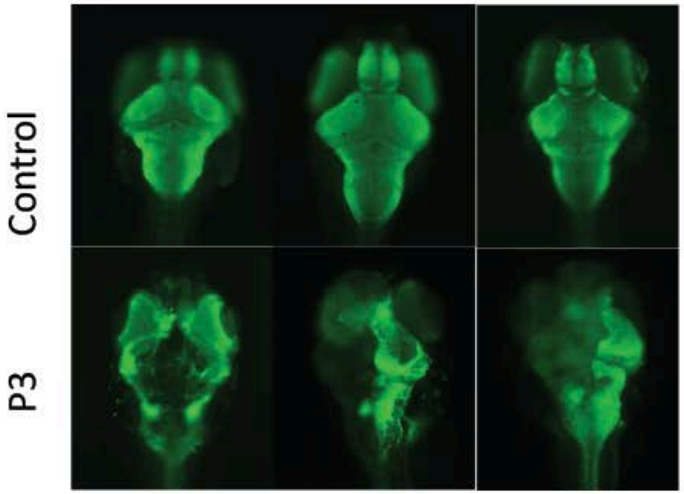
- Mild, but significant, decrease in the length of the trunk
- Histopathological assessment: **Altered axial muscle fibers, CNS, PNS, retina**

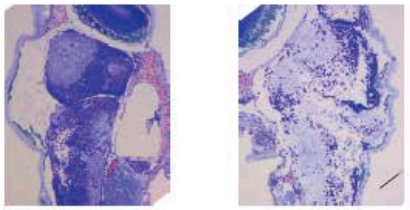
- In contrast with the severity of the lesions in organs with cholinergic innervation, such as CNS or muscle, non-cholinergic tissues such as liver, remained well preserved.

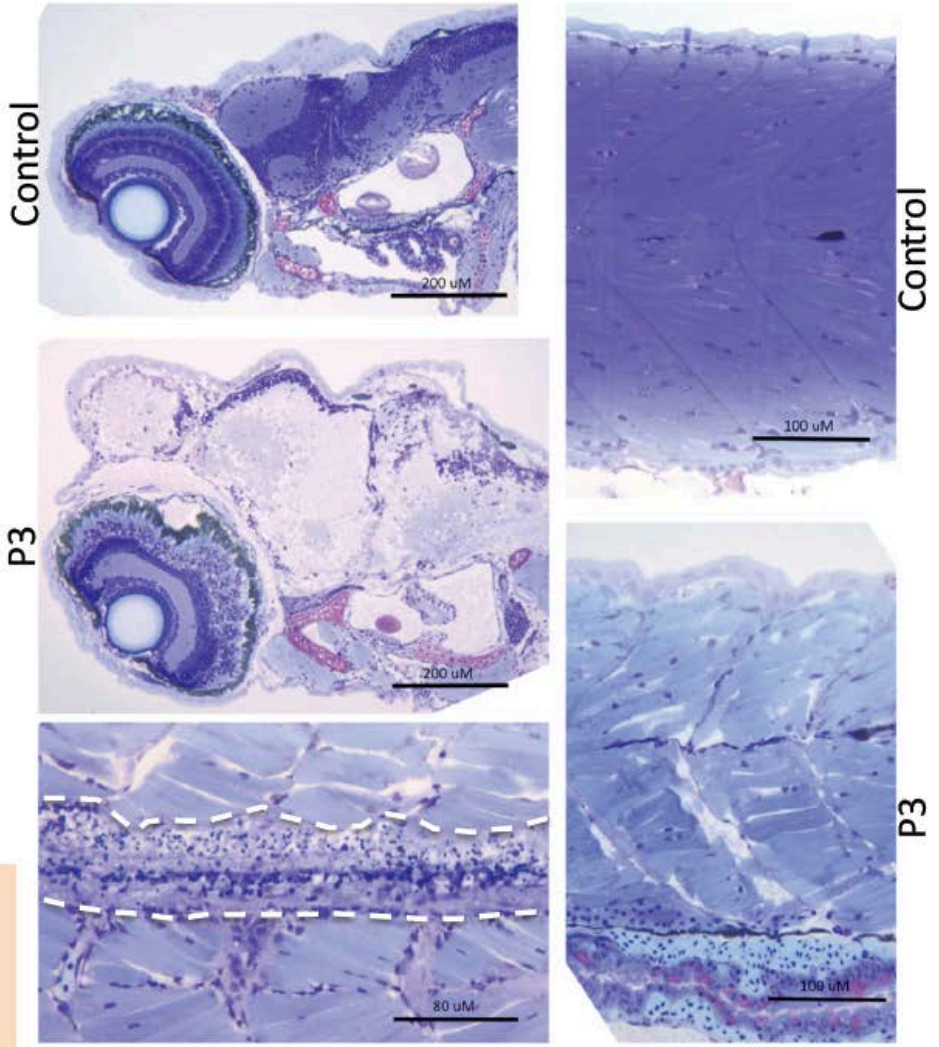


Severe OPP zebrafish model







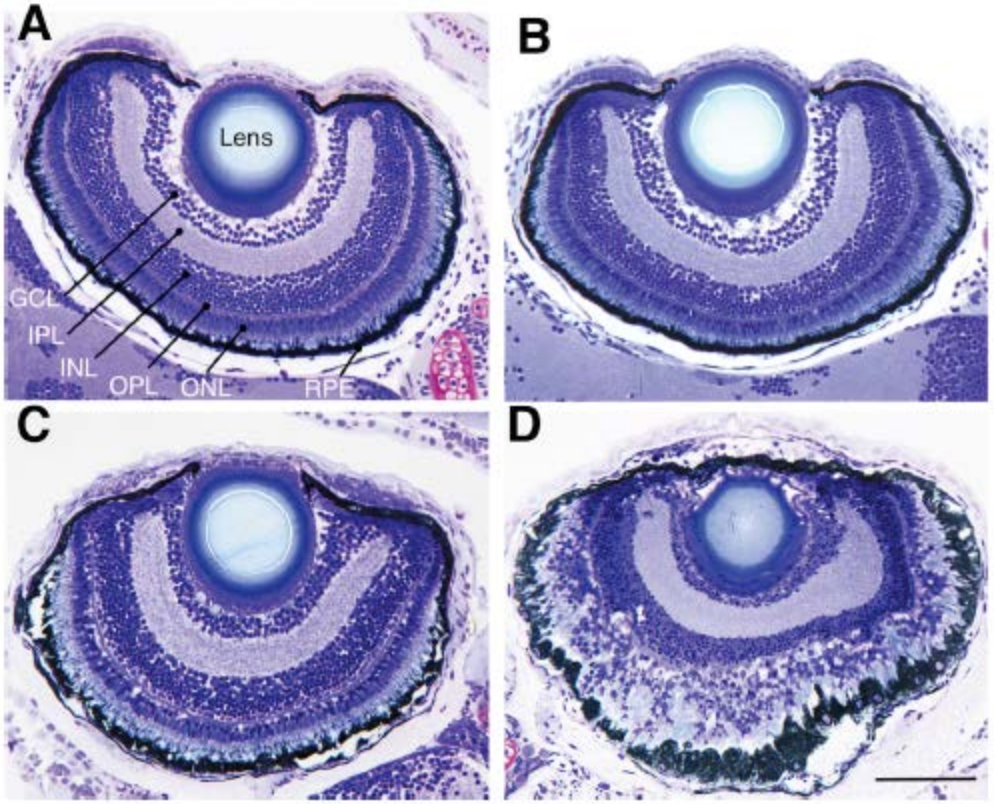


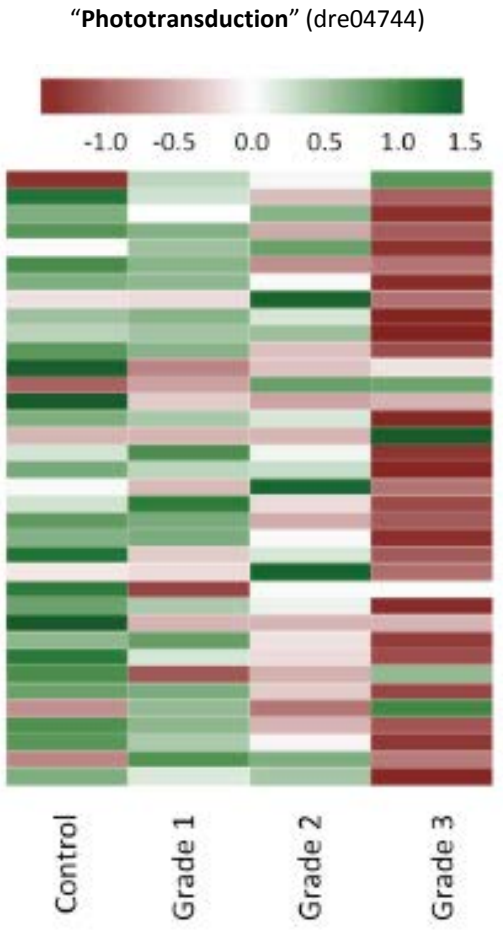
P3 (spinal cord)

Liquefactive necrosis of the brain
Spinal cord degeneration
Retina degeneration
Disrupted architecture of fast muscle fibers

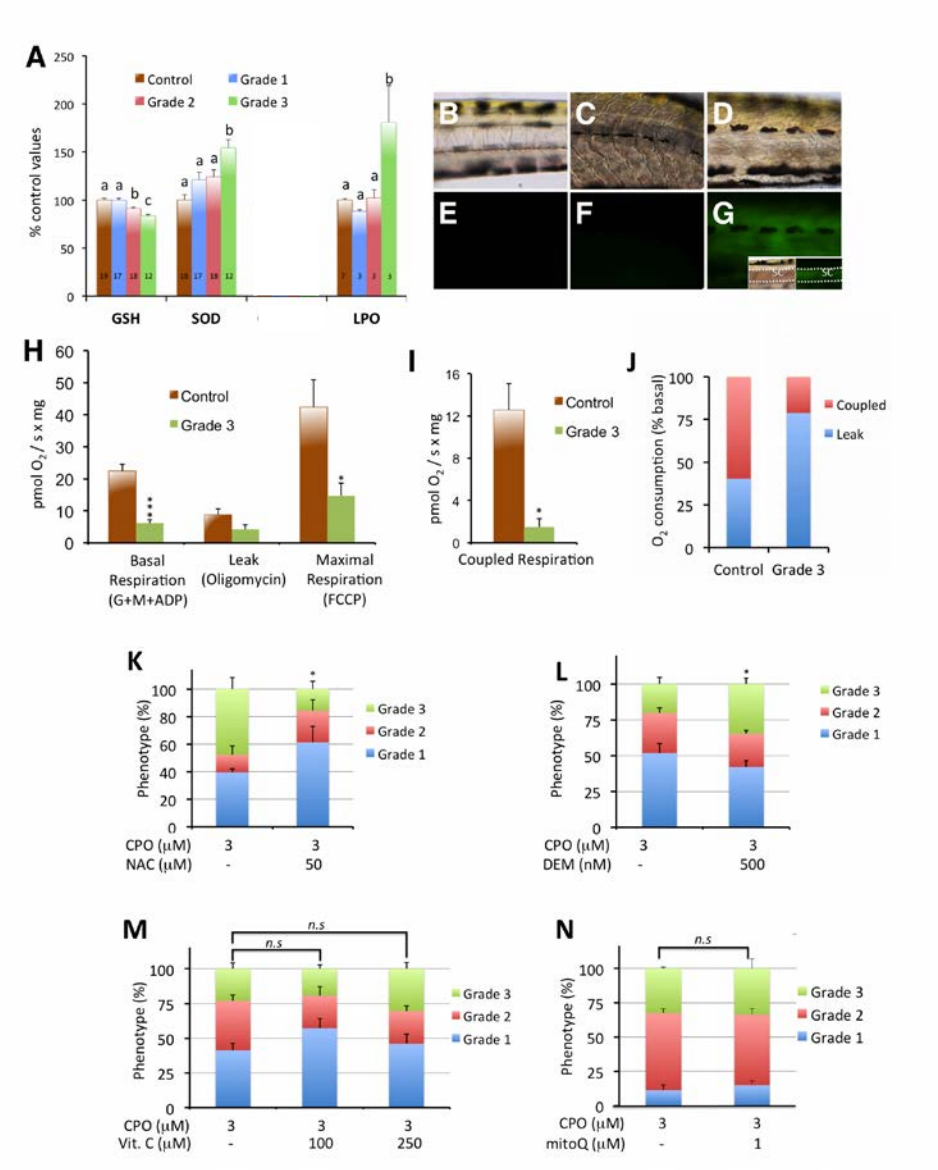
Severe OPP zebrafish model

- Mild, but significant, decrease in the length of the trunk
- Histopathological assessment: **Altered axial muscle fibers, CNS, PNS, retina**





Severe OPP zebrafish model



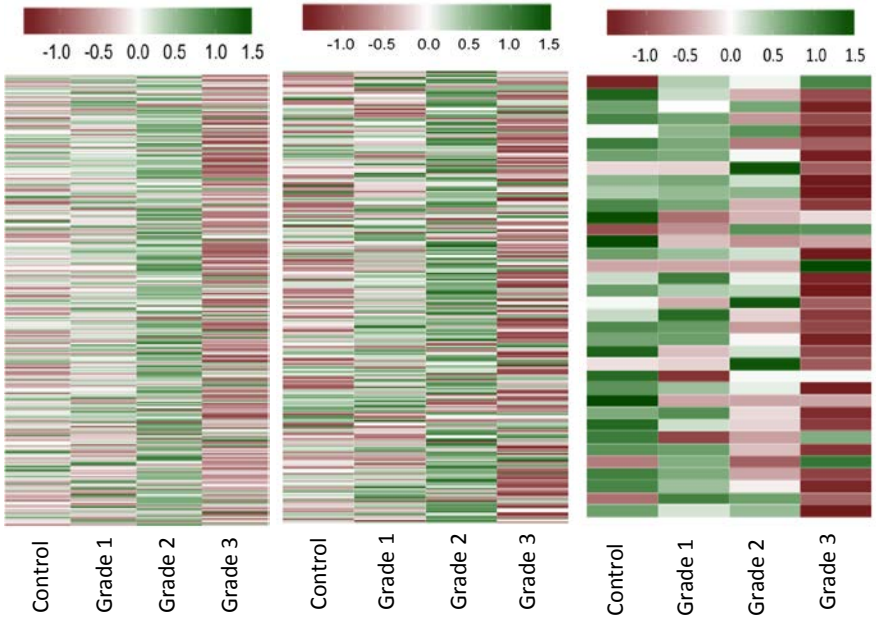
- ROS generation, LPO increased leading to oxidative stress
- Mitochondrial respiration decreased
- Phenotype is partially rescued by modulating GSH levels
- Antioxidants are not able to rescue the phenotype

Severe OPP zebrafish model

Transcriptomic analysis (RNAseq): 4,996 DEGs (FDR adjusted p≤0.05)

9 down-regulated KEGG pathways

34 up-regulated KEGG pathways



"Neuroactive ligand-receptor interaction"

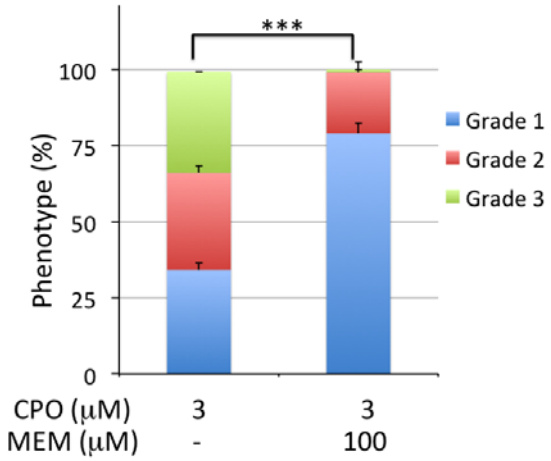
"Calcium signaling"

"Phototransduction"

Immune and inflammatory response

Proteosome (dre03050)
Salmonella infection (dre05132)
Citokine-cytokine receptor interaction (dre04060)
Toll-like receptor signaling pathway (dre04620)
NOD-like receptor signaling pathway (dre04621)
RIG-I like receptor (dre04622)
MAPK signaling pathway (dre04010)

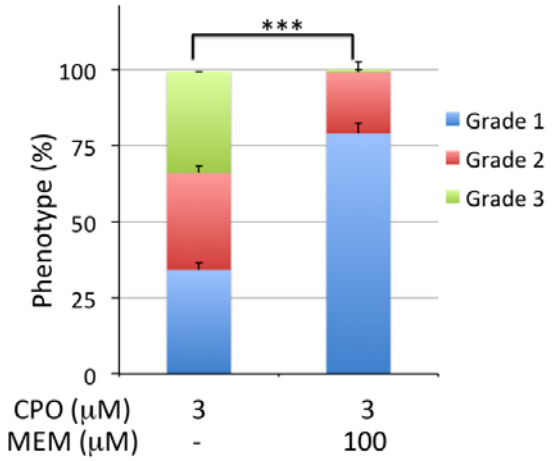
Severe OPP zebrafish model

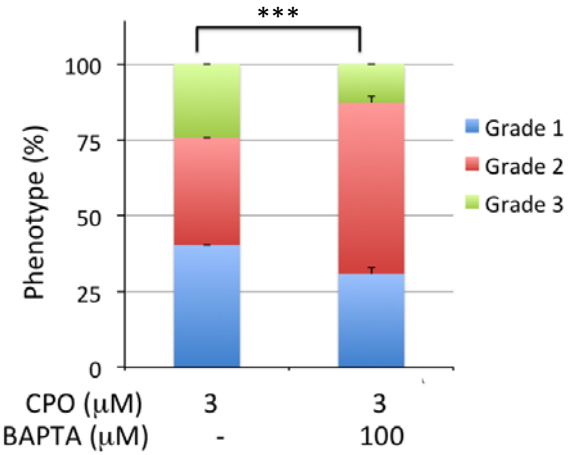


NMDA-receptor antagonists induce an almost total rescue of grade 3 phenotype

NMDA-receptor activation is a key event in the severe OPP pathophysiology in zebrafish

Severe OPP zebrafish model

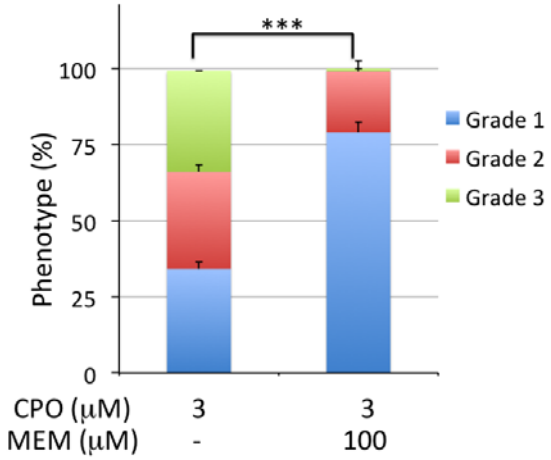


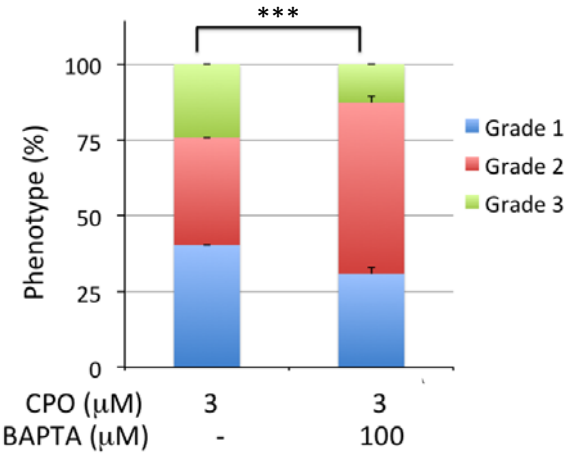


Intracellular Ca^{2+} levels are relevant for the pathophysiology of severe OPP in zebrafish

Permeable Ca^{2+} chelator BAPTA-AM induces a partial rescue of severe OPP phenotype (48% decrease)

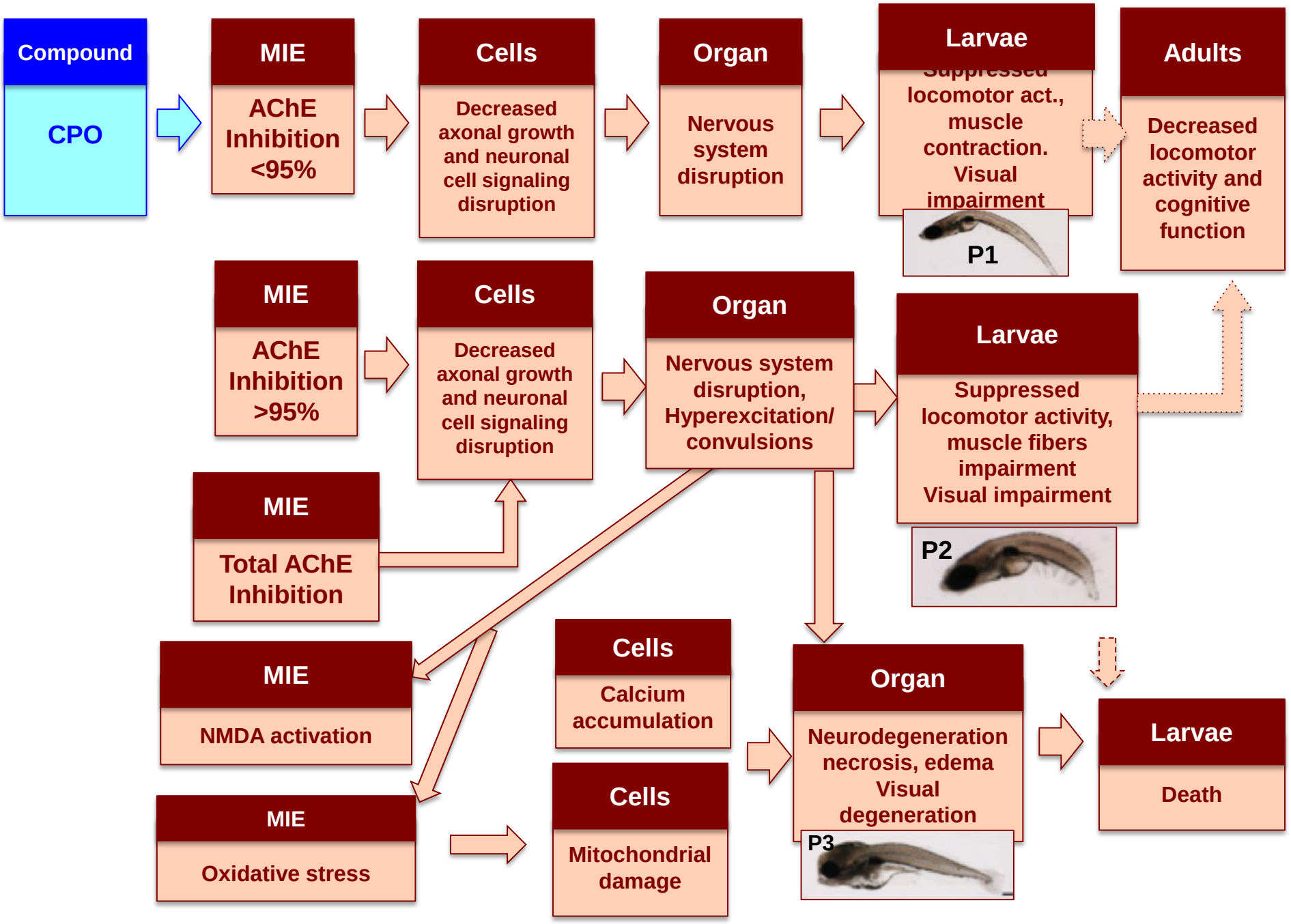
Severe OPP zebrafish model





NMDA-receptor activation is a key event in the severe OPP pathophysiology in zebrafish

Increase in intracellular Ca^{2+} is a key event in the severe OPP pathophysiology in zebrafish


Compound
CPO
MIE
AChE Inhibition <95%
Cells
Decreased axonal growth and neuronal cell signaling disruption
Organ
Nervous system disruption
Larvae
locomotor act., muscle contraction. Visual impairment
P1
Adults
Decreased locomotor activity and cognitive function
MIE
AChE Inhibition >95%
Cells
Decreased axonal growth and neuronal cell signaling disruption
Organ
Nervous system disruption, Hyperexcitation/ convulsions
Larvae
Suppressed locomotor activity, muscle fibers impairment Visual impairment
P2
MIE
Total AChE Inhibition
MIE
NMDA activation
MIE
Oxidative stress
Cells
Calcium accumulation
Cells
Mitochondrial damage
Organ
Neurodegeneration necrosis, edema Visual degeneration
P3
Larvae
Death

Conclusions

- **Chemical models** of mild, moderate and severe OPP can be **easily generated in zebrafish by exposing larvae from 7 to 8 dpf to** different concentrations of the prototypic OP compound **chlorpyrifos-oxon**
- Zebrafish models of OPP mimic most of the pathophysiological mechanisms behind human OPP, including AChE inhibition, NMDA-receptor activation, Ca^{2+} dysregulation as well as inflammatory and immune response.
- Zebrafish models of OPP can be classified as “partial models”
- Developed zebrafish models of OPP can be used for the identification of new antidotes or combinations of antidotes to fight against this toxidrome.

CSIC
Consejo Superior de Investigaciones Científicas

CID - CSIC
Demetrio Raldua
Eva Prats

CIB - CSIC
Eduardo Rial

UAB
Universitat Autònoma de Barcelona
Francesc Padros

IRB Barcelona
Institute for Research in Biomedicine
Antonio Zorzano
David Sebastian

CESAM
Centre for Environmental and Marine Studies
www.cesam.ua.pt
Amadeu Soares
Melissa Faria

ERDC
US Army Engineer Research and Development Center
Ed Perkins
Lynn Escalon

Mississippi State University
Mark Arick II

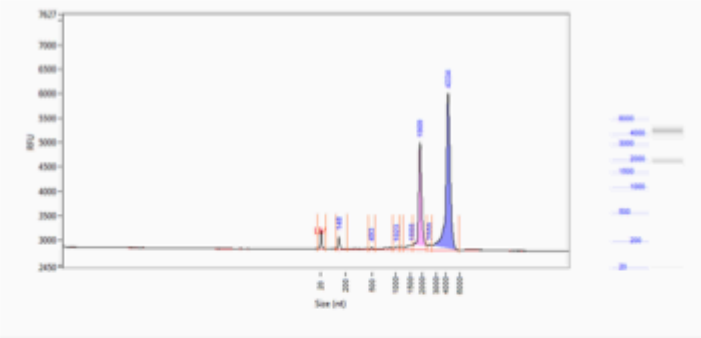
université de Bordeaux
MRGM
Patrick J Babin
Anja Knoll-Gellida
Guilaine Mathiu

EPOC, UMR CNRS 5805
Jerome Cachot
Florane Le Bihanic

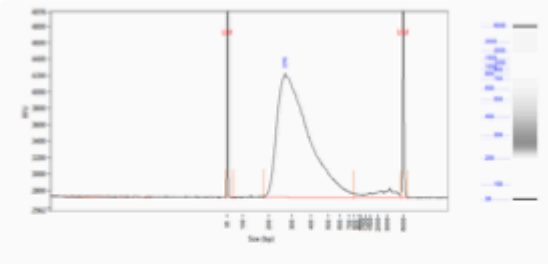
THIS WORK WAS SUPPORTED IN PART BY:
US Army ERDC-IRO (W912HZ-13-BAA-01)/ Environmental Quality Research Program/ NATO SfP project MD.SFPP 984777/ National Science Foundation EPSCOR Grant EPS-0903787/ Portuguese Foundation for Science and Technology (SFRH/BPD/78342/2011)/ Advanced Grant ERC-2012-AdG-320737 / Spanish Government (CTM2014-51985-R)

RNAseq Data Results

- 4 samples/phenotype
- Average 45 M reads/sample
- Very good quality data (QS>30)



High quality RNA
(RIN/RQN >9 for all samples)



RNA Seq libraries

HiSeq2500
RNA Seq Single Read 100bp sequencing
16samples / lane x4 lanes