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Induction of severe acute organophosphorus poisoning at the muscle level: what could we learn from zebrafish?

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International collaborative work

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- Universitat Autònoma de Barcelona, Spain (***Padrós Francesc***)

- Mississippi State University, USA (***Garcia-Reyero Natalia***)

- US Army Engineer Research and Development Center, Vicksburg, USA (***Arick II Mark***)

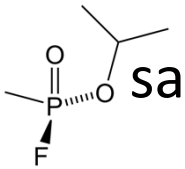
- CIB-CSIC, Madrid, Spain (***Rial Eduardo***)

- Universitat de Barcelona, Spain (***Sebastián David and Zorzano Antonio***)

- Université de Bordeaux, France (***Knoll-Gellida Anja and Babin Patrick J***)

Neuropathic organophosphorus (OPs) are used as:

- **Chemical warfare agents** (*e.g.* sarin, soman)

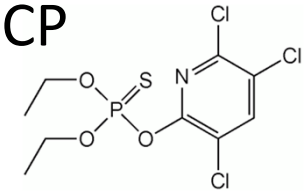


sarin

- Pesticides in agriculture (*e.g.* chlorpyrifos)

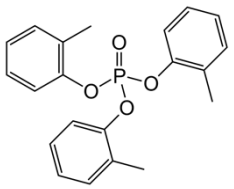
- Plasticizer and other applications in industry (*e.g.* tri-o-cresyl phosphate)

CP



“Toxic Industrial Chemicals” (TICs)

TOCP



1) Neuropathic OPs used as **chemical warfare agents**:

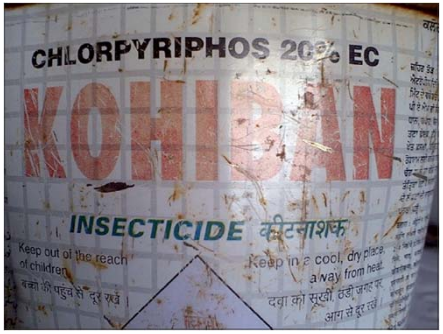
- *Military and terrorist threat:*

(**Sarin** : Syria civil war (2013); terrorist attack in Tokyo subway, 1995)

2) **Toxic Industrial chemicals (TICs)**:

OP pesticides = approximately 3 millions cases of acute severe poisoning and 300,000 deaths annually.

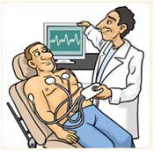
- *Accidental exposure*
- *Suicide*



Neuropathic OPs are potent **inhibitors** of:

1. **Acetylcholinesterase** → Short term toxicity

Cholinergic syndrome

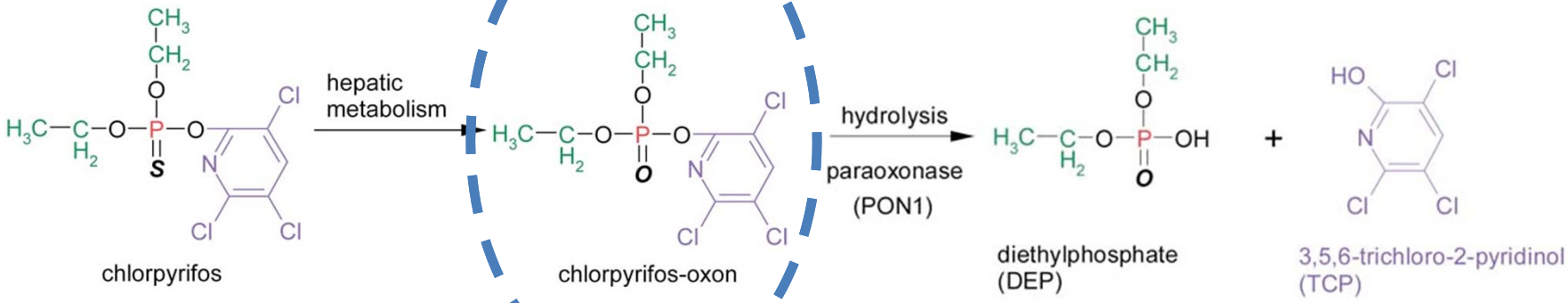


2. **Neuropathic Target Esterase** → Delayed toxicity

Organophosphorus induced delayed neuropathy (OPIDN)

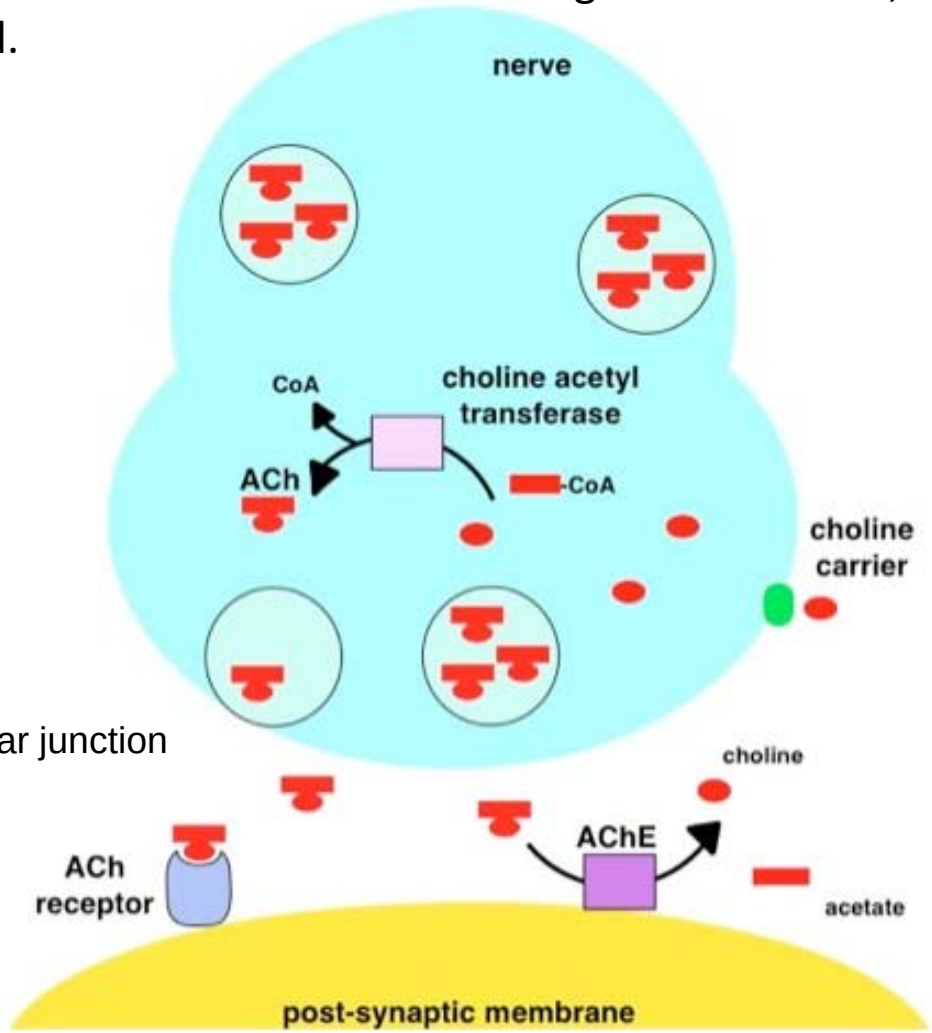
CHOLINERGIC TOXIDROME

ACETYLCHOLINESTERASE INHIBITION



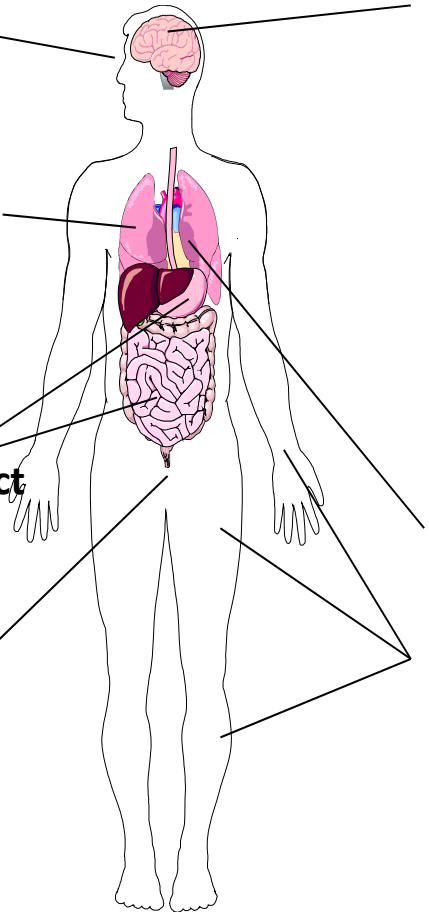
CPO

Mechanism of action of AChE. Each AChE molecule degrades about 25,000 molecules of acetylcholine per second.



Neuromuscular junction

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 System
Tachycardia
Increased Blood Pressure

Musculature
Weakness
Tremor
Fasciculations
Twitching
Cramps
Increased Sweating

General aim of the project

Zebrafish model(s) of cholinergic toxidrome

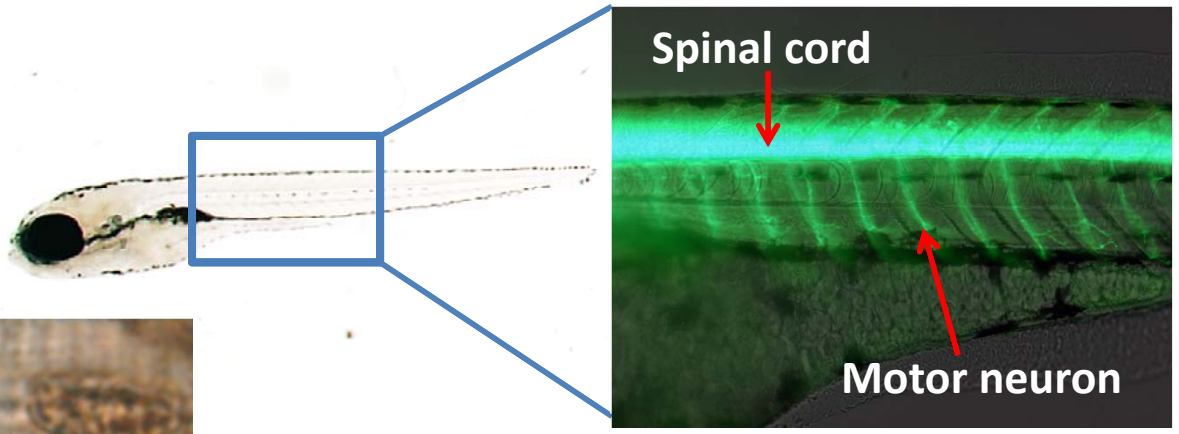
***In vivo* high-throughput screenings of small molecule libraries with zebrafish larva for identifying new drugs for multifunctional drug therapy against acute organophosphorus poisoning**

Zebrafish model of cholinergic toxidrome

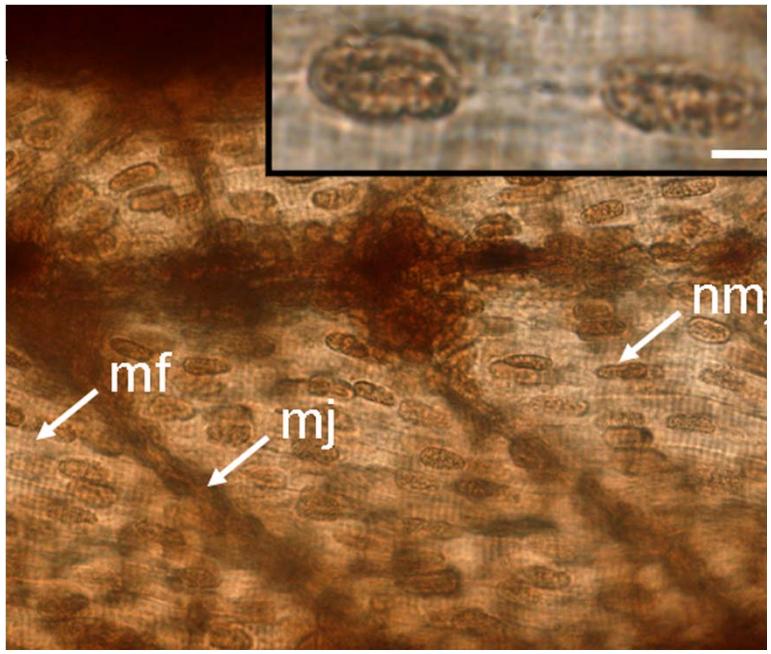
✓ Inherent advantages: low cost, short life cycle, transparency at the larval stages, high number of larvae to be used in microplates, conservation with human of the biological functions investigated, ….

Zebrafish model of cholinergic toxidrome

✓ Genetic and cellular tools



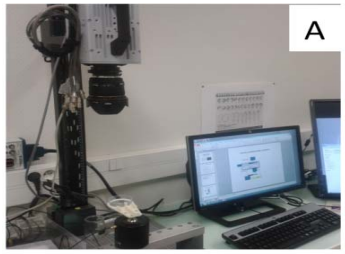
Live larva (*e.g.* transgenic line *hb9*)

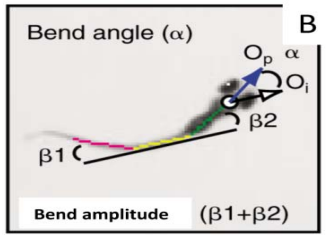


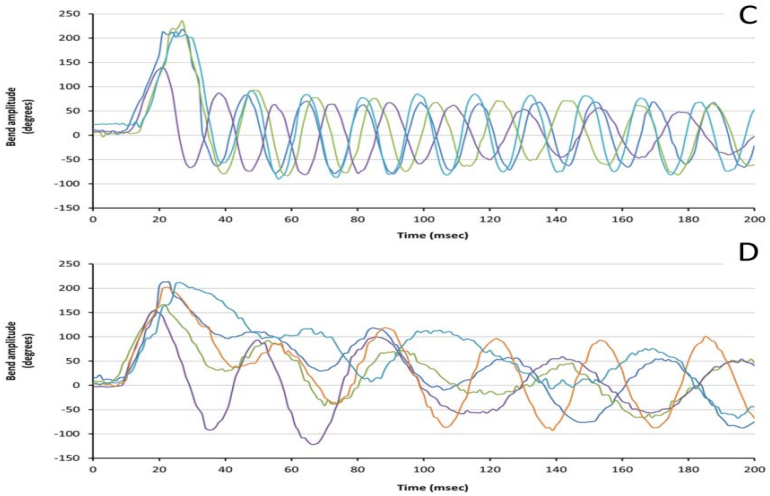
Neuromuscular junction pattern and muscular fiber organization

Zebrafish model of cholinergic toxidrome

✓ Locomotor function assessment



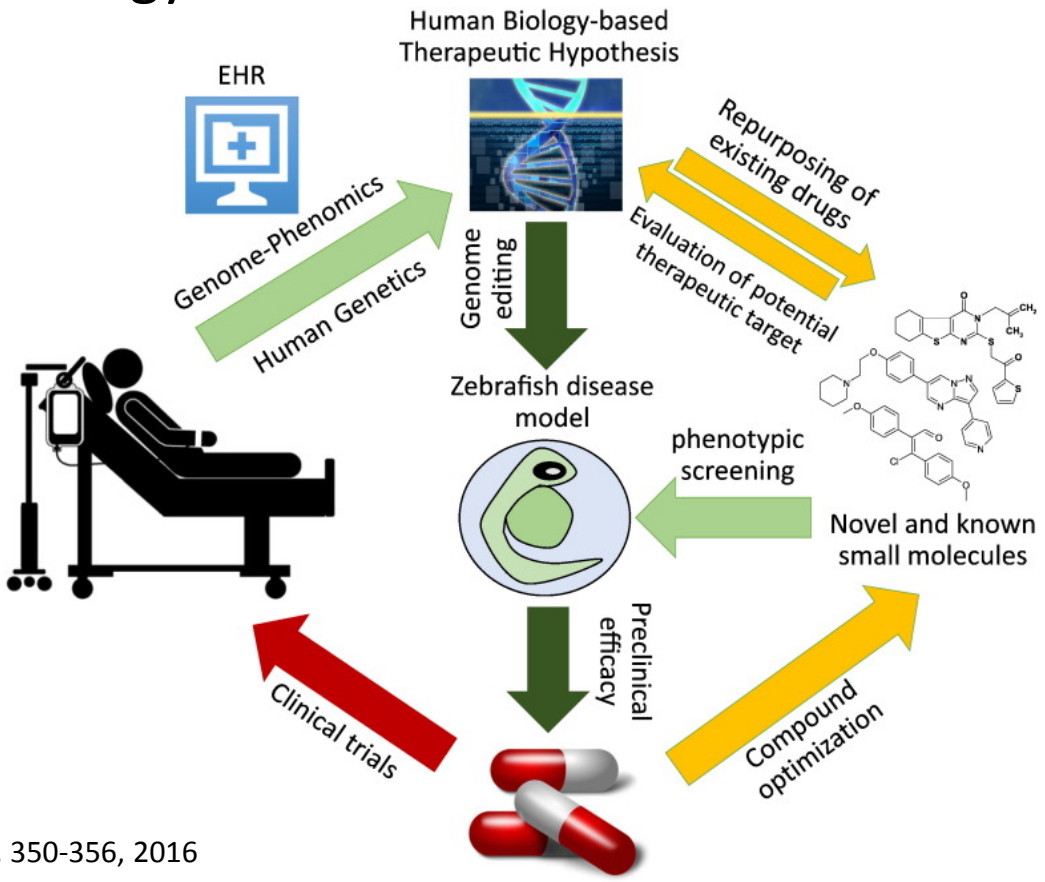




Acoustic/Electric/Touch escape response: kinematic analysis

Zebrafish model of cholinergic toxidrome

✓ Pharmacology and toxicology

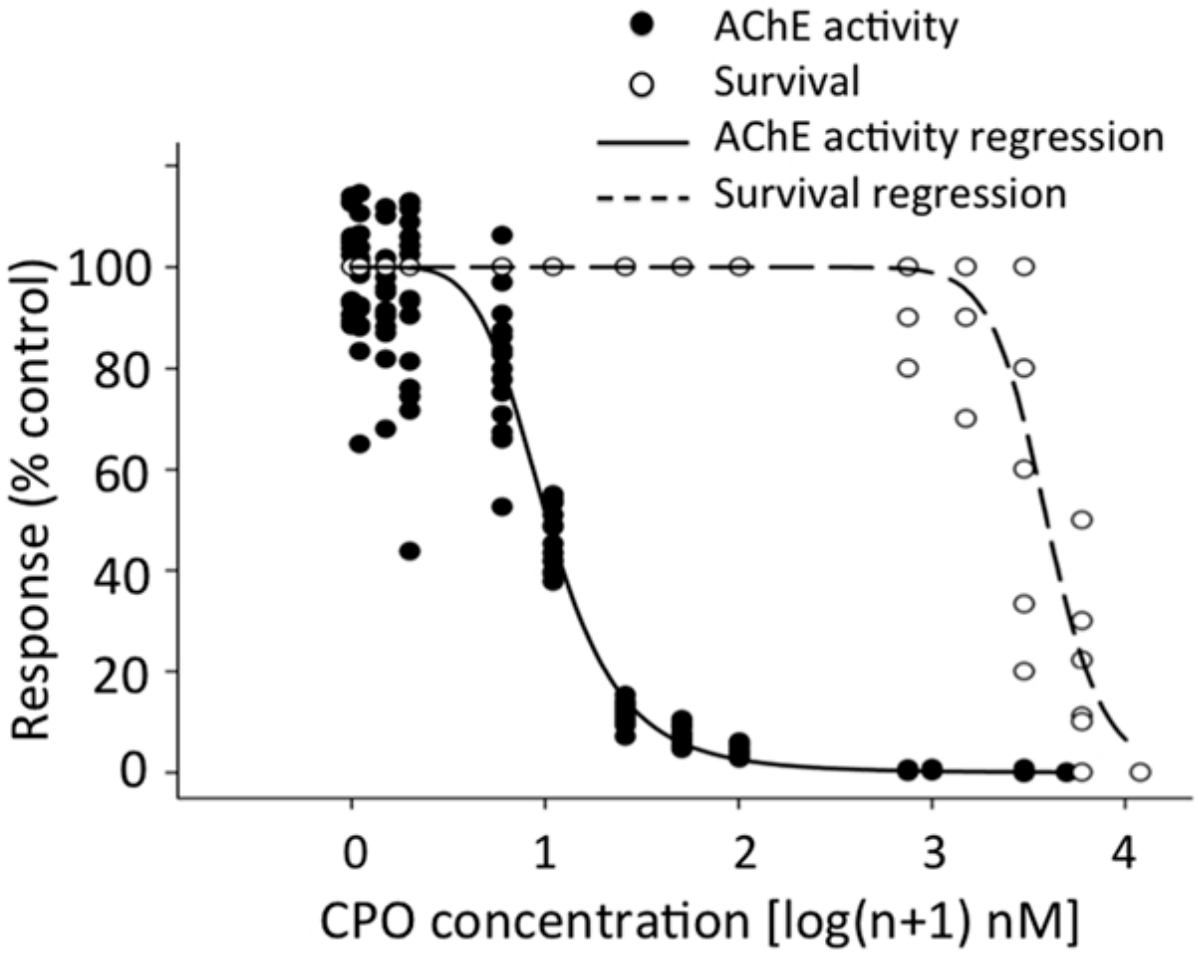


Willimans and Hong. *Comput. Struct. Biotechnol J.* 14, 350-356, 2016

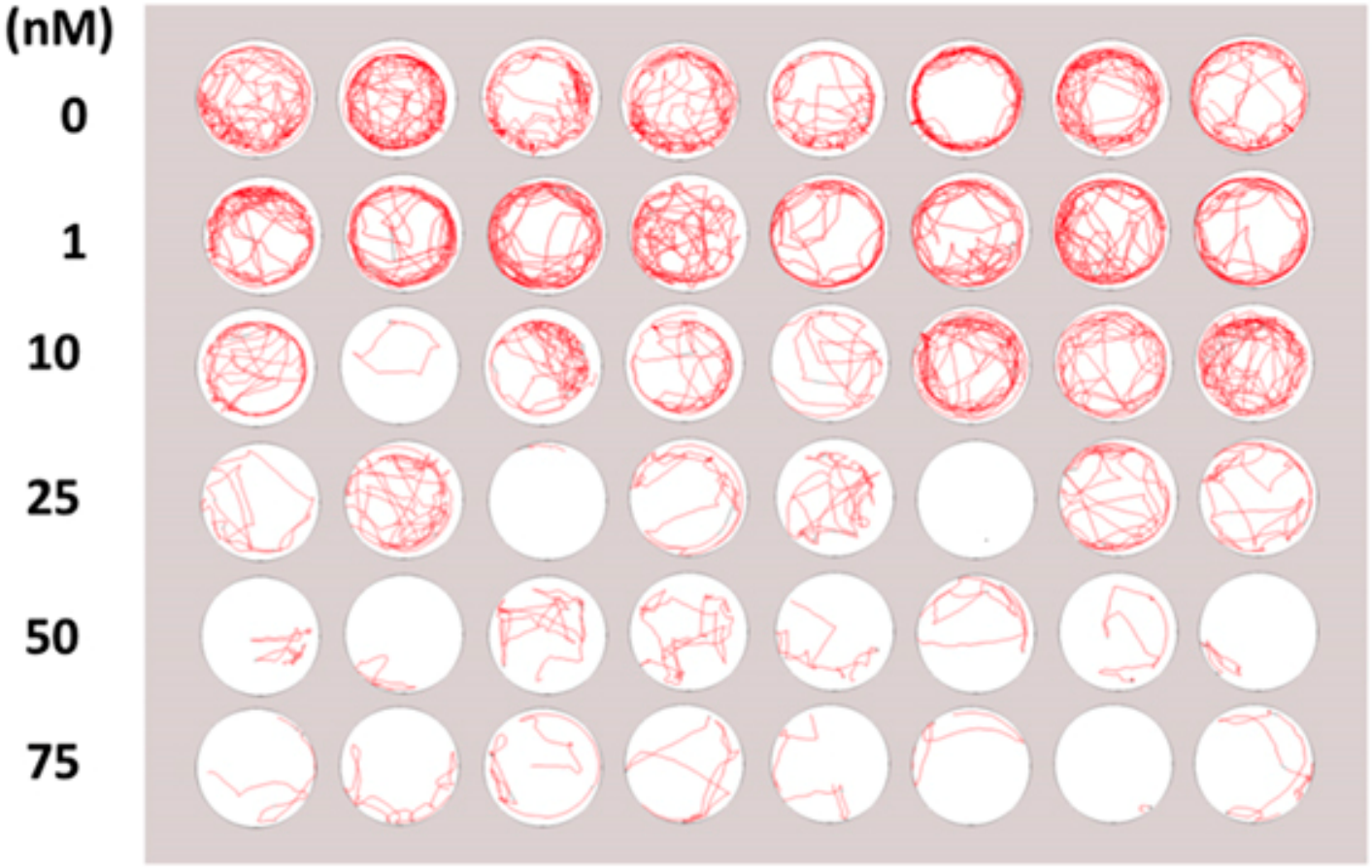
Zebrafish model of cholinergic toxidrome

Generation and validation of zebrafish models for mild, moderate and severe acute OP poisoning by exposing zebrafish larvae to different concentrations of the prototypic OP compound, chlorpyrifos-oxon (CPO)

CPO induces concentration-dependent inhibition of acetylcholinesterase (AChE)

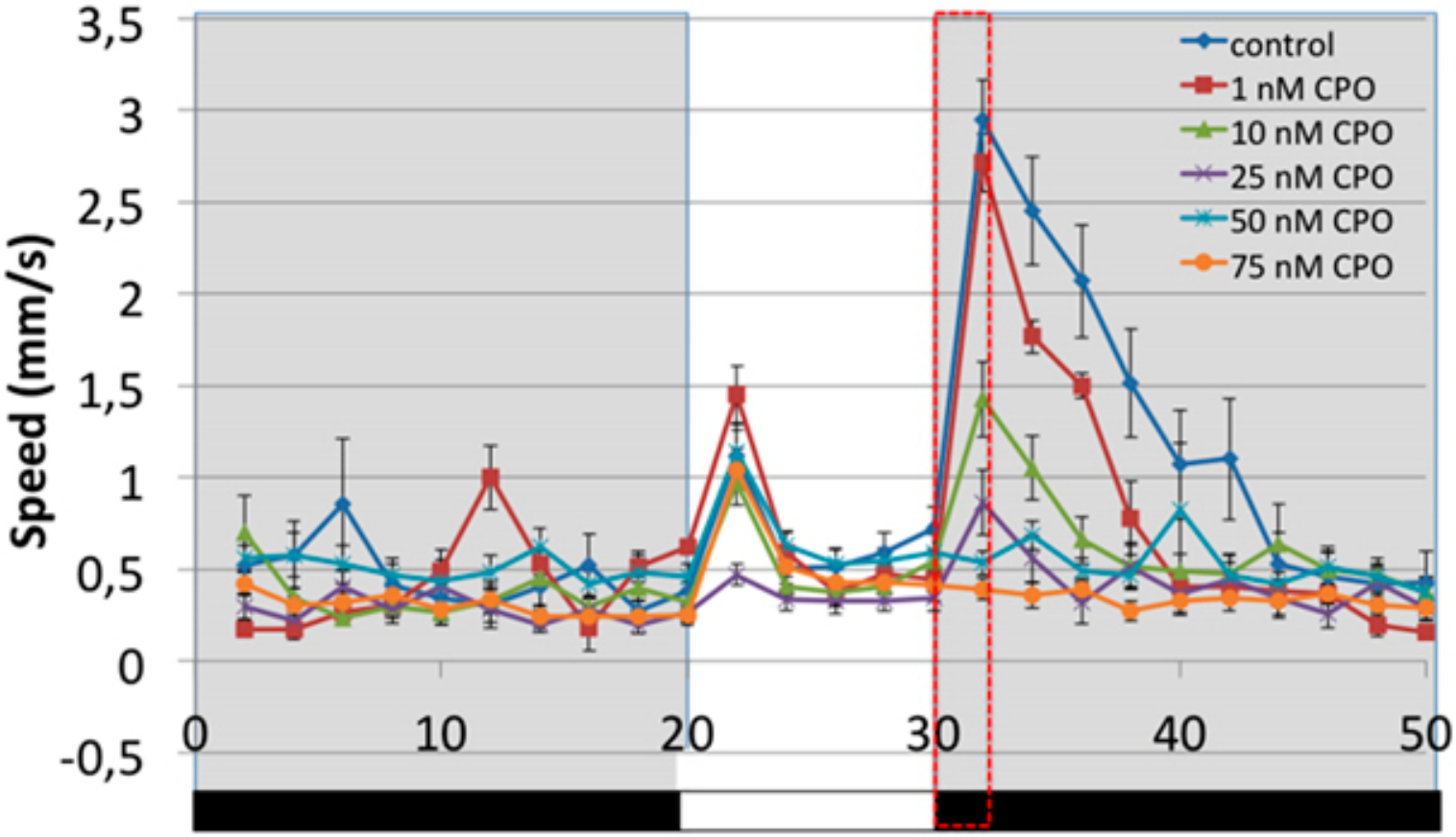


Motor behaviour is strongly impaired by CPO in zebrafish larvae



Locomotion tracking plots for the Visual Motor Response (VMR) assay in a 48-well behavioural arena

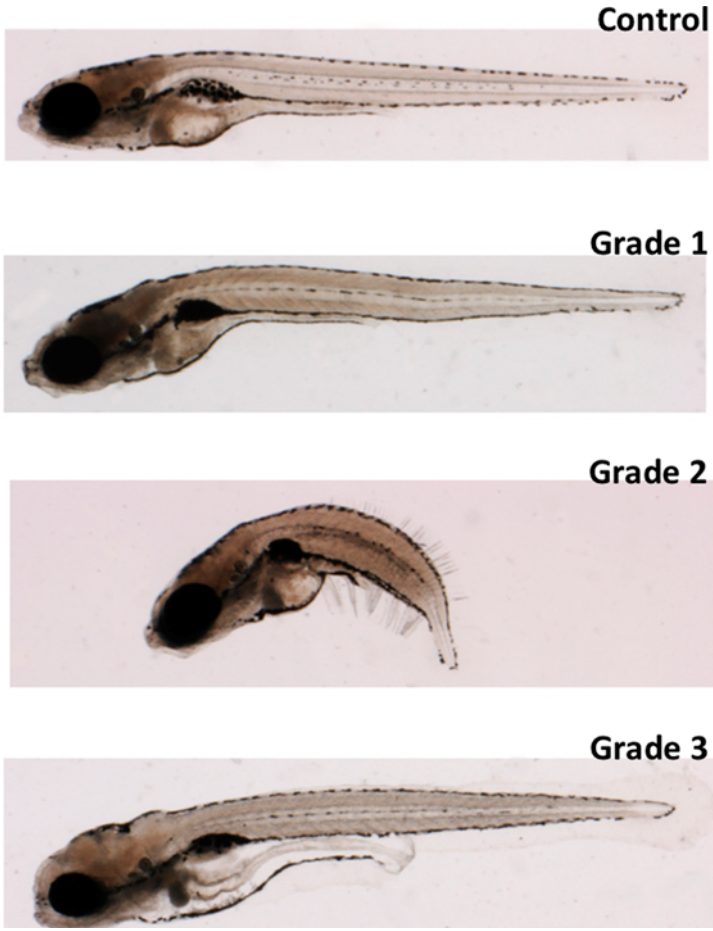
Motor behaviour is strongly impaired by CPO in zebrafish larvae



VMR profiles, showing that the hyperactivity peak evoked in response to sudden exposure to darkness is reduced until total abolition.

CPO induces the expression of three different phenotypes in zebrafish larvae according to CPO concentration

Day 8 larvae after
24 h exposure to CPO

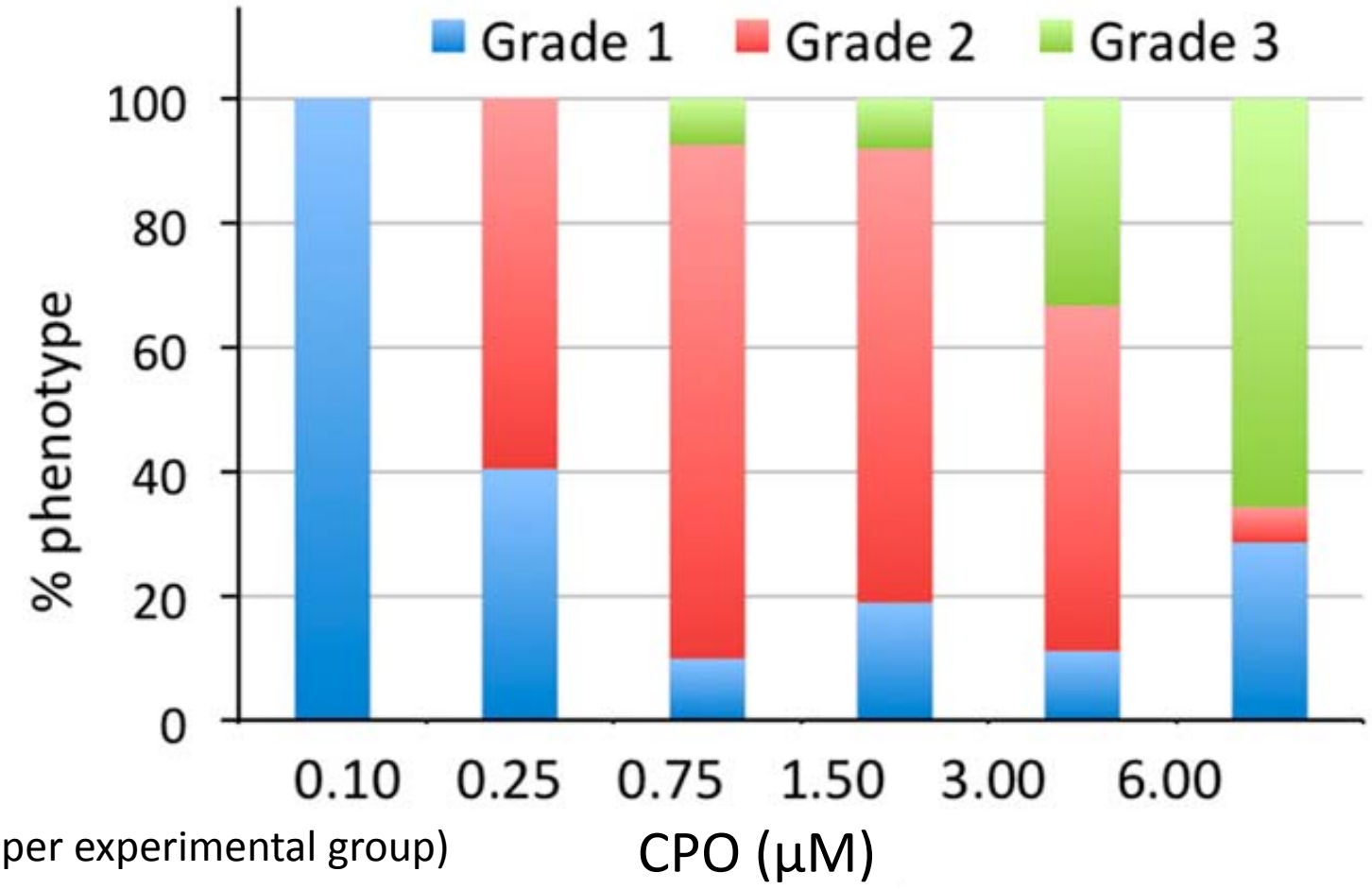


Mild

Moderate

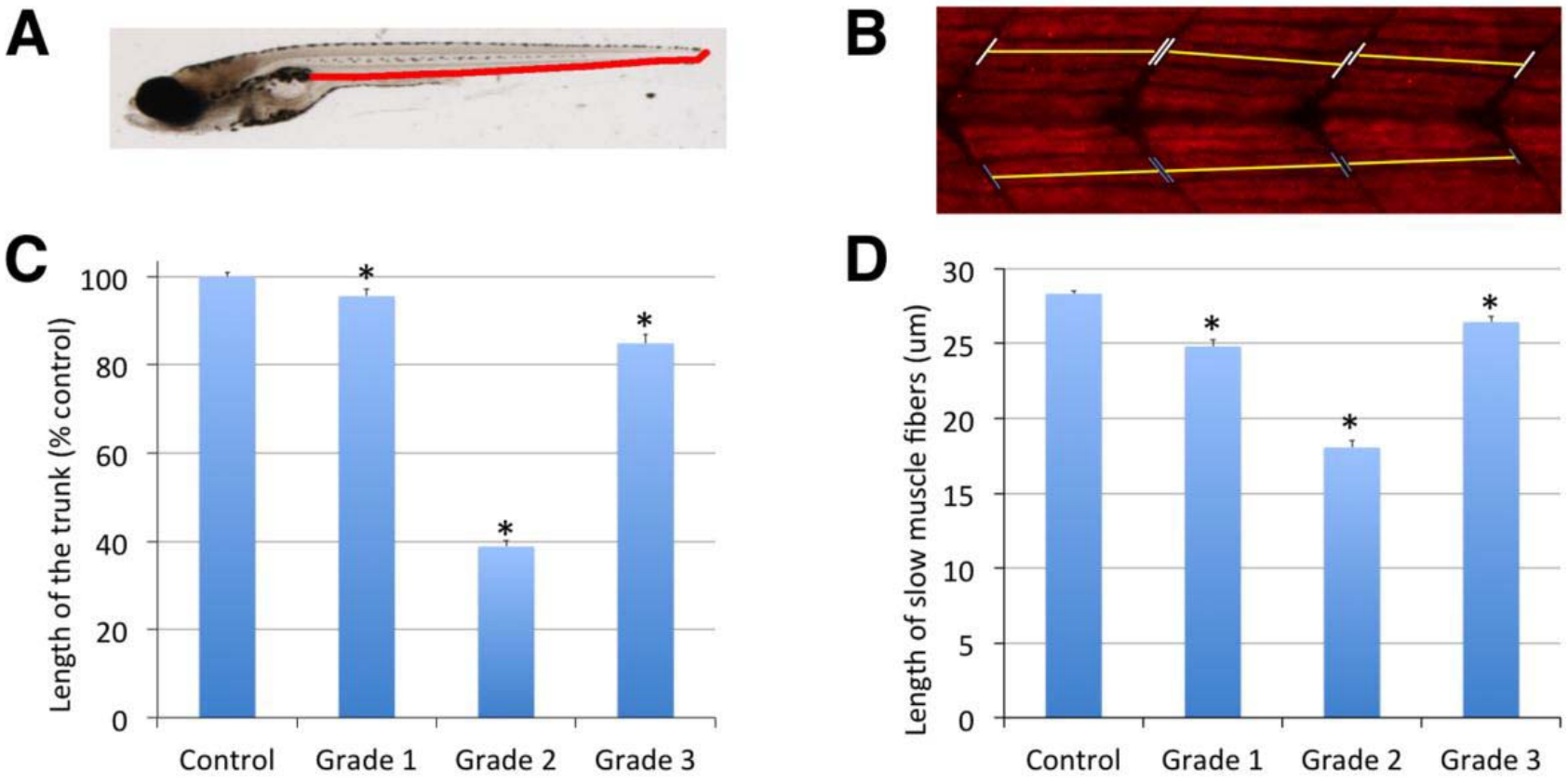
Severe

Prevalence of the three different phenotypes in response to different CPO concentrations



(48 larvae per experimental group)

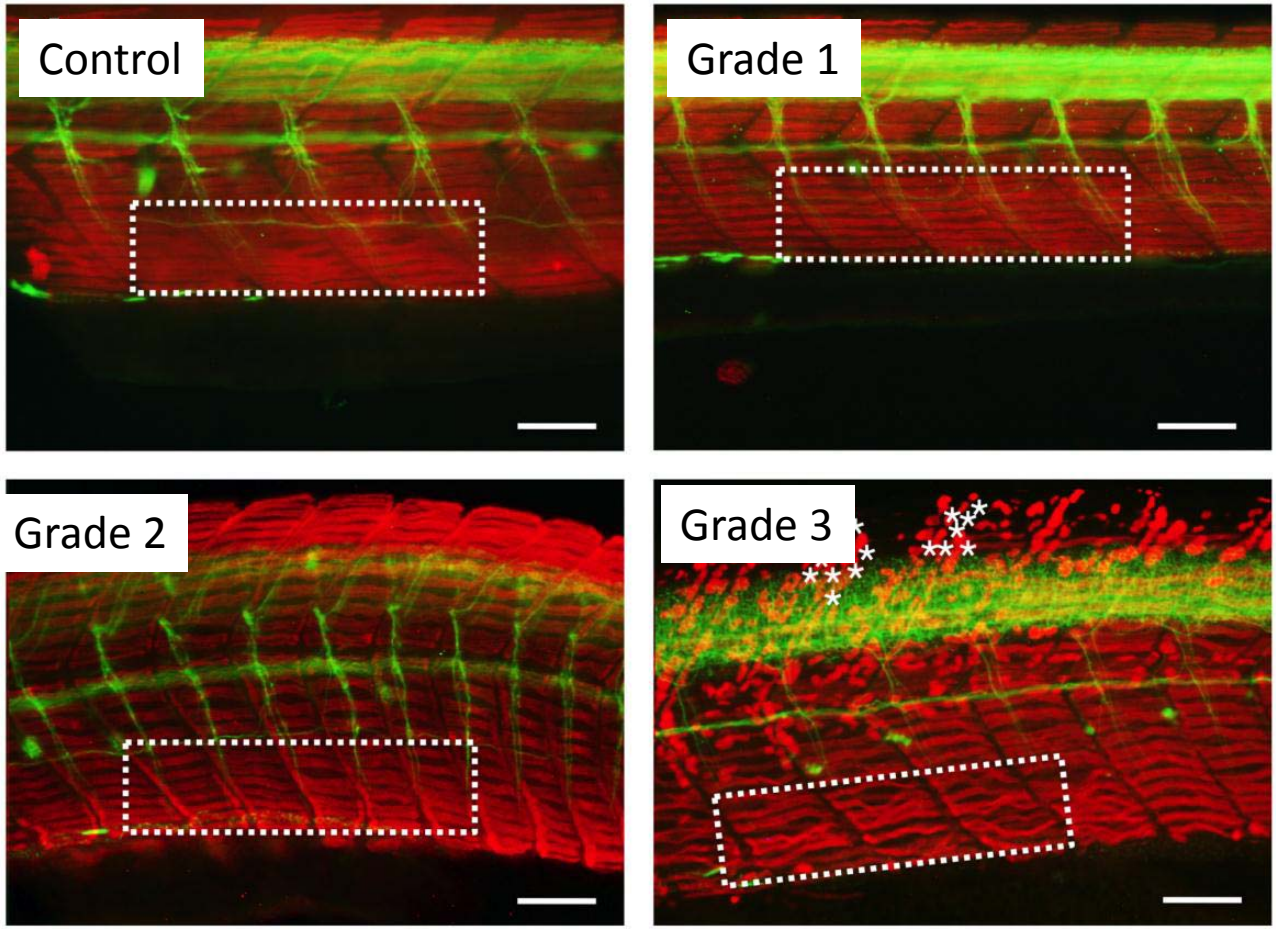
CPO induces reduction in the length of the trunk and axial muscle fibers in larvae



Characterization of pathological features at the trunk level in the different grades of severity of acute organophosphorus poisoning induced by chlorpyrifos-oxon in zebrafish larvae.

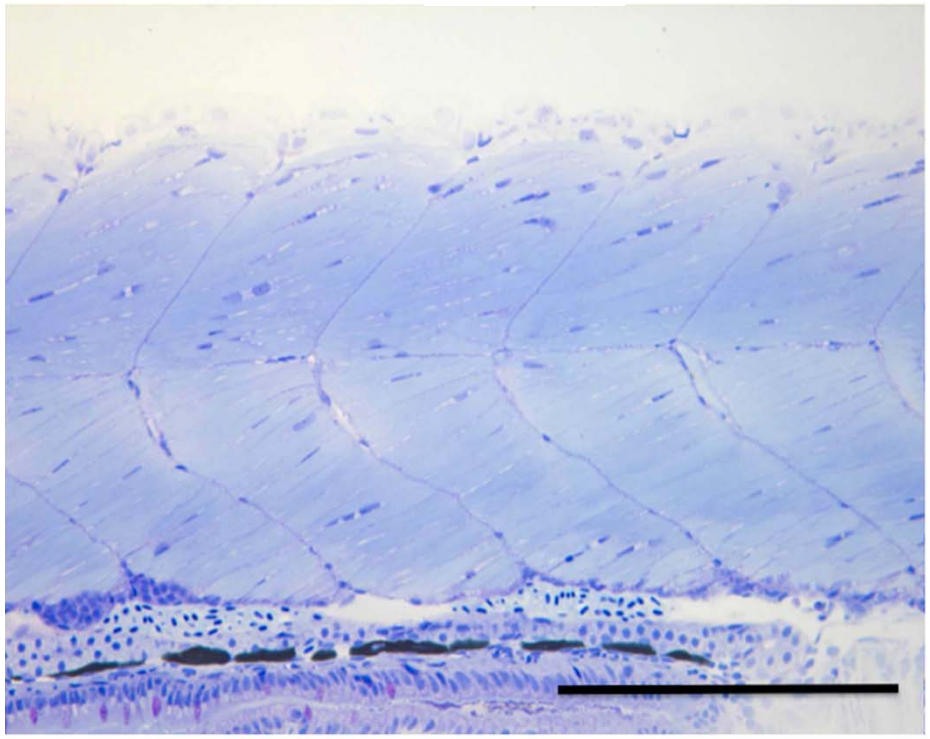
F59 (slow muscle fibers)

Acetylated α-tubulin (spinal axonal tracks)

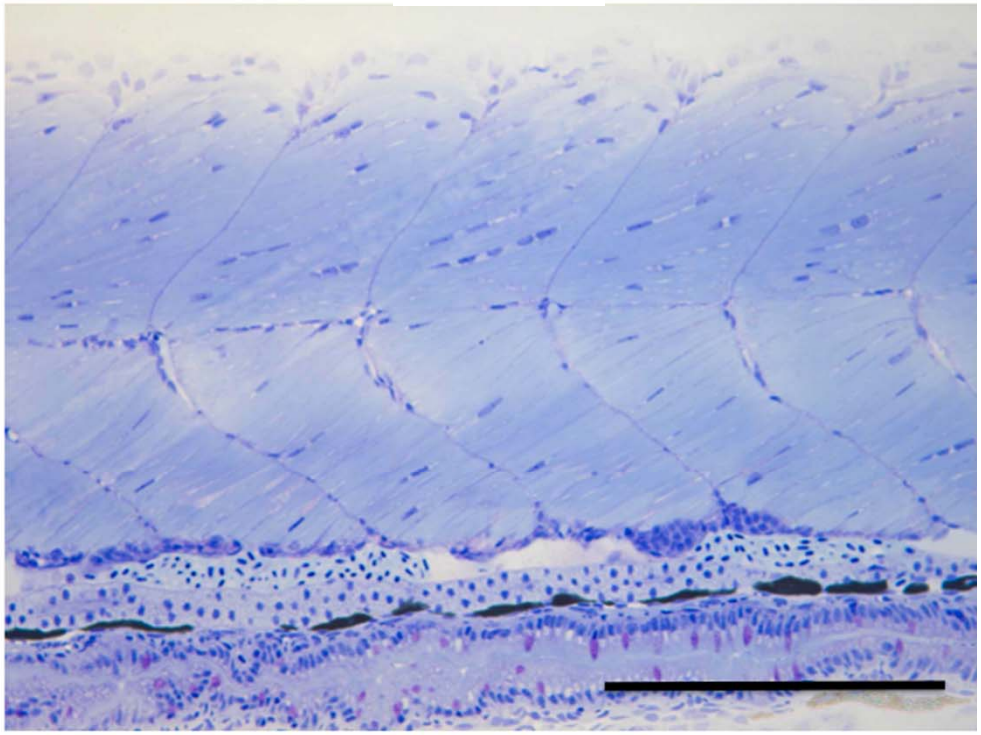


Histopathological analysis of grade 1 phenotype at the muscle level

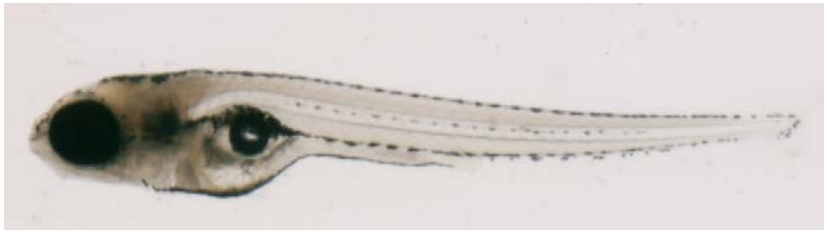
Control

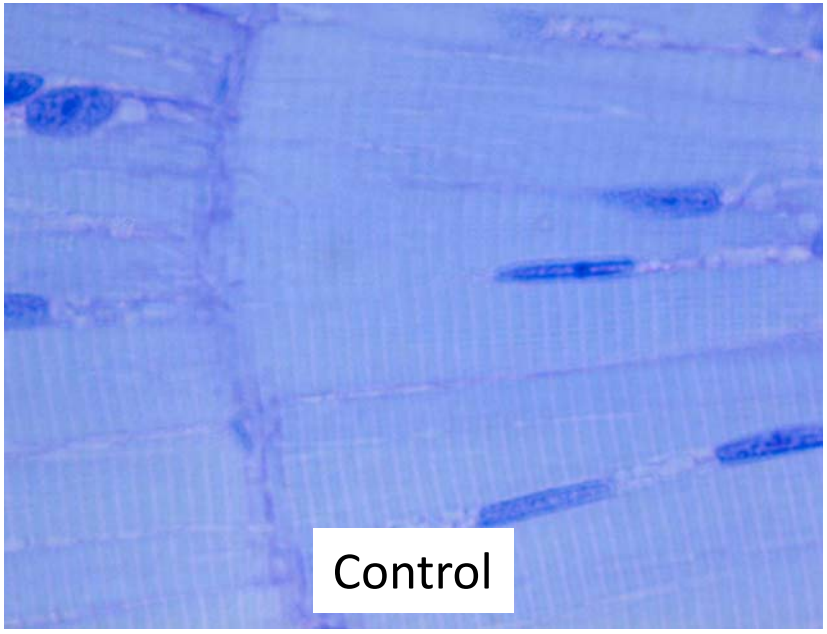


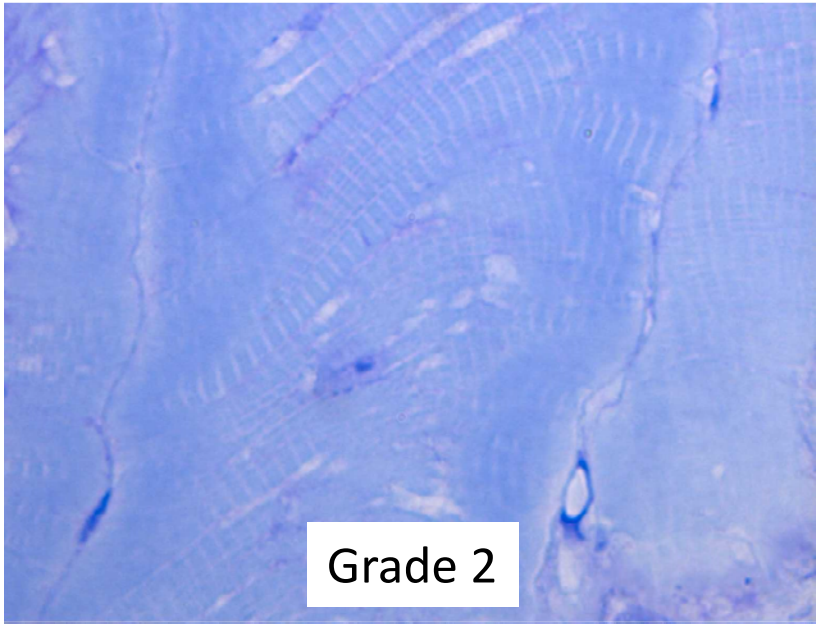
Grade 1



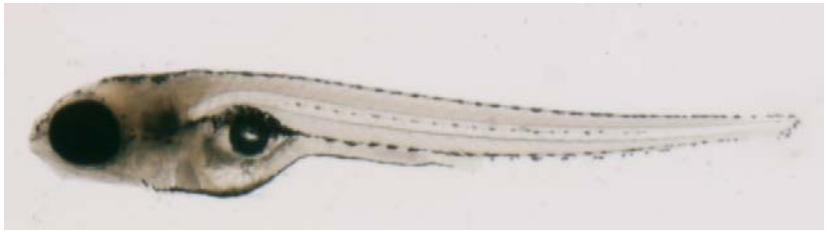
Hypercontracture of the axial muscles in grade 2 larvae

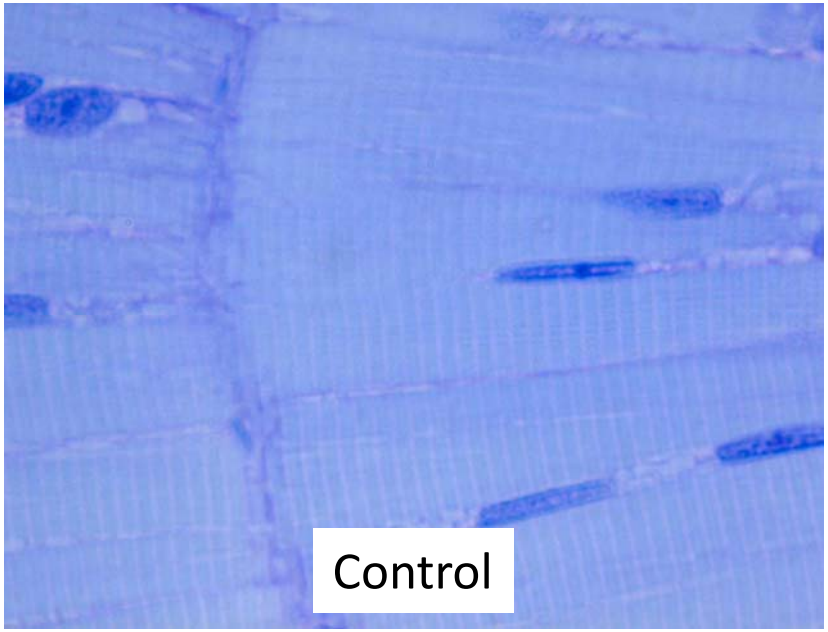


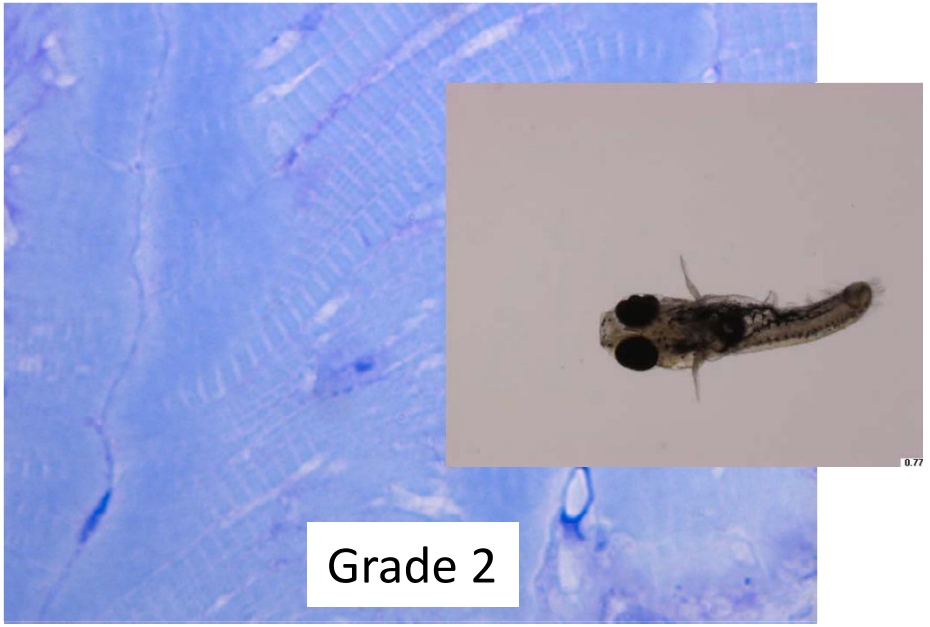




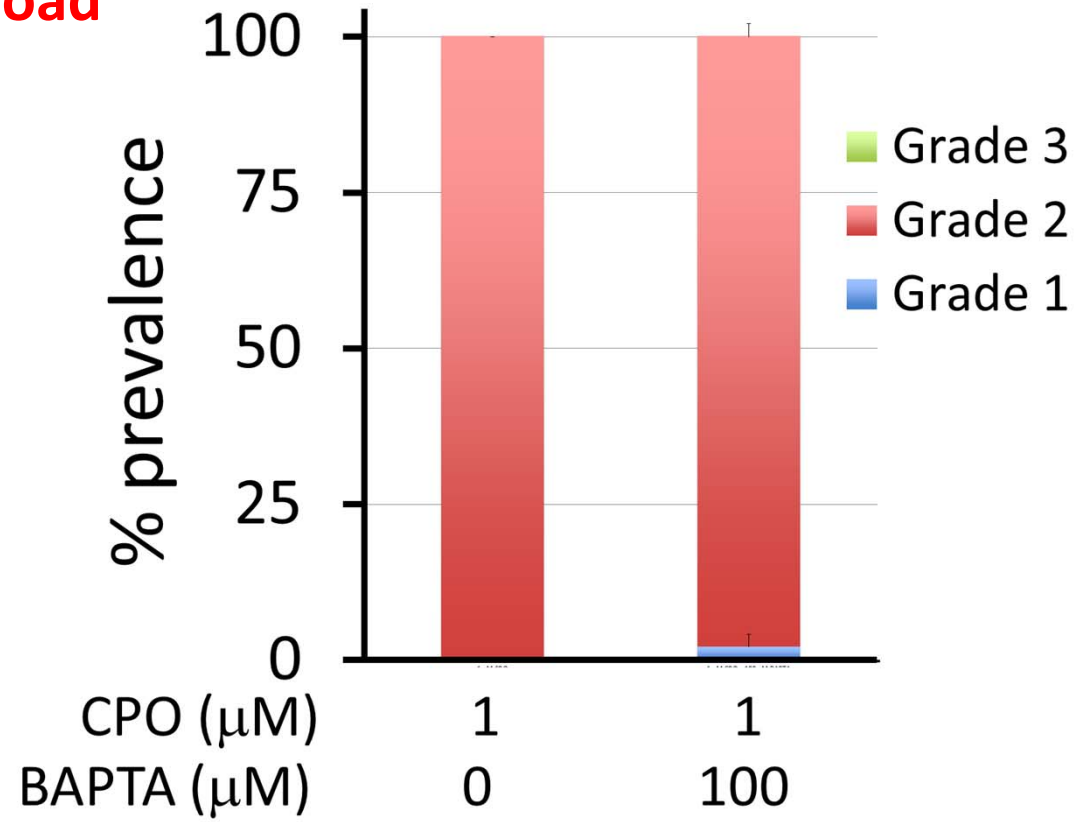
Hypercontracture of the axial muscles in grade 2 larvae





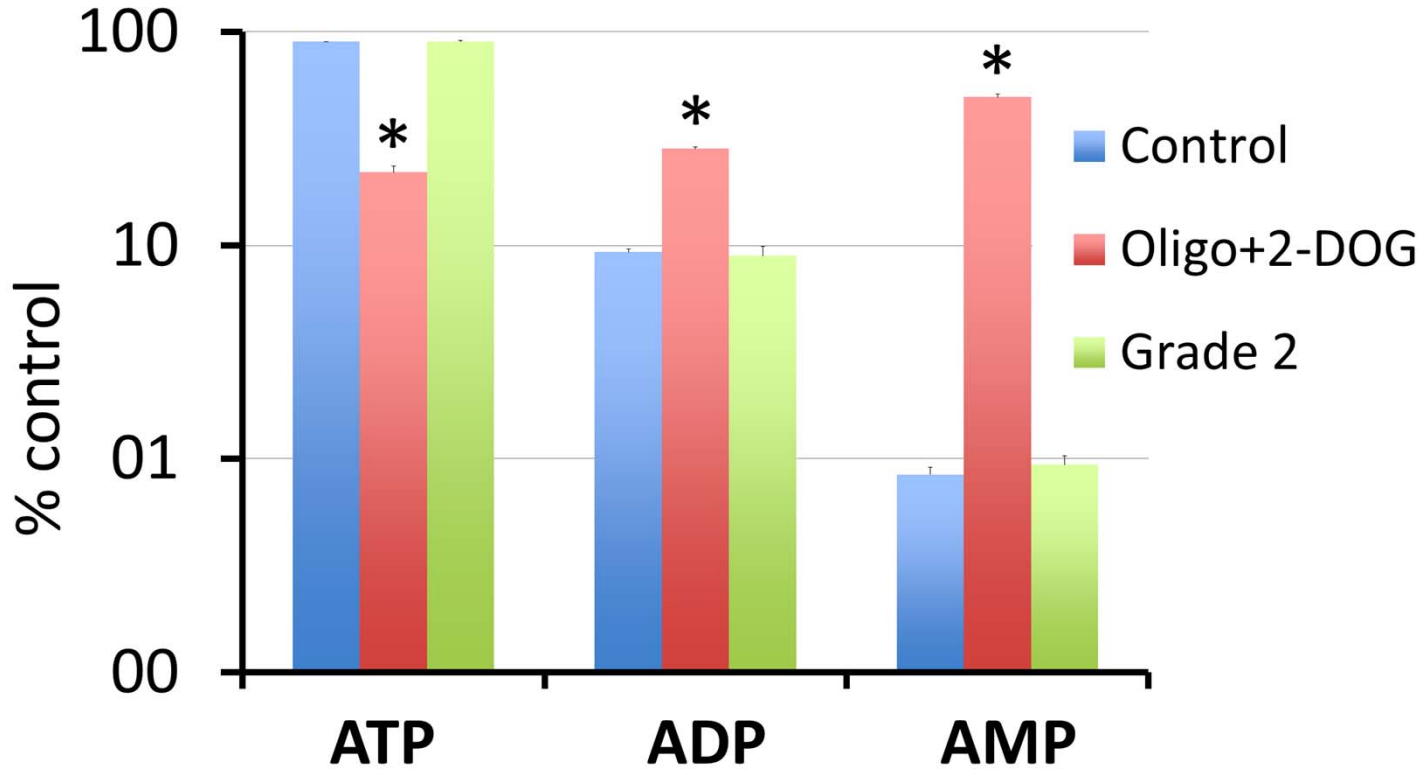


Hypercontracture of the axial muscles in grade 2 larvae is not related with calcium overload



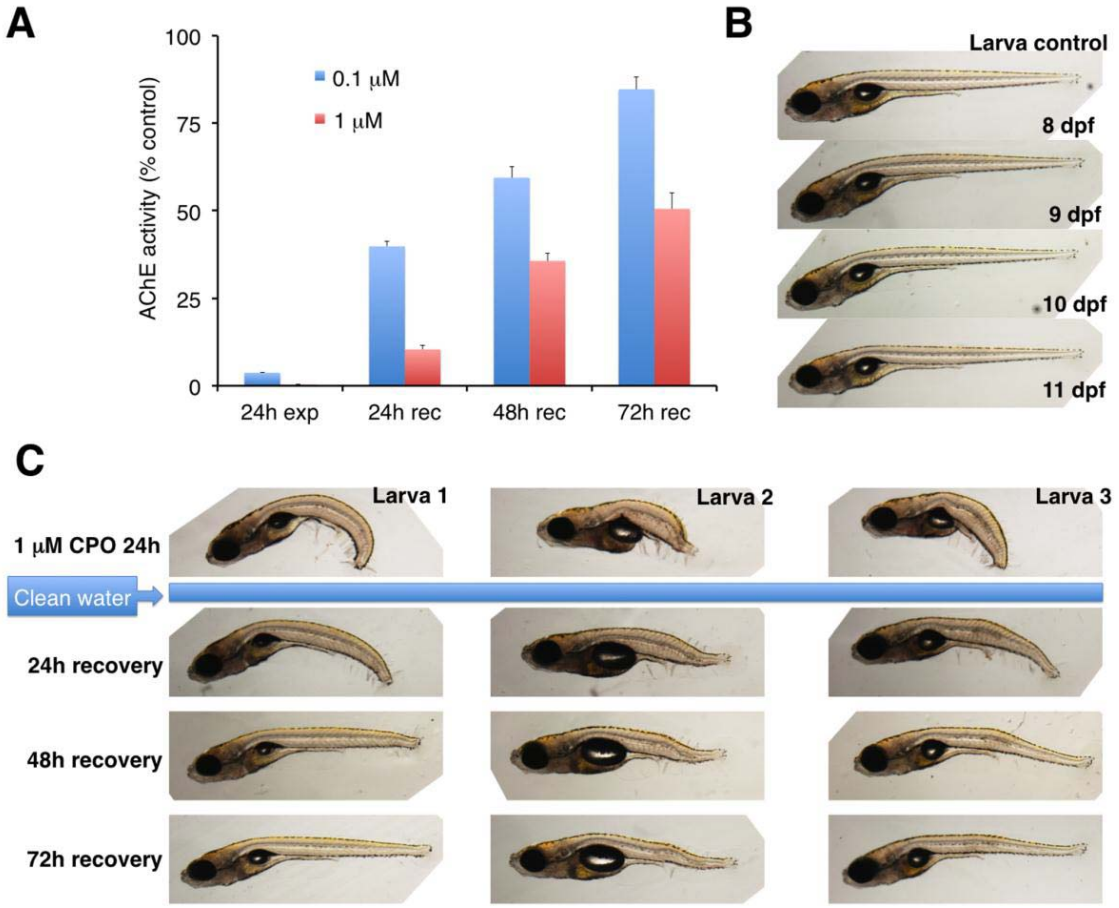
BAPTA-AM, a permeable calcium chelator, is not able to rescue the grade 2 phenotype, indicating that Ca^{2+} overload is not the mechanism leading to the hypercontracture.

Hypercontracture of the axial muscles in grade 2 larvae is not related with ATP depletion.



Oligomycin + 2-deoxyglucose (2-DOG)

Partial recovery of AChE activity and grade 2 phenotype in zebrafish larvae exposed to 1 µM CPO after the transfer to clean water.

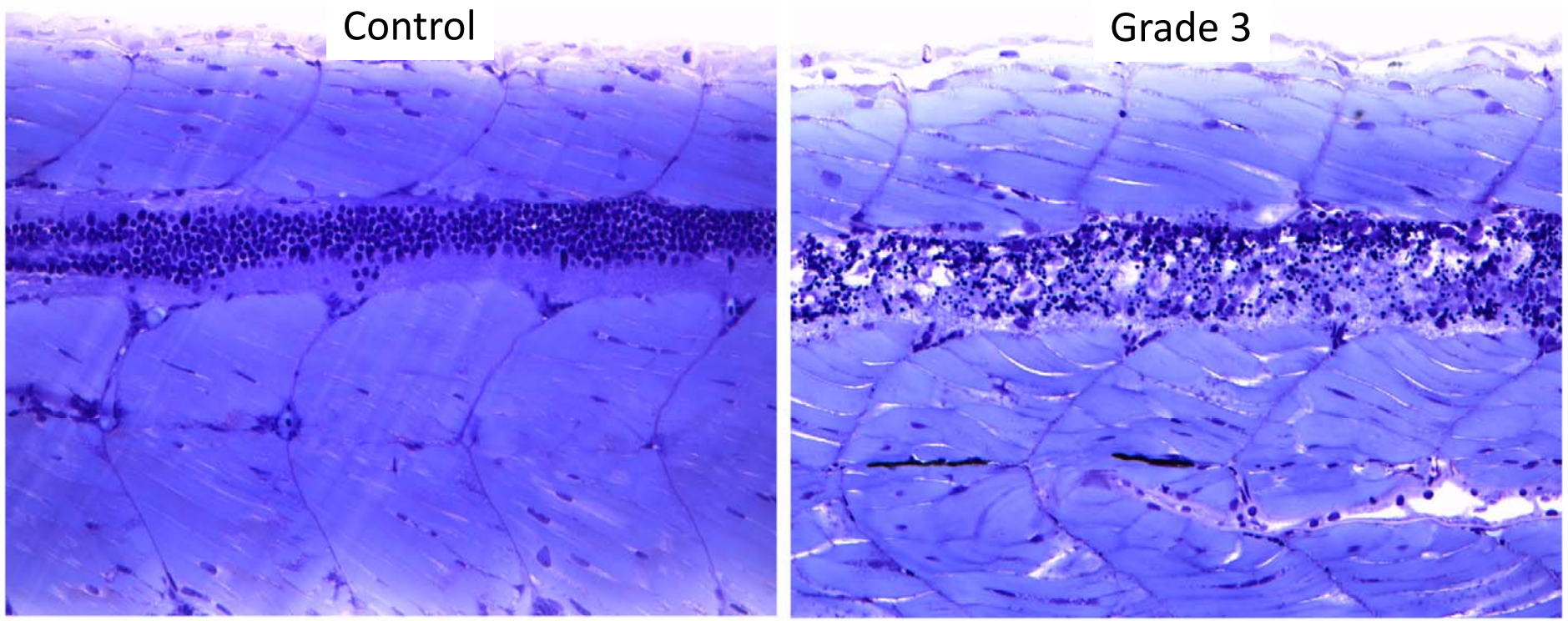


Grade 2 was the most prevalent phenotype within the 0.75–1.50 µM range of CPO concentrations. Larvae were unable to swim and their touch motor response was fully abolished.

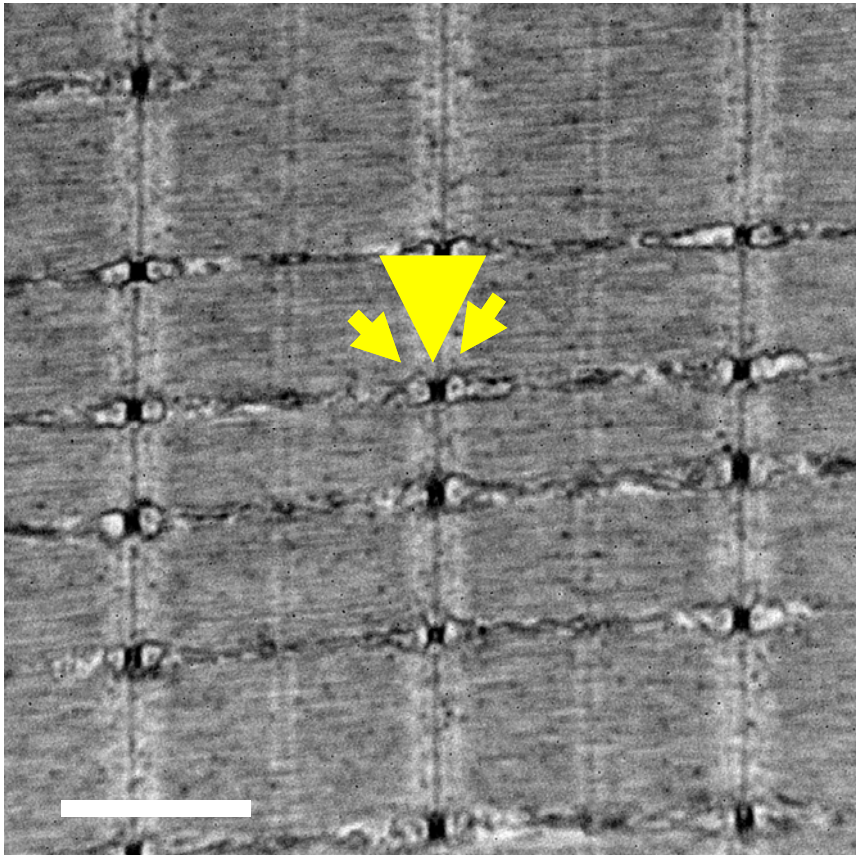
AChE inhibition was an upstream event in the toxic pathways that resulted in hypercontracture of the axial muscles and integrity impairment of axial muscle fibers which were not related with calcium overload or ATP depletion.

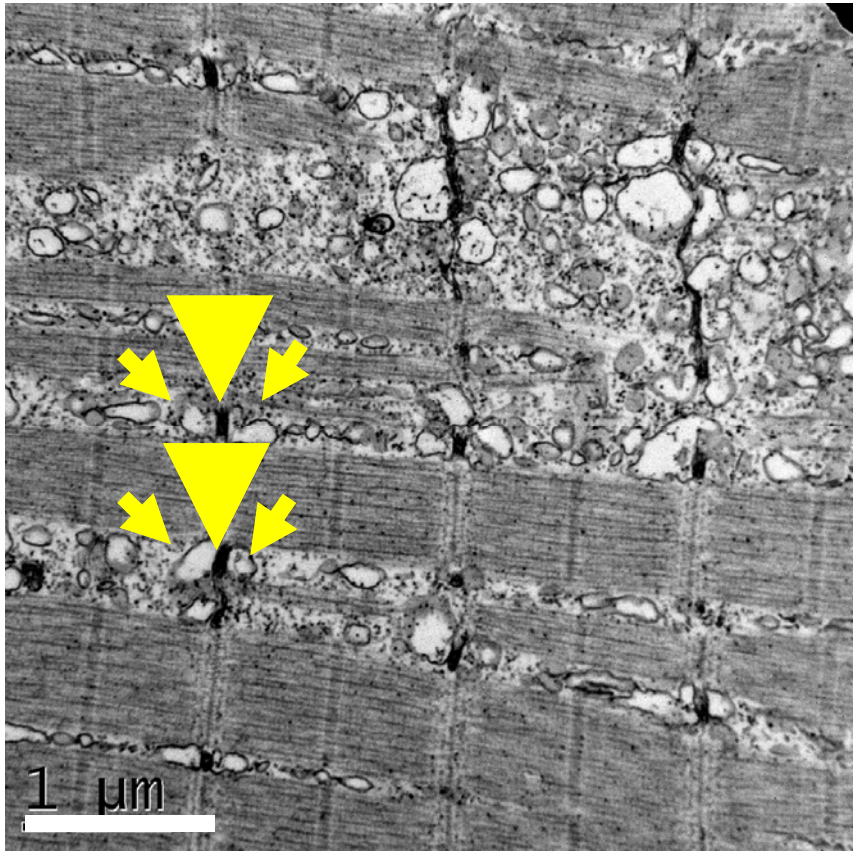
Reversibility, at least partial, of CPO's effects on body length and locomotor behavior may be observed after removing the compound from the water.

Histopathological assessment of grade 3 larvae shows severe lesions in muscle fibers

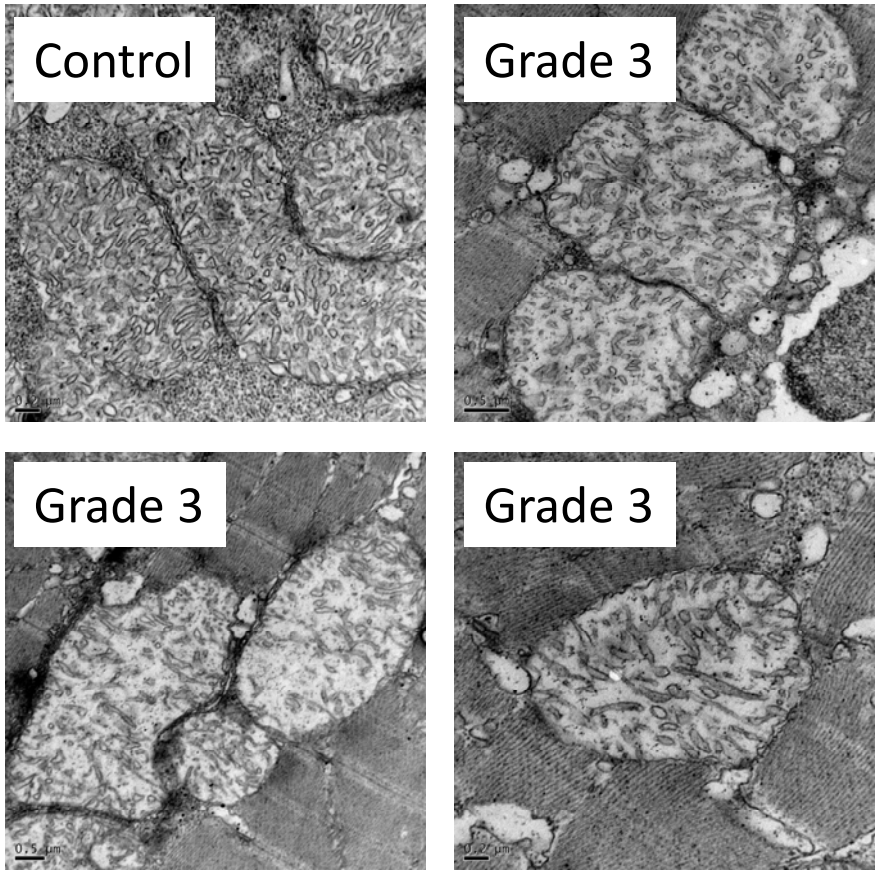


Histopathological assessment of grade 3 larvae shows severe lesions in muscle fibers

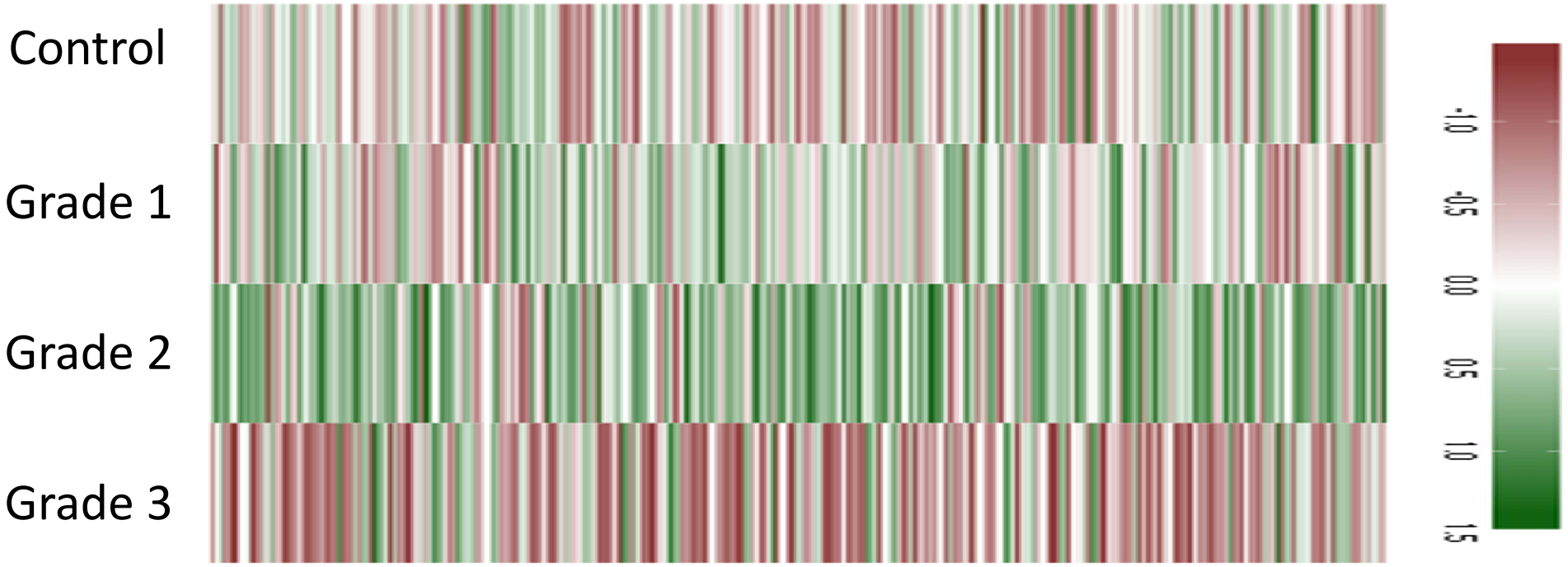




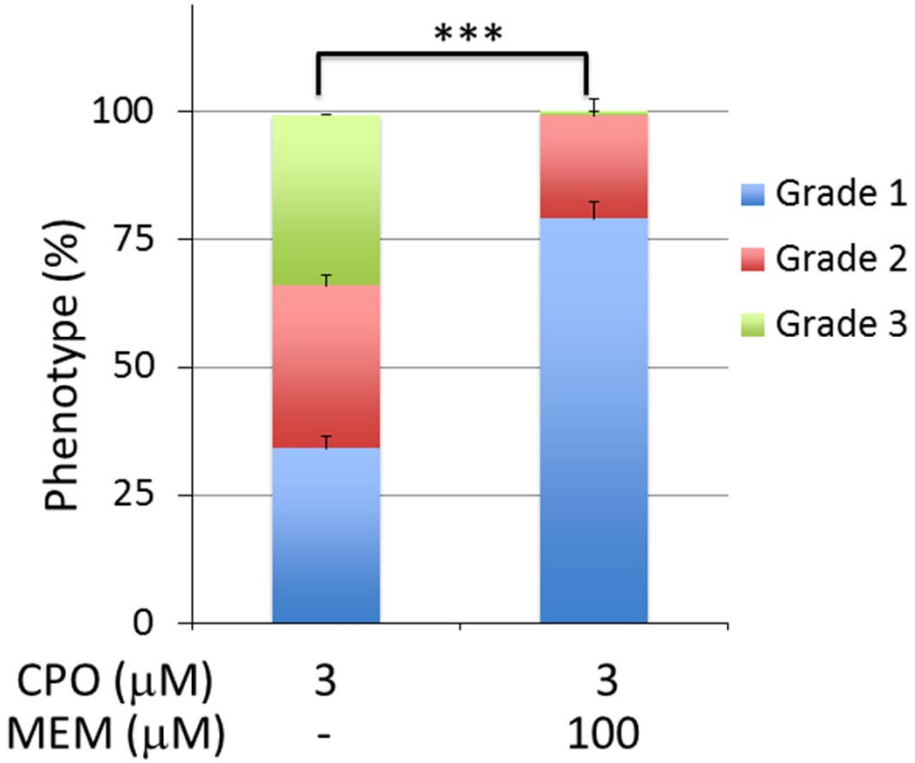
Swelling and loss of cristae in the mitochondria of the grade 3 larvae (fast-twitch axial muscle fibers)



Calcium dysregulation is central to grade 3 development. Heatmap of the **calcium signalling pathway** (dre04020) in control and the different grades of OPP, showing clear down-regulation of this pathway in grade 3 larvae.

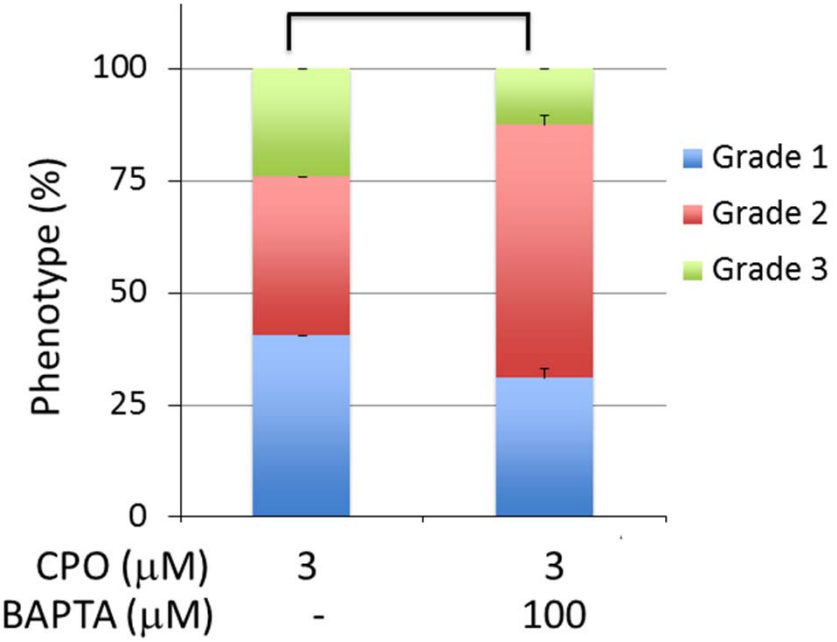


Calcium dysregulation is central to grade 3 development. Memantine, an antagonist of NMDA receptors, reduced the prevalence of the grade 3 phenotype dramatically.



Seven days post fertilization (dpf) larvae were pre-incubated with 100 µM memantine for 1 h followed by co-exposure with 100 µM memantine/3 µM CPO for 24 h.

Calcium dysregulation is central to grade 3 development. BAPTA-AM, a permeable calcium chelator, also reduced the prevalence of the grade 3 phenotype significantly**.**



Six days post fertilization zebrafish larvae were pre-incubated with 100 µM BAPTA-AM for 24 h followed by 24 h co-exposure with 3 µM CPO.

Grade 3 was the most prevalent phenotype at high CPO concentrations. It was characterized by an irreversible widespread necrosis of the axial muscle fibers. RNA-seq and pharmacological analyses supported the hypothesis that after initial cholinergic overstimulation, an influx of extracellular Ca2+ through NMDA receptors occurs and that the increase in the cytoplasmic levels of Ca2+ in animals with a compromised Ca2+ buffer capacity resulted in the uncontrolled activation of proteases, phospholipases and kinases.

General conclusion:

Our results show that zebrafish models may mimic pathophysiological mechanisms behind cholinergic toxidrome in humans. The suitability of the zebrafish larvae to *in vivo* high-throughput screenings of small molecule libraries makes these models a valuable tool for identifying new drugs for multifunctional drug therapy against acute organophosphorus poisoning including deleterious effect at the muscle level.