Towards the Standardization of the Dark/Light Transition Test with *Danio rerio* embryos
Preliminary Results from an Inter-laboratory Ring Test

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The DLT with zebrafish embryos

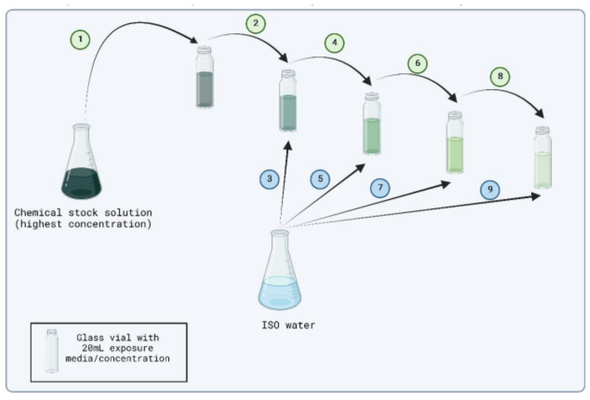
- One of the most commonly utilized behavioral assays with zebrafish embryos is the **dark/light transition test (DLTT)**. This test involves recording and quantifying the swimming activities of zebrafish eleutheroembryos under different light conditions.
- The DLTT **lacks standardization, and guidelines have yet to be established.** As a result, zebrafish laboratories employ in-house protocols to record the activity of zebrafish eleutheroembryos.
- Different statistical tests are employed to explain the results and analyze the output. Consequently, **comparing the results across different studies becomes a challenging task and poses a potential obstacle to bring these tests to regulators.**
- Prior to developing a universal and standard guideline, **it is necessary to characterize and understand the general variation in the DLTT.**

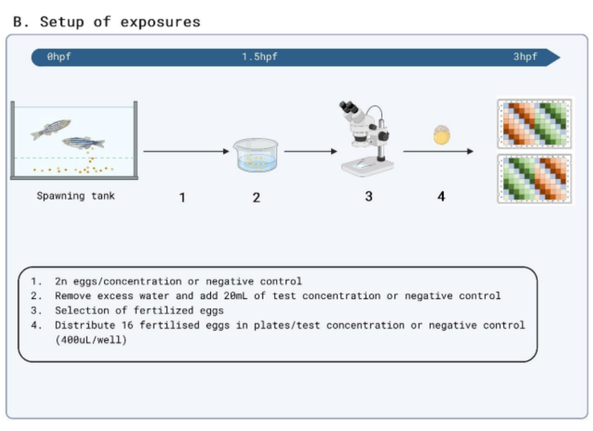
Goal of the Ring Test

To address the existing DLTT limitations, a ring test involving institutions from both the European Union (EU) and non-EU countries was organized in May 2023 and a SOP was distributed to all the participants in November 2023.

- **Determine the reproducibility of the DLTT with zebrafish embryos among different laboratories.**
- **Understand the potential experimental factors and test procedure key steps that contribute to observed variation in the general DLTT.**
- **Produce a comprehensive dataset that can be utilized to test different statistical approaches.**
- **Develop user-friendly data analysis pipeline for statistical analysis.**

The Standard Operation Procedure for the DLTT

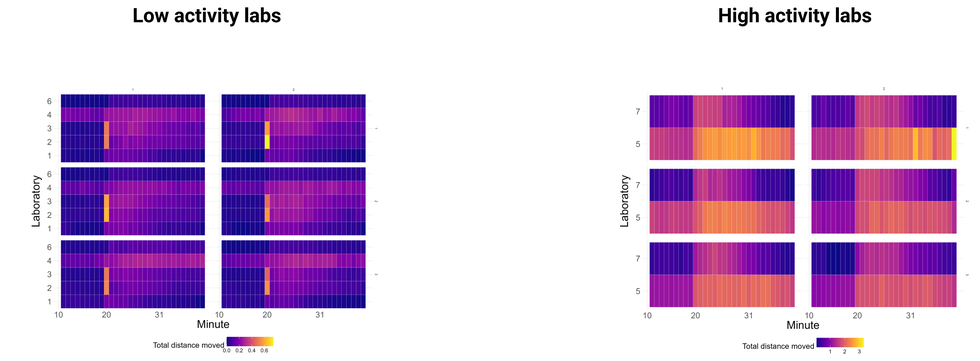


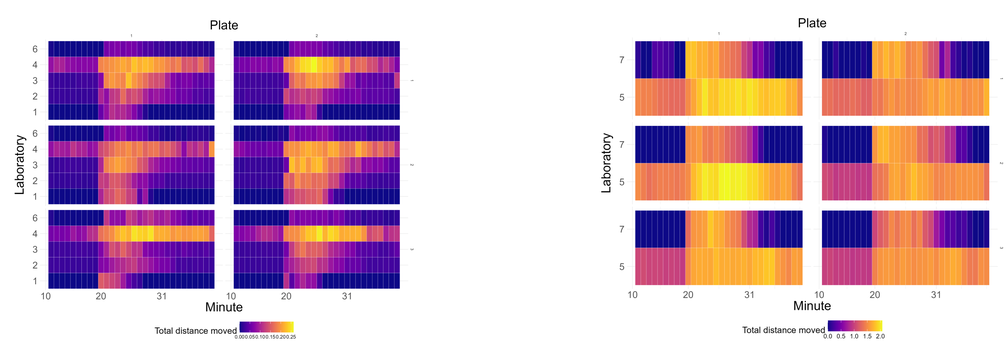


Overview on the testing strategy and exposure scenario

- Zebrafish eggs were exposed to **caffeine (CAF) and 3,4-dichloroaniline (34DCA)** from 3 to 120 hours post fertilisation (hpf) at 26 °C with a light: dark cycle of 14:10 hours.
- **Four apical observation indicators of lethality and developmental malformations** according to the OECD236 (i.e. coagulation, lack of somite, lack of tail detachment and lack of heartbeat) **were recorded at days 1, 4 and 5.**
- A standardized DLTT using automated observation chamber and behavioral tracking systems (Viewpoint and Noldus) **was performed at days 4 and 5.** Habituation: **5' ON - 5' OFF.** DLTT test: **10' ON - 20' OFF**
- **Five concentrations** and **three technical replicates** were tested with the final goal to obtain a sigmoidal dose/response curve for CAF and 34DCA with **at least 50% decrease of movement** during the dark phase at the highest tested concentration.

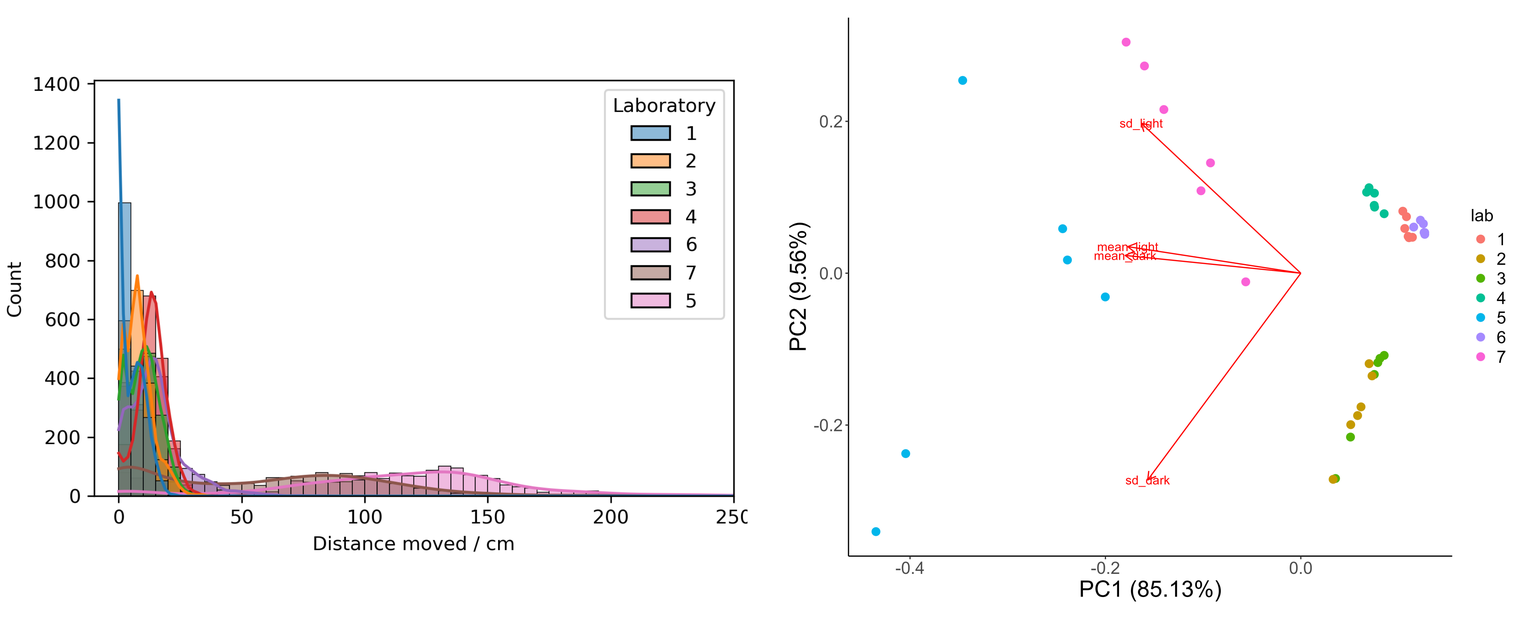
Behavioural profiles of the negative controls (day 5)





Heatmaps represent the behavioural profiles of the negative controls along the test (y-axes labs, x-axes test time in minutes). All three replicates are represented in the graphs. Data are represented as distance moved [cm]. Out of seven labs, two labs showed higher activity in the negative control along the DLTT.

General movement distribution and general technical variability among labs



Distribution curves for the movements among different labs and Principal Component Analysis (PCA) (Dark phase). Out of seven labs, two (labs 2+3) show higher activity as well as technical replicate variation respect to the others.

Outlook and next steps

Overall, **behavioural profiles of the negative controls show highly similar activity profiles among different labs**

Labs with higher activity show also a higher technical variability compared to the other labs. Consequently, these data need further investigation

We observed low technical variability among labs with similar behavioural profiles

The present results support the **reliability and robustness of the DLTT performed with the present SOP** but incorporation of more data is required for more robust evaluation of the current SOP

As next steps, it will be necessary to analyze the **exposed group** to confirm similar effect among labs. A further analysis including variables such as strain, age of the parents and swim bladder inflation will be performed within 2024