Experiences in developing the Amphibian Metamorphosis Assay for regulatory testing – a CRO perspective.

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Introduction

To support global chemical risk assessments for our clients, we have established and maintained a female *Xenopus laevis* colony and investigated the feasibility of amphibian testing at our laboratory at Scymaris Ltd. Frogs are housed in groups of 6 individuals and spawned every three months on rotation to ensure good egg production and the capacity to start studies monthly.

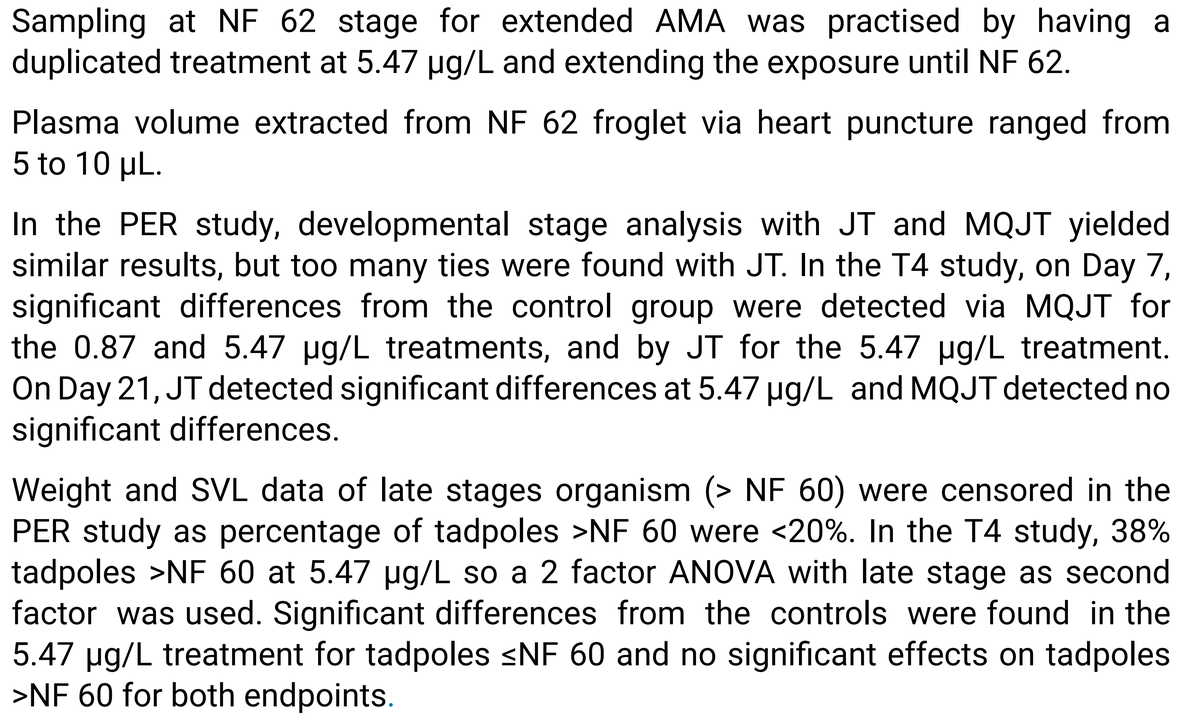
The Amphibian Metamorphosis Assay (AMA; Ref 1) is a conceptual framework level 3 screening assay to identify substances interfering with the hypothalamic-pituitary-thyroid (HPT) axis. Amphibian metamorphosis is modulated via thyroid dependent processes and *X. laevis* is a well-studied and easy to keep under laboratory conditions amphibian model. The observational endpoints for this assay are Hind Limb Length (HLL), Snout to Vent Length (SVL), developmental stage, wet weight, thyroid histology and mortality.

Xenopus culture

24 females on-site housed in groups of 6 and fed 3 times a week with Xenopus pellets. Each female individually identified and induced to spawn every three months.

Results

- Both PER and T4 were detected as thyroid active in the tests.
- Both studies were valid (<2 tadpole mortality per replicate in the control group).
- Sampling at NF 62 stage for extended AMA was practised by having a duplicated treatment at 5.47 µg/L and extending the exposure until NF 62.
- Plasma volume extracted from NF 62 froglet via heart puncture ranged from 5 to 10 µL.
- In the PER study, developmental stage analysis with JT and MQJT yielded similar results, but too many ties were found with JT. In the T4 study, on Day 7, significant differences from the control group were detected via MQJT for the 0.87 and 5.47 µg/L treatments, and by JT for the 5.47 µg/L treatment. On Day 21, JT detected significant differences at 5.47 µg/L and MQJT detected no significant differences.
- Weight and SVL data of late stages organism (> NF 60) were censored in the PER study as percentage of tadpoles >NF 60 were <20%. In the T4 study, 38% tadpoles >NF 60 at 5.47 µg/L so a 2 factor ANOVA with late stage as second factor was used. Significant differences from the controls were found in the 5.47 µg/L treatment for tadpoles ≤NF 60 and no significant effects on tadpoles >NF 60 for both endpoints.



Effect of PER on X. laevis HLL normalised by SVL growth (A), developmental stage (B). Effect of T4 on *X. laevis* HLL normalised by SVL growth (C), developmental stage (D).
*Significant difference ($p < 0.05$) from the dilution water control.

Methods

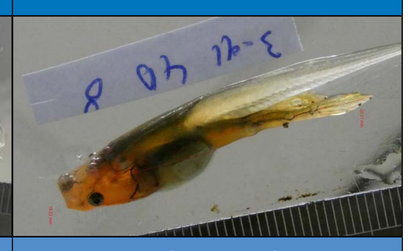
As part of test system validation, a reference toxicity test was performed. Fertilized embryos were kept in an incubator until Nieuwkoop and Faber (NF; Ref 2) stage 45 then transferred to pre-exposure tanks at 21 ± 2°C on flow-through regime at 50 mL/min until exposure start.

Test Animal	*Xenopus laevis* NF stage 51, selected with development stage and total length
Exposure period	21 days with interim sampling of 5 animals per vessel on Day 7
Exposure conditions	Flow-through regime, 25 mL/min in 9.5 L glass tanks. 20 tadpoles per test vessel, 4 replicate tanks per concentration and control DWC
Dilution water	Dechlorinated tap water with hardness, alkalinity and pH adjusted, UV sterilized, filtered to ≤5 µm. Measured Iodide concentration of 1.9 µg/L
Water parameters	22 ± 1 °C, pH 6.5-8.5, > 40% DO
Feed	Sera micron®, approximately 50% of guideline regime. Feed was screened for trace metals and pesticides
Lighting	12 h Light : 12 h Dark. 600 to 2000 lux
Test substances	(1) Sodium perchlorate (PER). Nominal concentrations: 62.5, 125, 250 and 500 µg/L (2) L-Thyroxine Sodium Salt Pentahydrate (T4). Nominal concentrations: 0.14, 0.87 and 5.47 µg/L
Endpoints analysed	Overall mortality. Day 7 and 21: Wet weight, SVL, HLL normalised by SVL, Developmental stage. Length measurements via image analysis (3)
Statistical analysis	Using ToxRat®(Ref 4). Developmental stage analysed with multi- quantal Jonckheere-Terpstra test (MQJT) and Jonckheere-Terpstra (JT). HLL, SVL and weight analysed with JT if monotonic dose-response or Dunnett's test (when normality and variance homogeneity achieved).

Conclusions Performance Criteria

Criterion	Acceptable limits	PER	T4
Mortality in controls	≤10% . <2 per rep	0 %	2.5% . 1 in 2 reps
Minimum median developmental stage of controls at end of test	NF 57	NF 59	NF 58
Spread of developmental stage in controls	10th-90th percentile ≤4	NF 58-60	NF 57-59
Dissolved oxygen	≥40% ASV	≥64.2%	≥57.1%
pH	6.5-8.5. Spread ≤0.5	7.00-7.83*	6.86-7.77*
Water temperature	22 ± 1°C. Spread ≤0.5°C	22.4-23.0°C*	21.8-22.5°C*
Test concentration without over toxicity	≥2	4 (all)	3 (all)
Replicate performance	≤2 reps per concentration compromised	0	0

*measured pH and temperature exceeded tight range specified in test guideline.
This is currently being investigated further for future testing.

PER Day 21 – 500 µg/L	Control Day 21	T4 Day 21 – 5.47 µg/L
		
Delayed Development	**Expected Development**	**Advanced Development**
↓ Developmental stage	NF 58-59	↑ Developmental stage
↓ HLL/SVL	HLL/SVL: 0.58-0.74 mm	↑ HLL/SVL (Day 7)
No effect on censored SVL	SVL: 21-25 mm	↓ SVL
No effect on censored Weight	Weight: 1.4-1.6 g	↓ Weight

References (1) OECD (2009). Guidelines for the Testing of Chemicals. TG 231: The Amphibian Metamorphosis Assay. OECD, Paris. (2) Nieuwkoop, P.D. and Faber, J. (1956) Normal table of *Xenopus laevis* (daudin): A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. North-Holland, Amsterdam. (3) CellSens Imaging Software (version 3.2) Olympus corporation, Shinjuku Monolith, 2-3-1 Nishi-Shinjuku, Shinjuku-ku, Tokyo 163-0914, Japan. (4) ToxRat Solutions (Version 3.3.0) GmbH & Co. KG, Naheweg 15, D-52477 Alsdorf, Germany.