

# Reproducibility of the OECD guideline 241: the Larval Amphibian Growth and Development Assay.

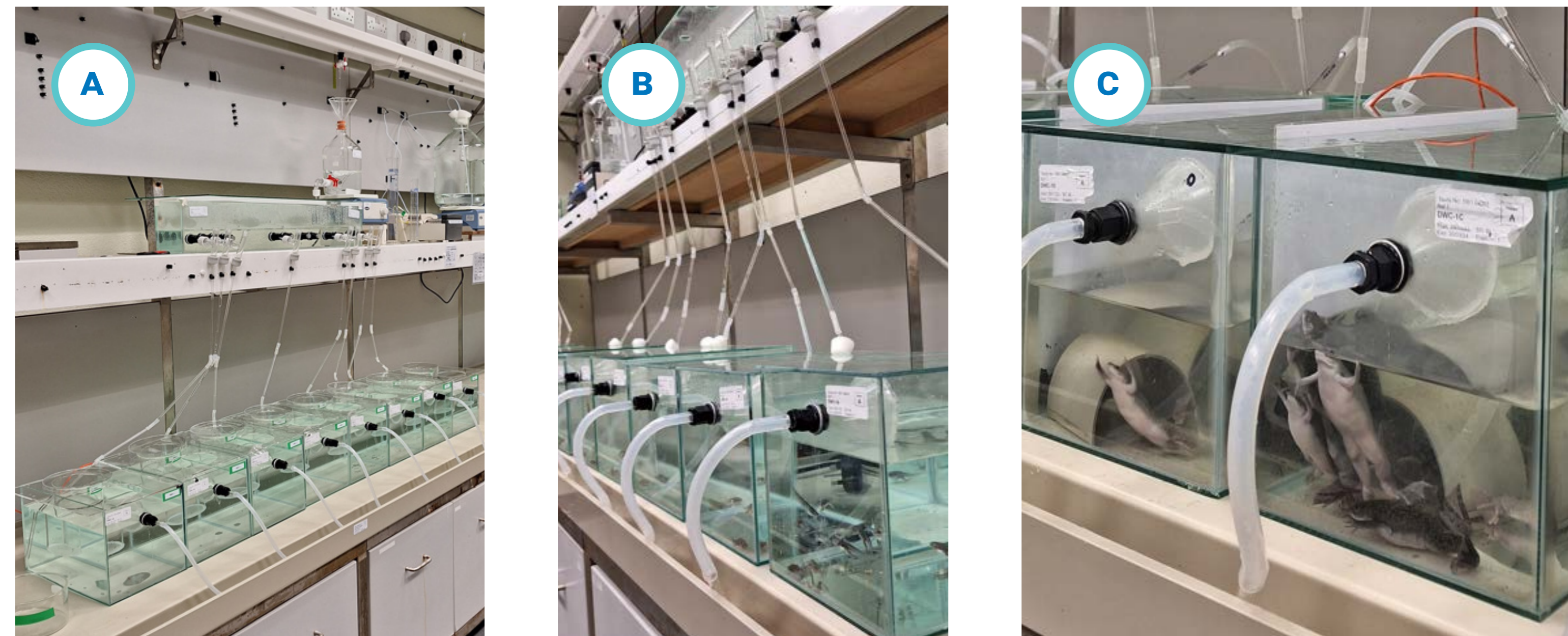
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## Introduction

The Larval Amphibian Growth and Development Assay (Ref 1) is a level 4 *in vivo* assay of the OECD Conceptual Framework on Endocrine Disrupters Testing and Assessment and provides data on adverse effects on the hypothalamus pituitary thyroid and gonadal axes using the model *Xenopus laevis*. The assay was adopted in 2015 with few chemicals tested during the validation process and little data on inter-laboratory reproducibility. So far, few studies have been conducted and few laboratories are able to perform this test. The aim of this study was to perform the LAGDA following the OECD 241 guideline to investigate the feasibility of passing the strict study validity criteria as well as the replicability of the test design.

## Methods



### Experimental design:

**A Larval phase;** Freshly fertilized embryos without jelly were placed in meshed beakers suspended in water and released in the test vessels when freely swimming. Embryos were observed daily. Airlines were installed in larval and juvenile phase to maintain water parameters.

**B Transition phase;** At NF stage 62, tailed froglets were transferred to another tank vessel of similar conditions and 5 were randomly subsampled per tank vessels. For PTU exposed tadpoles, as most tadpoles never reached NF 62, only 4 froglets were subsampled and 8 froglets transferred to the juvenile phase across the concentration.

**C Juvenile phase;** At NF stage 66, 10 froglets were left in each tank to grow out until test end (10 weeks after control median time to NF 62). Tanks were changed to 12.5 L to account for frog growth.

<b>Test Animal</b>	<i>Xenopus laevis</i> NF stage 8-10 (Ref 2), individually selected.
<b>Exposure period</b>	Up to 116 days.
<b>Exposure conditions</b>	Flow through regime, 35 mL/min in 9.5 L glass tanks. 20 embryos per test vessel, 4 replicate tanks per concentration and 8 for control. Tank vessels changed to 12.5 L during the juvenile phase.
<b>Dilution water</b>	Dechlorinated tap water with hardness, alkalinity and pH adjusted, UV sterilized, filtered to $\leq 5 \mu\text{m}$ . Iodide concentration of 1.9 $\mu\text{g/L}$ .
<b>Water parameters</b>	21 $\pm$ 1 °C, pH 6.5-8.5, > 40% DO.
<b>Feed</b>	Following guideline, mixture of trout feed starter with spirulina wafer and fish flakes as well as brine shrimps. <i>Xenopus</i> pellets during juvenile phase. Feed analysed for trace metals and pesticides.
<b>Lighting</b>	12 h Light : 12 h Dark. 600 to 2000 lux.
<b>Test substances</b>	6-Propyl-2-thiouracil (PTU). Nominal concentration 34 mg/L. Mean measured concentration: 32.2 mg/L.
<b>Endpoints analysed</b>	Daily mortality. At NF 62: Time to NF 62, Snout-Vent-Length (SVL), Weight, Thyroid histology At test end: Weight, SVL, Liver Somatic Index LSI, genetic/phenotypic sex ratio, Histopathology (gonads, reproductive ducts, kidney, liver).

## Results

- Frogs were sexed with visual gross morphological examination. Histopathology and sex ratio results are pending.
- At NF 62, PTU treated tadpoles were larger and displayed delayed metamorphosis compared to the control group, developed a red growth under snout and an enlarged belly inducing swimming difficulties during prometamorphosis.
- At test end, PTU exposed frogs were smaller with a lower LSI compared to the control group. Necropsies indicated an enlarged growth in neck region.

Treatment	Weight (g)	N	SVL (mm)	N	Time to NF 62	N
DWC	2.3 $\pm$ 0.4	20	25.5 $\pm$ 1.3	20	42 $\pm$ 1	142
PTU 34mg/L	6.3 $\pm$ 1.5	4	32.2 $\pm$ 3.5	4	71 $\pm$ 8*	17

**Table 1.** Results at NF 62. Mean  $\pm$  SD. \*As 90% tadpoles never reached NF 62 in PTU exposed treatment, the actual Time to NF 62 mean is >71.

Phenotypic sex	Treatment	Weight (g)	SVL (mm)	LSI
Female	DWC	21.4 $\pm$ 7.1	53.8 $\pm$ 8.5	0.063 $\pm$ 0.009
	PTU 34 mg/L	7.5 $\pm$ 4.8	44.4 $\pm$ 7.5	0.031 $\pm$ 0.008
Male	DWC	21.7 $\pm$ 5.9	52.8 $\pm$ 8	0.061 $\pm$ 0.006
	PTU 34 mg/L	7.4 $\pm$ 5.1	39 $\pm$ 9.9	0.035 $\pm$ 0.004

**Table 2.** Results at test end. Mean  $\pm$  SD.

## Validity Criteria

Criterion	Acceptable limits	Achieved	Comment
<b>Dissolved oxygen</b>	$\geq 40\%$ ASV	$\geq 11$	<40 on 6 days
<b>Water temperature</b>	21 $\pm$ 1 °C spread $\leq 1^\circ\text{C}$	20.6 – 21.8 °C	Mean: 21.4 °C
<b>pH</b>	6.5 – 8.5, spread $\leq 0.5$ units	6.7 – 7.8	Exceeded
<b>Test chemical concentration</b>	$\pm 20\%$ of mean measured value	88 – 110%	Achieved
<b>Mortality in controls</b>	$\leq 20\%$ in each control rep	7 out of 8 reps	1 exceeded
<b>Viability in spawn chosen</b>	$\geq 70\%$	89%	Achieved
<b>Median time to NF 62</b>	$\leq 45$ days	42 days	Achieved
<b>Mean weight at NF 62</b>	1.0 $\pm$ 0.2 g	2.3 $\pm$ 0.4 g	See below comments
<b>Mean weight at test end</b>	11.5 $\pm$ 3 g	21.6 $\pm$ 6.4 g	

DWC replicate	Percent mortality	Median time to NF 62
A	0	42
B	5	44
C	5	43
D	10	42
E	35	40
F	5	42
G	10	42
H	10	42
<b>Pooled</b>	10	42

- DO, mean weights and pH criteria all considered to be directly linked to feed/tank size/flow rate/cleaning.
- Mortality outside of criteria only in 1 of 8 replicates. No possibility to exclude 1 control replicate according to guideline.
- Temperature spread ( $\leq 1^\circ\text{C}$ ) criteria not met by 0.2 °C, an investigation is currently pending to improve this criteria.

## Technical considerations

- The transition phase lasted 46 days as 90% of PTU exposed tadpoles never metamorphosed. The TG does not indicate how unmetamorphosed individuals should be dealt with.
- DO drop after each feed during larval phase. 50% water siphoning twice daily and tank scraping every other day to maintain DO >40%, even after airlines added. Guideline feed promoted microbial growth impacting water quality, more so than Sera Micron® which is used for OECD 231. Water quality issues during larval phase linked to feeding guidelines induced high scoliosis frequency.
- Weekend feeding guidelines (1 feed of equal amount as 3 feeds) were not implemented due to welfare concerns. Welfare concerns regarding tank vessels size recommendations (4 – 10 L, equivalent to 1 juvenile frog per 0.4 – 1 L at test end). We recommend using 10 – 15 L tank vessels and adding enrichment when possible.
- Validity criteria related to weight at NF 62 and test end not achieved, believed to be due to guideline recommended feed and feeding regime. We recommend weighing juvenile frogs regularly and adjust feed to pass weight validity criteria.

## Conclusion

- Ethical concerns over number of animals used with reference to number of failed tests based on strict validity criteria
- Feeding guidelines need refining with additional recommendations for water quality management. We plan to run a feeding trial to fine tune the assay using Sera Micron® with focus on improving the water parameters.
- The TG refers to updating methods in the future based on lab experience after running the test – this should now be considered



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