Comparative vitellogenin expression in two alternative fish models using 17-α Ethynylestradiol

Ciaran Dummett[1], Matthew Baron[1*], Theodore Henry[2], Awadhesh Jha[1]

[1]School of Biological Sciences, Plymouth University, Plymouth, PL4 8AA
[2]Centre for Marine Biodiversity & Technology, Heriot-Watt University, Edinburgh, EH14 4AS

[*]matthew.baron@plymouth.ac.uk

Introduction

- Determining realistic organotypic responses in alternative testing methods (*in vitro* and *in vivo*): an important step towards reducing animal numbers required for chemical testing
- Zebrafish larvae (*D. rerio*) and 3-D rainbow trout liver spheroids (*O. mykiss*): two proposed alternative models in line with the objectives of the 3Rs
- Spheroids well established in mammalian toxicity studies, but novel in fish **[1]**. They can be used in acute exposures (≤ 96h), yet demonstrate potential for use in chronic toxicity and bioaccumulation studies
- Vitellogenin (Vtg), a biomarker in environmental toxicity testing is used to assess elicit estrogenic effects on aquatic organisms

Aims & objectives

- Validation of RNA extraction, reverse transcription and qPCR techniques for small tissue samples (liver spheroids) utilising existing methods developed for zebrafish larvae
- Optimise primers (housekeeping & target gene) for liver spheroids
- Develop and optimise exposure protocol for liver spheroids
- Optimise and validate exposure concentrations of 17-α Ethynylestradiol (EE2)
- Compare Vtg gene expression in both model systems over a time-course exposure (24-72 h)

Materials & methods

In situ trout liver perfusion; hepatic suspensions prepared

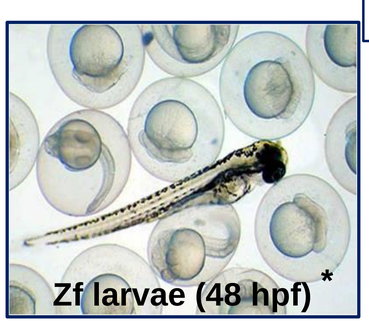
Cells cultured under gyratory-mediated conditions for 8 d to form spheroids **[1]**

Zebrafish eggs collected, cleaned and water quality monitored

Larvae collected at 48 h post-fertilisation

Transfer to respective exposure vessels (6-well micro-plates – spheroids; Pyrex® beakers – zebrafish larvae

Exposure to EE2 (24 - 72 h)



RNA extracted, counted and diluted appropriately

Reverse Transcription of RNA; cDNA counted and diluted appropriately

qPCR

Fig. 1. Experimental design for EE2 exposure and Vtg gene expression determination in liver spheroids and zebrafish larvae. * Image used with permission from Reinardy et al. 2013

- β-Actin 1 & 18S rRNA primers tested in both model systems for suitability as housekeeping genes. Zebrafish and rainbow trout liver Vtg primers optimised in each system
- Preliminary experiments determined appropriate exposure vessels for liver spheroids (glass beakers; 6, 24 & 96-well micro-plates)

Results & discussions

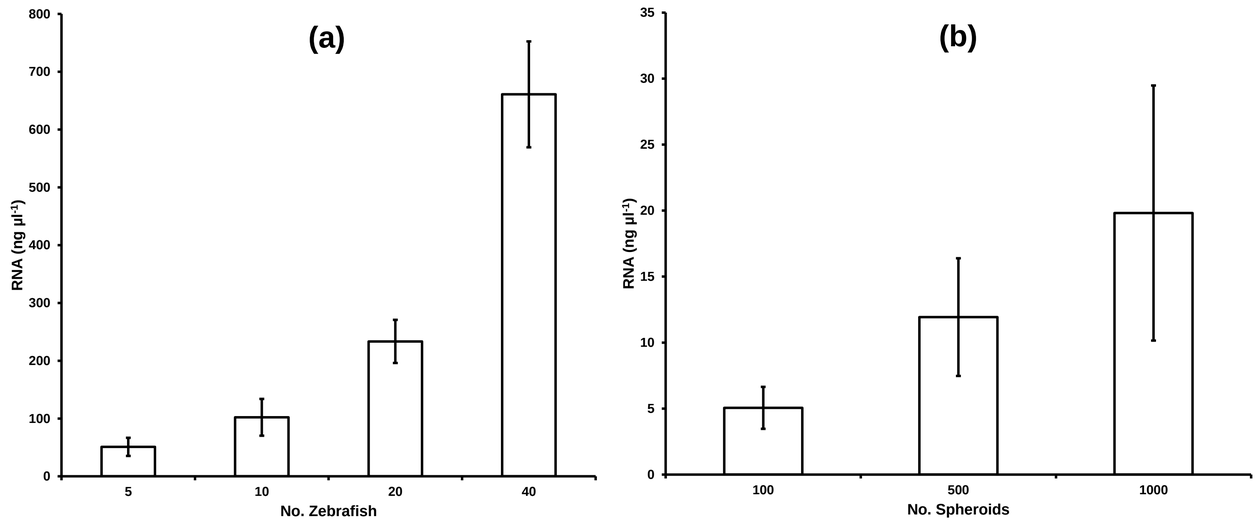


Fig.2. RNA counts from **(a)** zebrafish larvae (48 hpf) and **(b)** liver spheroids (8 d) at different seeding densities. Values expressed as mean ± SD (*n* = 5 beakers - zebrafish larvae; *n* = 7 wells; liver spheroids)

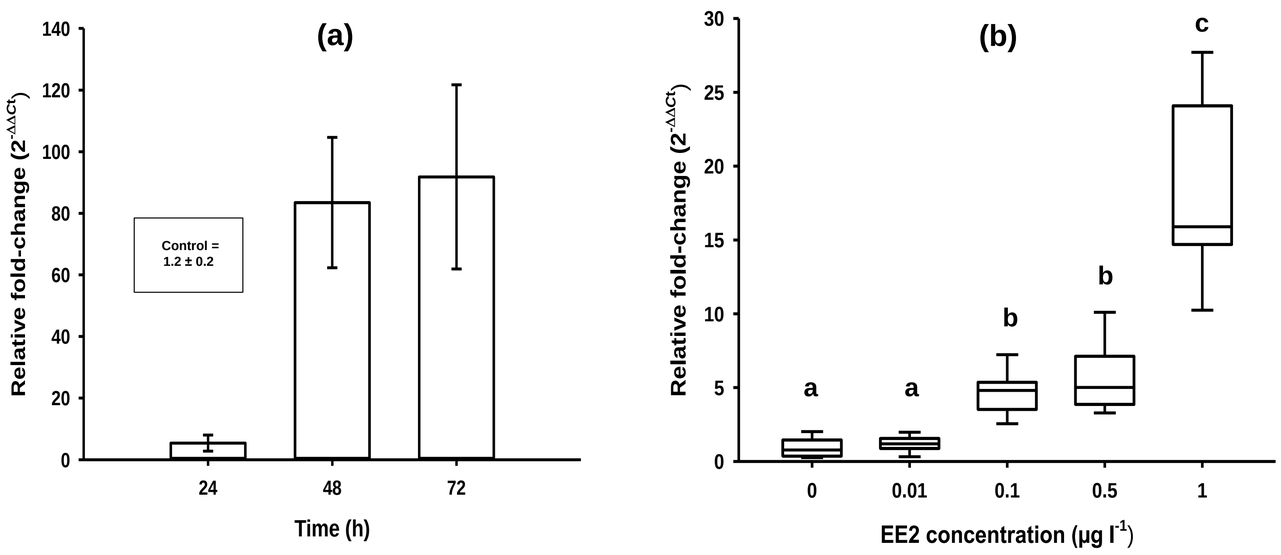


Fig.3. Vtg expression in **(a)** Zebrafish exposed ≤72 h to 0.5 µg l^{-1} EE2. Fold-change values for solvent controls were pooled (no significant difference determined between time-point experiments). Values at each respective time-point were significantly different to the solvent control ($P < 0.05$). **(b)** Trout liver spheroids exposed for 96 h to EE2 (≤ 1 µg l^{-1}). Values that do not share a letter are significantly different ($P < 0.01$)

- 18S rRNA was chosen as housekeeping gene for spheroids as the expression of β-Actin 1 was not stable
- Exposures in 24, 96-well micro-plates and glass bijous resulted in non-viable cultures. Spheroids exposed in pHEMA-coated 6-well micro-plates were viable for the entire exposure period (≤96 h)
- 30 (zebrafish) and 1000 (spheroids) were used for subsequent exposures based on RNA counts
- Vtg expression at 24 h in zebrafish larvae after exposure to 0.5 µg l^{-1} EE2 and detected after 96 h in liver spheroids (0.1 µg l^{-1})

Further work

- Further validation of liver spheroid Vtg expression over time-course and comparison with zebrafish
- Determination and comparison of cytochrome P450 1A expression in both model systems

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