A novel method for measuring catecholamine secretion levels in larval zebrafish (*Danio rerio)*

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Introduction

The stress response is a critical survival tool for almost all living organisms. Teleost fish respond to stressors by releasing catecholamines (i.e. adrenaline, noradrenaline and to a lesser extent dopamine) into the blood. These hormones are one of the key components in eliciting the "flight or fight" response of teleost fish as they are the endogenous ligands for ubiquitous adrenergic receptors (Steele 2011). The first aspect of this response consists of a neural component, via the sympathetic nervous system which stimulates the chromaffin tissue (teleost homologue to the adrenal medulla in mammals). This leads to increased catecholamine secretion into the blood. Catecholamine secretion is also regulated by a variety of humoral factors including angiotensin II, cortisol and vasoactive intestinal polypeptide (VIP). These hormones work in conjunction to produce the typical cardiorespiratory and metabolic effects known to comprise the stress response (Kastenhuber 2010). Since catecholamine secretion is the critical afferent limb of the acute adrenergic stress response, studies examining the stress response rely on measurements of circulating catecholamine levels. Such measurements are readily performed on large adult fish from which blood samples can be easily withdrawn. However, measuring circulating catecholamines in small or larval fish is not yet technically feasible.

Hypothesis

The objective of this project was to test a method for assessing the acute adrenergic stress response in larval zebrafish (*Danio rerio) by* measuring the appearance of catecholamines in the water. This was based on the tissue permeability properties of the larvae potentially permitting the diffusion of catecholamines into the surrounding environment.

Methods

- Fertilized zebrafish eggs were collected at day one and housed in petri dishes for four days.
- 200 larvae were placed into 300µl of dechrominated Ottawa tap water in a 1.5ml eppendorf tube.
- Fish were stressed by achieving hypoxia by gently aerating tubes with pure N_2 for 40 minutes in a 28° C flowing water bath at.
- Normoxic control followed the same procedure except atmospheric air was aerated into the water instead of N_2.
- N_2 and air flow were regulated by Cameron instruments GF3/MP Gas mixing flow meter.
- Post-aeration the larvae placed on ice and the supernatant was extracted.
- An internal standard of 2,3-Dihydroxybenzoic acid (DHBA) was added to each tube.
- Samples were purified using a modified version of a catecholamine extraction protocol as previously described (Woodward 1982)
- The main modifications of the protocol are the elimination of sodium bisulphite and the use of supernatant instead of fish homogenate.
- Extracts were measured using a Varain Prostar 410 HPLC (High Pressure Liquid Chromatography) with one of two Ultratechsphere ODS 5µ, 150mm columns coupled (X or Y) to an Antec Decade II electrochemical detector.
- 100µl of each individual extract was injected in sequence along with the corresponding standards using a Varian Prostar 410 auto sampler
- Electrochemical detector was ran at 2Na range, with a flow rate of 1ml/min and a pressure of either ~900 or ~1300 psi.
- All samples were injected using Cat-A-mobile phase with 0.1mg/ml 1-octanesulfonic acid as a delaying agent.

Fig 1:

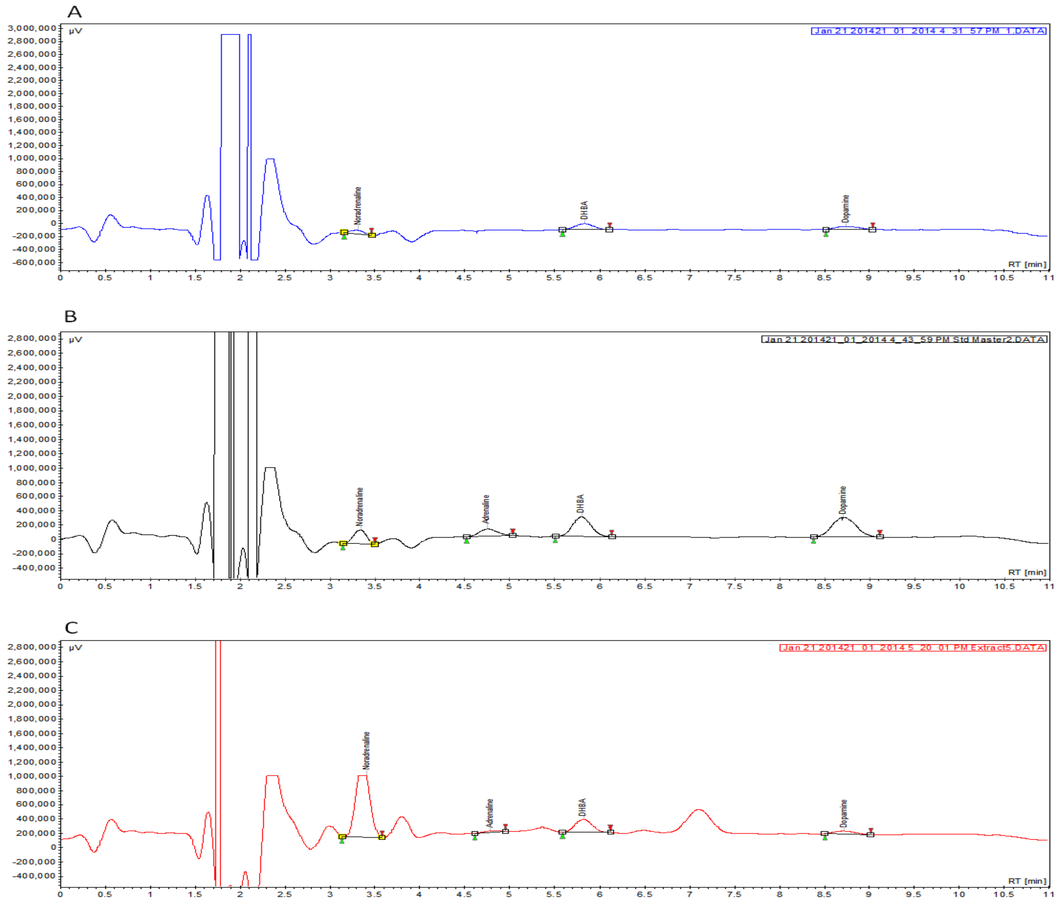
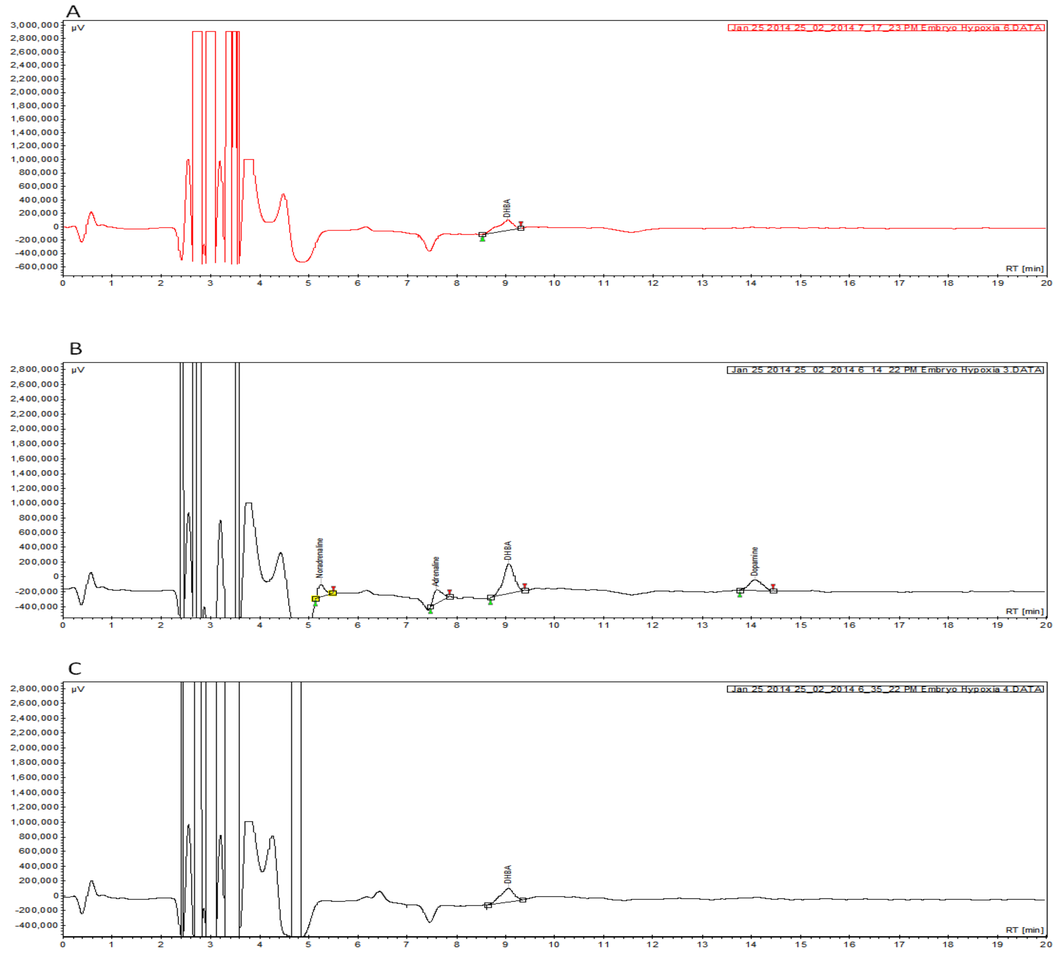


Fig. 2:



Results

	Noradrenaline (nmol/L)	Adrenaline (nmol/L)	Dopamine (nmol/L)
Normoxia	0.00	0.27	0.00
Hypoxia	104.73	1.55	4.64

Table 1: Catecholamine concentrations in 100µl of holding water after exposure of zebrafish larvae to hypoxia (N = 1) or Normoxia (N=2). Each individual graph is normalized using DHBA as an internal standard. The concentrations were averaged using unbiased arithmetic means.

Conclusion

This work provides preliminary evidence supporting detectable increase of noradrenaline in the water after hypoxic exposure when compared to normoxic conditions. Although the sample size is still too small to draw any concrete conclusions, this method does indicate that catecholamine release into the water can be detected using an HPLC. Some future stages of development would include comparing the net body homogenate catecholamine levels of the larvae both pre-experimentation and post-experimentation. This data would allow us to determine the average increase in blood catecholamines in the larvae, which if coupled with the supernatant concentration data pre and post experiment would allow the determination of the permissiveness of zebrafish larvae bodies to these hormones. To further determine tissue permissiveness, larvae could be allowed to soak in high catecholamine solutions and then assess the whole body catecholamine content to provide an alternative experiment to solidify the exact level of permissiveness. Once this is known, the environmental water could be used to determine the exact rise in catecholamines in the larvae due to a particular stressor to a very precise range (quite possibly the picomolar range). This would be a highly useful tool to allow the measurement of stress response in fish that are too small to sample for blood, allowing an early stress assessment method. This method could be further expanded to determine the permissivity of larvae at various stages of development, enabling an even greater breadth of applicability.

Figures:

Figure 1: HPLC graphs with µV shifts as a function of run time (min) for a blank (A), known standards (B) and Hypoxia extract (C). The area cordoned off underneath each peak represents the relative amount of catecholamine corresponding to the peak label. All runs A-C come from the same sequence on the Ultratechsphere column X.

Figure 2: HPLC graphs with µV shifts as a function of run time (min) for a blank (A), known standards (B) and Normoxia extract (C). The area cordoned off underneath each peak represents the relative amount of catecholamine corresponding to the peak label. All runs A-C come from the same sequence on the Ultratechsphere column Y.

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