Detection of Local Steroidogenic Enzyme Expression in Brown Anoles (*Anolis sagrei*)

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Introduction

The endocrine system in vertebrates responds to stress by releasing steroid hormones, mainly glucocorticoids (GC), which increase blood glucose levels to supply key organs and muscles with energy needed for survival (Sapolsky et al., 2000). Steroid hormones are synthesized via an enzymatic pathway that converts cholesterol into either GCs, androgens, or estrogens in a step-wise manner (Figure 1). The adrenal cortex is known to produce GCs, but evidence suggests that individual organs can also produce steroid hormones *de novo* in response to stress. This study aims to quantify gene expression of four steroidogenic enzymes, encoded by CYP19A1, CYP17A1, StAR, and HSD17ß3 genes, via qRT-PCR to determine if the stress of fasting in *Anolis sagrei* (brown anole) increases local steroidogenesis in comparison to fed organisms. RNA extraction protocol and CYP17A1 and StAR primer concentrations for qRT-PCR were optimized, paving the way for successful quantification of steroidogenic enzyme gene expression in the brown anole.

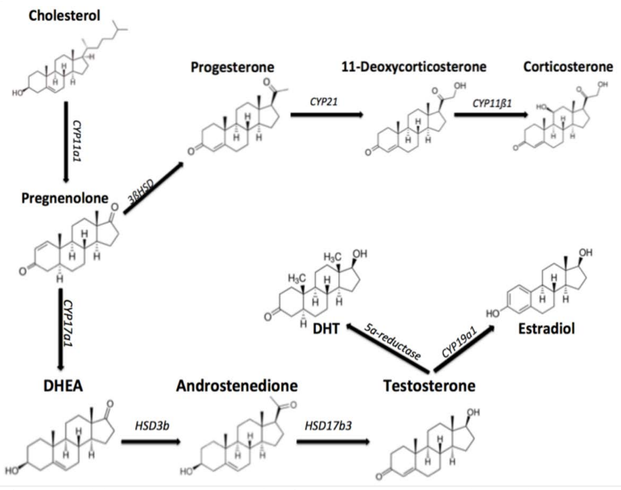

Figure 1. Synthesis of steroid hormones results from an enzymatic pathway converting cholesterol into androgens and estrogens. Gene names of enzymes are shown in italics and hormones or precursors are shown in bold. Enzymatic reaction substrates and products structures are shown.

Methods

RNA Extraction Optimization

- Brown anole tissue samples in RNAlater solution were homogenized in Trizol (1mL/50mg tissue, Thermo Fisher Scientific) at 5500 rpm for 20 sec with 2.3 mm zirconia/silica beads.
- RNA was extracted from tissues according to manufacturer's instructions with the addition of a centrifugation step (12,000 x g for 15 min at 4ºC) after the initial centrifugation.
- RNA integrity was tested via gel electrophoresis on a 1% bleach agarose gel.

Primer Specificity Testing via RT-PCR

- RT-PCR reactions were run with each primer set to amplify target genes (StAR, CYP17a1, CYP19a1, HSD17ß3, and ß-actin as a control).
- RT-PCR reactions were performed using OneTaq One-Step RT-PCR kit (NEB) according to manufacturer's instructions.
- PCR products were analyzed via gel electrophoresis on a 2% agarose gel.
- PCR products were sequenced to confirm amplification of target genes.

Primer Optimization for qRT-PCR

- RNA extracted from brown anole brain tissue was reverse-transcribed to cDNA using Oligo$(dT)_{20}$ primers.
- Various ratios of forward: reverse primer concentrations were tested via qRT-PCR for CYP17a1, StAR, and ß-actin genes to determine the ratio that produced amplification with the lowest Ct values and low levels of primer dimers.
- For ß-actin, 1:1, 1:7, 7:1, 7:7, 8:1, and 9:1 forward: reverse primer ratios were tested.
- For CYP17a1 and Star, 1:1, 1:7, 7:1, and 7:7 forward; reverse primer ratios were tested.
- qPCR data was analyzed via Step-One Plus Expression Suite software.

Results

RNA Extraction Optimization

Optimization of Trizol RNA extraction protocol resulted in high-yield, pure RNA from brain tissue, as compared to original protocol (Figure 2).

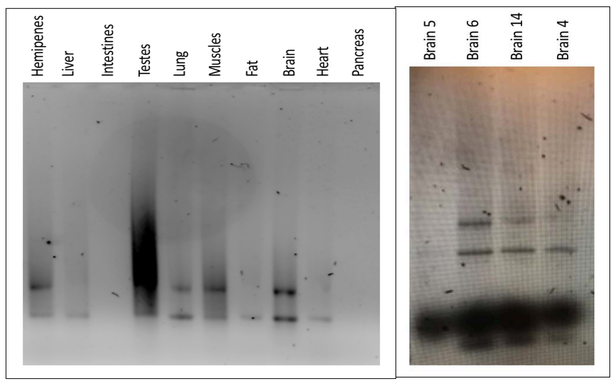

Figure 2. RNA extracted from brown anole brain tissue using optimized protocol (right) had much less contamination than samples extracted before procedural changes (left). Equal volumes of RNA were run on 1% bleach agarose gels

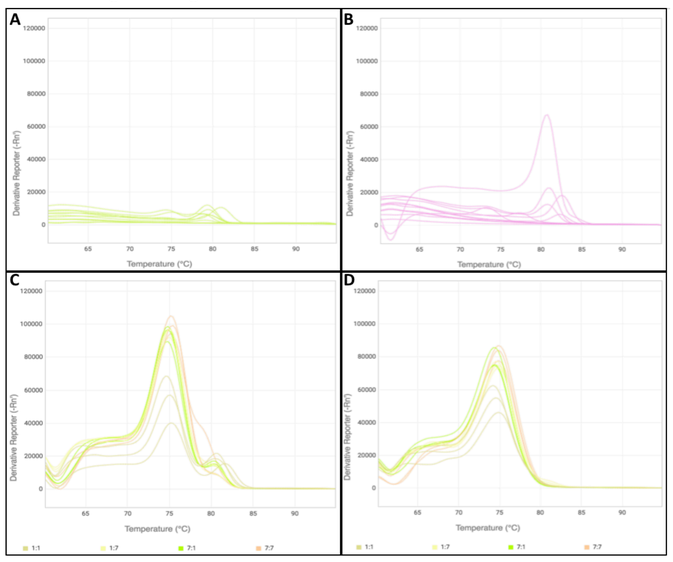

Figure 4. Melt curves from qPCR reactions using CYP17a1 and StAR primers (various concentrations) showed no contamination of NTC (A /B) and little to no primer-dimerization in template-containing reactions (C/D). Two-step qRT-PCR with standard conditions, using RNA from brown anole brain tissue, was used to amplify CYP17a1 and StAR genes. A) CYP17a1 NTCs had almost no amplification. B) StAR NTCs had small peaks around 80ºC. C) CYP17a1 template reactions had a peak around 75ºC. D) StAR template reactions had a peak around 75ºC.

Primer Specificity Testing via RT-PCR

RT-PCR using brown anole testes RNA successfully amplified CYP19a1, StAR, and ß-actin genes (Figure 3). Amplification of CYP19a1 and HSD17ß3 was unsuccessful. Sequencing confirmed the successful amplicons were homologous to target genes.

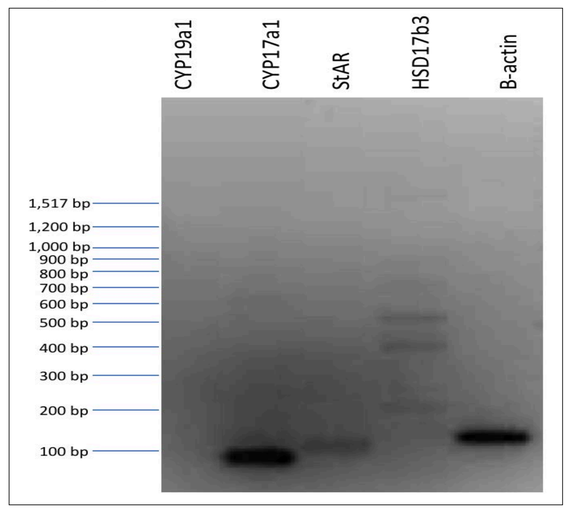

Figure 3. RT-PCR reaction with CYP17a1, StAR, and ß-actin specific primers produced amplicons of the expected sizes, while CYp19a1 was not amplified and HSD17ß3 primers produced multiple amplicons. Expected amplicon sizes: CYP17a1 (87 bp), StAR (112 bp), ß-actin (131 bp).

Primer Optimization for qRT-PCR

Optimal forward: reverse primer concentration ratios were found for CYP17a1 and StAR primer sets, with 7:7 producing the lowest Ct values and least amount of primer dimers for both target genes (Figure 4).

Discussion

While this study did not quantify gene expression of steroidogenic enzymes in brown anole tissue, the optimizations performed and imperfections detected in experimental design are essential for future research on local steroidogenesis. Addition of a centrifugation step and more conservative use of Trizol reagent in RNA extractions led to RNA free from phenol contamination, which improves the accuracy of concentration determination, an essential step in quantification via qRT-PCR. Primer design was also analyzed and it was determined that the use of degenerate primers may be the best option for designing primers for the brown anole genome, as this genome has not been sequenced or mapped. Finally, primer concentrations were optimized for the CYP17a1 and StAR primer sets, though not for the endogenous control ß-actin. This showed that qRT-PCR conditions were optimal and the primer sets for these two target genes seem to be sufficient for the brown anole template cDNA. Future studies on local steroidogenesis using these optimizations and findings could lead to the discovery of the mechanisms of this phenomenon, which could have clinical implications for diseases caused by malfunctions in or lack of steroid hormones.

Acknowledgments

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