Vacation scholarship report

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INTRODUCTION

The zebrafish (*Danio rerio*) is an important vertebrate laboratory model organism and is extensively used in many areas of research. Despite its wide use in research there still remains insufficient knowledge regarding the processes underlying zebrafish sexual development. Unlike mammals many vertebrate species including fish, do not have sex chromosomes and the genetic factors determining their sex are unknown. Epigenetic events are thought to play a number of key roles in the sex determination and differentiation pathways, through various levels of gene regulation including DNA methylation and histone modifications. In vertebrates, DNA cytosine methylation silences gene expression by interfering with the binding of specific transcription factors and through the recruitment of repressive chromatin machinery.

The aim of this project was to investigate the relationships between epigenetic DNA methylation patterns and sex differentiation, focusing on key liver and gonadal genes: *anti-müllerian hormone* (*amh*); *vitellogenin* (*vtg*); *estrogen receptor 1* (*esr1***)** and *aromatase* (*cyp19a*).

METHODS

In order to investigate the possible link between the methylation of candidate gene promoters, mRNA levels and sex, genomic DNA and RNA were isolated from liver and gonad tissue samples (4 adult male and 4 adult female zebrafish) using the TRI reagent method. In each sample the concentration of nucleic acid was verified using spectrometry before converting all unmethylated cytosines within the genomic DNA to uracil by sodium bisulfite conversion using Qiagen EpiTect kit. The bisulfite converted DNA was then amplified by methylation specific PCR (Promega Gotaq) using specifically designed methylation and unmethylation primers. Gel electrophoresis was used to visualise and to obtain both sex and gene specific DNA methylation profiles.

Real time quantitative PCR (RT-QPCR) was carried out on the mRNA obtained from liver and gonad tissue samples to calculate the relative *amh, vtg, esr1* and *cyp19a* gene expression by comparison against the expression of the control gene *rpl8*. Any potential DNA contamination was removed from the liver and gonadal mRNA samples by DNase exposure. The mRNA was then reverse transcribed into cDNA and used in RT-QPCR to obtain relative gene expression profiles for each of the candidate genes.

RESULTS AND DISCUSSION

1A.

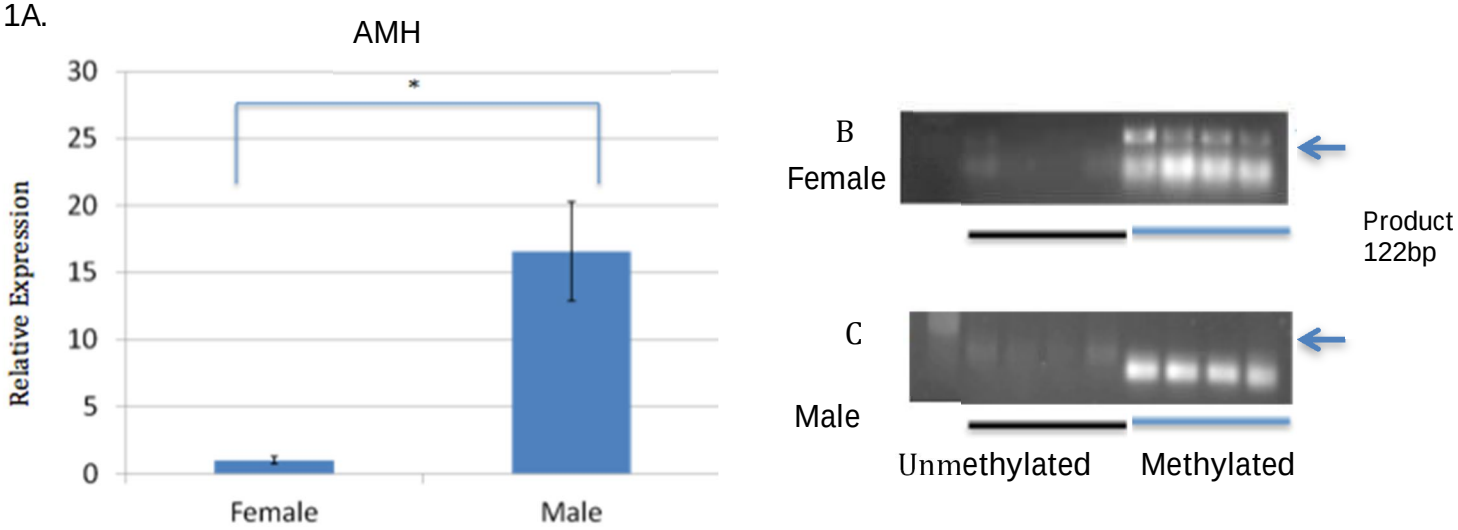

Fig. **1** – A - The relative level of *amh* gene expression measured in tissue collected from male zebra fish testis and female ovaries by RT-QPCR. *p*-value = 0.006 < 0.05 using an unpaired two-tailed T-test. (**P*<0.05).**B** and **C -** Methylation-specific PCR (MSP) analysis of genomic DNA from male and female gonadal tissue samples. Primers specific for the methylated *amh* and unmethylated promoters both amplify **a** band that is 122 bp.

Anti-müllerian hormone (amh) inhibits the expression of aromatase in developing gonads and functions as a negative modulator of leydig cell differentiation and function by down regulating several enzymes involved in the steroidogenic pathway. Methylation analysis of the *amh* promoter region indicates a sex related difference in methylation of the *amh* promoter region. The gene *amh* was shown to be highly expressed (fig.1A) and unmethylated in samples taken from the male testis (fig.1C), where as in female ovary methylation of the *amh* promoter region (fig.2B) correlated with either low or undetectable levels of mRNA expression levels(fig.1A).

There is a statistical difference between male and female zebrafish *amh* gene expression since *p*-value = 0.006 < 0.05 using an unpaired two-tailed T-test. (**P*<0.05). These results support the indication that *amh* is a key gene involved in reproductive functioning and in sex determination and differentiation in zebra fish. It also highlights DNA methylation as a key regulatory mechanism, regulating the spatial and temporal expression of specific genes.

Although MSP methylation profiles were obtained for *cyp19a*, *esr1* and *vtg* in gonadal tissues, differences in methylation were not reliably distinguishable. The lack of visual methylation differences of the candidate genes between male and female samples may be due to the heterogeneity of cells within gonadal tissues, which may have influenced the DNA and RNA profiles within the samples as well as the low accuracy of the gel electrophoresis methodology used in this project. As a result of these challenges associated with gel electrophoresis based DNA methylation analysis the research team in now continuing this work using pyrosequencing-based methylation detection and analysis.