

Differential Evolution of Regulatory Gene Networks in the Sex Determination of Turtles

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Abstract

Sexual reproduction in vertebrates requires the development of separate male and female sexes. Different species employ a variety of mechanisms used in sex determination. In turtles, and many other reptiles, the sex can be determined by the genotype (GSD) or environmentally (TSD) depending on the species. Here, we measured the expression of genes involved in sex determination in painted turtles incubated under fluctuating temperature conditions. Yolk and embryos were sampled at different developmental stages leading up to the temperature sensitive period. The comparison of our data with data from constant temperature support the hypothesis that *Sfi1* and *Wt1* might open the thermosensitive period in turtles.

Background

What if the gender of your child could be determined by how warm your heat your home? In some species of reptiles, including turtles, the temperature of the nest does impact the sex ratio of the offspring.

The long-term objective of this research is to investigate how and why sex can be determined by environmental temperature (ESD or TSD) in some species of turtles and by the genotype of sex chromosomes (GSD) in other species of turtles. "Sex determination is the irreversible commitment to the male or female developmental fate, which can be initially triggered by genetic factors such as those contained in sex chromosomes (GSD) or by environmental factors (ESD such as temperature (TSD)" (Valenzuela 2008).

This can be investigated by manipulating the nesting temperatures of both TSD and GSD turtles to see if there are differences in the amount and type of mRNA being produced during different developmental stages leading up to the thermo sensitive period (TSP) that determines if the developing gonad becomes an ovary or a testis. According to Valenzuela and Lance (2004), shift incubation experiments have revealed that temperature affects sex determination during a specific window of time during embryonic development (TSP), where as Fig. 1 shows a predetermined sex in GSD" (Valenzuela 2008). The gene pathway used in sex determination of in vertebrates are initiated by *Sfi1*

mRNA from different genes that are active at any given time and tissue can be collected and used to generate a stable DNA copy of them (cDNA) which can be measured using a technique, called Quantitative Polymerase Chain Reaction (QPCR). The number of PCR cycles is used to measure the quantity of RNA present at that stage, which helps us determine which genes were most active (produced more mRNA) at that stage and which genes were activated to initiate the thermosensitive period in TSD turtles.

By comparing painted turtles (TSD) with softshell turtles (GSD), the results of this experiment will help scientists understand how the regulatory gene networks differ among TSD and GSD turtles. This is crucial in understanding how the genome of turtle populations can be affected by ecology and to assist in developing turtle conservation strategies.

Fig. 1. Displays differences in Sex Determination among GSD and TSD Turtles during Development (modified from Valenzuela 2008).

	→ Life Cycle Development Over Time →		
Embryonic Stage	Stage 1-9	Stage 10-14	Stage 15 to Birth
Sex Determination in Turtle Species	Fertilized Zygote	Gastrulation	Gonad Differentiation and Development
GSD Soft Shell	XX or XY	XX or XY	XX or XY
TSD Painted	No sex ID	Temperature delays gonad differentiation.	

Objectives

- To investigate differences in gene expression as a result of manipulating incubation temperatures on painted turtles (TSD).
- To identify which gene is expressed to activate the thermo-sensitive period for sex determination in turtles.
- To explore the response of gene expression during development to fluctuating incubation temperature.

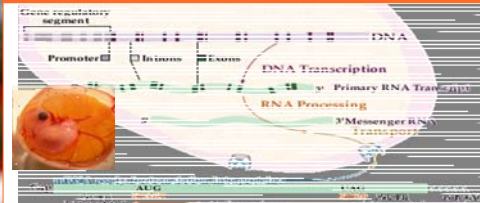
Materials and Methods

- Use the protocols established by Valenzuela (2008) to collect, label, randomly distribute, and incubate eggs from *Chrysemys picta*, the common Painted Turtle used as a model organism for TSD.
- Incubate eggs in treatments of female control 31, male control at 26, natural male, natural female, 26+/-3, 26+/-5, 31+/-3, and 31+/-5 (Valenzuela 2008).
- Collect yolk and embryo samples into 1 mL microfuge tubes using a sterile technique that minimizes RNA breakdown, previous to and during the TSP at stages 9, 12, 15, 18, and 21 (Valenzuela 2008). The yolk samples were preserved with tri reagent and embryos were preserved with RNAlater and both were frozen.
- Retrotranscribe mRNA from the embryos to synthesize stable cDNA (Valenzuela 2008). This reverses the transcribed mRNA found in the embryo back into stable copy DNA. Then perform quantitative fluorescent PCR in real time on the cDNA to measure the amount of RNA that was expressed of the six genes: *Wt1*, *Sfi1*, *Sox9*, *Dax1*, *Dmrt1*, and *Aromatase* during the different embryonic stages. The number of PCR cycles needed to bring the sample above a threshold where fluorescence is measurable is correlated with the amount of gene expression. The more cycles indicate that few initial copies of mRNA were present and thus that gene expression was low.

Fig. 2. Randomly distribution of eggs from *C. picta* into treatments at Dr. Valenzuela's lab at Iowa State University.



Fig. 3. Displays how mRNA can be used to measure the amount of gene expression in embryos. Image from www.ewa.cz/pages/1/813.htm



Results

A comparison of gene expression among the different developmental stages leading up to the thermosensitive period of the painted turtle found a significant difference in the expression of the *Wt1* and *Sfi1* genes at stage 9 prior to the thermosensitive period among the turtles incubated under fluctuating temperature conditions and constant temperature. In addition to this, painted turtles incubated at constant temperature showed an increased developmental rate.

Discussion

In TSD painted turtles, the thermo sensitive period is initiated in stage 9 by the expression of the *Wt1* gene that acts like a master switch turning on and off the *Sfi1* gene used to activate the thermo sensitive period in the TSD pathway for gonad development. The results indicate expression of both *Wt1* and *Sfi1* leading up to the thermosensitive period of embryonic development. Although it is found in stage 9, which is earlier than previously hypothesized, it could indicate that these genes are expressed to activate the thermosensitive period for sex determination in painted turtles.

It is suspected that *Wt1* is responsible for TSD and is also present in GSD turtles, but the mechanism it uses to stimulate *Sfi1* is inactive since *Sfi1* cannot activate the TSP in GSD turtles allowing their genotype to determine the sex. This suggests that GSD evolved after the TSD in turtles because the *Wt1* gene is vestigial in GSD turtles. Future research may study gene expression in GSD turtles.

"Heterochronic changes [Alberch et al., 1979] in the developmental timing of sex specification and determination and the overlap of sex differentiation with those steps in TSD taxa may confer developmental flexibility to the embryo, allowing it enough time to assess its environment before it commits to a sexual fate" (Valenzuela 2008). This adaptive "heterochronic change, defined as a developmental change in the timing of events, leading to changes in size and shape" (Wikipedia 2009) may allow eggs to develop female at female optimal temperatures and male at male optimal temperatures. The potential selection for sex phenotypic plasticity or flexibility acting at the individual and population level favors the evolution of a regulatory system of sexual development that enables the embryo to assess its environment and differentiate into the sex that will attain the maximum potential fitness given the external conditions [Charnov and Bull, 1977; Bull, 1984; Valenzuela, 2004].

However, we found that manipulating incubation temperatures on painted turtles with TSD we were able to identify differences in gene expression during developmental growth between turtles incubated at fluctuating and constant temperatures. In the painted turtles with TSD, the expression of *Wt1* and *Sfi1* was greater in treatments of fluctuating temperature conditions than constant temperature. This could suggest that development is a cumulative effect resulting required number of warm growth days and turtles experiencing fluctuating temperature receive less warm growth days. However, although the turtles incubated at constant temperature showed an increased developmental rate, they did not reach the thermosensitive period as quickly as turtles incubated at fluctuating temperature. One hypothesis is that temperature fluctuation varies the timing of the thermal sensitive period.

Additional studies could also look for factors such microRNA or micro-chromosome that could be regulating sex determination in turtles suggested in Martinez et al. (2008). Similarly, sex determination could also be affected by other variables studied by Radder et al. (2009) which identified genotypic sex determination reversal in lizards caused by altering yolk allocations as a possible means of altering evolutionary sex ratios.

Fig. 4 and 5. show the regulatory gene pathway used in vertebrate sex determination. Images from www.endotext.org and herkules.oulu.fi

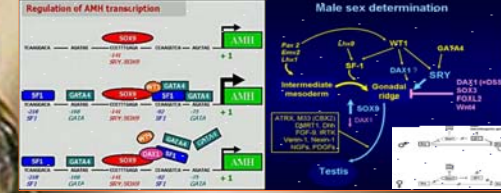


Fig. 6. Gene expression regulating TSD in male and female painted turtles at stage 12. Images courtesy of Valenzuela 2009.



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* Recommended online movies: www.pbs.org/wgbh/nova/miracle/program.html

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