

The Painted Turtle, *Chrysemys picta*: A Model System for Vertebrate Evolution, Ecology, and Human Health

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INTRODUCTION

Painted turtles (*Chrysemys picta*) are representatives of a vertebrate clade whose biology and phylogenetic position hold a key to our understanding of fundamental aspects of vertebrate evolution. These features make them an ideal emerging model system. Extensive ecological and physiological research provide the context in which to place new research advances in evolutionary genetics, genomics, evolutionary developmental biology, and ecological developmental biology which are enabled by current resources, such as a bacterial artificial chromosome (BAC) library of *C. picta*, and the imminent development of additional ones such as genome sequences and cDNA and expressed sequence tag (EST) libraries. This integrative approach will allow the research community to continue making advances to provide functional and evolutionary explanations for the lability of biological traits found not only among reptiles but vertebrates in general. Moreover, because humans and reptiles share a common ancestor, and given the ease of using nonplacental vertebrates in experimental biology compared with mammalian embryos, painted turtles are also an emerging model system for biomedical research. For example, painted turtles have been studied to understand many biological responses to overwintering and anoxia, as potential sentinels for environmental xenobiotics, and as a model to decipher the ecology and evolution of sexual development and reproduction. Thus, painted turtles are an excellent reptilian model system for studies with human health, environmental, ecological, and evolutionary significance.

BACKGROUND INFORMATION

The painted turtle, *C. picta*, (Fig. 1) (synonym: *Testudo picta* [Schneider 1783]) is classified as follows: kingdom Animalia, phylum Chordata, class Reptilia, subclass Anapsida, order Testudines, suborder Cryptodira, superfamily Testudinoidea, family Emydidae, subfamily Deirochelyinae (Bickman et al. 2007). Its common names include painted turtle (English), tortue peinte (Travis and French 2000), and tortuga pintada (Spanish). A full description of the biology of the painted turtle can be found in Ernst et al. (1994), Ernst and Barbour (1989), and an associated online resource, *Turtles of the World* (<http://nlbif.eti.uva.nl/bis/turtles.php>). A summary of the description and natural history of painted turtles is presented here.

Fossils of *C. picta* have been found in the midwest United States and eastern Canada, dating back to the late Miocene, Pliocene, Plistocene, and Holocene (for review, see Ernst et al. 1994). Currently, painted turtles are widely distributed in North America, from Nova Scotia to British Columbia, south to Georgia, west to Oklahoma, Colorado, Wyoming, Idaho, and Oregon. They also inhabit isolated areas in Texas, New Mexico, Arizona, Utah, and Mexico. Four subspecies are recognized, although their validity was recently reevaluated (Starkey et al. 2003): the eastern *C. p. picta* (Schneider 1783), the midland *C. p. marginata* (Agassiz 1857), the southern *C. p. dorsalis* (Agassiz 1857), and the western *C. p. bellii* (Gray 1831). Figure 2 outlines the current phylogenetic relationship hypothesis among species and subspecies of painted turtles. This freshwater turtle prefers slow-moving shallow water and



FIGURE 1. Adult *Chrysemys picta bellii*, Ames, IA, USA. (Photo by Nicole Valenzuela.)

is found in ponds, marshes, lakes, and creeks, particularly those containing soft substrate at the bottom along with abundant basking sites and aquatic vegetation.

The painted turtle karyotype consists of $2n = 50$ chromosomes, of which about half are macrochromosomes and half are microchromosomes (Stock 1972), with a total genome size of 2.6 pg (Shedlock 2006). *C. picta* exhibits sexual dimorphism, with males having elongated foreclaws and longer and thicker tails, while females reach larger body sizes. *C. picta* mature as a function of their body size more than their age. Males mature when they reach ~7-9.5 cm plastron length and females mature at ~9.0-16.5 cm plastron length (Ernst and Barbour 1989; Ernst et al. 1994). Courtship and mating occur mostly from March to mid-June but can extend to September (Ernst 1971a), and clutches may be sired by multiple males (Pearse et al. 2002). Females nest mostly from late May until mid-July in sandy or loamy soil in mainly open areas. Nests are approximately the size of a grapefruit and contain one to 23 eggs. Females may lay one to three clutches per year. Variation in these traits exists among subspecies and geographic locations. Eggs are typically elliptical in shape (~28-35 mm in length and ~16-23 mm in width), weigh ~4-9 g, and have a whitish, smooth, and flexible shell. The length of incubation varies from ~50 to 80 days depending on temperature; development proceeds at a faster rate at higher temperatures and is also affected by temperature fluctuations (e.g., Ernst 1971b; Les 2007). *C. picta* exhibits temperature-dependent sex determination, a mechanism that is common among reptiles (Valenzuela and Lance 2004). Eggs incubated at $\leq 26^{\circ}\text{C}$ produce exclusively males, those incubated at $\geq 30^{\circ}\text{C}$ produce exclusively females, and those incubated at $\sim 28^{\circ}\text{C}$ produce a balanced sex ratio (Bull and Vogt 1979; Ewert et al. 2004). Painted turtles are omnivorous but

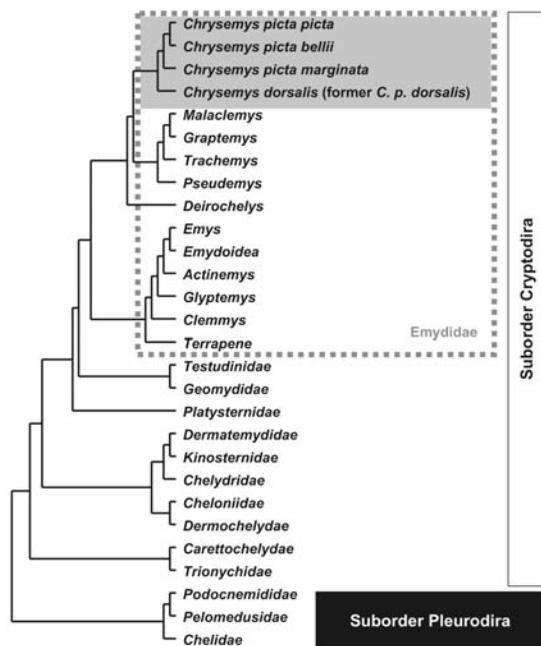


FIGURE 2. Current phylogenetic relationship hypothesis among species and subspecies of painted turtles (shaded area), and *Chrysemys* position in comparison to other genera within its family Emydidae (encircled by the dotted line), and to other chelonian families in both turtle suborders (Cryptodira or hidden-necked turtles, and Pleurodira or side-necked turtles). Based on data from Starkey et al. (2003), Near et al. (2005), and Iverson et al. (2007).

change from a carnivorous diet when young to a more herbivorous diet as they mature (Ernst and Barbour 1989; Ernst et al. 1994).

SOURCES AND HUSBANDRY

Painted turtles used for research are often collected from the wild using traps, although direct capture is also possible (Vogt 1980). Painted turtles are also available in the pet market and through biological suppliers, but the geographic origin of these turtles may be uncertain. Turtle eggs are commonly collected from natural nests in areas where females are observed nesting, preferably in open areas. Nesting marks are more easily detected within 24 h of oviposition. Eggs can also be obtained by oxytocin injection of females kept in captivity or trapped in the wild, at a dose of 1-4 U per 100 g of body weight (Ewert and Legler 1978). There is geographic variation in the biology of painted turtles; thus, the place of origin of eggs or individuals used for research may be important to know to enable comparisons of results to be made across studies.

As early as a within a day following oviposition, eggs develop an opaque spot or “white spot” on the top of the shell at the site where the vitelline membrane attaches to the inner shell membrane (Chan 1989). Once this white spot is visible, extreme caution should be taken to minimize egg movement. Keeping eggs with their white spot at the top helps avoid the mortality that results when the embryo is at the bottom of the egg where the yolk concentrates (Booth 2004). Eggs can be incubated at temperatures between 26°C and 31°C in boxes partially filled with moist substrate (e.g., sand with 4% water content, or medium to fine vermiculite set at –150 kPa, by adding 1.11 g of water per gram of vermiculite [Morris et al. 1983]). Eggs may be half or fully buried. Boxes should be rotated among shelves and positions inside the incubator to adjust for any thermal clines that may be present. Water should be replaced at least weekly to keep moisture conditions constant. Sterilized substrate, water, containers, and minimum handling should be used, and eggs lacking signs of proper development (e.g., the absence of a white spot, or lack of blood vessels when candled) should be removed to minimize fungal infection during incubation.

Hatchlings and larger individuals kept in captivity should be maintained at 24°C-27°C water temperature, and the aquaria or containers should be cleaned frequently to maintain water quality (see Johnson 2004 for general husbandry and medicine). Basking platforms and UV light sources must be provided to allow proper calcium metabolism. Commercial aquatic turtle food containing appropriate nutritional composition and pellet sizes for hatchlings, growing juveniles, and adults is available and can be fed to captive individuals.

RELATED SPECIES

Other turtles have been used extensively for research, particularly the slider turtle *Trachemys scripta*, which belongs to the same family, Emydidae, as *C. picta* and with which it shares many characteristics. Procurement of *T. scripta* eggs and individuals, as well as protocols for egg incubation and husbandry, are the same as for *C. picta*. Other species, such as the snapping turtle (*Chelydra serpentina*) and marine turtles, have also been used for developmental biology studies, and have the advantage of possessing larger clutch sizes (average of 20-40 eggs typically for snappers, and 50-110 for sea turtles). However, their much larger body sizes make it difficult to rear them in captivity. In addition, the aggressive behavior of snapping turtles makes it difficult to handle individuals. The threatened or endangered status of all sea turtles complicates using them for research. If snapper or sea turtle eggs are used, egg sampling and incubation follow the same protocol described above, except that sea turtle eggs can be successfully incubated between 25°C and 33°C with pivotal temperatures ranging from 29°C to 30°C (Girondot 1999).

USES OF THE *C. PICTA* MODEL SYSTEM

Painted turtles have been used to test evolutionary ecology models of life history trade-offs, such as those between offspring size and number (Rollinson and Brooks 2008). *C. picta* exhibits indeterminate growth, a trait that results in a higher reproductive output of females as they grow older, as reproductive frequency, egg size, and hatchling size within reproductive seasons increases with female age (Congdon et al. 2003). Female reproductive success also influences genetic population structure,

because gene correlations among individuals from nesting areas vary among years and are negatively correlated with the proportion of females that reproduce successfully each year (Scribner et al. 1993). The nesting ecology of painted turtles is very well documented (e.g., Congdon and Gatten 1989; Valenzuela and Janzen 2001; Morjan 2003; Rowe et al. 2005; Hughes and Brooks 2006).

The buoyancy control mechanism required by aquatic vertebrates has been investigated in painted turtles. In *C. picta* it involves management of lung volume and slight changes in stored water volume (e.g., Peterson and Gomez 2008 and references therein). Ecotoxicology studies suggest that, due to their ecology and life history, painted turtles might serve as model sentinels for environmental contaminants (e.g., Rie et al. 2005; Bergeron et al. 2007). For instance, some xenobiotics may cause deformities in *C. picta* embryos and adults (Bell et al. 2006), while compounds such as cadmium may reduce the proliferation of germ cells or delay their migration to the genital ridge during embryonic development and neonatal stages, thus potentially disrupting reproductive processes later in life (Kitana and Callard 2008).

OVERWINTERING

Painted turtles have served as a model system for a large body of research aimed at understanding the biology of overwintering, the process of surviving the unfavorable seasonal conditions of winter (e.g., Costanzo et al. 2004). Contemporary studies have focused on freeze tolerance (for review, see Packard and Packard 2004), the metabolic and gene expression mechanisms underlying this capacity (for reviews, see Storey 2006; Warren and Jackson 2008), and the cardiovascular control of hypoxia/anoxia, extending original cardiovascular research on turtles that began in the 19th century (for review, see Overgaard et al. 2007). Painted turtles remain submerged for extended periods while overwintering, aided by their extreme ability to tolerate anoxia. The physiological mechanisms responsible for this tolerance include metabolic depression, ion channel arrest, use of large glycogen stores, and extreme buffering capacity (for review, see Warren and Jackson 2008). Indeed, the painted turtle has become a model to understand anoxic survival in ectothermic vertebrates (Farrell and Stecyk 2007). The involvement of the liver in lactate buffering and metabolism is a unique feature of these turtles that prevents the dangerous dropping of pH in the body (Warren and Jackson 2008). Additionally, the shell and skeleton of painted turtles sequester lactate, and given the size of the shell, this amounts to a large portion of the lactate present in the body (Warren and Jackson 2008).

Subzero temperatures during overwintering present another physiological challenge for both hatchling and adult turtles (Costanzo et al. 2008). Unlike other organisms that employ colligative cryoprotectants as a defense against freezing, painted turtles use their high tolerance to anoxia and employ antioxidant defenses by up-regulating selected genes and proteins, such as those involved in iron sequestration, which allows them to undergo repeated freezing and thawing (for review, see Storey 2006). In addition, painted turtles possess a nonpolymerizing hemoglobin, a defense against freezing not found in other testudines (for review, see Reischl et al. 2007). Painted turtles preserve some of the highest cardiac muscle contractility during anoxia among vertebrates (Overgaard et al. 2007) and exhibit higher concentrations of myocardial phosphodiesterases than other less anoxia-tolerant turtles (Wasser et al. 1997). Clearing of any gastrointestinal contents prior to overwintering is viewed as an additional adaptation related to the supercooling capacity of painted turtle hatchlings (Packard and Packard 2006).

NEUROSCIENCE

The painted turtle has recently been used to study the physiology of the reptilian lymphatic system, which had previously received very little attention (for review, see Walker et al. 2008). Symmetrical lymph hearts, located on both sides of the spinal cord, appear to share neuronal control as indicated by their beating synchrony, which may be advantageous for reducing energy demands of fluid homeostasis (Walker et al. 2008). Painted turtles have also been the subject of neurological studies, including phylogenetic analyses of the structure of basal ganglia in vertebrates (for review, see Parent 1997). Studies indicate that glutamate receptors in the brain stem and cerebellum of painted turtles are distributed in a similar manner as in rats, and further that N-methyl D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors colocalize. Given that glutamate is a major neurotransmitter of the vertebrate central nervous system, these results not only have helped

reveal the organization of neural circuits that control motor behavior in turtles, but also highlight the potential common function of these neural circuits in vertebrates (Keifer and Carr 2000).

DEVELOPMENT AND SEX DETERMINATION

Painted turtles have become a model system to study the thermal ecology and evolution of temperature-dependent sex determination (TSD) in vertebrates, which is a common mechanism among turtles (Valenzuela and Lance 2004). Experimental analyses of the response of sex ratio to constant incubation temperatures described a female-to-male reaction norm with increasing temperature (i.e., TSD_{Ia}) such that values $\leq 26^{\circ}\text{C}$ result in 100% males, those $\geq 30^{\circ}\text{C}$ result in 100% females, and those $\sim 28^{\circ}\text{C}$ result in a 50:50 sex ratio (for reviews, see Bull and Vogt 1979; Ewert et al. 2004). Temperature, and hence sex ratio, in natural nests may be influenced by vegetation cover, although not predictably (Weisrock and Janzen 1999), and vegetation cover at nest sites is affected by female preferences that vary between populations (Morjan and Valenzuela 2001; Morjan 2003). Interestingly, the phenotypic consequences of female nesting decisions are not repeatable (Valenzuela and Janzen 2001), such that nest site selection is not likely the main target of selection for adaptive sex ratio manipulation (Escalona et al. 2009). Maternally allocated steroid hormones may affect the thermal response of the sex ratio in natural populations (Bowden et al. 2000).

Several aspects of the developmental biology of painted turtles beyond the effects of anoxia or temperature discussed above have been studied (e.g., Ewert 1985), including the description of normal embryonic development (Mahmoud et al. 1973). Phenotypic plasticity induced by environmental conditions experienced in the nest can significantly influence the phenotypes of painted turtle hatchlings. One example is soil moisture, which helps determine the mineral content (calcium and phosphorus) of neonates (Sternadel et al. 2006). Developmental studies have examined traits of phylogenetic importance for chelonians. For instance, cranial circulatory development in painted turtles is very similar to that of softshell turtles (*Apalone spp.*), and resembles that of adult *Trionychia*, suggesting that their divergent adult conditions may result from neotenic changes between clades (Jamniczky and Russell 2008).

More recent research in evolutionary developmental biology has explored the response to temperature during embryogenesis of the gene network underlying sexual differentiation in painted turtles (Fig. 3). These studies revealed two potential candidate genes (*Wt1* and *Sf1*) that might act as master thermal switches, alone or in combination, sensing the environmental temperature and activating the male- and female-differentiation pathways in a temperature-specific manner (Fig. 4; Valenzuela et al. 2006; Valenzuela 2008b). Additionally, comparing gene expression data from painted turtles to other turtles and vertebrates indicates that the fundamental difference between TSD and genotypic sex determination mechanisms is not driven by differences in *aromatase* expression (Valenzuela and Shikano 2007). This comparative approach also suggests that multiple TSD systems exist that differ in the molecular underpinnings of development at the level of the regulation of the gene network underlying sexual differentiation (Valenzuela 2008a).

GENETICS, GENOMICS, AND ASSOCIATED RESOURCES

Painted turtles are an emerging model system for evolutionary and ecological genomic research, aided by the increasing number of genomic resources available (Janes et al. 2008). Phylogenomic analysis using megabase-scale end-sequence scanning of the existing BAC library of painted turtles and sequences from other reptiles examined the genomic abundance, distribution, and phylogenetic structure of transposable elements of the family CR1-like LINE (Chicken Repeat 1-like Long Interspersed Nuclear Elements; Shedlock 2006). This approach showed that LINE and non-long-terminal-repeat (non-LTR) retrotransposon repeat types have become more abundant in reptilian lineages over evolutionary time, and also revealed that a nonlinear relationship exists between repeat diversity and basal metabolic rate (Shedlock 2006). Additionally, the pattern of retroelements and simple sequence repeats in reptiles is more similar to the mammalian than the minimalist avian pattern (Shedlock et al. 2007). Turtle, bird, and crocodylian genomes also exhibit distinctive phylogenetic lineages of CR1-like LINES and few AT-rich simple sequence repeats (Shedlock et al. 2007). End sequencing of the painted turtle BAC library was also used to develop primers for genetic analyses across chelonians to enable hypothesis testing about species delimitation in recent radiations (Shaffer and Thomson 2007).

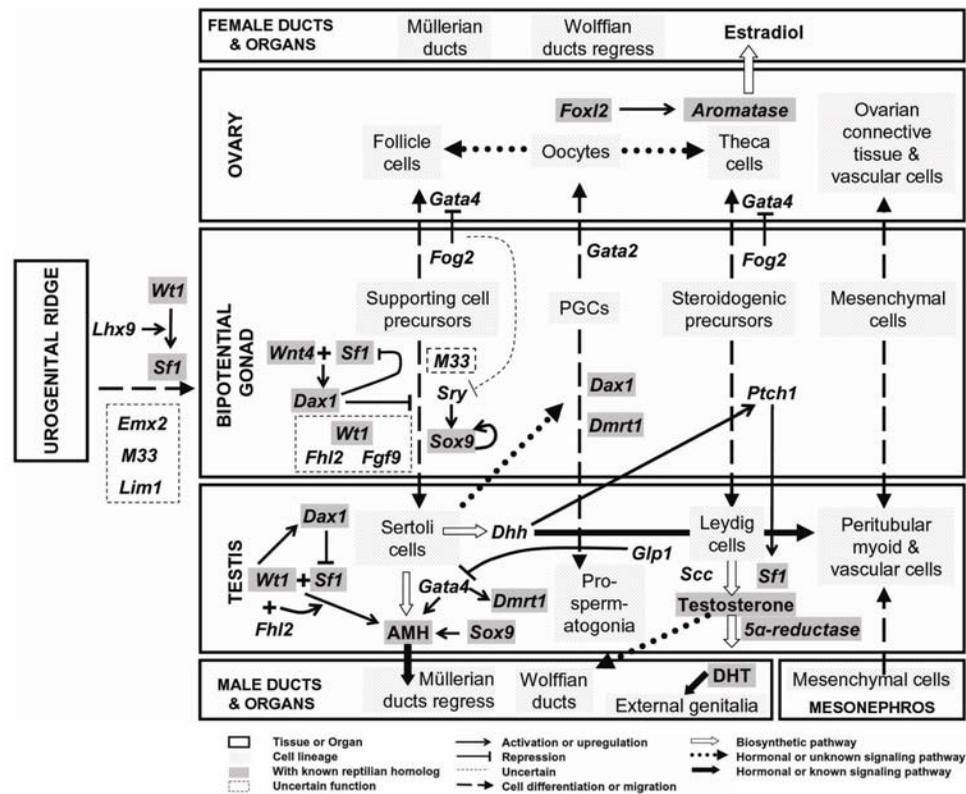


FIGURE 3. Gene network and cellular interactions underlying gonadogenesis in genotypic sex determination (GSD) mammalian model systems (data from Valenzuela 2008a; see references therein for additional sources). (Dark gray boxes) Network elements whose reptilian homologs have been identified. The list of elements of the gene network is not exhaustive. Key to symbols is found at the *bottom* of the figure. (Reproduced from Valenzuela 2008a, with permission from Oxford University Press © 2008.)

Genetic and genomic resources developed in other turtles can be used in painted turtles, but some significant efforts are rendering this one species an emergent model system. A BAC library was recently developed at the Joint Genome Institute, with 11.2X coverage and containing 210,816 clones with an average size of 143 kb. Details on the library are available at Genome Project Solutions (<http://genomeprojectsolutions.com/Chrysemys.html>) and Symbio Corporation (<http://www.symbio.com/>). Over 3460 BAC end-sequence reads generated from this library have been deposited in GenBank (CZ257443 to CZ253983) (Shedlock et al. 2007). This important resource will complement the upcoming sequencing project for the painted turtle (Janes et al. 2008). Additional genomic resources, such as EST and cDNA libraries, are currently being produced and will become available shortly. Other genetic resources include the sequenced mitochondrial genome (GenBank NC_002073) (Mindell 1999) and a variety of primers for the amplification of multiple mitochondrial and nuclear markers (e.g., Engstrom et al. 2007). From the nuclear genome, sequenced regions available in GenBank include various microsatellites, a series of genes and pseudogenes of the PTOR olfactory receptors, as well as full or partial mRNA sequences for *aromatase*, Wilm's tumor-associated gene (*Wt1*), RNA fingerprint protein 35 (*R35*), steroidogenic factor 1 (*Sf1*), the dosage-sensitive sex-reversal adrenal hypoplasia congenital on the X chromosome gene (*Dax1*), and the NCK-associated protein (*NCKAP*) gene. Protein sequence data available in GenBank include the above-mentioned genes plus the hemoglobin β , α -A, and α -D subunits, and the sperm protamine P1-type protein.

The use of genomic information from a wide array of species is proving useful in many areas of biomedical research. In particular, the National Institutes of Health, through the National Human Genome Research Institute, has emphasized the importance of using comparative genomic sequence analysis to understand the structure and function of the human genome and the biological processes at work in human health and disease (<http://www.genome.gov/17015353>). The strategy includes a mixture of whole genome sequencing, gene mapping, and sequencing of genomic regions, includ-

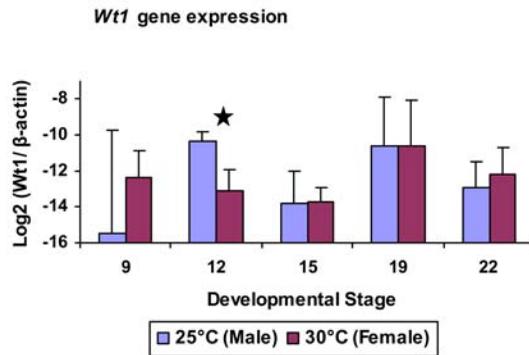


FIGURE 4. Developmental expression of *Wt1* in *Chrysemys picta* (TSD) (Modified from Valenzuela 2008b). Y-axis represents *Wt1* initial copy number normalized to β -actin (+ s.d.). Stages 15-22 correspond to *C. picta*'s thermosensitive period. (Star) Significant differential expression. See Valenzuela (2008b) for experimental details.

ing nonmammalian organisms, with the aim of deepening our understanding of human biology and evolution. Compared with mammalian embryos that are relatively inaccessible to experimental manipulation, nonplacental animal models offer distinct advantages that will improve our understanding of the biological processes important for human health, such as enabling the study of genes associated with human reproductive disorders. The painted turtle is an ideal emergent model system for such efforts.

TECHNICAL APPROACHES

Methods for incubating painted turtle eggs and collecting embryos for gene expression analysis are described in **Egg Incubation and Collection of Painted Turtle Embryos** (Valenzuela 2009).

For studies of gene expression related to sex determination, embryos should be collected at multiple developmental stages both prior to and during the thermosensitive period (TSP) (e.g., Valenzuela et al. 2006). To facilitate comparisons across studies, developmental stages of turtle embryos are determined according to the widely used criteria of Yntema (1968). For research aimed at characterizing and understanding variation among individuals, samples from individual embryos should be processed separately and analyzed without pooling them. RNA can be extracted from the adrenal-kidney-gonadal (AKG) complex or, whenever possible, from gonads and AK separately, using commercial kits (e.g. QIAGEN's RNeasy Kits), and digested with DNase I. RNA should be quantified using a spectrophotometer (e.g., NanoDrop ND-1000), and its quality should be assessed by the presence of ribosomal bands in agarose gels stained with ethidium bromide. Total RNA (e.g., 1 μ g per sample) may then be retrotranscribed with (dT)20 primers (or with gene-specific primers) using commercially available kits (e.g., Superscript III; Invitrogen). For samples that yield less than the desired amount of total RNA in the total eluted volume (e.g., 8 μ L), as much as the total eluate can be used for the reverse transcriptase-polymerase chain reaction (RT-PCR), and the total amount of RNA can be recorded and later standardized during the data analysis. Real-time PCR can be used to quantify transcript abundance of genes of interest using gene-specific primers designed from conserved regions of sequences available in public databases. Degenerate primers can be used as needed. Ideally, primers should span a region of 75-150 bp. A housekeeping gene, such as β -actin, should be used for normalization of gene expression. SYBR Green quantitative PCR (QPCR) mixes (e.g., Brilliant SYBR Green QPCR Master Mix; Stratagene) can be used for real-time PCR quantification of individual genes. ROX dye can be used as the reference dye for background correction. To quantify transcript abundance of unknown samples and to enable comparisons among plates and studies, generate standard curves using pure cloned DNA or PCR products of the gene of interest. Prepare serial dilutions from a known concentration and run them in duplicate in each QPCR to ensure technical repeatability of the results. Common samples should be run in all plates to test for plate-specific QPCR differences in amplification.

The initial template amount for each sample and each gene is calculated using standard-curve quantification. Multiple algorithms exist for this step, and some are incorporated in the software packages associated with the real-time PCR machines (e.g., Mx3000P; Stratagene). For comparison purposes and given the use of the standard curve, initial copy numbers can be calculated from the molecular weight of the fragments for each gene and standardized to a common initial amount of total RNA. Expression of target genes is then normalized using the housekeeping gene expression

data. It is important to log₂-transform the data to correct for the heteroscedasticity and non-normal distribution characteristic of amplification data (Valenzuela et al. 2006). Significance of the treatment effect (e.g., incubation temperature) can be determined by testing for differences in the mean values of gene expression at each sampled developmental stage using ANOVA.

ACKNOWLEDGMENTS

This contribution was funded in part by National Science Foundation grant IOS 0743284 to N. Valenzuela.

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