

Embryological ontogeny of *aromatase* gene expression in *Chrysemys picta* and *Apalone mutica* turtles: comparative patterns within and across temperature-dependent and genotypic sex-determining mechanisms

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Abstract Although the role of aromatase in many estrogen-dependent reproductive and metabolic functions is well documented in vertebrates, its involvement in the ovarian development of species exhibiting temperature-dependent sex determination (TSD) is incompletely understood. This is partly due to the conflicting temporal and spatial pattern of *aromatase* expression and activity across taxa. To help resolve this ongoing debate, we compared for the first time the embryological ontogeny of *aromatase* expression in turtles possessing genotypic sex determination (GSD) (*Apalone mutica*) and TSD (*Chrysemys picta*) incubated under identical conditions. As anticipated, we found no significant thermal differences in *aromatase* expression at any stage examined (prior to until the end of the thermosensitive period) in *A. mutica*. Surprisingly, the same was true for *C. picta*. When placed in a phylogenetic context, our results suggest that *aromatase* expression is evolutionarily plastic with respect to sex determination in reptiles, and that differences between reptilian TSD and GSD are not *aromatase*-driven. Further research across TSD and GSD species is warranted to fully decipher the evolution of functional differences among sex-determining mechanisms.

Keywords Sex differentiation · Estrogen · Reptiles · Development · Evolution

Introduction

Aromatase is the enzyme responsible for the conversion of androgens to estrogens, and as such it plays a key role in many biological functions that are estrogen-dependent, including sex differentiation of vertebrate developing embryos (reviewed in Place and Lance 2004). Well established differences exist between the most studied vertebrates, mammals and birds, both possessing genotypic sex determination (GSD). Estrogen seems critical for ovarian development in birds because *aromatase* is expressed exclusively in female gonads and the injection of aromatase inhibitors in ovo induces sex reversal of ZW females prior to visible gonadal sex differentiation. In contrast, early ovarian development in mammals appears independent of estrogen synthesis because of the late *aromatase* expression observed in mice (reviewed in Place and Lance 2004). However, the mode of action of aromatase during sex differentiation in vertebrates with temperature-dependent sex determination (TSD), such as reptiles, is not fully understood, especially because observations across taxa are somewhat conflicting with respect to the temporal and spatial pattern of *aromatase* expression and activity.

Indeed, differential aromatase activity or gene expression (“expression” hereafter) occurs in the developing gonad of turtles at female- and male-producing temperatures during the thermosensitive period (TSP) of *Emys orbicularis* (e.g., Desvages and Pieau 1992), *Dermochelys coriacea* (Desvages et al. 1993), and *Malaclemys terrapin* (Jeyasuria and Place 1998) turtles. These observations lead to the conclusion that *aromatase* is an important upstream element in the sexual differentiation cascade under TSD. By contrast, differential “expression” during the TSP was lacking in the TSD turtles *Trachemys scripta* (White and Thomas 1992; Willingham

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et al. 2000; Murdock and Wibbels 2003) and *Lepidochelys olivacea* (Salame-Mendez et al. 1998). Furthermore, differential *aromatase* “expression” in developing gonads late into or after the TSP was observed in two crocodylians (TSD), *Alligator mississippiensis* (Gabriel et al. 2001) and *Crocodylus porosus* (Smith and Joss 1994). This lack of endogenous gonadal *aromatase* “expression” until late in development lead to the conflicting hypotheses that estrogens are not involved in early ovarian differentiation in TSD species, or that tissues other than the gonads provide the site for primary thermal sensitivity, as well as the sources of estrogen for early ovarian development (e.g., Smith and Joss 1994; Murdock and Wibbels 2003; reviewed in Pieau and Dorizzi 2004).

Although crucial to understanding the functional differences between TSD and GSD systems and the evolution of sex-determining mechanisms, no study to date has inspected *aromatase* expression in closely related GSD and TSD reptiles. Here, we take a first step to resolve this apparent discrepancy by examining *aromatase* gene expression in two turtle species with contrasting sex-determining mechanisms, *Chrysemys picta* (TSD) and *Apalone mutica* (GSD). We then compare the sequence of the transcripts, examine the patterns of *aromatase* expression, and explore their evolutionary significance for the divergence of the developmental basis of sex determination by placing our results in a phylogenetic context.

Chrysemys picta is a TSD species that produces an increasing proportion of females with increasing temperature (TSDIa, Ewert and Nelson 1991). GSD is recognized in *A. mutica* from incubation experiments at multiple temperatures that yield 1:1 sex ratios (Janzen 1993). We analyzed *aromatase* gene expression using quantitative real-time PCR at several developmental stages from oviposition to the end of the TSP in embryos incubated simultaneously at male- and female-producing temperatures for *C. picta*. With this design, we characterized temperature-specific gene expression in a temperature-sensitive species (*C. picta*) and contrasted this with the gene expression profile of a temperature-insensitive species (*A. mutica*), which served as a negative control. We used these data to test whether differences between TSD and GSD mechanisms in reptiles are *aromatase*-driven and whether *aromatase* gene expression patterns are evolutionarily plastic in reptiles.

Materials and methods

Sample collection

Eggs were collected from freshly laid natural nests of *C. picta* and *A. mutica* and brought to the laboratory for

incubation at constant temperatures that produce ~100% male (25°C) and ~100% female (30°C) *C. picta* hatchlings. Eggs were divided equally among multiple plastic containers filled three fifths of the way with moisten vermiculite set at -150 kPa by adding 338 g of water per 300 g of vermiculite. Boxes containing only the substrate were weighed (± 0.1 g), and they were weighed again after adding the eggs and the weight was recorded. Eggs were partially buried in the substrate in random positions in the box. Prior to the sampling of any egg (or weekly otherwise), the boxes were reweighed and lost moisture (as determined by weight loss) was replaced. The new weight was recorded after the removal of any eggs. Egg containers were rotated at least weekly to control for the effect of any potential thermal cline in the incubators.

Embryos were collected at several developmental stages (sensu Yntema 1968) equivalent for both temperature treatments and species used: stage 9, before TSP; stage 12, before TSP; stage 15, TSP onset; stage 19, middle TSP; and stage 22, end TSP (Bull and Vogt 1981). Sampled embryos were placed immediately in 10 vol of RNAlater[®], stored at -20°C and subsequently at -80°C for later use following the manufacturer’s recommendations.

Cloning and quantitative PCR

RNA was extracted from the adrenal–kidney–gonadal complex (AKG) using Qiagen’s RNeasy kits, and the DNase I digestion step was followed to prevent any DNA contamination. Exceptions to this protocol were embryos at the earliest developmental stages (stages 9 and 12), for which whole embryos or trunks were used (respectively). RNA was quantified using a NanoDrop[®] ND-1000 spectrophotometer, and run in 1% agarose gel stained with ethidium bromide to check its quality by the presence of ribosomal bands. Individual samples were kept separate and analyzed without pooling. One microgram of total RNA per sample was retro-transcribed with (dT)₂₀ primers using the Superscript III kit from Invitrogen following the manufacturer’s protocol. For samples that yielded less than 1 μ g total RNA in 8 μ l elute volume, as much as 8 μ l was used for the reverse transcriptase-PCR, and the total RNA amount was recorded for standardization during the data analysis as described below.

Degenerate primers were designed for relatively conserved regions of vertebrate *aromatase* cDNA sequences as found in GeneBank (Table 1). These primers amplify partial cDNA fragments (818 bp for *C. picta*, 487 bp for *A. mutica*), which were used for cloning and sequencing in our two study species using the pGEM[®]-T Easy Vector System from Promega. The same procedure was employed to clone a 343-bp fragment of the β -actin cDNA (Valenzuela et al. 2006). This housekeeping gene was used to test for the

Table 1 Primers used for standard or quantitative real-time PCR (*qm*) amplification of *aromatase* and β -*actin* cDNA fragments in two study turtles

Primers	<i>Chrysemys picta</i>	<i>Apalone mutica</i>
β - <i>actin</i> forward	5'-CAGGTCATCACCATYGGCAA-3'	
β - <i>actin</i> reverse	5'-GCTTGCTGATCCACATCTGC-3'	
<i>Aromatase</i> forward	5'-ATGGGAATYGGTSCCCTC-3'	5'-GGGAGATTYCTBTGGATGGG-3'
<i>Aromatase</i> reverse	5'-GGGTGCTCTGCAATCAGT-3'	5'-GCCAMGCATCAAAGTAGTTCTG-3'
<i>qm</i> β - <i>actin</i> forward	5'-AAGCCCTCTCCAGCCAT-3'	5'-AAGCTCTCTCCAGCCCT-3'
<i>qm</i> β - <i>actin</i> reverse	5'-GACAGCACAGTGTGGCG-3'	
<i>qm</i> <i>Aromatase</i> for	5'-TGGGTTACAGTGCATTGGC-3'	5'-CAGGACTCGTGCGCATGA-3'
<i>qm</i> <i>Aromatase</i> rev	5'-GAGGCCTGGACCAGACAA-3'	5'-TCCCAGTCCAGCAGTCACATC-3'

Blank cells indicate that the same primers were used for both species because sequences were identical

quality of the extracted RNA during quantitative PCR (QPCR) and for the normalization of *aromatase* gene expression. Internal primers were designed to amplify an 111-bp (*C. picta*) and an 85-bp (*A. mutica*) *aromatase* cDNA fragment, as well as a common 124-bp β -*actin* cDNA fragment for the quantification of initial transcript abundance via QPCR, using the Brilliant® SYBR® Green QPCR Master Mix in an Mx3000P real-time PCR machine from Stratagene. 6-Carboxyl-*X*-rhodamine was used as the reference dye for background correction. Standard curves were generated from pure miniprep cloned DNA obtained above by diluting cut inserts at concentrations of 5, 1, 1×10^{-2} , 1×10^{-4} , 1×10^{-6} , 1×10^{-12} , 1×10^{-14} , and 1×10^{-16} ng/ μ l and run in duplicate in each QPCR. Conditions for the QPCR reactions were as follows: one cycle at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min, followed by a dissociation curve cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Fluorescent readings were taken at the onset of the QPCR, at the end of each annealing and extension cycle, and during the ramp upwards of the dissociation curve cycle. Readings at the end of the extension cycle were used for the quantification of the initial template amount as described below.

Data analysis

The initial template amount for each sample and each gene was calculated using standard-curve quantification via the algorithms implemented in Mx3000p v2.0 from Stratagene with background correction. Initial copy numbers were calculated from the molecular weight of the fragments for each gene, and standardized to 1 μ g of initial total RNA. Expression of *Sfl* was normalized using β -*actin* expression data and Log_2 transformed to correct for the heteroscedasticity and nonnormal distribution characteristic of amplification data. Statistical analysis was carried out on these normalized Log_2 -transformed data keeping individual values separate (Valenzuela et al. 2006). The significance of

the temperature treatment effect was determined by testing for differences in the mean gene expression values at each sampled developmental stage via an ANOVA as implemented in Jmp 5.1.2® (SAS Institute, 2004).

Results and discussion

Partial 818- and 487-bp cDNA *aromatase* fragments were cloned and sequenced from AKG tissue of *C. picta* and *A. mutica* embryos, respectively (GenBank DQ383800 and DQ457012), encompassing helices A–I from the predicted structure of *A. mississippiensis* (Gabriel et al. 2001) and the conserved domains corresponding to the substrate-binding loop and the distal alpha-helix (van Nes et al. 2005). These fragments exhibit high but not perfect homology to other vertebrate *aromatase* cDNAs (Table 2).

Aromatase cDNA of *C. picta* was almost identical to that of *T. scripta* (Murdock and Wibbels 2003), both TSD turtles. These sequences differ by only 6 out of 818 nucleotides (0.007% sequence divergence). On the other hand, an order of magnitude higher sequence divergence was found between *C. picta* and *A. mutica* (33 substitutions out of 487 nucleotides or 0.07% sequence divergence), a GSD turtle (Table 2). *Chrysemys picta*'s sequence was also 89% identical to that of alligator, 84% to that of chicken, and 76% to that of human. In contrast, *A. mutica*'s sequence appears more divergent, as it is only 86% identical to that of alligator, 82% to that of chicken, and 75% to that of human (Table 2). Primers specific to each taxon were used for the QPCR when sequences differ between species, although in the case of β -*actin*, they amplified the exact same portion of the cDNA fragments (*qm* primers in Table 1).

Differences were also found at the amino acid level (Table 3). The amino acid sequence of *C. picta* differs by two residues from that of *T. scripta*, but this variation did not encompass any change in amino acid polarity or charge, and thus, the partial sequences do not suggest functional

differences. However, *A. mutica*'s fragment differed by 13 amino acid substitutions from *C. picta*'s (they shared a substitution with respect to *T. scripta*), five of which encompassed changes in amino acid polarity or charge, but whose functional importance is uncertain as they do not

reside in the substrate-binding loop or the distal alpha-helix (van Nes et al. 2005).

Six *C. picta* embryos exhibiting non-*aromatase* amplification, as determined from the dissociation curves and product melting temperature, were excluded from further

Table 2 Comparative alignment of partial *aromatase* cDNA fragment sequences characterized in *Chrysemys picta* and *Apalone mutica* (GenBank DQ383800 and DQ457012) and several other vertebrates

	5	15	25	35	45	55
Trachemys	ATGGGAATTG	GTCCCCTCAT	TTCACATGGG	AGATTCCTTT	GGATGGGAAT	GGGAAATGCC
Chrysemys
ApaloneT.....	T.....G.....C.....
AlligatorC.....	.G.....T.....G.....	A..C.....
GallusG..T.....T..C.....G.....	A..T.....
HomoA.....	C..C..C..CG.....G.....	C..C.G.....

	65	75	85	95	105	115
Trachemys	TGCAACTATT	ACAATAAGAT	GTATGGAGAA	TTTATGAGAG	TCTGGATCAG	TGGAGAGGAA
Chrysemys
ApaloneC.....C.....G..C.....	C.....
Alligator	A.....G.....	T.....
GallusC.....C.....C	A.....G.....	T.....	C..T..A...
HomoC.....CCG.G.	A.....C.....C.....TC.....

	125	135	145	155	165	175
Trachemys	ACACTCATAA	TTAGCAAATC	CTCCAGTATC	TTCCATGTAA	TGAAACATGG	GCAATATATC
ChrysemysT.....
Apalone	..T...T.....	T.....	A.....
AlligatorT..T.....A..G.G	..T.....C...G..
Gallus	..T.T.....CG.A	..T..C.....CT.....	..A.C..CG.T
HomoT.....	..C...G..	..A...GCA...G..CAA	T..T..C.G.

	185	195	205	215	225	235
Trachemys	TGTAGATTG	GAAGCAAGCT	TGGGTTACAG	TGCATTGGCA	TGCATGAAAA	TGGTATTATA
Chrysemys
Apalone	.C.....A..	..A...A	..T.....
Alligator	.C.....	.G.....T.....
Gallus	.C.C.....	.G.....	..A.....T.....	..G.....
Homo	.C.C...C..	.C...A..	..C.G..C..T..G.....	A..C..C...

	245	255	265	275	285	295
Trachemys	TTTAATAATA	ACCCAGCACT	CTGGAGAGAA	ATTCGACCTT	TTTTACACAA	AGCTTTGTCT
ChrysemysA.....
ApaloneT...T.....	A...A.....T.....
AlligatorT...A.....	T...A.....	..G.....	..C.....
GallusC.....A.....	..AG..G	..C...C.....	..C.....CC.....
HomoC..C..	.T...AG..	..A.AC..	..C...C.....	..C..T.TG..C...A

	305	315	325	335	345	355
Trachemys	GGTCCAGGTC	TCGTGCGTAT	GATAGCAATT	TGTGTTGAAT	CAACGAAAGA	CCATCTGGAC
ChrysemysC.....
Apalone	..A...A.....C.....T.....
AlligatorT.....	.T..A.....T.....A.C...T.....
GallusT.....	.T...C.....C.....A.TT.T	T..C.....
Homo	..C..C..C..	.T..T.....	.G.CA..G.CC.....	..CCTC...AC	A.....

	365	375	385	395	405	415
Trachemys	AGATTGGAGG	ATGTGACAGC	TGGACTGGGA	AACATCAATG	TGCTGAATTT	TATGAGACAG
ChrysemysT.....
ApaloneT.....T.....C.....
Alligator	.A.....A.....	.G...CA.....	..A.....T...CC.....G.G.
Gallus	.A.C.A..A..	.A...CA.....	..A.G.A.....	..G.....	..T...CC.....	C.....G.

Table 2 (continued)

Homo	..G.....	.G.....CAA	..A.TC...C	T.TG.GG.C.	..T...CCC.	.C..C.T.GT

	425	435	445	455	465	475
Trachemys	ATCACACTGG	ACACTTCTAA	CACACTCTTT	CTAGGGATCC	CTTTGGATGA	AAATGCCATT
Chrysemys
ApaloneA.....A.....	..G...C.G
Alligator	...TGT.A.	.T.....	..A.....	..CA.....T...
Gallus	.T.TG.T.	...G.....	..AG.....G...G.....
Homo	G...TG....	...C.....	..G.....C	T.GA.....C...	..G...T..C

	485	495	505	515	525	535
Trachemys	GTGCTTAAAA	TTCAGAACTA	CTTTGATGCT	TGGCAAGCAC	TTTTGTTGAA	ACCTGACATC
ChrysemysC....
ApaloneG.
AlligatorCAC....
Gallus	..A....G.T.	..C.A.A.	G..C....A
Homo	..G.....	.C..AGGT.	T.....AT.	..CC.CA.C.	..A.....

	545	555	565	575	585	595
Trachemys	TTCTTTAAGA	TTTCTTGGCT	GTACAAGAAA	TATGAAAAGT	CAGTGAAGGA	TTTGAAAGAA
Chrysemys
Apalone
Alligator	..T..C....GG...	..C..A..G.
Gallus	..T.....	T.G.....GG..G	..CC.A..	C.....G.
Homo	A.....A..GG....	..T..C....T

	605	615	625	635	645	655
Trachemys	GCAATTGAAG	TCTTAATAGA	ACAGAAACGA	CAGAGACTTT	CCACTGTTGA	AAAATTGGAA
Chrysemys
Apalone
AlligatorG..TT	.A.....C.	G.....T	..AG....C...T
GallusG...AA.AG....G...T
Homo	..C..A....	.TC.G...C	.G.A...A..	.GC..GA..	..A.AA..	G...C....

	665	675	685	695	705	715
Trachemys	GAGCACATGG	ACTTTGCATC	ACAACCTGATT	TTTGACACAGA	GCCGTGGAGA	TCTGACTGGT
Chrysemys
Apalone
AlligatorG...A.A	A.....A.C.
GallusT.....	T..G.....	A.A.A....C.
Homo	..ATGT....CA.	TG.GT....	..A..G...	AA.....T..	C....AA.A

	725	735	745	755	765	775
Trachemys	GAGAATGTGA	ACCAGTGTGT	GTTGGAGATG	ATGATAGCTG	CACCTGACAC	ACTGTCTGTG
Chrysemys
Apalone
Alligator	..A..C..T.C.....C....	.C.....	..A..A...
GallusC.C.....T....	.T...T..	T....C...
HomoCA.	A.....A..	C...C..A.	.T.....	CA.....C

	785	795	805	815		
Trachemys	ACTCTCTTCT	TCATGCTAGT	ACTGATTGCA	GAGCACCC		
ChrysemysG...		
Apalone		
AlligatorT.		
Gallus	..A.....A	.T.....A.CG....		
Homo	T..T.G....T.	T..C.....	A.....		

analyses. Standard curves for β -actin and aromatase in *C. picta* had R^2 values of 0.97 and 0.89, respectively, and of 0.98 and 0.99, respectively, in *A. mutica*. As anticipated, we found no significant differences in gene expression between

embryos incubated at 25 and 30°C at any of the developmental stages examined in *A. mutica* (GSD; $P>0.05$; Fig. 1a). Importantly, however, no temperature-specific differences were detected in *C. picta* (TSD; $P>0.05$;

Table 3 Amino acid sequence alignment corresponding to the partial *aromatase* cDNA fragment characterized in *Chrysemys picta* and *Apalone mutica* and those of several other vertebrates. Position 1 corresponds to position 53 of the translated full *aromatase* cDNA sequence of *Trachemys scripta* [Genebank accession number AF178949 of Murdock and Wibbels (2003)]. The conserved domain corresponding to the substrate-binding loop is indicated in bold and that of the distal alpha-helix is indicated in bold italics (van Nes et al. 2005). Underlined bold amino acids represent nonsynonymous changes among turtles that result in changes of amino acid polarity or charge

	5	15	25	35	45	55
Trachemys	MGIGPLISHG	RFLWMGMGNA	CNYYNKMYGE	FMRVWISGEE	TLIISKSSSI	FHVMKHGQYI
Chrysemys
ApaloneL.V...L...	.V.....	.F.....K..
Alligator	...A.....V...I...	.V.....V	.Y....H.V
GallusV...T...	.V.....	.F.....VWN.V
HomoI.S.RV...M	..I...NH.S

	65	75	85	95	105	115
Trachemys	CRFGSKLGLQ	CIGMHENGII	FNNNPALWRE	IRPFFTKALS	GPGLVRMIAI	CVESTKDHL
ChrysemysK.
Apalone	S.....K.V...
Alligator	S.....Y.....T.K.	M.....S.T.....
Gallus	S.....Y.....H.K.IV...
Homo	S.....K...E..KT	T.....M....VTV	.A..L.T...

	125	135	145	155	165	175
Trachemys	<u>RL</u> ED VTAGLG	NINVLNFMRO	ITLDTSN TLF	LGIPLDENAI	VLKIQNY FDA	WQALLLLK PDI
Chrysemys
Apalone	.. D KS.L G	.X.....
Alligator	K..E..TE..L.R	.M..... K ..	.R.....
Gallus	K..E..TEV.	.V....L.R	.M..... K ..	.V....S..
Homo	..E..NES.	YVD..TLL.R	VM.....	.R....S..	.V...G....I....

	185	195	205	215	225	235
Trachemys	FFKISWLYKK	YEKSVKDLKE	AIEVLIEQKR	QRLSTVEKLE	EHMDFASQLI	FAQSRG DLTG
Chrysemys
Apalone
AlligatorE.....G	.MDL..Q...	.K.....D	.V...T...	..N....A
GallusC..	..EAA....G	.M.I.....	.K.....DN....A
HomoDAE..	R.I..E....	.C....TE..	L.EK....R
		
	245	255	265			
Trachemys	ENVNQCVLEM	MIAAPDTLSV	TLFFMLVLIA	EH		
ChrysemysV.....	..		
Apalone		
Alligator		
GallusI..I...	DD		
HomoI...	L.....M..	S.....F...	K.		

Fig. 1b). Gene expression detected in *C. picta* (Fig. 1) mimics previous reports of aromatase activity in *T. scripta* and *L. olivacea*, where no significant differences were detected between temperature treatments (putative sexes) during the TSP (Willingham et al. 2000; Salame-Mendez et al. 1998), but differs from reports in *E. orbicularis*, *D. coriacea*, and *M. terrapin*, where aromatase activity was significantly higher at female-producing temperatures during the TSP (Desvages and Pieau 1992; Desvages et al. 1993; Jeyasuria and Place 1998).

Aromatase catalyzes the conversion of androgens to estrogens, which are fundamental for many reproductive and metabolic processes. Although the relative importance of aromatase in sexual differentiation appears clear for birds

and mammals, the generality of its role for early ovarian development in TSD reptiles has been questioned due to conflicting results from studies across turtles and crocodylians (reviewed in Pieau and Dorizzi 2004). These discrepancies lead to the hypothesis that aromatase may be a downstream target in the sexual differentiation cascade instead of a key upstream developmental switch in TSD taxa (e.g., Murdock and Wibbels 2003).

Viewing our results in a phylogenetic context (Fig. 1c) helps elucidate this ongoing debate about the function and importance of *aromatase* in gonadal differentiation of reptilian embryos. For instance, the two contrasting “expression” patterns are found among closely related TSD turtles, such as sea turtles (*Dermochelys* and *Lep-*

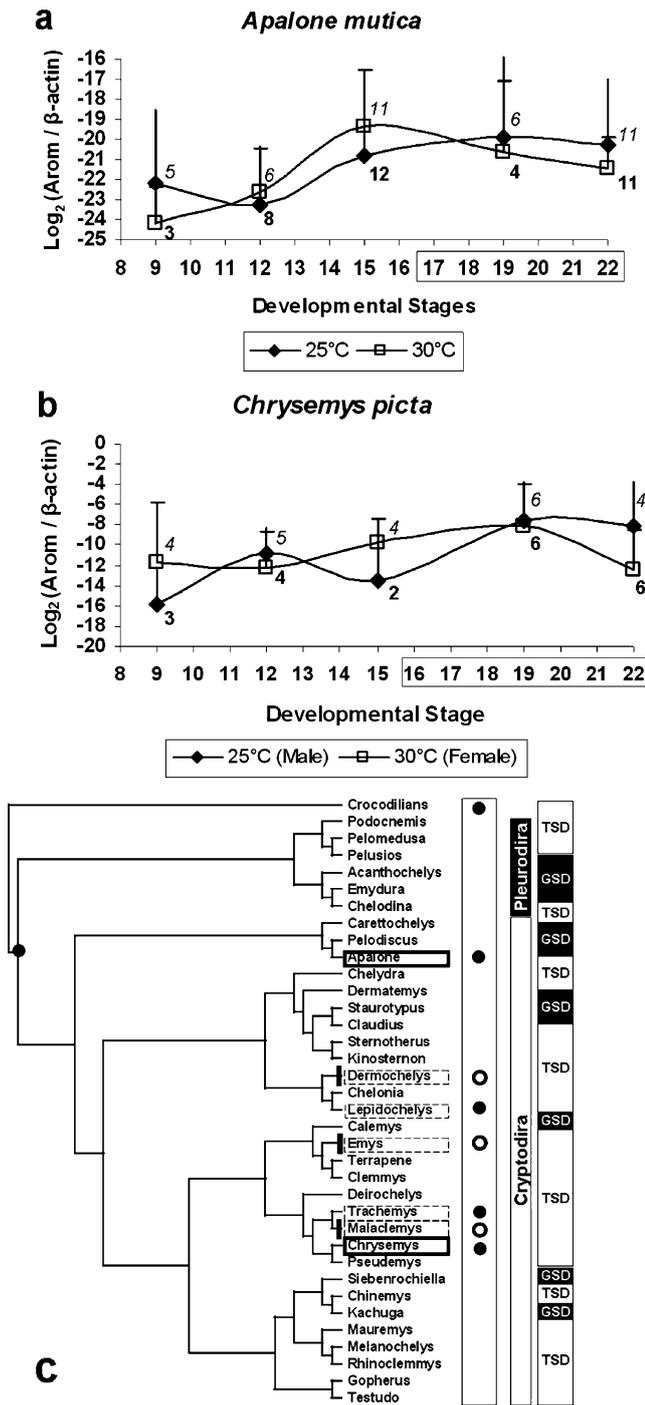


Fig. 1 Developmental expression of *aromatase* in *Chrysemys picta* (a) and *Apalone mutica* (b). The Y-axis represents *aromatase* initial copy number (+SD) normalized to *B-actin* initial copy number and transformed to Log_2 scale. Stages 9, 12, and 15–22 represent whole embryos, trunks, and AKG complexes, respectively. 25 and 30°C are male- and female-producing temperatures, respectively, for *C. picta*. Boxed stages are TSP of *C. picta*. Sample sizes at 25°C (*italics*) and at 30°C (*bold*) are presented by each sampling time. Panel c illustrates the phylogenetic relationship among species (modified from Janzen and Krenz 2004) with significant differential *aromatase* “expression” early during the TSP (*open circles*) and those without (*solid circles*), as detailed in the text. *Solid-line boxes*, this study; *dashed-line boxes*, previously reported and referenced in the text

idochelys), and several Emydides (*Emys*, *Trachemys*, *Malaclemys*, *Chrysemys*) (Fig. 1c). Interestingly, however, in *A. mutica* (GSD), we detected an almost identical pattern to that of *C. picta* (Fig. 1a,b). This suggests that the pattern found in *C. picta*, *T. scripta*, and *L. olivacea* might not reflect a different TSD underlying mechanism from that of *E. orbicularis*, *D. coriacea*, and *M. terrapin*, but instead a common pattern of expression across TSD/GSD boundaries. A lack of early differential steroidogenic activity during the TSP has also been found in crocodylians (Smith and Joss 1994; Gabriel et al. 2001) such that this pattern extends beyond turtles.

Analyzing gonadal tissue separate from adrenal/kidney complexes (AK) should account for the alternative that AK expression may mask subtle but biologically significant differential expression in the gonads alone (Pieau and Dorizzi 2004). However, several authors have detected no steroidogenic activity in the gonadal ridges alone until after the end of the TSP in multiple turtles (e.g., Thomas et al. 1992; White and Thomas 1992; Salame-Mendez et al. 1998). Therefore, this hypothesis may explain some but not all of the reported discrepancies among taxa, as the two patterns have been detected specifically in gonadal tissue. Furthermore, a study performed on these same embryos (Valenzuela et al. 2006) detected significant differential *Sfl* expression at stage 12, a gene also known to express in extragonadal tissue such as the adrenals and brain. Thus, detecting differential gene expression in our study was not necessarily precluded by the use of AKG tissue. Nonetheless, this important functional and evolutionary question remains open for future studies that analyze gonadal and nongonadal tissue separately across TSD and GSD species.

While further taxon sampling is needed, the data collected to date suggest that multiple gains and losses are required to characterize the evolutionary history of *aromatase* expression in reptiles. Based on this information, it therefore appears that *aromatase* expression is evolutionarily plastic with respect to sex determination in reptiles (Fig. 1c). Importantly, although *aromatase* remains an important regulator of sex differentiation, differences between reptilian TSD and GSD are not *aromatase*-driven. Also, while comparisons of several genes are necessary to elucidate the developmental basis of sex determination, we hypothesize that *aromatase* is a downstream element in TSD’s developmental cascade. This is consistent with our finding of differential *Sfl* expression prior to the TSP in *C. picta* using the same set of embryos (Valenzuela et al. 2006).

To our knowledge, ours is the first comparison of *aromatase* expression between closely related species with contrasting sex-determining mechanisms to try to understand some of the functional differences between TSD and GSD systems and the evolution of sex-determining mechanisms. The importance of a comparative approach is

illustrated by the fact that, in a phylogenetic context, the seemingly conflicting observations appear evolutionarily significant. Similar approaches across genes and taxa should prove useful to decipher if divergence of TSD systems has occurred by drift or selection resulting in mechanisms with distinct developmental bases.

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