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Comparative gene expression of steroidogenic factor 1 in *Chrysemys picta* and *Apalone mutica* turtles with temperature-dependent and genotypic sex determination

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SUMMARY Characterizing the molecular network underlying temperature-dependent (TSD) and genotypic (GSD) sex determination, including patterns across closely related taxa, is crucial to elucidate the still enigmatic evolution of sex determining mechanisms in vertebrates. Here we examined the expression of an important gene for sexual differentiation common to both systems, *Sf1*, at male- and female-producing temperatures, in TSD (*Chrysemys picta*) and GSD turtles (*Apalone mutica*). We tested the hypotheses that *Sf1* expression responds to temperature consistently across TSD turtles but is unaffected in GSD turtles, and that this differential expression starts no earlier than the onset of the thermosensitive period (TSP). As expected, *Sf1* expression was thermally insensitive in *A. mutica* (GSD). Although *Sf1* exhibited a differential expression by temperature in *C. picta*, the expression pattern differed from other TSD turtles (*Trachemys scripta*), perhaps reflecting divergence of the gene regulatory networks underlying sex determination over evolutionary time. Most notably, *Sf1* was differentially expressed in *C. picta* (significantly higher at the male-producing temperature) before the onset of the TSP, implying that in TSD taxa significant thermal effects may occur early in development. This result may reconcile field observations where temperatures experienced prior to the TSP have an effect on sex ratios, thus challenging traditional TSP models. Importantly, the molecular factors that render TSD mechanisms thermosensitive remain unknown, and potential candidates are genes that express differentially before the onset of the TSP (genes shaping or opening the TSP-window rather those acting once the TSP window has opened). Therefore, our findings make *Sf1* one such potential candidate.

INTRODUCTION

Sexual differentiation has paramount consequences for the evolution of a multitude of traits, such as sex allocation, sex ratio, sexual dimorphism, sexual selection, sexual conflict, chromosome and genome evolution, and ultimately, speciation and extinction (e.g., Berry and Shine 1980; Rice 1984; Lindholm and Breden 2002; Edwards et al. 2005). Important advances have been made in our knowledge of the molecular mechanism underlying genotypic sex determination (GSD) particularly in mammals and birds (reviewed in Morrish and Sinclair 2002; Place and Lance 2004). However, in comparison with GSD systems, much less is known about the molecular circuitry associated with environmental sex-determining (ESD) mechanisms in vertebrates (reviewed in Place and Lance 2004). ESD is commonly found among reptiles in the form of temperature-dependent sex determination or TSD (reviewed in Valenzuela 2004a; Valenzuela and Lance 2004). The evolutionary significance of TSD in many vertebrates remains a conundrum (reviewed in Valenzuela 2004b), partly because we lack a complete understanding of the functional mechanics of TSD at the molecular level and how it differs from GSD systems in closely related taxa. Additionally, deciphering the developmental dynamics of sexual differentiation from a molecular perspective in TSD taxa may help us reconcile observed discrepancies in sex ratio production between laboratory experiments and natural nests (e.g. Pieau 1982; Bull 1985; Schwarzkopf and Brooks 1985; Valenzuela et al. 1997; Valenzuela 2001) thus facilitating the study of sex ratio evolution under TSD.

Genes that are common to both GSD and TSD taxa that regulate the gonadal differentiation cascade have been identified (reptilian homologues of mammalian and avian genes) (e.g., *Sox9*, *Sf1*, *Wt1*, *Dax1*, *Dmrt1*, *AMH*, and *Aromatase*). Further, their influence on sex differentiation is well documented under laboratory conditions (reviewed in Place and Lance 2004). The steroidogenic factor 1 (*Sf1*), a nuclear orphan receptor also known as AD4BP and NR5A1, is an important gene for sexual differentiation in vertebrates. It is required for the formation of primary steroidogenic organs

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(adrenal gland and gonad) in mammals, and for the expression of steroidogenic enzymes therein (Parker and Schimmer 1997; Morohashi 1999). *Sf1* is a member of the nuclear hormone receptor superfamily of transcription factors (a homologue to Drosophila's transcription factor FTZ-F1 or Fushi tarazu factor 1). In species with GSD such as mammals and birds, *Sf1* has sexually dimorphic expression in differentiating gonads and is also expressed in other regions of the developing embryo. Differential expression has also been detected in TSD reptiles, and some similarities and differences have been detected (reviewed in Place and Lance 2004). For instance *Sf1* expression in chickens and American alligators is lower in males than females during sex differentiation (Smith et al. 1999a, b; Western et al 2000) whereas in mice, humans, and red-eared slider turtles its expression is higher in testis than ovaries (Achermann et al. 1999; Fleming et al. 1999; Crews et al. 2001; Fleming and Crews 2001).

Differential expression of genes involved in sex differentiation in reptiles with temperature-dependent sex determination (TSD) has been studied mainly from the onset of and until after a window of time when incubation temperature is believed to affect the resulting sex ratio of embryos (the thermosensitive period (TSP)). These studies have provided highly valuable information about the genetic basis of sexual differentiation across vertebrates. However, some fundamental questions about the nature and extent of diversity in expression patterns across taxa remain unaddressed. For instance, it is not known whether *Sf1* expression varies between closely related reptiles with environmental and GSD, yet in order to clarify the functional importance of genes associated to developmental processes a comparative approach across systems is crucial. Additionally, the gene(s) that render sex determination thermosensitive in TSD taxa remains unknown, and potential candidates are early acting genes that shape or open the TSP window, yet it is not known if differential expression occurs at developmental stages prior to or at the onset of the TSP.

In order to address these issues we performed a comparative developmental genetics study in which we cloned and characterized a partial cDNA fragment of *Sf1* in two turtle species with contrasting sex determining mechanisms, *Chrysemys picta* (TSD) and *Apalone mutica* (GSD). *C. picta* is a TSDIa species that produces an increasing proportion of females with increasing temperature (Ewert and Nelson 1991). *A. mutica* is a GSD species that consistently produces 1:1 sex ratios across incubation temperatures (Janzen 1993). We analyzed *Sf1* gene expression using quantitative real-time PCR at several developmental stages from oviposition to after 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. This design allowed us to test the hypothesis that temperature affects *Sf1* expression in the same manner across TSD turtles by comparing our results from *C. picta* to those reported previously for another TSD turtle, *Trachemys scripta*. We also tested the hypotheses that temperature has no effect on *Sf1* expression in GSD reptiles as it does in TSD species; and that differential *Sf1* expression starts at or after the developmental stages corresponding to the onset of the TSP of TSD taxa.

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 approximately 100% male (25°C) and approximately 100% female (30°C) *C. picta* hatchlings. Eggs were divided equally among multiple plastic containers filled 3/5 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 recorded. Eggs were partially buried in the substrate in random positions in the box. Before the sampling of any egg (or weekly otherwise), the boxes were re-weighted 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, 1976) equivalent for both temperature treatments and species studied: stage 9, before TSP; stage 12, before TSP; stage 15, TSP onset; stage 19, middle TSP; stage 22, end TSP (Bull and Vogt 1981). Sampled embryos were placed immediately in 10 vol of RNAlater® (Ambien Inc., Austin, TX, USA), 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 (AKG) complex using QIAGEN's RNeasy Kits (Qiagen, Valencia, CA, USA) and the DNAseI 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 (NanoDrop Technologies Inc., Wilmington, DE, USA), 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 (Carlsbad, CA, USA) following the manufacturer's protocol. For samples that yielded less than 1 μg total RNA in 8 μl elute volume, as much as 8 μl were used for the

RT-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 *Sf1* cDNA sequences as found in GenBank (Table 1). These primers amplify a partial (470 bp) cDNA fragment used for cloning and sequencing in our two study species using the pGEM®-T Easy Vector System from Promega (Madison, WI, USA). The same procedure was used to clone a 343 bp fragment of the *β-actin* cDNA. This housekeeping gene was used to test for the quality of the extracted RNA during QPCR, and for normalization of *Sf1* gene expression after validation by testing for temperature- and species-specific differences in *β-actin* expression levels. As the above-mentioned primers amplified >150 bp fragments which are not adequate for real time PCR, internal primers were designed to amplify a 126 bp *Sf1* and a 124 bp *β-actin* cDNA fragment for the quantification of initial transcript abundance via real time QPCR. We used the Brilliant® SYBR® Green QPCR Master Mix in an M×3000P real-time PCR machine from Stratagene (La Jolla, CA, USA) following manufacturer's recommendations. ROX was used as the reference die for background correction. Standard curves were generated from pure miniprep cloned DNA 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/μl and run in duplicate in each QPCR. Conditions for the QPCR reactions were as follows: 1 cycle at 95°C for 10 min, followed by 45 cycles of 95°C for 30 sec, 60°C for 1 min, 72°C for 1 min, followed by a dissociation curve cycle of 95°C for 1 min, 55°C for 30 sec, and 95°C for 30 sec. Fluorescent readings were taken at the onset of the QPCR, at the end of each annealing and extension cycle, and during the ramp upward of the dissociation curve cycle. Readings at the end of the extension cycle were used for quantification of initial template amount as described below.

Data analysis

Initial template amount for each sample and each gene was calculated using standard curve quantification via the algorithms implemented in M×3000p v2.0 from Stratagene with background correction. Initial copy numbers were calculated from the molecular weight of the fragments for each gene, and standardized to1ug of initial total RNA. Expression of *Sf1* was normalized using *β-actin* expression data, and Log_2 transformed to correct for the heteroscedasticity and non-normal distribution characteristic of amplification data. Statistical analysis was carried out on these normalized Log_2-transformed data.

The significance of the temperature treatment effect was determined by testing for differences in the mean gene expression values at each sampled developmental stage relative to individual variation via an ANOVA as implemented in Jmp 5.1.2© (SAS Institute Inc. 2004).

RESULTS

The cloned *Sf1* cDNA fragment (Table 2, GenBank accession numbers DQ295020 and DQ295021) corresponds to part of SF1's ligand-binding domain (Li et al. 1999). The *Sf1* cDNA fragment characterized for *C. picta* differed by only three out of 470 nucleotides (0.6% sequence divergence) from that of *T. scripta* (Cowan and Wibbels unpublished). On the other hand, a total of 46 nucleotide substitutions (9.8% sequence divergence) were found between *C. picta* and *A. mutica*, a turtle with GSD. Two of those were identical nucleotide changes between *C. picta* and *T. scripta*, two encompassed two consecutive nucleotides, and one other change encompassed three consecutive nucleotides. Primers specific for each species were used for the QPCR when sequences differ between species although they amplified the exact same portion of the *Sf1* cDNA fragments. The amino acid sequence of *C. picta* was identical to that of *T. scripta* (Table 3). However, *A. mutica*'s fragment differed by 12 amino acid substitutions, five of which encompassed changes in amino acid polarity or charge (one in two adjacent amino acids).

Standard curves for *β-actin* and *Sf1* had R^2 values of 0.97 and 0.99, respectively, for *C. picta*, and of 0.98 and 0.94, respectively, for *A. mutica*. No significant differences were found between male and female temperature treatments in the level of expression of *β-actin* at any developmental stage ($P>0.1$) in *C. picta* and *A. mutica*, and the level of expression of *β-actin* was very similar between both species (results not shown). Five *C. picta* embryos exhibiting non-*Sf1* amplifica-

Table 1. Primers used to amplify partial cDNA fragments of *Sf1* and β-actin in two turtle species used in this study

Primers	*Chrysemys picta*	*Apalone mutica*
β-actin forward	5′-CAGGTCATCACCATYGGCAA-3′	
β-actin reverse	5′-GCTTGCTGATCCACATCTGC-3′	
Sf1 forward	5′-CTGCTGCAGAACTGCTGGAG-3′	
Sf1 reverse	5′-ATGAGSAGGTTGTTGCARGGC-3′	
qmβ-actin forward	5′-AAGCCCTCTTCCAGCCAT-3′	5′-AAGCTCTCTTCCAGCCCT-3′
qmβ-actin reverse	5′-GACAGCACAGTGTTGGCG-3′	
qmSf1 forward	5′-CTTGCATTCGCTCCAGGT-3′	5′-CCTGCAGTCGCTCCAGGT-3′
qmSf1 reverse	5′-GCGTTGGCTTTCTCCTGA-3′	5′-GCGTTGGCCTTCTCCTGG-3′

Blank cells indicate that the same primers were used for both species because sequences were identical.
qm, designation of internal primers used for quantitative real-time PCR.

Table 2. Comparative alignment of partial cDNA fragment sequences characterized in *Chrysemys picta* and *Apalone mutica* (Genbank DQ295020-DQ295021) and several other vertebrates

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               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   5         15         25         35         45         55
Trachemys      CTGCTGCAGA ACTGCTGGAG TGAGCTGCTG GTGTTTGACC ATATCTACAG GCAGGTGCAG
Human          .......... .......... C......... .....C.... .C......C. C.....C...
Mouse          .......... ....T..... C......... .....G.... .C......C. C.....C...
Gallus         .......... .......... C......... .....C.... .CG.G...C. ....C.....
Alligator      ---------- ---------- ---------- ---------- ---------- ----------
Chrysemys      .......... .......... .......... .......... .......... ..........
Apalone        .......... .......... C......... .......... ........C. .T........

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   65         75         85         95        105        115
Trachemys      CACGGGAAGG AGCACAGCAT GCTGCTTGTG ACGGGCCAGG AGGTGGAGAT GGCGACAATC
Human          .....C.... ..GG...... C.....G..C ..C..G.... ........C. .A.C...G.G
Mouse          T....C.... .AG....... C.....G..T ..T..A.... ........C. .AGC...G.G
Gallus         .....C..A. .......TG. ......G..C ..C..G.... .......TC. .T.AG..G.G
Alligator      ---------- ---------- ---------- ---------- --.....C.. .T....CG.G
Chrysemys      .......... .......... .......... .......... .......... ..........
Apalone        .......... .......... .......... .......... .......C.. .......G.G

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  125        135        145        155        165        175
Trachemys      GCAGCTCAAG CAGGGTCCAA CCTGAATAAC CTGGTCCTGA GGGCACAAGA ACTGGTCCTA
Human          ..CA.C..G. .G..C..GCT G...C.C.G. .....GT..C ....G..G.. G.....G..G
Mouse          ..T.TG..G. .T..C...CT G...C.C.G. .....G...C ....C..... GT.A..G..C
Gallus         ..G..G..G. .G..C....T ....C.CTC. .....G...C .......G.. .........G
Alligator      ..G.TG.... .G..C....T ......C... .........C .C..C..G.. G..C.....G
Chrysemys      .......... .......... .......... .......... .......... G.........
Apalone        .....C.... .C..C....T ......CT.. .......... ....G..... G.....G..G

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  185        195        205        215        225        235
Trachemys      CACTTGCATT CGCTCCAGGT GGATCGACAA GAGTTTGTGT GTTTGAAGTT CCTGATACTC
Human          ..GC...T.G ....G...C. ...C..G..G ........C. .CC.C..... .A.C..C...
Mouse          ..G......G .A..G...C. ...C..C..G .....C..C. ..C.C..... ...C..C...
Gallus         ...C....C. .A.CG..... ...C..G..G .....C..C. .CC.C..A.. ......C...
Alligator      ..TA....C. .C........ C..C..G..G .....C..C. .CC.C..... ..........
Chrysemys      .......... .......... .......... .......... .......... ..........
Apalone        ...C....G. .......... ...CA..... .......... ..C....... ......C...

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  245        255        265        275        285        295
Trachemys      TTCAGTCTCG ATGTGAAATA TTTGGAAAAC CACAGCCTGG CGAAGGATGC TCAGGAGAAA
Human          .....C..G. ..T....G.T CC..A.T... ....T..... T..A..C... .........G
Mouse          .....C.... .........T CC..A.C... ........C. TA.....C.. C.....A..G
Gallus         .....C.... .......G.. CC....G... ...GCG.... .T.....C.. .........G
Alligator      .......... .......... CC....G... .......... .C.....C.. G........G
Chrysemys      .......... .......... .......... .......... .......... ..........
Apalone        .....C..G. .C.....G.G CC....G... .......... .......G.. C........G

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  305        315        325        335        345        355

Trachemys      GCCAACGCAG CTCTGTTGGA GTACACCATC TGCCACTACC CTCACGCCGC GGACAAGTTC
Human          ........C. .C...C.T.. C......C.G .......... .G...TG..G ......A...
Mouse          ........T. .C........ T......T.G ..T....... .A...TG..G ......A...
Gallus         .....T.... .C...C.... ......GG.G .......... .A...TG.A. A.........
Alligator      ........G. .G...C.... ......GG.G .......... .G......A. ..........
Chrysemys      .......... .......... .......... .......... .......... ..........
Apalone        ........G. .C...C.... ......GGC. .......... .G...T...G ..........

               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  365        375        385        395        405        415
Trachemys      CGTCAGCTCC TGCTGCGGCT GGCTGAGATC CGCTCCCTCA GCATGCAGGC CGAGGAGTAC
Human          .AG.....G. .....T.C.. ..TG...G.G ..GG....G. .......... .A........
Mouse          .AG...T.G. .AT..T.C.. ..TG...G.G ..GG....G. .......... .A........
Gallus         ..G.....G. .......... .A.....G.. ..GG.G..G. .......... A.........
Alligator      ..G....... .......... ...C...... ..GG....G. .......... ..........
Chrysemys      .......... .......... .......... .......... .......... ..........
Apalone        ..C......T .......... ...C...G.G ...G....G. .......... G.........

               ....|....| ....|....| ....|....| ....|....| ....|....|
                  425        435        445        455        465
Trachemys      CTCTACCACA AGCACCTGAG CGGGGAGGTG CCTTGCAACA ACCTGCTCAT
Human          ..G....... ........G. .AAC...A.. ..CC...... ..........
Mouse          ..G....... ....TT..G. .AAC...A.. ..CC...... ....T.....
Gallus         ..G....... .......... ...A...... ..C....... ....C.....
Alligator      ..G....... .......... .......... ..C....... ....C.....
Chrysemys      .......... .......... .......... ..C....... ....C.....
Apalone        .......... .......... ...C...... .......... ..........
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Trachemys, *Trachemys scripta*; Human, *Homo sapiens*; Mouse, *Mus musculus*; Gallus, Gallus *gallus*; Alligator, *Alligator mississippiensis*; Chrysemys, *Chrysemys picta*; Apalone, *Apalone mutica*.

Table 3. Alignment of amino acid sequences corresponding to the partial cDNA fragment characterized in *Chrysemys picta* and *Apalone mutica* and those of several other vertebrates

	355	365	375	385	395	405
Trachemys	LEVGDQMKLL	QNCWSELLVF	DHIYRQVQHG	KEHSMLLVTG	QEVEMATIAA	QAGSNLNNLV
Homo	~~~~~~~~..	....	....	..G.I.....	....LT.V.T	....L.HS..
Mouse	~~~~~~~~..	L	........Y.	..D.I.....	....LS.V.V	....L.HS..
Gallus	~~~~~~~~..	....	..V...L...	....V.....	...DLSAV..	....I.HS..
Alligator	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~~~~~~~~~	~~.D.S.V.V	....I.....
Chrysemys	~~~~~~~~..	....	....	....	....	....
Apalone	~~~~~~~~..	....	.....?....	....	...D...V..	....I..Y..

	415	425	435	445	455	465
Trachemys	LRAQELVLHL	HSLQVDRQEF	VCLKFLILFS	LDVKYLENHS	LAKDAQEKAN	AALLEYTICH
Homo	........Q.	LA..L.....	.....I....	..L.F.N..I	.V........	....D..L..
Mouse	........Q.	.A..L.....	....	....F.N...	.V........	....D..L..
Gallus	....	..P.......	....	.........A	....	.......V..
Alligator	.........M	....	....	....	....	.......V..
Chrysemys	....	....	....	....	....	....
Apalone	....	Q.........	....	....C.....	...E......	.......A..

	475	485	495	505	515
Trachemys	YPHAADKFRQ	LLLRLAEIRS	LSMQAEEYLY	HKHLSGEVPC	NNLLIEMLHA
Homo	...CG...Q.	...C.V.V.A	.....K....	....GN.M.R	....X-~~~~
Mouse	...CG...Q.	...C.V.V.A	.....K....	....GN.M.R	....X-~~~~
Gallus	...CT.....	.....T.V.A	....	....	....X-~~~~
Alligator	....T.....	.........A	....	....	....X-~~~~
Chrysemys	....	....	....	....	....X-~~~~
Apalone	...SG.....	.......V.A	....	....	....X-~~~~

Position 405: N = Asparagine (polar) to I = Isoleucine (non-polar)
Position 421: H = Histidine (positive charge?) to Q = Glutamine (polar)
Position 474: A = Alanine (non-polar) to S = Serine (polar)
Position 474: A = Alanine (non-polar) to G = Glycine (polar
Position 490: S = Serine (polar) to A = Alanine (non-polar)

Position numbers according to the translated full cDNA sequence of *Trachemys scripta* (Genbank accession number AF033833 of Cowan and Wibbels, unpublished). Shaded amino acids represent non-synonymous changes that result in changes of amino acid polarity or charge. Underlined amino acids represent residues that contact phospholipid ligands in mouse (Li et al. 2005).
Trachemys, *Trachemys scripta*; Human, *Homo sapiens*; Mouse, *Mus musculus*; Gallus, *Gallus gallus*; Alligator, *Alligator mississippiensis*; Chrysemys, *Chrysemys picta*; Apalone, *Apalone mutica*.

tion, as determined from the dissociation curves and product melting temperature, were excluded from further analyses (final samples sizes are presented in Fig. 1). Although *Sf1* expression in *C. picta* was higher at male producing temperature (25°C) during several developmental stages (Fig. 1A), these differences were not statistically significant except during stage 12 of development (the second sampling time before the onset of the TSP) ($P < 0.05$). This significance is not due to outliers as the result remains unchanged after removing the sample with the most extreme gene expression. As anticipated, *Sf1* expression in *A. mutica* did not differ by temperature treatments at any of the stages examined ($P > 0.05$, Fig. 1B).

DISCUSSION

The cloned *Sf1* cDNA fragment revealed a high but not perfect homology to other vertebrate sequences (Table 2). We found a large similarity between the *Sf1* cDNA fragments characterized for *C. picta* and *T. scripta* (Cowan and Wibbels, unpublished), both turtles with TSD. On the other hand, higher sequence divergence was detected in the *Sf1* cDNA fragment of *A. mutica*, a turtle with GSD. Some significant differences were also found at the amino acid level (Table 3). The amino acid sequence of *C. picta* was identical to that of *T. scripta*, and thus, they are functionally equivalent. However, five of the 12 amino acid substitutions found between *A.*

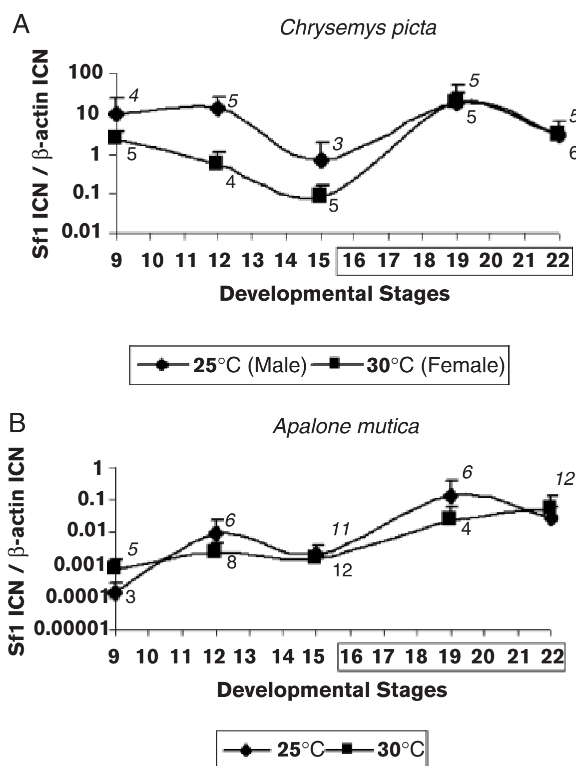

Fig. 1. Developmental gene expression of *Sf1* in *Chrysemys picta* and *Apalone mutica* expressed as the ratio of *Sf1* to β-actin initial copy number (ICN) per 1 μg of total RNA (+ SD) and scaled to comparable units. Stages 15–22 include adrenal–kidney–gonadal complexes while whole embryos or trunks were used for stages 9 and 12, respectively. Male, male-producing temperature for *Chrysemys picta* (25°C); female, female-producing temperature for *Chrysemys picta* (30°C). Developmental stages within the square box constitute the thermosensitive period for *C. picta*. Sample sizes at 25°C (italics) and at 30°C (bold) are presented by each sampling time.

mutica and *C. picta* encompassed changes in amino acid polarity or charge (one in two adjacent amino acids) which may be of potential functional importance since the fragment is part of *Sf1*'s ligand-binding domain (Li et al. 1999), and one of these five substitutions occurs in a residue adjacent to a residue known to contact a phospholipid ligand in mouse (Li et al. 2005). Further research is needed to determine if these changes are neutral or functional.

Sf1 is expressed in all vertebrates examined to date although the pattern of expression varies by taxonomic group. For instance, *Sf1* expression in chickens is lower in males than females during sex determination (Smith et al. 1999a, b) whereas in mammals its expression is higher in testis than ovaries. A homologue has been found in alligators and turtles that have TSD. Interestingly, *Sf1* expression is also differential in reptiles but *Sf1* expression in *Alligator mississippiensis* (TSD) resembles the pattern found in chicken (higher expression in females; Western et al. 2000) while expression in *T. scripta* (TSD) resembles the mammalian pattern (higher expression in males) (Fleming et al. 1999; Crews et al. 2001; Fleming and Crews 2001). In *T. scripta* turtles, *Sf1* expression at the pivotal temperature showed three distinct patterns in different individuals: male-like, female-like, and intermediate, and shifts from a male- to a female-producing temperature (or vice versa) during the TSP induces a corresponding change in gene expression with short-term overcompensation (Fleming and Crews 2001).

In the present study we investigated *Sf1* expression in a turtle with environmental sex determination, *C. picta*, and found the pattern of expression to be higher in males but to differ in its tempo from the expression profile reported for *T. scripta* (Fleming et al. 1999; Crews et al. 2001; Fleming and Crews 2001), as well as from the expression in the GSD turtle, *A. mutica*, used as negative control. As anticipated, *Sf1* expression in *A. mutica* did not differ by temperature treatment. In *C. picta*, *Sf1* expression was significantly higher at the male-producing temperature (25°C) during a developmental stage before the onset of the TSP. Contrary to expectation, *Sf1* expression during the TSP in *C. picta* was not statistically different between female- (30°C) and male-producing temperatures (25°C) (Fig. 1A).

Thus, it appears that temperature affects *Sf1* expression in a differing manner across TSD turtles. Concordant gene expression across species would be expected if TSD systems are pleisiomorphic and developmentally conserved in turtles as some phylogenetic studies suggest (Janzen and Krentz 2004). On the other hand, differences at the level of gene expression between TSD species as reported here, suggest that multiple TSD systems may exist that differ in their developmental or molecular underpinnings (sensu Abouheif and Wray 2002) perhaps resulting from divergence over evolutionary time (True and Haag 2001; Nuzhdin et al. 2004). The vast diversity of systems harbored within GSD (Wilkins 2002) indicates that such processes have operated in the evolution of sex determining mechanisms and thus should be expected within TSD as well. Furthermore, *Aromatase* gene expression also differs among TSD species (Desvages and Pieau 1992; White and Thomas 1992; Desvages et al. 1993; Smith and Joss 1994a; Jeyasuria and Place 1998; Willingham et al. 2000; Gabriel et al. 2001; Murdock and Wibbels 2003), but in that case, *C. picta* and *T. scripta* exhibit the same expression pattern (N. Valenzuela and Shikano unpublished data).

Alternatively, the lack of statistically significant differential during the TSP of *C. picta* may be explained because mRNA from AKG complexes was used in these experiments and both in situ hybridization and QPCR studies in *T. scripta* have revealed that *Sf1* expression in the adrenal tissue can be high enough to mask differences in expression from gonadal tissue (see Fig 2C in A. Fleming and D. Crews, 2001; and M. Ramsey and D. Crews unpublished data). Future in situ

hybridization studies should reveal the localization of this differential expression, as well as gene expression analysis where gonadal tissue is isolated from the adrenal–kidney tissue.

As predicted by our second hypothesis, no significant differences in *Sf1* expression between temperature treatments were detected in *A. mutica*. This is consistent with the expectation that temperature has no effect on *Sf1* expression in reptiles with GSD. The possibility of sex-specific differences in *Sf1* expression in *A. mutica* could not be addressed here as no molecular marker exists to sex embryos of this species. Thus, this question awaits the development of appropriate markers. *Sf1* expression and among-individual variation in *A. mutica* was lower than that of *C. picta* whereas *β-actin* expression was comparable. Gene expression variation in *C. picta* was more than an order of magnitude lower than levels detected for *Aromatase* in *A. mississipiensis* (TSD) (Gabriel et al. 2001). Differences in the amount of variation in gene expression across genes and species can be biologically significant as they may also result from diverging developmental regulation evolving under varying selection regimes or drift (True and Haag 2001; Nuzhdin et al. 2004).

Most notably, we found that *Sf1* expression in *C. picta* was significantly higher in males at a developmental stage before the onset of the TSP. This is the first instance in which such an early temperature-specific differential expression has been reported. This result contradicts the general expectation that differential *Sf1* expression begins at or soon after the onset of the TSP of TSD taxa. In reptiles, the TSP window of time is determined from sex ratio data obtained during egg-transfer experiments (reviewed in Valenzuela and Lance 2004). Specifically, embryos are incubated at male- and female-producing temperatures and sets of embryos are sequentially shifted to the reciprocal temperature during development. The disconcordance between expected sex ratio and temperature allows the onset and ending of the TSP to be determined. The TSP in turtles often falls within the middle third of incubation although among species variation has been reported (e.g. Souza and Vogt 1994; Valenzuela 2001). Because of the emphasis on this TSP definition, it has not been widely appreciated that thermal effects taking place before the onset of the TSP may still influence the outcome of sexual differentiation perhaps by enabling particular temperature-specific responses of the embryos during the TSP or by thermal effects on cell proliferation (Smith and Joss 1994b). For instance, observational studies of sex ratios and natural incubation temperatures of nests in the field have demonstrated that hatchling sex does not always correspond to the thermal conditions present during the TSP (Valenzuela 2001). Furthermore, hatchling sex ratios in the wild were associated with temperatures experienced prior to the TSP, suggestive of a significant thermal influence early in development, and challenging the existing TSP definition (Valenzuela 2001). Our finding that *Sf1* was differentially expressed in *C. picta* between thermal treatments offers a potential causal molecular link that could reconcile these previous field observations that appear to conflict with conventional TSP models. As such, it underscores the importance of profiling gene expression starting at very early stages of development when functionally important genes may be differentially affected by temperature in ways that could influence sex ratio production in TSD species. On the other hand, *A. mutica* appears to have lost the thermal sensitivity during GSD evolution, thus explaining the lack of differential *Sf1* expression at early stages as observed in *C. picta*.

Our finding of *Sf1* early expression is of paramount importance because the gene(s) that renders sex determining mechanisms thermosensitive in TSD species remains unknown, and potential candidates could be genes that exhibit differential expression before or exactly at the onset of the TSP (those genes that shape or open the window of TSP rather than those that act within the window once it opens). As early *Sf1* expression is needed for the formation of bipotential gonads (Wilkins 2002) *Sf1* would be a realistic candidate to be co-opted as the master thermal-switch in the evolution of TSD. The genital ridge may be present by stage 12 in the study species, as urogenital tissue is present by stage 12 in *T. scripta* (Spotila et al. 1998). As trunks were used at stage 12 in our study, there is the potential that the differential signal detected comes from an extragonadal site known to express *Sf1* such as the adrenal tissue (brain is known to express *Sf1* also but heads were excluded at stage 12). Irrespective of the tissue source, a differential pattern was detected in a temperature-specific manner, which in *C. picta* is also sex specific as each temperature used produces a single sex. By sampling equivalent developmental stages at low and high temperatures and by the use of a GSD negative control, we controlled for other thermal effects on developmental rate independent of sex differentiation. Thus, our findings open the door for future research to test this hypothesis by exploring the localization of *Sf1*'s early differential expression, to test whether other genes exhibit similar concurrent patterns of expression, and how this early developmental expression might enable other thermal responses during the TSP, all of which are important to understand sex ratio evolution under TSD. The alternative that the TSP (as traditionally defined), is active in *C. picta* by stage 12 is ruled out by previous observations in this species under similar thermal regimes (Bull and Vogt 1981). The fact that temperature in the lab before the TSP does not affect sex ratio is not irreconcilable with our findings, as the shift experiments used to define the TSP are designed to only detect sex ratio responses to the temperature experienced during this window of time, and are thus unable to detect the master switch responsible for shaping or opening that window. Our approach at the gene expression level is a more appropriate tool to target that question particularly when applied to early developmental stages preceding the onset of the TSP.

Elucidating the still puzzling evolutionary dynamics of sex determining mechanisms in vertebrates requires a comprehensive view of the molecular networks underlying sex determination and sex differentiation in TSD and GSD species. For instance, identifying additional elements that allow critical sex-differentiation events to be realized during the TSP remains essential, and most importantly, the network element(s) that opens the TSP window in TSD taxa is still elusive. Examining molecular networks across species in a phylogenetic context will allow us to elucidate the pattern of genetic architectures associated with TSD and GSD, and shed light on the evolutionary process that generated it. Our findings on *Sf1* expression contribute an initial step toward this understanding, and underscore the important role that early developmental stages may play in the evolution of TSD mechanisms.

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