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Adrenal–Kidney–Gonad Complex Measurements May Not Predict Gonad-Specific Changes in Gene Expression Patterns During Temperature-Dependent Sex Determination in the Red-Eared Slider Turtle (*Trachemys scripta elegans*)

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ABSTRACT Many turtles, including the red-eared slider turtle (*Trachemys scripta elegans*) have temperature-dependent sex determination in which gonadal sex is determined by temperature during the middle third of incubation. The gonad develops as part of a heterogenous tissue complex that comprises the developing adrenal, kidney, and gonad (AKG complex). Owing to the difficulty in excising the gonad from the adjacent tissues, the AKG complex is often used as tissue source in assays examining gene expression in the developing gonad. However, the gonad is a relatively small component of the AKG, and gene expression in the adrenal-kidney (AK) compartment may interfere with the detection of gonad-specific changes in gene expression, particularly during early key phases of gonadal development and sex determination. In this study, we examine transcript levels as measured by quantitative real-time polymerase chain reaction for five genes important in slider turtle sex determination and differentiation (AR, ERα, ERβ, aromatase, and *Sf1*) in AKG, AK, and isolated gonad tissues. In all cases, gonad-specific gene expression patterns were attenuated in AKG versus gonad tissue. All five genes were expressed in the AK in addition to the gonad at all stages/temperatures. Inclusion of the AK compartment masked important changes in gonadal gene expression. In addition, AK and gonad expression patterns are not additive, and gonadal gene expression cannot be predicted from intact AKG measurements. *J. Exp. Zool. 307A:463–470, 2007.*

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Reptiles with temperature-dependent sex determination (TSD) are useful model organisms for studying genes involved in sex determination and sex differentiation because the gonad is truly bipotential until a temperature cue acts to determine sex. Many reptiles, including the red-eared slider turtle (*Trachemys scripta elegans*), exhibit TSD, where the sex of the developing embryo is determined by egg incubation temperature during a critical temperature-sensitive period (TSP) of development (Bull, '80; Wibbels et al., '91). The mechanism by which temperature is transduced into a sex-determining cue is not known, although much current research in TSD reptiles examines differential gene expression in the gonad before or at the onset of the TSP (Fleming et al., '99; Kettlewell et al., 2000; Western et al., 2000; Maldonado et al., 2002; Murdock and Wibbels, 2003a,b; Ramsey et al., 2007; Shoemaker et al., 2007).

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In the slider turtle, gonadal development can be divided into three main phases: bipotential (stages 13–16), sex determination (stages 17–19/21), and sex differentiation (stages 21–23). The TSP encompasses the bipotential and sex determination phases of development. The reptilian gonad arises as a bipotential genital ridge along the medial ventral mesonephros, and is part of a heterogenous tissue complex that also includes the developing adrenal gland in addition to the mesonephros (from which the Wolffian and Mullerian ducts develop) and later, metanephros which will become the kidney (Raynaud and Pieau, '85). For the sake of convention, this organ complex will be referred to as the adrenal–kidney–gonad (AKG) complex. Gene expression from other compartments could mask gonad-specific changes in gene expression during development, including during the TSP while sex is being determined. However, because of the difficulty in separating the gonad from the surrounding mesonephros tissue, the AKG complex is frequently used as the tissue source in gene expression studies looking at sex determination and differentiation in reptiles (Spotila et al., '98; Kettlewell et al., 2000; Murdock and Wibbels, 2003a,b; Takada et al., 2004). Many of genes involved in amniote gonadal sex determination and differentiation are also expressed in adjacent adrenal and kidney tissue (Vainio and Lin, 2002; Parviainen et al., 2007; Shoemaker et al., 2007), and early, subtle gonad-specific changes in gene expression may be diluted by expression in other compartments. In addition, there may be unexpected sex differences in developmental adrenal or kidney gene expression, as has been measured in mouse fetal adrenal (Mukai et al., 2002) and newborn and adult mouse kidney (Janmohamed et al., 2004; Rinn et al., 2004).

Here, we report gene expression levels in intact AKG tissue for five genes important in gonadal sex determination and differentiation in the slider turtle, and compare these expression patterns with those for isolated adrenal–kidney (AK) and gonad tissue sources. The genes measured include the steroid hormone receptors: androgen receptor (AR), estrogen receptor α (ERα), estrogen receptor β (ERβ), as well as aromatase, the enzyme that converts testosterone to estradiol, and steroidogenic factor-1 (*Sf1*), a master regulator for steroidogenic enzymes. Our results indicate that all five genes exhibit different patterns of expression depending on the tissue source of measurement.

MATERIALS AND METHODS

Tissue collection

Red-eared slider turtle (*T. scripta*) eggs were purchased from Robert Clark (Clark Turtle Farms, Hammond, LA) within 24 hr of laying and randomized on site to control for clutch effects. Eggs were held at room temperature in Texas until candled to establish viability. Eggs were then further randomized, placed into trays containing 1:1 vermiculite and water mix and placed in incubators (Precision, Chicago, IL) at 26°C (male-producing temperature) or 31°C (female-producing temperature). Incubator temperatures were monitored continuously with HOBO recording devices (Onset Computer Corporation, Bourne, MA) and verified daily with calibrated shelf thermometers. To avoid small temperature gradient effects, the egg trays were also rotated daily within the incubator. Eggs were dissected at regular intervals to assess developmental stage (Yntema, 1968). Embryos were harvested at stages 17, 19, 21, and 23. AKG, AK or isolated gonad tissue was rapidly dissected and processed for experimental use.

Total RNA preparation and cDNA synthesis

For the AK and gonad measurements, we dissected gonad tissue off the AK component of the AKG for processing. Therefore, the AK and gonad samples represent tissue from the same individuals. The gonad is a very small component of the AKG, therefore to ensure adequate material for q polymerase chain reaction (PCR) assays, tissue from multiple individuals (30–50/sample) were pooled for AK and gonad measurements. AKG tissue was pooled from 10–30 individuals. After dissection, AKG, AK, or gonad tissue was placed in RNAlater (Ambion, Austin, TX) for overnight incubation at 4°C followed by storage at −80°C, or placed directly into Promega RNAgents Total RNA Isolation kit denaturing solution and stored at −80°C. Total RNA was extracted using RNAgents Total RNA Isolation kit (Promega, Madison, WI) following the manufacturer's protocol. Abundance and purity was assessed through spectrophotometry readings at 260 and 280 nm.

Total RNA was treated with DNase to eliminate any genomic DNA contamination in the RNA samples using Turbo DNA-Free kit (Ambion) following the manufacturer's protocol. Single-stranded cDNA was reverse-transcribed (1 μg total

RNA/20 μl reaction concentration) using Superscript First Strand Synthesis for real-time (RT)-PCR kit (Invitrogen, Carlsbad, CA). cDNA synthesis was primed with both oligo-dT and random hexamers using a modified manufacturer's protocol (Invitrogen).

Real-time qPCR

Gene sequences, RT-PCR primer sequences and amplicon sizes have been described (Ramsey and Crews, 2007; Ramsey et al., 2007). For all genes, specificity of target was verified by gel electrophoresis. Expression levels were normalized to PP1 (protein phosphatase 1 γ) housekeeping gene. PP1 was chosen based on stability of expression under experimental conditions and relative level of abundance compared with AR, ERα, ERβ, aromatase, and *Sf1* (Ramsey et al., 2007).

RT-qPCR experiments were conducted on ABI Prism 7900 RT-PCR machine (Applied Biosystems, Foster City, CA). Reaction conditions have been described (Ramsey and Crews, 2007; Ramsey et al., 2007). Relative gene expression levels were assessed using SYBR green detection chemistry. Dissociation curve analysis was performed after each assay to determine target specificity. Each sample was run in triplicate. For each gene, AKG, AK, and gonad samples were run on the same plate to eliminate inter-run variability.

RT-qPCR data analysis

Assay results were first analyzed using the Applied Biosystems Sequence Detection System software (SDS 2.2.1). Relative quantification was performed by a modified comparative critical threshold (CT) method that corrects for different PCR amplification efficiencies among primer pairs (Simon, 2003). Gene expression normalized to PP1 housekeeping is given as mean normalized expression $= (E_{\mathrm{pp1}} \hat{} \,\mathrm{meanCT}_{\mathrm{pp1}})/(E_{\mathrm{exp\ \ gene}} \hat{} \,\mathrm{meanCT}_{\mathrm{exp\ \ gene}})$, where $E = \mathrm{PCR}$ efficiency $(E = 10 \hat{} \,\text{-}(1/\mathrm{slope}))$ (Pfaffl, 2001), mean CT is the average CT across the three replicates, and exp gene = AR, ERα, ERβ, aromatase, or *Sf1*. Normalized gene expression levels were then calibrated to a baseline measurement (stage 17 measurement at the female-producing temperature).

Statistical analysis of qPCR expression patterns

Regression analyses were performed for each gene, comparing expression patterns for AKG (X) versus AK (Y) and AKG (X) versus isolated gonad (Y) compartments using the Excel Data Analysis module. For each gene, expression patterns produced from the mean triplicate value for stages 17, 19, 21, 23 at both male and female-producing temperatures were used to assess whether AKG patterns could predict either AK patterns (Table 1) or isolated gonad patterns (Table 2). For the regression of AK onto AKG analysis (Table 1), the stage 23 31° group was omitted from the analysis owing to interwell variability within the AK samples (presumably owing to RNA degradation in the original preparation).

RESULTS

AR, ERα, ERβ, aromatase, and *Sf1* transcript abundance was measured using quantitative real-time RT-PCR (qPCR). Intact AKG, isolated AK, and isolated gonad tissue was used as template, and the resulting patterns were compared between temperatures and across stages.

Adrenal–kidney–gonad complex

AR, ERα, ERβ, aromatase, and *Sf1* are all expressed in the AKG (Fig. 1A, D, H, K, N).

TABLE 1. AKG expression patterns can predict AK patterns for some genes. Depicted below is a regression analysis of AK (Y) patterns onto AKG (X) patterns

Gene	R^2	F	P
AR	0.313	2.279	0.191
ERα	0.531	5.658	0.063
ERβ	0.237	1.554	0.268
Aromatase	0.504	5.089	0.074
Sf1	0.797	19.683	0.007

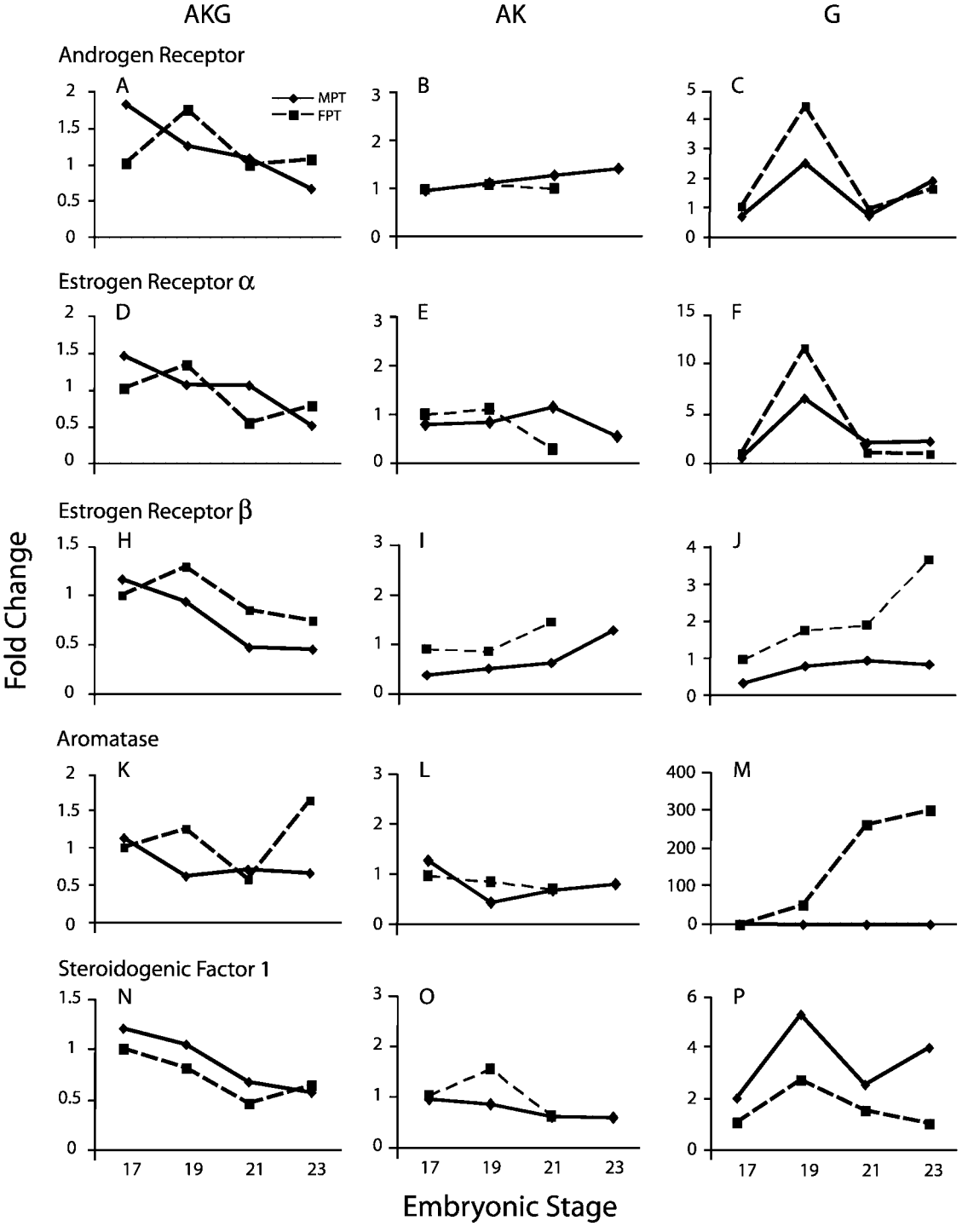
R^2 values indicate that AKG can predict AK patterns for *Sf1* at a significance of $P < .05$.
AR, androgen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; AK, adrenal–kidney; AKG, adrenal–kidney–gonad.

TABLE 2. AKG expression patterns do not predict G patterns. Depicted below is a regression analysis of isolated gonad (Y) patterns onto AKG (X) patterns

Gene	R^2	F	P
AR	0.120	0.820	0.400
ERα	0.158	1.124	0.330
ERβ	0.013	0.078	0.790
Aromatase	0.138	0.959	0.365
Sf1	0.023	0.140	0.721

Low R^2 values indicate that AKG and gonad parameters do not predict each other reliably.
AR, androgen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; AKG, adrenal–kidney–gonad; G, gonad.

AR and ERα expression patterns are similar in the AKG. AR is slightly higher at the male-producing temperature during the TSP (stage 17), but expression at the male-producing temperature steadily declines throughout the phases of gonadal commitment and differentiation (Fig. 1A), whereas at the female-producing temperature there is a moderate increase during gonadal commitment before declining. Similarly, ERα is slightly higher in the middle of the TSP (stage 17) at the male-producing temperature, declining slightly throughout gonadal differentiation (Fig. 1D); at the female-producing temperature ERα expression remains unchanging throughout the



TSP (stages 17–19), and then declines slightly as ovarian differentiation commences (stage 21) but remains level through stage 23.

ERβ and aromatase are both expressed slightly higher at the female-producing temperature, but with differing patterns of expression. At both incubation temperatures ERβ expression is equivalent at stage 17, but expression drops steadily at the male-producing temperature until leveling out at the onset of testis differentiation (stage 21; Fig. 1H); at the female-producing temperature ERβ increases slightly as ovarian commitment begins (stage 19), but then declines as differentiation continues. Aromatase is also equivalent between the two temperatures during the TSP (stage 17), and then exhibits a drop in expression at the male-producing temperature as sex is being determined and then remains stably expressed as testis differentiation continues (Fig. 1K). At the female-producing temperature, aromatase expression is unchanged through the TSP (stages 17–19), and after a brief decline as the ovary is being determined and ovarian differentiation commences (stage 21), expression begins a female-specific increase as ovarian differentiation is completed (stage 23).

*Sf*1 expression is slightly higher at the male-producing temperature throughout the TSP and into gonadal commitment and differentiation, but levels at both temperatures decline slightly throughout development (Fig. 1N).

Adrenal–kidney

AR, ERα, ERβ, aromatase, and *Sf1* are all expressed in the AK compartment of the AKG (Fig. 1B, E, I, L, and O). For all genes, Table 1 lists results from a regression analysis of AKG versus AK component expression patterns. For one gene, *Sf1*, AKG predicts AK patterns at a significance of $P < .05$.

AR gene expression is not differential by temperature in the AK (Fig. 1B). ERα is expressed at a constant level throughout AK development at the male-producing temperature (Fig. 1E). At the female-producing temperature, levels are steady during the TSP, but drop slightly at the female-producing temperature between stages 19 and 21.

ERβ expression is also relatively constant through development in the AK compartment (Fig. 1I). As with the AKG measurement, levels are slightly higher at the female-producing temperature. At both temperatures, ERβ increases slightly after the TSP has closed, although the increase at the female-producing temperature can be seen at stage 21, whereas male-producing temperature levels do not increase until stage 23. Like AR, aromatase levels are not differential by temperature in the AK (Fig. 1L).

The SF1 AK expression pattern in unique and does not match either the AKG or gonad patterns (Fig. 1N, O, and P), although AKG patterns do predict AK (Table 1). Expression is steady at the male-producing temperature, but there is a slight peak at the female-producing temperature at stage 19. For all five genes, expression levels for AK stage 23 at the female-producing temperature were eliminated owing to interwell variability caused by low target abundance in the preparation.

Gonad

AR, ERα, ERβ, aromatase, and *Sf1* are all expressed in the gonad compartment of the AKG (Fig. 1C, F, J, M, P). Gonad-specific gene expression patterns have been described for all five genes (Ramsey and Crews, 2007; Ramsey et al., 2007). For each of the five genes, Table 2 lists results

Fig. 1. Quantitative real-time polymerase chain reaction (qPCR) analysis of AR, ERα, ERβ, aromatase, and *Sf1* expression and female and male-producing temperatures using different compartments of the developing adrenal–kidney–gonad (AKG) complex patterns during temperature-dependent sex determination in the red-eared slider turtle (*Trachemys scripta elegans*). Gene expression patterns were compared between AKG complex, adrenal–kidney (AK), and isolated gonad (**G**) tissues. AKG (**A**, **D**, **H**, **K**, **N**) patterns do not predict isolated gonad (**C**, **F**, **J**, **M**, **P**) patterns although AK (**B**, **E**, **I**, **L**, **O**) did not differ by temperature across development. Target tissue for qPCR analyses consisted of pooled AKG, AK, or gonad tissue from 30 to 50 individuals/stage/temperature. Levels were normalized relative to PP1 housekeeping gene (see Materials and Methods) and then calibrated across temperatures to the normalized stage 17 FPT value for each gene. For each gene, stage 17 FPT = 1, and other values reflect the ratio of gene expression level/temp and stage to stage 17 FPT. G, gonad; FPT, female-producing temperature (31°C) and is represented by the dashed line in all graphs; MPT, male-producing temperature (26°C); AR, androgen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β. Data points for all five genes for AK tissue at stage 23 at the female-producing temperature are absent owing to interwell variability, presumably because of degradation in the original total RNA preparation.

from a regression analysis of AKG versus isolated gonad component expression patterns. In all cases, AKG tissue does not predict gonad-specific gene expression patterns.

For AR, expression is equivalent during the middle of the TSP (stage 17) rather than higher at the male-producing temperature (Fig. 1C, A) as measured with AKG tissue. The female-specific peak in expression as ovarian commitment occurs (stage 19) is much higher in the gonad-only assay (the peak is twice as high in females as compared with males in isolated gonad vs. a normalized difference of only .5 for the same comparison in AKG).

Like AR, ERα exhibits a female-specific peak at stage 19 in the isolated gonad tissue (Fig. 1F and D) that is barely perceptible in the AKG measurement (five-fold higher peak at the female-producing temperature according to gonad measure vs. .3 normalized difference in the AKG measure).

ERβ patterns exhibit two primary differences between gonad and AKG measurements. In the isolated gonad tissue, ERβ is higher at the female temperature during the middle of the TSP (Stage 17), whereas in the AKG measurement levels are equivalent between (Fig. 1H and J). Also, the female-specific increase in ERβ as the ovary is differentiating (stage 23) is specific to the gonad measurement and is not detectable in AKG.

Aromatase exhibits a much different pattern in gonad versus AKG tissue. In both, aromatase levels are equivalent midway through the TSP (stage 17), but whereas aromatase increases throughout ovarian development when measured in isolated gonad tissue, the AKG measurements at the female-producing temperature decline at stage 21 and only show a modest increase at stage 23 (Fig. 1M and K).

Like ERβ, *Sf1* follows a qualitatively similar pattern in gonad and AKG tissue. However, the magnitude of difference is attenuated in the AKG measurement, and the male-temperature-specific peaks observed at stages 19 and 23 are not detected in AKG (Fig. 1P and N).

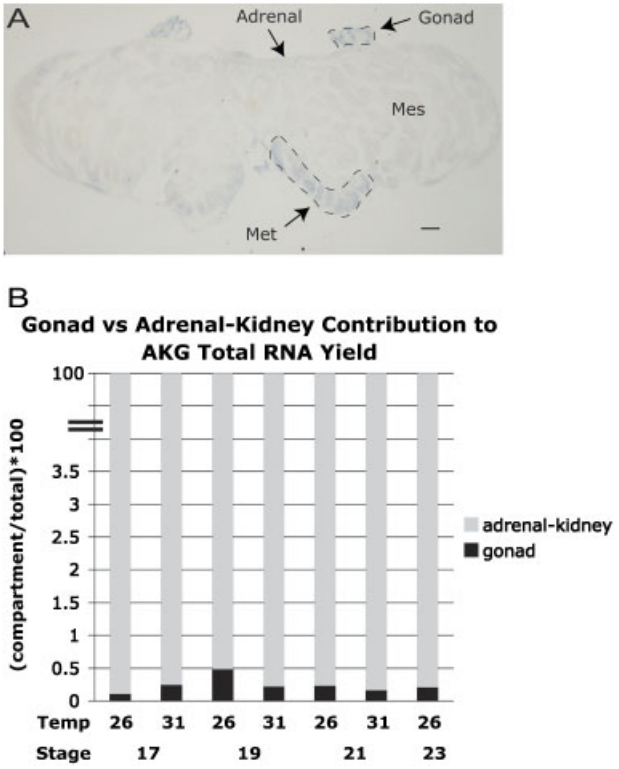


Fig. 2. The gonad represents a relatively small proportion of the adrenal–kidney–gonad (AKG) complex. (**A**) A representative photomicrograph of sectioned AKG tissue shows the relative size of gonad versus embryonic kidney (mesonephros, Mes), developing adult kidney (metanephros; Met), and adrenal components of the AKG. Here, digoxigenin-labeled androgen receptor (AR) probe was hybridized to stage 19 26° (male-producing temperature) whole mount AKG tissue and then sectioned (see Ramsey and Crews, 2007 for in situ hybridization methods). Purple staining represents AR expression in the gonad and developing metanephros. The adrenal component is not clearly defined in this individual, but arrow points to region where the adrenal gland is developing relative to the gonad and kidney. Bar = 100 μm. (**B**) The relative contribution of gonad versus adrenal–kidney (AK) tissue to total RNA extracted from pooled individuals for each group. For each stage/temperature, gonads were dissected off the AK component and each pooled to produce template for qPCR experiments, so AK and gonad samples represent tissue from the same individuals (see Methods). For each group, gonad and AK total RNA yields were added to produce the AKG total yield. Relative contribution for gonad versus AK was then derived by dividing each component by the AKG total yield (gonad yield/total AKG, or AK yield/total AKG) and multiplying by 100 to get the percentage contribution of each compartment. Stage 23 31° values were omitted owing to degradation of the AK total RNA prep for that group.

DISCUSSION

The gonad represents a minute part of the AKG complex (Fig. 2), yet AKG tissue is frequently used as source tissue for experiments looking at temperature-specific changes in gonadal gene expression (Spotila et al., '98; Kettlewell et al., 2000; Murdock and Wibbels, 2003a,b; Takada et al., 2004). During the TSP, the AKG contains not just the developing gonad, adrenal, and embryonic kidneys, but also the Wolffian and Mullerian ducts (Raynaud and Pieau, '85; Wibbels et al., '99)—all potential sources of differential gene expression that cannot be distinguished in an

AKG tissue preparation. Here, we show that expression patterns for AR, ERα, ERβ, aromatase, and *Sf1* do not correspond in isolated gonad versus AKG measurements. Temperature-specific differences in gonadal gene expression are at best attenuated, and at times are completely undetectable in AKG tissue measurements.

Isolated gonad tissue exhibits different patterns of expression than AKG tissue in all five genes examined here. In all cases, measuring gene expression in isolated gonad tissue versus AKG tissue results in more interpretable results. For example, AR and ERα demonstrate quantitative differences (higher at the female-producing temperature) late in the TSP and as sex is being determined (Fig. 1C,F). In both cases, the increase at the female temperature can be seen in the AKG measurement, but cannot be distinguished from expression at the male-producing temperature (Fig. 1A and D).

Aromatase was the only gene with an increase at the female-producing temperature that was consistent across AKG and isolated gonad measurements, but the AKG increase was later in development than the gonad measurement (Fig. 1K and M) and, in general, AKG did not reliably detect aromatase gonadal gene expression patterns (Table 2). Temperature-based differential aromatase activity has been hypothesized as an upstream marker of ovarian commitment in TSD reptiles (Crews et al., '94; Pieau et al., '99) and aromatase assays using isolated gonad tissue in the pond turtle (*Emys orbicularis*) measured increased aromatase activity at female-producing temperatures late in the TSP (Desvages and Pieau, '92). However, other researchers measuring aromatase expression using AKG target tissue did not detect differences in aromatase expression or activity during the TSP in the slider turtle (Willingham et al., 2000; Murdock and Wibbels, 2003a) or in the alligator (Smith et al., '95; Gabriel et al., 2001). Using isolated gonad tissue as target, we were able to detect higher levels of aromatase expression during the TSP while sex is not yet determined (Ramsey et al., 2007), adding evidence to support aromatase as an early responder to female-producing temperature in TSD reptiles.

This loss of temporal resolution in temperature-specific gonadal gene expression can also be seen with *Sox9*, a gene involved in male sexual development across vertebrate groups. Early reports using AKG tissue did not detect a difference in *Sox9* expression until late in male development (Spotila et al., '98). In isolated gonads, higher *Sox9* expression can be detected at the male-producing temperature during the TSP itself (Shoemaker et al., 2007). Differences in gene expression are attenuated until later in development even in cases where AKG and gonad expression patterns are qualitatively similar. For example *Dmrt1*, a highly conserved gene involved in male sexual development, can be detected at higher levels in the male-producing temperature early in the TSP using AKG tissue (Kettlewell et al., 2000; Murdock and Wibbels, 2003b), but differences are more profound in isolated gonad tissue (Shoemaker et al., 2007).

In AK tissue, only *Sf1* demonstrated temperature-based changes in expression during the sex-determining phase of the TSP. In the AK, *Sf1* was higher at the female-producing temperature at a stage when it was higher at the male-producing temperature in the gonad. Our qPCR gonad-specific measurements are consistent with earlier work reporting male-specific *Sf1* gonadal expression (Fleming et al., '99). Gonadal expression of *Sf1* is attenuated in total AKG measurements, most likely owing to a masking effect of differential expression in the developing AK. For AR, ERα, ERβ, and aromatase, the discordance between patterns of expression the AKG and isolated gonad during the TSP do not seem to result from temperature-specific differences in AK component gene expression. In the absence of temperature-specific expression differences in the AK compartment, any temperature-specific differences in gene expression in the AKG measurements should be because of gonad compartment contribution. However, the AKG patterns do not reflect the patterns in gonad tissue, presumably owing to dilution of the gonadal contribution to the AKG transcript pool.

These results suggest that AKG assays can detect genes such as *Dmrt1* that display large differences between male and female-producing temperatures, but may miss more subtle temperature-specific changes taking place in the gonad. Taken together these results indicate that measurements based on AKG tissue can obscure important temporal relationships between temperature and gene expression within the gonad.

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