A Novel Candidate Gene for Temperature-Dependent Sex Determination in the Common Snapping Turtle

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ABSTRACT Temperature-dependent sex determination (TSD) was described nearly 50 years ago. Researchers have since identified many genes that display differential expression at male- vs. female-producing temperatures. Yet, it is unclear whether these genes (1) are involved in sex determination *per se*, (2) are downstream effectors involved in differentiation of ovaries and testes, or (3) are thermo-sensitive but unrelated to gonad development. Here we present multiple lines of evidence linking *CIRBP* to sex determination in the snapping turtle, *Chelydra serpentina*. We demonstrate significant associations between a single nucleotide polymorphism (SNP) (c63A > C) in *CIRBP*, transcript levels in embryonic gonads during specification of gonad fate, and sex in hatchlings from a thermal regime that produces mixed sex ratios. The A allele was induced in embryos exposed to a female-producing temperature, while expression of the C allele did not differ between female- and male-producing temperatures. In accord with this pattern of temperature-dependent, allele-specific expression, AA homozygotes were more likely to develop ovaries than AC heterozygotes, which, in turn, were more likely to develop ovaries than CC homozygotes. Multiple regression using SNPs in *CIRBP* and adjacent loci suggests that c63A > C may be the causal variant or closely linked to it. Differences in *CIRBP* allele frequencies among turtles from northern Minnesota, southern Minnesota, and Texas reflect small and large-scale latitudinal differences in TSD pattern. Finally, analysis of *CIRBP* protein localization reveals that *CIRBP* is in a position to mediate temperature effects on the developing gonads. Together, these studies strongly suggest that *CIRBP* is involved in determining the fate of the bipotential gonad.

KEYWORDS genetics of sex; cold-inducible RNA-binding protein; temperature-dependent sex determination; genetic association

SEX determination in vertebrates is divided into two broad categories, either genotypic or environmental. Genotypic sex determination (GSD) occurs at fertilization and is governed by an individual's genotype. GSD species often, but not always, display morphologically distinct sex chromosomes, as observed in mammals, birds, snakes, some lizards, and some turtles. While some GSD systems are monogenic, other GSD systems involve two or more genes (Moore and Roberts 2013; Bachtrog *et al.* 2014). Environmental sex determination occurs when extrinsic factors influence whether an

embryo develops into a female or a male. Various abiotic and biotic factors determine sex in metazoans, but temperature is the only environmental factor known to influence sex determination in amniotes (Bull 1983; Janes *et al.* 2010; Merchant-Larios and Díaz-Hernández 2013). This phenomenon is referred to as temperature-dependent sex determination (TSD) and is observed in many lizards, numerous turtles, and all crocodilians studied to date (Ewert *et al.* 1994, 2004; Lang and Andrews 1994; Viets *et al.* 1994; Deeming 2004; Harlow 2004). However, GSD and TSD are not as distinct as this simple description suggests. It has long been recognized that genes and the environment interact to determine sex in TSD species (Bull *et al.* 1982; Janzen 1992; Rhen and Lang 1998) and that TSD and GSD can coexist in the same species (Conover and Heins 1987). More recent work has shown that extreme temperatures can override GSD in a species with heteromorphic sex chromosomes (a ZZ/ZW system) and that an evolutionary transition from GSD to TSD can occur in one

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generation with loss of the W chromosome (Quinn *et al.* 2007; Holleley *et al.* 2015).

The key event during sex determination in gonochoristic vertebrates is irreversible commitment of the bipotential gonads to testicular or ovarian fate. In reptiles with TSD, sex is determined during the middle of embryogenesis when gonads are responsive to changes in ambient temperature (Yntema 1976, 1979; Bull 1987; Mrosovsky and Pieau 1991; Wibbels *et al.* 1991; Lang and Andrews 1994; Merchant-Larios *et al.* 1997, 2010; Burke and Calichio 2014; Rhen *et al.* 2015). A substantial number of genes are differentially expressed in developing gonads at male- vs. female-producing temperatures (reviewed in Place and Lance 2004; Shoemaker and Crews 2009; Rhen and Schroeder 2010). Such genes are candidate sex-determining genes. However, differential expression alone does not reveal whether genes (1) are involved in sex determination *per se*, (2) are downstream effectors involved in differentiation of ovaries and testes, or (3) are thermo-sensitive but unrelated to sex determination or gonad differentiation. Consequently, the mechanism that transduces temperature into a biological signal for ovary vs. testis development has not been found in any species. In fact, we do not even know whether there is a single master TSD gene or whether TSD is polygenic (a.k.a. “parliamentary system”) (Crews and Bull 2009; Georges *et al.* 2010). Here, we define “TSD gene” as a thermosensitive gene involved in determining the fate of the bipotential gonad (*i.e.*, thermosensitive sex-determining gene).

Genetic association and linkage studies have been used to identify sex-determining genes or sex-linked chromosomal regions in several species with GSD (Anderson *et al.* 2012; Hattori *et al.* 2012; Kamiya *et al.* 2012; Myosho *et al.* 2012; Yano *et al.* 2012; Wilson *et al.* 2014). Segregating variation for sex determination has been reported in a TSD lizard (Rhen *et al.* 2011) and two fish with TSD (Vandeputte *et al.* 2007; Yamamoto *et al.* 2014), suggesting that DNA-based genetic analyses could be used to distinguish TSD genes from other classes of genes (*i.e.*, downstream genes involved in gonad differentiation or thermo-sensitive genes that play no part in sexual differentiation of the gonads). Studies that integrate DNA sequence variation, environmental perturbations, and expression data are a particularly powerful way to identify loci causally related to phenotype (Cordell and Clayton 2005; Schadt *et al.* 2005; Ott *et al.* 2011; Lewis and Knight 2012; Gagneur *et al.* 2013). Yet, genetic analyses of this sort have never been conducted in a reptile with TSD.

The common snapping turtle, *Chelydra serpentina*, displays several characteristics that make genetic studies of TSD feasible. Females develop at low and high incubation temperatures, while males are produced at intermediate temperatures (Yntema 1976; Rhen and Lang 1994; Ewert *et al.* 2005). Shifting eggs from a male-producing (26.5°) to a female-producing (31°) temperature for 5 days of a 65-day incubation period is sufficient to induce ovary development in all embryos (Rhen *et al.* 2015). This brief

temperature-sensitive period (TSP) provides a unique opportunity to identify TSD genes because shorter exposures to 31° reveal variation in thermo-sensitivity: *i.e.*, exposure to 31° for 2.5 days causes some individuals to develop ovaries while other individuals develop testes. Controlled breeding studies demonstrate that susceptibility to this temperature shift is genetically determined and that genetic variance is entirely additive (T. Rhen, unpublished results).

Variation in TSD pattern in snapping turtles incubated at constant temperatures also supports the premise that TSD has a genetic basis. Studies within populations have found among-family variation in sex ratio at temperatures that produce mixed sex ratios, which suggests that TSD pattern is heritable (Janzen 1992; Rhen and Lang 1998). Ewert *et al.* (2005) describe a dramatic latitudinal cline for TSD pattern across North America, indicating that snapping turtles are genetically differentiated across distinctive thermal environments. Turtle embryos from Minnesota and Texas also differ in their response to shifts between female- and male-producing temperatures (Rhen *et al.* 2015). These observations provide compelling evidence that a balance between migration and selection maintains the observed cline in TSD pattern, in close accord with theoretical predictions for geographic clines (Haldane 1948; Fisher 1950; Conover and Heins 1987; Schulte *et al.* 2000; Umina *et al.* 2005). In short, segregating variation for sex determination within populations and population differentiation make genetic association studies a practical and powerful approach to find TSD genes.

Cold-inducible RNA-binding protein (CIRBP) was first identified as a candidate TSD gene based on differential expression of messenger RNA (mRNA) in embryonic gonads at male- vs. female-producing temperatures (Rhen and Schroeder 2010). The molecular, cellular, and biological function of CIRBP in other species makes this a promising gene for further study. CIRBP has a glycine-rich carboxy terminus and a single RNA recognition motif (RRM) near its amino terminus that plays a crucial role in mRNA processing, RNA export, and translation (Dreyfuss *et al.* 2002; Maris *et al.* 2005). CIRBP is known to regulate temperature-dependent cellular processes through translational repression and mRNA stabilization (Yang *et al.* 2006; De Leeuw *et al.* 2007; Xia *et al.* 2012; Liu *et al.* 2013). Furthermore, research suggests that CIRBP is involved in reproduction, although its precise function remains unclear (Elliot 2004; Xia *et al.* 2012).

Here, we conduct experiments spanning multiple levels of biological organization to test the hypothesis that CIRBP plays a role in TSD. We confirm the temporal pattern of CIRBP mRNA expression described in an earlier article (Rhen and Schroeder 2010) and provide new data on protein localization in embryonic and hatchling gonads. More importantly, we identify a single nucleotide polymorphism at codon 63 in CIRBP (c63A > C). Although these variants are synonymous, they exhibit allele-specific expression. Differences in expression of alternative alleles can have important phenotypic consequences (Knight *et al.* 2003; Pickrell *et al.* 2010). While expression of one CIRBP allele (A) is thermosensitive, the

other allele (C) is not. We also detect significant associations between *CIRBP* genotype, *CIRBP* expression in embryonic gonads, and sex determination at the individual, family, and population level. In contrast, adjacent loci were not associated with sex determination when tested via multiple logistic regression. Taken together, these studies meet all criteria for establishing the veracity of genetic associations, including moderate sample size, replication, and protection from bias due to genotyping error or population stratification (Ioannidis *et al.* 2008). To our knowledge, this is the first genetic association between a specific gene and sex to be reported in any TSD reptile. As such, it sets the stage for genome-wide association studies to elucidate the genetic architecture underlying TSD in the snapping turtle (*i.e.*, the number of loci, magnitude of gene effects, allele frequencies, allelic effects, and interactions among sex-determining genes).

Materials and Methods

Collection of eggs and adults

We collected eggs from snapping turtles nests in June 2008, 2009, and 2010. Adult turtles were collected in June, July, and August of the same years. Collection sites extended from the Iowa border to the Canadian border within the state of Minnesota. Adult snapping turtles were also collected on the Lake Lewisville U.S. Army Corps of Engineers property, Lewisville, Texas, in May of 2013 and 2014. Eggs and adults were transported to the animal quarters in the Biology Department at the University of North Dakota. Experiments were carried out according to a protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota (Protocol #0905-1).

Egg incubation and tissue collection

Eggs were washed in tepid water and candled to separate fertile and infertile eggs. Eggs from each clutch were randomly assigned to various experimental treatments, placed in plastic containers, and completely covered in moist vermiculite (1 part vermiculite:1 part water by mass). Egg containers were placed in foam box incubators set to a nominal temperature of 26.5°, which produces exclusively male hatchlings. Depending upon the particular study, eggs were either maintained at 26.5° throughout incubation or shifted to 31° for varying periods of time during the TSP. Embryos from each clutch were periodically sampled to monitor developmental rate and ensure embryos were at the desired stages for treatments [staging according to Yntema (1968)]. Equal (or roughly equal) numbers of eggs from each clutch were randomly assigned to each experimental group within each study. Thus, we were able to use clutch identity as a blocking factor/independent variable in all studies.

This experimental design is based on an extensive set of studies where eggs were shifted between different female- and male-producing temperatures at various stages of development to define the TSP for sex determination (Rhen *et al.* 2015). The entire TSP broadly encompasses Yntema stage 14

to stage 21 (Supplemental Material, Figure S1), but varies with the order of temperature shifts (male to female vs. female to male), the precise temperatures used (some temperatures are more potent at masculinizing or feminizing embryos than other temperatures), as well as the duration and timing of exposure to different temperatures. Those studies uncovered a brief sex-determining period in snapping turtles exposed to a specific thermal regime. Moving embryos from a male temperature (26.5°) to a female temperature (31°) for 5 days of a 65-day incubation period induces ovarian development in all stage 17 embryos. The sex-determining period for this thermal regime in snapping turtles (~8% of embryogenesis) is comparable to the sex-determining period in mice (~10% of embryogenesis) (Figure S1).

We used this temperature shift paradigm to produce mixed sex ratios instead of a constant pivotal temperature for two major reasons. First, we can focus on a brief period when high temperature (31°) first specifies (days 1–3 of the shift) and then determines (days 4–5 of the shift) ovarian fate (exposure starting at stage 17–17.5). Gonads in embryos kept at 26.5° during this same 5-day period are fated to become testes. This facilitates identification of causal TSD genes by allowing analysis of molecular changes precisely when temperature specifies gonad fate. In contrast, we would have to sample embryos over a 2-week period from stage 14 to stage 21 (the entire TSP) if eggs were incubated at a constant pivotal temperature. Second, incubation of eggs at a constant pivotal temperature throughout development is unnatural. Eggs in nature normally experience temperature fluctuations on various temporal scales (Wilhoft *et al.* 1983; Packard *et al.* 1985; Congdon *et al.* 1987; Kolbe and Janzen 2002). Thus, a temperature-shift paradigm is arguably a more natural way to produce mixed sex ratios and reveal phenotypic and genetic variation in temperature sensitivity.

Euthanasia of embryos and hatchlings was by rapid decapitation. Adrenal–kidney–gonad complexes were quickly dissected from embryos and hatchlings and either placed in RNAlater or fixed in 4% paraformaldehyde. Tissues in RNAlater were kept at –20° for long-term storage. Tissues in 4% paraformaldehyde were kept overnight at 4°, then washed in 1× PBS, and transferred to 70% ethanol for long-term storage.

RNA extraction from gonads and Complementary DNA synthesis

Gonads were microdissected from the underlying adrenal–kidney complex as previously described (Rhen *et al.* 2007). We then extracted total RNA from gonad pairs of individual embryos using the PicoPure RNA Isolation Kit (Life Technologies). A subset of samples from one study was extracted with the RNeasy Micro Kit (Qiagen) for comparison to the PicoPure Kit. Purified RNA (150 ng) from each gonad pair was reverse-transcribed in 20-μl reactions using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Gonad complementary DNA (cDNA) was diluted to the equivalent of 1.25 ng input RNA/μl for use in real-time PCR reactions. Thus,

we measured gene expression in pure gonad preparations that were completely separated from adrenal and kidney tissue.

DNA extraction from kidneys

Genomic DNA was isolated from kidneys using TRI Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati). Genomic DNA was isolated from blood using a phenol/chloroform/isoamyl alcohol protocol available online at http://www.genome.ou.edu/protocol_book/protocol_partIII.html#III.H. The concentration and purity of DNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The integrity of DNA was determined by agarose gel electrophoresis. Purified genomic DNA was stored at -20° until used for genotyping.

PCR methods for measuring gene expression and for genotyping

We used dye-based and probe-based PCR to measure *CIRBP* expression and to determine *CIRBP* genotypes. Real-time PCR was performed using the Bio-Rad CFX 384 Real-Time PCR System with SsoFast EvaGreen supermix according to the manufacturer's instructions (Bio-Rad). Each 10- μ l PCR reaction contained 5 μ l of the 2 $\times$ EvaGreen supermix, 0.3 μ l of the forward primer (0.3 μ M final concentration), 0.3 μ l of the reverse primer (0.3 μ M final concentration), 2.4 μ l of water, and 2 μ l of cDNA (input = 2.5 ng total RNA). Standard curves were made via serial dilution of purified PCR products as described in Rhen *et al.* (2007).

We designed Taqman probes (Life Technologies) and primers to distinguish the SNP at position c63A > C in the open reading frame of snapping turtle *CIRBP*. SsoFast probes supermix (Bio-Rad) was used for allele-specific expression assays and genotyping. We isolated PCR products that contained only the A allele or only the C allele. We empirically determined the optimal annealing and extension temperature (64.3°) for distinguishing these alleles. We then made standard curves that were specific for each *CIRBP* allele: the A probe was hydrolyzed only when the A template was present (Figure S2A), while the C probe was hydrolyzed only when the C template was present (Figure S2B). Slopes and intercepts for standard curves were statistically homogeneous. We could therefore directly and quantitatively compare the abundance of transcripts for each allele and accurately genotype individuals.

We also designed primers for high-resolution melt temperature (HRM) analysis of PCR products to confirm *CIRBP* genotypes for the SNP at position c63A > C. We determined optimal conditions for distinguishing AA homozygotes, AC heterozygotes, and CC homozygotes using 10 ng of genomic DNA as template. Amplification was via three-step PCR. Samples were heated to 98° for 2 min and then subjected to 40 PCR cycles: 98° for 5 sec (denature), 53° for 1 sec (anneal), and 60° for 5 sec (extend). After 40 cycles, we collected HRM data from 60° to 85° at 0.2° increments and 10-sec read times. HRM data were imported into Bio-Rad Precision Melt Analysis Software. Optimal settings for *CIRBP* genotyping

were determined using pure A standard as template, pure C standard as template, and an equal mix of A and C standards as template (pre-melt temperature = 70.4 – 70.5° ; post-melt temperature = 75.7 – 75.8° ; temperature shift bar height = 0.20; melt shape = 35; T_m = melt temperature difference = 0.25°).

In addition, we designed primers for HRM genotyping of loci presumably linked to *CIRBP*. The snapping turtle genome has not yet been sequenced so we used comparative genomics to identify seven protein-coding genes that display conserved gene order in human, chicken, painted turtle, and Chinese softshell turtle genomes: *STK11*, *ATP5D*, *MIDN*, *CIRBP*, *C19orf24*, *EFNA2*, and *CAMK4L*. Given the synteny across mammals, birds, and divergent turtle species, these genes are most likely linked in the snapping turtle. We used Illumina sequencing data from the snapping turtle to identify SNPs within these genes (more details are provided in *Sequencing and Bioinformatics, SNP identification, and RNA-Seq* below). We optimized HRM genotyping for these SNPs as described above. Sequences for PCR primers and probes are provided in Table S1.

Temperature shifts to characterize *CIRBP* mRNA and protein expression patterns

We conducted an experiment to define the time course for *CIRBP* mRNA induction. We collected gonads from embryos incubated at 26.5° and from clutch mates shifted from 26.5° to 31° at stage 16. Approximately equal numbers of embryos from each temperature were sampled at 6, 12, 24, and 48 hr of the temperature shift. Tissue was collected and RNA extracted from embryonic gonads as described above.

A similar experiment was conducted to examine *CIRBP* protein expression during and after the sex-determining period. Eggs were incubated at 26.5° until embryos reached stage 16. At that time, half of the eggs were kept at 26.5° to produce males, while half were shifted to 31° for 6 days to produce females. Embryos were sampled from both temperatures on days 1, 2, 3, 4, 5, and 6 of the shift. We also sampled embryos after eggs at 31° were returned to 26.5° (days 11, 21, 31, and at hatch). Adrenal–kidney–gonad complexes were fixed in 4% paraformaldehyde for 24 hr at 4° and then transferred to 70% ethanol for long-term storage.

***CIRBP* expression–sex ratio correlation study**

We conducted an experiment to examine the relationship between *CIRBP* mRNA expression in gonads during the TSP and sex ratio at hatching. Snapping turtle eggs from nine randomly collected clutches were incubated at 26.5° until embryos reached stage 17.5 of development. At stage 17.5, eggs were shifted to 31° for 2.5 days and then shifted back to 26.5° for the rest of development. This is the developmental stage when snapping turtle embryos are most sensitive to the feminizing effect of high temperatures (Rhen *et al.* 2015). Roughly equal numbers of embryos from each of the nine clutches were sampled at 24 and 48 hr of the shift to 31° . Tissue was collected and RNA was extracted from embryonic gonads as described above. A subset of eggs from each clutch was allowed to hatch to

determine sex ratios for each clutch. The sex of hatchlings was diagnosed based on gross morphology of gonads and reproductive tracts as previously described (Rhen and Lang 1994).

Sequencing

We used cDNA from the gene expression–sex ratio correlation study described above as template for PCR amplification of the entire CIRBP-coding sequence in multiple individuals. Purified PCR products were Sanger-sequenced using BigDye as previously described (Rhen *et al.* 2007). Sequencher software was used to align sequences and search for SNPs.

We also collected 12 clutches from southern Minnesota and 13 clutches from northern Minnesota for RNA-Seq (range in latitude = 43.511° North to 48.720° North). Approximately equal numbers of eggs from these clutches were separated into two experimental groups. One group of eggs was incubated at 26.5° throughout development to produce males. The other group of eggs was incubated at 26.5° until stage 17.5, and eggs were then shifted to 31° for 6 days to produce females and returned to 26.5° for the rest of development. Embryos from both groups were sampled on days 1, 2, 3, 4, and 5 of the temperature shift. Tissue was collected and RNA was extracted from embryonic gonads as described above.

We combined an equivalent amount of total RNA from gonad pairs from individual embryos to make 20 pools for RNA-Seq: 2 temperatures × 5 days × 2 biological replicates. Total RNA from 25 embryos was pooled for each biological replicate. All together, we sampled 500 embryos from 25 clutches collected across a wide geographic range to capture as much genetic variation as possible (5° range in latitude from the southern-most clutch to the northern-most clutch within Minnesota). Ten pools (2 temperatures × 5 days) were from eggs collected in southern Minnesota (12 clutches). The other 10 pools (2 temperatures × 5 days) were from eggs collected in northern Minnesota (13 clutches). Clutches of eggs from northern and southern Minnesota were separated by latitude of 46° 55' N. Total RNA was sent to the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign for sequencing. Twenty individual cDNA libraries were prepared with unique barcodes and sequenced on Illumina HiSeq 2000 (100 bp, single-end reads; 156.4 million reads).

Additional sequence data using tissues derived from all three germ layers enabled *de novo* assembly of a more complete snapping turtle transcriptome. The data described above were supplemented with sequence data from eight hypothalamus/pituitary libraries (50 bp, single-end Illumina reads; 172.2 million reads), two intestine libraries (50 bp, single-end Illumina reads; 31.8 million reads), as well as normalized embryonic gonad libraries (454 Sequencing, average read length = 350 bp; 2.8 million reads).

Individual and population-based genetic association studies

Snapping turtle eggs from another 15 clutches from northern Minnesota were incubated at 26.5° until stage 17.5 of

development. Eggs were shifted to 31° for 2.5 days and then shifted back to 26.5°. Similar numbers of embryos were sampled from each clutch at 24 and 48 hr of the exposure to 31°. Tissue was collected and RNA was extracted from embryonic gonads as described above. A subset of eggs from each clutch was allowed to hatch to determine sex ratios and to collect tissue for genotyping. Hatchling sex was diagnosed, adrenal–kidney–gonad complexes were dissected, snap-frozen, and stored at –80° until genomic DNA was isolated from kidneys. Hatchlings were genotyped using TaqMan MGB probes. Genotypes were verified using a different set of PCR primers and high-resolution melt temperature analysis.

Equal numbers of adult turtles were collected from the southern half and the northern half of Minnesota to compare allele frequencies in meta-populations experiencing gene flow, but differing slightly in TSD pattern. A small number of turtles from Texas were available for comparison to a geographically distinct population unlikely to experience gene flow with turtles in Minnesota. We drew blood from the subcarapacial vein of adult turtles as described by Moon and Hernandez Foerster (2001). Whole blood was transferred to a microfuge tube and kept on ice until genomic DNA was extracted (within a few hours).

We genotyped adult snapping turtles captured in northern Minnesota ($n = 28$), southern Minnesota ($n = 28$), or northern Texas ($n = 9$). Genotypes for 481 individual embryos and hatchlings were also available from 15 clutches collected in northern Minnesota (*i.e.*, from the genetic association study). To avoid pseudoreplication (*i.e.*, siblings within a clutch are not independent samples), we used genotype frequencies within families to infer parental genotypes. Most clutches ($n = 14$) exhibited genotype frequencies consistent with Mendelian inheritance from a single mother and a single father. One clutch displayed genotype frequencies, suggesting multiple paternity. Thus, we inferred 31 genotypes for parents of embryos and hatchlings from the genetic association study.

Bioinformatics, SNP identification, and RNA-Seq

We used CLC Genomics Workbench (CLC bio, Cambridge, MA) for read mapping, SNP verification, and RNA-Seq analyses. We mapped reads to full-length CIRBP cDNA and determined the number of reads that were mapped and not mapped from each cDNA library (20 total gonad libraries). We used the SNP detection tool to identify polymorphisms in CIRBP cDNA. Parameters for SNP detection required a minimum coverage of 20 reads for each SNP and a minimum variant frequency of 10%. Illumina reads uniquely mapped to CIRBP were then used to estimate allele frequencies in populations from northern and southern Minnesota. A recent article shows that pooled RNA-Seq data can be used to estimate allele frequencies with accuracy similar to pooled genome sequencing (Konczal *et al.* 2013). As indicated in the previous section, we also conducted a completely independent study in which we extracted DNA and genotyped adult and hatchling turtles using traditional methods.

Figure 1 Expression of *CIRBP* mRNA increased in gonads of snapping turtle embryos shifted from 26.5° to 31°, but not in gonads from embryos kept at 26.5°. (A) qPCR results for embryos sampled at 6, 12, 24, and 48 hr of the temperature shift. (B) RNA-Seq results for pooled samples from embryos at 1, 2, 3, 4, and 5 days of the temperature shift. Sample sizes in bars indicate the number of individual embryos (A) or pooled samples (B) in each experimental group.

The same procedure was used to identify SNPs in a set of genes flanking *CIRBP*. The snapping turtle genome has not yet been sequenced so we used comparative genomics to identify genes likely to be linked to *CIRBP*. Six protein-coding genes are syntenic with *CIRBP* in human, chicken, painted turtle, and Chinese softshell turtle genomes in the following chromosomal arrangement: *STK11*, *ATP5D*, *MIDN*, *CIRBP*, *C19orf24*, *EFNA2*, and *CAMK4L*. These genes span ~650 kb in a painted turtle scaffold (gi|371559100|gb|JH584733.1) and exhibit highly correlated physical distances in the homologous Chinese softshell turtle scaffold (gi|351669362|gb|JH209284.1) ($r = 0.9988$, $n = 7$). We used sequence data from snapping turtle gonads, hypothalamus/pituitary, and intestine to identify 12 common SNPs in these genes.

We used Illumina single-end reads to measure allelic-specific *CIRBP* expression. We normalized gene expression using reads per kilobase of exon per million mapped reads (RPKM) (Mortazavi *et al.* 2008). We explicitly estimate allele-specific expression by counting only those reads that contain the SNP (Jiang and Wong 2009). We calculated expression values for alternative alleles using uniquely mapped reads ($n = 4,744$) that included position 63 within the *CIRBP*-coding sequence. We used 199 bp as the length of the transcript for allele-specific RPKM calculations because Illumina reads from gonads were 100 bp long and had to overlap the SNP to be informative. Other methods, such as RNA-Seq by Expectation-Maximization, use uninformative reads to calculate expression values for splice isoforms (or alleles) by assigning fractions of uninformative reads in proportion to counts from informative reads (Li *et al.* 2010). The assumption that uninformative reads are from a particular isoform (or allele) is unfounded and could lead to biased expression estimates for splice variants and for alternative alleles. Thus, we have chosen to be conservative and only use informative, uniquely mapped reads for our analysis of allelic-specific expression. Two other SNPs were detected in the 3' UTR of *CIRBP*. Although we can unambiguously map reads containing these SNPs, we cannot determine linkage relationships

among SNPs separated by >100 bp from single-end RNA-Seq data. We therefore analyzed each SNP separately.

Linkage analysis

We used PCR and HRM analyses to determine genotypes for markers in *STK11*, *ATP5D*, *MIDN*, *CIRBP*, *C19orf24*, *EFNA2*, and *CAMK4L* in 56 adult snapping turtles from Minnesota. We assigned numerical values of 0, 1, or 2 based to the genotype at each locus: homozygotes for rare allele = 0, heterozygotes = 1, and homozygotes for common allele = 2. We excluded *ATP5D* from further analysis because the frequency of the rare allele was too low (0.027) to be useful as a marker. We calculated pairwise correlations among genotypes for all other loci and squared this value (r^2) to get a standardized measure of LD as outlined by Rogers and Huff (2009).

Immunohistochemistry

Paraformaldehyde-fixed tissues were embedded in paraffin, sectioned at 6 μ m, and placed on microscope slides. Sections were de-paraffinized and rehydrated using standard immunohistochemistry protocols. The primary *CIRBP* antibody was diluted 1:500 (caalog no. ARP40348_P050, Aviva Systems Biology, San Diego). Negative controls were incubated in blocking solution without the primary antibody. The Vecta-Stain ABC Elite kit (Vector Laboratories) was used to detect the primary/secondary antibody complex. Staining was performed using fresh 3, 3'-diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin. Images were captured with an Olympus BX-51 microscope equipped with an Infinity 2 digital camera (Lumenera, Ottawa) using Rincon HD software (Imaging Planet, Goleta, CA).

Statistical analyses

We used three-way analysis of variance (ANOVA) with clutch, temperature, and day as main effects to analyze *CIRBP* mRNA expression in the quantitative PCR (qPCR) study. Significant main or interaction effects were followed by comparisons among specific groups of interest. The Dunn-Sidak method was used to correct for multiple comparisons. The nominal

Figure 2 Spatial distribution of CIRBP protein in gonads from embryos shifted from 26.5° to 31° (A–C) and from embryos kept at 26.5° (D–F). There was no detectable staining in the negative control when 1° antibody to CIRBP was omitted (inset of A). The overall pattern of CIRBP expression was similar in gonads throughout the TSP, including on day 6 of the temperature shift (A and D). However, CIRBP expression diverged in nascent ovaries and testes on day 21 with prominent staining of medullary cords in males (E) but a lack of cord staining in females (B). The expression pattern for CIRBP was quite distinct in ovaries and testes on day 31: staining was heavy in the cortex of ovaries and in the seminiferous tubules of testes near hatching (C and F).

significance level was calculated as $\alpha' = 1 - (1 - \alpha)^{1/k}$, where k is the number of comparisons for an experiment-wise $\alpha = 0.05$. Spearman's rank correlation was used to test for a correlation between *CIRBP* mRNA expression and sex ratio in clutch mates. This nonparametric test is appropriate when data do not meet assumptions for the Pearson product-moment correlation (*i.e.*, a linear relationship between variables). We used likelihood-ratio (LR) chi-square tests or Fisher's Exact Tests for variation in sex ratios among clutches and for associations between genotype and sex. In another test for association between *CIRBP* genotype and sex, we used a matched-pairs *t*-test to control for genetic background: allele frequencies in males and females were compared after pooling clutches with very similar numbers of males and females (Risch and Teng 1998; Teng and Risch 1999). We used the Cochran–Mantel–Haenszel test to compare allele frequencies between northern and southern Minnesota populations while controlling for temperature effects on transcript levels in the RNA-Seq study. In other words, data were stratified by temperature, thus allowing comparison of northern and southern Minnesota populations. Statistics for gene expression and genetic associations were performed using JMP 5.0.1.2 software (SAS Institute, Cary, NC). Effects were considered significant at $P \leq 0.05$. Throughout the text, means are given $\pm$ SEM ($\pm$ SE).

Data availability

Data files are uploaded as supplementary files: Data for Figure 1 contain raw and processed expression data for Figure 1. Data for Figure 3 contain clutch sex ratios and mean expression data for Figure 3. Data for Figure 3 contain raw expression data for Figure 3. Data for Figure 4A contain read counts

for alternative CIRBP alleles from RNA-Seq study and summary data plotted in Figure 4A. Data for Figure 4B contains raw expression data and summary data plotted in Figure 4B. Data for Figure 4C, Table 2, and Figure S2 contain individual genotype and phenotype data for those figures and table. Data for Figure 5 contain raw data presented in Figure 5. Data for Figure 6 and Figure 7 contain individual genotype data for those figures.

Results

CIRBP mRNA and protein expression patterns

We incubated three clutches of eggs at 26.5° until embryos reached stage 16. Half of the eggs from each clutch were then shifted to 31°, while half were kept at 26.5°. We sampled embryos at 6, 12, 24, and 48 hr to measure *CIRBP* mRNA expression. Temperature (ANOVA, $F_{1,130} = 23.0$, $P < 0.0001$), time (ANOVA, $F_{3,130} = 70.8$, $P < 0.0001$), and the temperature $\times$ time interaction (ANOVA, $F_{3,130} = 7.9$, $P < 0.0001$) all influenced *CIRBP* mRNA levels. Expression of *CIRBP* mRNA did not differ between male and female temperatures at 6 hr ($P = 0.65$) or 12 hr ($P = 0.97$), but was significantly different at 24 hr ($P = 0.005$) and 48 hr ($P < 0.0001$) (Dunn-Sidak $\alpha' \leq 0.005$ for 10 comparisons; Figure 1A).

We tested for developmental changes within each temperature. At 31°, there was no detectable change from 6 to 12 hr ($P = 0.75$). Expression of *CIRBP* mRNA increased significantly from 6 to 24 hr ($P = 0.0002$) and from 6 to 48 hr at 31° ($P < 0.0001$) (Figure 1A). At 26.5°, there was no detectable change from 6 to 12 hr ($P = 0.95$) or from 6 to 24 hr ($P = 0.19$). However, *CIRBP* expression did increase significantly from 6 to 48 hr at 26.5° ($P < 0.0001$) (Figure 1A).

Figure 3 Correlation between *CIRBP* mRNA expression and sex ratio. Siblings were either sampled as embryos to measure *CIRBP* expression in gonads or allowed to hatch to determine sex ratio for each clutch. (A) Sex ratio of hatchling snapping turtles from nine clutches of eggs incubated at 26.5° for most of development. Eggs from each clutch were exposed to 31° for 2.5 days during the TSP. The number of hatchlings from each clutch is shown within each bar. (B) Correlation between *CIRBP* expression and sex ratio in siblings exposed to 31° for 2.5 days. Gene expression values represent the mean for embryos from each clutch. Sex ratios for each clutch are from A.

We also examined data from an independent RNA-Seq study in which embryos were sampled on days 1, 2, 3, 4, and 5 of the temperature shift (Figure 1B). Temperature (ANOVA, $F_{1,10} = 313.2$, $P < 0.0001$), time (ANOVA, $F_{4,10} = 9.5$, $P = 0.002$), and the temperature $\times$ time interaction (ANOVA, $F_{4,10} = 7.9$, $P = 0.004$) were all significant. Expression of *CIRBP* mRNA was higher in 31° RNA libraries compared to 26.5° RNA libraries on day 1, but the difference was not significant ($P = 0.01$) after the Dunn-Sidak correction ($\alpha \leq 0.005$ for 10 comparisons). Expression of *CIRBP* was significantly higher in 31° RNA libraries than in 26.5° RNA libraries on days 2–5 ($P < 0.0001$ for all comparisons; Figure 1B). There was a significant increase in *CIRBP* expression in 31° embryos from day 1 to days 2–5 ($P = 0.002$, $P = 0.0001$, $P < 0.0001$, respectively) (Figure 1B). In contrast, there was no detectable change in *CIRBP* expression between day 1 and days 2–5 at 26.5° ($P = 0.70$, $P = 0.59$, $P = 0.91$, respectively). These data indicate that a steady-state difference in *CIRBP* expression between male- and female-producing temperatures is established within 48 hr.

We next used immunohistochemistry to examine the spatial pattern of *CIRBP* protein expression during sex determination (days 1–6 of a temperature shift) and during gonad differentiation (days 11, 21, 31, and hatch). Eggs were incubated at 26.5° throughout embryogenesis to produce males or were exposed to 31° for 6 days to produce females. Staining for *CIRBP* was very strong in embryonic gonads (compare Figure 2A to the negative control shown in the inset). Expression patterns were very similar at female- and male-producing temperatures during the TSP (Figure 2, A and D, respectively). *CIRBP* was expressed in the primary sex cords and in cells of the coelomic epithelium. Expression patterns began to diverge after the temperature shift on day 21. While staining of medullary sex cords was no longer evident in differentiating ovaries, staining of these structures remained prominent in differentiating testes (Figure 2, B and E, respectively). The spatial pattern of *CIRBP* expression differed even more dramatically on day 31: staining clearly defined the ovarian cortex in female embryos and seminiferous tubules in male embryos (Figure 2, C and F, respectively).

Family-based correlation between *CIRBP* expression and sex ratio

Nine clutches of eggs were incubated at a male-producing temperature (26.5°) for most of development and exposed to a female-producing temperature (31°) for 2.5 days during the TSP. Exposure to 31° started at stage 17.5 and produced an overall sex ratio of 30% males (34 males:80 females). There was significant variation in sex ratio among clutches, ranging from one clutch that produced exclusively females to one clutch that produced a male-biased sex ratio (LR $\chi^2 = 25.9$, d.f. = 8, $P = 0.0011$; Figure 3A).

A subset of embryos from each clutch was sampled on day 1 or 2 of the temperature shift to measure *CIRBP* expression. Levels of *CIRBP* mRNA were affected by clutch identity (ANOVA, $F_{8,78} = 2.06$, $P = 0.05$) but not by day of sampling (ANOVA, $F_{1,78} = 2.19$, $P = 0.14$) or the clutch $\times$ day interaction (ANOVA, $F_{8,78} = 1.74$, $P = 0.10$). We used RNA extraction kit as an independent variable in the model because we used two different kits for this experiment (ANOVA, $F_{1,78} = 13.6$, $P = 0.0004$). We also used 18S ribosomal RNA (rRNA) as a covariate (ANOVA, $F_{1,78} = 9.65$, $P = 0.003$) to control for variation in RNA extraction efficiency. We observed a negative correlation between *CIRBP* mRNA expression and sex ratio among clutches (Spearman's Rho = -0.70, $P = 0.0358$; Figure 3B). Clutches with lower *CIRBP* mRNA expression produced more males, while clutches with higher *CIRBP* mRNA expression produced more females.

SNP identification and verification

We sequenced the *CIRBP*-coding sequence in four clutches with the most extreme sex ratios to search for polymorphisms that might explain this familial correlation. We identified a SNP at position c63A > C in the open reading frame for *CIRBP*. Offspring from female-biased clutches were AA homozygotes ($n = 8$ individuals), while offspring from male-biased clutches were either AA homozygotes ($n = 6$ individuals) or AC heterozygotes ($n = 5$ individuals). Genotype frequencies differed among groups even though sample size was small (Fisher's Exact Test, d.f. = 1, $P = 0.0445$).

We used high-throughput sequence data from an independent RNA-Seq study to verify the c63A > C polymorphism and to search for other polymorphisms and splice variants. Of

Figure 4 Allele-specific *CIRBP* expression and temperature-dependent sex determination. (A) Incubation temperature had different effects on expression of alternative *CIRBP* alleles in gonads from snapping turtle embryos. RNA-seq was used to measure *CIRBP* expression in pooled RNA samples. The number of pooled samples is shown in each bar. (B) Allele-specific expression of *CIRBP* in gonads of embryos exposed to 31° for 2.5 days. TaqMan probes were used to measure *CIRBP* expression in individual embryos. Least-squares means and standard errors are from two-way ANOVAs using genotype and sampling day as main effects and 18S rRNA as a covariate. The number of embryos of each genotype is shown within the bars. (C) Association between *CIRBP* genotype and sex in hatchling snapping turtles exposed to 31° for 2.5 days during the TSP. The number of hatchlings of each genotype is shown within the bars.

156.4 million Illumina reads from embryonic gonads, 176,583 reads mapped uniquely to *CIRBP* cDNA. This analysis revealed that the SNP c63A > C was fairly common: 3939 reads mapping to this position contained an A, while 805 reads contained a C. These variants are synonymous (TCA to TCC) for serine 21 in the primary structure of CIRBP. Two additional SNPs were found in the 3' UTR at position 1147 (5756 reads with G; 3423 reads with A) and position 2092 (3919 reads with T; 1026 reads with A) of full-length *CIRBP* cDNA. Finally, we recovered two isoforms produced by alternative splicing of exons at the carboxy terminus of *CIRBP*. These isoforms are conserved among snapping turtles, chickens, and humans.

Temperature-dependent, allele-specific expression of *CIRBP*

We utilized RNA-Seq data to test for temperature effects on expression of alternative *CIRBP* alleles and splice variants. Both splice variants displayed the same expression pattern (results not shown). Temperature had different effects on expression of alternative *CIRBP* alleles at position 63 as indicated by a significant temperature $\times$ allele interaction (ANOVA, $F_{1,20} = 14.7$, $P = 0.001$). The A allele was induced by shifting embryos to 31°, while expression of the C allele did not differ between embryos at 31° and 26.5° (Figure 4A). A similar pattern was observed for SNPs at position 1147 and position 2092 within the 3' UTR: exposure to 31° increased expression of the common allele, but not the rare allele (results not shown). These data reveal temperature-dependent, allele-specific expression of *CIRBP* in gonads during the TSP.

Genetic association between *CIRBP* and sex determination

We used TaqMan MGB probes to directly test for associations between *CIRBP* c63A > C genotype, allele-specific expression, and sex in individual embryos and hatchlings (rather than pooled samples as for RNA-Seq). We repeated the study in which eggs were incubated at 26.5° and exposed to 31° for 2.5 days during the TSP to produce mixed sex ratios. Embryos from 15 clutches were sampled on day 1 or 2 of the

temperature shift to measure allele-specific expression or allowed to hatch to test for associations between genotype and sex. Homozygotes for the A allele had higher *CIRBP* expression than homozygotes for the C allele when exposed to the female-producing temperature (Figure 4B). The A allele in heterozygotes was expressed at roughly half the level observed in AA homozygotes (Figure 4B). Expression of the C allele in heterozygotes was about half the level observed in CC homozygotes (Figure 4B). Although mean levels of the A and C alleles did not differ in heterozygotes, expression of the A allele was significantly more variable than the C allele when we compared their variance ($F_{49,49} = 6.8$, $P < 0.00001$; Figure 4B). These results were consistent with the allele-specific pattern of expression in the RNA-Seq study.

The 2.5-day exposure to 31° produced a sex ratio of 72% males (200 males:77 females). Sex ratio was more male-biased in this study than in the previous study, but there was still significant variation in sex ratio among clutches (LR $\chi^2 = 83.4$, d.f. = 14, $P < 0.0001$). In fact, one clutch produced exclusively females while another clutch produced only males (Figure S3). There was a significant association between hatchling genotype and sex (LR $\chi^2 = 25.4$, d.f. = 2, $P < 0.0001$): 65% of A/A homozygotes were males, 85% of A/C heterozygotes were males, and 100% of C/C homozygotes were males (Figure 4C).

Because TSD could be polygenic, siblings may share alleles for other sex-determining genes. One way to mitigate the potentially confounding effect of genetic similarity at other loci involves pooling families with the same structure (i.e., the same number of affected and unaffected siblings) and testing for a difference in allele frequency between affected and unaffected individuals within groups (Risch and Teng 1998; Teng and Risch 1999). We pooled 15 families into nine groups with very similar sex ratios (i.e., differences between pooled clutches were 1, 6, 1.9, 0, 0.9, and 1.4%). Groups with exclusively males or females were excluded because both sexes were not available for comparison of allele frequencies. However, it is interesting to note offspring in the clutch that produced all males were homozygotes for the C allele (clutch 15 in Figure S3), while offspring in the clutch that produced

Figure 5 Sex ratio in hatchling snapping turtles as a function of incubation temperature and population origin. Eggs were collected from natural nests in southwestern Minnesota ($n = 8$ nests in the Pipestone area) and north central Minnesota ($n = 24$ nests near Lake Itasca). Eggs were brought to the University of North Dakota for incubation under identical conditions. Sex ratios for snapping turtles from Claiborne Parish, Louisiana, are from data reported by Ewert *et al.* (2005). Bars represent binomial standard errors.

all females were homozygotes for the A allele (clutch 36 in Figure S3). The distribution of families among the remaining groups was quite even (*i.e.*, two families/group for six groups and one family in the last “group”). Sample size was very similar for each group so we applied a matched pairs *t*-test. The mean difference in frequency of the C allele between males and females (0.026 ± 0.009 SE, $n = 7$) was significantly different from zero (t -ratio = 2.8, d.f. = 6, $P = 0.03$). The C allele was found at a higher frequency in males than in females, even after excluding two clutches that could have driven the genetic association at the individual level. These findings demonstrate that the C allele is associated with testis determination while the A allele is associated with ovary determination.

Population-based genetic association between *CIRBP* and sex determination

We used RNA-Seq data to test for a population difference in frequency of *CIRBP* c63A > C variants. Using the Cochran–Mantel–Haenszel test to control for temperature effects on transcript levels, we found that the C allele was more common in northern Minnesota (~ 0.204) than it was in southern Minnesota (~ 0.135) ($LR \chi^2 = 38.25$, d.f. = 1, $P < 0.0001$). In agreement with this result, allele frequencies also differed between southern and northern populations within each temperature (at 26.5°, $LR \chi^2 = 45.58$, d.f. = 1, $P < 0.0001$; at 31°, $LR \chi^2 = 5.27$, d.f. = 1, $P = 0.02$). These populations differ by $\sim 0.5^\circ$ in their pivotal temperature (*i.e.*, the constant temperature that produces a 1:1 sex ratio) (Figure 5).

Because estimation of allele frequencies from RNA-Seq data is a relatively new approach (Konczal *et al.* 2013), we also estimated *CIRBP* allele frequencies in a completely independent study using traditional DNA-based genotyping.

Table 1 Summary of *CIRBP* genotype frequencies for adult snapping turtles from different populations

Population	<i>CIRBP</i> genotype	Frequency
Northern Minnesota	AA	21
Northern Minnesota	AC	7
Northern Minnesota	CC	0
Northern Minnesota (parents of eggs)	AA	21
Northern Minnesota (parents of eggs)	AC	8
Northern Minnesota (parents of eggs)	CC	2
Southern Minnesota	AA	25
Southern Minnesota	AC	3
Southern Minnesota	CC	0
Texas	AA	9
Texas	AC	0
Texas	CC	0

Adult turtles were collected in northern Minnesota, southern Minnesota, and Texas. Additional genotypes for adults in northern Minnesota were inferred from genotypes in 15 clutches of eggs used in the genetic association study.

Genotypes for 96 individuals from three populations are shown in Table 1. Samples from northern Minnesota were combined for subsequent analyses because there was no difference in genotype frequencies between adults that were directly genotyped and inferred parental genotypes (based on 481 embryo and hatchling genotypes from 15 clutches) ($LR \chi^2 = 2.69$, d.f. = 2, $P = 0.26$). In agreement with the RNA-Seq study, the C allele was more common in northern (~ 0.161) than in southern Minnesota (~ 0.054) (Fisher’s Exact Test, d.f. = 1, $P = 0.0348$). Moreover, the C allele was not detected in Texas turtles. Differences in *CIRBP* allele frequencies among northern Minnesota, southern Minnesota, and Texas turtles reflect small- and large-scale latitudinal differences in TSD pattern (Figure 5).

Linkage disequilibrium, association, and expression analyses for genes adjacent to *CIRBP*

Although the genetic associations described above are robust (*i.e.*, significant at the individual, family, and population levels), it is possible that *CIRBP* c63A > C is in linkage disequilibrium with a distinct causal locus (*i.e.*, another SNP or even another sex-determining gene). To test this hypothesis, we examined genes in the region surrounding *CIRBP*. All of the SNPs detected were either synonymous or found in noncoding regions: *STK11* (silent), *ATP5D* (silent), *MIDN* (silent), *CIRBP* (silent and 3’ UTR), *C19orf24* (3’ UTR and 3’ UTR), *EFNA2* (5’ UTR, 5’ UTR, silent, and 3’ UTR), and *CAMK4L* (5’ UTR). A hypothetical physical map of these markers, based on the painted turtle scaffold, is shown in Figure 6.

We used PCR and HRM analyses to determine genotypes for these markers in 56 adult snapping turtles from Minnesota. We detected significant linkage disequilibrium (LD) among some markers (6 of 55 pairwise comparisons) in unrelated adults from across Minnesota, indicating that these genes are indeed linked in the snapping turtle (Figure 6). However, LD decayed rapidly with hypothesized physical distance (Figure 7). This suggests that enough recombination has occurred historically to allow mapping of the causal variant to a fairly small interval.

Figure 6 Hypothetical map of 12 SNP markers in the snapping turtle genome. Physical distances among markers are based on the region of the painted turtle genome that contains *CIRBP*. Red lines connect markers that display significant LD in a random sample of unrelated adult snapping turtles from Minnesota. Different scale bars are shown for the entire region (50,000 bp) vs. the expanded segments for closely linked markers (1000 bp).

We therefore determined genotypes for a subset of markers in hatchling turtles from the genetic association study described above (*i.e.*, 15 clutches). Simple logistic regression revealed that *CIRBP* c63A > C was strongly associated with sex and that the strength of associations for flanking markers declined with distance from *CIRBP* c63A > C (Table 2). We also used multiple logistic regression (*i.e.*, composite interval mapping) to test for associations while controlling for genotype at other loci (Table 2). Our original marker, *CIRBP* c63A > C, displayed a significant association with sex while flanking markers were either not associated with sex (*STK11*, marker in *CIRBP* 3' UTR) or were weakly associated with sex (*MIDN*, *EFNA2*).

Finally, we used RNA-Seq data to test whether temperature affected expression of genes flanking *CIRBP*. Incubation temperature did not influence expression of *STK11* ($F_{1,10} = 0.4$, $P = 0.54$), *ATP5D* ($F_{1,10} = 0.5$, $P = 0.5$), or *MIDN* ($F_{1,10} = 0.92$, $P = 0.4$) in embryonic gonads. However, we did observe small but significant temperature effects on expression of *c19orf24* ($F_{1,10} = 11.4$, $P = 0.007$), *EFNA2* ($F_{1,10} = 10.8$, $P = 0.008$), and *CAMK4L* ($F_{1,10} = 7.4$, $P = 0.02$). Overall, *c19orf24* expression was slightly higher at 26.5° (mean = 28.7 ± 0.5 RPKM, $n = 10$) compared to 31° (mean = 26.1 ± 0.5 RPKM, $n = 10$). Although expression of *CAMK4L* was barely detectable, it was higher at 26.5° (mean = 2.12 ± 0.14 RPKM, $n = 10$) compared to 31° (mean = 1.60 ± 0.14 RPKM, $n = 10$). In contrast, expression of *EFNA2* was slightly higher at 31° (mean = 8.1 ± 0.5 RPKM, $n = 10$) compared to 26.5° (mean = 5.8 ± 0.5 RPKM, $n = 10$). When we used *CIRBP* expression as a covariate, temperature effects on *c19orf24*, *EFNA2*, and *CAMK4L* expression were no longer significant. Conversely, temperature still had a strong effect on expression of *CIRBP* even when *c19orf24*, *EFNA2*, or *CAMK4L* were used as covariates.

Discussion

Temperature-dependent sex determination is found in many reptiles and has been studied for several decades. Yet, the mechanism underlying this mode of sex determination remains

obscure. Here we demonstrate, for the first time, genetic associations between a specific gene and sex in a TSD reptile. We provide multiple lines of evidence that *CIRBP* is involved in determining gonad fate in the snapping turtle, *C. serpentina*. Significant associations between *CIRBP* genotype and gonadal phenotype (sex determination) were detected in independent experiments at the individual, family, and population levels. Additional analyses of LD, genetic association, and expression of flanking genes suggest that *CIRBP* c63A > C could be the causal variant or is close to it. In fact, *CIRBP* c63A > C was associated with sex while a second SNP within the 3' UTR of *CIRBP* (~2200 bp downstream) was not associated with sex when tested via multiple logistic regression.

Moreover, expression of adjacent genes either was not affected by incubation temperature (*STK11*, *ATP5D*, and *MIDN*) or was weakly affected by temperature (*c19orf24*, *EFNA2*, and *CAMK4L*). Flanking genes not affected by temperature were on one side of *CIRBP* while weakly affected genes were on the other side of *CIRBP*, suggesting that the temperature effect on expression of adjacent genes was due to a shared chromatin domain rather than a direct temperature effect. Such regional effects of chromatin structure are known to contribute to co-expression patterns of neighboring genes (Chen *et al.* 2010). In agreement with this idea, adjacent genes were not affected by temperature when *CIRBP* expression was used as a covariate. The converse was not true. Together these observations indicate that temperature had a direct effect on *CIRBP* expression and an indirect effect on adjacent genes.

We provide evidence that the association between *CIRBP* and TSD is due to temperature-dependent, allele-specific expression. Quantitative PCR and RNA-Seq studies show that one *CIRBP* allele (A) was induced when embryos were exposed to a female-producing temperature, while expression of the other allele (C) was not affected by the change in temperature. The first allele was associated with ovary determination and the second with testis determination. We also defined the spatial and temporal expression pattern of

Figure 7 Plot of linkage disequilibrium (r^2) vs. hypothetical distance among SNPs within the snapping turtle genome. Physical distances among markers are based on the region of the painted turtle genome that contains *CIRBP*. Red diamonds indicate SNP pairs that display significant linkage disequilibrium in unrelated adult snapping turtles from Minnesota. Black diamonds indicate SNP pairs that are in linkage equilibrium. The black line is the regression of r^2 vs. $\log_{10}$ of distance.

CIRBP protein within developing turtle gonads. These data show that *CIRBP* is physically in a position to mediate temperature effects on the bipotential gonads. Protein expression patterns diverged after sex determination, suggesting that *CIRBP* may be involved in other aspects of ovary and testis development. For example, staining for *CIRBP* was pronounced in the cortex but was less intense in the medulla of differentiating ovaries. Given the consistency of the relationship between genotype, transcript levels, and phenotype, we are confident that the association between the *CIRBP* genotype and gonadal sex is real.

Elevated expression of *CIRBP* may be involved in specification of ovarian fate in snapping turtles because it precedes the appearance of key granulosa cell markers. Independent experiments (short-term qPCR study, long-term qPCR study, and RNA-Seq study spanning both time frames) indicate that a slight, but significant, expression difference emerges 24 hr after a temperature shift from a male to a female temperature. A twofold steady-state difference is established 48 hr after the shift to a female temperature (this study; Rhen and Schroeder 2010). We have previously shown that *FoxL2* and aromatase genes are not induced until day 3 of the shift to a female-producing temperature (Rhen *et al.* 2007). Granulosa cells, which derive from the coelomic epithelium in other species (Sawyer *et al.* 2002; Mork *et al.* 2012), are critical for steroid production and normal ovary development. We detected a significant genetic correlation between *CIRBP* expression and sex ratio in siblings briefly exposed to 31° (Figure 3B). In contrast, aromatase expression during the shift was not correlated with sex ratio (Figure S4). These results indicate that *CIRBP* is upstream while aromatase and *FOXL2* are downstream effectors in the network for ovary determination. The increase in *CIRBP* expression in embryos shifted to a female temperature also precedes a decline in *Sox9* and *PdgfrB* expression (testis-determining genes), which starts 3 days after the shift (Rhen *et al.* 2007, 2009).

We do not know how differences in *CIRBP* expression influenced sex determination, but there are intriguing hints

from the literature. Proteins in the RRM family, which includes *CIRBP*, regulate splicing and translation of target mRNAs involved in a range of biological processes. One of the best-characterized members of this family is *Sex-lethal*, the master sex-determining gene in *Drosophila melanogaster* (Salz 2011). SXL protein regulates splicing of *Transformer* mRNA to produce female-specific Tra protein. In turn, Tra and its binding partner Tra2 (also RRM family members) regulate female-specific splicing of *Doublesex* mRNA. This splicing cascade is a shared feature of sex determination in insects (Salz 2011).

We hypothesize that *CIRBP* might play a similar regulatory role in RNA processing during sex determination in snapping turtles. Studies in other vertebrates show that *CIRBP* is involved in germ-cell development and reproduction (Elliot 2004; Xia *et al.* 2012). Paralogs of *CIRBP* in humans include *TRA2α*, *TRA2β*, RNA-binding motif protein 3 (*RBM3*), X-linked RNA-binding motif protein (*RBMX*), and the Y-linked RNA-binding motif protein family (*RBMYS*). In *Xenopus*, *CIRBP* displays nucleocytoplasmic shuttling and has distinct roles in each cellular compartment (Aoki *et al.* 2002). Nuclear *CIRBP* is associated with cell growth and differentiation (Lleonart 2010; Emmanuel *et al.* 2011; Masuda *et al.* 2012). In response to various environmental stressors, *CIRBP* moves from the nucleus to the cytoplasm where it associates with stress granules to regulate RNA metabolism (Yang and Carrier 2001; De Leeuw *et al.* 2007). It would be particularly informative to determine whether *CIRBP* interacts with transcripts from other sex-determining genes. For example, we recently reported that incubation temperature rapidly influences expression and splicing of *WT1* in snapping turtle embryos: the ratio of +KTS:–KTS isoforms is very precisely regulated in gonads (Rhen *et al.* 2015).

Homozygotes for the A allele had higher *CIRBP* expression than homozygotes for the C allele when shifted to 31°. As expected based on dosage, the A and C alleles in heterozygotes were expressed at about half the level of the corresponding homozygotes. There was also a significant association between

Table 2 Association of SNPs and sex in hatchling snapping turtles exposed to 31° for 2.5 days during the TSP (15 clutches, 248 hatchlings)

Gene (SNP position)	Single-locus analysis		Multiple-locus analysis	
	LR χ^2	P-value	LR χ^2	P-value
STK11	0.02	0.88	3.60	0.058
MIDN	6.12	0.013	5.92	0.015
CIRBP (c63A > C)	23.36	0.000001	10.16	0.0014
CIRBP (3' UTR)	13.72	0.0002	2.53	0.11
EFNA2 (+250,230)	7.40	0.0065	4.92	0.027

SNPs are found within genes that display synteny with *CIRBP* in human, chicken, painted turtle, and Chinese softshell turtle genomes. Simple logistic regression tested for association with single genes. Multiple logistic regression was tested for associations while controlling for genotype at all other loci.

CIRBP genotype and hatchling sex: AA homozygotes were more likely to develop ovaries than AC heterozygotes, which, in turn, were more likely to develop ovaries than CC homozygotes. Turtles were nearly four times more likely to be female for each A allele they carried (Unit Odds Ratio = 3.9; 95% C.I. 2.1–8.3), which indicates a relatively strong effect on sex determination. We estimate that this SNP accounts for ~25% of the heritability for TSD pattern (T. Rhen, unpublished data). This association was not due to a structural change in *CIRBP* because sequence variants are synonymous. Polymorphisms in adjacent genes were synonymous or in noncoding regions, further indicating the association between *CIRBP* and sex results from a *cis* regulatory SNP. In short, temperature-dependent expression of the A allele appears to play a role in transducing high temperatures into a signal for ovary determination.

Whatever the molecular mechanism, the association between *CIRBP* genotype and sex determination extends to the population level. A continent-wide cline in TSD pattern suggests that snapping turtles are genetically differentiated across their species range (Ewert *et al.* 2005). Here we show that this cline is evident at smaller spatial scales: turtle populations separated by 3° latitude within Minnesota differ by 0.5° in their pivotal temperatures. If *CIRBP* were causally involved in TSD, we would expect variation in allele frequencies among populations due to a balance between gene flow and selection on TSD pattern. In accord with this prediction, two independent experiments using very different methodologies (RNA-Seq of pooled samples and direct genotyping of individuals) revealed population differences in *CIRBP* allele frequencies. Moreover, the direction of the difference is consistent with the TSD pattern: the C allele (less responsive to feminization) was more common in the population with a higher pivotal temperature while the A allele (more responsive to feminization) was more common in the population with a lower pivotal temperature.

To the best of our knowledge, this is the first study to demonstrate genetic associations between a specific candidate gene and gonadal sex within a TSD reptile. However, recent analysis of *Dmrt1* evolution among species has revealed a relationship between a pair of amino acid changes and a

transition from TSD to GSD (Janes *et al.* 2014). Evidence that *CIRBP* plays a role in TSD spans multiple levels of biological organization: we present molecular, cellular, developmental, organismal, and population analyses supporting this hypothesis. RNA-binding proteins like *CIRBP* are capable of regulating numerous mRNAs in a coordinated fashion (Bandziulis *et al.* 1989; Keene 2007) and play a role in sex determination in other organisms. Taken together, the data described here suggest that *CIRBP* is involved in transducing temperature into a molecular signal for ovary vs. testis determination in the snapping turtle. Further research is required to experimentally test whether *CIRBP* actually mediates temperature effects on sex determination. The ultimate test will involve manipulation of *CIRBP* expression and gonad development at male- and female-producing temperatures. While *CIRBP* explains substantial variation in thermosensitive sex determination (~25% of the heritability for gonadal sex), additional genes must be involved in sensing temperature because there was not perfect concordance between genotype and sex. This observation provides the first suggestion that TSD is polygenic. The current study also provides proof of principle that genome-wide association studies could be used to identify other TSD genes and unravel the genetic architecture of this unusual mode of sex determination. Whether *CIRBP* plays a role in TSD in other species remains an open question.

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A Novel Candidate Gene for Temperature-Dependent Sex Determination in the Common Snapping Turtle

Anthony L. Schroeder, Kelsey J. Metzger, Alexandra Miller, and Turk Rhen

Sry expression (2 days)

Mouse

E10

E10.5

E12

E12.5

E19-21

Genital ridge
is formed

Bipotential
gonad
develops

Gonadal sex is
determined

Morphological
differentiation
is evident

Birth
or
Hatching

Snapping
turtle

Y13

Y14

Y18-20

~Y20

Y26

Thermosensitive period
(5 to >20 days, depending on temperature)

Gene	Primer/Probe	Primer Sequence	Position (relative to c63A>C)
<i>STK11</i> (silent)	HRM Forward Primer	5'-TGTGGTCCTCCGCTCTCAG-3'	-274,429
<i>STK11</i> (silent)	HRM Reverse Primer	5'-GGGTCATATTCAAGCATCCCTCTAAGTAG-3'	-274,429
<i>ATP5D</i> (silent)	HRM Forward Primer	5'-CCACTTTGGACATGCTGGATC-3'	-176,911
<i>ATP5D</i> (silent)	HRM Reverse Primer	5'-TCCAGATTTCGACTTAGCAGCAG-3'	-176,911
<i>MIDN</i> (silent)	HRM Forward Primer	5'-AAGGTGGAGCGCCTGC-3'	-113,221
<i>MIDN</i> (silent)	HRM Reverse Primer	5'-CAGCCTCTTCTGCTGCATCA-3'	-113,221
<i>CIRBP</i> (c63A>C)	Probe Forward Primer	5'-GGTGGACTGAGTTTTGATACCAATG-3'	0
<i>CIRBP</i> (c63A>C)	Probe Reverse Primer	5'-TTCACCACGACAACCTTCAGAGATC-3'	0
<i>CIRBP</i> (c63A>C)	Probe Forward (Fam)	5'-AACAGTCA <u>A</u> CTGGAGCAA-3'	0
<i>CIRBP</i> (c63A>C)	Probe Reverse (Vic)	5'-AACAGTC <u>C</u> CTGGAGCA-3'	0
<i>CIRBP</i> (c63A>C)	HRM Forward Primer	5'-GGACTGAGTTTTGATACCAATGAA-3'	0
<i>CIRBP</i> (c63A>C)	HRM Reverse Primer	5'-CGTATTTAGAAAAGACTTGCTCCA-3'	0
<i>CIRBP</i> (3'UTR)	HRM Forward Primer	5'-TGGGTGTAAATTGGAAGGCA-3'	+2,187
<i>CIRBP</i> (3'UTR)	HRM Reverse Primer	5'-AGCAAGTTTCTCTTTAAGTATATCAAGG-3'	+2,187
<i>CIRBP</i> (near poly-A)	Real-Time Forward Primer	5'-CAAGTGAACAATCTGACTTGTAACAG-3'	+4,075
<i>CIRBP</i> (near poly-A)	Real-Time Reverse Primer	5'-TTTTTACATCGATCTTTCTTGCAT-3'	+4,075
<i>C19orf24</i> (3'UTR)	HRM Forward Primer	5'-GGACTGAAGTGTTGCAGCCA-3'	+17,756
<i>C19orf24</i> (3'UTR)	HRM Reverse Primer	5'-TTGACTAGATTTTCGTAGGCAGGC-3'	+17,756
<i>C19orf24</i> (3'UTR)	HRM Forward Primer	5'-AATTCTCTCTAATCTGTTTGGGG-3'	+21,407
<i>C19orf24</i> (3'UTR)	HRM Reverse Primer	5'-TGAGTAGTTCAATGCCAGTAC-3'	+21,407
<i>EFNA2</i> (5'UTR)	HRM Forward Primer	5'-AGGCTGTGTTTGCAGGTGTG-3'	+72,433
<i>EFNA2</i> (5'UTR)	HRM Reverse Primer	5'-CCCCATTCAGAGTTGCTGCTG-3'	+72,433
<i>EFNA2</i> (5'UTR)	HRM Forward Primer	5'-AGGACTTTCATCCACCGCTG-3'	+73,077
<i>EFNA2</i> (5'UTR)	HRM Reverse Primer	5'-TGATCTGGTTTTATTGGTGTAGGTGG-3'	+73,077
<i>EFNA2</i> (silent)	HRM Forward Primer	5'-CGACCAACAAATGACTCCCTG-3'	+250,230
<i>EFNA2</i> (silent)	HRM Reverse Primer	5'-TGGTGAAAATAGGCTCCGGA-3'	+250,230
<i>EFNA2</i> (3'UTR)	HRM Forward Primer	5'-AGCGGTTTGCCATTGCTTGT-3'	+251,220
<i>EFNA2</i> (3'UTR)	HRM Reverse Primer	5'-TCCAGGTGGCTCTGAGGC-3'	+251,220
<i>CAMK4L</i> (5'UTR)	HRM Forward Primer	5'-CCATCCTCAATGTGCAGGAATATTTG-3'	+381,765
<i>CAMK4L</i> (5'UTR)	HRM Reverse Primer	5'-CCCCGCACATAAGGAGAGAC-3'	+381,765