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Embryonic Brain-Gonadal Axis in Temperature-Dependent Sex Determination of Reptiles: A Role for P450 Aromatase (CYP19)

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ABSTRACT Sex determination in egg-laying amniotes may be fundamentally different from that of placental mammals. The mammalian ovary differentiates normally in the absence of estrogen, whereas estrogen seems to be crucial for proper ovarian development in birds, reptiles, and lower vertebrates. Estrogens are produced normally by the biosynthetic conversion of androgens by the enzyme aromatase (CYP19), which is the sole mediator of this reaction. Aromatase inhibitors are capable of reversing females to males in turtles and chickens; therefore, a role for aromatase as the female sex determinant has been postulated for species in which sex determination is temperature-dependent.

The entire aromatase coding sequence (1,509 base pairs) from adult terrapin ovaries was cloned, and Northern analysis indicates a single transcript (2.4 kb) for adult ovaries, whereas male and female brains express a 2.4-kb as well as a 9.6-kb transcript. Using a sensitive (attomole sensitivity) competitive RT-PCR technique, aromatase transcript abundance was quantified during embryonic development for embryos treated with and without estrogen. Aromatase is transcribed, well before the temperature-sensitive, (stage 12), at both male and female temperatures in the brain. There is a switch to lower aromatase transcript abundance in the female brain concurrent with an exponential rise of aromatase transcript in the putative ovary. Transcripts remain below the detection limits in the putative testes but exhibit female levels of aromatase transcript when treated with estrogen. Aromatase mRNA levels are generally reduced in the brain by estradiol application. On the basis of these findings, we have postulated a model based on the competition between 5α-reductase and P450 aromatase for androgen substrate in both the brain and the undifferentiated gonad to explain the TSD phenomenon in reptiles. *J. Exp. Zool. 281:428–449, 1998.*

The process of sex determination "is essentially a decision of cell fate. In such decisions, a cell or group of cells has to choose between alternate pathways and establish a specific pattern of gene expression that will provide the characteristic properties of the final cell type. In simplistic terms, we can envision two steps, an initiation, driven by either extrinsic factors (e.g., temperature or perhaps neuroendocrine signals) and/or internal 'switches,' and stabilization, which will depend on regulatory loops (within or between cells) and chromatin structure reorganization to maintain the pattern of gene expression" (Lovell-Badge, '97).

In all vertebrates, the gonad begins as an indifferent organ made up of several cell lineages, each of which can be considered bipotential, with a different fate in testes versus ovaries. These include the supporting cell precursors, which give rise to Sertoli cells in testes and follicle (or granulosa) cells in ovary; the steroidogenic cell lineage, which give rise to Leydig cells in testes and theca cells in ovaries; the connective tissue cell lineage, which organizes the male- and female-specific patterns; and the primary germ cells, which respond to their (somatic cell) environment by entering meiosis early, giving oocytes, or arresting in mitosis and beginning the process of spermatogenesis.

In mammals, evidence exists for the involvement of at least two transcription factors in the development of a bipotential gonad: WT-1 and SF-1. WT-1, the tumor suppressor gene for Wilms' tumor, is expressed in the glomeruli of the kidney and the gonadal ridge of the developing gonad prior to the onset of sex determination. The function of WT-1 is suggested to be involved in the

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thickening of the mesoderm to give rise to a bipotential gonad. SF-1 (steroidogenic factor-1, also known as adrenal 4 binding protein AD4BP) is a member of a group of orphan nuclear receptors that are involved in transcriptional regulation of the genes necessary for steroid synthesis. SF-1 binds to a TCAAGGTCA motif and is a key regulator of adrenal and gonadal steroidogenesis. SF-1 knockouts in mice result in the loss of adrenals and gonads (Lou et al., '94). SF-1, like WT-1, is involved very early in gonadal development and is expressed prior to the onset of sex determination at about 9 days post conception (d.p.c.) in mice (Parker and Schimmer, '97).

In mammals, the fate of the bipotential gonad depends on the activity of an internal switch for initiation. This gene *SRY,* located on the Y chromosome, is present in males. *SRY* encodes a DNA binding protein and is likely to be involved in gene expression (Gubbay et al., '90; Koopman et al., '90; Sinclair et al., '90). It is active within the indifferent gonads (or genital ridge) and is responsible for triggering the differentiation of testes rather than ovaries. The testes then secrete two factors, anti-Müllerian hormone (AMH, also called MIS) and testosterone, which masculinizes the rest of the embryo (Lee and Donahoe, '93).

The search for the testis-determining factor (TDF) had been the Holy Grail for researchers in mammalian sex determination. The strategy for searching for TDF was based on the assumption that the Y chromosome encodes a control gene that regulates the expression of all other genes in the genome that are involved in the male pathway of development. Through deletion mapping of XX females and XY females and finer restriction mapping using the first two maps, a region of the Y-chromosome has been narrowed down that contains TDF. This region contains the gene for the testis-determining factor and is termed the sex-determining region of the human Y-chromosome (*SRY*) (Koopman et al., '89; Gubbay et al., '90). The *SRY* gene codes for a protein with an 80 amino acid region that is homologous to the high-mobility group proteins (HMGs) recently recognized as a family of transcription factors (Laudet et al., '93). Much evidence exists showing that *SRY* acts specifically within the supporting cell precursors. As these cells differentiate into Sertoli cells, they signal the other lineages to follow the male pathway. *Sry* is expressed for a brief period, only from 10.5 to about 12.0 d.p.c. in mice; hence, it is responsible only for initiating the Sertoli cell differentiation and not involved in the maintenance of the Sertoli cell-specific gene expression (Koopman et al., '90). There are differences, however, when we look at ruminant versus mouse model systems. In the ovine system, *Sry* expression continues after the initial Sertoli cell differentiation and persists after full testis determination (Payen et al., '96). Whether the persistence of *SRY* is functioning in maintenance mode of sex determination in sheep is unknown.

Two other genes involved in gonad cell lineage determination are the *SRY*-related gene *SOX9* and an unusual member of the nuclear hormone receptor superfamily, *DAX-1*. Mutations in the *SOX9* gene cause skeletal disorders and lead to severe dwarfism in humans. Thirty percent of patients with this condition, campomelic dysplasia (CD), are 46 XY females (Wagner et al., '94). In the mouse, *Sox9* is expressed at a low level in the very early genital ridge in both XX and XY embryos. Coincident with the first appearance of *Sry* transcripts *Sox9* levels increase in the XY genital ridge, while expression is turned off in XX genital ridges. The high level of expression is maintained in the testis, specifically within the Sertoli cells, whereas the gene is never active in follicle cells. The high level of *Sox9* expression also correlates with Sertoli cell differentiation in chick genital ridges. *Sry* has not been found in any avian species. It is thought that *Sox9* is part of an underlying vertebrate sex-determining mechanism and functions as a critical Sertoli cell differentiation factor (Morais da Silva et al., '96).

Why is *Sox9* turned off in the XX genital ridge, coincident with the stage at which *Sry* comes on in the male? This implies the presence of a repressor that becomes active at this time only in females. *DAX-1* is at present the only candidate gene for this repressor. *DAX-1* is a gene that when mutated cause adrenal hypoplasia congenita (AHC) in humans, an X-linked disorder in which the adrenal fails to develop. *DAX-1* is also implicated in sex determination, as AHC is also associated with a failure to mature properly sexually at puberty, a condition known as hypogonadotropic hypogonadism (HHG) (Muscatelli et al., '94). It maps to the X chromosome and, when duplicated, causes female development in XY individuals. When deleted, XX individuals develop as males. In the mouse, *dax1* was found to be expressed at the same stage as *Sry*, but in the developing genital ridges of both sexes, and then to be maintained within the ovary, while it is turned off in the testis at the

first signs of cord formation (as reviewed in Werner et al., '96).

This scheme (Fig. 1) puts *SOX9* at a pivotal point in sex determination of the male, with *SRY* and *DAX-1* competing to control its activity. In normal female development, *SRY* is absent, so *DAX-1* prevails, whereas in normal male development, *SRY* predominates, but it can be out-competed when *DAX-1* is present in a double dose or if *Sry* expression is low.

Downstream from the differentiation of the testis, i.e., the onset of *Sry* expression, Müllerian-inhibiting substance (MIS) expression is initiated to cause regression of female reproductive structures. The timing of testicular formation normally occurs just after the migration of the primordial germ cell into the gonadal ridge, a ridge of tissue apical to the developing kidney or mesonephros. The regression of the cortex and the onset of medullary cords in the developing gonad signify the development of a testes, whereas the regression of the medulla and the proliferation of the cortex signifies ovarian development. Within the testes, the Leydig cells of the interstitia between the developing tubules differentiate secondarily, while the Sertoli cells within the tubules are differentiating. In higher vertebrates and some fish, this also involves the regression of accessory ducts that are involved in the transportation of gametes (eggs and sperm) from the gonad. In the female pathway, the Müllerian ducts become the oviducts and the Wollfian ducts (which normally become the vasa deferentia) regress. In the male pathway, the opposite takes place.

Molecular mechanism for SRY *function*

SRY binds to regions 5´ of the TATA boxes in both aromatase and in Müllerian-inhibiting substance (MIS) (Haqq et al., '93). There is also conservation of these DNA binding sites (SRYe) in the aromatase gene in both the chicken and the rat. This suggests that these sequences are evolutionarily conserved and may be found in the reptiles as well. The positions of these SRYe sites are different in MIS and in aromatase. This difference may translate to a difference in the function of *SRY* in either activating or inhibiting the expression of these genes.

When *Sry* is expressed, *Dax-1* levels are the same in male and female gonads suggesting that *Sry* is not directly suppressing *Dax-1* expression and is probably interfering with its downstream activity (Swain et al., '96), which might be the repression of *Sox-9,* as earlier suggested.

Besides *SOX9, DAX-1,* and *MIS,* SF-1 is also implicated in sex determination of the bipotential gonad. The *MIS* gene has a binding domain for SF-1 and is expressed at the same time that SF-1 is expressed during male development. There is a sexually dimorphic expression of SF-1 in rats, which correlates to the expression of *MIS* in rats. At E13 in the rat embryo, SF-1 levels precede that of *MIS* and peak at the end of Müllerian duct regression. *MIS* expression follows that of SF-1 closely in the male. In the female, SF-1 is low at these stages (E13-E20), as is MIS in the rat. When a truncated SF-1 (lacking the ligand binding domain) was co-expressed with a wild-type *MIS* reporter construct, the *MIS* activity was enhanced in both HeLa and R2C cells, suggesting that a Sertoli cell specific ligand was required for SF-1 function as SF-1 by itself was unable to enhance *MIS* activity. The removal of the putative ligand binding site is thought to eliminate the requirement for this factor (Shen et al., '94).

Similarly, SF-1 and *DAX-1* expression co-localizes in multiple cell lineages (Swain et al., '96). In mouse embryos, *SF-1* expression in the urogenital ridge and brain either preceded or coincided with *DAX-1* expression. SF-1 binds specifically to a conserved sequence in the *Dax-1* 5´ flanking region but this region is not necessary for *Dax-1* expression. Moreover, *Dax-1* expression appears unimpaired in knockout mice lacking *SF-1*. Recently, it was shown that *DAX-1* interacts directly with *SF-1* in in vitro binding studies and inhibits *SF-1* mediated transactivation. The inhibitory domain of *DAX-1* was localized to the carboxy terminal region (Ito et al., '97).

Fetal steroidogenic expression in mammals

The function of *SF-1* in the expression of MIS in the Sertoli cells and precursors are not the only function of *SF-1* in gonadal differentiation. *SF-1* is also expressed in the Leydig cells at E12 in the mouse coincident with the formation of testicular cord (Parker and Schimmer, '97). The onset of *SF-1* expression in the Leydig cells of the developing testis must be involved in testosterone production through the general upregulation of steroidogenesis. Cholesterol side chain cleavage ($P450_{SCC}$ or CYP11A), steroid 17α hydroxylase (P45017α or CYP17) and aromatase ($P450_{AROM}$ or CYP19) are under *SF-1* control (Parker and Schimmer, '97). Thus, the fetal interstitial Leydig cells produce testosterone that virilizes the Wolffian duct derived male structures such as the epididymis, vas deferens, and seminal vesicles. The external genitalia

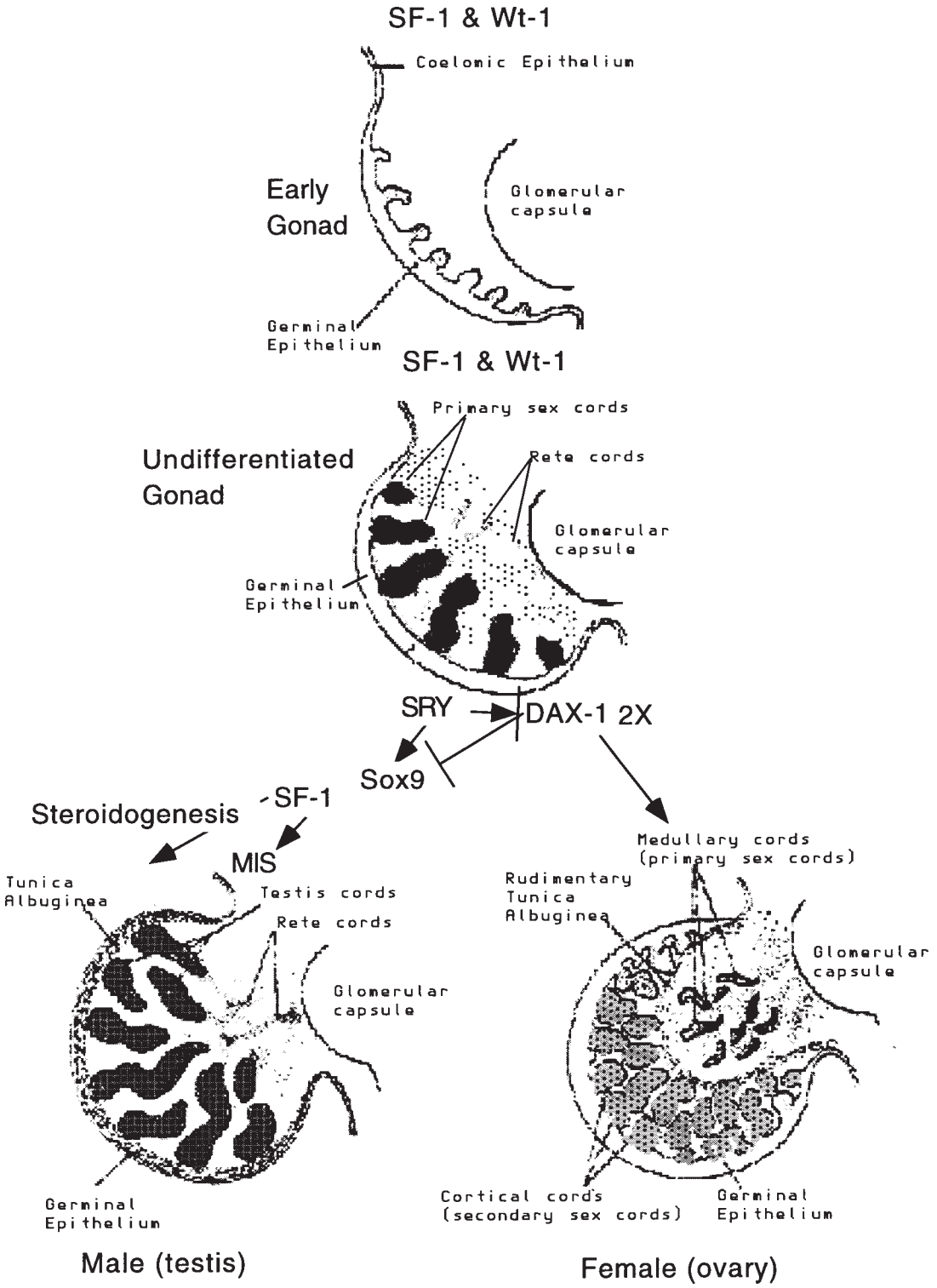

Fig. 1. A schematic representation of the developing mammalian gonad showing the developmental path taken during commitment to an ovary versus a testis. Also shown are the genes thought to be associated with this differentiation.

are virilized further by the more potent androgen dihydrotestosterone (DHT) that is derived from testosterone in a reaction catalyzed by 5α-reductase (Parker and Schimmer, '97). *SF-1* expression ceases or is greatly reduced in the developing mouse ovary, which suggests that the ovary can develop and differentiate without the production of estrogen (Takayama et al., '95). The ontogeny of steroidogenic enzyme gene expression shows that the fetal mouse ovary does not express three key enzymes, including aromatase, indicating again that estrogen is not necessary for ovarian differentiation or development (Greco and Payne, '94). In fact, excess estrogen appears to cause fetal death in mice (Mahendroo et al., '97). In knockout mice for 5α-reductase (Type I) fetal death occurs between gestation days 10.75 and 11. Administration of an estrogen receptor antagonist or inhibitors of aromatase reverse the high rate of fetal death (Mahendroo et al., '97) implicating estrogen toxicity as a major cause of fetal death.

Sex determination in non-mammalian amniotes

Gonad differentiation in non-mammalian amniotes is morphologically similar to that of mammals and it is likely that the same genes (Fig. 1) are involved in testis and ovary formation. Papers in this symposium have already established that *Wt-1* and *SOX-9* as well as *SF-1* are important in sex determination of reptiles. However, in the non-mammalian amniotes, there appears to be no sex specific *SRY*-like gene. Using a probe from the conserved HMG box region of *SRY,* Tiersch et al. ('91) found an equal signal in male and female DNA from birds, reptiles, amphibians, and fish. Similarly, attempts to clone an *SRY*-like gene from chicken, gecko, alligator, and turtle have revealed a whole family of *Sox* genes: 10 in the turtle (Spotila et al., '94), 17 in the alligator, and 18 in the chicken (Coriat et al., '94). None of these *SRY*-like genes appears to be sex-specific. Graves and Foster ('94) suggest that quite different genes may control sex determination in non-mammalian vertebrates and sex in mammals. The model of Jost ('53) in which maleness is imposed on the neutral, or default, female phenotype may not apply. There is evidence in birds, for example, that femaleness is imposed on the default or neutral, male phenotype (Graves and Foster, '94; Clinton, this symposium).

The extant crocodilians and the birds are believed to have evolved from a common archosauran ancestor (Gauthier et al., '88). Protein sequence data (de Jong et al., '85) and peptide hormone sequence data (Lance et al., '84; Rodriguez-Bello et al., '93; Tohohiko et al., '93; Wang and Conlon, '93) lend strong support to this relationship. There are no sex chromosomes in crocodilians (Cohen and Gans, '70), and all crocodilians exhibit temperature-dependent sex determination (Lang and Andrews, '94). All birds show genetic sex determination (GSD) but not all birds have heteromorphic sex chromosomes. The more primitive ratites have morphologically identical sex chromosomes (de Boer, '80).

THE DIAMONDBACK TERRAPIN *(MALACLEMYS TERRAPIN)*

Natural history

The diamondback terrapin, *Malaclemys terrapin*, is an estuarine emydid turtle that ranges from the Gulf Coast of Texas to Cape Cod, Massachusetts. It lives in salt and brackish waters of the eastern United States. The animals show sexual dimorphism, with females being larger and having a rounder head, deeper shell, and shorter tail than males. Females may reach 9 inches, although the carapace is usually 7–8 inches in length. Males are about 5 inches in length (Roosenburg, '91). Throughout its range, the terrapin nests on coastal dunes or narrow sandy beaches where raised sand dunes are not available. Nesting season in the Chesapeake Bay region begins in late May and continues until late July. Female terrapins come ashore and deposit an average of 13 eggs in a shallow nest. Once nesting is completed, the female leaves the nest to incubate in the sand without any further parental care. After 60–120 days, hatchlings emerge from the nest and enter the nearest water (Roosenburg, '91). Experimental nest sites followed in 1989 and 1990 produced only male turtles in particular beaches on the Patuxent River (Roosenburg, '92).

TSD in diamondbacks

The sex of the developing embryo of *Malaclemys terrapin* is environmentally determined by the temperature at which the eggs are incubated. Incubation at constant high temperatures, above approximately 29°C, produces mainly females, whereas incubation at low temperatures, below 29°C, produces mainly males (Roosenburg, '92; Jeyasuria et al., '94). Temperature experiments in our laboratory have shown that 100% of the offspring are female at a temperature of 31°C, and all offspring are male at 27°C with very low levels of mortality. We have

also shown that the pivotal temperature in this animal is slightly above 29.5°C. Animals that have been reared in captivity for up to 8 years have not shown any signs of sex reversal.

TEMPERATURE-DEPENDENT SEX DETERMINATION (TSD) IN REPTILES

Most researchers now envision a cascade mechanism for sex determination as illustrated in Figure 1. Crews argued that cascade in the sex-differentiation process of TSD is similar to that in GSD, except that temperature has an effect on the gene regulation of enzymes, hormones, or receptors upstream of this process (Crews et al., '94). It should be kept in mind, however, that the sex-determination pathway can be extremely malleable. An example of the plasticity of sex-determining mechanisms can be found in *Caenorhabditis elegans*, in which a sex to autosomal chromosome ratio is the normal primary signal required for sex determination. A transfer of a gain of function mutant of *tra-1* from a sex to autosomal location in this organism produces a ZZ/ZW sex-determining system (i.e., sex is dependent on a single female-determining gene). Similarly, temperature-sensitive mutations can essentially produce a temperature-dependent sex determination (Hodgkin et al., '89).

TSD has been documented in 28 turtle species (Ewert and Nelson, '91), some lizards (Bull, '87; Viets et al., '93), and all crocodilians studied (Deeming and Ferguson, '89; Lang and Andrews, '94). The temperature profiles on sex determination in these different orders, however, are varied. Alligators produce 100% females at 30°C and 100% males at 33°C (pattern IB in Fig. 2), whereas the converse takes place in sea turtles, with males being produced at the lower temperature (27°C) and females at the higher temperature (30°C) (pattern IA in Fig. 2). In crocodiles and some turtle species, 100% females are produced at low and high temperatures (pattern II in Fig. 2), and males are predominantly produced at intermediate temperatures. Pieau ('96) suggested that this profile encompasses both of the previous profiles, as there is a larger temperature range.

TSD appears to be an ancestral character in crocodilians (Lang and Andrews, '94); it may be ancestral or derived in chelonians (Ewert and Nelson, '91); and it appears to be derived in lizards (Viets et al., '93). The temperature-sensitive period (TSP) in a leopard gecko, *Eublepharis macularium*, begins at about stage 32/33, which coincides and corresponds to stage 15 in the turtle and stage 20/22 in the alligator (Lang and Andrews, '94). Unlike the

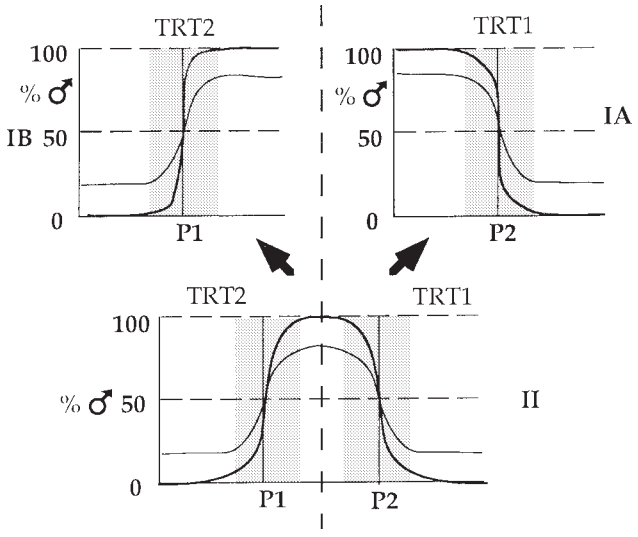

Fig. 2. The different temperature patterns and profiles found in reptiles with temperature-dependent sex determination. The temperature profiles in type I (Ia and Ib) profiles could be derived from the type 2 profile. [Reproduced by permission from Pieau, C. (1996) Temperature-dependent sex determination in reptiles. BioEssays, *18:*19–26.]

TSP in turtles, the TSP in lizards and alligators occurs shortly after oviposition because eggs are laid in a fairly advanced stage of development (Janzen and Pakustis, '91). In this light, generalizations about the universality of the mechanism(s) of TSD may be premature.

Thus far, most of the temperature studies done on reptiles have involved constant incubation temperatures in the laboratory. In some field studies, the average temperature of the nest has favored one sex over the other. Theories for the underlying molecular mechanisms behind TSD are difficult to propose for the simple reason that different sex-determining mechanisms exist in the class Reptilia. A large number of reptiles show heteromorphic sex chromosomes, suggesting a heterogametic sex-determining system. In fact, there is postulation that TSD functionally overrides an underlying gene-dependent system, and some researchers believe this is what happens around the intermediate temperatures where both sexes are being produced at a 1:1 ratio. J. Lang (personal communication) has demonstrated that the ratio of males to females at these intermediate temperatures is related to the clutch from which the eggs are derived. This so-called clutch effect suggests additional genetic factors may be involved.

Other environmental factors may influence sex ratios. Eggs of *Trachemys scripta* incubated at different oxygen tensions have significantly al-

tered incubation periods but unaltered sex ratios (Etchberger et al., '91). At higher carbon dioxide levels, the incubation times are increased (equivalent to using a lower male-producing temperature), but the sex ratios are altered in favor of females. Etchberger et al. ('92) suggested that this is a function of the lower pH at the higher carbon dioxide levels.

Temperature dependence and the critical sex-determining period in turtles

A concise staging of embryonic development is known only for *Chelydra serpentina* (Yntema, '68) to which all other work with turtles is compared. The *temperature-sensitive period* (TSP) for sex determination in turtles is defined by temperature-shift experiments. In *Chelydra,* this period is between stages 14 and 19 (Yntema, '76, '79) and coincides with the development of the limb bud into an early paddle stage. The TSP for male development in *Trachemys scripta* lies between stages 16 and 21, whereas that for females lies between stages 17 and 19. TSPs have been defined for four other turtle species (*Emys orbicularis, Chelydra serpentina, Graptemys ouchitensis, Carretta caretta*), for which the critical period for gonadal sex determination tends to lie between stages 14 and 20 (see Raynaud and Pieau, '85; also reviewed in Janzen and Paukstis, '91). The potency of temperature on the sex of turtles can be seen in temperature-shift experiments in which the eggs are first incubated at a male-producing temperature (26°C) and then shifted to one of two different female-producing temperatures (31°C and 32.5°C). The female bias is greater in the 32.5°C shift than in the 31°C shift. The higher temperature has a higher potency for female production than the lower temperature.

The *estrogen-sensitive period* is the developmental period in which the embryos are still capable of reversing sex to female at male-producing temperatures when treated with estrogen. Wibbels et al. ('91b) placed this period between stages 14 and 21. There is a synergistic effect of both temperature and estradiol; i.e., at a higher male-producing temperature (closer to a female-producing temperature), the dose of estradiol required to cause sex reversal is lower (Crews et al., '91; Wibbels et al., '91a). Reversal of sex in the turtle, therefore, seems to be a dose-dependent phenomenon. The experimental evidence above tends to indicate that gene products are expressed quantitatively rather than qualitatively and that sex is not only stage-specific but also depends on the dose of external stimuli received during the sex-sensitive period.

The temperature-sensitive and estrogen-sensitive periods fall within the same period as gonadal differentiation. Cord formation begins at about stage 15. By stages 18 and 19, medullary sex cords are well organized, and some cords have enclosed germ cells. The cords proliferate at these stages, and, at stage 21, the testes are separated from the mesonephros by a narrow stalk of connective tissue, and the young seminiferous tubules begin to proliferate whereas the cortical region is reduced to a single cell thickness (Wibbels et al., '91b).

Female differentiation involves the regression of the medullary region (sex cords) and the proliferation of the cortex (Fig. 1). At stages 18–19, a membrane has formed, separating the medulla from the cortex. The organization of the medullary region breaks down between stages 18 and 20. There is a proliferation of the cortex between stages 20 and 26 (Wibbels et al., '91b).

Fetal steroidogenic expression in non-mammalian amniotes

In both birds and reptiles, the synthesis of estrogen in the developing embryo plays an important role in sex differentiation. Simply blocking estrogen synthesis in female chick embryos by injecting an aromatase inhibitor results in phenotypically male hatchlings that are capable of spermatogenesis when sexually mature (Elbrecht and Smith, '92; Wartenberg et al., '92, Shimada, '98). Similar results have been found in turtles with temperature-dependent sex determination (TSD). Eggs incubated at a temperature known to produce female hatchlings produced male hatchlings when treated with aromatase inhibitors. Conversely, eggs incubated at a temperature known to produce male hatchlings produced female hatchlings when treated with estrogen (Crews and Bergeron, '94; Jeyasuria et al., '94; Lance and Bogart, '94; Pieau et al., '94; Wibbels et al., '94; Richard-Mercier et al., '95).

It has not been possible to fully sex-reverse genetically male chick embryos using hormonal or drug treatments (Samsel et al., '82). Male chick embryos that are treated with estrogens exhibit feminized gonads, but these feminized gonads revert to normal male structures some weeks after hatching (Wolff and Ginglinger, '35; Burns, '61).

Researchers have treated eggs with different hormones and specific hormone antagonists to decipher a possible mechanism for sex determination in TSD as well as in GSD. The extent to which hormones play a role in sex determination seems to depend very much on the taxa. In many

teleost fish and amphibians (see Hayes, '98), the sex of larvae can be sex reversed using steroids (Zohar, '89; Burns '61, as reviewed in Lance and Bogart, '92). Several researchers have shown that the sex of reptiles can be reversed by treating eggs with estrogen at a male-producing temperature in TSD species (Gutzke and Bull, '86; Bull et al., '88; Crews et al., '89, '91; Lance and Bogart, '91, '92; Jeyasuria et al., '94). The effect of estrogen treatments is enhanced as incubation temperatures approach the pivotal temperature (the temperature at which equal numbers of males and females are produced) (Wibbels et al., '91a, '93).

The reversal of females to males using androgens at female-producing temperatures, however, has not been possible to date in TSD species. The treatment of eggs with testosterone at male-producing temperatures will, in certain cases (high doses), produce some females. This is probably the result of the biochemical conversion of testosterone to estradiol by aromatase. The effect of using a non-aromatizable androgen (i.e., an androgen that is incapable of converting to an estrogen via the aromatase reaction) has an effect only at the pivotal temperatures. In *Trachemys scripta*, a dose of 10 μg of DHT (dihydrotestosterone, a non-aromatizable androgen) applied in 5 μl of ethanol will nudge the sex ratio to 100% male at pivotal temperatures (Wibbels and Crews, '95). DHT has, however, reduced the percentage of males in other experiments at pivotal temperatures (Rhen and Lang, '94) and had no effect in alligators.

At female-producing temperatures, the addition of a non-steroidal aromatase inhibitor (letrozole, CGS 20267) lowered the aromatase activity in the gonads of embryos (stage 15) of *Emys orbicularis* incubated at female temperatures (Richard-Mercier et al., '95). In 13 of the 27 letrozole-treated embryos that were observed, the aromatase activity of the gonads was equivalent to the testicular levels produced at male temperatures. Morphologically, the treated gonads looked like testes or ovotestes. The cortex of the ovotestes normally regresses after hatching, producing a normal testes (Richard-Mercier et al., '95). The Müllerian duct, in many cases, persists after aromatase inhibitor application. In birds, estrogens will feminize males with a reduction in the cortical area of the gonad, but testes development proceeds with maturation of the bird (Wolff, '36 as reviewed in Lance and Bogart, '92). All these studies are consistent with the expression of aromatase being required for sex determination of the female.

AROMATASE AND TSD

The embryonic levels of steroidogenic enzymes at male- and female-producing temperatures have been studied together with the levels of various steroids in different tissues during development in the turtle. White and Thomas ('92) have shown that in early embryogenesis, steroidogenesis takes place in the adrenals, the mesonephros, and the liver, with some steroidogenic activity in the gut. There is little steroidogenesis in the genital ridge and the gonads of very early embryos. Dorizzi et al. ('93), however, showed that estrogen levels in the turtle embryo gonad are greater at female-producing temperatures than at male-producing temperatures at stage 26 (sea turtle staging, equivalent to stage 21 of *Chelydra*). This estrogen level is correlated with the level of aromatase activity in the gonads at this stage.

Gonadal aromatase activity increases during development at female-producing temperatures (Desvages and Pieau, '92a). Aromatase activity begins at about stage 16 and continues to increase until peaking at stage 24. The increase in activity is dramatic after stage 20. This is also the period of medullary cord regression. The levels of aromatase in presumptive testes show a low basal level of activity throughout this sex-determining period. The temperature potency effect has also been shown by performing temperature shifts and then examining aromatase activity levels (Desvages and Pieau, '92b). Gonads from embryos shifted to a higher temperature from a male-producing temperature show a greater increase in aromatase activity.

The levels of aromatase activity in the gonads of sea turtles and terrestrial turtle species show a very similar trend during embryogenesis, suggesting a key role for endogenous estrogen and the enzyme producing it, aromatase, in sexual differentiation of the ovary (Desvages et al., '93). In contrast, Smith and Joss ('94) have shown with alligators that aromatase activity begins to be expressed after sex determination and continues to increase after hatching. This also points to a difficulty in providing a single mechanism for sex determination in reptiles.

Aromatase (the gene)

The cDNA encoding aromatase P450 has been isolated and sequenced from the human (Corbin et al., '88; Harada, '88; Toda et al., '89), rat (Lephart et al., '90), mouse (Terashima et al., '91), pig (Corbin et al., '95), rabbit (Delarue et al., '96), cow

(Hinshelwood et al., '93), chicken (McPhaul et al., '88), zebra finch (Shen et al., '94), trout (Tanaka et al., '92), medaka (Fukada et al., '96), and catfish (Trant, '94). These aromatase cDNA sequences show that approximately 50% of amino acids in fishes have human homologues (Terashima et al., '91). Within a phylogenetic lineage, the homology is much closer. The deduced amino acid sequence for medaka is 75% homologous to that for trout (Tanaka et al., '92). The similarity of the bovine P450 aromatase sequence with that of the mouse, rat, chicken, and trout are 79%, 76%, 71%, and 57% respectively (Hinshelwood et al., '93).

In the human, the gene for aromatase is particularly complex, consisting of 10 introns (Fig. 3). The heme binding region and the entire 3´ untranslated region are encoded in the tenth exon. The size of the gene is estimated to be at least 72 kb. The size of the medaka gene for $P450_{AROM}$, the smallest yet, is 2.6 kb (Tanaka et al., '95).

Aromatase is expressed in the ovary, placenta, adipose, bone, and brain tissue of humans. Tissue-specific expression results from the use of different promoter regions in the same gene. Primer extensions and S1 nuclease protection assays show that transcripts from different tissues have unique 5´ untranslated regions. In the ovary, a proximal promoter close to the start site of transcription is used. The placenta uses a promoter that is at least 40 kb upstream of the start site. The placenta also uses a minor promoter that is 9 kb from the start site. In all cases, the amino acid sequences are identical for each transcript. The alternate promoters are spliced into a common intron/exon boundary 5´ to the start site of translation. Promoters I.1 and I.2 (Fig. 4) (1% of transcripts) are used for transcription of the placental aromatase transcript, whereas promoter II, is used by the ovary. Promoter I.3 is transcribed in the ovary as detected by RT-PCR (Harada et al., '93) and is not discernible in Northern analysis (Simpson et al., '93).

Adipose tissue in humans is a major source of estrogen production in post-menopausal women. The aromatase expression occurs in stromal tissue that surrounds the adipocytes (Simpson et al., '97). Different regions of the body in human females—buttocks, abdomen and thighs—use a different combination of promoters and therefore the transcripts used are varied. The promoters used in the adipose tissue of the buttocks are promoters II, I.3, and I.4, whereas only promoters I.3 and I.4 are used in the thighs and abdomen. Promoter I.4 is used as the major promoter in the buttocks and the thighs, whereas promoters I.4 and I.3 are used in equally in the abdomen (Fig. 4) (Agarwal et al., '97).

Multiple transcripts are also seen in the rat (Hickey et al., '90), pig, and horse (Hinshelwood et al., '95). The porcine aromatase has two isoforms, one that is ovarian and another that is placental. The ovarian form of aromatase is less active and is also expressed in the fetal adrenal and testis (Conley et al., '96). To date, there has only been one published transcript found in the fishes and the birds. However, the goldfish has more than one transcript and appears to be the result of an additional locus for aromatase (Callard and Tchoudakova, '97).

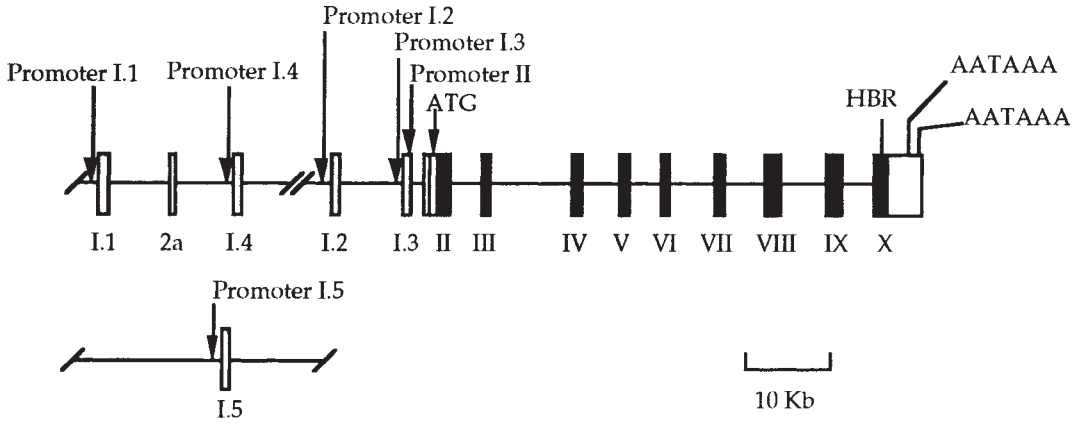

Fig. 3. Gene structure of the human aromatase gene. The open rectangles signify untranslated exons, and the closed rectangles represent translated sequences. The HBR(heme binding region) is on the last exon, which also contains the 3´ untranslated region. The line through the open bar in exon II indicates the acceptor splice site for the exons produced by the promoters upstream of promoter II. Promoter I.5 is used in fetal lung, liver, and intestine. The position of I.5 has not as yet been determined. [Redrawn from Simpson, E.R., et al. (1994) Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocrinol. Rev., *15:*342–355.]

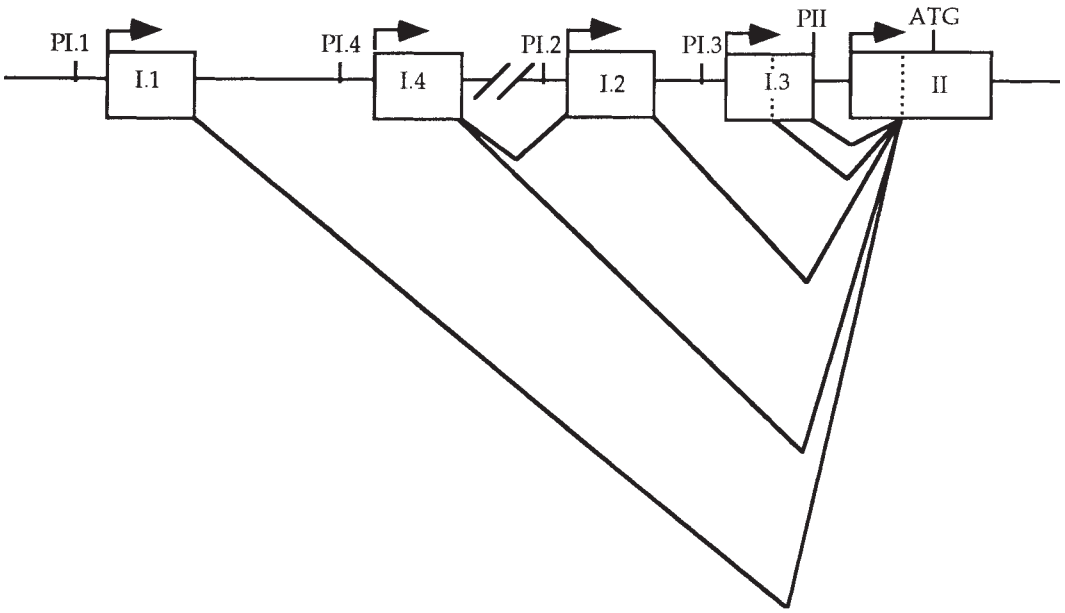

Fig. 4. Alternative promoters used by adipose tissue. The diagram is meant to show the alternative splice pattern that produce the alternative transcripts. The coding regions for all of these transcripts are similar. The region between exons I.4 and I.2 has not been linked and is at least 35 kb, putting the size of the aromatase gene at over 70 kb in length.

Gene regulation of aromatase transcription

The sequences –301 from the placenta-specific exon 1 of the human gene are sufficient for placenta-specific transcription (Yamada et al., '95). The element that is involved in the transcription of the placenta-specific transcript is trophoblast specific element (RNCCTNNNRG), which is bound to by the TSE-binding protein. TSE and TSE-binding protein are found also in the promoter for the α subunit of human chorionic gonadotropin (HCG). An element has been isolated between –2141 and –2115 of exon I.1 that binds NF-IL6 (nuclear factor-interleukin 6 also termed LAP and C/EBPβ) and is involved in TPA (tetradecanoyl phorbol acetate)-mediated enhancement of gene expression. Using DNase I protection assays, another element that binds nuclear factors termed hATRE 2 (human aromatase transcription response element 2) has been found. When used via a bacteriophage expression cDNA library, hATRE 2 allowed isolation of NF-IL6 (Toda et al., '95).

In human adipose tissue, promoter I.4, a TATA-less promoter is used. The expression is stimulated by the jak/STAT signaling pathway. Adipose stromal cells maintained in the presence of serum and glucocorticoids exhibit aromatase expression. The serum component can be replaced with IL-6, IL-11, oncostatin M, and leukemia inhibitory factor (LIF). STAT3 is phosphorylated and binds to a γ interferon activation site (GAS) element. The stimulation of cells by these factors is followed by a rapid phosphorylation of Jak1 and not of Jak2 or Jak3. The Jak/STAT signaling pathway together with sp-1 and glucocorticoids seems to be the main route via which estrogen is synthesized in the elderly (Zhao et al., '95).

The brain transcript in the human isolated by 5′ RACE (rapid amplification of cDNA ends) is yet another tissue-specific transcript. The promoter region for this transcript isolated from a genomic clone contains a putative TATA box and CAAT box. Downstream of the TATA box are a potential *SF-1* site (Ad4 BP) and a potential upstream androgen/glucocorticoid responsive element (Honda et al., '94). Androgens are known, at least in the rat, to regulate aromatase expression in the hypothalamus and preoptic regions of the brain. Aromatase activity is also seen in the amygdala but is not modulated by androgens. The transcripts found in the amygdala and in the hypothalamus are similar (Honda et al., '94). Abdelgadir and coworkers ('94) found similar results to those above, but they also showed that estradiol increases aromatase activity in the preoptic area with no increase in mRNA levels. The increased activity is a result of control at a postranscriptional level. Castrated rats exhibit a decrease in aromatase activity in the preoptic area and in the mediobasal hypothalamus, but activity remains the same in the amygdala. Treatments with test-

osterone or dihydrotestosterone are capable of restoring the aromatase activity and mRNA levels in the preoptic areas and medial basal hypothalamus to the uncastrated levels.

The role of aromatase in TSD of the diamondback

Using a partial terrapin cDNA clone (Jeyasuria et al., '94) as a template, we have isolated full-length cDNA's (~2.4 kb) from a primer-extended library of a terrapin ovary (Jeyasuria et al., '96). The three transcripts have the same coding region but different 5´ untranslated regions suggesting alternate 5´ splicing. A Northern blot probed with the 433-bp fragment (within the coding region but outside the heme binding region) of turtle P-450arom showed that only the ovary and brains (both male and female) gave signals. The ovary showed a band at 2.4 kb for both small and large oocytes mRNAs. A brain-specific band (9.6 kb) was observed for both male and female mRNA, and a 2.4-kb transcript, similar to the one found in the ovary, was also observed in both sexes. The 9.6-kb band exhibited the same intensity for both the male and female brain tissue when standardized to total RNA loaded and therefore an equivalent transcript content. The ovarian 2.4-kb transcript from the large oocytes was five times more abundant than the brain transcripts based on densitometry. There is one smaller band in the ovary that is about 1.4 kb, but this band probably does not produce a functional aromatase protein considering that the aromatase transcript itself is 1,512 bp.

In order to quantify the abundance of aromatase mRNA, we developed a sensitive (<0.01 attomole) competitive RT-PCR technique to study the ontogeny of aromatase transcript levels throughout development in single adrenal/kidney/gonadal (AKG) complexes and brains (Jeyasuria and Place, '97). Consistent with an important role for aromatase in embryonic ovarian differentiation, we find an exponential rise in transcript abundance for AKG complexes at female-producing temperatures during the temperature-sensitive period (TSP). At stage 14, the transcript level is below the detectable limit of the assay (0.001 atmol/μg). Aromatase transcript abundance rises to 1 atmol/organ at stage 18 and reaches levels 10- to 30-fold higher at stages 20–22. The aromatase transcript abundance in AKGs of males lies below the detection limits of the assay at all stages.

According to Desvages and Pieau ('92a), the basal level of aromatase activity in the developing male gonad is about 0.03 to 0.14 femtomole/gonad/hour, which is 500-fold lower than the activity seen in the developing female gonad. Hence, the level of transcript parallels the aromatase activity profiles established by Desvages and Pieau ('92a) for *Emys orbicularis* and those measured by us for *Malaclemys terrapin*. The close relationship between the aromatase activity and the transcript abundance would suggest that, at least in the ovary, estrogen synthesis is under transcriptional control during embryogenesis.

A comparison of aromatase transcript abundance in the male and female brain (Fig. 5) finds that there is greater abundance at the beginning of the TSP in the female brain. Transcript abundance increases exponentially during the TSP but generally remains lower than that found in the female AKG. Aromatase transcript abundance in the male brain is lower at the beginning of the TSP but becomes greater than the female brain in the second half of the TSP. Estradiol treatment at stage 14 reduces the transcript level in the brain at stage 18, which suggests that estradiol exerts a negative feedback on the brain. The early rise in aromatase transcript in the brain at 31°C may actually stimulate estrogen synthesis early, which initiates the AKG exerting a negative feedback on itself (the brain) and a positive feedback on the AKG aromatase expression. When estradiol is added to the embryo at 31°C, the brain aromatase reaches levels that are equivalent to a stage-26 embryo at stages 22–25. This is probably related to an acceleration in the differentiation of the ovary and an earlier second-phase rise in aromatase levels in the brain. The effect of estradiol in the male brain is a depression of transcript abundance between stages 21 (within the TSP) and 25 consistent with the lower levels seen at female-producing temperatures.

In mammals, the synthesis of estrogen in the brain is localized and controlled by different promoters. (Lephart, '96). The brain transcript in the rat is tissue-specific, and the promoter region contains an upstream *SF-1* site (Ad4 BP) and an androgen-responsive element. Androgens are known, at least in the rat, to regulate aromatase expression in the hypothalamus and preoptic regions of the brain. Aromatase activity is also seen in the amygdala but is not modulated by androgens; the transcripts found in the amygdala and the hypothalamus are, however, similar (Honda et al., '94). Levels of aromatase activity are enhanced in embryonic day-13 mice by stimulation of α1-adrenergic receptors and through the activation of protein kinase C and G (Abe-Dohmae et al., '96).

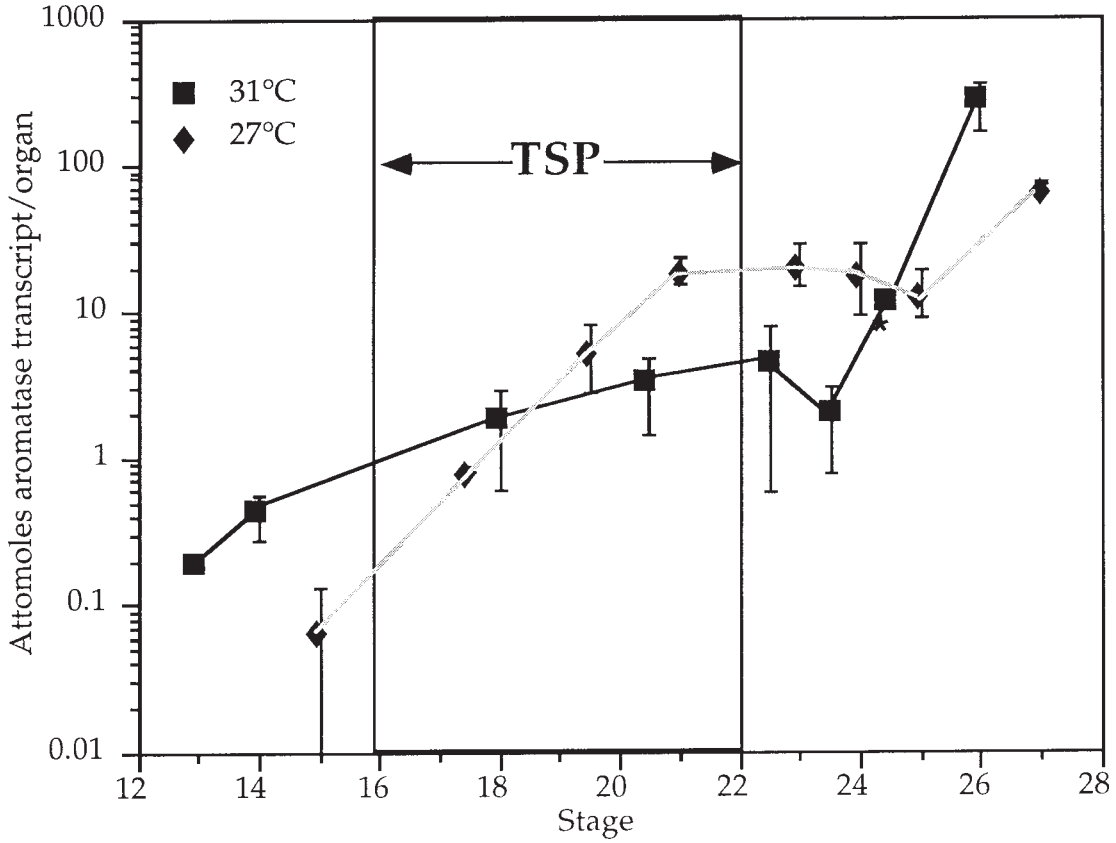

Fig. 5. A comparison of the abundance of aromatase transcripts (attomoles/organ) in the brains of embryos incubated at male (27°C) and female (31°C) temperatures with respect to the stage of embryonic development. The asterisk (*) represents a point with a higher variation that can be represented in this graph (SE = 11.99). A comparison of aromatase abundance for different incubation temperatures versus stages was done for stage groups rather than discrete stages because of different staging of the two temperatures. Differences were grouped for stages 17.5–19, 20.5–22, 22.5–23, and 23.5–24 (P = 0.48, P =0.011, P = 0.048, and P = 0.019, respectively). Boxed area represents the temperature-sensitive period (TSP).

In mammals, local estrogen formation in the brain plays an important role in the sexual differentiation of the brain. In rats and mice, fetoneonatal estrogen binding protein (FEBP, α-fetoprotein) mops up maternal estrogen in the fetal circulation, but testosterone is able to transverse the brain and is then aromatized to estrogen (Abe-Dohmae et al., '96). Roselli et al. ('94) showed that estradiol increases the aromatase activity in the preoptic area, but mRNA levels remain the same as the untreated individuals, which suggests that increased activity is a result of a postranslational mechanism.

Interestingly, the transcripts found in brains of both male and female adult terrapins includes the ovarian transcript as well as the 9.6-kb brain transcript. Whether this transcript is translatable to a functional product has not been determined at this time. If this transcript turns out to be a differentially spliced nonfunctional transcript or an unspliced precursor of the ovarian transcript, then it may explain the differences between the sexes. It would be tempting to suggest that a temperature-dependent splicing event is involved in aromatase expression in the reptile brain.

SOME MODELS FOR TSD

It has been shown very clearly that estradiol will reverse the sex of males to females at male-producing temperatures for turtles (Pieau, '74; Gutzke and Bull, '86; Bull et al., '88; Crews et al., '89; Jeyasuria et al., '94) and alligators (Lance and Bogart, '92). In accordance with this finding, aromatase enzymatic activities are high in gonads at female-producing temperature, but they are low at male-producing temperatures (Desvages and Pieau, '92a). We find similar results with the diamondback terrapin. The mean ovary and testes aromatase activities in stage-23 embyros were 126 ± 4.34 (n = 6) fmol/hr · gonad and 0.067 ± 0.131 fmol/hr · gonad, respectively. The female and male

brain aromatase activities were 4.6 ± 0.32 fmol/hr · brain and 7.34 ± 1.5 fmol/hr · brain, respectively. The aromatase mRNA levels in gonads correlate with the measured aromatase activities (Jeyasuria and Place, unpubl. data). The brain transcript levels are roughly proportional to the activity measurements, although the male brain has 10 times greater transcript abundance.

Because estrogens reverse the sex of males, it would be expected that blocking estrogen synthesis would cause female sex reversal. Aromatase inhibitors are capable of reversing the sex of females to males especially at female-biased temperatures that are close to the pivotal temperature (Crews et al., '94; Richard-Mercier et al., '95). This female-to-male sex reversal is enhanced by the addition of both aromatase inhibitor and testosterone, making reversal possible at female-producing temperatures farther from the pivotal temperature. This implies a function for androgens in male gonadal differentiation (Crews et al., '94; Jeyasuria et al., '94). Dihydrotestosterone (DHT), a non-aromatizable androgen, is capable of favoring male development at a female temperature bias close to the pivotal temperature (Crews et al., '94). The conversion of testosterone to DHT is under the control of the enzyme 5α-reductase, and the use of 5α-reductase inhibitors at pivotal temperatures has the expected effect of producing a higher proportion of females (Crews et al., '94).

The presence of aromatase activity and transcripts in the presumptive ovary early in the TSP suggests that estrogen not only has a role at the beginning of ovarian development but it also has a role in the maintenance of cortical proliferation and the regression of the sex cords as suggested by Pieau ('96). Estrogen receptors have been shown to be transcribed in gonads of both presumptive sexes prior to the onset of aromatase mRNA in the genotypic female gonad (Smith et al., '97). The aromatase transcript level in the brain shows a similar increase at this stage, though it is not as dramatic as in the AKG. The levels are, however, threefold higher in the male than in the female brain. The increased transcription of aromatase and its attendant increase in aromatase activity and estrogen synthesis in the presumptive ovary may initially be controlled directly from the brain.

Aromatase mRNA is present in the brain at both male and female temperatures prior to presence of aromatase mRNA in the presumptive ovary. There is a lower level of aromatase mRNA in the male brain (27°C) than in the female brain (31°C) during the early part of the sex-determining period, but this level is reversed during the latter part of the sex-determining period (Fig. 5). The reversal of these levels in the brain coincides with the exponential rise in aromatase transcript abundance in the presumptive ovary. Higher levels of aromatase may reduce 5α reduction of androgen substrates at physiological levels (an important consideration with experiments involving the applications of larger doses of steroids).

Steroids also may not act through classical intracellular steroid receptor-based mechanisms but may act directly on membranes. 5α and 5β-pregnan-3α-ol-20-one bind directly to $GABA_A$ receptors, consequently increasing the current through associated Cl^- channels. Both 5α and 5β reductase are found at high levels in areas of the zebra finch brain that lack steroid receptors (Vanson et al., '96). The presence of unmyelinated axon bundles found in the early differentiating ovary of *Lepidochelys olivacea* (Pacific ridley turtle) (Merchant-Larios et al., '89) may suggest a direct innervative role of the brain on gonadal development. The gonads of turtles that have been organ-cultured undergo differentiation in vitro but are not affected by male and female incubation temperatures, again implying that the signal for differentiation is under temperature control and comes from a source other than the gonad itself (Merchant-Larios and Villalpando, '90). If the message to develop into an ovary or a testis comes from elsewhere, it is quite feasible that this message could come from the brain because the brain shows a sexual dimorphic expression of aromatase before the gonad. However, early studies with hypophysectomies of young embryos of lizards (Raynaud and Pieau, '85), have demonstrated that sexual differentiation of the gonads proceeds normally in decapitated embyros. The gonadal anlagen differentiates into testes or into ovaries with normal Müllerian duct regression in males. Whether these studies with genotypic sex-determined species are applicable to environmentally sex-determined species is unclear. The brain/gonad interdependence could be uncoupled by a sex chromosome locus.

It is also possible that the signal for sex determination could come from the yolk. Conley et al. ('97) have determined that the yolk of alligators contains appreciable reserves of estradiol, androstenedione, and, to a lesser extent, testosterone. These steroids decline markedly, coincident with gonadal differentiation. The authors suggest that,

at female temperatures, estradiol initiates aromatase expression and stimulates ovarian differentiation. They also suggested that this reservoir of variable estradiol provides a significant, epigenetic maternal contribution to the observed effect of clutch on sex ratios. It is difficult, however, to use this argument to explain the bimodal temperature dependence of snapping turtles and crocodiles.

A critical finding in our studies through competitive RT-PCR quantification of aromatase mRNA levels in the brain is that it is reduced by estrogen treatment at 27°C during the temperature-sensitive period. This finding is a twist on the balance of steroid ratios model initially suggested by Bogart ('87). Estrogen production is reduced in the brain in female development, but it is increased in the gonads. The balance of aromatase activity versus 5α-reductase activity may play this balancing role both in the gonad and the brain. Added progesterone (5 μM) to aromatase activity assays of gonads (Jeyasuria et al., '94) results in enhancement (nearly twofold) of activity and can be attributed to providing an alternate substrate for 5α-reductase activity, which would otherwise reduce the substrate available for aromatase. The enhancement seen in the brain is not as great, suggesting a lower 5α-reductase in the brain (Jeyasuria et al., '94). 5α-reductase activity has been shown to be extremely high in turtle embryo gonads (Desvages and Pieau, '91). A schema summarizing our current findings on aromatase expression is presented in Figure 6.

What is the role of temperature in this process? At this point, we would like to hypothesize some possible mechanisms based on the available data in other species about how temperature might influence sex in reptiles.

MODEL 1: TEMPERATURE-DEPENDENT DIFFERENTIAL SPLICING

As we have suggested, the different levels of transcripts seen in the brain during development may be due in part to the differential splicing of the aromatase transcripts in the brain. The study by Harry et al. ('90) on the loggerhead turtle showed some differences in heterogeneous nuclear ribonucleoprotein particles (hnRNPs) in male and female temperatures in urogenital tissue. A higher level of hnRNPs was seen at male temperatures than at female temperatures at early stages (stage 23–26). The reverse occurs at stages 27 and 28, when the expression was seen in the female. This difference in hnRNPs is based on a mammalian antibody cross-reactivity of a HSP90 antibody. The difference in hnRNPs was seen in the urogenital tissues, but hnRNPs are probably involved in differential splicing in the brain, and these splicing complexes may be temperature-sensitive.

It is conceivable that the large brain transcript we observe may be due to a long 3′ UTR, which would be similar to the bovine aromatase transcript that has a 3.5-kb 3′ UTR. The bovine aromatase 3′ UTR is homologous to the human 3′ UTR but has lost the polyadenylation signal (PAS) that is found in the human transcript. It has been suggested that the 3′ end is longer because a downstream PAS is used (Vanselow and Furbass, '95). It is thus possible that translation of the large brain transcript in the terrapin is repressed by the binding of hnRNPs to the 3′ UTR. This repression again may be temperature-sensitive. It is known that hnRNPs form presplicing complexes that keep splicing repressed until a mild heat shock allows completion of the splicing. The reverse may occur in the brain, where the aromatase mRNA in the developing brain is repressed. Future work in these areas will help delineate whether these are viable options.

Competition of 5α-reductase and aromatase activity in the brain

Bogart ('87) hypothesized that a balance of androgens to estrogens would decide the male/female outcome, which we adopt as a basic premise for our models. However, this balance would have different outcomes, depending on which organ gives the master signal, the brain or the gonad. In the brain, a high estrogen balance would initiate a male developmental pathway, whereas a low estrogen balance would initiate a female developmental pathway. The reverse estrogen balance would operate in the gonad. This balance between estrogens and androgens is largely modulated by activities of two enzymes, aromatase and 5α-reductase, given that steroidogenesis is already operative. Mammalian 5α-reductase has two isoforms. Isoform 2 (type 2 5α-reductase) from the human has a very sharp pH activity profile (Wilson, '92) with an optimum at pH 5.5. This same pH profile is seen in rats (Poletti et al., '96), where the two isoforms have been expressed in a yeast system. Activity rapidly decreases on either side of the optimum. In humans and rats, the target tissues for androgens like the prostrate have a pH of 5.5 (Wilson, '92; Poletti et al., '96). These two isoforms have not yet been demonstrated in lower vertebrates, but 5α-reductase activity has been demonstrated in all vertebrates.

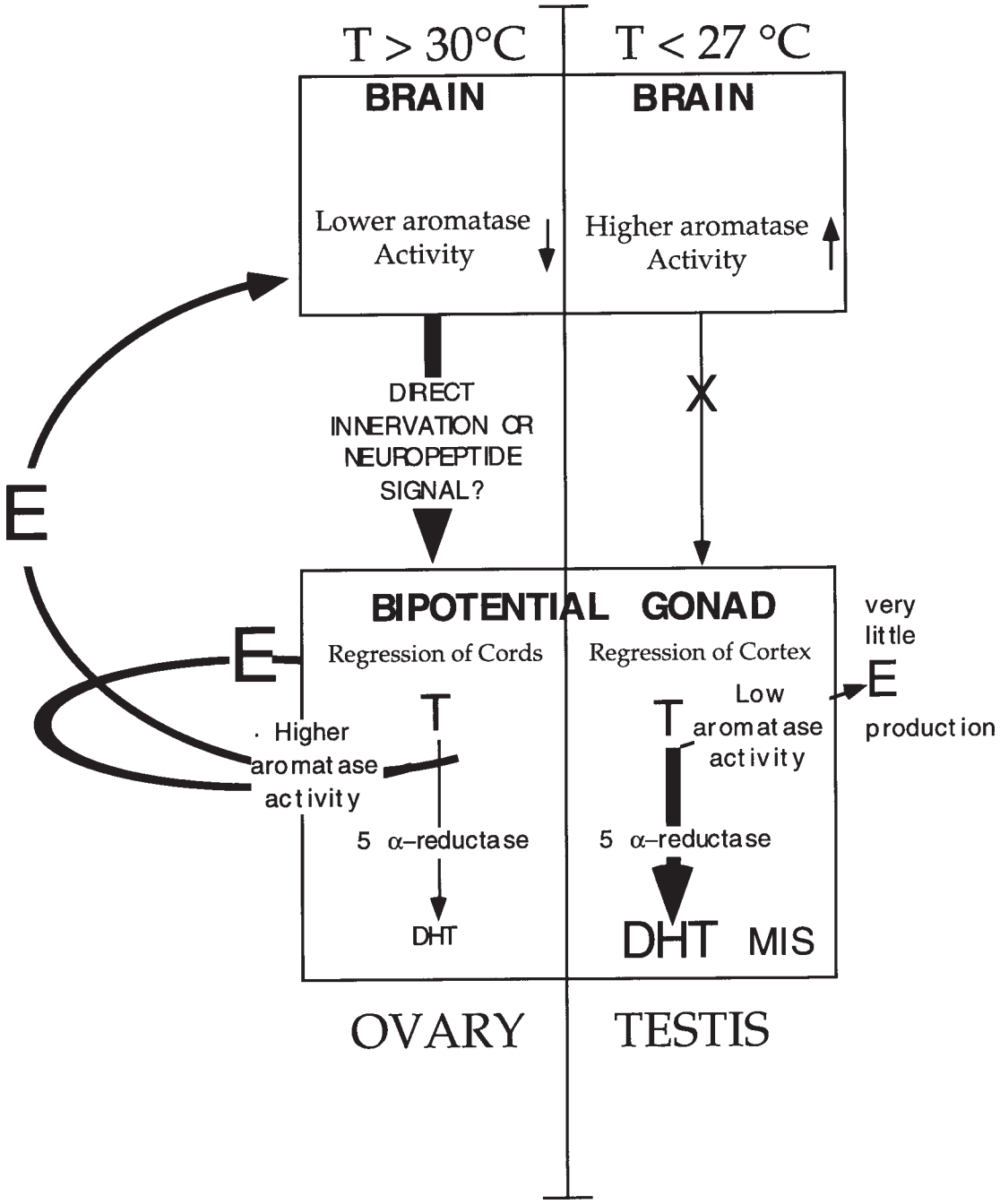

Fig. 6. A schema showing how competing aromatase and 5a-reductase and their respective products estrogen and dihydrotestosterone could be involved in the sex-determining process of reptiles.

Temperature control of 5α-reductase activity in the brain

Within the cytosol of poikilotherms, pH is affected by alterations in temperature: a decrease in pH of about 0.017 pH units per degree centigrade (Hochachka and Somero, '84). Raising the incubation temperature would decrease the pH intracellularly in the terrapin embryo, and thus even a small shift in pH would alter 5α-reductase activity significantly because of a strong pH dependence curve. Higher temperatures would also decrease the pH by increasing the metabolic rate and thus the O_2 consumption, which would raise the CO_2 levels and further lower the pH. *Trachemys scripta* eggs incubated at 29°C and a CO_2 concentration of 0% produced 20% females.

When the CO_2 levels were increased to 5%, 10%, and 15%, however, there was a shift to a female bias of 63%, 80%, and 63%, respectively. Etchberger et al. ('92) argued that the increase in CO_2 levels lowers the cellular pH, which is similar to what would happen at higher female-producing temperatures where the blood CO_2 levels increase because of increased metabolism.

We have shown that both male and female brains express aromatase before the temperature-sensitive period in turtles. We expect similar findings will be found for 5α-reductase. If we assume that reptiles possess a Type 2 5α-reductase isoform with a narrow bell-shaped pH dependency of activity and that aromatase possesses a much broader pH dependency than the Type 2 5α-reductase (which preliminary data support), then the following model can be proposed for temperature-dependent sex determination.

In order for male brains to have a high estrogen

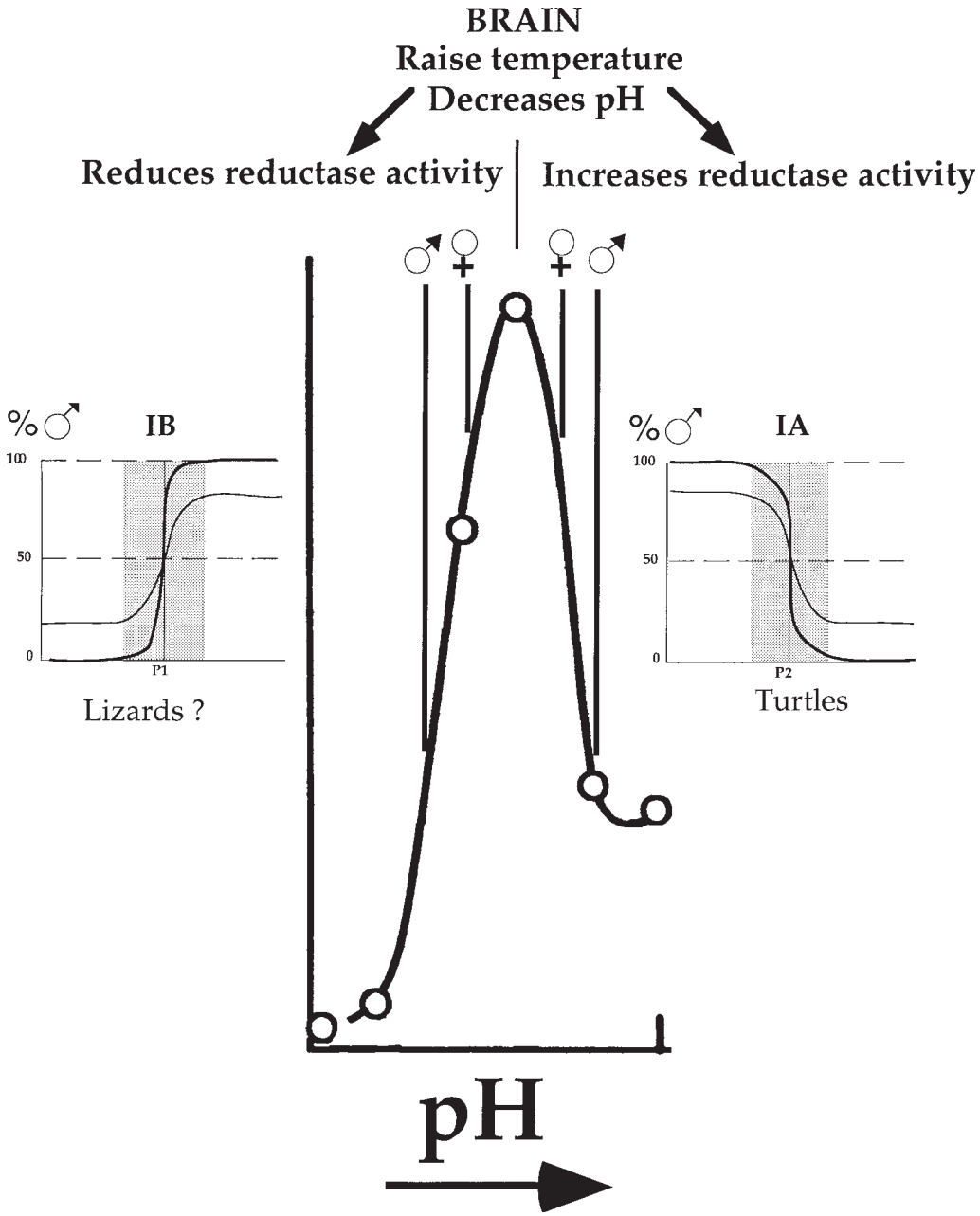

Fig. 7. Model using 5α-reductase pH optimum curve to show how reductase activity would increase or decrease in the brain with respect to an increase in temperature. Patterns IA and patterns IB are derived from this curve, but pattern II cannot be derived from this model.

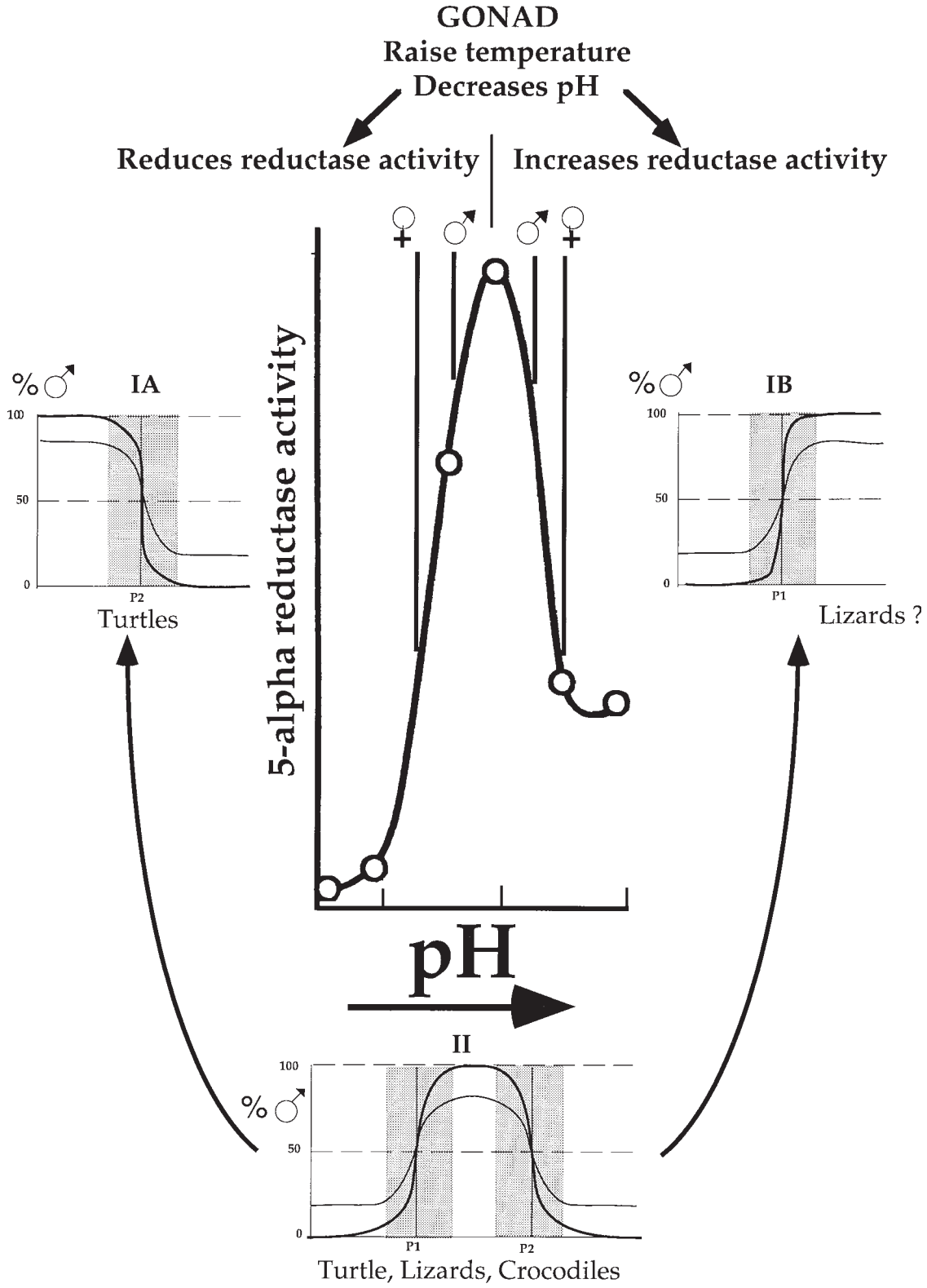

Fig. 8. Schematic of 5α-reductase activity pH optimum curve (derived from mammalian literature) and the effect of a pH change with temperature (hypothetical) on both sides of the optimum curve. Also shown are the different temperature patterns found in reptiles and how they may be derived from this pH optimum curve. All known patterns of temperature-dependent sex determination can be predicted from this model.

balance at male incubation temperatures, the 5α-reductase activity in the males brain will be as shown in Figure 7. The 5α-reductase activity for males will be either on the right side (Pattern IB) or on the left side (Pattern IA) of the bell curve, with that for female being closer to the pH optima. Increases in incubation temperature would decrease cellular pH in the embryo and result in higher 5α-reductase activity for turtles and lower activity for lizards. This would then be consistent with the sex-ratio patterns seen for these species. For turtles, secondarily produced neurotransmitters could then signal the development of an ovary either by direct innervation or via the hypothalamic-pituitary-gonadal axis. The developing ovary would express aromatase mRNA, resulting in increasing aromatase activity (Desvages and Pieau, '92a,b) and estrogen production. The circulating estrogen levels could then exert a negative feedback on the transcription of aromatase in the brain. Unfortunately, this model is incompatible with the Pattern II of sex ratios found in all crocodiles and some turtles like the snapping turtle.

Temperature control of 5α-reductase activity in the gonad

Our model could also be applied to the gonad (Fig. 8) where DHT production by 5α-reductase would compete with estrogen production for androgen substrates and increase proliferation of the medullary region of the gonad forming testicular cords. For this to happen in turtles, male 5α-reductase activity would be on the left side of the bell curve closer to the pH optima; raising the temperature would then lower the activity and thus increase the estrogen balance and produce females. Conversely, in lizards, higher temperatures would result in increased 5α-reductase activity and produce males. By having high 5α-reductase activity associated with males, all known patterns of temperature-dependent sex determination can be predicted, including Pattern II. The model calls for both these enzymes, aromatase and 5α-reductase, to compete with each other to initiate and maintain an ovary or a testes respectively.

From our results, it is apparent that a developing ovary expresses aromatase mRNA within the temperature-sensitive period of sex determination. It is also apparent that the brain aromatase mRNA levels are under negative control by estradiol. Furthermore, brain aromatase mRNA is expressed early in development, and female levels are lower than male levels at a critical period in the TSP (approximately stage 18). In the gonad, aromatase transcripts are quantifiable at female temperatures by stage 14, whereas they are undetectable at male temperatures throughout the temperature-sensitive period.

Estrogen is efficient at reversing the sex of females to males because its feminizing effects operate in two areas, the brain and the gonad. Aromatase inhibitors have mixed success because, while their effects in the gonad are to prevent estrogen production and produce a male, the effect in the brain is just the reverse. The relative ineffectiveness of 5α-reductase inhibitors is also due to the contrary effect they have on the brain and the gonad. Blocking 5α-reductase drives sex phenotype in the female direction in the gonad but may drive it in the male direction in the brain.

In conclusion, this work has shown the importance of aromatase expression in temperature-dependent sex determination and the negative control of the transcription of aromatase by estrogen in the brain. The model that seems most parsimonious is that aromatase and 5α-reductase compete for a similar androgen substrate in the gonad and that 5α-reductase activity is under a unique temperature/pH control in the gonad. It is hypothesized that the sex differentiation process in TSD requires cross-talk between the brain and the gonad. The messenger for this brain-gonadal axis is estrogen in the gonad-brain direction, and the messenger for the brain-gonad direction is unknown but is hypothesized to be a direct nervous connection.

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