Commentary

Temperature, steroids and sex determination

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Soon after the sex steroid hormones were first identified, isolated and synthesized, they were found to have potent effects on sex determination and sexual differentiation in various fish, amphibians, reptiles and, to a lesser extent, birds (reviewed in Burns 1961). However, there was no evidence that these chemicals could influence gonadal differentiation in mammals, although they were recognized to mediate differentiation of accessory and secondary sex characteristics. Indeed, conventional wisdom today holds that steroid hormones play no role in sex determination in mammals, and it is only following gonadal differentiation that steroid hormones produced by the ovaries or testes sculpt the characters that distinguish males from females. Thus, with the exception of limited research in aquaculture and poultry science, the study of the role of sex hormones in vertebrate sex determination essentially ceased by midcentury, with the result that current texts focus on the molecular genetics of sex determination in mammals, and restrict discussion of sex steroid hormones to their influence on the sexual differentiation.

Although the research from common laboratory and domesticated mammals, including humans, supports this principle of steroid hormone independence of sex determination, the singularity of chromosomal control of mammalian sex determination may not be so absolute. Indeed, evidence that we may be overlooking something important has existed for many years. For example, in laboratory mammals, gonadal primordia from genetic female fetuses transplanted under the kidney capsule of adult male hosts develop into ovotestes (for review see George & Wilson 1994). Electron microscopic examination of the testicular portion of these ovotestes reveals various types of testicular somatic cells including Sertoli and Leydig cells. In metatherian mammals, administration of exogenous oestrogen will sex-reverse male embryos (reviewed in Burns 1961). Because mammals arose from progenitors common to other vertebrate classes, it seems reasonable that vestigial remnants of a sex hormone mediation of sex determination might be present in mammals.

Does sex steroid hormone control of sex determination reflect an ancestral condition?

The hypothesis that steroid hormone determination of gonadal sex may have preceded chromosomal or genotypic

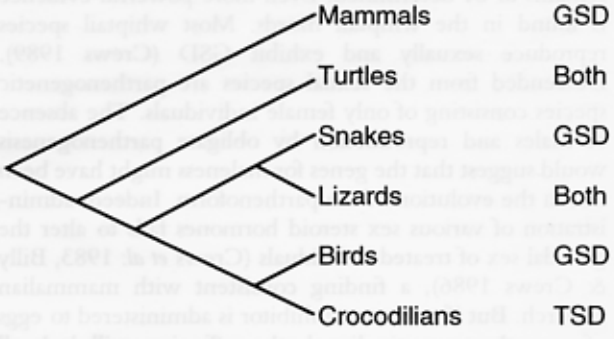

FIGURE 1. Phylogeny of sex-determining mechanisms in amniote vertebrates. In lizards and turtles both genotypic sex determination (GSD) and temperature-dependent sex determination (TSD) have been documented.

determination of gonadal sex (=genotypic sex determination or GSD) in vertebrate evolution can be tested. Reptiles are amniotes and occupy a pivotal position in the evolution of both mammals and birds; the therapsid reptiles gave rise to mammals and ancient crocodilians gave rise to birds (Fig. 1). It stands to reason then that mammals would share the basic amniote pattern of sex determination, but have evolved regulatory genes to control the initiation of sex determination (e.g. in eutherian mammals a single copy gene *SRY* on the Y chromosome encodes the testis-determining factor leading to testicular differentiation and development of a male phenotype). One hypothesis is that these regulatory genes have been superimposed onto an otherwise reptilian system. However, *SRY* is part of a large family of genes, and comparative studies in a variety of vertebrate species, including reptiles, having either GSD or temperature-dependent sex determination (TSD) do not find *SRY*-like genes to be sex-linked (Tiersch *et al.* 1991). Thus, an alternative hypothesis is that *SRY*-like genes evolved independently of TSD. In this scenario sex determination initially was regulated by steroid hormones and *SRY* was not associated with a particular sex, only subsequently becoming associated with one sex and acquiring a sex-determining function. Finally, it is possible that an *SRY*-like gene evolved early but was regulated by steroid hormones. Later, this gene moved

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to a heritable sex-specific chromosome with attendant loss of steroid regulation.

The best evidence that steroid hormones may be important in sex determination in species with genotypic sex-determining mechanisms is found in recent experiments with birds and lizards having GSD. Administration of aromatase inhibitor to genetic female chicken embryos results in phenotypic male hatchlings complete with testes (Elbrecht & Smith 1992, Wartenberg *et al.* 1992); whether these sex-reversed individuals will reproduce as males remains to be determined. Even more powerful evidence is found in the whiptail lizards. Most whiptail species reproduce sexually and exhibit GSD (Crews 1989). Descended from the sexual species are parthenogenetic species consisting of only female individuals. The absence of males and reproduction by obligate parthenogenesis would suggest that the genes for maleness might have been lost in the evolution of the parthenoform. Indeed, administration of various sex steroid hormones fails to alter the gonadal sex of treated individuals (Crews *et al.* 1983, Billy & Crews 1986), a finding consistent with mammalian research. But if aromatase inhibitor is administered to eggs of a parthenogenetic lizard, the offspring will lack all female characteristics and have testes, vasa differentia and hemipenes, and produce motile sperm as adults (Wibbels & Crews 1994). Taken together, these results suggest that the administration of aromatase inhibitor to the embryo in some manner circumvented those regulatory genes that normally trigger ovarian development, yet activated the co-ordinated genetic cascade that results in a male-typical phenotype.

What is TSD?

In the last 20 years we have learned that many (but by no means all) egg-laying reptiles lack sex chromosomes and, instead, exhibit TSD (for reviews see Bull 1980, Raynaud & Pieau 1985, Ewert & Nelson 1991, Crews *et al.* 1994, Janzzen & Paukstis 1991, Pieau *et al.* 1994); it is perhaps significant that all viviparous reptiles appear to exhibit GSD. In TSD, the temperature experienced by the embryo during the middle third of incubation determines the sex of the offspring, rather than the genotype established at fertilization (Fig. 2). Experiments indicate that the spectrum of temperature is slight, usually extending over no more than 5–7 °C, and that the transitional temperature range (those temperatures yielding a mixed sex ratio rather than all males or all females) can be extremely narrow, as little as 1 °C. Further, the effect of temperature during this window of sensitivity is quantitative, having a cumulative effect on sex determination.

The manifold effects of incubation temperature

The temperature experienced by the embryo not only determines gonadal sex, but also influences sexual differentiation. Indeed, it appears that much of the variation observed within the sexes of TSD reptiles can be traced back to the incubation temperature of the eggs, much like intrasexual variation in polytocous mammals can be traced to the position of the fetus relative to the sex of its neighbours *in utero*. This is demonstrated particularly well in the leopard gecko. In this species, females are produced at extreme incubation temperatures with males being produced at intermediate incubation temperatures (pattern C in Fig. 2). If individuals from known incubation temperatures are followed to adulthood, we will find that sexually dimorphic morphology, physiology and behaviour are affected profoundly by the temperature experienced *in ovo*. For example, in the leopard gecko the male is the larger sex. However, females from a male-biased incubation temperature grow faster and larger than do females from a female-biased incubation temperature, growing as rapidly and as large as males from lower, female-biased incubation temperatures (Tousignant & Crews 1992). When the effects of gonadal sex and incubation temperature are experimentally dissociated using oestrogen to sex-reverse eggs incubating at a male-biased temperature, we find that oestrogen-determined females grow according to the incubation temperature rather than their gonadal sex.

The onset of sexual maturity and plasma hormone levels in adult leopard geckos also varies as a function of the incubation temperature of the egg. Associated with these

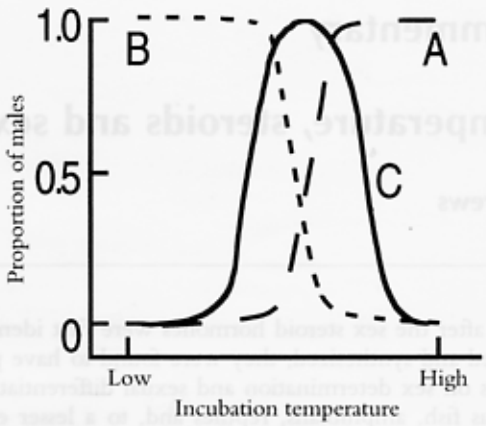

FIGURE 2. Response of hatchling sex ratio to incubation temperature in various egg-laying reptiles. These graphs represent only the approximate pattern of the response and are not drawn according to any single species. The three patterns recognized presently are (A) only females produced from low incubation temperatures, males at high temperatures, (B) only males produced from low incubation temperatures, females at high temperatures, and (C) only females produced at the temperature extremes, with male production at the intermediate incubation temperatures. Genotypic sex determination also occurs in reptiles with the result that the hatchling sex ratio is fixed at 1:1 despite incubation conditions.

sex and temperature differences in growth rate are differences in the circulating concentrations of thyroxine (Coomber *et al.* 1994). Further, females from a male-biased incubation temperature take longer to reach sexual maturity (Tousignant & Crews 1992) and have significantly lower circulating concentrations of oestradiol (OE_2) and significantly higher testosterone levels than females from all-female or female-biased incubation temperatures (Gutzke & Crews 1988). Similarly, males from a female-biased incubation temperature have significantly higher levels of OE_2 as adults than do males from a male-biased incubation temperature (Tousignant *et al.* 1994).

Incubation temperature also affects adult sociosexual behaviour. Aggression appears to be a male-typical trait in the leopard gecko. Females usually show little or no aggression in response to males, whereas males will posture and often attack another male as he approaches; males rarely attack females. Females from female-biased incubation temperatures are less likely to be aggressive toward male stimulus animals compared with females from a male-biased incubation temperature (Flores *et al.* 1994). If oestrogen is used to sex-reverse eggs incubated at a male-biased temperature, as adults these oestrogen-determined females will be as aggressive as the rare temperature-determined females from the same male-biased temperature. This suggests that the aggressive behaviour in a female leopard gecko is less affected by ovarian hormones than by incubation temperature.

Attractiveness to males is a female-typical trait. However, females from a male-biased incubation temperature are less attractive to males than are females from female-biased incubation temperatures; oestrogen-determined females from a male-biased incubation temperature are both attractive and aggressive (Flores *et al.* 1994). Taken together, these findings suggest that in TSD, sexual differentiation, as sex determination, results from a cascade of events initiated during early development in which the effects of incubation temperature has primacy over the effects of steroid hormones.

Are sex steroid hormones the physiological equivalent of incubation temperature?

How does temperature determine sex? Pieau (1974) was the first to demonstrate that if OE_2 is administered to turtle eggs incubating at a male-producing temperature, all of the offspring will have ovaries (Fig. 3). Subsequently a number of researchers have replicated this finding with a variety of turtles, crocodilians and lizards with TSD; recent evidence indicates that this oestrogen-induced female sex determination is permanent, resulting in individuals that lay eggs as adults (for reviews see Raynaud & Pieau 1985, Crews *et al.* 1994, Pieau *et al.* 1994, Wibbels *et al.* 1994). The window of hormone sensitivity corresponds to the window of temperature sensitivity or during the middle third of incubation. In addition, there is a synergism between oestrogen sensitivity and incubation temperature such that the closer an egg is to an all-female producing temperature, the less exogenous OE_2 is required to override the effects of incubation temperature. These and other findings led to the suggestion that incubation temperature may exert its action on sex determination via sex steroid hormones (Fig. 4).

We might predict therefore that if oestrogen will override the effects of a male-producing incubation temperature, then administration of exogenous androgens to eggs incubating at an all-female temperature should result in male hatchlings (Fig. 3). This is not the case. Indeed, if testosterone is administered to eggs at a male-producing temperature, about half will hatch as females. This is probably due to the aromatization of the testosterone to OE_2, as administration of both testosterone and aromatase inhibitor to eggs incubating at a male-producing temperature results in only male offspring (Crews & Bergeron 1994). If a non-aromatizable androgen such as dihydrotestosterone (DHT) is administered to eggs incubating at an all-female producing temperature, there is no discernable effect on the ovarian nature of the gonad. However, if DHT is administered to eggs incubating at an intermediate incubation temperature that produces a mixed sex ratio, most or all of the hatchlings will be male (Wibbels & Crews 1993) (Fig. 3). Unlike with OE_2, there is no apparent synergism between incubation temperature and DHT sensitivity. Taken together, these data indicate that male sex determination is not a default state but is under the influence of androgenic hormones in much the same way as female sex determination is oestrogen-sensitive.

Other research with steroidogenic enzymes and their inhibitors show clearly that steroids must be involved in the mediation of the temperature induction of sex determination. Steroidogenic enzyme activity is influenced by incubation temperature in turtles with TSD. Pieau (1973) demonstrated 3β-hydroxysteroid dehydrogenase (HSDH) activity in the undifferentiated gonads of the European pond turtle, with activity being the most intense in embryos incubating at a male-producing temperature. There appear to be species-differences, as significant 3β-HSDH activity is present in the adrenals and mesonephros but not in the undifferentiated gonads of the Olive ridley sea turtle (Merchant-Larios *et al.* 1989), the saltwater crocodile (Smith & Joss 1994) and the red-eared slider (Thomas *et al.* 1992).

Levels of the enzyme aromatase increase at the end of the temperature-sensitive window in embryos of the European pond turtle incubating at a female-producing incubation temperature, but not in embryos incubating at a male-producing incubation temperature (Desvages & Pieau 1992*a*). Similar results have been obtained for the saltwater crocodile (Smith & Joss 1994). This increase does not appear to be due to temperature modulation of aromatase activity, but rather to temperature-induced

Chemical Manipulations

Steroid hormones	Synthetic ligands	Enzyme inhibitors	Peptides	Steroid antibodies
Male-producing temperature				
♀ Estradiol-17β	♀ R2858	- Cyanoketone	- EGF	* Androgen antiserum
♀ Estradiol benzoate	♀ DES	Reductase inhibitor		
♀/ Testosterone	♀ PCBs	- 4MA		
- Corticosterone	♀/ Norethindrone	- MK906		
- Cholesterol	♀,♀/ Tamoxifen	Aromatase inhibitor		
- Dihydrotestosterone	♀/ Clomiphene citrate	- ATD		
- Progesterone	♀/ Nafoxidine	- 4-OHA		
	- Hydroxyflutamide	- CGS 16949A		
	- Metyrapone	- CGS 20267		
	- R5020			
	- RU486			
	- Aminoglutethimide			
	- Cyproterone acetate			
	- ICI M164384			
Intermediate temperature (mixed sex ratio)				
♀/,I Testosterone	♀ Norethindrone	Reductase inhibitor		
♂ Dihydrotestosterone	♀ Tamoxifen	♀ 4MA		
♀ Estradiol-17β		♀ MK906		
I DHT + E2		Aromatase inhibitor		
		♂ CGS 16949A		
		♂,I CGS 20267		
Female-producing temperature				
- Testosterone	* Norethindrone	- Cyanoketone	- MIS	* Estrogen antiserum
- Dihydrotestosterone	-,I Tamoxifen	Reductase inhibitor		
- Progesterone	♂/ Aminoglutethimide	- 4MA		
- Estradiol-17β	- R1881	- MK906		
- Estradiol benzoate	- Nafoxidine	Aromatase inhibitor		
- Cholesterol	- Depoprovera	♂ CGS 16949A		
	- Cyproterone acetate	♂ CGS 20267		
	- ICI M164384	- ATD		
		- 4-OHA		

FIGURE 3. Effects of various chemical manipulations *in ovo* on the hatchling sex ratio in reptiles with temperature-dependent sex determination. Summary of experiments with steroid hormones, synthetic steroid ligands, steroidogenic enzyme inhibitors and steroid hormone antibodies. Minus indicates no observed effect and sex was concordant with incubation temperature. Symbol indicates direction of sex determination; symbol followed by diagonal indicates that only some individuals were affected by the treatment. I indicates intersex. Asterisk indicates that normal sexual differentiation, but not gonadal sex, was disrupted by treatment. Multiple symbols reflect differences in results of experiments using different species. Note that some chemicals (e.g. norethindrone) known to be antagonists in mammals and birds act as agonists in temperature-dependent sex determination.

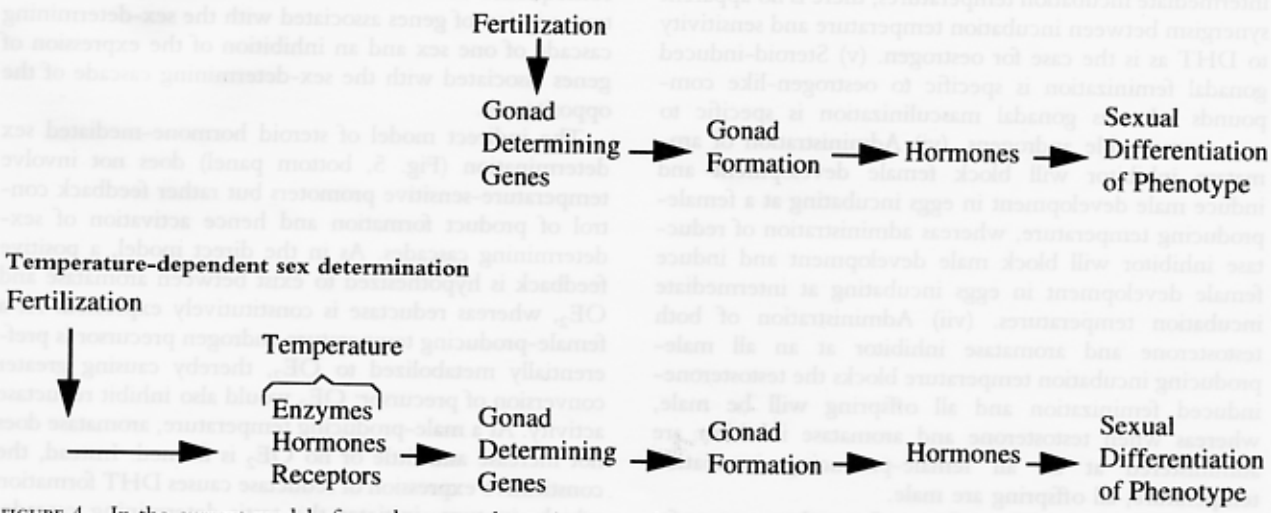

FIGURE 4. In the current model of vertebrate sex determination and sexual differentiation (top panel), gonadal sex is fixed at fertilization by specific chromosomes, a process known as genotypic sex determination (GSD). Only after the gonad is formed do hormones begin to exert an influence, modifying specific structures that eventually will differ between the sexes. Research on reptiles with temperature-dependent sex determination (TSD) indicates that sex determination in these species is fundamentally different in at least one way (bottom panel). Gonadal sex is not irrevocably set by the genetic composition inherited at fertilization, but rather depends ultimately on which genes encoding for steroidogenic enzymes and hormone receptor are activated during development by temperature. Incubation temperature modifies the activity as well as the temporal and spatial sequence of enzymes and hormone receptors such that sex-specific hormone milieux, created in the urogenital system of the developing embryo, determine gonad type. The ability to manipulate sex in TSD by both physical and chemical stimuli provides a degree of unparalleled control for mechanistic studies of the co-ordinated gene expression underlying sex determination that is not possible with mammals or other amniote vertebrates having GSD.

increases in expression of the aromatase gene. Shifting eggs from a male-producing to a female-producing incubation temperature greatly increases aromatase activity, and complementary shifts from a female-producing to a male-producing incubation temperature only gradually decrease aromatase activity (Desvages & Pieau 1992*b*, Desvages *et al.* 1993).

Administration of aromatase inhibitors to eggs incubating at an all female-producing temperature results in male offspring, whereas the administration of reductase inhibitors to eggs incubating at a male-biased intermediate incubation temperature results in female offspring (Crews & Bergeron 1994) (Fig. 3). Further, administration of both testosterone and aromatase inhibitor to eggs incubating at a female-producing temperature results in male offspring, again suggesting that male sex determination is not a default condition of female sex determination. Finally, the administration of DHT and OE_2 together to eggs incubating at a pivotal temperature, or the temperature that produces a 50:50 sex ratio, results in individuals having ovotestes (Wibbels & Crews 1993). Incubation temperature also appears to modulate the quantity of hormone receptors in the gonad, enabling detection of the hormonal milieux created by the sex-specific temperatures (J Bergeron & D Crews, unpublished data).

Two models for the mechanism of action of incubation temperature

It is clear that in TSD, male and female sex determination are separate processes that are differentially affected by incubation temperature rather than the organization/default system characteristic of genotypic sex determination (Jost 1961). That is, females presumably result from the activation of an ovary-determining cascade and simultaneous inhibition of a testis-determining cascade; similarly, males presumably result from the activation of a testis-determining cascade and simultaneous inhibition of an ovary-determining cascade. Any model for the mechanism of action of incubation temperature must account for the following facts to be satisfactory. (i) The effect of incubation temperature or exogenous steroids is all-or-none (i.e. individuals are either male or female). (ii) Intersexes can be formed experimentally by the simultaneous administration of DHT and OE_2 to eggs incubating at a pivotal temperature. (iii) Exogenous OE_2 will override the effects of a male-producing temperature, and there is a correlation between oestrogen sensitivity and temperature sensitivity. (iv) Exogenous DHT cannot overcome the effects of a female-producing temperature, although DHT will induce male development in eggs incubating at

intermediate incubation temperatures; there is no apparent synergism between incubation temperature and sensitivity to DHT as is the case for oestrogen. (v) Steroid-induced gonadal feminization is specific to oestrogen-like compounds whereas gonadal masculinization is specific to non-aromatizable androgens. (vi) Administration of aromatase inhibitor will block female development and induce male development in eggs incubating at a female-producing temperature, whereas administration of reductase inhibitor will block male development and induce female development in eggs incubating at intermediate incubation temperatures. (vii) Administration of both testosterone and aromatase inhibitor at an all male-producing incubation temperature blocks the testosterone-induced feminization and all offspring will be male, whereas when testosterone and aromatase inhibitor are administered at an all female-producing incubation temperature, all offspring are male.

Two different models can be put forward to account for how incubation temperature determines sex in TSD reptiles (Fig. 5). Central to both models is the fact that testosterone serves as a precursor molecule destined for conversion to DHT (via reductase) or oestrogen (via aromatase). Similarly, in both models incubation temperature is hypothesized to activate genes encoding for steroid hormone receptors (e.g. male-producing temperature up-regulating androgen receptor (AR) and female-producing temperature up-regulating oestrogen receptor (ER)).

In the direct model of steroid hormone-mediated sex determination (Fig. 5, top panel) it is hypothesized that temperature-sensitive promoters are associated with the genes encoding for steroidogenic enzymes. At a female-producing temperature it is possible that the gene(s) encoding for aromatase are activated and the gene(s) encoding for reductase inhibited. Similarly, at a male-producing temperature the gene(s) encoding for reductase are activated and the gene(s) encoding for aromatase inhibited. Consequent effects on the concentrations of steroid hormone in turn feed back to increase the amount and activity of steroidogenic enzymes at the level of the urogenital sinus, thereby creating temperature-specific hormonal milieux. For example, Pieau and colleagues (reviewed in Pieau *et al.* 1994) have documented the existence of a positive feedback between OE_2 and aromatase at a female-producing temperature in the European pond turtle. As yet unproven, a similar feedback relationship may exist between DHT and reductase, although the lack of a dose–response of the reductase inhibitor and the absence of a synergism between incubation temperature and DHT sensitivity indicates that the feedback may be negative in nature. The resulting hormonal milieu would lead to DHT or OE_2 binding to specific high-affinity nuclear receptor proteins (AR and ER respectively), which in turn activate the receptors such that the hormone–hormone receptor complex binds to the sex-specific hormone response elements on the DNA. The consequence of such events would be a stimulation of the transcription of genes associated with the sex-determining cascade of one sex and an inhibition of the expression of genes associated with the sex-determining cascade of the opposite sex.

The indirect model of steroid hormone-mediated sex determination (Fig. 5, bottom panel) does not involve temperature-sensitive promoters but rather feedback control of product formation and hence activation of sex-determining cascades. As in the direct model, a positive feedback is hypothesized to exist between aromatase and OE_2, whereas reductase is constitutively expressed. At a female-producing temperature androgen precursor is preferentially metabolized to OE_2, thereby causing greater conversion of precursor; OE_2 would also inhibit reductase activity. At a male-producing temperature, aromatase does not increase and little or no OE_2 is formed. Instead, the constitutive expression of reductase causes DHT formation which, in turn, initiates the testis-determining cascade. The activity of *SRY*-like genes in turn feedback to inhibit aromatase expression further. Thus, incubation temperature would act only indirectly via the testis determining gene(s) having a negative feedback on the regulation of aromatase gene expression, leading to suppression of OE_2. Support for this hypothesis comes from Haqq *et al.* (1993) who demonstrated recently in the rat that *SRY* may control male development through regulation of aromatase and Müllerian inhibiting substance genes. The mechanism by which this biochemical switching system could operate has been modelled by Jackson (1993).

What can TSD tell us about GSD?

It has long been thought that, in GSD, steroid hormones (from the mother or embryos) are not involved in gonad formation. In TSD, incubation temperature initiates a cascade of events involving steroid hormones, culminating in sex determination. It has been suggested that TSD may represent the evolutionary precursor to GSD. Thus, the work with TSD reptiles indicates that the conclusion that sex determination in mammals is independent of sex steroid hormones may be premature. Indeed, should reptilian TSD and mammalian GSD prove not to be basically similar, then a major problem in evolution must be accounted for.

The fact that reptiles and mammals descended from amniote ancestors suggests that effects of temperature on sex determination and sexual differentiation may be present, but masked, even in homeotherms. Alternatively, temperature sensitivity could be obvious but, because it has not been investigated in mammals, is as yet unknown. Indeed, temperature has not been adequately investigated as a factor in steroid hormone action despite numerous studies documenting how both hormone responsiveness as well as hormone action depend completely on temperature (Licht 1986). It is remarkable that virtually all studies of

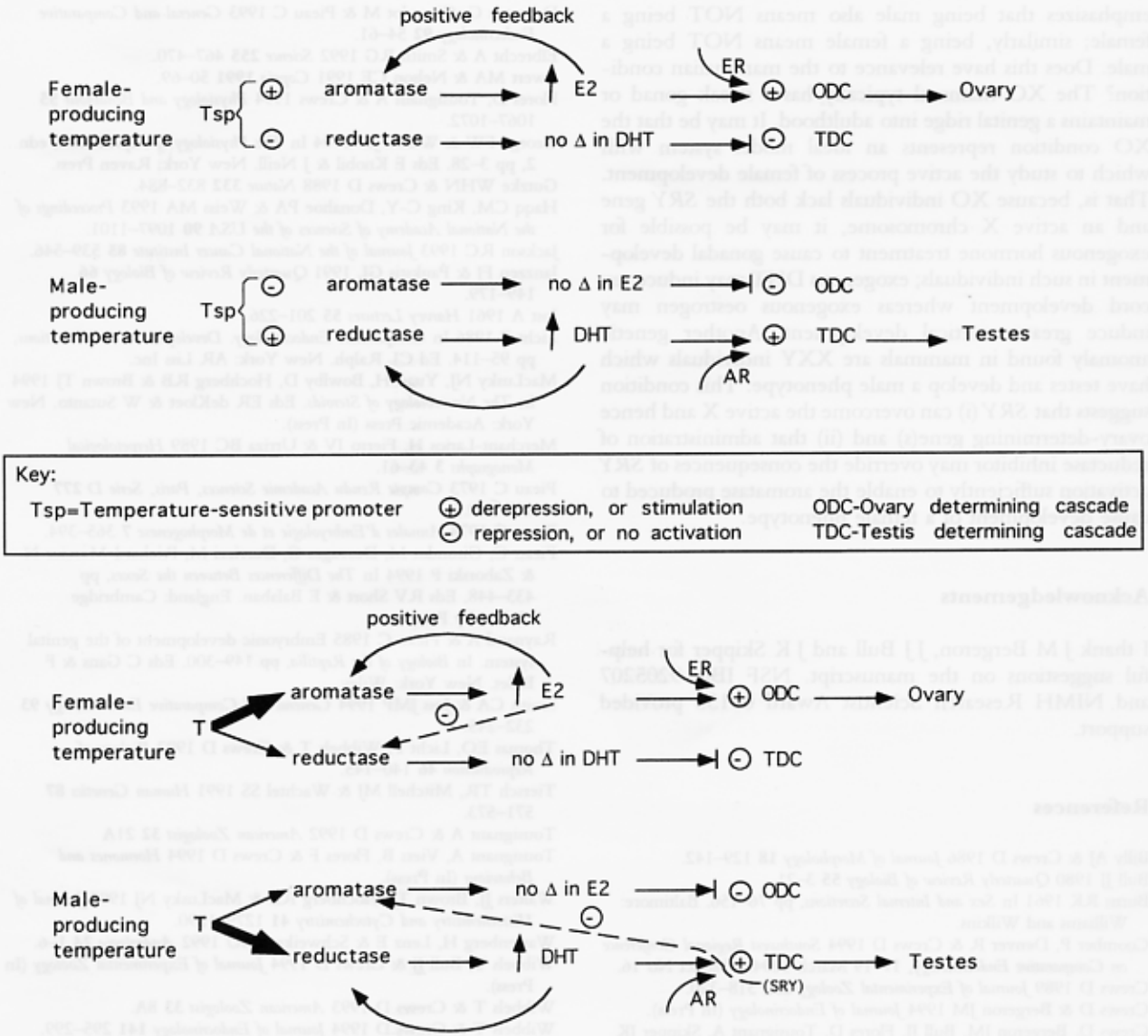

FIGURE 5. Two models of the mechanism of action of incubation temperature in temperature-dependent sex determination. In both, the temperature experienced during the middle third of incubation initiates the cellular and molecular cascades that result in male or female offspring. In addition to the effects on genes encoding for steroidogenic enzymes, incubation temperature is hypothesized to activate genes encoding for steroid hormone receptors (e.g. male-producing temperature up-regulating androgen receptor and female-producing temperature up-regulating oestrogen receptor). The top panel depicts a direct action of incubation temperature on temperature-sensitive promoters on genes encoding for steroidogenic enzymes. The bottom panel depicts an indirect action of incubation temperature.

the interaction of steroid hormones and their receptors have been conducted at 4 °C. Techniques employing physiological temperatures (e.g. Walters *et al.* 1993, MacLusky *et al.* 1994) will probably yield more realistic answers to questions of the relationship between the effects of physical and physiological factors on hormone receptors and the actions of steroid hormones in reproductive responses.

Another reason TSD is important to our understanding of mammalian sex determination is that it speaks directly to the issue of female sex determination. Most effort today focuses on elucidating the mechanisms underlying male development; female development has been relegated to a passive or default state (Jost 1961). Studies of reptiles with TSD show that female development must be an active process just as is male development. That is, this work

emphasizes that being male also means NOT being a female; similarly, being a female means NOT being a male. Does this have relevance to the mammalian condition? The XO mammal typically has a streak gonad or maintains a genital ridge into adulthood. It may be that the XO condition represents an ideal model system with which to study the active process of female development. That is, because XO individuals lack both the *SRY* gene and an active X chromosome, it may be possible for exogenous hormone treatment to cause gonadal development in such individuals; exogenous DHT may induce sex cord development whereas exogenous oestrogen may induce greater cortical development. Another genetic anomaly found in mammals are XXY individuals which have testes and develop a male phenotype. This condition suggests that *SRY* (i) can overcome the active X and hence ovary-determining gene(s) and (ii) that administration of reductase inhibitor may override the consequences of *SRY* activation sufficiently to enable the aromatase produced to cause development of a female phenotype.

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