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Sex determination: where environment and genetics meet

David Crews

Section of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA
Correspondence (e-mail: crews@mail.utexas.edu)

SUMMARY In mammals and birds the genetic constitution established at the time of fertilization determines the type of gonad that develops, whereas in all crocodilians and many turtles it is the temperature experienced during the mid-trimester of embryogenesis that initiates gonadal differentiation. Research with the red-eared slider suggests considerable conservation in the genetic cascades that underlie the sex determination process in vertebrates and, further, that the patterns of expression of these genes appear to reflect phylogenetic relationships, with turtles being more similar to mammals than they are to birds and crocodilians. After the determination and differentiation of an individual's gonadal sex, epigenetic forces shape those morphological, physiological, and behavioral traits that characterize each individual's unique sexuality. Research with the leopard gecko thus relates to the fundamental question of what factors determine individual variability, particularly as it relates to sexually dimorphic behaviors. Taken together, this research illustrates how sexuality depends on sex, but sex should not be confused with sexuality. That is, sex is merely a means of categorizing individuals or gonads, whereas sexuality serves as a descriptor of concordant traits each of which is typically sexually dimorphic in its expression.

INTRODUCTION

Ecology and genetics intersect in the concept of phenotypic plasticity. How these two fields relate to organismal development has had a fragmented past (Sarkar 1999; Sarkar and Fuller, this issue), the waxing and waning of enthusiasm apparently due more to the changing fads in the scientific enterprise than to the resolution of its central questions. As a consequence, our understanding of the process by which the internal milieu and the environment induce different phenotypes from a given genotype remains rudimentary. This is particularly true for our understanding of the mechanisms that control behavior. For example, as a behavioral neuroscientist I have long grappled with the problem of how and why individuals of the same sex, whether from inbred strains of rodents or species of genetically identical lizards, differ in their behavior. If we can learn about the forces that shape this individuality, perhaps we will achieve a greater understanding of variation as the raw material for evolutionary change. Progress is being made as molecular neuroscientists come to realize that organisms, not genes, are the unit of selection in evolution, and behavioral neuroscientists investigate how changes in the internal and external environments throughout an individual's lifespan shape its behavior.

SEX VERSUS SEXUALITY

Before proceeding it is first necessary to be clear on what I mean by sex and how it is different from sexuality. Sex refers to discrete traits such as the genetic complement and/or gonad type that categorize individuals. Sexuality, on the other hand, goes beyond the components of sex and represents the continuously variable suite of traits that emerge during the organism's lifetime, making each individual unique. Usually these are morphological, physiological, and behavioral traits that are sexually dimorphic in their expression. However, there is no reason why this terminology may not be extended to the molecular realm, because even here within- and between-sex differences exist in the nature and pattern of gene expression as well as gene sequences themselves.

The process by which an individual goes from being sexual to a sexual being can be divided into two distinct phases (Crews 1999). I have termed the first phase primary organization and it refers to the genetic and hormonal determination of the primary and secondary sex structures. This includes the sexual differentiation process that follows gonad determination when the morphological, physiological, behavioral, and neural traits that will become the elements of sexuality are first formed.

But what determines gonadal sex need not dictate sexuality. Sexuality results from nongenomic factors and heritable genetic variation and its sequelae. Some of these factors include, but are not limited to, activational effects of sex steroid hormones, the behavioral and physiological condition of the mother, the environments encountered throughout life, and age and sociosexual experiences. This process I have called secondary organization.

So sexuality emerges as the organism accumulates expe-

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riences through the life cycle and also as it undergoes the morphological and physiological transformation that establishes the individual as a reproductive adult. Thus three sources of stimuli comprise experience: stimuli that arise endogenously, stimuli from the immediate environment, and stimuli generated by the organism as it interacts with its inner and outer environments. It is this latter aspect, the dynamic relationship between the organism and its internal milieu and external environment that often shapes the developmental trajectory of the individual.

The fields of behavioral endocrinology and behavioral neuroscience have provided us with an ample tool kit with which to study the development of sexuality. But we tend to view the development of brain and behavior through the lens of the activation/organization hypothesis (Arnold and Breedlove 1985). In its simplest form this paradigm states that differences between males and females can be traced to sex steroid hormones early in life organizing the substrates of body, brain, and behavior that are in turn activated in adulthood by changes in the nature and pattern of secretion of sex steroid hormones. This, of course, is an oversimplification and glosses over the many subtleties that have been uncovered, but I believe it can be argued that the focus on sex differences has hindered the study of the development of sexuality.

Another problem arises from the animal model systems that we tend to use. Many of the guiding concepts in behavioral neuroscience were developed on mammals and birds, many of them highly domesticated. Such preparations have been very useful in studies of primary organization, or sex determination and sexual differentiation. But are these animal models suitable for dissecting the genetic from the environmental components of sexuality. In other words, are there more suitable model systems to examine the component processes of secondary organization?

MECHANISMS OF SEX DETERMINATION

The trigger initiating the sex determination process can be either genetic or heritable to the animal, as is the case in animals with genotypic sex determination, or external to the animal, as is the case with environmental sex determination. In mammals and birds the genetic constitution established at the time of fertilization determines the type of gonad that develops, whereas in some other vertebrates it is a change in environment in which the adult or the embryo finds itself that is responsible for the resulting sex (Crews 1993). For example, in sex-changing fish the presence and behavior of conspecifics can cause an individual to transform itself into the opposite sex, whereas in all crocodilians, many turtles, and several lizards it is the temperature experienced during the mid-trimester of embryogenesis that determines the gonadal sex of the individual. This latter process is known as temperature-dependent sex determination (see also Godwin, this issue) and has been suggested as the evolutionary precursor to genotypic sex determining mechanisms (Janzen and Paukstis 1991). This ability to manipulate sex in temperature-dependent sex determination by both physical and chemical stimuli provides a degree of unparalleled control for mechanistic studies of the coordinated gene expression underlying sex determination that is not possible with other amniote vertebrates having genotypic sex determination.

We have been using the red-eared slider turtle as a model system to understand how the physical stimulus of temperature is transduced into a physiological and ultimately a molecular signal that determines the type of gonad that will be formed (Crews et al. 1996, 2001). Our results to date as well as those of other laboratories indicate that temperature effects the activation of steroidogenic enzymes as well as the genes that encode them. This in turn creates temperature-(= gonad) specific hormonal milieus at the gonadal ridge and regulates genes coding for various nuclear transcription factors, including the receptors of sex steroid hormones.

Although the triggers for initiating sex determination may differ among vertebrates, there is considerable conservation in the genetic cascades that underlie the sex determination process. Not only are the gene sequences coding for transcription factors and proteins important in sex determination similar across vertebrates, but also the patterns of expression of these genes appear to reflect phylogenetic relationships, with turtles being more similar to mammals and crocodilians more similar to birds (Fig. 1). For example, the pattern of expression of steroidogenic factor-1 (SF-1) is similar in mammals and turtles in that it is up-regulated in both genetically and temperature-determined (respectively) males during early development, whereas in crocodilians and birds it is down-regulated. A similar relationship occurs in the timing of SOX9 expression that also reflects phylogeny; in mammals and turtles SOX9 expression precedes Mullerian inhibiting substance (MIS) expression, whereas in the alligator and the chicken the reverse pattern applies.

Although information on the patterns of gene expression and their correlations with gonadal destiny are informative, it is equally important to link these levels of analysis by assessing the activity of the protein that appears to be responsible for estrogen-induced ovarian development. In the red-eared slider turtle, if eggs incubating at a low male-producing temperature are switched to a high female-producing temperature during the middle third of incubation (= the thermosensitive period), they will hatch as females (Crews 1996; Crews et al. 2001). The masculinizing effects of a low incubation temperature effects can also be overridden by applying estrogen or aromatizable androgens to the egg during the thermosensitive period of sexual development. Estrogenic ligands feminize embryos via an estrogen-specific receptor in a dose dependent manner. Indeed, exogenous estrogen syn-

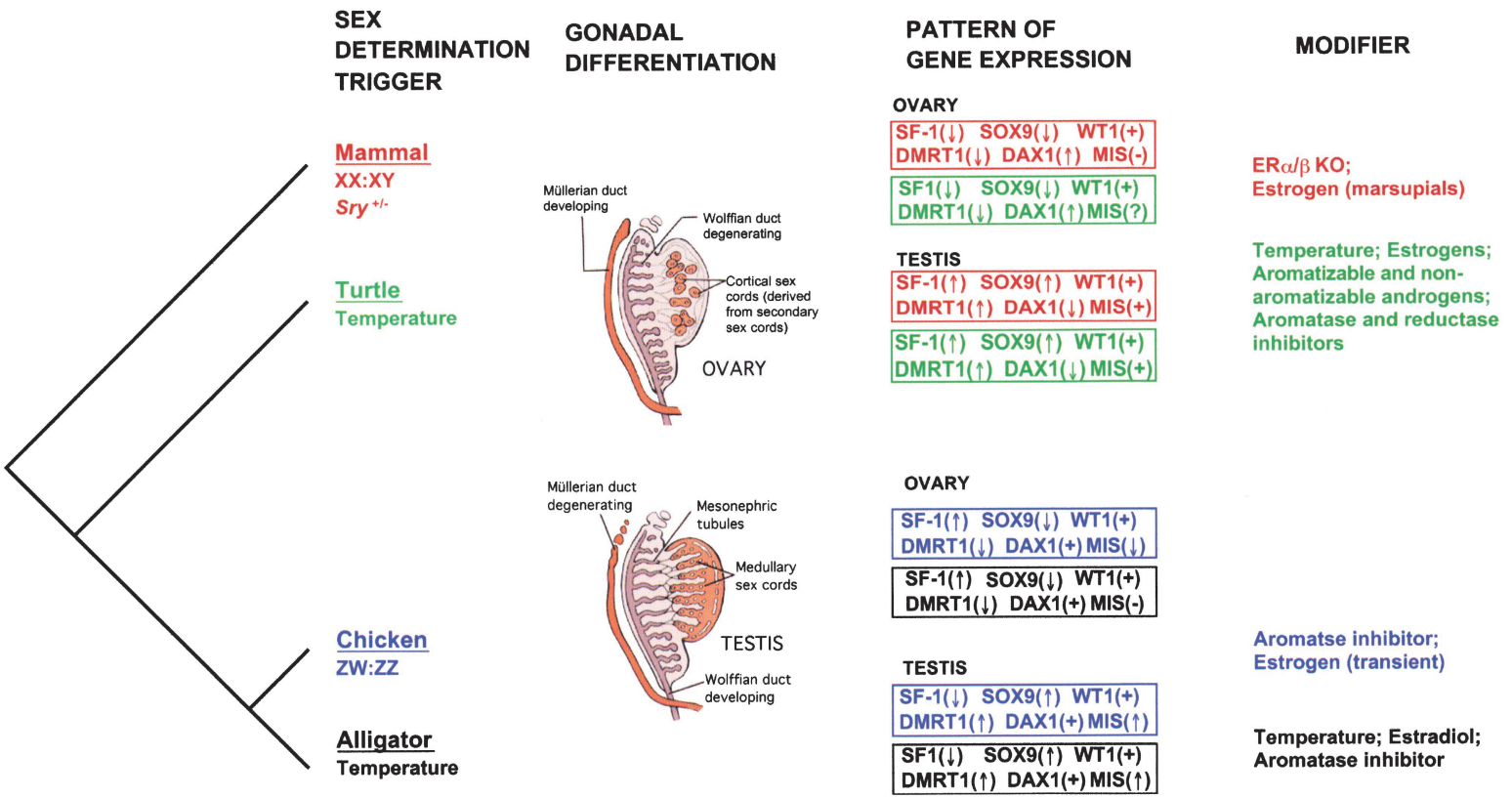

Fig. 1. The genes involved in sex determination in amniote vertebrates are highly conserved as is their patterns of expression. On the left is the phylogeny of amniote vertebrates based on evidence from fossils, gene sequences, proteins, and morphology (Eernisse and Kluge 1993); to this can now be added patterns of gene expression. Depicted are some of the genes (in boxes) involved in the differentiation of genital ridge into an ovary (upper schematic) or testis (lower schematic) in each vertebrate group studied to date. Color coding as follows: mouse in red, red-eared slider turtle in green, chicken in blue, and alligator in black. Note that the same genes are involved in this process in species that exhibit genotypic sex determination (mammals and birds) and those that exhibit temperature-dependent sex determination (turtles and crocodilians). To the right (Modifier) are some of the manipulations (environmental, genetic, and chemical) that have been demonstrated to sex-reverse individuals in the various groups. DAX1, dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X chromosome; DMRT1, DM-related transcription factor one; MIS, Mullerian inhibiting substance; SF-1, steroidogenic factor one; SOX9, SRY-related HMG box nine; SRY, sex-determining region on the Y chromosome; WT1, Wilms tumor one; ER α/β KO, double knockout for estrogen receptor α and estrogen receptor β; +, presence; −, absence; ↑, up-regulation; ↓, down-regulation.

ergizes with incubation temperature to induce ovarian differentiation, indicating that temperature and estrogen are acting at different steps in a single pathway. Aromatase, the enzyme that converts testosterone to estradiol, is also involved in ovarian determination. Administration of aromatase inhibitors blocks ovarian development in embryos incubating at a female-producing temperature, and instead the young hatch as males. Aromatase activity in the gonadal complex only increases after the fate of the gonads has been determined. However, during the thermosensitive period, aromatase activity in the brain of embryos destined to become females is significantly higher than in the brain of embryos destined to become males. After this, the level of aromatase activity in both male and female brains decreases. Such results point to the brain as a site of aromatase response to temperature in this species and suggest that the product of aromatase activity, estradiol, may induce alterations in the neuroendocrine axis controlling gonadal sex steroid hormone production.

But the very nature of genotypic sex determination makes it difficult to distinguish epigenetic from genetic contributions to sexuality. That is, the presence of sex chromosomes makes it difficult to study the development of sexuality in conventional avian and mammalian model systems. Consider, for example, behaviors displayed by both sexes but at different frequencies. To what extent are differences observed between adult males and females due to their differences in sex chromosomes, differences in gonads and their attendant differences in the nature and pattern of hormone secretion, or differences in nongenomic yet heritable factors such as maternal influences and sex-typical experiences? Species with temperature-dependent sex determination enable one to at least remove one of these sources, namely sex-specific chromosomes. For this reason my laboratory has also been studying the functional outcomes as well as the causal mechanisms of temperature-dependent sex determination.

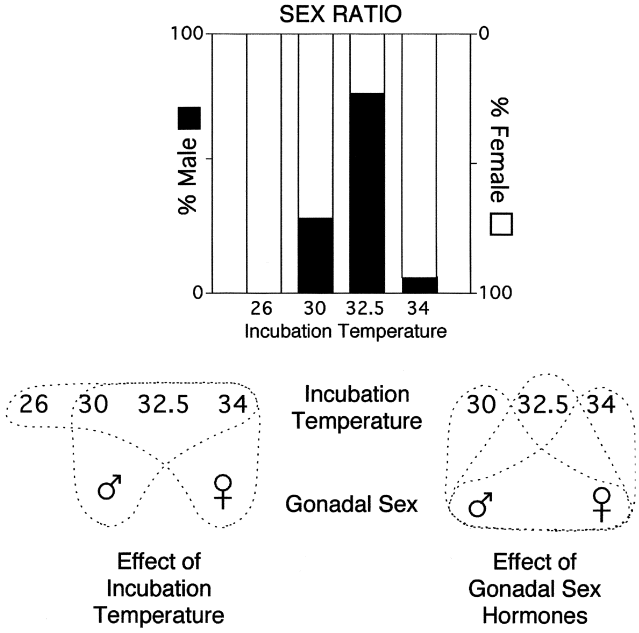

Fig. 2. Pattern of temperature-dependent sex determination in the leopard gecko. Middle panel shows the effect of incubation temperature on sex ratio: Extreme temperatures produce only or mostly females, whereas intermediate temperatures produce different sex ratios. Because the effects of incubation temperature and gonad-typical hormones covary, any difference between individuals could be due to the incubation temperature of the egg, the gonadal sex hormones of the individual, or both factors combined. To assess the contribution of each, they must be dissociated. The dotted lines indicate groupings that distinguish between the effects of temperature (study of same-sex animals that differ only in their incubation temperature; lower left), whereas the effects of gonadal sex hormones are revealed by comparing males and females from the same incubation temperature (lower right). (Modified from Coomber et al. 1997.)

FUNCTIONAL OUTCOMES OF TEMPERATURE-DEPENDENT SEX DETERMINATION

Because each egg has the potential to become either a gonadal male or a gonadal female, species with temperature-dependent sex determination have become particularly useful preparations with which to study phenotypic plasticity. We have been using the leopard gecko (*Eublepharis macularius*) as a model system to investigate how events early in life not only determine an individual's gonadal sex but also shape its sexuality (Crews 1999; Crews et al. 1998; Godwin and Crews 2002). In the leopard gecko high and low incubation temperatures produce only or mostly females, whereas intermediate incubation temperatures produce different sex ratios (Fig. 2). That is, 26°C and 34°C are female-producing incubation temperatures, whereas 30°C produces a female-biased sex ratio (approximately 75:25) and 32.5°C produces a male-biased sex ratio (approximately 25:75).

Although incubation temperature and gonadal hormones are linked in temperature-dependent sex determination, this association is neither fixed nor fundamental as it is in species with genotypic sex determination. Rather, the effect of incubation temperature and gonadal hormones on the phenotype can be dissociated; because there are females produced at "female" incubation temperatures and females at "male" incubation temperatures, comparing males and females from the same incubation temperature will reveal the effect of gonadal sex hormones, whereas comparing males (or females) from the different temperatures illustrates the effects of incubation temperature. Thus, in the first instance the focus is on sex differences, whereas in the latter instance the focus is on the development of sexuality. Hereafter "gonadal hormone effects" will refer to comparisons between the sexes from the same incubation temperature, whereas "temperature effects"

Table 1. Incubation temperature during embryonic development not only determines the type of gonad that forms in the leopard gecko (*Eublepharis macularius*) but also shapes the adult phenotype

Sex determination	Testes	Ovaries
Growth	+	+
Sex hormone levels	+	+
Sexual behavior	+	+
Aggressive behavior	+	+
Neurophenotype		
Preoptic area		
Neurochemistry	+	+
Volume	+	+
Ventromedial hypothalamus		
Neurochemistry	+	+
Volume	−	+
Amygdala		
Neurochemistry	−	+
Nucleus sphericus		
Neurochemistry	+	+
Sensitivity to sex hormones	+	+

Depicted are different traits that are influenced by incubation temperature. +, significant effect; −, indicates no effect.

will refer to comparisons between same-sex individuals from different incubation temperatures.

In a series of experiments we have demonstrated that incubation temperature not only establishes the gonadal sex of the individual, but also directly affects the morphology, growth, endocrine physiology, neurobiology, and aggressive and sexual behavior of the adult (Crews et al. 1998) (Table 1). For example, there is an obvious sex difference in size; males in general grow more rapidly and are larger than females from the same incubation temperature. However, females from the male-biased incubation temperature grow as rapidly and become as large as males from the female-biased incubation temperature. As in other vertebrates there is also a sex difference in the circulating concentrations of sex steroid hormones; males exhibit higher androgen-to-estrogen ratios than females both early in life and in adulthood. However, the levels of sex hormones in the circulation within a sex also vary according to the temperature experienced during embryonic development; in both sexes the androgen-to-estrogen ratio is significantly higher in animals from the male-biased incubation temperature compared with female-biased incubation temperatures. Finally, adult males and females from different incubation temperatures respond differently to the administration of exogenous sex steroid hormones (Flores and Crews 1995; Rhen and Crews 1999). This suggests that incubation temperature not only influences the amount of hormone secreted but also is responsible for how the individual responds to sex steroid hormones in adulthood.

Incubation temperature influences the nature and frequency of the behavior displayed by the adult leopard gecko (Crews et al. 1998). Females generally respond aggressively only if attacked, whereas males will posture and then attack other males but rarely females. Yet females from a male-biased incubation temperature are significantly more aggressive toward males than are females from a low or female-biased incubation temperature. Within each sex, however, there is significant variation that can be accounted for by incubation temperature. For example, females from a male-biased incubation temperature show the male-typical pattern of offensive aggression and are less attractive to males than are females from lower incubation temperatures. Similarly, males from the female-biased incubation temperature are more sexually active and less aggressive toward females than are males from the male-biased incubation temperature (Rhen and Crews 1999). These differences in behavior suggest that incubation temperature directly influences the neuroendocrine mechanisms regulating the reproductive behavior of adult animals.

Because the nature and pattern of growth, hormone secretion, and behavior ultimately are expressions of brain activity, it stands to reason that neural phenotypes must also exist and, further, that these might be sensitive to incubation temperature. Our experiments suggest that, indeed, incubation temperature has direct organizational effects on the development of brain nuclei that are independent of the effects of gonadal hormones. For example, research over the last 30 years indicates that in vertebrates with genotypic sex determination, including lizards, the volume of certain brain nuclei such as the preoptic area and the ventromedial hypothalamus differs between males and females. Surprisingly, there are no statistically significant sexual dimorphisms in these nuclei between males and female leopard geckos at those incubation temperatures that produce both sexes (Crews et al. 1998). There are, however, significant differences in the size of limbic brain areas across incubation temperatures within each sex. For example, the volume of the preoptic area is larger in both males and females from the male-biased incubation temperature compared with animals from the female-biased incubation temperature. The opposite pattern is found for the ventromedial hypothalamus. That is, the volume of the ventromedial hypothalamus is larger in females from low incubation temperature compared with females from the male-biased incubation temperature.

Incubation temperature also influences the metabolic capacity of certain forebrain nuclei in adult leopard geckos, and these differences correlate with the latent temperature effects on sexual and agonistic behavior. For example, males from the female-biased incubation temperature are not only more sexually active (Rhen and Crews 1999), they also have greater metabolic capacity in the preoptic area than their counterparts from the male-biased incubation temperature (i.e., less sexually active males) (Crews et al. 1996, 1998). Such a temperature effect in metabolic capacity in the preop-

tic area is consistent with the established functional relationship between neuronal activity in this brain region and male sexual behavior (Sakata et al. 2000). Incubation temperature also influences metabolic capacity in other behaviorally relevant nuclei. In those vertebrate species studied to date, the septum, anterior hypothalamus, and medial amygdala/nucleus sphericus are all areas involved in the regulation of agonistic behavior. Male leopard geckos from the male-biased incubation temperature, which are very aggressive, have higher metabolic capacity in these brain areas compared with males from the female-biased incubation temperature, which are relatively docile.

CONCLUSION

Phenotypic plasticity refers to the process by which the environment induces different phenotypes from a given genotype. The mechanisms underlying plasticity can be *either* committed and fixed *or* labile and reversible. Further, they can vary among or within individuals. When we consider that single gene mutations can sex-reverse individuals even in species with sex chromosomes, it becomes apparent that the process of sex determination and sexual differentiation represents a form of phenotypic plasticity.

Research with the red-eared slider suggests considerable conservation in the genetic cascades that underlie the sex determination process in vertebrates in general and, more specifically, that the patterns of expression of these genes appear to reflect phylogenetic relationships, with turtles being more similar to mammals than they are to birds and crocodilians. Using the leopard gecko as another animal model system, my laboratory has explored how the experience of temperature during embryogeny affects the phenotype of the adult organism. Results to date indicate that incubation temperature accounts for much of the variation observed among individuals of the same sex in morphology, growth, endocrinology, neural activity, and neuroanatomy. Some sociosexual behaviors and brain measures are affected directly by incubation temperature, whereas both incubation temperature and gonadal hormones influences others. Together these animal models allow dissection of environmental effects from those imposed by the genetically determined sex of the individual.

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