

# Sex Determination in Reptiles

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## SUMMARY

The sex-determining mechanisms (SDMs) of reptiles are remarkably diverse, ranging from systems that are under complete genetic control to those that are highly dependent upon temperatures that embryos experience during development. Because reptiles exhibit a remarkable diversity of SDMs, this group provides excellent models for addressing critical questions about the proximate mechanisms of sex determination, as well as its ecology and evolution. The goal of this chapter is to integrate studies from different disciplines (e.g., ecology, evolution, physiology, molecular biology) to broadly summarize the current understanding of reptilian sex determination. This chapter is divided into six topics that cover the (1) diversity of reptilian SDMs, (2) taxonomic distribution of SDMs, (3) molecular and physiological mechanisms underlying sex determination, (4) timing of sex determination during embryogenesis, (5) ecology and evolution of reptilian SDMs, and (6) current gaps in our understanding of this field and where future research should be directed.

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## 1. INTRODUCTION

In sexually reproducing species, the sex of an individual is arguably one of the most important aspects of its phenotype. Whether an embryo develops as a male or a female will have profound consequences on its life history, behavior, physiology, morphology, and ultimately its fitness. Indeed, differences between the sexes are among the most spectacular sources of phenotypic variation within populations. It is not surprising, then, that factors governing sexual development are of primary interest to scientists and have been debated for over three millennia. Over this expanse of time, our understanding of sex determination has transformed from mythological speculations to explanations based on hard-won scientific evidence (reviewed in Mittwoch, 2000). The end of the 19th century marked a critical point at which advances in cytogenetics enabled the discovery of sex chromosomes and their relationship to sex determination (reviewed in Brown, 2003). After this discovery, it was widely accepted that the sexual phenotype

of most organisms is determined by genetic factors located on sex-specific chromosomes.

Research has since revealed far greater diversity in sex-determining mechanisms (SDMs). Karyological studies have revealed a variety of sex-specific chromosomal arrangements (e.g., male vs. female heterogamety (Mittwoch, 1996)). In many species, heteromorphic sex chromosomes do not exist, but instead sex-determining factors lie on autosomes. In other organisms, sex is determined by the ratio of X chromosomes to autosomes or the ploidy level of the zygote (Cook, 2002). During the latter half of the 20th century, the ubiquity of these genetic systems was challenged by studies that demonstrate a role of environmental factors in the sex-determination process. Indeed, in many species, environmental conditions experienced during embryogenesis (rather than genotypic factors) trigger the developmental cascade that eventually leads to the male or female phenotype. This environmental sex determination (ESD) has since been shown to exhibit remarkable diversity in itself (Bull, 1983). It is now well established that SDMs range from those under complete genetic control to those that are highly dependent upon environmental parameters.

The diversity in SDMs is intriguing since the division of the sexes is so similar throughout most animals. Indeed, without knowledge of such diversity, one might expect that a single mechanism would have been stabilized at an early stage of evolution. Why, then, have so many different mechanisms evolved as a means to produce males and females? How do the proximate mechanisms of different SDMs vary? Which SDMs are ancestral and how have new mechanisms arisen? Answers to these and many related questions are continuously sought after by biologists in many disciplines. The ideal approach for tackling such questions requires an integrative examination of closely related organisms that vary in their SDMs. The class Reptilia is a group that satisfies this requirement. Reptiles show spectacular diversity in SDMs, ranging from systems that have a strong genetic basis to those that are under almost complete environmental influence

(Bull, 1980). In many cases, closely related species differ in fundamental aspects of their SDMs, making these taxa well-suited for comparative analyses. Many characteristics of reptiles make them especially amenable to experimental manipulation, which facilitates tests of theoretical predictions about the evolution of alternative SDMs (Janzen & Paukstis, 1991a). Not surprisingly, reptiles have received considerable research attention, and have provided critical insights toward our understanding of sex determination. Our understanding, however, is far from complete, and reptiles will undoubtedly continue to serve as excellent models for addressing fundamental issues in this field.

The primary objective of this chapter is to provide an overview of our current understanding of sex determination in reptiles. Given that the vast literature on this topic spans several decades, specific details on all aspects of such a broad topic cannot be covered in a single review. The author's hope is that this review will provide a sense of the current state of the field and a framework that will guide research in specific directions that warrant further study. An additional goal of this review is to provide an awareness of different perspectives on reptilian sex determination (e.g., viewing SDMs as a dichotomy vs. a continuum), as well as to identify gaps in our knowledge and finally to provide suggestions for future research.

This chapter is divided into six major sections that focus on the 'what,' 'who,' 'how,' 'when,' 'why,' and 'where' questions of reptilian sex determination. The first section begins by introducing the diversity of SDMs (the 'what' question) found within reptiles by detailing the specifics of genotypic sex determination (GSD) and the variations in reaction norms that describe temperature-dependent sex determination (TSD). Secondly, a brief overview of the taxonomic and phylogenetic distribution of alternative SDMs (the 'who' question) is provided, and the evolutionary transitions between systems discussed. The third section addresses the proximate mechanisms involved in reptilian sex determination (the 'how' question). This section discusses the molecular and hormonal aspects of sex determination and evaluates commonalities and differences among mechanisms. The fourth section explores the timing of sexual lability during embryonic development (the 'when' question), and compares different methods for addressing this issue. The fifth section explores the ecology and evolution of reptile SDMs (the 'why' question). This section establishes the existence of TSD in nature and evaluates hypotheses of adaptive significance and micro-evolutionary potential of SDMs. The final section briefly points out emergent themes and critical gaps in this field, and suggests avenues of future research that warrant further investigation (the 'where' question).

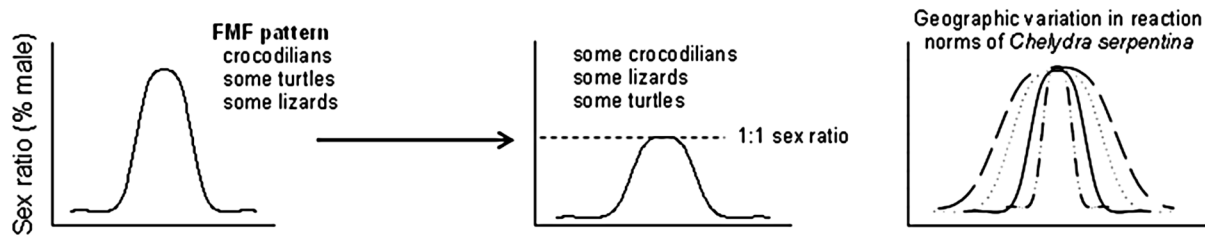
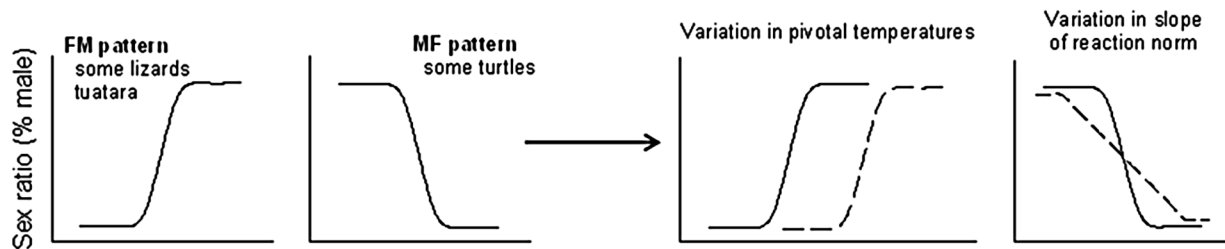
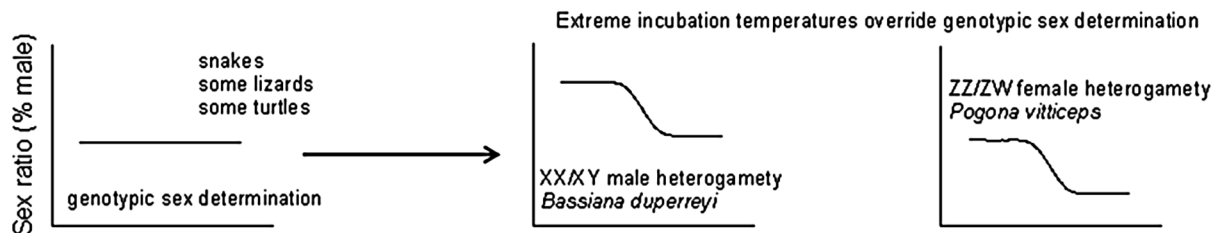
## 2. WHAT MECHANISMS OCCUR IN REPTILES? DIVERSITY OF SEX-DETERMINING MECHANISMS (SDMs) AND PATTERNS

Reptilian SDMs are traditionally placed into two main categories: one in which sex is determined solely by genetic factors (i.e., GSD) and the other a form of ESD in which sex is determined primarily by the temperature that embryos experience during development (i.e., TSD). Intriguingly, within both of these sex-determining systems, we see remarkable diversity in patterns. For example, under GSD, both male and female heterogamety evolved independently multiple times within reptiles. Under TSD, shapes of reaction norms that describe the relationship between developmental temperature and sex determination vary considerably among taxa (Figure 1.1). Moreover, evidence is accumulating that elements of both TSD and GSD may co-occur in some species, even within single populations (e.g., Shine, Elphick, & Donnellan, 2002). In this section, the current knowledge on the diversity of sex-determining patterns of reptiles is reviewed. To ease discussion, GSD and TSD are treated independently in many parts of this review, but recent arguments suggest that this dichotomy may oversimplify the complexity of SDMs and that TSD and GSD may not be alternative mechanisms but instead represent endpoints of a continuum (Sarre, Georges, & Quinn, 2004).

### 2.1. Patterns of Genotypic Sex Determination

Genotypic sex determination is a system in which offspring sex is irreversibly fixed by its own (or its parent's) genotype (Bull, 1980; Janzen & Paukstis, 1991a). That is, genetic factors inherited from the parents determine the sex of the offspring. These genetic factors may reside on sex chromosomes, which differ from autosomes in their size, number, and gene content, and are elements of the genome that segregate during meiosis. Importantly, sex-determining genes located on sex chromosomes direct the pathways that lead to male or female development. The two most common types of GSD are male and female heterogamety. Under male heterogamety, offspring that inherit the Y chromosome from the father develop into males (XY) and those that inherit the father's X chromosome develop into females (XX). In many species, this system is reversed and females are the heterogametic sex; sex chromosomes in this system are referred to as Z and W (i.e., males are ZZ, and females ZW).

Although GSD is common in reptiles, many species that have been karyotyped show no evidence of heteromorphic sex chromosomes (Table 1.1), presumably because homomorphic chromosomes have been retained, or have changed very little, from the ancestral state (Ohno, 1967).

**(a) Females produced at both extremes****(b) Females produced at one extreme****(c) Both sexes produced at all temperatures**

**FIGURE 1.1** Diversity of sex-determining patterns in reptiles (modified from Warner & Janzen, 2010). All graphs show sex ratio (% male) as a function of increasing egg incubation temperature (x-axes). The three major patterns of sex determination with respect to incubation temperature are shown to the left of the arrows. Patterns to the right of the arrows are variants of those patterns. (a) Pattern of temperature-dependent sex determination (TSD) in which females are produced at both temperature extremes, and males at intermediate temperatures (FMF pattern). In some species, intermediate temperatures produce mixed sex ratios, and other species show geographic variation in the shapes of reaction norms (e.g., the lines illustrate population-specific reaction norms in the common snapping turtle, *Chelydra serpentina* (Ewert, Lang, & Nelson 2005)). (b) Patterns of TSD in which males and females are produced at one or the other temperature extreme (FM and MF patterns). Pivotal temperature (i.e., temperature that produces 1 : 1 sex ratio) varies considerably among species, populations, and embryos produced by different females within populations. Additionally, considerable diversity occurs in the transitional range of temperatures (i.e., range of temperatures that yields mixed sex ratios) among and within species. (c) Sex ratio is not influenced by incubation temperature (genotypic sex determination). Recent studies demonstrate that extreme incubation temperatures reverse genotypic females to phenotypic males (Radder, Quinn, Georges, Sarre, & Shine, 2008) and vice versa (Quinn et al., 2007) in certain lizard species.

Nevertheless, reptiles exhibit remarkable variation in the degree of sex chromosome differentiation (Solari, 1994), which may reflect different stages in the evolutionary transitions between homomorphic and heteromorphic systems (Marshall-Graves & Shetty, 2001; Charlesworth, D., Charlesworth, B., & Marais, 2005). To detect GSD in species with apparently homomorphic chromosomes, experimental approaches are needed to verify that sex determination is unresponsive to environmental parameters (e.g., temperature) (Valenzuela, Adams, & Janzen, 2003). Indeed, incubation temperature does not influence primary sex ratios in many turtle and squamate species that lack differentiated chromosomes (e.g., Bull & Vogt, 1979;

Uller, Mott, Odierna, & Olsson, 2006; Uller, Odierna, & Olsson, 2008). A caveat, however, is that most cytological studies define sex chromosomes only when they are morphologically distinguishable and often do not attempt to detect differences in gene content. Unfortunately, cytological techniques used for karyotyping (e.g., C-banding, reverse fluorescent staining) vary in their ability to detect sex chromosomes that are not highly differentiated. As a result, seemingly homomorphic chromosomes may actually exhibit some differentiation, which can be detected by more advanced techniques. For example, early work on the lizard *Pogona vitticeps* suggested homomorphic sex chromosomes (Witten, 1983), but more advanced techniques

**TABLE 1.1** Summary of sex-determining mechanisms (SDMs) found in reptilian families. Family classifications and number of extant species correspond to Pough et al. (2004). XY, XX, XXY are forms of male heterogamety; ZW, ZZW, ZWW are forms of female heterogamety. Experimental evidence for a thermally sensitive SDM comes from laboratory-based egg incubation studies. Under the MF pattern, males are produced at cool incubation temperatures and females at warm temperatures; this pattern is reversed under the FM pattern. Under the FMF pattern, females are produced at extreme incubation temperatures and males at intermediate temperatures

Order Family	Number of extant species	Karyological evidence for morphologically differentiated sex chromosomes			Experimental evidence for a thermally sensitive SDM		
		Number shown/ examined	Heterogametic system(s)	Reference <sup>a</sup>	Number shown/ examined	TSD pattern(s)	Reference <sup>a</sup>
Chelonia							
Podocnemidae	8	0/8	-	15	3/3	MF	8,22
Pelomedusidae	18	0/5	-	15	2/2	FMF	8,22
Chelidae	50	3/23	XY	6,13,14,15	0/9	-	8,22
Carettochelyidae	1	0/1	-	15	1/1	MF	8,22
Trionychidae	27	1/8	ZW	15	0/3	-	8,22
Kinosternidae	22	2/16	XY	15	15/18	MF, FMF	8,22
Dermatemydidae	1	0/1	-	15	1/1	MF	8,22
Dermochelyidae	1	0/1	-	15	1/1	MF	8,22
Cheloniidae	6	0/5	-	15	6/6	MF	8,22
Chelydridae	3	0/3	-	15	2/2	FMF	8,22
Emydidae	40	0/30	-	15	23/24	MF	8,22
Testudinidae	40	0/15	-	15	6/6	MF	8,22
Bataguridae	65	2/33	XY, ZW	15	8/8	MF, FMF	8,22
Crocodylia							
Alligatoridae	8	0/8	-	15	6/6	FMF	8,22
Crocodylidae	13	0/13	-	15	6/6	FMF	8,22
Gavialidae	2	0/1	-	15	1/1	FMF	22
Rhynchocephalia							

	2	0/2	-	15	2/2	FM	22
Sphenodontidae							
Squamata (Sauria)							
Gekkonidae	1050	13/144	XY, XXY, ZW	15	20/27 <sup>b,c</sup>	FMF	2,7,22,23
Dibamidae	10	1/1	XY	15	0/0	-	-
Scincidae	1260	6/118	XY	15,19	4/6 <sup>b</sup>	MF, FM <sup>d</sup>	7,12,19,22
Xantusiidae	18	0/12	-	15	0/0	-	-
Gerrhosauridae	35	0/12	-	15	0/0	-	-
Cordylidae	42	0/11	-	15	0/0	-	-
Teiidae	125	1/61	XY	15	0/3	-	22,23
Gymnophthalmidae	150+	5/22	XY, XXY	15	0/0	-	-
Lacertidae	250	42/103	ZW, ZZW	15	1/5 <sup>c,d</sup>	-	4,7,22,23
Varanidae	40	4/22	ZW	15	0/2 <sup>c</sup>	-	7,23
Lanthanotidae	1	0/0	-	-	0/0	-	-
Shinisauridae	1	0/0	-	-	0/0	-	-
Anguidae	110	0/13	-	15	0/1 <sup>c</sup>	-	7,22
Helodermatidae	2	0/1	-	15	0/0	-	-
Xenosauridae	6	0/1	-	15	0/0	-	-
Chamaeleonidae	130	1/50	ZW	15	0/2 <sup>c</sup>	-	1,7,23
Agamidae	380+	2/89	ZW	5,15,20,21	13/25 <sup>b</sup>	FM, FMF <sup>d</sup>	7,21,22
Iguanidae	915+	76/250	XY, XXY	15	0/5 <sup>c</sup>	-	7,22,23
Squamata (Amphisbaenia)							
Amphisbaenidae	135	0/25	-	15	0/0	-	-

(Continued)

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Order Family	Number of extant species	Karyological evidence for morphologically differentiated sex chromosomes			Experimental evidence for a thermally sensitive SDM		
		Number shown/ examined	Heterogametic system(s)	Reference <sup>a</sup>	Number shown/ examined	TSD pattern(s)	Reference <sup>a</sup>
Rhineuridae	1	0/1	-	15	0/0	-	-
Trogonophidae	6	0/2	-	15	0/0	-	-
Bipedidae	3	1/3	ZW	15	0/0	-	-
Squamata (Serpentes)							
Anomochilidae	2	0/0	-	-	0/0	-	-
Aniliidae	1	0/0	-	-	0/0	-	-
Xenopeltidae	2	0/0	-	-	0/0	-	-
Tropidophidae	31	0/1	-	15	0/0	-	-
Bolyeriidae	2	0/0	-	-	0/0	-	-
Acrochordidae	3	0/2	-	15	0/0	-	-
Boidae	74	6/29	ZW	15	0/1	-	18
Uropeltidae	55	0/0	-	-	0/0	-	-
Xenopeltidae	2	0/1	-	15	0/0	-	-
Loxocemidae	1	0/1	-	15	0/0	-	-

Leptotyphlopidae	90	0/1	-	15	0/0	-
Anomalepididae	15	0/0	-	-	0/0	-
Typhlopidae	200	0/6	-	15	0/1	17
Colubridae	1800+	57/140	ZW	15	0/7 <sup>c</sup>	3,9,11,23,24
Atractaspididae	18	0/0	-	-	0/0	-
Elapidae	300	77/110	ZW, ZZW, ZWW	15	0/2	10,16
Viperidae	228	38/61	ZW	15	0/0	-
<b>Total</b>	<b>7803 +</b>	<b>338/1466</b>			<b>121/186</b>	

Note: Thermal effects on sex determination may have been evaluated in more squamate species than reported in this table, but offspring sex ratios are seldom reported.

<sup>a</sup>To reduce the number of citations, most references refer to literature reviews rather than original publications.

<sup>b</sup>Some species contain both heteromorphic chromosomes and thermal-sensitivity of sex determination.

<sup>c</sup>Thermal effects on sex determination are reported in some species, but evidence for TSD is unconvincing (see Viets, Ewert, Talent, & Nelson, 1994; Harlow, 2004).

<sup>d</sup>Sex ratios of some species are 1 : 1 at one extreme temperature but sex-biased at the other extreme.

<sup>1</sup> Andrews (2005); <sup>2</sup> Blumberg, Lewis, and Skoloff (2002); <sup>3</sup> Du and Ji (2002); <sup>4</sup> Du and Ji (2006); <sup>5</sup> Ezaz et al. (2005); <sup>6</sup> Ezaz et al. (2006); <sup>7</sup> Harlow (2004); <sup>8</sup> Janzen and Paukstis (1991a); <sup>9</sup> Ji and Du (2001a); <sup>10</sup> Ji and Du (2001b); <sup>11</sup> Ji, Du, and Xu (2001); <sup>12</sup> Ji, Du, and Xu (2006); <sup>13</sup> Martinez, Ezaz, Valenzuela, Georges, and Marshall-Graves (2008); <sup>14</sup> McBee, Bickham, Rhodin, and Mittermeier (1985); <sup>15</sup> Olmo and Signorino (2005); <sup>16</sup> Shine (1989); <sup>17</sup> Shine and Webb (1990); <sup>18</sup> Shine, Madsen, Elphick, and Harlow (1997); <sup>19</sup> Shine, Elphick, and Donnellan (2002); <sup>20</sup> Uller, Mott, Odierna, and Olsson (2006); <sup>21</sup> Uller, Odierna, and Olsson (2008); <sup>22</sup> Valenzuela (2004b); <sup>23</sup> Viets, Ewert, Talent, and Nelson (1994); <sup>24</sup> Webb, Brown, and Shine (2001).

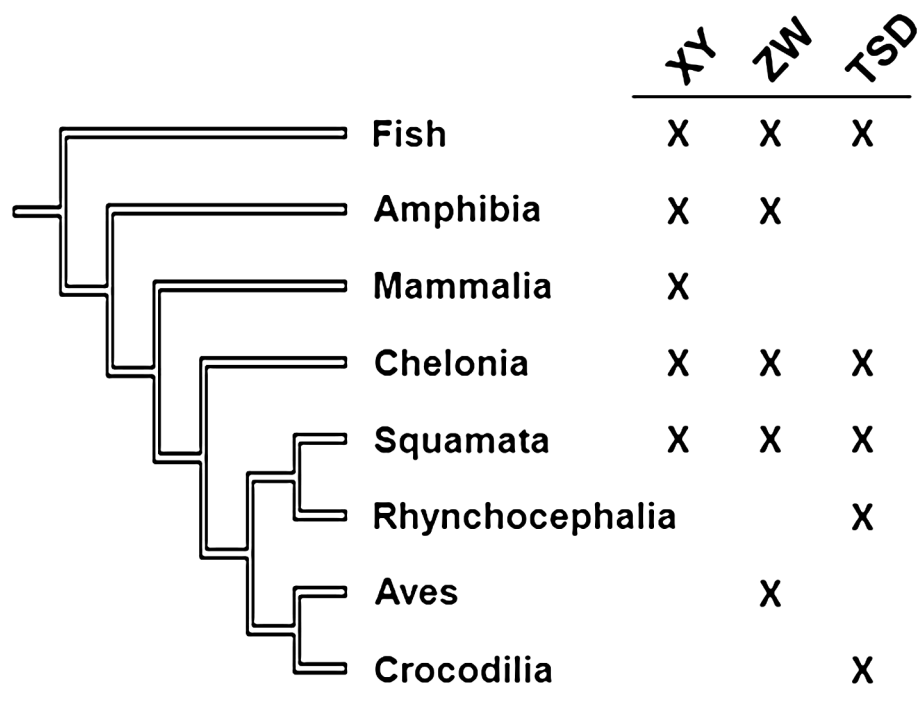
(i.e., comparative genomic hybridization) reveal a ZZ/ZW sex microchromosome system in this species (Ezaz et al., 2005). This reclassification of chromosomal systems is also illustrated in two turtles with GSD (Ezaz et al., 2006; Martinez, et al., 2008). Considering that early studies have not identified heteromorphic chromosomes in many GSD reptiles (Table 1.1), advanced techniques may reveal more chromosomal differentiation than was detected in past studies.

Some squamate species exhibit multiple sex chromosomes and more than one chromosome sort according to sex, resulting in different diploid numbers for males and females (Solari, 1994). For example, male heterogamety has been identified in the lizard *Sceloporus poinsettia*, but diploid numbers differ between the sexes (male  $2n = 31$ , female  $2n = 32$ ; a system referred to as  $X_1X_2Y \text{ ♂} / X_1X_1X_2X_2 \text{ ♀}$  (Cole, Lowe, & Wright, 1967)). Similar sex differences in diploid numbers occur in several other squamate reptiles (Olmo, 1986; Olmo & Signorino, 2005), including species with female heterogamety (e.g., in some lizards of the genus *Lacerta*, females differ from males in diploid number (Odierna et al., 1996)). These multiple sex chromosome systems may have resulted from the fusion of an autosome with a sex chromosome (Solari, 1994), and there is no evidence that they cause any fundamental change in the SDM (Bull, 1980). Therefore, these multiple

sex chromosome systems will be treated as either XX/XY or ZZ/ZW systems accordingly.

### 2.2. Patterns of Temperature-dependent Sex Determination

Under TSD, offspring sex is irreversibly determined by the temperature embryos encounter during development. All of the extant reptilian orders contain some members with TSD, but its prevalence varies among the major groups (Figure 1.2). The presence of TSD is identified experimentally when eggs are incubated under a variety of constant temperatures in the laboratory. The resultant offspring are then sexed and the effect of incubation temperature on the primary sex ratio is evaluated. Experiments must rule out the possibility of sex biases in embryonic mortality, which can cause skews in secondary sex ratios and give the impression of TSD. Such differential embryonic mortality has been described in snakes (Burger & Zappalorti, 1988) and birds (Eiby, Wilmer, & Booth, 2008). Primary sex ratios of TSD species vary dramatically depending upon incubation regimens. Typically, a narrow range of temperatures (sometimes just 1–2°C) produces mixed sex ratios, and temperatures above or below this range yield all of one or the other sex; this range



**FIGURE 1.2** Phylogenetic distribution of genotypic (XY and ZW) and temperature-dependent sex determination (TSD) in vertebrates, with particular attention given to reptiles. Temperature-dependent sex determination in Amphibia has never been demonstrated in nature, but thermal effects on offspring sex are well documented and are often referred to as a thermal sex reversal (see Dournon, Houillon, & Pieau, 1990; Chardard, Penrad-Mobayed, Chesnel, Pieau, & Dournon, 2004).



is called the transitional range of temperatures (TRT) and is an important parameter that describes the shape of the TSD reaction norm. The constant incubation temperature that produces a population-wide 1 : 1 sex ratio is called the pivotal temperature; this temperature expressed at the individual level is called the threshold temperature (Mrosovsky & Pieau, 1991).

Perhaps the most spectacular aspect of reptilian sex determination is the diversity in TSD patterns (Figure 1.1). Each pattern contains at least one pivotal temperature and at least one TRT, but these parameters can vary considerably among taxa and populations (threshold temperatures can even vary among individuals within populations) (Ewert, Jackson, & Nelson, 2004; Ewert, et al., 2005; Warner, Lovern, & Shine, 2008). Accordingly, evidence suggests that the pivotal temperature has a heritable genetic basis (Bull, Vogt, & Bulmer, 1982a; Janzen, 1992; Rhen & Lang, 1998; Janes & Wayne, 2006). Given these features, pivotal temperatures have the potential to evolve in response to selective pressures, which may explain the diversity of TSD patterns. Most descriptions of TSD discuss three types of reaction norms that describe the relationship between constant-temperature incubation and sex ratio (Head, May, & Pendleton, 1987; Pieau, 1996; Kraak & Pen, 2002), but in reality these reaction norms are broad generalizations of a variety of patterns (Figure 1.1). Under one pattern, males are produced at low incubation temperatures and females at high temperatures (male–female or ‘MF’ pattern); this is characteristic of many turtle and lizard species (Ewert, Etchberger, & Nelson, 1994; Harlow, 2004). Although rare, the reverse pattern is found in some species (female–male or ‘FM’ pattern), whereby low incubation temperatures produce females and high temperatures produce males (i.e., tuatara and some lizards (Harlow, 2004; Mitchell et al., 2006)). Another pattern yields female offspring at extreme incubation temperatures and males at intermediate temperatures (female–male–female or ‘FMF’ pattern). This pattern contains two pivotal temperatures and, hence, has two TRTs. The FMF pattern is found in all crocodylians and in several lizard and turtle species. The literature often refers to these TSD patterns as type Ia, type Ib, and type II, respectively, but here the less ambiguous letters are adopted to symbolize the sex produced at low or high temperatures.

Even within these three patterns, we see remarkable diversity in the shapes of the reaction norms (Figure 1.1). The pivotal temperature varies considerably among species, such that incubation temperatures that produce predominantly males in one species will produce predominantly females in another species. In some North American turtles, variation in pivotal temperatures is explained by latitude (Bull, Vogt, & McCoy, 1982b; Ewert et al., 1994; 2004; 2005). For example, in *Chelydra*

*serpentina*, a species with an FMF pattern of TSD, the upper pivotal temperature is greater in northern populations than in southern populations (Ewert et al., 2005). Threshold temperatures and the slope of the sex-determining reaction norm (determined by the width of the TRT) can vary substantially among clutches within the same population (Rhen & Lang, 1998; St. Juliana, Bowden, & Jansen, 2004; Warner et al., 2008). In the alligator snapping turtle (*Macrochelys temminckii*), for example, the sex-determining response of embryos from some clutches has a steep relationship with incubation temperature, whereas embryos from other clutches are relatively unresponsive to temperature, resembling a pattern expected under GSD (Ewert et al., 1994). Considerable among-clutch variation has also been shown among populations of painted turtles (*Chrysemys picta*) and snapping turtles (*Chelydra serpentina*) (Ewert et al., 2004; 2005).

To add to this diversity, some species exhibit a pattern in which no incubation temperature produces exclusively male offspring. For example, in the Australian jacky dragon (*Amphibolurus muricatus*), extreme incubation temperatures produce all females but intermediate temperatures yield about 1 : 1 sex ratios, and often these sex ratios are slightly female-biased (Harlow & Taylor, 2000; Warner, Lovern, & Shine, 2007); hence, the pivotal temperature is not readily identifiable under this pattern. Moreover, family effects on sex ratios in *A. muricatus* can vary irrespective of developmental temperature; that is, some clutches have balanced sex ratios whereas others are extremely male- or female-biased when incubated at temperatures expected to produce 1 : 1 sex ratios (Warner et al., 2008). Additionally, sex ratios of many kinosternid turtles and crocodylians never achieve 100% males across any constant-temperature incubation regime (Lang & Andrews 1994; Ewert et al., 2004). Intuition would suggest that such patterns would yield highly female-biased primary sex ratios in nature, possibly leading to unstable population demographics (Girondot et al., 2004). However, field data supporting this idea are scarce and it is possible that more males are produced in nature than one would expect from the results of artificial incubation experiments (see Section 6, on ecology and evolution).

The evolution of this diversity in TSD patterns is not entirely clear, but it has been proposed that the FMF pattern was the ancestral state from which the other patterns evolved (Deeming & Ferguson, 1988; Pieau, 1996). Perhaps selection favored shifts in the sex-determining response along the temperature range until lethal extremes precluded sex determination (Pieau, 1996). Such changes in thermal sensitivities could result in the FM or MF patterns depending on the direction of the shift. Given the evidence for variable and heritable pivotal temperatures (Bull, Vogt, & Bulmer, 1982a; Janzen, 1992; Rhen & Lang,

1998; Dodd, Murdock, & Wibbels, 2006), such a scenario is possible. Additional information on species-specific patterns of TSD, as well as resolved phylogenies to which these patterns can be mapped, will enable robust tests of this hypothesis.

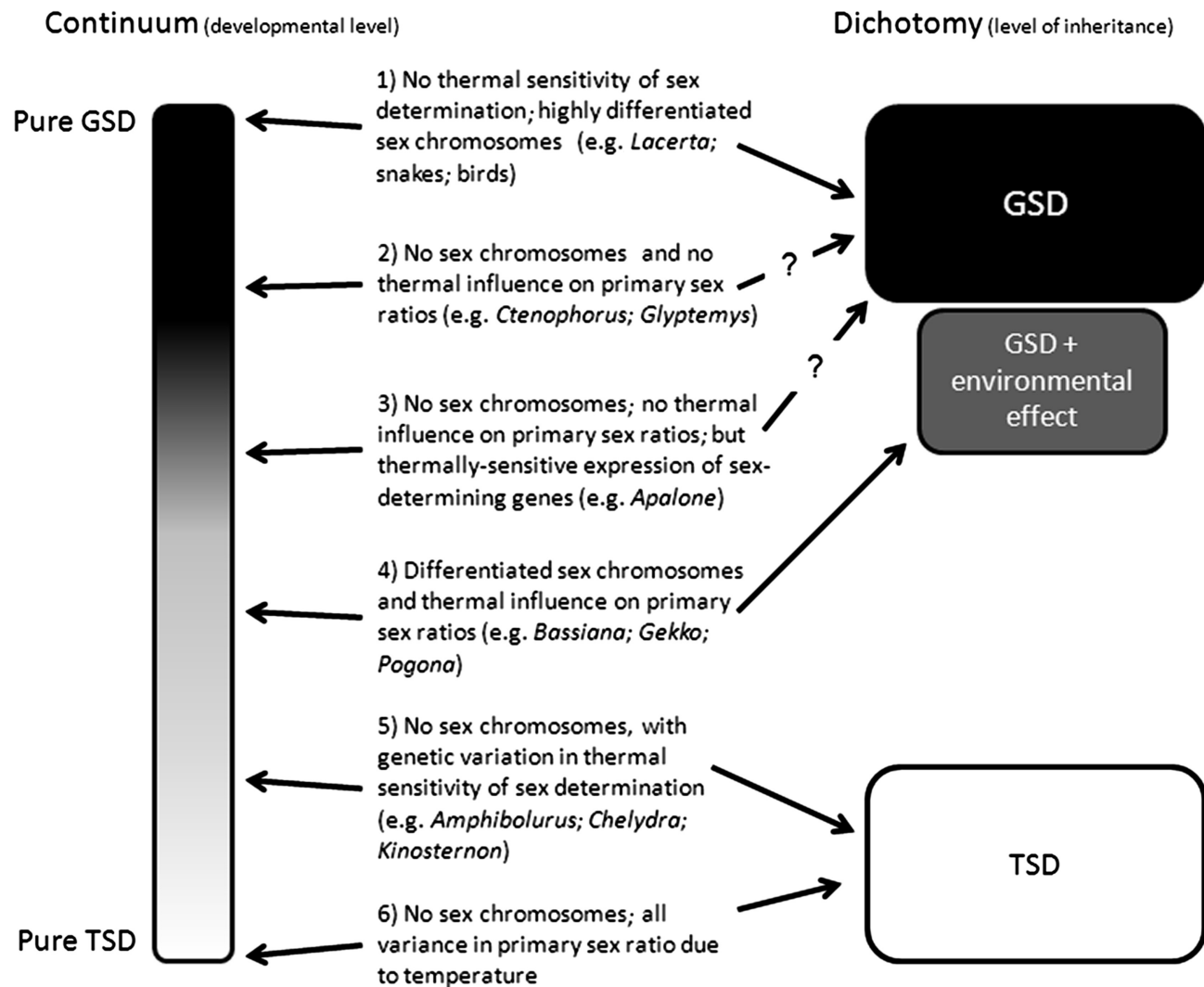
### 2.3. Sex-determining Mechanisms as a Dichotomy or a Continuum?

Traditional classification schemes place reptilian SDMs into one of two discrete categories: GSD or TSD. This dichotomous perspective implies that these two mechanisms are fundamentally different from each other in terms of process and are mutually exclusive (Bull, 1983). Indeed, the inheritance of sex and sex-linked traits is fundamentally different under TSD systems, which lack sex chromosomes, vs. GSD systems, with sex chromosomes. However, evidence is accumulating that the underlying mechanisms that shape the sexual phenotype of offspring in GSD and TSD systems are similar (Place & Lance, 2004), and that these mechanisms may represent a continuum (Sarre et al., 2004; Barske & Capel, 2008). One end point of the continuum is *pure* GSD, where sex is determined by genetic factors; *pure* TSD is at the other endpoint, where sex is determined by developmental temperature. At intermediate points along the continuum, sex is determined by variable levels of genetic and environmental contributions. Much discussion of reptile SDMs now treats these two perspectives (dichotomous vs. continuous) as opposing schools of thought that have very different impacts on our understanding of the proximate mechanisms and evolution of SDMs. The goal here is to briefly discuss the basis for both perspectives and illustrate that the source of conflict between ideas may involve confusion about the level at which SDMs are viewed (i.e., evolutionary genetic level vs. developmental level) (Figure 1.3).

It is important to first establish what is meant by GSD and TSD at both the evolutionary genetic level and the developmental level (Valenzuela, 2008a). At an evolutionary genetic level, GSD is defined by the presence of sex-specific heritable factors (e.g., heteromorphic or homomorphic sex chromosomes), whereas TSD is defined by the absence of consistent heritable genetic differences between the sexes (as in Valenzuela et al., 2003). Under TSD, male and female genomes are identical, but sex differences are induced by a thermal trigger that influences sex-specific gene expression (see Section 4 on proximate mechanisms). Given these definitions, the distinction between GSD and TSD is whether or not one sex contains a single copy of a sex-determining gene(s). If sex chromosomes are present, the genetic mechanism by which sex is determined is transmitted between generations in a sex-dependent manner (i.e., one of the parents can contribute

two different types of chromosome). Clearly, the presence (GSD) or absence (TSD) of sex chromosomes will have fundamentally different consequences on population genetics, sex-ratio evolution, sex-linked traits, and autosomal traits (Bull, 1983; Reeve & Pfennig, 2002). Thus, when SDMs are viewed this way, a dichotomous classification appears appropriate. Importantly, however, thermo-sensitivity of sexual differentiation can also be inherited (Bull, et al., 1982a), which may occur in many species with sex chromosomes (e.g., Radder, Quinn, Georges, Sarre, & Shine, 2008; Luchenback, Borski, Daniels, & Godwin, 2009; Nakamura, 2009). Another consideration is that sex chromosomes have not been identified in many GSD species; if sex chromosomes do not occur in these taxa, then inheritance of sex chromosomes may not always exist in GSD taxa. Clearly, more research is urgently needed to evaluate the co-occurrence, stability, and genetic inheritance of both TSD and GSD mechanisms within single populations under natural developmental conditions (e.g., Radder et al., 2008; Bull, 2008).

From a developmental perspective, biologists are interested primarily in the proximate developmental mechanisms involved in sexual differentiation (as in Sarre et al., 2004). Under *pure* GSD, the embryo's genotype triggers the developmental pathway that leads to the male or female phenotype. Under *pure* TSD, this pathway is triggered by developmental temperature. Under these definitions, the boundary between GSD and TSD is blurred because elements of both sex-determining systems co-occur within many species (Barske & Capel, 2008). For example, we see remarkable genetic variation among and within species in their sensitivity to incubation temperature, suggesting that many species do not exhibit *pure* TSD or *pure* GSD. Instead, different degrees of thermal sensitivity of sex determination occur in the background of GSD. Support for this idea is widespread; thermally sensitive SDMs coexist with differentiated sex chromosomes in many reptilian species (Shine et al., 2002; Sarre et al., 2004; Quinn et al., 2007). Evidence also illustrates that the molecular pathways involved in TSD and GSD are quite similar (Bull, Hillis, & O'Steen, 1988a; Servan, Zaborski, Dorizzi, & Pieau, 1989; Sarre et al., 2004; Valenzuela, 2007), indicating that the processes involved are not discrete. Intriguingly, chromosomal sex determination in some invertebrate (Cook, 2002), fish (Baroiller, Chourrout, Fostier, & Jalabert, 1995; Kraak & Pen, 2002; Luckenback et al., 2009), and amphibian (Dournon et al., 1990; Kraak & Pen, 2002) populations shows sensitivities to temperature, indicating that this is not just a reptilian phenomenon. Overall, the evidence suggests that there is a common underlying mechanism that guides sexual differentiation in 'GSD' and 'TSD' reptiles, and that categorizing mechanisms may simplify the complexity of these systems when viewed from a developmental perspective.



**FIGURE 1.3** Two contrasting views about reptile sex-determining mechanisms (SDMs). The traditional perspective is that genotypic (GSD) and temperature-dependent sex determination (TSD) are fundamentally different and that the mechanisms are mutually exclusive. This viewpoint is derived from an evolutionary genetics level, where sex-determining factors (e.g., sex chromosomes) are inherited among generations. Species that exhibit thermally sensitive SDMs and contain sex chromosomes represent an intermediate state; in this case environment affects sex, but the genetic variation in sex determination is still inherited on sex chromosomes (dichotomous view to the right) (Valenzuela, N., Adams, D. C., & Janzen, F. J., 2003). Importantly, however, under this dichotomous perspective, cases 2 and 3 are traditionally classified as GSD, but if no sex chromosomes exist then this poses classification problems when using definitions at the level of inheritance (indicated by question marks). A more recent perspective from a developmental standpoint posits that the distinction between GSD and TSD is not clear-cut, and these two mechanisms represent endpoints of a continuum (continuous view to the left) (Sarre, S. D., Georges, A., & Quinn, A., 2004). A continuous perspective provides a different understanding of how elements of both GSD and TSD coexist to determine the sexual phenotype of offspring.

In summary, GSD and TSD are traditionally classified as discrete mechanisms when limiting our view to the level of inheritance, but these SDMs represent a continuum when extending our view to the developmental level. Valenzuela et al. (2003) make clear distinctions between systems at the level of inheritance, but still recognize that a combination of SDMs lies at intermediate states along a continuum (Figure 1.3). For example, in organisms containing both heteromorphic sex chromosomes and thermally sensitive

sex determination (Baroiller et al., 1995; Shine et al., 2002; Kozielska, Pen, Beukeboom, & Weissing, 2006), there is a genetic component that determines sex differences under some circumstances, and this component is inherited on a sex chromosome (Valenzuela et al., 2003). However, at the developmental level, both mechanisms coexist and affect the sexual phenotype of offspring, which places this system at an intermediate state in the GSD to TSD continuum. This state is termed GSD + environmental

effect by Valenzuela et al. (2003) and lies between the endpoints of the GSD to TSD continuum according to Sarre et al. (2004). Regardless of whether these intermediate states are stable or transient (Bull, 2008), recognition of a continuum will broaden our understanding of the evolutionary transitions among SDMs.

### 3. WHO EXHIBITS GENOTYPIC SEX DETERMINATION (GSD) AND TEMPERATURE-DEPENDENT SEX DETERMINATION (TSD)? TAXONOMIC AND PHYLOGENETIC DISTRIBUTION

Despite the extensive literature on SDMs in reptiles, we have only scratched the surface in terms of our understanding of their taxonomic distribution. To date, sex-determining mechanisms have been identified in less than 6% of the extant reptile species (Table 1.1). This statistic reveals our ignorance in many respects, but it also signifies that much exciting and fruitful research is yet to come. Nevertheless, much has been learned to date, and we have gained a detailed understanding of many aspects of the distribution of alternative SDMs in reptiles. For example, we know that SDMs are non-randomly distributed throughout reptiles. Recent evidence suggests that GSD evolved independently in three lineages of extinct Mesozoic marine reptiles (e.g., Sauropterygia, Mosasauroidea, Ichthyosauria), which may have been partially responsible for radiations within these clades (Organ, Janes, Meade, & Pagel, 2009). In extant reptiles, both species of tuatara exhibit the FM pattern of TSD (Mitchell et al., 2006) and all crocodylians that have been studied (13 of 23 species) exhibit the FMF pattern (Lang & Andrews, 1994; Deeming, 2004). The ZZ/ZW chromosomal system is ubiquitous in avian reptiles (sister group to Crocodylia). Because SDMs are conserved within these lineages, insights into their evolution are limited based on studies of these groups. Much more can be learned about the evolution of SDMs by focusing on taxa that exhibit considerable diversity, such as the Chelonia and Squamata. Indeed, both of these groups contain species that exhibit XX/XY and ZZ/ZW chromosomal systems, as well as TSD (Figure 1.2). Hence, this section will focus on these two lineages and follow with a brief overview of evolutionary transition between SDMs.

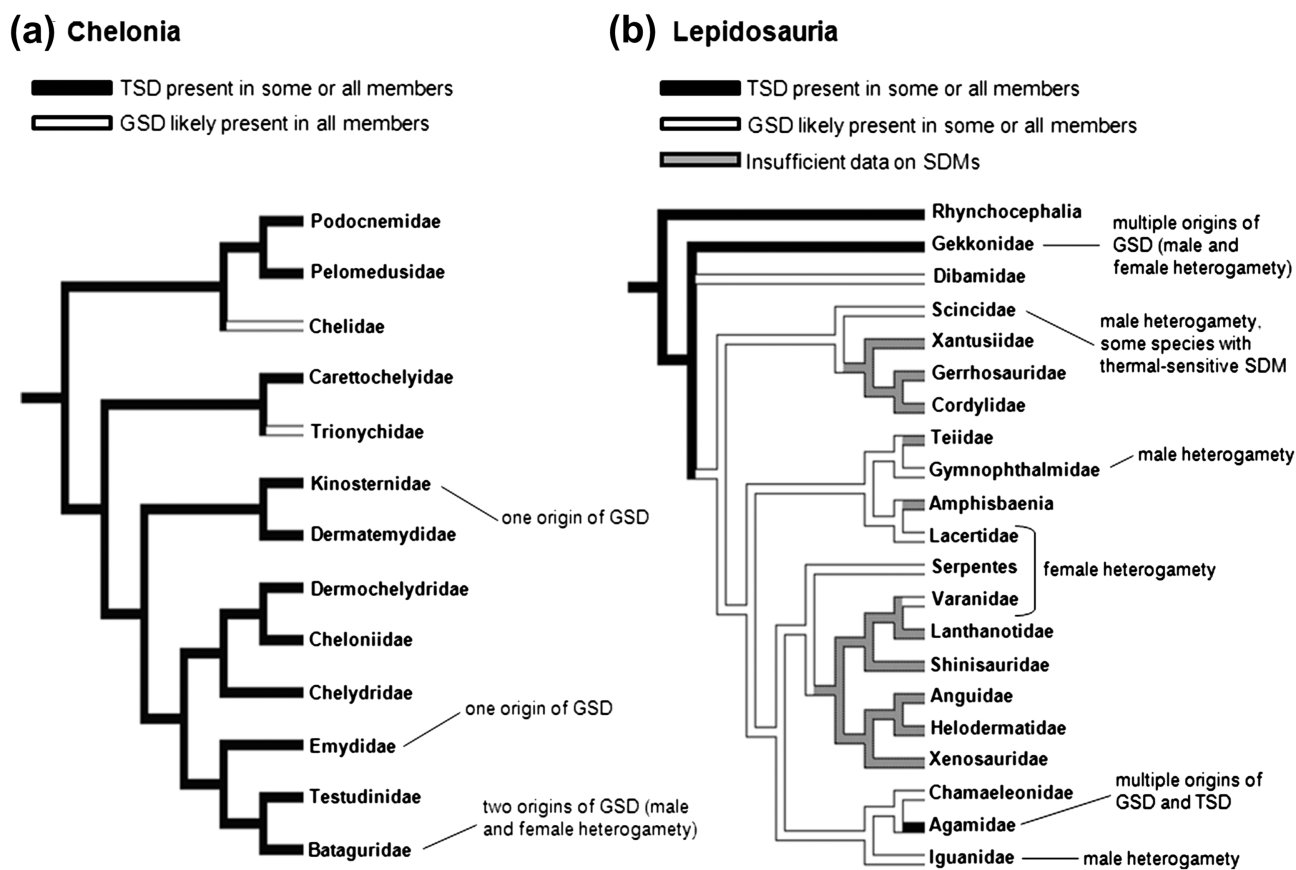
#### 3.1. Chelonian and Squamate Sex Determination

Comparative analyses demonstrate that TSD is the ancestral state for turtles (Janzen & Krenz, 2004; Organ & Janes, 2008) (Figure 1.4a). Of the 149 turtle species that

have been karyotyped, sex chromosomes have been identified in only eight species (Table 1.1). Egg incubation experiments showing an absence of temperature effects on sex have further identified GSD in several species that lack differentiated sex chromosomes (e.g., Bull & Vogt, 1979; Bull, Legler, & Vogt, 1985). In most species examined (81%; see Table 1.1), however, incubation experiments found evidence for TSD with both the FMF and MF patterns, suggesting that TSD is the most prevalent SDM within turtles. Based on our current knowledge of SDMs and turtle phylogeny, comparative analyses suggest that GSD has arisen at least six times in this group (Janzen & Krenz, 2004). Independent origins of sex chromosomes occur twice (XY and ZW systems) in the Bataguridae (Carr & Bickham, 1981; Olmo & Signorino, 2005) and once in Kinosternidae (Sites, Bickham, & Haiduk, 1979). Based on experimental evidence from egg incubation studies, GSD arose once in the Emydidae (*Glyptemys insculpta*) and is likely ubiquitous in the Chelidae and Trionychidae. Sex chromosomes have been identified in three members of the Chelidae (Ezaz et al., 2006; Martinez et al., 2008) and one member of the Trionychidae (Olmo & Signorino, 2005).

As in turtles, comparative analyses suggest that TSD is likely the ancestral condition in squamates (Janzen & Krenz, 2004; Pokorná & Kratochvíl, 2009; but see Organ & Janes, 2008), from which both XY and ZW chromosomal systems have evolved independently multiple times (Figure 1.4b). Of the squamate species that have been karyotyped, less than 26% exhibit differentiated sex chromosomes, most of which are snakes or belong to the lizard genera *Anolis*, *Sceloporus* (Iguanidae), or *Lacerta* (Lacertidae) (but see Table 1.1 for other examples). Incubation experiments provide additional support for GSD in many taxa that lack differentiated sex chromosomes (e.g., Uller et al., 2006; 2008). In contrast, many species have retained (or independently evolved) the ancestral state of TSD, and all three patterns (FM, MF, and FMF) occur in lizards. Based on reliable evidence, rather than anecdotal observations that are occasionally reported (see Harlow, 2004), TSD appears to be confined to only two or three lineages (Agamidae, Gekkota, and probably Scincidae). Importantly, however, thermal effects on sex determination have not been studied in the vast majority of squamate species (Table 1.1).

Phylogenetic reconstructions of the evolution of lizard SDMs are relatively unclear, as well-resolved trees and extensive taxon sampling are currently lacking. At present, at least three phylogenetic hypotheses for relationships of the major lizard families have been proposed (Estes, de Queiroz, & Gauthier, 1988; Townsend, Larson, Louis, & Macey, 2004; Vidal & Hedges, 2005), and the evolution of SDMs varies depending on which phylogeny is used (Pokorná & Kratochvíl, 2009). Adding to this

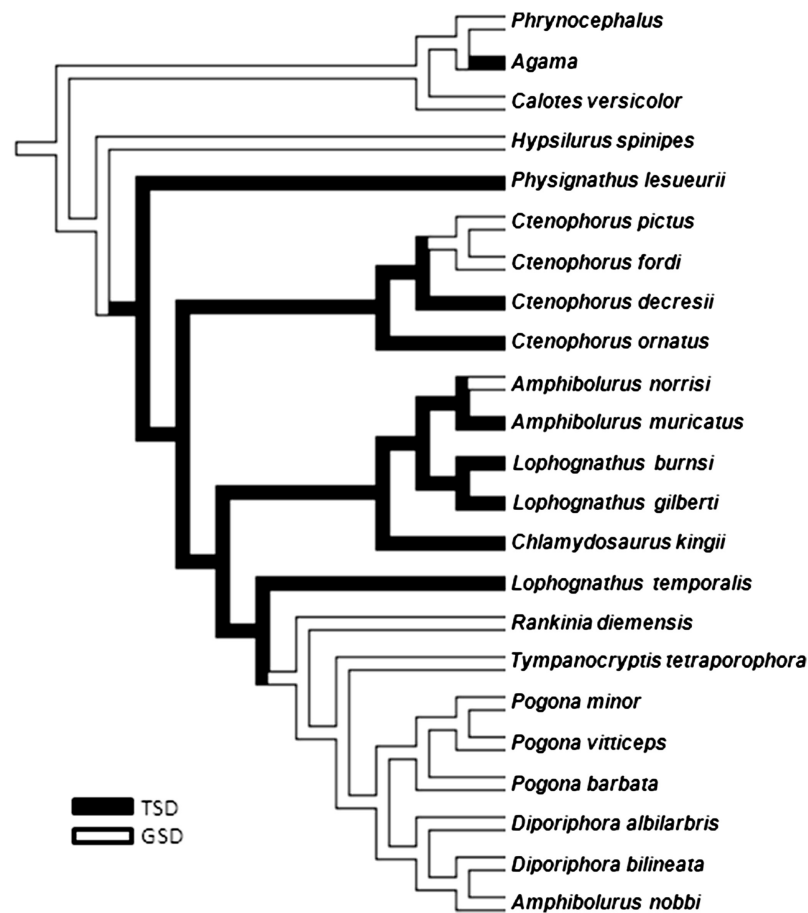


**FIGURE 1.4** Phylogenetic distribution of sex-determining mechanisms (SDM) in extant families of (a) Chelonia and (b) Lepidosauria (Squamata + Rhynchocephalia). Family classifications correspond to Pough et al. (2004). The chelonian phylogenetic hypothesis is based on nuclear DNA (Krenz, Naylor, Shaffer, & Janzen, 2005) and the squamate phylogenetic hypothesis is based on nuclear and mitochondrial DNA (Townsend et al., 2004). Temperature-dependent sex determination (TSD) is ancestral in turtles, from which genotypic sex determination (GSD) evolved at least six times. The evolutionary reconstruction of squamate SDMs is not resolved due to extremely low taxon sampling both among and within families. Nevertheless, evidence suggests that TSD is ancestral for this clade (Janzen & Krenz, 2004; Pokorná and Kratochvíl, 2009) and may have evolved independently in the Agamidae. Both male and female heterogamety have multiple independent origins in squamates. Because the MacClade phylogenetic analysis program used here (Maddison & Maddison, 2001) produces the most parsimonious topology, the phylogeny indicates that ancestors of some squamate families exhibit GSD (white branches); however, this conclusion is premature because of insufficient data on squamate SDMs. Additional details on SDMs within families are given in Table 1.1. See Organ and Janes (2008) and Pokorná and Kratochvíl (2009) for more detailed phylogenetic analyses of reptile SDMs.

uncertainty, phylogenetic relationships among species within families are currently not resolved (e.g., Han, Zhou, & Bauer, 2004; Smith, Sadlier, Bauer, Austin, & Jackman, 2007; Hugall, Foster, Hutchinson, & Lee, 2008). Another problem is the lack of research on SDMs of lizards (SDMs are known in < 5% of the species), as well as questionable reports of TSD in several species. Indeed, anecdotal observations of sex-ratio skews in response to incubation temperatures have been reported in many families (e.g., Chamaeleonidae, Iguanidae, Lacertidae, and Varanidae (Viets et al., 1994)), but the evidence for TSD in these groups is unconvincing due to low sample sizes or inconclusive evidence (see review by Harlow, 2004). Because of these issues, the ‘phylogenetic reconstruction’ of squamate SDMs presented in Figure 1.4b excludes

these reports and is extremely generalized, but it none the less provides information on some important aspects of the evolution of squamate SDMs. For example, (1) both male heterogamety (e.g., iguanids, gekkonids, scincids) and female heterogamety (e.g., snakes, gekkonids, lacertids) have arisen multiple independent times; (2) SDMs are evolutionarily labile within the Agamidae and Gekkonidae; and (3) thermally sensitive SDMs may have been retained (or independently arisen) in the Scincidae despite the presence of sex chromosomes in this lineage.

Perhaps the most compelling evidence of TSD in squamates occurs in the family Agamidae (Harlow, 2004). Both TSD and GSD have evolved independently multiple times within this group (Pokorná & Kratochvíl,



**FIGURE 1.5** Phylogenetic reconstruction of sex-determining mechanisms (SDMs) in the lizard family Agamidae. The agamid phylogeny is based on hypotheses proposed by Schulte, Melville, & Larsan, (2003) and Hugall et al. (2008). Parsimony analysis was performed with MacClade Software (Maddison and Maddison, 2001). The phylogeny suggests that genotypic sex determination (GSD) was the ancestral state in this family. Temperature-dependent sex determination (TSD) evolved two independent times, accompanied by at least three independent reversals back to GSD. This analysis is treated as preliminary given that SDMs are known for only 26 of the 380+ extant species of Agamidae.

2009) (Figure 1.5). In some cases, sister taxa differ in SDMs, suggesting a recent evolution of either GSD or TSD (e.g., *Amphibolurus* and *Ctenophorus*). Most species exhibit the FMF pattern, but some have the FM pattern (e.g., *Lophognathus temporalis* (Harlow, 2004)). Because of this diversity within a single family, the Agamidae provides excellent model systems for exploring ecological and evolutionary aspects of vertebrate SDMs (Warner & Shine, 2005; Uller & Olsson, 2006; Quinn et al., 2007; Warner, Uller, & Shine, 2009). However, as SDMs have only been identified in 26 of the 380+ agamid species worldwide, much exciting work is yet to be done.

The Gekkonidae is another family that exhibits considerable diversity in SDMs (Pokorná and Kratochvíl, 2009). Temperature-dependent sex determination occurs in three lineages of this family (all FMF pattern: Dipodactylinae, Eublepharinae, and Gekkoninae) (Viets et al.,

1994), from which GSD has evolved independently at least three times (Janzen & Krenz, 2004; Pokorná and Kratochvíl, 2009). Intriguingly, comparative analyses indicate that male heterogamety evolved multiple times in some gecko lineages, whereas female heterogamety evolved in others (Pokorná and Kratochvíl, 2009). Considering the current diversity of SDMs that has been described in such a small fraction of the extant gekkonids (SDMs are known in only about 33 of 1050+ species), it would not be surprising if far more origins of alternative SDMs occur in this family than in others, and that both SDMs may occur within a single species. For example, strongly differentiated sex chromosomes occur in *Gekko japonicus*, but sex determination is also highly sensitive to incubation temperature in this species (Yoshida & Itoh, 1974; Tokunaga, 1985).

Recent laboratory experiments and correlative data from the field provide convincing evidence of thermal

sensitivity of sexual differentiation in scincid lizards. For example, the Australian skink, *Bassiana duperreyi*, exhibits male heterogamety, but eggs incubated at cool temperatures that mimic thermal regimens of nests overproduce males, whereas warmer temperatures produce a 1 : 1 sex ratio. Maternal factors, such as yolk allocation (i.e., egg size), can also interact with incubation temperature to affect offspring sex ratios in this species (Shine et al., 2002; Radder, Pike, Quinn, & Shine, 2009). Sex is also sensitive to temperature in three distantly related viviparous scincid lizards (*Eulamprus tympanum*, *Niveoscincus ocellatus*, and *Sphenomorphus indicus*). Experimental manipulations of basking conditions, and hence maternal thermoregulation, have profound effects on offspring sex ratios. In *E. tympanum*, warm gestation temperatures overproduce male offspring and cool temperatures produce 1 : 1 sex ratios (Robert & Thompson, 2001), but this pattern is reversed in the viviparous skink *N. ocellatus* (Wapstra et al., 2004; 2009). Skewing of sex ratios in response to gestation temperature are more substantial in the viviparous skink *S. indicus* (Ji et al., 2006) than that reported in *E. tympanum* or *N. ocellatus*.

### 3.2. Transitions between Sex-determining Mechanisms (SDMs)

The evolution of reptilian SDMs involves multiple transitions between TSD systems and GSD systems with well-differentiated sex chromosomes. It is generally accepted that sex chromosomes arise from an autosomal ancestor when a gene involved in ESD acquires a mutation that consistently directs either male (under XY systems) or female (ZW systems) development (Ohno, 1967). After this primordial sex-determining locus emerges, the pair of autosomes that contain that locus become sex chromosomes. During early stages of chromosome evolution, sex chromosomes are essentially identical except at the sex-determining locus, where they differ only in gene content and not morphology (Matsuda et al., 2007). This sex-determining locus resides only on the Y or W chromosomes, and recombination with its homolog becomes increasingly limited (Charlesworth, 1991). As a result, the sex-linked differential segment is free to diverge from the homologous region, causing sex chromosomes to become progressively differentiated from each other (Rice, 1996; Vallender & Lahn, 2004; 2006). The homogametic chromosome (X or Z) maintains its size and gene content, while the heterogametic chromosome (Y or W) loses these features (Vallender & Lahn, 2004; Charlesworth et al., 2005).

Intriguingly, the complete range of chromosome differentiation occurs in snakes (Modi & Crews, 2005;

Matsubara et al., 2006). Sex chromosomes are only slightly differentiated in basal species (Boidae) and fully differentiated in the more derived viperid snakes. Members of the family Colubridae exhibit intermediate levels of chromosome differentiation. Given this diversity within snakes, this group provides an excellent model for comparative studies of sex chromosome evolution. In addition, comparative gene mapping demonstrates that the sex chromosomes of snakes, mammals, and birds were derived independently from different autosomal ancestors (Marshall-Graves & Shetty, 2001; Matsubara et al., 2006). Given the evolutionary lability of GSD, it is possible that this process has also occurred multiple times within reptiles, as has already been shown in turtles (Sites et al., 1979; Carr & Bickham, 1981).

What about transitions in the opposite direction, from GSD to TSD? Some authors argue that highly differentiated sex chromosomes place constraints on reversals back to homomorphic chromosomes; that is, once chromosomal differentiation has begun, the probability of subsequent change in the SDM is greatly diminished (Marín & Baker, 1998). In support of this model, a recent phylogenetic reconstruction of the evolution of SDMs in squamates suggests that the transition from TSD to GSD is unidirectional and that sex chromosomes are an ‘evolutionary trap’ preventing subsequent evolution back to TSD (Pokorná & Kratochvíl, 2009). Whether this is truly the case remains to be seen, as sampling of more taxa and better resolution of phylogenies are needed (TSD may have arisen from GSD in agamid lizards (Figure 1.5)). Indeed, one phylogenetic reconstruction of reptilian SDMs suggests that transitions between XY systems and TSD occur in both directions, but a ZW system is less likely to give rise to TSD, perhaps because of a greater instability of XY systems (Organ & Janes, 2008). Importantly, transitions back and forth between TSD and GSD may involve very few changes that could occur at different locations in the sex-determining pathway, which in turn can affect master thermal switches (Sarre et al., 2004; Barske & Capel, 2008; Valenzuela, 2008b). Transitions from heteromorphic to homomorphic systems can occur by a translocation of a piece of an autosome onto the Y (or W) chromosome (Rice, 1996), particularly if little chromosome differentiation has occurred. Additionally, sex chromosomes could become extinct at the end of chromosome degeneration (Charlesworth & Charlesworth, 2000; Charlesworth et al., 2005), resulting in homomorphic chromosomes, which could lead to a thermally sensitive SDM. Moreover, Bull (1981) provides a theoretical framework illustrating how ESD can evolve from male-heterogametic systems. Overall, we cannot ignore the possibility that transitions between TSD and GSD occur in both directions, which may explain the impressive diversity and distribution of SDMs in reptiles.

#### 4. HOW IS SEX DETERMINED? PROXIMATE MECHANISMS

Our understanding of the molecular and physiological underpinnings of reptilian sex determination is far from complete. Research on this topic has focused primarily on species with TSD to understand how interactions among genes, steroid hormones, and temperature affect sex determination and gonad differentiation. Since the gene regulatory networks and physiological pathways that influence key events during gonadal differentiation are highly conserved and have common elements among all vertebrates (Place & Lance, 2004), our in-depth understanding of mammalian systems offers some critical insights into GSD and TSD in reptiles. As many reviews have already covered this material in depth (Crews et al., 1994; Crews, 1996; Lance, 1997; Pieau, Dorizzi, & Richard-Mercier, 1999; Morrish & Sinclair, 2002; Elf, 2004; Pieau & Dorizzi, 2004; Place & Lance, 2004), this section will provide only a brief overview of gonadal differentiation and its molecular and physiological basis under GSD and TSD.

##### 4.1. Gonadal Differentiation and Gene Expression

To understand proximate mechanisms, a distinction between sex determination and sexual differentiation is useful. Sex determination is the process that guides the undifferentiated gonads to develop into testes or ovaries. This process can be initiated by a master switch, which is either under genetic control (under *pure* GSD), environmental control (under *pure* TSD), or has elements of both mechanisms. Sexual differentiation, on the other hand, refers to the development of specialized sex organs (i.e., testes or ovaries). Under these definitions, the process of sex determination guides sexual differentiation and ends when gonadal development is irreversibly committed to becoming either a testis or an ovary.

Although the specific details of gonadal development (i.e., gonadogenesis) vary among vertebrate taxa, many general patterns are conserved (see Place & Lance, 2004). Prior to gonadogenesis in mammals, male and female embryos develop similarly and the rudiments of the testes and ovaries (genital ridges) are at first indistinguishable. At this early stage, embryos contain two sets of ducts: the Wolffian ducts (male reproductive tract) and the Müllerian ducts (female reproductive tract). In embryos that develop into males, the genital ridges begin to develop into seminiferous tubules, which indicate testis development. The embryonic testes produce two important hormones that influence sexual development of males. The first is anti-Müllerian hormone (AMH) (also known

as Müllerian-inhibiting substance (MIS)), which causes the Müllerian ducts to degenerate. The second hormone, testosterone, stabilizes the Wolffian ducts, which eventually develop into seminal vesicles, epididymis and vas deferens. In the absence of these two hormones, the Wolffian ducts degenerate and the Müllerian ducts develop into oviducts and the uterus (Mittwoch, 1996; Place & Lance, 2004).

Each stage of gonadal differentiation is governed by numerous genes that perform different roles and interact with each other in complex ways (Table 1.2). In mammals, a major sex-determining switch (*Sry* gene located on the Y chromosome) sets the testicular developmental pathway in motion (Sinclair et al., 1990). The *Sry* gene triggers expression of the downstream gene *Sox9*, which is expressed in the Sertoli cells (but not in the ovary) and is the key regulator of the *Amh* gene in mammals (Arango, Lovell-Badge, & Behringer, 1999). Other genes (e.g., *Wtl* and *Sfl*) also contribute to testis formation by activating the transcription of the *Amh* gene, the product (AMH) of which initiates the degeneration of the Müllerian duct. In contrast, ovaries develop in the absence of the *Sry* gene. The gene *Dax1* is expressed in ovaries, which suppresses the activation of *Wtl* and *Sfl*, enabling the retention of the Müllerian ducts (Nachtigal et al., 1998). Thus, expression of this gene will lead to female development. Another gene that is likely involved in testis formation is *Dmrt1*. This gene is expressed in the bipotential gonads of both sexes, but at later stages it is only expressed in the testes. Recent research using RNA interference to knock down *Dmrt1* shows convincing evidence that this gene is required for testis determination in the chicken (Smith et al., 2009), but its role in sex determination is largely unknown in non-avian reptiles. Indeed, no upstream or downstream targets of *Dmrt1* have been identified.

The roles of the genes discussed above are described primarily from a mammalian perspective, but many of these genes have homologs in reptiles with GSD and TSD (Bull et al., 1988a; Smith, McClive, Western, Reed, & Sinclair, 1999; Pieau & Dorizzi, 2004; Place & Lance, 2004). At present, a master sex-determining switch (e.g., *Sry* in mammals) has not been identified in reptiles with GSD or TSD. Instead, expression of many of the genes involved in gonadal differentiation is temperature-sensitive in TSD species. For example, in American alligators (*Alligator mississippiensis*) and leopard geckos (*Eublepharis macularius*), *Sox9* expression is upregulated in gonads of embryos exposed to male-producing temperatures, but this expression occurs after the initiation of male gonadal differentiation (Western, Harry, Graves, & Sinclair, 1999a; Valleley, Carwright, Croft, Markham, & Coletta, 2001). Interestingly, *Amh* expression precedes *Sox9* expression in alligators, suggesting that, unlike the



**TABLE 1.2** Genes, steroids, and enzymes involved in sex determination in vertebrates. With the exception of the *Sry* gene, all factors listed are found in all vertebrate taxa, and serve similar functions under temperature-dependent sex determination (TSD) and genotypic sex determination (GSD)

Sex-determining factor	Full name	Definition or function for sexual development
Gene		
<i>Sry</i>	Sex-determining region Y	Major testis-determining factor located on the Y chromosome. Found only in placental mammals
<i>Sox9</i>	SRY-like HMG box	Regulates transcription of the anti-Müllerian hormone gene in mammals. Involved in testes differentiation
<i>Sf1</i>	Steroidogenic factor 1	Involved in the formation of the primary steroidogenic organs (adrenal glands and gonads). Plays a role in testis development and regulation of <i>Amh</i>
<i>Wt1</i>	Wilms' tumor suppressor gene	Transcription factor involved in urogenital development. Necessary for maintenance of seminiferous tubules and Sertoli cells in mammals
<i>Amh</i> or <i>Mis</i>	Anti-Müllerian hormone or Müllerian-inhibiting substance	Regulates anti-Müllerian hormone, which inhibits development of the Müllerian ducts and hence is important in testis development
<i>Dmrt1</i>	Doublesex- and mab-3-related transcription factor	Transcription factor involved in gonadal differentiation, but its role is poorly understood
<i>Dax1</i>	Dosage-sensitive sex reversal	May be antagonist of <i>Sry</i> function in mammals. Represses <i>Wt1</i> and <i>Sf1</i> expression (i.e., suppresses testis formation) and important in ovarian differentiation
Steroid hormones and steroidogenic enzymes		
DHT	Dihydrotestosterone	Non-aromatizable androgen involved in testis development
T	Testosterone	Androgen involved in testis development
E <sub>2</sub>	Estradiol	Estrogenic steroid hormone involved in ovarian development
Aromatase	-	Enzyme that converts androgens to estrogens
Reductase	-	Enzyme that converts testosterone to dihydrotestosterone

regulation of *Amh* by *Sox9* in mammals, other genes may regulate *Amh* in alligators (Western, Harry, Graves, & Sinclair, 1999b). In contrast, *Sox9* expression precedes *Amh* expression in the TSD turtle *Trachemys scripta* (Shoemaker, Ramsey, Queen, & Crews, 2007). Expression of *Wtl* occurs during the early stages of gonadogenesis and at both male- and female-producing temperatures in alligators (Western, Harry, Marshall-Graves, & Sinclair, 2000). However, levels of expression of *Wtl* are greater at male- than at female-producing temperatures in turtles (*T. scripta* and *C. picta*) that exhibit TSD (Spotila, Spotila, & Hall, 1998; Valenzuela, 2007). Moreover, *Wtl* has been shown to regulate *Sfl* in mammals (Wilhelm & Englert, 2002)—another gene involved in gonad formation. The expression of *Sfl* also varies depending upon incubation temperature in turtles (Crews, Fleming, Willingham, Baldwin, & Skipper, 2001). Indeed, both *Wtl* and *Sfl* are implicated as candidates for master thermal switches in TSD turtles (Valenzuela, LeClere, & Shikano, 2006; Valenzuela, 2007; 2008b). *Dax1* and *Dmrt1* expression occur in both sexes prior to gonadal differentiation in alligators and turtles, but their expression is temperature-sensitive during the embryonic stages that coincide with sexual differentiation (Smith et al., 1999; Kettlewell, Raymond, & Zarkower, 2000; Shoemaker et al., 2007; Valenzuela, 2008b). Nevertheless, the exact roles of these genes and their position in the regulatory network require more investigation to fully understand the molecular basis for GSD and TSD. Excellent reviews by Shoemaker and Crews (2009) and Ramsey and Crews (2009) provide more details of the regulatory network.

Thermal sensitivity of gene expression has recently been demonstrated in a turtle with GSD. In the softshell turtle *Apalone mutica*, expression of the *Wtl* gene is sensitive to incubation temperature, similar to that shown in TSD species (Valenzuela, 2007). Expression of *Dax1* is also sensitive to temperature in *A. mutica*, presumably because it is regulated by *Wtl* (Valenzuela, 2008b). Despite this thermal sensitivity, sex ratios remain unaffected by incubation temperature in this species. A possible reason for this involves the thermal insensitivity of the *Sfl* gene, which lies directly downstream from *Wtl* and *Dax1* (Valenzuela et al., 2006; Valenzuela, 2008b). Because *Sfl* is not sensitive to these effects, thermally sensitive expression of *Wtl* and *Dax1* is not functional in sex differentiation, indicating that there may be no selection operating on the thermal sensitivity of gene expression in GSD taxa. This thermal sensitivity of gene expression in *A. mutica* is likely a characteristic that is retained from a TSD ancestor, suggesting that this species may exhibit an intermediate state in the transition to or from GSD (Figure 1.3). It has been proposed that molecular switches that affect the thermal sensitivity of SDMs may occur at many different

levels in the regulatory network, and the molecular pathways for TSD (and possibly GSD as well) could differ among species (Sarre et al., 2004; Valenzuela, 2008b). Thus, even within TSD (or GSD), multiple processes may reveal a similar pattern, further illustrating that all TSD and all GSD systems are not comprised of single mechanisms. From this perspective, SDMs cannot be grouped into dichotomous categories.

#### 4.2. Physiological Mechanisms under Temperature-dependent Sex Determination (TSD)

It is generally accepted that temperature exerts its effect on sex determination in TSD species by acting on genetic mechanisms that govern steroid production, steroidogenic enzymes, or steroid hormone receptors (see Table 1.2 for list of hormones and enzymes). Such an effect will change the hormonal environment of the embryo, thereby directing development in a male or female direction. Indeed, levels of steroid hormones in reptilian egg yolks decline dramatically during development and the degree of this decline can depend on incubation temperatures (Conley et al., 1997; Elf, 2004); such effects may direct gonadal development. This notion is supported by experiments that manipulate the hormonal environment of the developing embryo. For example, administration of the estrogen 17 $\beta$ -estradiol ( $E_2$ ) to eggs of many reptile species induces female development even at male-producing temperatures (Bull, Gutzke, & Crews, 1988; Crews, 1996; Freedberg, Bowden, Ewert, Sengelau, & Nelson, 2006). The ability of  $E_2$  to counteract the effects of temperature is greatest during the developmental window that corresponds with gonadogenesis (Gutzke & Chymiy, 1988; Wibbels, Bull, & Crews, 1991) (see also Section 5). Perhaps this effect occurs via an influence on gene expression, as exogenous  $E_2$  application to eggs of *T. scripta* results in downregulation of *Sfl* and *Dmrt1* expression (potentially repressing testis formation) at male-producing temperatures (Fleming & Crews, 2001; Murdock & Wibbels, 2006). Moreover,  $E_2$  has an increasingly potent effect on ovarian development as incubation temperatures move from strictly male-producing towards the threshold temperature (Crews & Bergeron, 1994). Administration of  $E_2$  to eggs can also impact sexual differentiation in species with GSD (Bull et al., 1988b). Despite male heterogamety in the turtles *Staurotypus triporcatus* and *Staurotypus salvinii*,  $E_2$  exposure during development permanently induces female development (Freedberg et al., 2006). In both TSD and GSD reptiles, these hormonal manipulations lead to fully functional females similar to those from unmanipulated eggs (Crews et al., 1994). Clearly,  $E_2$  is the primary

steroid that is required for ovarian differentiation in reptiles (Pieau & Dorizzi, 2004).

Another key player involved in directing sexual differentiation of the gonad is aromatase. Aromatase is an enzyme that is responsible for converting androgens into estrogens in both TSD and GSD species (Crews et al., 1994; Pieau & Dorizzi, 2004). Aromatase activity or *aromatase* gene expression in the gonad is greatest at female-producing incubation temperatures during the period of gonadal differentiation in several TSD species such as *Emys orbicularis* and *Malaclemys terrapin* (Desvages & Pieau, 1992; Jeyasuria & Place, 1998), but not in *T. scripta* or *C. picta* (Murdock & Wibbels, 2003; Valenzuela et al., 2006). The initial trigger of aromatase activity has not been identified, but it may involve thermal sensitivity of genes that lie upstream in the regulatory network (Lance, 1997; Valenzuela & Shikano, 2007). At the onset of gonadal differentiation, aromatase activity is orders of magnitude greater in female gonads than in male gonads (Lance, 1997), indicating that the aromatization of androgens provides an important source of estrogens needed for ovarian development. Indeed, the application of testosterone (T)—a precursor to  $E_2$ —to eggs has feminizing effects on some developing embryos of *T. scripta* when exposed to female-producing temperatures; this non-intuitive result is presumably due to the aromatization of T to  $E_2$  (Crews & Bergeron, 1994; Crews, 1996). Another line of evidence that illustrates the importance of aromatase is provided by studies that block  $E_2$  synthesis with aromatase inhibitors. By applying aromatase inhibitors to eggs, embryos develop into males when incubated at female-producing temperatures. This has been demonstrated in numerous reptilian species that exhibit TSD (e.g., Crews et al., 1994; Rhen & Lang, 1994; Warner & Shine, 2005). Moreover, aromatase inhibitors can induce male development in eggs of all-female parthenogenetic lizards (i.e., *Aspidoscelis uniparens*) (Wibbels & Crews, 1994). These manipulations result in phenotypic males with similar gonadal morphology and similar behaviors to naturally produced males; they are also capable of spermatogenesis as adults (Elbrecht & Smith, 1992; Wennstrom & Crews, 1995; Shine, Warner, & Radder, 2007; Warner & Shine 2008a).

Many other chemicals (e.g., hormones, enzyme inhibitors, receptor antagonists) applied to eggs have varying effects on sexual development (reviewed in Crews et al., 1994; Crews, 1996). Unlike  $E_2$  application at male-producing temperatures, androgens cannot overcome the effects of female-producing incubation temperatures. For example, dihydrotestosterone (DHT), a non-aromatizable androgen, only induces male development at temperatures near the threshold temperature for sex determination. Nevertheless, manipulations of  $5\alpha$ -reductase (the enzyme that converts T to DHT) provide evidence that androgens

are important for male development. For example, by blocking DHT synthesis using  $5\alpha$ -reductase inhibitors, male development is also blocked, suggesting that DHT is important for male development (Crews & Bergeron, 1994). Moreover, simultaneous administration of T and a  $5\alpha$ -reductase inhibitor to eggs results in a greater number of female hatchlings than control treatments in the red-eared slider turtle (*T. scripta*) (Crews & Bergeron, 1994), whereas simultaneous application of both T and an aromatase inhibitor results in nearly 100% male offspring. Overall, these results illustrate that steroidogenic or steroid-metabolizing enzymes (aromatase and  $5\alpha$ -reductase) play a critical role in sex determination and differentiation.

The manipulative studies described above clearly demonstrate critical roles for steroid hormones in sex determination, but what about hormone levels that occur naturally prior to gonadogenesis? Indeed, embryonic production of steroids during initial stages of gonadal development is negligible in the reptiles examined (White & Thomas, 1992). Yet, both  $E_2$  and T are plentiful in egg yolks at the time of oviposition, long before embryos are capable of producing these steroids (Bowden, Ewert, & Nelson, 2000; Bowden, Ewert, Freedberg, & Nelson, 2002; Elf, Fivizzani, & Lang, 2002; Lovern & Wade, 2003). Recent research demonstrates that these steroid hormones are maternally derived and deposited into the yolks of eggs during vitellogenesis (reviewed in Elf, 2004). Moreover, variation in yolk steroids is largely explained by among-clutch differences (i.e., clutch effects (Elf, 2004; Warner et al., 2008)), and this variation often reflects levels of circulating hormones in females (Callard, Lance, Salhanick, & Barad, 1978; Janzen, Wilson, Tucker, & Ford, 2002). Given these patterns, maternal condition could influence the hormonal environment of developing embryos (Kratochvíl, Kubička, & Landová, 2006; Warner et al., 2007; Lovern & Adams, 2008), thereby influencing sexual differentiation. As expected, offspring sex of several reptilian species is associated with maternally derived  $E_2$  or T when eggs are incubated at pivotal temperatures (reviewed in Radder, 2007). Indeed, eggs from clutches with relatively high levels of  $E_2$  tend to produce female offspring in a turtle with TSD (*C. picta*) (Bowden et al., 2000), and eggs with relatively high levels of T tend to produce male offspring in a lizard with GSD (*Anolis carolinensis*) (Lovern & Wade, 2003). Maternally derived corticosterone also influences offspring sex ratios in the lizard *Amphibolurus muricatus* (Warner et al., 2007), but experimental work suggests that elevated levels of corticosterone in eggs in this species yields female-biased secondary sex ratios via differential embryonic mortality (Warner, Radder, & Shine, 2009a). Despite these important maternal effects, in some species an association between maternally derived steroids and offspring sex is not

detected (*C. serpentina* (St. Juliana et al., 2004); *B. duperreyi* (Radder, Ali, & Shine, 2007); *A. muricatus* (Warner et al., 2007)). In these cases, perhaps the levels of maternally derived steroids are too low to have a significant effect on sex determination. Nevertheless, steroids deposited into yolk represent an important maternal contribution, and their role in modulating sex determination requires further investigation.

## 5. WHEN IS SEX DETERMINED? TIMING OF EMBRYONIC SEXUAL LABILITY

Under *pure* GSD, only the genetic makeup of the embryo determines the direction of gonadal development, and accordingly natural external factors should have no influence on sexual differentiation. Hence, the genetic sex of the zygote is determined at fertilization under this system, but the process of gonadal sex differentiation begins when sex-determining genes are expressed. In most cases of GSD, the genetic constitution of the zygote will determine offspring sex. However, in some GSD taxa, external factors (e.g., temperature, hormones) can irreversibly direct gonadal differentiation at some point after fertilization (Crews, 1996; Shine et al., 2002; Freedberg et al., 2006; Quinn et al., 2007). If the role of sex chromosomes in sex determination is overridden by an environmental factor, then phenotypic sex (i.e., the gonad) is not determined solely by the genetic constitution of the embryo but by an interaction with the environment it experiences during the critical time of development. The effect of environmental factors in many GSD species is dependent upon the dosage of that factor (e.g., hormones, temperature) and has a permanent effect on gonadal sex (Freedberg et al., 2006; Radder et al., 2008). Thus, the distinction between TSD and GSD becomes blurred from a developmental perspective.

Under *pure* TSD, sex is determined strictly by environmental parameters regardless of genotype. In this system, embryonic sex is not determined by genetic factors inherited at fertilization, but instead temperature triggers the developmental cascade of events that direct gonadal differentiation during a critical window of development. During this window, embryonic sex remains labile and can be modified by environmental cues, namely temperature. This interval is commonly referred to as the thermosensitive period (TSP) (Mrosovsky & Pieau, 1991). Although the TSP occurs at roughly the middle third of development in TSD reptiles, it has not been evaluated in most TSD species. This section evaluates the different methods used to identify the TSP and explores the ecological significance of the variation in TSPs that occurs among reptiles.

### 5.1. Methods for Establishing the Thermosensitive Period (TSP)

Most studies that quantify the onset and duration of the TSP in TSD reptiles have relied upon temperature-shift experiments (Bull & Vogt, 1981; Pieau & Dorizzi, 1981; Bull, 1987; Lang & Andrews, 1994; Valenzuela, 2001). Under these experiments, researchers incubate eggs at a constant temperature that produces some known sex ratio, typically 100% of one sex. Eggs are then moved during specified developmental intervals to thermal conditions that are expected to generate the other sex. Investigators can then manipulate the timing and duration of the temperature shifts among treatments to identify the timing and size of the developmental window in which sex determination processes are responsive to temperature. These experiments involve either a one-step or a two-step temperature shift design to identify the onset and duration of the TSP.

Although shift experiments have been instructive in identifying the TSP in many species, such designs are not without complications. First, switching temperature modifies developmental rates, rendering it difficult to quantify the percentage of the developmental period during which sex is responsive to external cues. Secondly, shift experiments may fail to elicit sex reversal even if the embryo is potentially able to respond, because the stimulus for change may not always be sufficiently strong. Related to this, because results from shift experiments depend on the duration and magnitude of the shifts (Lang & Andrews, 1994), prior knowledge of the appropriate temperatures and length of exposure for inducing a response is needed. To avoid these problems, recent work by Shine et al. (2007) used chemical manipulation of eggs to identify the window of embryonic sexual lability. Specifically, the researchers applied an aromatase inhibitor to eggs of *A. muricatus* and *B. duperreyi* at specified times during embryonic development. By inhibiting aromatase activity during development, the conversion of T to E<sub>2</sub> is blocked, thereby inducing male development at female-producing temperatures (Crews et al., 1994). Chemically manipulating eggs at different times of embryogenesis and identifying the sex of the resultant offspring provides information on when embryonic sex is responsive to external cues without affecting developmental temperature and, thus, developmental rates.

Do temperature-shift experiments and chemical-application experiments (e.g., application of aromatase inhibitors or E<sub>2</sub>) yield similar conclusions? Studies in which exogenous estrogens were applied to eggs at different stages of development suggest that embryo sensitivity to these chemicals coincides with the TSP (Gutzke & Chymiy, 1988; Wibbels et al., 1991). However, caution is needed because embryo responses to

developmental temperatures and chemical applications depend on temperature magnitude and chemical dosage, respectively, and these two approaches may provide differing results. Moreover, chemical manipulations may reverse embryo sex at times of development when normal thermal cues are unable to do so. Another concern is that chemicals may remain in the egg during times when embryos are insensitive to such cues, thereby rendering it difficult to determine the exact timing of the response. Thus, although chemical-application experiments may identify the window of sexual lability, this may not always equate to the TSP. Indeed, results of a preliminary temperature-shift experiment on eggs of the jacky dragon (*A. muricatus*) indicate that onset of the TSP may be later in development than that suggested by chemical-application experiments (application of an aromatase inhibitor; Figure 1.6). These contrasting results may be due to (1) differing ‘dosage effects’ of the aromatase inhibitor vs. incubation temperature or (2) the aromatase inhibitor applied early in development may not take effect until a later period that corresponds with the actual critical sex-determining period. Both types of experiment supply useful information about windows of sexual lability during development, but only temperature-shift experiments can accurately identify the TSP.

## 5.2. Variation in the Thermosensitive Period (TSP) and its Ecological Implications

The developmental timing of the TSP is relatively similar among reptiles with TSD, but some variation exists (Figure 1.7). To date, most of our knowledge of the TSP has been derived from studies of turtles. For instance, in the European turtle *Emy orbicularis*, the TSP begins at a stage just prior to the onset of gonadal differentiation (stage 16), and the duration of the TSP, in terms of developmental stages, depends on incubation temperature (Pieau & Dorizzi, 1981). As a consequence, male differentiation occurs slightly earlier and may last longer than female differentiation. Similar patterns occur in the North American turtles *Graptemys ouachitensis* and *C. picta* (Bull & Vogt, 1981). In general, the TSP extends throughout much of the middle third of development coinciding with gonadal differentiation (Bull and Vogt, 1981; Pieau & Dorizzi, 1981). Similar patterns occur in other turtle families (Yntema, 1979; Yntema & Mrosovsky, 1982; Valenzuela, 2001).

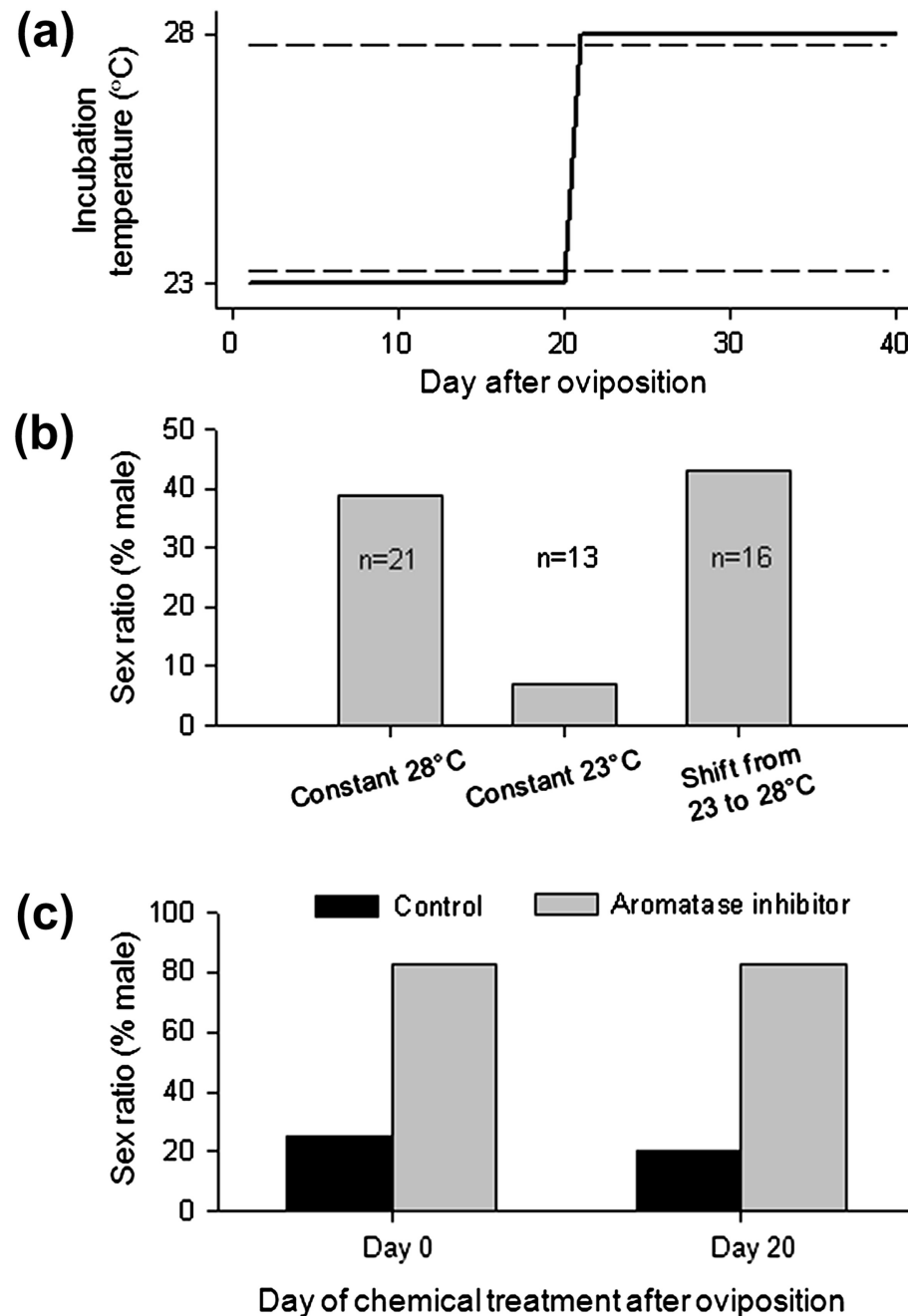
For crocodylians, the TSP begins at a later period in development compared to that of turtles, but still coincides with gonadal differentiation (Deeming & Ferguson, 1988). Detailed shift experiments on *A. mississippiensis* indicate that sex ratios are labile from embryonic stages 21 to 24 (Lang & Andrews, 1994), which encompass roughly the third quarter of embryonic development. Differences

among eggs from different clutches also explain a significant amount of the variation in the TSP, hinting at a genetic component to the timing of sexual differentiation. Similar experiments on eggs of the leopard gecko show that sex is determined within stages 32–37, which occurs shortly after oviposition and extends to about half of the incubation period. For comparison, embryonic morphology is similar at the onset of the TSP for geckos, turtles, and crocodylians (Bull, 1987). The TSP in tuatara is likely to be similar to that in other TSD reptiles. Although embryos were not staged, the estimated TSP for tuatara occurs between 25% and 55% of the total incubation period, which likely corresponds closely with the period of gonadogenesis (Mitchell et al., 2006).

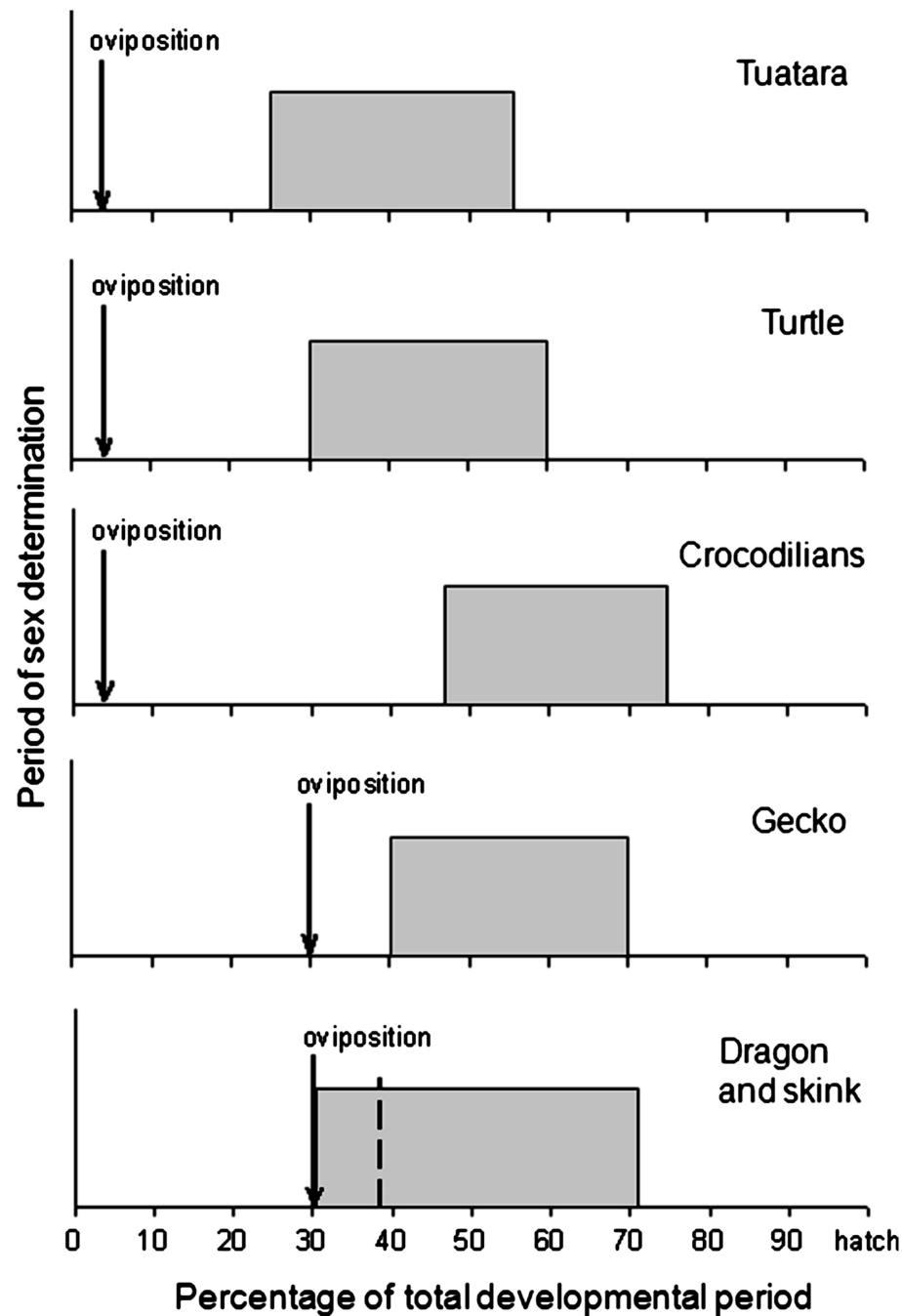
The major reptilian lineages vary considerably in the degree of embryonic development that occurs *in utero* prior to oviposition (Andrews, 2004) (Figure 1.7). Crocodylians, turtles, and tuatara oviposit during very early stages of development prior to neurulation (e.g., gastrula or neurula stages), whereas most oviparous squamates retain developing embryos for about 30% of the total period of development (Andrews & Mathies, 2000). Thus, comparisons of the squamate TSP with that of other reptilian lineages can vary depending on whether the TSP is expressed in terms of total developmental period or total incubation period subsequent to oviposition (Shine et al., 2007).

The timing of the TSP relative to oviposition has important consequences on maternal control of the sex ratio in TSD species. Because squamates typically oviposit at advanced stages of embryonic development, this may enable reproductive females to predict the thermal conditions of potential nest sites during the TSP of their embryos. Hence, squamates may have considerable control over offspring sex ratios via active nest-site selection (Warner & Shine, 2008b). Intriguingly, viviparity will enable even more maternal control over the sex ratio via active thermoregulatory behaviors (Robert & Thompson, 2001; Wapstra et al., 2004; Ji et al., 2006). This type of control in many viviparous squamates may enable mothers to facultatively adjust sex ratios in adaptive directions (Robert, Thompson, & Seebacher, 2003; but see Allsop, Warner, Langkilde, Du, & Shine, 2006). Due to the greater temporal separation of the TSP and oviposition in turtles, crocodylians, and tuatara, similar maternal control is unlikely in these species, as predictive ability of future nest conditions is low.

The timing of thermal effects on fitness-relevant phenotypes (other than sex) of offspring has important consequences for the evolution of the TSP. In squamates, for example, incubation temperature soon after oviposition (i.e., near the time of the TSP) has significantly greater impacts on offspring phenotypes than do temperatures later in development (Shine & Elphick, 2001; Andrews, 2004). Since incubation temperatures



**FIGURE 1.6** Comparison of two methods for identifying the onset of the thermosensitive period (TSP) of sex determination in embryos of the lizard *Amphibolurus muricatus*. (a) Experimental design for a one-step temperature-shift experiment. Dotted lines represent two treatments where eggs were incubated at a constant 23°C (female-producing temperature) or 28°C (1 : 1 sex ratio temperature). The dark line represents a third treatment where eggs were shifted from 23°C to 28°C on day 20 after oviposition. (b) Sex ratios produced from each treatment ( $\chi^2=7.5$ ,  $P=0.006$ ). As expected, predominately females were produced under a constant 23°C, but the shift treatment yielded 1 : 1 sex ratios similar to that of the constant 28°C treatment, suggesting that the TSP begins at some point after the first 20 days of incubation (from an unpublished dataset obtained by D. Warner, R. Radder, and R. Shine). (c) Results from aromatase-inhibitor applications given to eggs at day 0 and day 20 after oviposition (graph derived from data in Shine, R., Warner, D. A., & Radder, R., 2007). By manipulating the hormonal environment of embryos, sex determination is affected at very early stages of development (before or at day 20 of incubation). Hence, temperature-shift and chemical-application experiments provide different conclusions about the onset of the TSP.



**FIGURE 1.7** Thermosensitive periods (TSP) of sex determination in reptiles (modified from Shine, R., Warner, D. A., & Radder, R., 2007). In tuatara, turtles, and crocodilians, oviposition occurs at very early stages of embryonic development (Andrews, 2004), but in lizards oviposition occurs later in development at a stage close to the onset of the TSP. The TSPs for the top four graphs were estimated using temperature-shift experiments. However, the bottom graph for the dragon and skink was derived from studies using a chemical-application experiment (Shine et al., 2007). Interestingly, temperature-shift experiments suggest that the TSP may begin at some point after the dashed line (see Figure 1.6) rather than immediately after oviposition for the dragon, *Amphibolurus muricatus*.

during this early developmental phase are important in shaping offspring phenotypes (e.g., size, locomotor performance), we might expect selection to move the TSP to coincide with this important phase of incubation (Bull, 1987). Indeed, an overlap of the TSP with the phase of

incubation that affects fitness-relevant phenotypes would be critical in how incubation temperature shapes sex-specific phenotypes, a condition that would support models for the adaptive significance of TSD (Shine, 1999) (more details in Section 6).

## 6. WHY DO REPTILES EXHIBIT A DIVERSITY OF SEX-DETERMINING MECHANISMS (SDMS)? ECOLOGY AND EVOLUTION

Theoretical and empirical studies have provided an important foundation for understanding the selective pressures operating on SDMs (Charnov & Bull, 1977; Bulmer & Bull, 1982; Bull & Charnov, 1989; Warner & Shine, 2008a). These studies have revealed that the evolution of SDMs can be highly dynamic, can be facilitated or constrained by pre-existing features of an organism's biology, and that selection can operate at multiple levels (Uller, Pen, Wapstra, Beukeboom, & Komdeur, 2007). To accurately understand the evolution of reptilian SDMs, it is important to first establish that TSD is not just a laboratory phenomenon. This section will begin by evaluating the evidence that TSD exists in nature and subsequently discuss models that explain when selection would favor TSD over GSD (and vice versa) and argue that different SDMs evolved in different reptilian lineages under different selective pressures. Lastly, the micro-evolutionary potential of TSD in response to changing environments will be examined.

### 6.1. Ecological Relevance: Does Temperature-dependent Sex Determination (TSD) Occur in Nature?

Temperature-dependent sex determination is usually, if not always, determined via laboratory experimentation that fails to mimic the complexities of natural thermal regimens. Additionally, sex-determining reaction norms are rarely evaluated from natural nests. This raises two important questions concerning the presence of TSD in nature. First, do the complex thermal regimens of natural nests have predictable impacts on sex ratios in TSD species? Secondly, are all locations along the laboratory-generated reaction norm ecologically relevant?

In nature, eggs that experience mean daily temperatures above the laboratory-established pivotal temperature are expected to yield one sex, whereas eggs exposed to mean daily temperatures below the pivotal temperatures should yield the other sex. Although many field studies are in broad agreement with these expectations (Bull & Vogt, 1979; Janzen, 1994a), sex ratios are not always related to the mean temperature within nests (Warner & Shine, 2009). For example, a study on painted turtles (*C. picta*) in Canada shows that sex ratios are not related to mean temperatures in natural nests (Schwarzkopf & Brooks, 1985). In the European pond turtle (*Emys orbicularis*), mean daily temperatures above the pivotal temperature produce the opposite sex from that predicted from laboratory studies (Pieau, 1982). Research on map turtles (genus *Graptemys*)

demonstrates that both the mean and variance of nest temperatures are important in predicting sex ratios (Bull, 1985). In the jacky dragon (*A. muricatus*), the average of the daily temperature range throughout incubation is the best predictor of offspring sex ratios (Warner, 2007). These examples demonstrate that the mean nest temperature is often inadequate in fully explaining variation in sex ratios produced under natural conditions. What, then, are the appropriate thermal parameters that we need to measure to accurately predict offspring sex ratios?

To accurately predict offspring sex ratios from natural thermal regimens, several characteristics of the nests' thermal patterns and the embryos' developmental patterns must be considered (Shine & Harlow, 1996; Georges, Doody, Beggs, & Young, 2004). First, because warm temperatures accelerate embryonic development (Andrews, 2004), more development will occur when daily temperatures fluctuate above, rather than below, the pivotal temperature (Shine & Harlow, 1996). Consequently, sexual differentiation will depend on the relative proportion of development taking place above or below the pivotal temperature. Temperatures below the 'developmental zero' will have no impact on sexual differentiation because development is arrested when thermal fluctuations drop this low. Secondly, only temperatures during the TSP should be considered, as temperatures before or after this critical window have no impact on sex determination (Mrosovsky & Pieau, 1991). Because daily fluctuations and seasonal trends in nest temperatures prevent a constant rate of embryonic development, identifying the onset and end of the TSP in nature is not straightforward.

To address these issues, models have been developed for evaluating the influence of natural thermal regimens of nests on offspring sex ratios (Georges, 1989; Georges et al., 2004; Georges, Beggs, Young, & Doody, 2005). These models propose that, if similar amounts of development (during the critical sex-determining stages) occur above the pivotal temperature as below it, then both sexes will be produced. However, if more than half of development is spent above the pivotal temperature during each day of incubation, then males (for FM pattern) or females (for MF pattern) will be produced. That is, the key predictor of sex ratios from natural nests is not the mean temperature or its variance, but instead the temperature above and below which half of development occurs, calculated on a daily basis. This predictor statistic is referred to as the constant temperature equivalent (CTE), and produces temperature values that are equivalent to a constant incubation temperature in an incubator. This CTE approach has received strong support in several studies of turtles and tuatara because the CTE calculated from natural nest temperatures accurately predicts the sex ratios expected under constant temperature incubation in the laboratory



(Georges, 1989; Georges, Limpus, & Stoutjeskijk, 1994; Demuth, 2001; Mitchell et al., 2006). However, a disadvantage of this approach is that it only applies to species that have a single pivotal temperature (i.e., those with either the MF or FM pattern of TSD).

Predicting sex ratios from natural nests in species with the FMF pattern of TSD has been problematic for two reasons. First, the CTE models cannot be applied to species with this pattern because two pivotal temperatures exist (Georges et al., 2004). Although warm and cool temperatures produce females, embryonic sensitivity of the sex-determining response to these two extremes will differ dramatically (i.e., more development occurs at temperatures above the upper pivotal temperature than at temperatures below the lower pivotal temperature). Moreover, the proximate mechanisms involved in TSD may differ between embryos experiencing high vs. low incubation temperatures, even though these extremes produce similar sex ratios. Indeed, the amount of developmental time spent at female-producing temperatures (high and low temperatures combined) does not predict offspring sex ratio in *A. muricatus*, a lizard with the FMF pattern of TSD (Warner & Shine, 2009). A second problem involves the functional significance of one of the pivotal temperatures in the FMF pattern. Field data from nests of the snapping turtle (*Chelydra serpentina*) and Amazonian river turtle (*Podocnemis expansa*) (both with FMF pattern) illustrate that temperatures towards the lower pivotal temperature are rarely experienced in nature, suggesting that only the MF pattern is realized in natural nests (Valenzuela, 2001; Janzen, 2008). Similarly, mean nest temperatures beyond the upper pivotal temperatures may not occur in the lizard *A. muricatus* (compare Harlow and Taylor (2000) with Warner and Shine (2008b)), yet daily extremes still fluctuate well beyond the upper pivotal temperature. These issues are not accounted for in current CTE models, and thus the development of sophisticated models is needed to fully understand the existence of the FMF pattern in nature.

Overall, the existence of TSD in nature is well supported for turtles (Bull, 1985), crocodylians (Ferguson & Joanen, 1982), and tuatara (Mitchell et al., 2006). For lizards, however, few field data exist, but there is no evidence against its existence in this group (see Doody et al., 2006; Warner and Shine, 2008b; Wapstra et al., 2009). In reptiles generally, field data clearly demonstrate that warm and cool nests produce substantially different sex ratios (Bull & Vogt, 1979; Janzen 1994a; 1994b) and that daily and seasonal thermal fluctuations contribute to this variation (Bull, 1985; Georges et al., 2004). Moreover, laboratory experiments that mimic the thermal fluctuations of natural nests also illustrate the importance of temperature variance, not only on offspring sex (Valenzuela, 2001; Les, Paitz, & Bowden, 2007) but also on a variety of

fitness-relevant phenotypes (Du & Ji, 2006; Mullins & Janzen, 2006).

## 6.2. Adaptive Significance of Sex-determining Mechanisms (SDMs)

Theoretical models of sex ratio evolution predict that reproductive females invest equally into each sex when the cost of producing a son equals that of a daughter (Fisher, 1930). Indeed, balanced primary sex ratios are considered evolutionarily stable because frequency-dependent selection will favor the rarer sex if population sex ratios are perturbed from unity. Consequently, selection should favor SDMs that ensure equal numbers of sons and daughters. In many reptiles, and most other animals, GSD provides a convenient mechanism by which 1:1 sex ratios are produced due to random segregation of chromosomes during meiosis. Hence, selection will favor GSD when the fitness returns of producing a son are equal to that of a daughter.

In many situations, however, one sex may provide greater fitness returns than the other, thereby shifting the selective pressures that operate on SDMs. In such cases, maternal control over the sex ratio will enhance parental fitness by enabling overproduction of the sex that benefits most from prevailing conditions (Trivers & Willard, 1973; Cockburn, Legge, & Double, 2002). For example, many female adders (*Vipera berus*) in a Swedish population produce only one litter in their lifetimes, and that litter consists of equal numbers of sons and daughters. Because of intense competition among males, mothers that survive to produce a second litter reduce competition between their already-produced sons and their current offspring by overproducing daughters in their second litter (Madsen & Shine, 1992). Although sex-biased investment has been demonstrated in numerous reptiles that exhibit GSD (Madsen & Shine, 1992; Lovern & Wade, 2003; Calsbeek & Sinervo, 2004; Calsbeek & Bonneaud, 2008), the precise mechanisms that enable these shifts are not well understood. Theoretical and empirical research suggests that mechanisms for sex-ratio adjustment likely occur at multiple levels (Uller et al., 2007) and through multiple pathways (Pike & Petrie, 2003; Uller & Badyaev, 2009). Unfortunately, reptiles have received relatively little attention as models for empirical studies of sex allocation theory (Wapstra et al., 2007; Wapstra & Warner 2010). Undoubtedly, more research in this area will provide new insights into the selective forces responsible for diversity of SDMs in reptiles.

Sex allocation theory (recently reviewed in West, 2009) provides a useful framework for explaining the adaptive significance of ESD, particularly for TSD in reptiles. As described above, models of sex allocation theory propose that if some conditions are more

conducive towards one sex than the other, maternal fitness would be enhanced if the sex best-suited for the given conditions was over-produced (Trivers & Willard, 1973; Charnov, 1982). These ideas have been extended by Charnov and Bull (1977) to explain when selection will favor ESD over GSD. In terms of TSD, their model proposes that, if male and female offspring have different optimal developmental temperatures, selection will favor a SDM that enables each sex to be produced at their respective optimal developmental temperature. Indeed, experimental studies on a variety of reptilian species demonstrate that egg incubation temperatures affect offspring phenotypes in ways that could influence their fitness (e.g., Andrews, Mathies, & Warner, 2000; Van Damme, Bauwens, Braña, & Verheyen, 1992), and some of these effects are long-lasting (Freedberg, Stumpf, Ewert, & Nelson, 2004; Elphick & Shine, 1998). If those traits differentially impact the fitness of sons vs. daughters (e.g., Gutzke & Crews, 1988; Warner & Shine, 2005), selection will favor an SDM that is sensitive to temperature so that each sex develops at its optimal temperature. Thus, under these situations, TSD would be favored and maintained by selection because it would confer higher parental fitness than GSD (Bull, 1983).

The matching of offspring sex with developmental temperature via selection can occur through multiple pathways. Hence, the Charnov–Bull model comprises several differential-fitness hypotheses that link incubation temperature with sex-specific fitness to explain the adaptive significance of TSD. Several of these hypotheses are outlined in Table 1.3, and since previous reviews have covered these at length (Shine, 1999; Valenzuela, 2004a; Janzen & Phillips, 2006), they will not be detailed here. Despite numerous hypotheses for the adaptive value of TSD, however, satisfactory empirical support has been elusive and TSD has remained an enigma in reptiles (Bull, 1983). Most attempts to empirically test the Charnov–Bull model have been impeded by the following factors: (1) Most reptiles with TSD are poorly suited for lifetime fitness studies. The vast majority of research on this topic has been conducted on turtles and crocodylians (Joanen, McNease, & Ferguson, 1987; Janzen, 1995), and measuring reproductive success (fitness) of these late-maturing, long-lived species is logistically difficult. (2) Offspring sex is often difficult to identify without sacrificing animals, which would preclude any measurement of fitness. (3) The effects of incubation temperature and offspring sex are naturally confounded. In order to evaluate the sex-specific effect of incubation temperature on offspring fitness, both sexes need to be produced across a broad range of incubation temperatures—an obvious problem with most TSD species. (4) As discussed above, incubation temperature may differentially affect fitness in males vs. females via multiple

complex pathways (shown in Table 1.3). Thus, any attempt to test the Charnov–Bull model must examine a wide range of variables, thereby posing a substantial challenge to comprehensive empirical analysis.

Recent work on an Australian lizard (*A. muricatus*) with TSD has overcome these obstacles to provide the first substantial support for the Charnov–Bull model in reptiles (Warner & Shine, 2005; 2008a). In this study, the authors incubated eggs across a range of temperatures that naturally occur in the field and applied an aromatase inhibitor to a subset of eggs in each incubation treatment. This manipulation blocked the conversion of T to E<sub>2</sub> to produce male offspring at female-producing temperatures, thereby decoupling the confounded effects of sex and incubation temperature. After eggs hatched, the offspring were harmlessly sexed by manual eversion of hemipenes on males and then raised under semi-natural conditions among six replicated field enclosures for four years. Because *A. muricatus* matures within one year of hatching and has a short life span (likely three to four years) relative to other TSD reptiles, the authors were able to measure near lifetime reproductive success of the offspring that were produced under the controlled incubation conditions. With the use of microsatellite DNA markers, parentage of all second generation offspring was assigned over three reproductive seasons, enabling a direct measure of reproductive fitness (i.e., lifetime number of offspring produced). Remarkably, the results provide strong support for the theoretical predictions of the Charnov–Bull model. Males that hatched from naturally male-producing temperatures sired more offspring than did sex-reversed males from female-producing temperatures. The reverse was true for females; temperatures that exclusively produce daughters were optimal for females (Warner & Shine, 2008a).

Although this pattern supports the Charnov–Bull predictions, the mechanism(s) by which incubation temperature differentially affects the fitness of sons and daughters remain unclear. Hence, subsequent studies that address the alternative hypotheses outlined in Table 1.3 are needed. Although all hypotheses have not been tested, the current data on *A. muricatus* provide support for hypothesis 4, which proposes that TSD enables each sex to hatch at its own optimal time of the season. Indeed, developmental temperature strongly affects the timing of hatching, which in turn has long-lasting impacts on fitness (Warner & Shine, 2005; 2007). Because early hatching likely benefits one sex more than the other, perhaps TSD evolved to create an adaptive match between the timing of hatching and the appropriate sex (Warner et al., 2009b); this pattern is similar to the scenario seen in the Atlantic silverside fish whereby earlier hatching benefits females more than males (Conover, 1984; Warner et al., 2009b). Nevertheless, in *A. muricatus*, the interactive effect of sex and incubation temperature on offspring fitness remains

**TABLE 1.3** Summary of seven differential-fitness hypotheses that fall within the Charmov–Bull (1977) framework. All hypotheses propose adaptive values or mechanisms for the maintenance of temperature-dependent sex determination (TSD)

Hypothesis	Role of incubation temperature	Prediction if adaptive	References
1. Different optimal egg size for sons vs. daughters	Enables mother to adjust clutch sex ratios via nest-site selection	Sons and daughters produced from different sized eggs, and sex-specific relationship between egg size and fitness	14, but see 11
2. Different phenotypic optima for sons vs. daughters	Induces changes to phenotype independent of sex	Incubation temperature affects phenotypes, and the phenotypic determinants of fitness are sex-specific	6, 10, 13, but see 20
3. Different norms of reaction for sons vs. daughters	Influences phenotypes and/or fitness of hatchlings, but differently in sons and daughters	Significant interaction between incubation temperature and sex on fitness-related phenotypes	4, 15, 16, 19
4. Different optimal hatching times for sons vs. daughters	Induces variation in time of hatching, and seasonal variation in sex ratio	Seasonal variation in offspring sex ratio production, and sex-specific relationship between time of hatching and fitness	2, 7, 18
5. Natal homing and nest-site philopatry	Induces variation in thermal quality of nesting sites	Females return to natal nest sites for oviposition; fitness of daughters is enhanced when hatching at natal site but male fitness is unaffected	12, but see 17
6. Sexual size dimorphism	Induces variation in growth rate	Sex with larger adult body size is produced at temperatures that induce rapid growth, thereby generating sexual size dimorphism	5, 8, but see 9
7. Differential mortality	Induces embryonic mortality, but differently in male vs. female embryos	Female and male embryos have different sensitivities to incubation temperature	1, 3

<sup>1</sup> Burger and Zappalorti (1988); <sup>2</sup> Conover (1984); <sup>3</sup> Eiby, Wilmer, and Booth (2008); <sup>4</sup> Elphick and Shine (1999); <sup>5</sup> Ewert and Nelson (1991); <sup>6</sup> Gutzke and Crews (1988); <sup>7</sup> Harlow and Taylor (2000); <sup>8</sup> Head, May, and Pendleton (1987); <sup>9</sup> Janzen and Paukstis (1991b); <sup>10</sup> Langkilde and Shine (2005); <sup>11</sup> Morjan and Janzen (2003); <sup>12</sup> Reinhold (1998); <sup>13</sup> Rhen and Lang (1995); <sup>14</sup> Roosenburg (1996); <sup>15</sup> Shine, Elphick, and Harlow (1995); <sup>16</sup> Shine, Elphick, and Harlow (1997); <sup>17</sup> Valenzuela and Janzen (2001); <sup>18</sup> Warner and Shine (2005); <sup>19</sup> Warner and Shine (2008a); <sup>20</sup> Warner, Woo, Van Dyk, Evans, and Shine (2010).

significant whether or not analyses are corrected for the timing of hatching (Warner & Shine, 2008a), which suggests that another mechanism could be involved. For example, perhaps continuous selection for males at certain temperatures and females at others results in developmental processes being optimized at different temperatures for each sex regardless of the timing of hatching. Additional work that evaluates the level at which temperature optimizes development is needed to address this hypothesis.

A single adaptive explanation for TSD in reptiles is unlikely to exist (Ewert & Nelson, 1991; Valenzuela, 2004a; Warner et al., 2009b). Given the diversity in life histories, mating systems, demographics, and developmental patterns of TSD reptiles, it is more likely that TSD and GSD evolved in different taxa for different reasons. For example, thermally sensitive SDMs in viviparous taxa may have evolved to enable mothers to facultatively adjust offspring sex ratios (via active thermoregulation) in adaptive directions (Robert et al., 2003; Wapstra et al., 2004). Alternatively, TSD may have evolved to create an adaptive match between the timing of hatching and sex in short-lived species, as described above (Conover, 1984; Harlow & Taylor, 2000; Warner et al., 2009b); such a match, however, is less likely to be important in longer-lived reptiles with TSD. In long-lived species, long-term effects of developmental temperature on adult reproduction are likely to be swamped out over time, suggesting that TSD may have evolved via different mechanisms in these taxa (Ewert & Nelson, 1991). In fact, models suggest that TSD may be maintained in long-lived species despite negative impacts of extreme sex-ratio fluctuations, which is a demographic feature associated with this SDM (Bull & Bulmer, 1989; Freedberg & Taylor, 2007). Other explanations concerning the adaptive value and maintenance of TSD relate to inbreeding avoidance, group selection (Ewert & Nelson, 1991; but see Burke, 1993), and cultural inheritance of nest sites (Freedberg & Wade, 2001); neutral hypotheses also have been proposed for different taxa (see Valenzuela, 2004a).

### 6.3. Evolutionary Potential of Temperature-dependent Sex Determination (TSD)

After TSD is established in populations, how will natural selection maintain viable offspring sex ratios in a changing environment? That is, what is the microevolutionary potential of TSD, and what factors might constrain sex-ratio evolution? Theoretical models predict that microevolution of the sex ratio can occur via selection on either (1) the thermal sensitivity of embryonic sex determination or (2) maternal choice of the nest site (Bulmer & Bull, 1982; Morjan, 2003a). Importantly, evolution of these two

key factors depends upon the amount of heritable genetic variation that is present. Although empirical studies have indeed shown that both of these factors have genetic components, genetic and non-genetic maternal factors may alter their evolutionary potential.

Variation in pivotal temperatures is often considered an index for variation in the thermal sensitivity of embryonic sex determination. Empirical studies demonstrate that most reptilian species with TSD exhibit remarkable variation in pivotal temperatures at the among-population level, and in threshold temperatures among clutches within populations. For example, pivotal temperatures shift geographically in some North American turtle species, implying that the sex-determining response of embryos may be adapted to local climates (Ewert et al., 1994; 2005) (Figure 1.1). However, the direction of this trend is opposite from that expected (i.e., pivotal temperatures increase with latitude (Bull et al., 1982b; Ewert et al., 2004)). Additional studies illustrate impressive variation in threshold temperatures among clutches within populations, implying a strong genetic component (Rhen and Lang, 1998; Janes and Wayne, 2006; Warner et al., 2008). Indeed, constant-incubation experiments suggest that the heritability of sex ratios produced at pivotal temperatures can be quite high, but heritability may be substantially muted under field conditions because of environmental noise imposed by natural thermal regimens among nests (Bull et al., 1982a; Janzen, 1992). Non-genetic maternal effects, such as yolk hormones or maternal nutrition (Bowden et al., 2000; Warner et al., 2007), also may place constraints on the evolutionary potential of the pivotal temperature.

Maternal nest-site choice also plays an important role in the microevolutionary potential of TSD (Bulmer & Bull, 1982). The availability of suitable nest sites varies substantially both within and among populations, and selection has apparently shaped maternal nesting behaviors so that females choose nest sites with favorable thermal conditions for their developing embryos (Janzen & Morjan, 2001; Warner & Andrews, 2002; Doody et al., 2006). Although laboratory experiments on eublepharine geckos with TSD demonstrate relatively little variation in nest-site selection (Bull, Gutzke, & Bulmer, 1988c; Bragg, Fawcett, Bragg, & Viets, 2000), field studies on other TSD reptiles show substantial within-population variation in thermal characteristics of nests chosen by females (Janzen & Morjan, 2001; St. Juliana et al., 2004; Kamel & Mrosovsky, 2005; Warner & Shine, 2008b). Moreover, field studies of the painted turtle (*C. picta*) illustrate strong individual repeatability for choice of specific microhabitat characteristics, such as the amount of vegetative cover over nest sites (Janzen & Morjan, 2001). Indeed, vegetation cover, which influences the nest thermal environment, is a strong predictor of clutch sex ratios in *C. picta* (Janzen, 1994a).

In fact, vegetative cover has consistently been shown to be an important cue driving nest-site selection in many TSD reptiles (Morjan, 2003b; Kolbe & Janzen, 2002; St. Juliana et al., 2004; Warner & Shine, 2008b), but heritability of maternal choice of nest microhabitat has not yet been demonstrated. Perhaps the most convincing evidence for the evolutionary potential of TSD involves the nesting behavior of the water dragon, *Physignathus lesueurii* (Doody et al., 2006). This species has a broad latitudinal range spanning 19° along the eastern coast of Australia, and hence occupies habitats that vary considerably in microclimate. Extensive work by Doody et al. (2006) clearly demonstrates that these lizards compensate for climatic differences by selecting low-vegetated nest sites (which receive more solar radiation) in cool locations of its geographic range, and vice versa in warm locations.

Adjustments in nesting phenology may also be important in the microevolution of TSD. Evidence suggests that the timing of the nesting season may shift in response to spatial or temporal variation in climate (e.g., Ewert et al., 2005; Doody et al., 2006; Schwanz & Janzen, 2008; Tucker, Dolan, Lamer, & Dustman, 2008). Importantly, whether these responses have a heritable genetic basis or are due to behavioral plasticity, shifts in nesting phenology may maintain viable offspring sex ratios in response to changing climates. However, recent analyses suggest that shifts in nesting phenology may not be enough to compensate for the predicted changes in climate (Schwanz & Janzen, 2008; Telemeco, Elphick, & Shine, 2009). Nevertheless, heritable variation in all three of these factors (changes in pivotal temperatures, nesting behavior, and nesting phenology) warrant further investigation, as such research will provide important insights into how TSD reptiles may cope with changes in habitat and global climate (Janzen 1994b; Telemeco et al. 2009).

#### 6.4. Comments on the Coexistence of Temperature-dependent Sex Determination (TSD) and Sex Chromosomes

Theoretical models predict that a stable sex ratio will evolve and be maintained regardless of whether sex is determined strictly by genetic mechanisms or by temperature (Bull, 1981; 2008). These models, as well as empirical evidence, suggest that elements of TSD and GSD can coexist within single populations (Lagomarsino & Conover, 1993; Baroiller et al., 1995). However, genetic models suggest that the coexistence of TSD and GSD is selected against only when GSD systems contain differentiated sex chromosomes (Bull, 1980; 1983). For example, consider a system with male heterogamety (XX/XY) and TSD. In

this system, phenotypic sex of some individuals will not match their chromosomal constitution. That is, developmental temperatures will override genotypic sex determination to produce some XX males and some XY females, whereas others in the population will maintain their usual condition (i.e., XY males and XX females). Consequently, XY females will mate with XY males, and a fourth of their progeny will inherit the YY genotype; this genotype is predicted to be non-viable due to the accumulation of nonfunctional genes on the Y chromosome. Frequency-dependent selection will then favor XX females as they will produce more progeny than their XY-female counterparts, thereby selecting against TSD in this system. This argument posits that TSD and GSD represent two distinct peaks in an adaptive landscape and that intermediate states would be selected against. See Bull (1980; 1983; 2008) for more discussion on this topic.

Despite this argument against the coexistence of TSD and sex chromosomes, the multiple evolutionary transitions between SDMs in reptiles suggest that some degree of coexistence must occur even if during a brief transient period. Intriguingly, recent discoveries of TSD and sex chromosomes in populations of two Australian lizards have challenged the incompatibility of these systems (Shine et al., 2002; Quinn et al., 2007). In these species, the production of YY (or WW) individuals does not occur because the environmental effect on sex determination occurs only at one temperature extreme. For example, in the male-heterogametic skink *Bassiana duperreyi*, cool temperatures override GSD so that predominately males are produced, but relatively warm nests produce balanced sex ratios, as expected under GSD. Because this thermal effect on sex determination occurs only under cool temperatures, this creates a mismatch between phenotypic and genotypic sex for some males, but not for females. That is, XX and XY males are produced, but XY females never occur, implying that YY progeny will not be produced in this system. Intriguingly, the thermal effect on sex determination is reversed (i.e., females are produced at high temperatures) in the female-heterogametic agamid *Pogona vitticeps* (Quinn et al., 2007), hence no WW progeny will be produced in this species. Similar co-occurrences of sex chromosomes and thermal effects on sex determination have been observed in other reptiles, fishes, amphibians, and invertebrates (e.g., Dournon, et al., 1990; Kozielska et al., 2006; Luckenback et al., 2009). Together, these systems provide evidence that TSD and GSD may be more compatible than previously considered and that these systems represent some intermediate states in the TSD to GSD continuum (Figure 1.3). Whether this coexistence is accidental, transient, or adaptive raises additional questions that warrant further empirical and theoretical investigation (see Bull (2008) for discussion).

## 7. WHERE TO GO NEXT: FUTURE RESEARCH AND CONCLUSIONS

Decades of theoretical and empirical research have provided many critical insights into the diversity of SDMs in reptiles, their phylogenetic distribution, proximate mechanisms, and evolutionary significance. This review has touched on many aspects of these issues and has attempted to provide a general consensus of where the field stands, the emerging directions, and what gaps need to be filled. Here, some of the emergent directions of the field will be reiterated and major gaps of inquiry where future research should be directed will be pointed out.

A major component of this review explores two different perspectives on reptilian SDMs: the traditional perspective divides reptilian SDMs into discrete categories, whereas the emerging alternative perspective treats TSD and GSD as endpoints of a continuum (Valenzuela et al., 2003; Sarre et al., 2004). Commonalities in proximate mechanisms and direct evidence of the co-existence of both SDMs within populations suggest greater complexity in sex-determining systems than is embodied in a dichotomous perspective. These two perspectives have fundamentally different consequences for our understanding of reptilian SDMs in terms of proximate mechanisms, ultimate explanations, and evolutionary transitions. Under a dichotomous view, SDMs are divided based on the presence or absence of consistent heritable genetic differences between the sexes, and hence are forced into one or the other category. This view may bias our perception of underlying mechanisms and limit our understanding of evolutionary transitions. On the other hand, a continuous view makes less of a distinction among SDMs, and therefore provides a logical understanding of why commonalities exist in TSD and GSD systems, which may give insights into why evolutionary transitions between SDMs have occurred so frequently in reptiles. More empirical and theoretical research is urgently needed to further understand the frequency and stability of co-existing SDMs in natural populations.

A second theme of this review involves our ignorance of the phylogenetic distribution of SDMs. At present, SDMs have been identified in less than 6% of reptile species, and this clearly limits the scope for evaluating the evolutionary history of SDMs. In addition, new technologies will help in identifying cryptic sex chromosomes that have escaped detection in early studies. Both karyological and experimental studies are needed to identify the relative influences of genotype and environment (and their interaction) on sex determination in the vast majority of reptilian species. Coupled with better-resolved phylogenies, these studies will undoubtedly change our current understanding of the phylogenetic distribution of SDMs

and provide insights into the origins of and transitions among SDMs.

More exploration into the mechanistic underpinnings of TSD and GSD is needed. We have only a crude understanding of the molecular regulatory networks and hormonal pathways involved in sex determination and sexual differentiation. Comparative work that simultaneously evaluates the gene networks in closely related TSD and GSD taxa will provide critical insights into the evolution or loss of master thermal switches involved in reptilian sex determination. Additionally, research on the thermal sensitivities of gene expression during gonadogenesis under fluctuating temperature regimens will provide a better understanding of how these systems work in nature. The role of maternally derived steroids in egg yolks in modulating sexual differentiation has only recently been given research attention. Hence, much work is needed to fully understand the factors that influence maternal allocation of steroids into yolk, as well as their interaction with incubation temperature and sex-determining genes.

Additional research on TSD under natural conditions is urgently needed. At present, almost all studies describe sex-determining reaction norms based on constant-temperature incubation in the laboratory. Experiments that mimic natural thermal regimens will be critical not only in identifying how these complex conditions affect sex ratios but also in our understanding of how natural thermal conditions affect sex-specific traits that are relevant to fitness. More work is needed to evaluate the long-term effects of natural incubation regimes on fitness (i.e., reproductive success) in nature. Certainly, short-lived reptiles with TSD (e.g., agamid lizards) will facilitate such research, but work is also needed on longer-lived species (e.g., turtles and crocodylians) to evaluate generalities or lack thereof. These types of experiment will provide critical insights into how Charnov–Bull (1977) predictions explain the adaptive significance of TSD in nature. Further, sophisticated CTE models, similar to those developed by Georges et al. (2004; 2005), are needed to successfully predict offspring sex ratios from natural nest temperatures in species with the FMF pattern of TSD. Lastly, in the face of human-induced habitat modifications and global climate change, more research is needed to understand the microevolutionary potential of both GSD and TSD. Experiments designed to evaluate variation in and heritabilities of embryonic sex-determining reaction norms and maternal nest-site selection will provide critical insights into how TSD reptiles will deal with rapid environmental changes.

The field of reptilian sex determination has a very dynamic history and its future will undoubtedly continue in this way as more information is gathered (Bull, 2004). To fully understand the functional and evolutionary aspects of SDMs, future research needs to take a comprehensive

integrative approach that utilizes skills from investigators in multiple disciplines. Given the diversity of SDMs within reptiles and their feasibility as model organisms, this group will continue to serve as a model for our general understanding of the proximate mechanisms and evolution of sex-determining mechanisms.

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## ABBREVIATIONS

<b>AMH</b>	Anti-Müllerian hormone
<b>CTE</b>	Constant temperature equivalent
<b>Dax1</b>	Dosage-sensitive sex reversal gene
<b>DHT</b>	Dihydrotestosterone
<b>Dmrt1</b>	Doublesex and Mab-3 related transcription factor
<b>E<sub>2</sub></b>	17 $\beta$ -estradiol
<b>ESD</b>	Environmental sex determination
<b>FM pattern</b>	Females produced at low temperatures, males at high temperatures
<b>FMF pattern</b>	Females produced at extreme temperatures, males at intermediate temperatures
<b>GSD</b>	Genotypic sex determination
<b>MF pattern</b>	Males produced at low temperatures, females at high temperatures
<b>MIS</b>	Müllerian-inhibiting substance
<b>SDM</b>	Sex-determining mechanism
<b>Sfl</b>	Steroidogenic factor 1
<b>Sox9</b>	Sry-like HMG box
<b>Sry</b>	Sex-determining region on the Y chromosome
<b>T</b>	Testosterone
<b>TRT</b>	Transitional range of temperatures
<b>TSD</b>	Temperature-dependent sex determination
<b>TSP</b>	Thermosensitive period
<b>Wt1</b>	Wilm's tumour suppressor gene

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