



# HORMONES AND REPRODUCTION OF VERTEBRATES

## Reptiles

Second Edition

Volume 3

Editors

David O. Norris and Kristin H. Lopez





# Hormones and Reproduction of Vertebrates, Volume 3

## Reptiles

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Hormones and Reproduction of Vertebrates, Volume 1—Fishes  
Hormones and Reproduction of Vertebrates, Volume 2—Amphibians  
Hormones and Reproduction of Vertebrates, Volume 3—Reptiles  
Hormones and Reproduction of Vertebrates, Volume 4—Birds  
Hormones and Reproduction of Vertebrates, Volume 5—Mammals

# Hormones and Reproduction of Vertebrates, Volume 3

Reptiles

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Edited by

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# Dedication

## Professor Louis J. Guillette, Jr. (1954–2015)

This second edition of these five volumes on *Hormones and Reproduction of Vertebrates* is dedicated to the memory of Professor Louis J. Guillette, Jr. (1954–2015) for bringing national and international attention to the effects of chemical pollution acting through the endocrine system and altering development and reproduction of vertebrate animals. Lou received his doctorate degree at the University of Colorado at Boulder and was a professor at Wichita State University (1981–85) and the University of Florida (1985–2010). In 2010, he became a Professor of Obstetrics and Gynecology and of Public Health Sciences at the Medical University of South Carolina (MUSC) in Charleston. He also held an Endowed Chair of Marine Genomics at MUSC and the Hollings Marine Laboratory in Charleston. Lou alerted the scientific community and general public to the deleterious effects of pesticides and other chemicals on development and reproduction through his teaching, lecturing, training of graduate and postdoctoral students, and many research collaborations, especially those dealing with alligators. He and his alligator research even became the subject of a murder mystery *What Becomes* by Roger Newman. In 2011, Lou received the Heinz Foundation Environment Award for his contributions to the development and reproduction of vertebrates, which are echoed throughout these volumes. Lou never met a stranger, and we remember him as a friend, a prolific scientist, a visionary, and a champion of the environment.

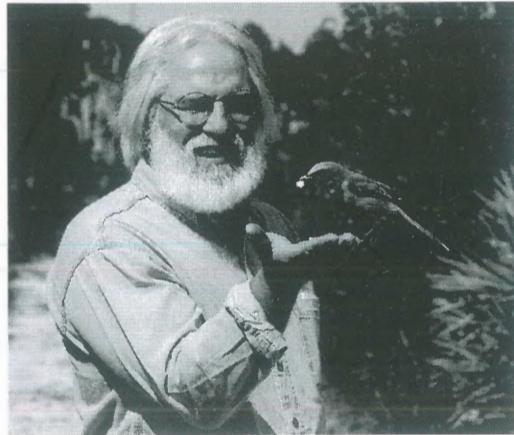


Photo courtesy of Elizabeth A. Guillette



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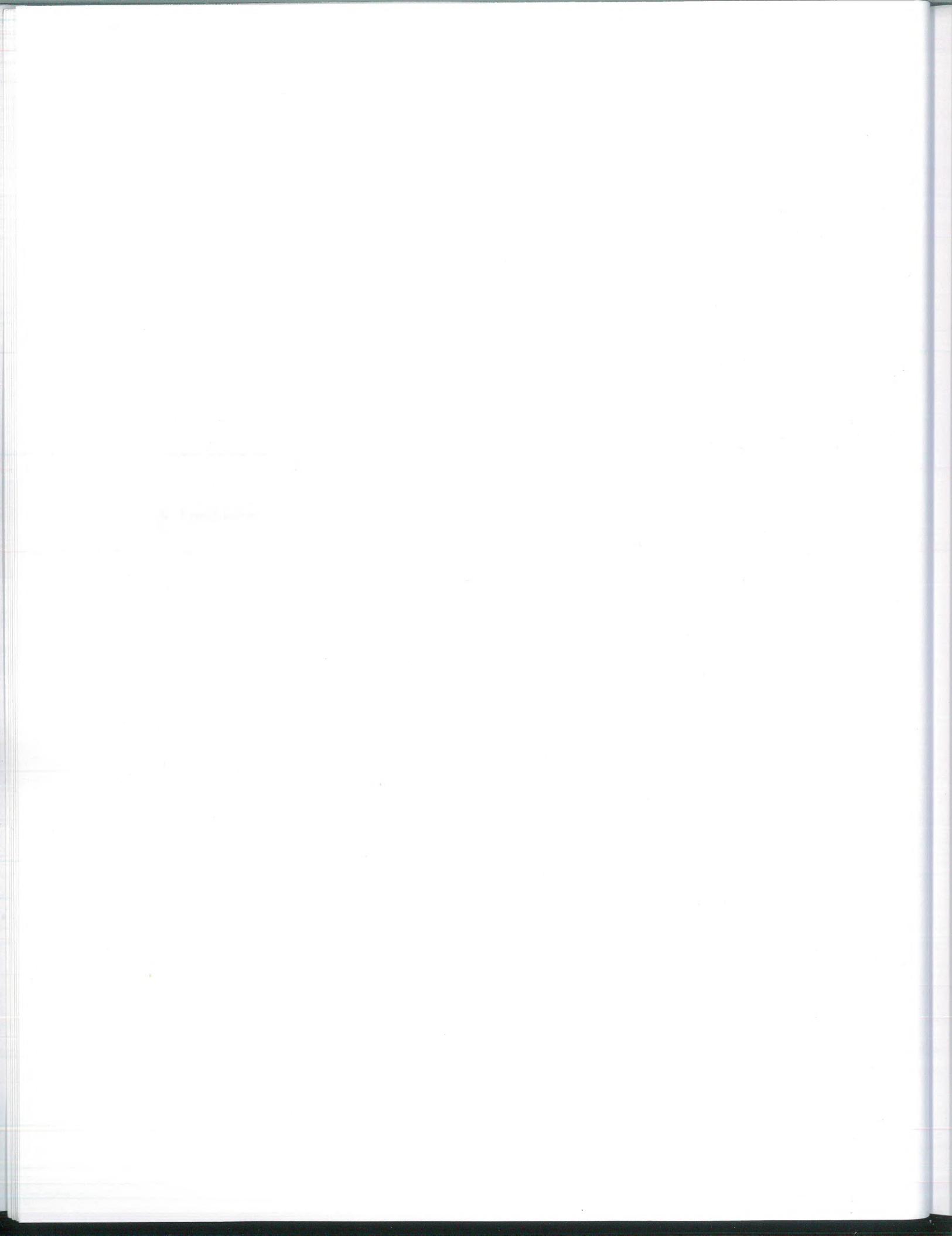
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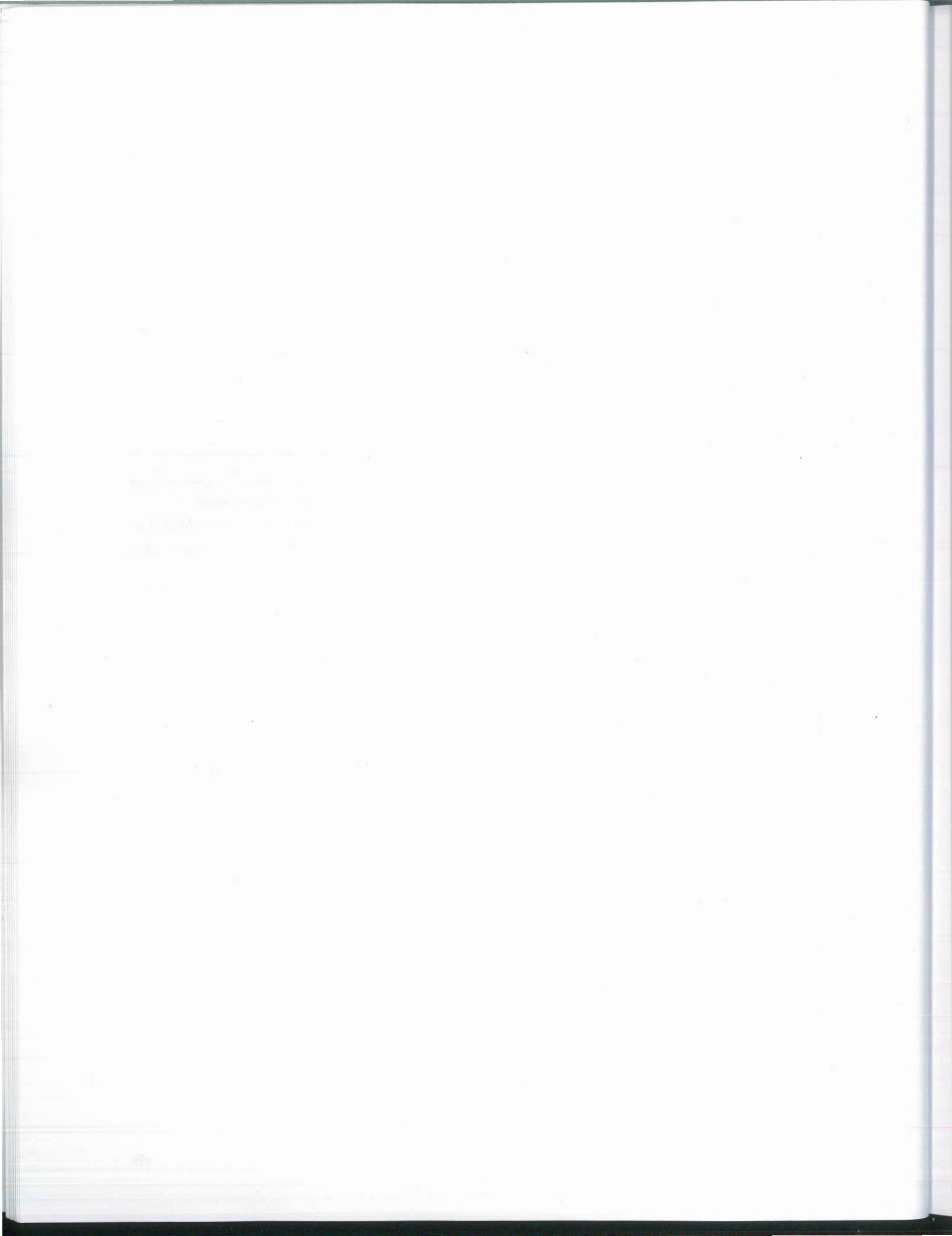
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# Preface to the series

Every aspect of the physiology and behavior of vertebrates is either regulated directly by hormones or modified by their actions, as exemplified by the essential and diverse roles of hormones in reproductive processes. Central to the evolutionary success of all vertebrates are the regulatory chemicals secreted by cells that control sex determination, sexual differentiation, sexual maturation, reproductive physiology, reproductive behavior, and the roles of environmental factors that influence these processes. To understand these processes and their evolution in vertebrates, it is necessary to employ an integrated approach that combines our knowledge of endocrine systems, genetics, and environmental factors in a comparative manner. In addition to providing insight into the evolution and physiology of vertebrates, the study of comparative vertebrate reproduction has had a considerable impact on the biomedical sciences and has provided a useful array of model systems for investigations that are of fundamental importance to human health as well as to the conservation of nonhuman vertebrates. The purpose of this series on hormones and reproduction of vertebrates is to bring together our current knowledge of comparative reproductive endocrinology in one place as a resource for scientists involved in general and reproductive endocrinology and for students who are just becoming interested in this field.

In this series of five volumes, we have selected authors with broad perspectives on reproductive endocrinology representing 18 countries on 4 continents. These authors are especially knowledgeable in their specific areas of interest and are familiar with the historical aspects of their fields and the cutting edge of today's research. We have intentionally included many younger scientists to bring fresh viewpoints to these reviews. Topics in each volume include sex determination, neuroendocrine regulation of the hypothalamic-pituitary-gonadal axis, separate discussions of testicular and ovarian functions and control, stress and reproductive function, hormones and pheromones associated with reproductive behaviors, and comparisons of reproductive patterns. Emphasis on using model species is balanced

throughout the series with comparative treatments of reproductive cycles of major taxa.

Chemical pollution and climate change pose serious challenges to the conservation and reproductive health of wildlife populations and humans in the 21st century, and these issues must be part of our modern perspective on reproduction. Consequently, we have included chapters that specifically deal with the impact of environmental factors such as temperature and photoperiod as well as the influences of endocrine-disrupting chemicals (e.g., pharmaceuticals, pesticides, persistent organic pollutants, and heavy metals) and nanoplastics as anthropomorphic factors that influence critical functions of our reproductive systems. Many authors throughout the series have also provided information connecting reproductive endocrinology to problems of species conservation in the face of climate changes and pollution.

The series consists of five volumes, each of which deals with a major traditional grouping of vertebrates: fishes, amphibians, reptiles, birds, and mammals. Each volume is organized in a similar manner so that themes can be easily followed across the volumes. We have attempted to standardize terminology and abbreviations to reflect the more common usage by endocrinologists working with this diverse assembly of organisms that we identify as vertebrates. Additionally, we have provided indices that allow readers to locate terms of interest and particular species. A glossary of abbreviations used is provided in each chapter.

Finally, and most importantly, we thank the many contributors to this work for their willingness to share their expertise, for their timely and thoughtful submissions, for their patience with our interventions and requests for revisions, and for the process of coordinating the production of five volumes. We also appreciate the contributions of the authors who provided the first edition versions, as their chapters provided a blueprint for the revisions. Some chapters were revised by the original authors, other chapters were completely rewritten by new authors, and several new

chapters were added. In a few cases, original chapters were reprinted with the addition of a modest number of newer references. We think you will find this new series of chapters especially useful.

And, of course, we must extend our special thanks to Editorial Project Manager, Andrea Dulberger of Academic

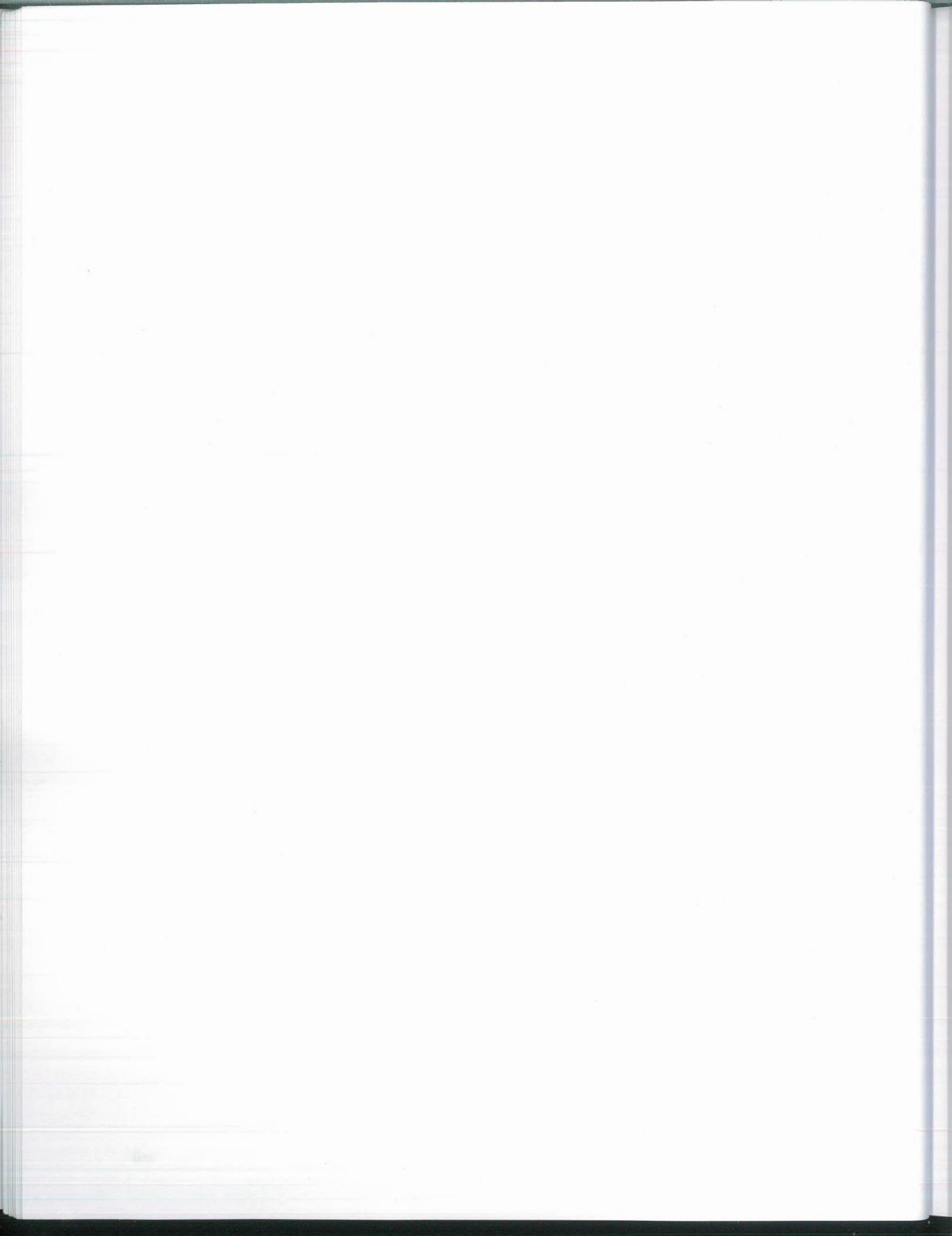
Press (Elsevier), for her help in keeping us all on track and overseeing the incorporation of these valuable contributions into these books.

**David O. Norris  
Kristin H. Lopez**

# Preface to Volume 3: Reptiles

Reptiles evolved from primitive amphibians and are the first vertebrate group to successfully separate themselves from the aquatic environment. Later, separate reptilian groups gave rise to birds and mammals. This volume provides a background on the development and functioning of the reproductive systems of reptilian vertebrates with an emphasis on the roles of hormones and pheromones, as well as environmental factors in directing formation and activities of the hypothalamic-pituitary-gonadal (HPG) axis. Where possible, the topics have been arranged similarly to the other volumes in this series to facilitate the efforts of readers looking for specific comparative data on reproductive processes. The role of pheromones is a special topic of interest for reptilian reproduction. Furthermore, the role of stress hormones associated with the hypothalamic-pituitary-adrenal (HPA) axis and other factors affecting HPG axis functions and reproductive behavior are emphasized

in several chapters. Reproductive cycles are also described for the major reptilian groups: Chelonia (turtles, tortoises), Crocodilia (crocodiles, alligators, caimans), and Squamates (lizards and snakes). Finally, there is a chapter dealing with the impacts of environmental disruption on the reptilian reproductive system by climate change, microplastic pollution, and hormone mimics and antagonists appearing in the environment at nonlethal levels as a consequence of widespread chemical pollution. This chapter is crucial for conservationists, ecologists, toxicologists, and physiologists who are concerned about how organisms interact with and adapt to their environments and how chemical pollution at environmentally relevant levels may affect individual species or affect reptiles as a group. Those with a special interest in the evolution of viviparity in vertebrates should read the new Chapter 5 in Volume 5 of this series.



## Chapter 1

# Sex determination and differentiation in reptiles

Amanda W. Carter<sup>a</sup>, Ryan T. Paitz<sup>b</sup>, and Satomi Kohno<sup>c</sup>

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## ABBREVIATIONS

BPA	bisphenol A
CDC2	cell division cycle protein 2
CTE	constant temperature equivalent
<i>cyp19</i>	cytochrome P450 19 aromatase
<i>dax 1</i>	dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DHT	dihydrotestosterone
<i>dmrt1</i>	doublesex and mab-3 related transcription factor 1
E <sub>2</sub>	17 $\beta$ -estradiol
EDCs	endocrine-disrupting chemicals
EE <sub>2</sub>	17 $\alpha$ -ethinylestradiol
ESD	environmental sex determination
FM	females and males produced at low and high temperatures, respectively
pattern	
FMF	females produced at low and high temperatures and males at intermediate temperatures
pattern	
GSD	genotypic sex determination
<i>kdm6b</i>	lysine demethylase 6B
MF	males and females produced at low and high temperatures, respectively
pattern	
POPs	persistent organic pollutants
SDMs	sex-determining mechanisms
<i>sox9</i>	Sry-box transcription factor 9
<i>Sry</i>	sex-determining region Y
T	testosterone
TCDD	tetrachlorodibenzoparadioxin
TRT	transitional range of temperatures
TSD	temperature-dependent sex determination
TSP	thermosensitive period
<i>wt1</i>	Wilm's tumor gene

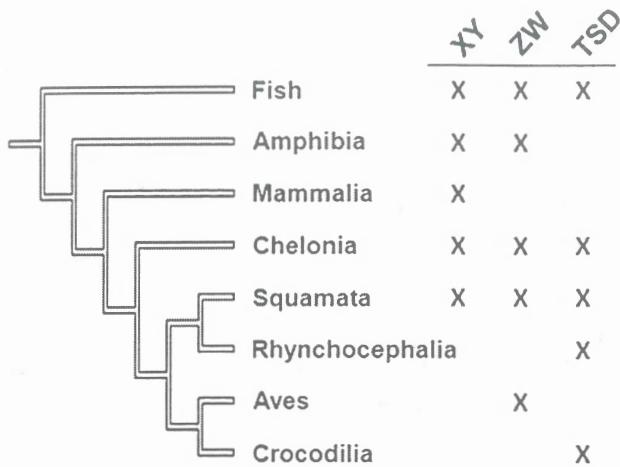
## 1 INTRODUCTION

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 female will have profound consequences on its life history, behavior,

physiology, morphology, and, ultimately, fitness. Indeed, differences between the sexes are among the most spectacular sources of phenotypic variation within populations. Unsurprisingly, factors governing sexual development are of primary interest to scientists and have been debated for over three millennia. Over time, our understanding of sex determination has transformed from mythological speculations to explanations based on hard-won scientific evidence (reviewed in Mittwoch, 2000). The turn of the 20th century marked a critical point in 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 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 versus female heterogamety; Mittwoch, 1996). In many species, heteromorphic sex chromosomes do not exist; instead, sex-determining factors lie in 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 demonstrating the role of environmental factors in the sex-determining process. Indeed, environmental conditions experienced during embryogenesis (rather than genotypic factors) trigger the developmental cascade that eventually leads to the male or female phenotype in many species. This environmental sex determination (ESD), including temperature-dependent sex determination (TSD) exhibits remarkable diversity (Bull, 1983) (see Fig. 1). It is now well established that SDMs range from being under complete genetic control to highly dependent upon environmental parameters.

The diversity in SDMs is intriguing since the division of the sexes is highly conserved throughout most animals. Indeed,



**FIG. 1** Phylogenetic distribution of genotypic (XY and ZW) and temperature-dependent sex determination (TSD) in vertebrates, with particular attention 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 Chardard et al., 2004; Dournon et al., 1990).

without knowledge of such diversity, one might expect that a single mechanism would have been stabilized at an early stage of evolution. Why have so many different mechanisms evolved 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. In many cases of reptiles, 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 provided critical insights into our understanding of sex determination. Our understanding, however, remains incomplete, and reptiles will undoubtedly continue to provide excellent opportunities to address 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. 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 as well as to identify gaps in our knowledge to provide suggestions for future research.

## 2 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. SDMs have been identified in less than 6% of the extant reptile species (Table 1). This statistic reveals our ignorance in many respects, but it also signifies that much exciting and fruitful research is yet to come. Nevertheless, we have gained a detailed understanding of many aspects of the distribution of alternative SDMs in reptiles. For example, we know that SDMs are not randomly distributed throughout reptiles. Recent evidence suggests that genotypic sex determination (GSD) evolved independently in three lineages of extinct Mesozoic marine reptiles (e.g., Sauropterygia, Mosasauridae, Ichthyosaura), which may have been partially responsible for radiations within these clades (Organ et al., 2009). In extant reptiles, both species of tuatara (Mitchell et al., 2006) and all crocodilians that have been studied (13 of 23 species) (Deeming, 2004; Lang & Andrews, 1994) exhibit a TSD (Fig. 1). The ZZ/ZW chromosomal system is ubiquitous in birds (avian reptiles, sister group to Crocodilia). 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 groups contain species that exhibit XX/XY and ZZ/ZW chromosomal systems, as well as TSD (Fig. 2). Hence, this section will focus on these two lineages, followed by a brief overview of the evolutionary transition between SDMs.

### 2.1 Chelonian and squamate sex determination

Comparative analyses demonstrate that TSD is the ancestral state for turtles (Janzen & Krenz, 2004; Organ & Janes, 2008) (Fig. 2A). Of the 149 turtle species that have been karyotyped, sex chromosomes have been identified in only eight species (Table 1). Egg incubation experiments showing no temperature effects on sex have further identified GSD in several species that lack differentiated sex chromosomes (e.g., Bull, 1985; Bull & Vogt, 1979). However, in most species examined (81%; see Table 1), incubation experiments found evidence for TSD, 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 et al., 1979). Based on experimental evidence from egg incubation studies, GSD arose once in the Emydidae

**TABLE 1** Summary of sex-determining mechanisms (SDM) found in reptilian families. Family classifications and number of extant species correspond to Pough et al. (2004). XY, XXXY 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	# of extant species	Karyological evidence for morphologically differentiated sex chromosomes			Experimental evidence for a thermally sensitive SDM		
		# shown /# examined	Heterogametic system(s)	Reference <sup>a</sup>	# shown /# examined	TSD pattern(s)	Reference <sup>a</sup>
Chelonia							
Podocnemidae	8	0/8	—	Olmo and Signorino (2005)	3/3	MF	Janzen and Paukstis (1991a) and Valenzuela (2004b)
Pelomedusidae	18	0/5	—	Ezaz, Stiglec, et al. (2006), Ezaz, Valenzuela, et al. (2006), Martinez et al. (2008), McBee et al. (1985), and Olmo and Signorino (2005)	2/2	FMF	—
Chelidae	50	3/23	XY	Olmo and Signorino (2005)	0/9	—	—
Carettochelyidae	1	0/1	—	Olmo and Signorino (2005)	1/1	MF	—
Trionychidae	27	1/8	ZW	Olmo and Signorino (2005)	0/3	—	—
Kinosternidae	22	2/16	XY	Olmo and Signorino (2005)	15/18	MF, FMF	—
Dermatemydidae	1	0/1	—	Olmo and Signorino (2005)	1/1	MF	—
Dermochelyidae	1	0/1	—	Olmo and Signorino (2005)	1/1	MF	—
Chelonidae	6	0/5	—	Olmo and Signorino (2005)	6/6	MF	—
Chelydridae	3	0/3	—	Olmo and Signorino (2005)	2/2	FMF	—
Emydidae	40	0/30	—	Olmo and Signorino (2005)	23/24	MF	—
Testudinidae	40	0/15	—	Olmo and Signorino (2005)	6/6	MF	—
Bataguridae	65	2/33	XY, ZW	Olmo and Signorino (2005)	8/8	MF, FMF	—
Crocodylia							
Alligatoridae	8	0/8	—	Olmo and Signorino (2005)	6/6	FMF	Janzen and Paukstis (1991a) and Valenzuela (2004b)
Crocodylidae	13	0/13	—	Olmo and Signorino (2005)	6/6	FMF	—
Gavialidae	2	0/1	—	Olmo and Signorino (2005)	1/1	FMF	Valenzuela (2004b)

Continued

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Order Family	# of extant species	Karyological evidence for morphologically differentiated sex chromosomes			Experimental evidence for a thermally sensitive SDM		
		# shown /# examined	Heterogametic system(s)	Reference <sup>a</sup>	# shown /# examined	TSD pattern(s)	Reference <sup>a</sup>
Rhynchocephalia							
Sphenodontidae	2	0/2	—	Olmo and Signorino (2005)	2/2	FM	Valenzuela (2004b)
Squamata (Sauria)							
Cekkonidae	1050	13/144	XY, XXX, ZW	Olmo and Signorino (2005)	20/27 <sup>b,c</sup>	FMF	Blumberg et al. (2002), Harlow (2004), Valenzuela (2004b), and Viets et al. (1994)
Dibamidae	10	1/1	XY		0/0	—	—
Scincidae	1260	6/118	XY	Olmo and Signorino (2005) and Shine et al. (2002)	4/6 <sup>b</sup>	MF, FM <sup>d</sup>	Harlow (2004), Ji and Du (2001a), Shine et al. (2002), and Valenzuela (2004b)
Xantusiidae	18	0/12	—	Olmo and Signorino (2005)	0/0	—	—
Cerphosauridae	35	0/12	—		0/0	—	—
Cordylidae	42	0/11	—		0/0	—	—
Teiidae	125	1/61	XY		0/3	—	Valenzuela (2004b) and Viets et al. (1994)
Gymnophthalmidae	150+	5/22	XY, XXX		0/0	—	—
Lacertidae	250	42/103	ZW, ZZW		1/5 <sup>c,d</sup>	—	Du and Ji (2006), Harlow (2004), Valenzuela (2004b), and Viets et al. (1994)
Varanidae	40	4/22	ZW		0/2 <sup>c</sup>	—	Harlow (2004) and Viets et al. (1994)
Lanthanotidae	1	0/0	—		0/0	—	—
Shinisauridae	1	0/0	—		0/0	—	—

Anguidae	110	0/13	-	Olmo and Signorino (2005)	0/1 <sup>c</sup>	-	Harlow (2004) and Valenzuela (2004b)
Helodermatidae	2	0/1	-		0/0	-	-
Xenosauridae	6	0/1	-		0/0	-	-
Chamaeleonidae	130	1/50	ZW		0/2 <sup>c</sup>	-	Andrews (2005), Harlow (2004), and Viets et al. (1994)
Agamidae	380+	2/89	ZW	Ezaz et al. (2005), Olmo and Signorino (2005), and Uller et al. (2006, 2008)	13/25 <sup>b</sup>	FM, FMF <sup>d</sup>	Harlow (2004), Uller et al. (2008), and Valenzuela (2004b)
Iguanidae	915+	76/250	XY, XXXY	Olmo and Signorino (2005)	0/5 <sup>c</sup>	-	Harlow (2004), Valenzuela (2004b), and Viets et al. (1994)
Squamata (Amphisbaenia)							
Amphisbaenidae	135	0/25	-	Olmo and Signorino (2005)	0/0	-	-
Rhineuridae	1	0/1	-		0/0	-	-
Tropidophiidae	6	0/2	-		0/0	-	-
Bipedidae	3	1/3	ZW		0/0	-	-
Squamata (Serpentes)							
Anomochilidae	2	0/0	-		0/0	-	-
Aniliidae	1	0/0	-		0/0	-	-
Xenophidiidae	2	0/0	-		0/0	-	-
Tropidophiidae	31	0/1	-	Olmo and Signorino (2005)	0/0	-	-
Bolyeriidae	2	0/0	-		0/0	-	-
Acrochordidae	3	0/2	-	Olmo and Signorino (2005)	0/0	-	-
Boidae	74	6/29	ZW		0/1	-	Shine, Madsen, et al. (1997)
Uropeltidae	55	0/0	-		0/0	-	-
Xenopeltidae	2	0/1	-	Olmo and Signorino (2005)	0/0	-	-
Loxocemidae	1	0/1	-		0/0	-	-
Leptotyphlopidae	90	0/1	-		0/0	-	-
Anomalepididae	15	0/0	-		0/0	-	-

Continued

**TABLE 1** Summary of sex-determining mechanisms (SDM) found in reptilian families. Family classifications and number of extant species correspond to Pough et al. (2004). XY, 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—cont'd

Order Family	# of extant species	Karyological evidence for morphologically differentiated sex chromosomes			Experimental evidence for a thermally sensitive SDM		
		# shown /# examined	Heterogametic system(s)	Reference <sup>a</sup>	# shown /# examined	TSD pattern(s)	Reference <sup>a</sup>
Typhlopidae	200	0/6	—	Olmo and Signorino (2005)	0/1	—	Shine and Webb (1990)
Colubridae	1800+	57/140	ZW		0/7 <sup>c</sup>	—	Du and Ji (2002), Janzen and Paukstis (1991a), Ji and Du (2001b), Valenzuela (2004b), and Webb et al. (2001)
Atractaspididae	18	0/0	—	—	0/0	—	—
Elapidae	300	77/110	ZW, ZZW, ZWW	Olmo and Signorino (2005)	0/2	—	Ji and Du (2001b) and Shine (1989)
Viperidae	228	38/61	ZW		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. See text for explanation of abbreviations.

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

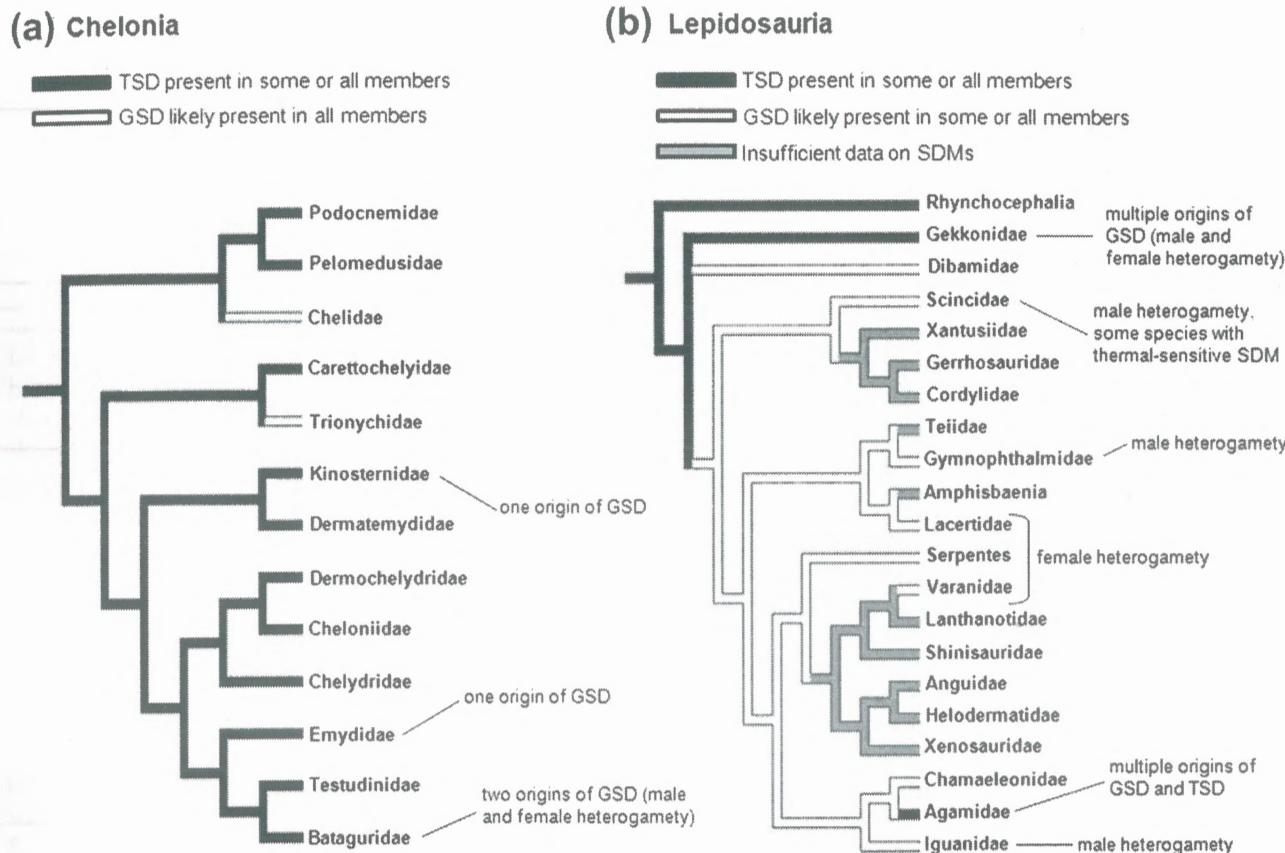
<sup>b</sup>Some species contain both heteromorphic chromosomes and thermal-sensitivity of sex determination.

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

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

(*Glyptemys insculpta*) and is likely ubiquitous in the Chelidae and Trionychidae. Sex chromosomes have been identified in three members of the Chelidae (Ezaz, Stiglec, et al., 2006; Ezaz, Valenzuela, 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; Organ & Janes, 2008; Pokorná & Kratochvíl, 2009) from which both XY and ZW chromosomal systems have evolved independently multiple times (Fig. 2B). Of the squamate species that have been karyotyped, less than



**FIG. 2** Phylogenetic distribution of sex-determining mechanisms (SDM) in extant families of (A) Chelonia, (B) Lepidosauria (Squamata + Rhynchocephalia), and (C) Agamidae. Family classifications correspond to Pough et al. (2004). The chelonian phylogenetic hypothesis is based on nuclear DNA (Krenz et al., 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 & 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 in Table 1. See Organ and Janes (2008) and Pokorná and Kratochvíl (2009) for a more detailed phylogenetic analysis of reptile SDMs. The agamid phylogeny is based on hypotheses proposed by Schulte et al. (2003) and Hugall et al. (2008). Parsimony analysis was performed with MacClade Software (Maddison & 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.

(Continued)

## (C) Agamidae

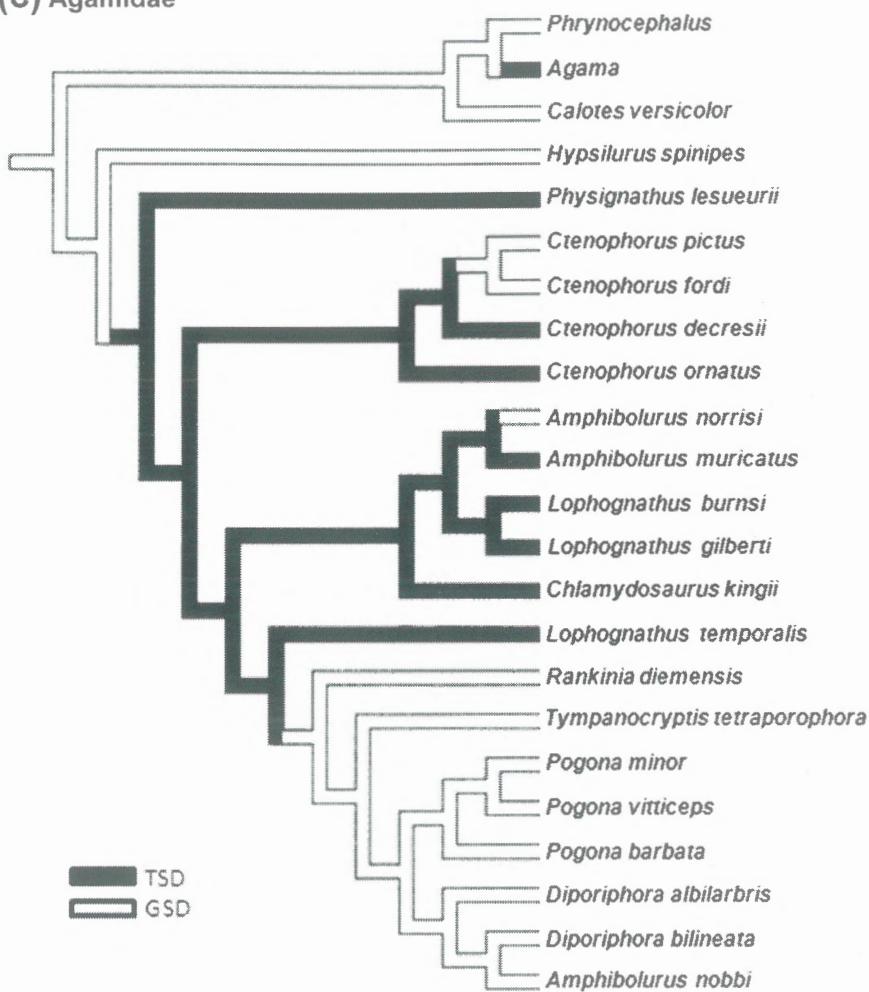


FIG. 2, CONT'D

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 for other examples). Incubation experiments support GSD in many taxa that lack differentiated sex chromosomes (Uller et al., 2006, 2008). In contrast, many species have retained (or independently evolved) the ancestral state of TSD in lizards. Based on reliable evidence, rather than anecdotal observations that are occasionally reported (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).

Phylogenetic reconstructions of the evolution of lizard SDMs could be more evident, 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 et al., 1988; Townsend et al., 2004; Vidal & Hedges, 2005), and the evolution of SDMs varies depending on which phylogeny is used (Pokorná & Kratochvíl, 2009). Adding to this uncertainty, phylogenetic relationships among species within families are unresolved (Han et al., 2004; Hugall et al., 2008; Smith et al., 2007). Another problem is the lack of research on SDMs of lizards (SDMs are known in <5% of the species) and questionable reports of TSD in several species. Indeed, anecdotal observations of sex-ratio skewness in response to incubation temperatures have been reported in many families (e.g., Chameleidae, Iguanidae, Lacertidae, and Varanidae) (Viets et al., 1994). However, 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 Fig. 2B excludes these reports. It is

extremely generalized, but it nonetheless 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.
3. Thermally sensitive SDMs may have been retained (or independently arisen) in the Scincidae despite the presence of sex chromosomes in this lineage.

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, 2009) (Fig. 2C). In some cases, sister taxa differ in SDMs, suggesting a recent evolution of either GSD or TSD (e.g., *Amphibolurus* and *Ctenophorus*). Because of this diversity within a single family, the Agamidae provides excellent model systems for exploring ecological and evolutionary aspects of vertebrate SDMs (Quinn et al., 2007; Uller & Olsson, 2006; Warner, Radder, & Shine, 2009; Warner & Shine, 2005; 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 & Kratochvíl, 2009). TSD occurs in three lineages of this family, Diplodactylinae, Eublepharinae, and Gekkoninae (Viets et al., 1994), from which GSD has independently evolved at least three times (Janzen & Krenz, 2004; Pokorná & Kratochvíl, 2009). Intriguingly, comparative analyses indicate that male heterogamety evolved multiple times in some gecko lineages, whereas female heterogamety evolved in others (Pokorná & 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 (Tokunaga, 1985; Yoshida & Itoh, 1974).

Recent laboratory experiments and correlative data from the field provide convincing evidence of the 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 (Radder et al., 2009; Shine et al., 2002). Sex is also sensitive to temperature in three distantly related viviparous Scincid lizards (*Eulamprus tympanum*, *Niveoscincus ocellatus*, and *Sphenomorphus indicus*), which give live birth. 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; Wapstra et al., 2009). Skewing of sex ratios in response to gestation temperature is more substantial in the viviparous skink *S. indicus* (Ji et al., 2006) than that reported in *E. tympanum* or *N. ocellatus*.

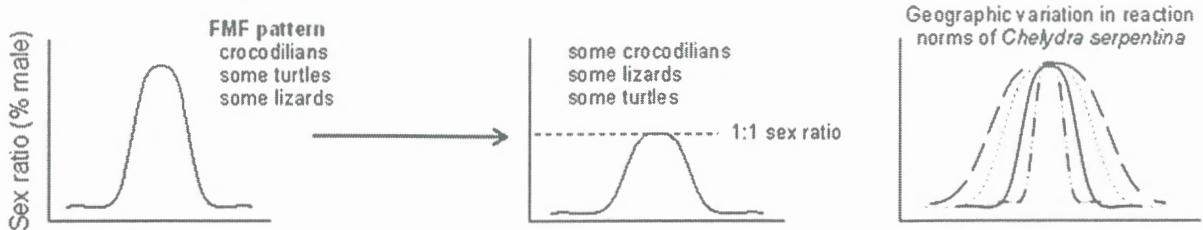
### 3 DIVERSITY OF SDMS AND PATTERNS

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, both sex-determining systems show a remarkable diversity in their 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 (Fig. 3). Moreover, evidence is accumulating that elements of both TSD and GSD may co-occur in some species, even within single populations (Shine et al., 2002). In this section, we review the current knowledge on the diversity of sex-determining patterns of reptiles. 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 strategies but instead represent endpoints of a continuum (Sarre et al., 2004).

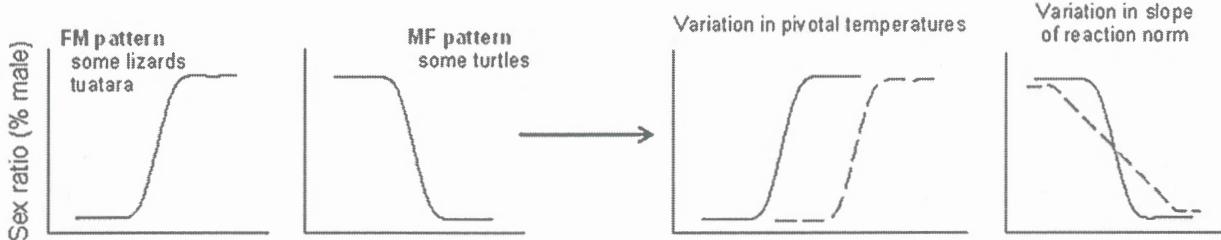
#### 3.1 Patterns of genotypic sex determination

GSD 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

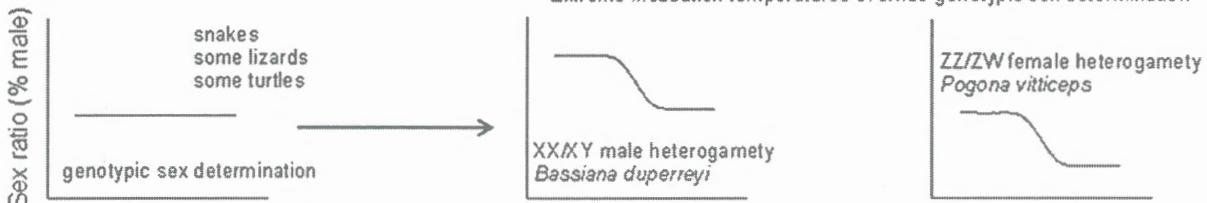
## (a) Females produced at both extremes

Geographic variation in reaction norms of *Chelydra serpentina*

## (b) Females produced at one extreme



## (c) Both sexes produced at all temperatures



**FIG. 3** Diversity of sex-determining patterns in reptiles. All graphs show the 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 shapes of reaction norms (e.g., the lines illustrate population-specific reaction norms in the common snapping turtle, *Chelydra serpentina*; Ewert et al., 2005). (B) Patterns of TSD in which males and females are produced at one or the other temperature extremes (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 (TRT) (i.e., the range of temperatures that yield mixed sex ratios) among and within species. (C) The 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 et al., 2008), and vice versa (Quinn et al., 2007) in certain lizard species. (Modified from Warner, D. A., & Janzen, F. J. (2010). Diversity of sex-determining mechanisms. In D. F. Westneat & C. W. Fox (Eds.), Evolutionary behavioral ecology. Oxford University Press.)

autosomes in 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 are 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), presumably because homomorphic chromosomes have been retained or changed very little from the ancestral state (Ohno, 1967). 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 (Charlesworth et al., 2005; Marshall Graves & Shetty, 2001). In GSD species with apparently homomorphic chromosomes, experimental approaches are needed to verify that sex determination is unresponsive to

environmental parameters (e.g., temperature) (Valenzuela et al., 2003). Indeed, incubation temperature does not influence primary sex ratios in many turtle and squamate species that lack differentiated chromosomes (Bull & Vogt, 1979; Uller et al., 2006, 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 exhibit some differentiation, which more advanced techniques can detect. For example, early work on the lizard *Pogona vitticeps* suggested homomorphic sex chromosomes (Witten, 1983), but more advanced techniques (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, Stiglec, et al., 2006; Ezaz, Valenzuela, et al., 2006; Martinez et al., 2008). Although early studies have not identified heteromorphic chromosomes in many GSD reptiles (Table 1), advanced techniques may reveal more chromosomal differentiation than in past studies.

Some squamate species exhibit multiple sex chromosomes and more than one chromosome sorts 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  $X_1X_2Y \delta^{\circ}/X_1X_1X_2X_2 \varphi$ ) (Cole et al., 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.

### 3.2 Patterns of temperature-dependent sex determination

Under TSD, offspring sex is irreversibly determined by the temperature embryos encounter during development. All extant reptilian orders contain some members with TSD, but its prevalence varies among the major groups (Fig. 2).

The presence of TSD is experimentally identified when eggs are incubated under various 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 et al., 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 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 (Fig. 3). 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 et al., 2004, 2005; Warner et al., 2008). Accordingly, evidence suggests that the pivotal temperature has a heritable genetic basis (Bull, Vogt, & Bulmer, 1982; Bull, Vogt, & McCoy, 1982; Janes & Wayne, 2006; Janzen, 1992; Rhen & Lang, 1998). 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 characterize the relationship between constant-temperature incubation and sex ratio (Head et al., 1987; Kraak & Pen, 2002; Pieau, 1996), but in reality, these reaction norms are broad generalizations of a variety of patterns (Fig. 3). Under one pattern, males are produced at low incubation temperatures and females at high temperatures (male-female, “MF pattern,” or TSD type Ia); this is characteristic of many turtle and lizard species (Ewert et al., 1994; Harlow, 2004). Although rare, the reverse pattern is found in some species (female-male, “FM pattern,” or TSD type Ib), 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, “FMF pattern,” or TSD type II). This pattern contains two pivotal temperatures and, hence, has two

TRTs. The FMF pattern is found in both species of tuatara (Mitchell et al., 2006), all crocodilians (Deeming, 2004; Lang & Andrews, 1994), and several lizard and turtle species (Harlow, 2004; Viets et al., 1994). Even within these three patterns, we see remarkable diversity in the shapes of reaction norms (Fig. 3). The pivotal temperature varies considerably among species, such that incubation temperatures that produce predominantly males in one species will produce predominantly females in another. In some North American turtles, variation in pivotal temperatures is explained by latitude (Bull, Vogt, & Bulmer, 1982; Bull, Vogt, & McCoy, 1982; Ewert et al., 2004, 1994, 2005). For example, in *Chelydra serpentina*, a species with an FMF pattern of TSD, the upper pivotal temperature is higher 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 et al., 2004; Warner & Shine, 2008b). 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. In contrast, 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 (*C. serpentina*) (Ewert et al., 2004, 2005).

In addition 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 & 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; 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, the sex ratios of many kinosternid turtles and crocodilians never achieve 100% males across any constant-temperature incubation regime (Ewert et al., 2004; Lang & Andrews, 1994). Intuition would suggest that such patterns would yield highly female-biased primary sex ratios in the wild, possibly leading to unstable population demographics (Girondot et al., 2004). However, field data supporting this idea are scarce, and more males may be produced in nature than expected from the results of artificial incubation experiments (see Section 6).

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 FM or MF patterns depending on the direction of the shift. Given the evidence for variable and heritable pivotal temperatures (Bull, Vogt, & Bulmer, 1982; Bull, Vogt, & McCoy, 1982; Dodd et al., 2006; Janzen, 1992; Rhen & Lang, 1998), such a scenario is possible. Additional information on species-specific patterns of TSD and resolved phylogenies to which these patterns can be mapped will enable robust tests of this hypothesis.

## 4 PROXIMATE MECHANISMS

Our understanding of the molecular and physiological underpinnings of reptilian sex determination has substantially advanced in the past ten years (Paitz et al., 2022). Research on this topic has focused primarily on species with TSD to understand how temperature regulates gene expression in gonadal 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 (Capel, 2017; Place & Lance, 2004), our in-depth understanding of mammalian and avian systems offers some critical insights into GSD and TSD in reptiles. Technological advances such as genome sequencing and RNA-seq have provided opportunities to characterize the sequence of events that transduce incubation temperature into a change in gene expression during reptilian sex determination. Processes such as histone methylation and intron retention are now known to play critical roles in mediating how temperature epigenetically regulates gene expression to control gonadal fate.

### 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 committed to becoming either a testis or an ovary.

Although the specific details of gonadal development (i.e., gonadogenesis) can 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 differentiate into seminiferous tubules, which indicate testis development. The embryonic testes produce two important hormones that influence the sexual development of males. The first is anti-Müllerian hormone (AMH=MIS, Müllerian-inhibiting substance), which causes the Müllerian ducts to degenerate. The second hormone, testosterone (T), stabilizes the Wolffian ducts, eventually developing into seminal vesicles, epididymis, and vas deferens. Without 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 2). In mammals, a primary sex-determining switch on the Y chromosome (*Sry* gene located on the Y chromosome) sets the testicular developmental pathway in motion (Sinclair et al., 1990). The *sry* gene triggers the gene expression of the critical transcription factors, such as DMRT1 and SOX9, that function together to establish sex-specific chromatin landscapes to coordinate the expression of many genes involved in testis development (Garcia-Moreno et al., 2019; Lindeman et al., 2021). Genes such as *wt1* and *sf1* contribute to testis formation by activating the transcription of *amh* gene, the product (AMH) of which initiates the degeneration of the Müllerian duct. In contrast, ovaries develop in the absence

**TABLE 2** Genes, steroids, and enzymes are 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 TSD and GSD.

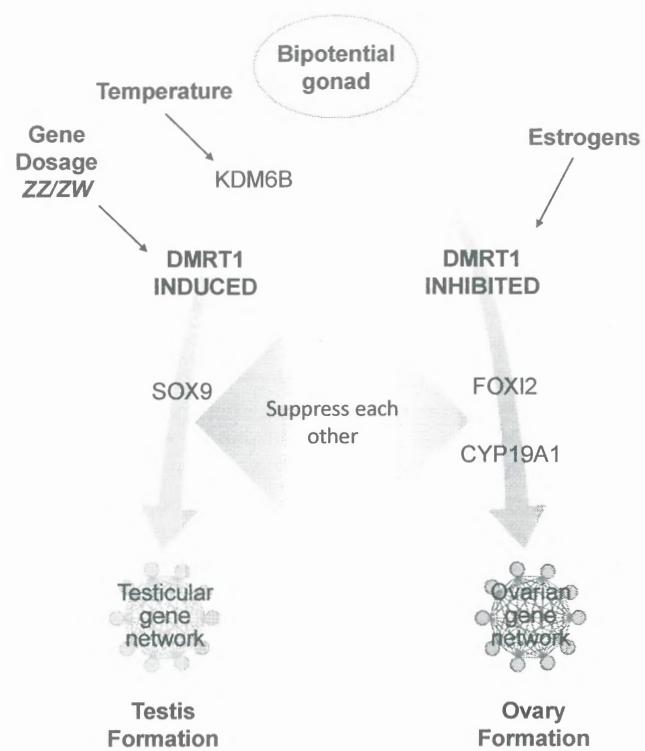
Sex-determining factor	Full name	Definition or function for sexual development
<b>Gene</b>		
<i>Sry</i>	Sex-determining region Y	Major testis-determining factor located on the Y chromosome. Found only in placental mammals
<i>sox9</i>	SRY-like HMG box	Regulates transcription of the anti-Müllerian hormone 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>	Wilm's tumor suppressor gene	Transcription factors 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>	Double sex- 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 SRY function in mammals. Represses WT1 and SF1 expression (i.e., suppresses testis formation), and is important in ovarian differentiation
<b>Steroid hormones and steroidogenic enzymes</b>		
DHT	Dihydrotestosterone	Nonaromatizable 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

See text for explanation of abbreviations.

of the *sry* gene. The gene *dax1* is expressed in ovaries, which suppresses the activation of WT1 and SF1 proteins, enabling the retention of the Müllerian ducts (Nachtigal et al., 1998). The expression of aromatase (CYP19), the enzyme responsible for converting androgens into estrogens, is also elevated in developing ovaries and can subsequently inhibit genes in the testis pathway (Capel, 2017).

Sex determination in birds is also chromosomally based, where males are the homogametic sex that possesses two Z chromosomes, and females possess one Z chromosome and one W chromosome (Smith et al., 2009). *dmrt1* is considered the male sex determinant because it is located on the Z chromosome, and a higher dosage of DMRT1 protein results in testis formation (Ioannidis et al., 2021; Smith et al., 2009). This system is also applied to reptilian GSDs in the ZZ/ZW system. A dysfunction of DMRT1 protein results in ovary formation (Ioannidis et al., 2021). Similar to mammals, ovary formation in birds is marked by the increased expression of *cyp19* (Guo et al., 2022). These patterns of gene expression in mammals and birds highlight the conserved processes that underlie how testes and ovaries develop (Capel, 2017).

Many of the genes discussed above have homologs in reptiles with TSD (Bull, Gutzke, & Crews, 1988; Bull, Hillis, & O'Steen, 1988; Pieau & Dorizzi, 2004; Place & Lance, 2004; Smith et al., 1999). Initial research on gene expression patterns underlying TSD focused on these conserved homologs. 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 (Valleley et al., 2001; Western et al., 1999). More recent work has used transcriptome-level quantification to identify differentially expressed genes at different incubation temperatures (Czerwinski et al., 2016; Radhakrishnan et al., 2017; Yatsu et al., 2016). One commonality of these transcriptome-level studies is that hundreds of genes are differentially expressed at incubation temperatures, resulting in ovary development, compared to temperatures resulting in testis development. These findings parallel work in mammals (Small et al., 2005) and birds (Ayers et al., 2015), showing hundreds of genes differentially expressed between the developing ovary and testis. Whether species possess GSD or TSD, it is clear that the process of gonadal differentiation involves a cascade of events culminating in a large number of genes being differentially expressed between the developing ovary and testis. While the role of *sry* in mammals and *dmrt1* in birds as factors that initiate these different cascades has been established, we are just now starting to understand how incubation temperature initiates these different cascades in species with TSD (Paitz et al., 2022).



**FIG. 4** Gonadal differentiation in reptiles can be regulated by genotype, temperature, and steroids. Various points in the cascade may respond to these factors to affect whether testis or ovary development occurs. The relative effects of these different factors underlie patterns of SDMs ranging from pure GSD (temperature and steroids have no effect) to pure TSD (genotype has no effect).

TSD appears to operate via thermo-sensitive protein function resulting in epigenetic regulation of critical transcription factors such as *dmrt1* (Ge et al., 2018; Haltenhof et al., 2020) (Fig. 4). Work in *T. scripta* has demonstrated that DMRT1 is necessary and sufficient to initiate testis development (Ge et al., 2017). Thermosensitive expression of DMRT1 arises through epigenetic regulation of the chromatin state involving the histone demethylase (*kdm6b*) (Ge et al., 2018). At incubation temperatures that induce testis development, *kdm6b* expression is elevated and functions to eliminate the trimethylation histones near the promoter of *dmrt1* to induce *dmrt1* expression subsequently (Ge et al., 2018). *kdm6b* expression is regulated by temperature via thermosensitive protein function. Specifically, CDC2-like kinases are active at cool incubation temperatures and phosphorylate RNA-binding proteins at these temperatures to mediate changes in RNA splicing (Haltenhof et al., 2020). *kdm6b* exhibits alternative splicing at different incubation temperatures in various TSD species (Bock et al., 2020; Deveson et al., 2017; Marroquín-Flores et al., 2021). Overall, this work is starting to identify processes through which thermosensitive proteins can regulate

the expression of critical transcription factors like DMRT1 to initiate the cascade of gene expression necessary for gonadal development.

By incorporating studies on thermosensitive protein function into TSD research (Haltenhof et al., 2020; Weber et al., 2020), our understanding of how incubation temperature is transduced into a cue for gonadal development is quickly advancing. Future work on processes of protein phosphorylation, alternative splicing, histone methylation, and DNA methylation will continue to identify how incubation temperatures regulate chromatin landscapes and gene expression in species with TSD. TSD involves a wide array of processes that must occur to complete gonadal differentiation. It is likely that genetic variation in these underlying processes, as well as environmental sensitivity of these processes, could manifest as variation in how/if incubation temperatures affect sex ratios.

## 4.2 Steroid signaling and TSD

While research is beginning to identify mechanisms through which incubation temperature regulates gene expressions in gonadal differentiation, other factors can regulate gene expression and influence sex determination. Numerous steroids have been shown to influence the expression of genes involved in gonadal differentiation and resulting sex ratios (see Table 2 for a list of hormones and enzymes). This notion is supported by experiments that manipulate the hormonal environment of the developing embryo. For example, administrating the estrogen, 17 $\beta$ -estradiol ( $E_2$ ) to eggs induces ovarian development, even at male-producing temperatures in many reptile species (Bull, Gutzke, & Crews, 1988; Bull, Hillis, & O'Steen, 1988; Crews, 1996; Freedberg et al., 2006). The ability of  $E_2$  to counteract the effects of temperature is most significant during the developmental window that corresponds with gonadogenesis (Gutzke & Chymiy, 1988; Wibbels et al., 1991). Moreover,  $E_2$  has an increasingly potent effect on ovarian development as incubation temperatures move from strictly male-producing temperatures towards the pivotal temperature (Crews & Bergeron, 1994). Administration of  $E_2$  to eggs can also impact sexual differentiation in species with GSD (Bull, Gutzke, & Crews, 1988; Bull, Hillis, & O'Steen, 1988). Estrogen induces ovarian development via estrogen receptor 1 (ESR1 or ER-alpha), which is one of two estrogen receptor isoforms in both TSD (Kohno et al., 2015) and GSD species (Li et al., 2022). In both TSD and GSD reptiles, these hormonal manipulations lead to fully functional females similar to those from unmanipulated eggs (Crews et al., 1994). Another line of evidence that illustrates the importance of estrogens 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 inhibiting aromatase has been demonstrated in numerous reptilian species that exhibit TSD (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 behaviors to naturally produced males, capable of spermatogenesis as adults (Elbrecht & Smith, 1992; Shine et al., 2007; Warner & Shine, 2008a; Wennstrom & Crews, 1995). Thus,  $E_2$  appears to be the primary steroid required for ovarian differentiation in reptiles (Pieau & Dorizzi, 2004).

There are several sources of estrogens that can affect gonadal differentiation. The first source is maternally derived estrogens present in the egg yolk when the egg is laid (Bowden et al., 2000; Carter et al., 2017; Conley et al., 1997; Elf, 2004; Paitz & Bowden, 2009, 2013). Both  $E_2$  and T are plentiful in egg yolks at oviposition, long before embryos can produce these steroids (Bowden et al., 2002, 2000; Elf et al., 2002; Lovorn & Wade, 2003). 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 (Janzen et al., 2002). As expected, the offspring sex of several reptilian species is associated with maternally derived  $E_2$  or T when eggs are incubated at pivotal temperatures (reviewed in Bowden & Paitz, 2018; Radder, 2007). Indeed, eggs from clutches with relatively high levels of  $E_2$  tend to produce female offspring in turtles with TSD (*C. picta*, Bowden et al., 2000; *T. scripta*, Carter et al., 2017). Variation in yolk  $E_2$  is primarily driven by seasonal patterns where a female's first clutch of a nesting season contains significantly lower yolk  $E_2$  concentrations than her second clutch (Bowden et al., 2000, 2004; Carter et al., 2017; Paitz & Bowden, 2009). As for other steroids, eggs with relatively high T levels tend to produce male offspring in a lizard with GSD [*Anolis carolinensis*; (Lovorn & Wade, 2003)]. Maternally derived corticosterone also influences offspring sex ratios in the lizard *A. muricatus* (Warner et al., 2007). However, experimental work suggests that elevated levels of corticosterone in eggs in this species yield female-biased secondary sex ratios via differential embryonic mortality (Warner, Radder, & Shine, 2009; Warner, Uller, & Shine, 2009). 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 et al., 2009, *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.

There is a substantial amount of work on the mechanisms underlying how yolk estradiol might influence gonadal differentiation in species with TSD. In several species with TSD, once eggs are laid, the concentrations of E<sub>2</sub> in the yolk decline rapidly (Bowden et al., 2002; Conley et al., 1997; Elf et al., 2002; Paitz & Bowden, 2009). In *T. scripta*, this decline results from E<sub>2</sub> being metabolized into estrone sulfate (Paitz et al., 2012; Paitz & Bowden, 2013). As E<sub>2</sub> levels decline, levels of estrone sulfate rise (Paitz & Bowden, 2013). Importantly, maternally derived estrone sulfate is also present in eggs when they are laid, with second clutches containing higher concentrations than those of first clutches (Paitz & Bowden, 2013). Exogenous manipulations of estrone sulfate result in an increased production of female hatchlings in the same manner that manipulations of E<sub>2</sub> influence sex ratios (Marroquín-Flores et al., 2022; Paitz et al., 2012; Paitz & Bowden, 2013). These results highlight a potentially important role for E<sub>2</sub> metabolites, such as estrone sulfate, in gonadal differentiation (Bowden & Paitz, 2021).

Since exogenous E<sub>2</sub> and estrone sulfate can induce ovaries to develop in reptilian species with TSD and GSD, numerous studies have examined the mechanisms underlying this effect. Some studies manipulate hormones at the onset of development to mimic variation in maternally derived levels (Marroquín-Flores et al., 2022; Paitz et al., 2012; Paitz & Bowden, 2013), while others manipulate hormones later in development to mimic the gonadal production of steroids (Ge et al., 2018; Wibbels et al., 1991). Several studies have investigated the effect of estrogens on *kdm6b* and *dmrt1* expression. Applying E<sub>2</sub> to *T. scripta* eggs at the onset of the thermosensitive period (TSP) inhibits the expression of *kdm6b* (Ge et al., 2018) and *dmrt1* (Murdock & Wibbels, 2006). Applying estrone sulfate to *T. scripta* eggs at oviposition does not affect *kdm6b* expression but inhibits *dmrt1* expression (Marroquín-Flores et al., 2022). It appears that even though E<sub>2</sub> and estrone sulfate can induce the development of ovaries, the two estrogens may differ in how they elicit their effects; E<sub>2</sub> may regulate *dmrt1* expression via *kdm6b*, while estrone sulfate appears to regulate *dmrt1* expression independent of *kdm6b*. Conversely, the timing of estrogen manipulation may affect whether *kdm6b* is involved in mediating how estrogens regulate *dmrt1*. More research is necessary to decipher the mechanisms underlying the effect of yolk estrogens on gonadal differentiation compared to the effect of gonadally produced estrogens later in development.

Many other chemicals (e.g., hormones, enzyme inhibitors, receptor antagonists) applied to eggs have varying effects on sexual development (reviewed in Crews, 1996; Crews et al., 1994). Unlike E<sub>2</sub> application at male-producing temperatures, androgens cannot overcome the effects of

female-producing incubation temperatures. For example, dihydrotestosterone (DHT, a nonaromatizable androgen) only induces male development at temperatures near the pivotal temperature for sex determination. Nevertheless, manipulations of 5α-reductase (the enzyme that converts T to DHT) prove that androgens are essential for male development. For example, by blocking DHT synthesis using 5α-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 5α-reductase inhibitor to eggs results in more 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 5α-reductase) play a critical role in sex determination and differentiation.

## 5 ENDOCRINE DISRUPTIONS

The recognition that environmental pollutants called endocrine-disrupting chemicals (EDCs) adversely affect animal reproductive physiology can be traced back to 1992 (Bern, 1992). After three decades of research, it is essential to evaluate the long-term nonlethal effects of EDCs to fully assess their environmental impacts.

There are two ways to alter endocrine signals: directly acting on endocrine receptors and indirectly affecting hormone metabolism. Any chemical that can potentially alter endocrine signaling can be an EDC (Diamanti-Kandarakis et al., 2009). Thus, EDCs may influence reptilian sex determination via the endocrine system. For example, bisphenol A (BPA), a primary component of polycarbonate plastics, binds to estrogen receptors (Crain et al., 2007) and also alters estrogen metabolism in the red-eared slider turtle, *T. scripta* (Clairardin et al., 2013).

Several investigations have been published using either field studies or controlled laboratory experiments to reveal the effects of EDCs on sex determination in several species that are not threatened or endangered (Table 3). This section discusses the potential impact of EDCs at an environmentally realistic concentration on sex determination in reptiles, mostly in species exhibiting TSD.

As discussed in previous sections, endocrine signals play critical roles in reptilian sex determination. Two cases of endocrine disruptions in the reproduction of wild reptiles have been identified in the American alligators at Lake Apopka, Florida, USA (Guillette et al., 2000), and in painted turtles (*C. picta*) at Cape Cod, Massachusetts, USA (Rie et al., 2000; Rudel et al., 1998) due to *p,p'*-dichlorodiphenyldichloroethylene (DDE)

**TABLE 3** Effects of developmental exposure to EDCs on reptilian sex ratios in the Lab experiments and environmentally identified concentrations of EDCs.

Contaminates	Species	Experimental concentration (ppb)	Temperature	Results	Environmental concentrations in egg (ppb)	Reference
17 $\alpha$ -Ethynodiol (EE <sub>2</sub> )	American alligator, <i>Alligator mississippiensis</i>	300	MPT	Female†		Matter et al. (1998)
Bisphenol A (BPA)	Broad-snouted caiman, <i>Caiman latirostris</i>	140,000	MPT	Female†		Stoker et al. (2003)
<i>p,p'</i> -Dichlorodiphenyl dichloroethylene (DDE)	Painted turtle, <i>Chrysemys picta</i>	10	MPT	Ovary-like structure†		Jandegan et al. (2015)
	<i>A. mississippiensis</i>	100	F-M Pivotal Temperature	Female†		Milnes et al. (2005)
	Red-eared slider, <i>Trachemys scripta</i>	5.7	Male-biased	Female†		Willingham and Crews (1999)
	<i>A. mississippiensis</i>	N/A			5800	Heinz et al. (1991)
	Hawksbill sea turtle, <i>Eretmochelys imbricata</i>	N/A			2.072	Salyarani et al. (2023)
	Common snapping turtle, <i>Chelydra serpentina</i>	N/A			852	De Solla et al. (2001)
	<i>C. serpentina</i>	N/A			340	Struger et al. (1993)
	Eastern spiny softshell turtles, <i>Apalone spinifera</i>				267	De Solla et al. (2003)
	<i>A. mississippiensis</i>	10	MPT	Female†		Matter et al. (1998)
	<i>C. serpentina</i>				0.007	De Solla et al. (2001)
	Polychlorinated biphenyl (PCB) (Aroclor 1242)	0.2	Male-biased	Female†		Willingham and Crews (1999)
	<i>C. serpentina</i>	N/A			12,067	Struger et al. (1993)
	<i>A. mississippiensis</i>	N/A			170	Heinz et al. (1991)

Continued

**TABLE 3 Effects of developmental exposure to EDCs on reptilian sex ratios in the Lab experiments and environmentally identified concentrations of EDCs—cont'd**

Contaminates	Species	Experimental concentration (ppb)	Temperature	Results	Environmental concentrations in egg (ppb)	Reference
Total PCB (the sum of 59 PCB congeners)	<i>A. spiniferus</i>				1490	De Solla et al. (2003)
	<i>C. serpentina</i>				737,683	De Solla et al. (2001)
trans-Nonachlor	<i>T. scripta</i>	11.2	Male-biased	Female†		Willingham and Crews (1999)
	<i>A. mississippiensis</i>	110	N/A			Heinz et al. (1991)
	<i>C. serpentina</i>		N/A	23		Struger et al. (1993)
	<i>A. spiniferus</i>			27.88		De Solla et al. (2003)
cis-Nonachlor	<i>C. serpentina</i>		Male-biased	Female†		De Solla et al. (2001)
	<i>T. scripta</i>	7.2				Willingham and Crews (1999)
	<i>A. mississippiensis</i>	0.07	N/A			Heinz et al. (1991)
	<i>A. spiniferus</i>				5.5	De Solla et al. (2003)
	<i>C. serpentina</i>					Heinz et al. (1991)
Chlordane	<i>T. scripta</i>	9.1	Male-biased	Female†		De Solla et al. (2001)
	<i>A. mississippiensis</i>		N/A			Willingham and Crews (1999)
	<i>A. spiniferus</i>				90	Heinz et al. (1991)
	<i>C. serpentina</i>				0.26	De Solla et al. (2003)
	<i>C. serpentina</i>				27	De Solla et al. (2001)
					73	Struger et al. (1993)

MPT, male-producing temperature; See text for explanation of abbreviations.

and alkylphenol contaminations, respectively. Alterations in the reproductive functions were associated with the contaminations of these sites.

### 5.1 Estrogenic EDCs

$17\alpha$ -Ethinylestradiol (EE<sub>2</sub>) is a synthetic estrogen used in oral contraceptive pills and can directly activate estrogen receptors. Developmental exposure to EE<sub>2</sub> at 300 ppb induced ovarian development at male-producing temperature in the American alligator, *A. mississippiensis* (Matter et al., 1998) (Table 3). EE<sub>2</sub> was measured at 20–78 ppb concentrations in wild fish in Canada (Al-Ansari et al., 2010) and 0.031 ppb in wastewater treatment plant effluent (Tang et al., 2021).

BPA is also a synthetic compound used in polycarbonate plastics, epoxy resins, and dental materials, and it has weak estrogenic activity (Vom Saal et al., 2012). Exposure of *Caiman latirostris* to 140,000 ppb BPA at male-producing temperature induced ovarian development, but the histology of BPA-induced ovaries was disorganized (Stoker et al., 2003). BPA exposure at 10 ppb induced an ovary-like structure, but there was no sex reversal in the painted turtle (Jandegian et al., 2015). Environmental concentrations of BPA were measured at 182 and 149,200 ppb in the sewage (Fromme et al., 2002) and industrial effluent (Huang et al., 2012), respectively. In the Laurentian Great Lake tributaries, the highest BPA concentration recorded was 380 ppb in the sediment (Elliott et al., 2017). Therefore, estrogenic EDCs, EE<sub>2</sub>, and BPA are unlikely to induce sex reversal in the wild, although the temperature and timing of exposure may alter the estrogenic potential of EE<sub>2</sub> or BPA *in ovo*.

### 5.2 Nonestrogenic EDCs

A nonestrogenic EDC, *p,p'*-DDE, is a metabolite of dichlorodiphenyltrichloroethane (DDT) and is a persistent organic pesticide. It is known to have antiestrogenic or antiandrogenic effects; however, the molecular mechanisms of these actions are not fully understood. A primary contaminant in Lake Apopka, *p,p'*-DDE, was tested in *A. mississippiensis* embryos (Milnes et al., 2005). An exposure to 100 ppb *p,p'*-DDE induced more females at the lower pivotal temperature, although this effect was not observed at male-producing temperatures (Milnes et al., 2005). In *T. scripta*, *p,p'*-DDE exposure at 5.7 ppb also induced ovarian development at male-biased temperatures (Willingham & Crews, 1999). *p,p'*-DDE is highly persistent and bioaccumulative in the reptilian egg, and *p,p'*-DDE concentrations were 267–5800 ppb in reptilian eggs (De Solla et al., 2001; Heinz et al., 1991; Salvarani et al., 2023; Struger et al., 1993) (Table 3). These results suggest that

temperature enhances the effect of *p,p'*-DDE exposure on ovarian induction at environmentally relevant concentrations, and environmental *p,p'*-DDE at a pivotal temperature has a potency to alter the sex ratio of reptiles in the wild. Other persistent organic pollutants (POPs), including 2,3,7,8-tetrachlorodibenzodioxin (TCDD), polychlorinated biphenyl (PCB), *trans*-Nonachlor, *cis*-Nonachlor, and Chlordane, also induced ovarian development at a male-biased temperature in the lab (Table 3). These POPs were measured in reptilian eggs at concentrations higher than those needed to induce ovarian development. Therefore, environmentally relevant POP exposures have the potential to skew the sex ratio of reptiles in the wild.

Due to their life history and site affinity, reptiles often serve as prominent sentinel species of pollutants in local environments. However, the complexity and plasticity of their sex determination currently prevent a comprehensive understanding of the effects of EDCs on sex ratios in reptiles. With a better understanding of the baseline biological systems, we could fully assess the impact of EDCs in reptiles.

## 6 ECOLOGY AND EVOLUTION

### 6.1 Ecological relevance: TSD under natural temperatures

Historically, TSD has been studied using incubation temperatures that fail to mimic the complexities of natural incubation conditions (Bowden et al., 2014). The majority of laboratory TSD research has relied on constant incubation temperatures. In natural systems, however, incubation temperatures can vary considerably because of diurnal temperature cycles, prevailing weather, and season, in addition to specific nest characteristics that effectively filter the ambient conditions embryos experience (e.g., nest depth, vegetative shade, nest material type, and oviposition date). While failing to capture the thermal variation experienced by embryos in natural nests has hindered our ability to predict sex ratios in the field, advances from variable incubation studies in the last decade are starting to close this gap. Here, we summarize the approaches used to study TSD under more natural incubation temperatures, the physiological insights these studies have provided, and the modeling advances in sex ratio predictions that have been spurred.

While constant temperature incubation regimens have fallen out of favor, their importance to the foundation of our present-day understanding of TSD cannot be overstated. Constant temperature studies allowed SDMs to be mapped onto phylogenies, the characterization of different patterns of TSD, and descriptions of key features of sex ratio reaction norms like the pivotal temperature. Even in the heyday of constant temperature incubations, researchers systematically introduced temperature variation in the form of constant

temperature shifts. In this incubation regimen, eggs experience a constant temperature for most development and a second constant temperature for a subset of development. Though these conditions do not mimic natural systems, they helped broadly demarcate the TSP and allowed for some of the first comparisons of the TSP across species (Figs. 1 and 2). However, as the field of TSD grew, the need for more ecologically relevant incubation studies became increasingly apparent.

The main source of variation that has been captured in incubation studies to date is diurnal temperature fluctuation. Though diurnal temperatures were not regularly used in incubation studies until about 2010, the importance of diurnal temperatures to sex determination has been appreciated since the early 1980s. Paukstis et al. (1984) provided one of the earliest attempts to simulate diurnal temperatures by holding eggs at a constant 18 and 31°C for 4 h each with steadily ramping temperatures for 8 h in between each extreme. As incubation technology has advanced, so has the precision of simulated diurnal temperatures. These incubation regimens are characterized by symmetrical, sinusoidal temperature fluctuations on a 24-h cycle for the entirety of development. The use of these diurnal conditions impelled arguably one of the biggest revolutions in the study of TSD since its discovery in reptiles by Charnier in 1966 (Charnier, 1966).

Sex ratios produced under variable temperatures do not match the sex ratios produced under constant temperatures of the same average. To better understand why fluctuating temperatures produce different sex ratios from constant temperatures, several characteristics of the embryos' developmental patterns must be considered (Georges et al., 2004; Shine & Harlow, 1996). 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 median temperature (i.e., the pivotal temperature). Temperatures above or below the range conducive to development will have no impact on sexual differentiation because development is arrested when temperatures fluctuate outside of this range. Second, 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).

Models have been developed to evaluate the influence of natural thermal regimens of nests on offspring sex ratios (Georges, 1989; Georges et al., 2005, 2004). 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. The key predictor of sex ratios from natural nests is not the mean temperature or its variance but the temperature above and below which half of the development occurs, calculated daily. This predictor statistic, called the constant temperature equivalent (CTE), produces temperature values that permit sex ratio comparisons between diurnally fluctuating temperatures and constant temperatures (Georges, 1989; Georges et al., 2004, 2005). Though developed for species with a single pivotal temperature, the CTE may also aid sex ratio predictions in FMF species, provided incubation temperatures do not drop into the cooler range of female-producing temperatures (Massey et al., 2019). The CTE performs best when there is homogeneous thermal variation around a stationary mean (Carter et al., 2018; Georges, 1989; Georges et al., 1994). However, because daily fluctuations and seasonal trends in nest temperatures prevent a constant rate of embryonic development, delimiting the bookends of the TSP in nature—and therefore, the broadest subset of temperatures that should be used to calculate a CTE to predict sex ratios—is not straightforward.

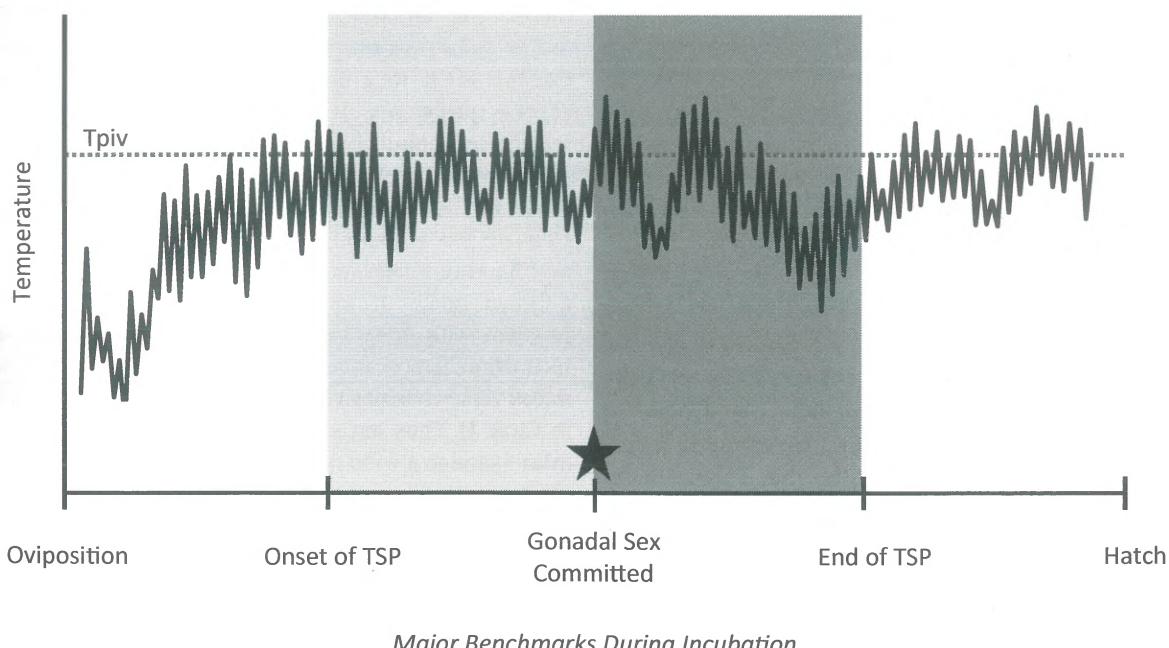
Increasingly, studies of TSD under variable incubation temperatures in the lab and field have emphasized the importance of more precisely predicting sex ratios using only the subset of temperatures contributing to sex determination (Breitenbach et al., 2020; Carter et al., 2018; Carter, Bodensteiner, et al., 2019; Carter, Paitz, & Bowden, 2019; Girondot et al., 2018; Massey et al., 2019). Most of these efforts have focused on the TSP. Defined using constant temperature shift incubations, the TSP is a population-level trait that describes the developmental period when sex can be influenced by temperature. The TSP roughly coincides with the middle third of development, from the onset of gonad development to the oldest stage at which sexual fate can be committed. Under variable temperatures, the TSP is a moving target since embryonic growth rates are temperature-dependent (Georges et al., 2005; Girondot et al., 2018). Modeling development rates under variable temperatures have aided TSP predictions by reflecting that warmer temperatures shorten and cooler temperatures lengthen the TSP by a few days depending on the specific conditions, respectively (Girondot et al., 2018; Girondot & Kaska, 2014). While better delimiting the TSP under variable temperatures has aided sex ratio predictions from natural temperature traces, gains in predictive power remain modest (Massey et al., 2019). This limitation is likely because the precise developmental stage at which sexual fate is committed within the TSP can vary significantly from one individual to the next (Carter et al., 2018; Carter, Bodensteiner, et al., 2019; Carter, Paitz, & Bowden, 2019).

While the TSP sets limits for the developmental stages over which sex can be determined in a *population*, the point at which an *individual's* sex is irreversibly committed can occur at nearly any developmental stage within the TSP, complicating sex ratio predictions. By shifting eggs from a diurnally fluctuating male-producing temperature to a diurnally fluctuating female-producing temperature for short periods of incubation, Carter et al. (Carter et al., 2018; Carter, Bodensteiner, et al., 2019; Carter, Paitz, & Bowden, 2019) demonstrate that gonadal fate can be triggered in as few as 5 days at a female-producing temperature within the broader ~20-day TSP. Moreover, after 8 days at a female-producing temperature, gonadal fate was committed in roughly half of the *T. scripta* hatchlings. Accordingly, sex ratio estimates that fail to ignore the temperatures irrelevant to sex determination, including those within the TSP that follow gonadal commitment, will be unreliable predictors of sex ratios (Carter et al., 2018; Carter, Bodensteiner, et al., 2019; Carter, Paitz, & Bowden, 2019) (Fig. 5). Outside of the initial attempts by Carter et al. (2018), there is no model that reliably predicts when gonadal fate is committed as a function of temperature and delimits the

narrowest subset of temperatures that should be used to predict sex ratios within the TSP. Such an endeavor will require careful integration of underlying mechanisms (e.g., temperature-dependent action of *kdm6b*) to predict this cryptic trait (Weber et al., 2020). In addition to aiding sex ratio predictions, further exploration of gonadal commitment as a function of prevailing incubation temperature may also provide new insights into the responses of TSD species to climate change, for example, by revealing novel targets of natural selection (Carter et al., 2018, Carter, Bodensteiner, et al., 2019, Carter, Paitz, & Bowden, 2019).

## 6.2 Adaptive significance of sex-determining mechanisms

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



**FIG. 5** A hypothetical example illustrating the importance of considering when gonadal fate is committed within the thermal sensitive period (TSP) for sex ratio modeling. TSP delimits the developmental stages when sex can be influenced by incubation temperature (highlighted by the combination of light and dark gray shading). The TSP can be estimated using temperature traces based on species- or population-level temperature-dependent development rates. At the individual level, however, gonadal fate can be committed at any point within the TSP (star symbol) and is highly influenced by the prevailing temperatures and an individual's thermal sensitivity (Carter et al., 2018; Carter, Bodensteiner, et al., 2019; Carter, Paitz, & Bowden, 2019). In this example, temperatures shaded in light gray presumably contribute to the sex determination of this individual, whereas temperatures shaded in dark gray do not, despite falling within the TSP. At present, most sex ratio models use temperatures across the entire TSP to predict sex ratios and, thus, often incorporate temperatures that do not actually contribute to sex ratios in many individuals. Though highly cryptic, understanding when and how gonadal fate is committed at the individual level within the TSP (and the amount of variation in this trait among individuals) should help advance sex ratio modeling.

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 the 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, shifting the selective pressures operating on SDMs. In such cases, maternal control over the sex ratio will enhance parental fitness by enabling the over-production of the sex that benefits most from prevailing conditions (Cockburn et al., 2002; Trivers & Willard, 1973). For example, many female adders (*Vipera berus*) in a Swedish population produce only one litter in their lifetimes, consisting 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 (Calsbeek & Bonneaud, 2008; Calsbeek & Sinervo, 2004; Lovren & Wade, 2003; Madsen & Shine, 1992), 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 the diversity of SDMs in reptiles.

Sex allocation theory (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 is enhanced if the sex best suited to given conditions is over-produced (Charnov, 1982; Trivers & Willard, 1973). These ideas have been extended by Charnov and Bull (1977) to explain when selection will favor ESD over GSD. Regarding TSD, their model proposes that if male and female offspring have different optimal developmental temperatures, selection will favor 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 (Andrews et al., 2000; Van Damme et al., 1992), and some of these effects are long-lasting (Elphick & Shine, 1998; Freedberg et al.,

2004). If those traits differentially impact the fitness of sons versus daughters (Gutzke & Crews, 1988; Warner & Shine, 2005), then 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).

How selection matches the sex and developmental temperature of offspring 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 4, and since previous reviews have covered these at length (Janzen & Phillips, 2006; Shine, 1999; Valenzuela, 2004a), they will not be detailed here. Despite numerous hypotheses for the adaptive value of TSD, adequate empirical support has yet to be offered, 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 not well suited for lifetime fitness studies. The vast majority of research on this topic has been conducted on turtles and crocodilians (Janzen, 1995; Joanen et al., 1987), and measuring the reproductive success (fitness) of late-maturing, long-lived species is logically difficult. (2) Offspring sex is often brutal to identify without sacrificing animals, which would preclude any measurement of fitness. (3) The effects of incubation temperature and offspring sex are naturally confounded. 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 versus females via multiple complex pathways (shown in Table 4). Thus, any attempt to test the Charnov-Bull model must examine a wide range of variables, posing a substantial challenge to comprehensive empirical analysis.

Studies on an Australian lizard (*A. muricatus*) with TSD have 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 occurred 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 were hatched, the offspring were harmlessly sexed by manual eversion of hemipenes on males and then raised under seminatural conditions among six replicated field enclosures for 4 years. Because *A. muricatus* matures within

**TABLE 4** Summary of seven differential-fitness hypotheses that fall within the Charnov-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 sizes 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	Morjan et al. (2003) and Roosenburg (1996)
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	Gutzke and Crews (1988), Langkilde and Shine (2005), Rhen and Lang (1995), and Warner et al. (2010)
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	Elphick and Shine (1999), Shine et al. (1995), Shine, Elphick, and Harlow (1997), and Warner and Shine (2008a)
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	Conover (1984), Harlow and Taylor (2000), and Warner and Shine (2005)
5.	Natal homing and nest-site philopatry	Induces variation in thermal quality of nesting sites	Females return to natal nest sites for oviposition, and fitness of daughters is enhanced when hatching at natal site, but male fitness is unaffected	Reinhold (1998) and Valenzuela and Janzen (2001)
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	Ewert and Nelson (1991), Head et al. (1987), and Janzen and Paukstis (1991b)
7.	Differential mortality	Induces embryonic mortality, but differently in male versus female embryos	Female and male embryos have different sensitivities to incubation temperature	Burger and Zappalorti (1988) and Eiby et al. (2008)

1 year of hatching and has a short life span (likely 3–4 years) relative to other TSD reptiles, the authors were able to measure the near-lifetime reproductive success of the offspring 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 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 produce exclusively 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 4 are needed. Although all hypotheses have not been tested, the current data on *A. muricatus* support 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 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, Radder, & Shine, 2009; Warner, Uller, & Shine, 2009); this

pattern is similar to the scenario seen in the Atlantic silverside fish whereby earlier hatching benefits females more than males (Conover, 1984; Warner, Radder, & Shine, 2009; Warner, Uller, & Shine, 2009). Nevertheless, in *A. muricatus*, the interactive effect of sex and incubation temperature on offspring fitness remains significant whether or not analyses are corrected for the timing of hatching (Warner & Shine, 2008a), which suggests 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, Radder, & Shine, 2009; Warner, Uller, & Shine, 2009). 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, Radder, & Shine, 2009; Warner, Uller, & Shine, 2009); 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). Models suggest that TSD may be maintained in long-lived species despite the negative impacts of extreme sex-ratio fluctuations, 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 have also been proposed for different taxa (see Valenzuela, 2004a).

## 7 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 their evolutionary significance. This review touched on many aspects of these issues as we have attempted to provide a consensus of where the field stands, emerging directions, and what gaps need to be filled. Here, we will reiterate some of the emergent directions of the field and point out significant gaps of inquiry where future research should be directed.

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 (Holleley et al., 2015; Sarre et al., 2004; Valenzuela et al., 2003). Commonalities in proximate mechanisms and direct evidence of the co-existence of both SDMs within populations suggest greater complexity in sex-determining systems than 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. A continuous view makes less of a distinction among SDMs, providing a logical understanding of why commonalities exist in TSD and GSD systems, which may provide insights into why evolutionary transitions between SDMs have occurred so frequently in reptiles. More empirical and theoretical research is urgently needed to understand further the frequency and stability of co-existing SDMs in natural populations.

High-throughput RNA sequencing and epigenetic analyses have provided insights into 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 steroid hormones 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 the maternal allocation of steroid hormones into yolk and 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 (i.e., agamid lizards) will facilitate such research, but work is also needed on longer-lived species (e.g., turtles and crocodilians) to evaluate generalities or lack thereof. These experiments will provide critical insights into how Charnov-Bull (Charnov & Bull, 1977) predictions explain the adaptive significance of TSD in nature. Furthermore, sophisticated models are needed to predict offspring sex ratios from natural nest temperatures successfully. Such an endeavor will benefit from integrating, at least conceptually, advances from our understanding of the putative mechanisms underlying sex determination (e.g., temperature-sensitive action of *kdm6b*). Lastly, in the face of human-induced pollution, 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.

One anthropogenic effect is pollution, including EDCs. It is imperative to analyze the contaminants in the eggs to evaluate their effects on sex determination in reptiles in natural systems. Further investigation can connect EDC occurrences in the wild and laboratory observations. However, alterations in the sex ratio at the contaminated sites have not been elucidated due to difficulties in assessing the sex of hatchlings in noninvasive ways. Red-eared slider and loggerhead hatchlings could be sexed using a drop of blood in Western blots of AMH protein (Tezak et al., 2020). This method allows us to noninvasively assess the sex ratio of hatchlings of reptiles, although the methods need to be improved in sensitivity and applicability for various TSD species. Without evaluating the sex ratios of hatchlings in the wild population of reptiles, the effects of EDCs on sex determination would not be assessed thoroughly.

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 SDMs.

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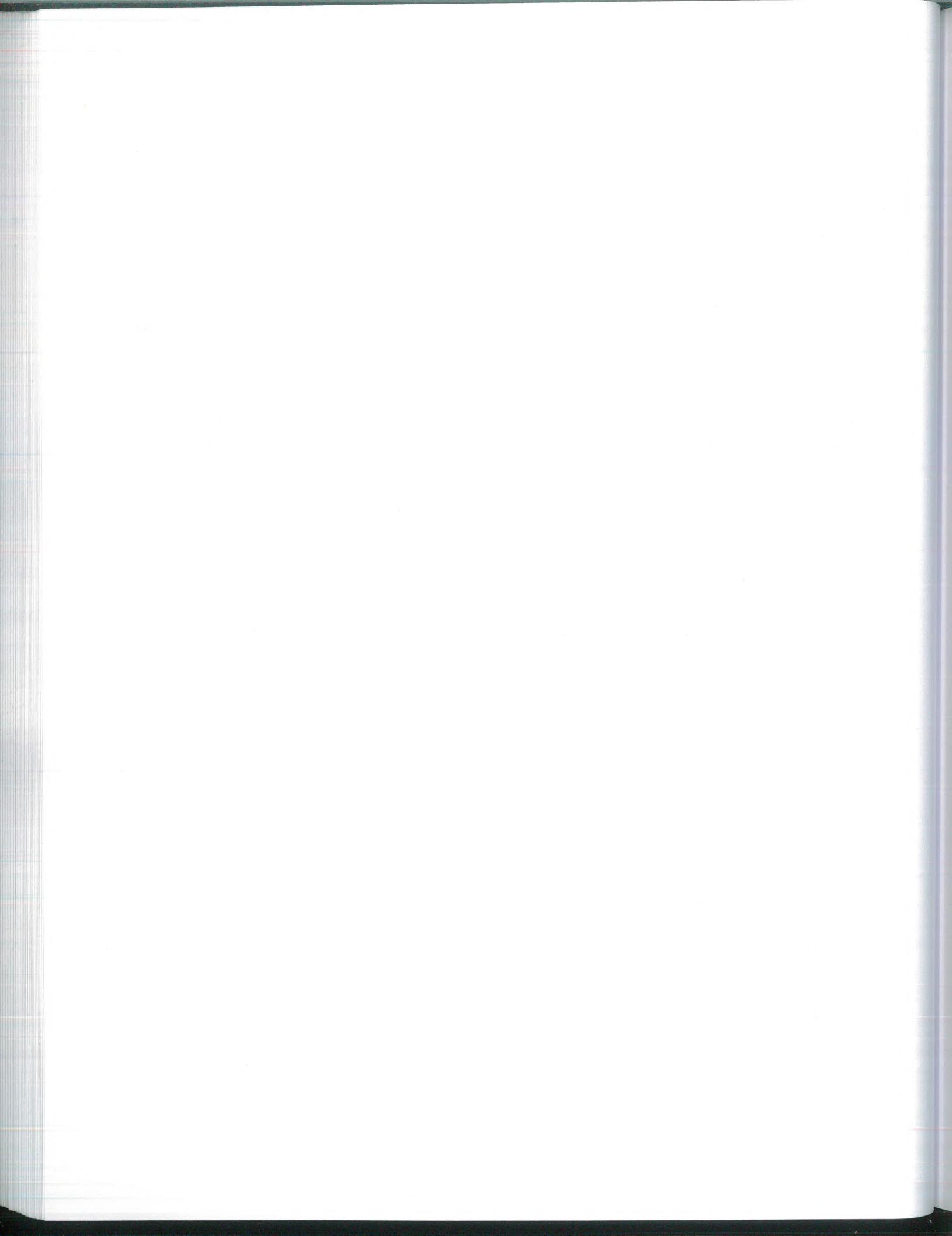
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## Chapter 2

# Neuroendocrinology of reptilian reproductive behavior

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## ABBREVIATIONS

AH-POA	anterior hypothalamus-preoptic area
AmbX	nucleus ambiguus
AMY	amygdala
AR	androgen receptor
BS	breeding season
CORT	corticosterone
DHT	5 $\alpha$ -dihydrotestosterone
E <sub>2</sub>	estradiol
ER $\alpha$	estrogen receptor alpha
GnRH	luteinizing hormone releasing hormone
NBS	nonbreeding season
NS	nucleus sphericus
P <sub>4</sub>	progesterone
POA	preoptic area
PR	progesterone receptor
RPM	retractor penis magnus
SC	sphincter cloacae
T	testosterone
TH	thyrotropin-releasing hormone
TPN	transversus penis
VMH	ventromedial hypothalamus

## 1 INTRODUCTION

With their remarkable diversity of sexual behavior, morphology, and physiology, reptiles provide ideal opportunities for addressing many questions regarding the neuroendocrinology of reproduction. A particularly useful strategy for identifying factors critical in reproductive evolution involves comparisons at multiple taxonomic levels, across diverse organisms that exist under a variety of ecological and physiological constraints. At the broadest level, evaluating traits across vertebrate groups facilitates our understanding of which mechanisms are fundamental and which have evolved based on particular features of organisms and their environments. Comparisons within animal classes allow the examination of organisms that

differ in general features of reproduction, offering more direct ways of testing hypotheses on the function or regulation of those features. Finally, studies within species can be especially valuable in determining relationships between structure and function and the mechanisms controlling them. These intraspecific investigations might include analyses between males and females, among morphs within a sex in a polymorphic species, and across seasons within individuals.

Reptiles offer a spectacular set of natural experiments. These species exhibit an impressive degree of variation in many behavioral traits, including foraging modes, antipredation strategies, and thermoregulatory behaviors, but none are more diverse than the traits associated with reproduction in this group (e.g., Van Dyke et al., 2020). In particular, courtship and copulatory strategies, modes of sex determination, and types of gestation provide exceptional models for understanding the factors that regulate reproduction. In addition, many squamates, and lizards in particular, can readily be studied in both their natural environments, which allow the full range of natural behavior, and in laboratory settings, which allow more controlled manipulations.

Despite this diversity, there remain relatively few reptilian model systems for which we have extensive information regarding the neuroendocrine regulation of reproductive behaviors. The reptile with the longest and most extensive history of neuroendocrine study is the green anole (*Anolis carolinensis*, Fig. 1). Publications describing the behavior of this species date as far back as the 1800s (e.g., Monks, 1881), and substantial work on its endocrine control began in the 1940s (Evans & Clapp, 1940; Noble & Greenberg, 1941a, 1941b). Investigations on the neural and muscular systems critical for the display of reproductive behaviors started about 30 years later and continue today, focusing on the integrated field of neuroendocrinology. Although more recent studies of neuroendocrine mechanisms in



**FIG. 1** Male *Anolis carolinensis*, Louisiana, USA. (Photo by M. Johnson.)

reptiles have encompassed a broader taxonomic range, *A. carolinensis* remains the species for which the largest quantity of detailed and diverse information is available. Therefore, we have framed the information presented in this chapter in the context of this species, including relevant material from other reptiles as available.

This chapter primarily focuses on the features associated with displays of sexual behavior, drawing mostly from research on the species that have been most extensively studied. We consider behavioral displays in their ecological contexts, associated seasonal and hormonal changes, the muscles and elements of the nervous system critical for the production of the behaviors, and the development and plasticity of reproductive traits. Finally, we attempt to integrate across organismal levels and discuss relationships among morphology, behavior, and the endocrine and other mechanisms regulating these traits. Aggression and dominance (and the stress associated with these social behaviors) are also critical components of reproductive success that involve neuroendocrine mechanisms, including an important role for neurotransmitters [e.g., serotonin and dopamine (reviewed in Overli et al., (2007))]. This literature is described in detail in Tokarz and Summers (2011) and Korzan and Summers, Chapter 7 (this volume); the focus here is on the sexual behaviors themselves.

## 2 BEHAVIORAL DISPLAYS IN ECOLOGICAL CONTEXT

### 2.1 *Anolis* lizards and the ecology of visual reproductive displays

*A. carolinensis* is a member of the highly diverse and speciose genus *Anolis*, or anoles. Anoles form a group of approximately 400 arboreal lizard species whose members occur throughout the southeastern United States, the Caribbean, and Central and South America (Nicholson et al., 2005; Schwartz & Henderson, 1991), in addition to an invasive range that includes tropical and temperate regions around the globe [e.g., Hawaii (Mautz & Shaffer, 2011), Japan (Sugawara et al., 2015)]. In general, anole species naturally occurring in the Caribbean have been studied more extensively than those on the mainland, with the exception of the well-studied *A. carolinensis*, whose native range extends throughout the southeastern United States. Robust phylogenies exist for the *Anolis* clade (e.g., Poe et al., 2017), and the genomes of *A. carolinensis* (Alföldi et al., 2011; Eckalbar et al., 2013) and recently, the brown anole, *Anolis sagrei* (Geneva et al., 2022), have been sequenced and annotated. Thus, this is a group well-positioned for comparative and molecular studies of behavior.

Among anoles, structures involved in reproduction are highly variable. In particular, the morphology of an extendable throat fan called the dewlap is remarkably diverse, with most species exhibiting a unique combination of dewlap color, pattern, and size (Nicholson et al., 2007). The dewlap is a structure involved in courtship and other social communication, and the variation of sexual dimorphism in dewlap morphology is extensive. Although in most species of anoles, the dewlap is substantially larger in males than females, species also exist in which the two sexes have similarly sized dewlaps (in some cases diminutive in both males and females, whereas in others the dewlap is sizable in both sexes; Johnson & Wade, 2010). The natural experiments offered by the diversity in ecology and morphology across anole species provide a unique opportunity for a wide variety of investigations on the function and regulation of dewlap displays in reproduction and other contexts.

Despite the impressive diversity of anoles, comprehensive data on male and female reproductive behaviors and the neural circuits that produce them are most extensively available for *A. carolinensis*. These seasonally breeding lizards reproduce in the field from approximately April through July; long days and warm temperatures induce testicular and ovarian growth (Licht, 1967a, 1967b; see also Lovren, Holmes, & Wade, 2004). The gonads become refractory to environmental conditions in late summer, followed by a period of modest testicular (although minimal

ovarian) growth during the winter. Outside of the breeding season (BS), individuals aggregate in concealed locations but may bask on warm days. Growth of the gonads is completed in the early spring, and secretions of these organs activate the display of sexual behaviors.

The sequence of social behaviors displayed by male green anoles is stereotyped and easily observed in both field and laboratory conditions. Male courtship displays consist of headbobs, push-ups, and extensions of the bright reddish-pink dewlap (Crews & Greenberg, 1981; Decourcy & Jenssen, 1994; Greenberg & Noble, 1944). Female green anoles may respond to (or initiate) courtship by bobbing their heads in a manner similar to males, but generally without dewlap extensions (Nunez et al., 1997). Males also perform similar displays in aggressive encounters with conspecific males, and more rarely, females occasionally perform aggressive bobbing displays toward males or females. As aggression escalates, dewlap extension, headbob, and push-up displays are often accompanied by the formation of a black spot behind each eye, the raising of a crest along the back, and postural changes (Greenberg, 2003); these modifiers are rarely used in the context of courtship.

If a female is receptive to (or perhaps solicits) copulation, she will remain stationary and allow the male to mount, and she will often bend her neck to facilitate his grasping the skin over the neck between his teeth (Crews, 1980). The male green anole then will mount the female, maneuver his tail under hers, evert one of two hemipenes from inside his cloacal vent, intromit, and ejaculate (Crews, 1980). This possession of bilateral, independently controlled hemipenes is typical of squamates, and males often alternate which hemipenis is used in consecutive intromissions (Shine et al., 2000).

## 2.2 Reproductive communication in other reptilian taxa

### 2.2.1 Differences between males and females

Courtship behaviors provide key information related to mate selection. The modes of communication used in courtship are species-specific and extremely variable among reptiles, in terms of signal complexity and the sensory systems associated with interpretation of the displays [reviewed in Johnson et al. (2019)]. Most iguanian lizards (including anoles) communicate primarily through visual signals [reviewed in Ord et al. (2002)], but many reptiles primarily use chemical and/or auditory signals to identify and attract potential mates, and to detract rivals.

In red-sided garter snakes (*Thamnophis sirtalis parietalis*), male courtship is facilitated by a female pheromone. Interestingly, some males produce this pheromone, potentially

allowing them to gain access to females in mating aggregations and increase their reproductive competence (Mason & Crews, 1985). The composition of the pheromone differs among individuals, allowing males to discriminate between large and small females (LeMaster & Mason, 2002), as well as identifying females from their own versus another population (LeMaster & Mason, 2003).

Male leopard geckos (*Eublepharis macularius*; a species in which sex is determined by egg incubation temperature) also use chemical signals during courtship. When approaching a female, a male will explore for female pheromones by licking the substrate or the air (i.e., performing “tongue-flicks”), a behavior common among chemical-sensing reptiles. Male leopard geckos can also recognize individual females based on these chemical cues (LaDage & Ferkin, 2006). When females are detected, male geckos use auditory signals as well, generating a buzzing sound by rapidly vibrating their tails (Godwin & Crews, 2002).

Individuals of several genera, including *Sceloporus*, *Aspidoscelis*, and *Urosaurus*, transfer sex pheromones from femoral pores to the substrate. These chemicals can send a variety of messages important in mate choice. For example, in female *Sceloporus graciosus*, the sagebrush lizard, femoral pore secretions can communicate physiological condition and reproductive status (Martins et al., 2006) or receptivity, as females produce more secretions when exposed to a male performing courtship displays than a male performing aggressive displays (Kelso & Martins, 2008). Femoral gland secretions from males of the Iberian rock lizard (*Lacerta monticola*; Martin et al., 2007) and the lizard *Liolaemus monticola* (Labra, 2006) indicate social dominance status.

The ability to perceive different types of sensory information in courtship may also be associated with differences in brain morphology and activity, as suggested by the differences exhibited between male and female *Podarcis hispanica* and *Podarcis liolepis*, species in which males have larger accessory olfactory bulbs, and in parallel are more responsive to chemical cues, than females (Font et al., 2012; Sampedro et al., 2008). The same pattern appears across lizard species. In an analysis of brain morphology across a group of six species (two of which were primarily visual, two primarily chemical, and two that used both visual and chemical communication) species that performed higher rates of visual displays had a denser lateral geniculate nucleus (a region involved in processing color and spatial information), and species that used a higher proportion of chemical displays had larger somas in the nucleus sphericus (a region that receives projections from the vomeronasal organ; Robinson et al., 2015).

Parallel to the diversity in courtship structures, the morphology of male copulatory organs (hemipenes) is strikingly

diverse among many reptilian species, particularly in snakes (Dowling & Savage, 1960) and lacertids (e.g., Arnold, 1986), in which the structures may be covered with features such as spines, ridges, and fleshy protuberances. In fact, in *Anolis* lizards, hemipenis morphology evolves faster than other rapidly evolving nongenital traits (limb length, dewlap size, and body size; Klaczko et al., 2015). Yet, copulatory behavior across reptiles appears generally conserved. The function of copulation—sperm delivery from the male to the female—is identical across species, and morphological constraints on this function have likely directed the evolution of copulatory behavior as well. Despite this, the duration of copulation varies widely across species, although outside of anoles (Martin et al., 2021), few data are available on the timing of copulation.

### 2.2.2 Variation within sexes: Alternative reproductive strategies

The taxa described above provide many opportunities to compare reproductive structure and function between males and females, but reptiles also allow for examinations of the differences among groups *within* a sex. For example, males within some species exhibit generally nonoverlapping phenotypic variation (with differing phenotypes often called “morphs”). In such species, one morph frequently displays the full range of male-typical morphological and behavioral traits and another is more female-typical in some of these traits [reviewed in Knapp (2003) and Stuart-Fox et al. (2021)]. All male types are generally able to produce functional sperm, but, as described below, they may use different behavioral tactics to achieve reproduction (Knapp, 2003). Male types, or morphs, are often given descriptive names by the scientists who study them (e.g., territorial, satellite, sneaker), but we should be cautious in interpreting their behaviors solely within the social context of these descriptions (see Kamath & Wesner, 2020). For example, terms such as “satellite” assume that the social structure of the population is defined by the “territorial” males, and “sneakers” are generally interpreted to “steal” copulations from those territorial *males*, a description that ignores any role a female may play in the copulation process. In this chapter, we use the descriptions of male types from the scientific articles we cite, but we suggest that readers consider the potential limitations of traditional terminology.

Two species of iguanid lizards in particular have become model systems in which to examine relationships between reproductive behavior and neuroendocrine traits in alternative morphs—*Urosaurus ornatus*, the tree lizard, and *Uta stansburiana*, the side-blotched lizard. *U. ornatus* male morphs are distinguished by their dewlap coloration. Although up to nine variations exist, most populations of this species have two general types: males with orange-blue dewlaps, which aggressively defend territories that typically

overlap the home ranges of multiple females, and males with orange dewlaps, which are less aggressive and may switch between sedentary satellite and nomadic behavior (Moore et al., 1998). Results from a common garden experiment (an experiment in which all individuals are raised in a common, controlled environment) indicate that morph type is genetically based (Thompson et al., 1993), and behavioral and morphological differences between morphs appear very early in male development (Moore et al., 1998). Yet, more recent work has questioned whether *U. ornatus* throat color can be classified into discrete groups, reporting that color traits (as quantified via spectrophotometry) are highly repeatable, yet continuous in both males and females (Paterson & Blouin-Demers, 2017). Whether or not morphs in this species are discrete, the covariation in color and mating behaviors offer a system that is useful in exploring the mechanisms underlying variation in reproductive traits.

Male *U. ornatus* exhibit behavioral courtship displays similar to those of *Anolis* lizards, including dewlap extensions, pushups, and lateral compression, which present throat coloration and bright blue ventrolateral (belly) patches. Independent of morph, the size of this patch predicts bite force, an important indication of fighting ability (Meyers et al., 2006). Larger belly patches are preferred by some female *U. ornatus* (Hamilton & Sullivan, 2005), but female preferences for male traits can differ between females with yellow or orange throats (Lattanzio et al., 2014).

*U. stansburiana* males exhibit three alternative morphs, also distinguished by dewlap color: orange, blue, and yellow-throated males, that differ in reproductive and aggressive behaviors (Hazard et al., 2019). Orange males are aggressive and defend territories encompassing multiple females; less aggressive blue males diligently guard females on their territories; and yellow males are nonterritorial sneakers, or deceptive female mimics that obtain copulations when the other males are inattentive (Sinervo & Clobert, 2003; Sinervo & Lively, 1996; Sinervo, Miles, et al., 2000). These morphs are genetically determined, and multiple lines of evidence indicate that this variation is based on a single Mendelian gene (Sinervo et al., 2001). The three morphs are maintained because of frequency-dependent selection that favors the rarest morph in a classic rock-paper-scissors “game” in which each morph can be reproductively out-competed by another (Zamudio & Sinervo, 2000).

The same locus that determines male morphs in *U. stansburiana* also determines female reproductive strategies that differ in resource allocation to offspring. Orange-throated females have been described as r-strategists, laying larger clutches of smaller eggs, and yellow-throated females are K-strategists, laying smaller clutches of larger eggs

(Sinervo & Licht, 1991; Sinervo, Svensson, & Comendant, 2000). Multiple, conflicting selective pressures play a role in the tradeoffs experienced by different morphs, contributing to the maintenance of rare morphs in a population (Lancaster et al., 2014).

Males of the lacertid lizard *Podarcis melisellensis* may exhibit reproductive morphs based on throat (and abdomen) color as well. White, yellow, and orange males differ from one another in body size and bite force, but these males do not differ in activity budgets (Huyghe et al., 2007). These differences may underlie different reproductive strategies (Huyghe et al., 2007), although morphs do not differ in reproductive success (Huyghe et al., 2014).

Several species exhibit size-based male phenotypes determined largely by male age; as a male grows, he adopts a different reproductive strategy. In Galapagos marine iguanas (*Amblyrhynchus cristatus*), the smallest (and youngest) males look very similar to females, and because they may be indistinguishable from females by larger males, these small males live on territories of other males and use a sneaking strategy to mate with unattended females. As sneakers grow larger, they adopt a satellite strategy, living around other males' territories and attempting to mate with females as resident males leave these territories. The largest, oldest males in the population defend territories in which females live and they court these females using head-bobbing displays (Wikelski et al., 1996, 2001). Similarly, collared lizard (*Crotaphytus collaris*) males exhibit developmentally plastic reproductive strategies, as they mature from smaller, younger nonterritorial males to larger, dominant territory-holding males (Baird & Hews, 2007). Green anoles, which have been classified into "heavyweight" (large heads, large dewlaps, high bite force) and "light-weight" males [small heads, small dewlaps, weak bite force; reviewed in Lailvaux and Husak (2014)], also use a size (and likely age) based reproductive strategy such that heavyweight males defend territories and lightweight males adopt a sneaker or satellite strategy.

### 2.3 Summary

The diversity of reproductive behaviors exhibited by reptiles provides many interspecific and intraspecific comparisons of courtship and copulatory structure and function. Because all behaviors are performed in an ecological context, consideration of the social and physical environments in which reproductive behaviors have evolved is necessary for informed investigations of the mechanisms underlying these behaviors. Having described these general features of reproductive behaviors in reptiles, we will now consider the relevant mechanisms associated with their control.

## 3 HORMONAL CONTROL OF REPRODUCTIVE BEHAVIORS IN ADULTHOOD

Reproductive behaviors in many species of vertebrates are influenced by sex steroid hormones. Testosterone (T), primarily produced by the testes, and its metabolites are particularly important for males across many animal taxa. For example, increases in T in males within a species can often be associated with more sexual and aggressive behavior, higher dominance, larger or more colorful ornaments, and greater endurance [reviewed in Miles et al. (2007)]. In parallel, female behaviors such as sexual receptivity are routinely facilitated by ovarian hormones, such as estradiol (E<sub>2</sub>) and progesterone (P<sub>4</sub>) (Blaustein & Erskine, 2002; Crews & Silver, 1985).

### 3.1 Hormonal control of anole reproduction

Consistent with the general patterns described above, T activates reproductive behaviors in male green anoles. Castration diminishes courtship and copulation, and treatment with T prevents and/or reverses that effect (Adkins & Schlesinger, 1979; Crews et al., 1978; Rosen et al., 2002; Winkler & Wade, 1998). It is common among mammalian and avian species that, for an animal to produce male reproductive behaviors, T must be metabolized into E<sub>2</sub> or 5 $\alpha$ -dihydrotestosterone (DHT) within the brain (Hull et al., 2002; Ball & Balthazart, 2002). However, in green anoles T itself is likely the primary factor responsible for the activation of male sexual behaviors, as systemic treatment with E<sub>2</sub> and inhibition of E<sub>2</sub> synthesis have little or no effect on male sexual behavior (Crews et al., 1978; Mason & Adkins, 1976; Winkler & Wade, 1998). Yet some evidence exists that E<sub>2</sub> may serve a facilitatory role, such that E<sub>2</sub> treatment in conjunction with T treatment increases male mounting behavior (Latham & Wade, 2010). DHT is also not sufficient to activate the display of male reproductive behaviors, but metabolism of T into DHT is required for maximal behavioral performance (Rosen & Wade, 2000). Interestingly, the effects of these hormones depend on the season, as the ability of exogenous T to activate male sexual behavior is increased in the BS compared to non-breeding season (NBS) (Neal & Wade, 2007a). In *A. sagrei*, E<sub>2</sub> is also not effective in activating sexual behaviors in castrated males to the levels seen in intact males (Tokarz, 1986), and treatment of brown anoles with anti-androgens produces variable results: cyproterone acetate (which prevents androgen from binding to its receptor and suppresses LH) inhibits aspects of male sexual behavior, but flutamide (which also prevents androgen binding to AR) does not (Tokarz, 1987).

The most detailed description of the reproductive cycle in female green anoles indicates that individuals ovulate a single egg about every 14 days alternately from each of the two ovaries (Jones et al., 1983). More recent work suggests that eggs can be laid in the laboratory as frequently as every 7 days (Lovern, Holmes, & Wade, 2004). Regardless, this pattern results in females carrying a single egg in the oviduct through much of a cycle, but a period of a few days exists in which a shelled egg is soon to be laid while another recently ovulated one is in the early stage of development in the other oviduct. Interestingly, removal of one ovary in green anoles causes compensatory follicular development on the other side and influences hypothalamic catecholamine asymmetries, likely through both neural and hormonal signals (Jones et al., 1997). When an egg is present in each oviduct, adult female plasma levels of E<sub>2</sub> and P<sub>4</sub> are both around 2.2 ng/mL, whereas in the one-egg condition, P<sub>4</sub> levels are slightly higher and E<sub>2</sub> is substantially lower (~600 pg/mL) (Jones et al., 1983). On average, across stages of the ovulatory cycle, yolked follicles contain roughly 800 pg/mL of E<sub>2</sub>, while the level in the plasma of females carrying these follicles is less than half of that (Lovern & Wade, 2001). Levels of T are high in yolked follicles (~6 ng/mL) but far lower in the plasma of these females (less than 200 pg/mL) (Lovern & Wade, 2001).

Experimental manipulations confirm that both E<sub>2</sub> and T play an important role in activating female receptivity in green anoles. For example, E<sub>2</sub> alone can facilitate female sexual behaviors (Winkler & Wade, 1998), and sub-threshold doses of E<sub>2</sub> can prime P<sub>4</sub> to activate the behavior (Wu et al., 1985). Parallel to the effects of T on male sexual behavior, E<sub>2</sub> with P<sub>4</sub> priming is more effective in the BS than the NBS (Wu et al., 1985). Administration of the anti-estrogen CI-628 can inhibit receptivity activated by E<sub>2</sub> treatment in green anole females (Tokarz & Crews, 1980). T also facilitates receptivity in green anole females, a function at least in part due to aromatization (Winkler & Wade, 1998). Parallel to patterns observed in males, circulating T is increased in reproductively active compared to inactive females, and among breeding females, those with a large yolked follicle have increased T compared to those with one or more eggs in the oviduct (Lovern & Wade, 2001). Thus, T is maximal when females are most likely to display sexual behavior.

Prostaglandin F2α can inhibit receptivity in estrogen-treated female anoles (Tokarz & Crews, 1981). In contrast, luteinizing hormone releasing hormone (GnRH) and thyrotropin-releasing hormone (TRH) facilitate the display of sexual behaviors in E<sub>2</sub>-primed individuals (Alderete et al., 1980). Although specific mechanisms are unclear, these data suggest that the reproductive behaviors of green anole females are directly or indirectly modulated by a variety of extra-gonadal factors.

### 3.2 Hormonal control in species with alternative reproductive strategies

In *U. ornatus*, T and P<sub>4</sub> also play important roles in the activation of male reproductive behaviors (Weiss & Moore, 2004). Although male morphs differ in adult behavior, territorial orange-blue males and nonterritorial orange males do not differ in their levels of circulating T (Moore et al., 1998). The variability in behavior may be the consequence of differential sensitivity to hormones resulting from early organizational effects (Weiss & Moore, 2004; see below). In addition, a general relationship exists between T and behaviors related to territoriality, such that animals with higher levels of T exhibit higher frequency and intensity of displays (Kabelik et al., 2006), and these male behaviors are activated by both T and P<sub>4</sub> (Weiss & Moore, 2004).

Collared lizard males exhibit age- and size-related morphs (territorial vs. nonterritorial) that also do not differ in T, DHT, or the stress-related hormone corticosterone (CORT) (Baird & Hews, 2007). Although courtship behaviors are more frequent in territorial males, there is no clear activational effect of steroid hormones on these behaviors; in fact, nonterritorial males who move into a recently vacated territory increase their display rates, but exhibit decreases in T and CORT (Baird & Hews, 2007).

Unlike tree and collared lizards, side-blotched lizard male morphs differ in sex steroid hormone levels; territorial orange males have higher levels of T than blue and yellow males (Sinervo, Miles, et al., 2000). Further, experimentally increasing T in yellow and blue males causes an increase in functions typical of orange males, including higher endurance, general activity, and control over access to females (Sinervo, Miles, et al., 2000).

Hormones may also affect female reproductive strategies in side-blotched lizards. Treatment with FSH increases clutch size, but at the expense of offspring size (Sinervo & Licht, 1991), demonstrating the classic r (fewer, larger offspring) versus K (more, smaller offspring) strategy tradeoff. These results indicate that clutch size is influenced by circulating plasma GTHs (Sinervo & Licht, 1991). Further, in crowded social conditions during the BS, both yellow (K-strategist) and orange (r-strategist) females have higher levels of CORT, but yellow females have higher levels than orange females (Comendant et al., 2003). If this increase in CORT enhances the females' competitive abilities, yellow females should experience the greater benefit. However, CORT appears to provide strategy-specific benefits to both morphs, such that the hormone improves offspring quality in K-strategist females and increases reproductive rates in r-strategist females (Lancaster et al., 2007).

### 3.3 Hormonal control of reproduction in other reptilian taxa

A substantial body of work on the role of hormones in reproduction has been conducted in whiptail lizards, focusing mainly on two species, *Aspidoscelis inornatus* and *Aspidoscelis uniparens*. The former is gonochoristic (consists of both males and females); the latter is a direct descendant of *A. inornatus* and is composed only of females that reproduce by parthenogenesis (Crews, 1989). Control of male sexual behaviors in whiptail lizards is similar to green anoles. DHT, and particularly T, activate sexual behaviors in male *A. inornatus* (Wade et al., 1993). P<sub>4</sub> can also activate the display of sexual behaviors in approximately one-third of male *A. inornatus* (Lindzey & Crews, 1992). This effect can be facilitated by low doses of DHT, and this androgen is more effective in activating the behaviors in P<sub>4</sub>-sensitive compared to P<sub>4</sub>-insensitive males (Lindzey & Crews, 1992), suggesting the potential for interactions between androgens and progestins in the regulation of male behaviors in this species.

This ability of P<sub>4</sub> to facilitate reproductive behaviors in some *A. inornatus* is particularly intriguing, because the all-female *A. uniparens* also display male-like sexual behaviors (including mounting and assuming a stereotypical donut-shaped copulatory posture), and they do so predominantly during the postovulatory phase of their cycles when circulating P<sub>4</sub> is high (Moore, Whittier, Billy, & Crews, 1985; Moore, Whittier, & Crews, 1985). In contrast, they tend to display female-typical receptivity before ovulation, when they have large yolked follicles and relatively high levels of E<sub>2</sub> (Crews, 1980). The pattern of receptivity is comparable in females of the gonochoristic species, *A. inornatus*, and E<sub>2</sub> treatment of gonadectomized females of both species activates receptivity (e.g., Wade et al., 1993). Interestingly, even months after ovariectomy, T activates male-like behavior in female *A. inornatus* and parthenogenetic *A. uniparens* (Wade et al., 1993).

Like other reptiles, in leopard geckos E<sub>2</sub> activates sexual receptivity in females (Rhen & Crews, 2000). Ovariectomy in adulthood causes females to be significantly less attractive to potential mates, and treatment of ovariectomized females with T decreases their attractiveness further, to the point where many T-treated females are attacked by males (Flores & Crews, 1995; Rhen et al., 1999). These results suggest that female pheromones (the major component of attractiveness) are affected by circulating hormones. Further, unmanipulated females never perform tail vibrations, but treatment with T causes about half of females to exhibit this typical male courtship behavior (Flores & Crews, 1995).

The role of steroid hormones in female nesting behavior has also been a focus of neuroendocrine work in turtles, as these behaviors can directly affect the sex of offspring. In addition to the role of estrogens, androgens are an important factor in female reproduction, as indicated by the presence of AR in the turtle oviduct (Selcer et al., 2005). An examination of hormone levels in green sea turtles (*Chelonia mydas*) comparing females who successfully oviposited to those who built nests but did not oviposit found that P<sub>4</sub> is higher in successful than unsuccessful females, whereas T, E<sub>2</sub>, and CORT did not differ between these groups (Al-Habsi et al., 2006). Further, although nesting female green sea turtles experience clear metabolic changes, hormones generally involved in metabolism (epinephrine, norepinephrine, and CORT) did not change across nesting stages, a finding that may indicate that the actions of these metabolic hormones are modified to facilitate reproduction (Jessop & Hamann, 2004).

In contrast to many other reptiles (and other vertebrates), red-sided garter snakes (*T. s. parietalis*) exhibit a dissociated breeding pattern, in which the display of sexual behaviors occurs at a time when gonadal hormones are low. These behaviors are restricted to a few weeks following emergence from winter hibernation, a time when the gonads have not yet recrudesced and circulating steroid levels are reduced. Male courtship behavior is not activated by gonadal androgens, but rather by the increase in temperature following hibernation (Crews et al., 1984). This behavior appears to be “organized” by testicular function the previous summer (Crews, 1991). Steroid hormones are also low in females at the time of mating. In these animals, mating stimulates a neuroendocrine reflex that causes a rise in prostaglandins (Whittier & Crews, 1989) and then a surge in E<sub>2</sub> (Mendonça & Crews, 1990). Unlike males, females normally will not exhibit sexual behavior on emergence from winter dormancy if they have been gonadectomized, but treatment with E<sub>2</sub> just before emergence will reinstate receptivity (Mendonça & Crews, 1996). Further, the production of female sex pheromones is activated by E<sub>2</sub> (Parker & Mason, 2012) and inhibited by T (Parker & Mason, 2014). Thus, similar to many other vertebrates, including a variety of lizard species, T is important for male sexual behaviors, and E<sub>2</sub> activates receptivity in females. However, the timing of gonadal development and steroid hormone production is asynchronous with the actual behavioral displays.

### 3.4 Evolution of hormones and reproductive behavior

Steroid hormones (and to a lesser extent, their receptors; see Section 4), have also been measured at larger taxonomic

scales (Vitousek, Johnson, & Husak, 2018), such that the evolution of endocrine traits within reptiles is emerging as an area of exciting work. For example, in a study of 18 Caribbean *Anolis* species, Husak and Lovorn (2014) found that aggression levels in anoles were not generally associated with circulating T in the expected pattern; three of the four island assemblages studied exhibited higher T in species with lower aggression, whereas in the fourth assemblage (comprised of species in Puerto Rico), species with higher T were more aggressive than those with lower T. These results showed that similar social behaviors can evolve due to different underlying mechanisms.

In a larger comparative study of 57 species of reptiles (and 37 species of amphibians, although results for amphibians will not be discussed here), Eikenaar et al. (2012) found that average circulating levels of T in the BS were higher in reptile species with shorter BSs, and that species with higher T also had higher CORT. They interpreted this to indicate that higher T facilitates the more intense male-male competition during shorter BSs and that higher CORT supports the increased energetic demands of the short season.

As studies of hormones and behavior have accumulated, several research teams (e.g., Eikenaar et al., 2012; Vitousek, Johnson, Donald, et al., 2018) have compiled datasets of circulating hormone measures across reptiles, providing the base for a wide range of comparative studies of endocrine traits. In the recently compiled database *HormoneBase*, measures of T and CORT were collected from 91 species of free-living reptiles [3 crocodilians, 15 turtles, and 73 squamates; (Johnson, Francis, et al., 2018)]. Nonavian reptiles were underrepresented in this database compared to birds and mammals, which aimed to compile all published measures of T and CORT in free-living vertebrates up to 2015, yet reptiles were better represented than amphibians and fish (Johnson, Francis, et al., 2018). Initial comparative analyses using *HormoneBase* have focused on patterns *across* vertebrates (e.g., Vitousek et al., 2019; Husak et al., 2021). Thus, there is great potential for future evolutionary analyses exploring the relationship between steroid hormones and reproduction in reptiles, both to determine these relationships within reptiles, and to compare patterns in reptiles with those in other clades.

### 3.5 Summary

Reptilian species provide particularly valuable models for the study of the role of sex steroid hormones in reproduction for at least two reasons. Investigations of hormones in reptiles support the general importance of T for male sexual behaviors and E<sub>2</sub> plus P<sub>4</sub> for female sexual behaviors, a

pattern observed in many vertebrate taxa. However, details such as the role of particular metabolites can differ across species, allowing for exploration of their role in the evolution of diversity in hormonal mechanisms critical for the display of reproductive behaviors. These steroid hormones act primarily on neural tissues to influence reproductive behavior. Critical regions of the brain are considered next.

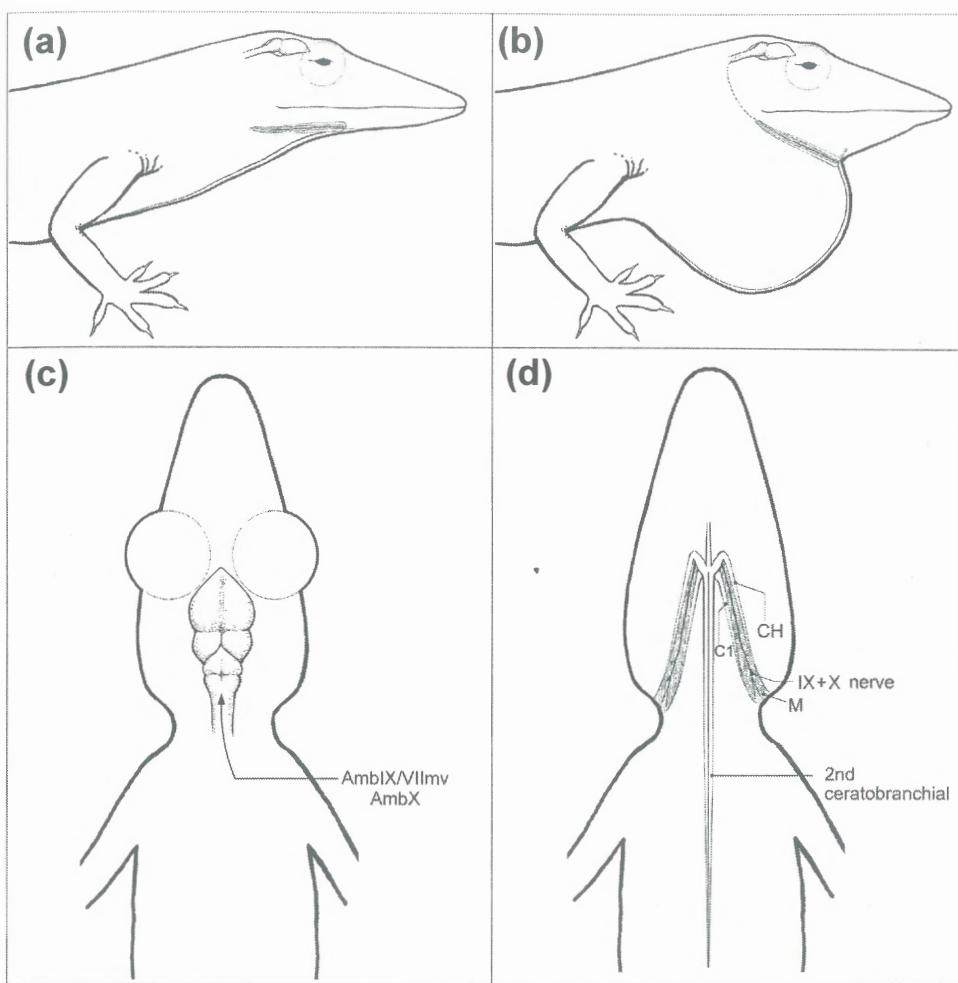
## 4 NEURAL CONTROL OF REPRODUCTIVE BEHAVIORS

Regions of the brain are highly conserved across vertebrate groups, particularly regions of the forebrain involved in producing social and reproductive behaviors, (the “social behavior network”; Newman, 1999; Goodson, 2005). Included in this network are the ventromedial hypothalamus (VMH), preoptic area (POA), and amygdala (AMY), regions with clear homologies in mammals, birds, and reptiles (Goodson, 2005). Whereas regions outside the forebrain are also involved in reproductive behavior, the forebrain is by far the most-studied region of the brain in reptiles to date.

### 4.1 Neural and muscular control of anole reproduction

In green anoles, far more is known about the neural control of male than female sexual behaviors. In the forebrain, lesion studies have shown that the anterior hypothalamus-preoptic area (AH-POA) (Wheeler & Crews, 1978) and ventromedial nucleus of the AMY (Greenberg et al., 1984; equivalent to the posterior portion of the medial AMY; Bruce & Neary, 1995) are important for male courtship and copulation. Implants of either DHT or E<sub>2</sub> into the AH-POA in gonadectomized males can facilitate courtship (Crews & Morgentaler, 1979), and peripheral administration of E<sub>2</sub> increases male mounting attempts in gonadectomized males (Latham & Wade, 2010). Manipulations with this degree of specificity have not been conducted in female green anoles, but destruction of the basal hypothalamus impairs receptivity (Farragher & Crews, 1979). A variety of sex differences in the morphology of these regions exist in green anoles, but they largely depend on the animals’ endocrine environment and are therefore discussed below (see Section 6).

The mechanics of dewlap extension have been studied in *A. carolinensis* and *Anolis equestris*. The simplicity of dewlap control makes it particularly amenable to investigations of relationships between structure and function, as well as the mechanisms involved in regulating them. Key



**FIG. 2** Components of dewlap extension in the green anole. Panels (A) and (B) show a lateral view of a male before and during dewlap extension, respectively. Panel (C) shows a dorsal view of the general location within the brain of the motoneurons that extend the dewlap. These cells exist in two regions: the vagal component of the nucleus ambiguus (AmbX) and the region containing the glossopharyngeal portion of nucleus ambiguus and the ventral motor nucleus of the facial nerve (AmbIX/VIIImv). Panel (D) provides a ventral view of the muscles and cartilage that control dewlap extension. M, ceratohyoid muscle; C1, first ceratobranchial cartilage; CH, ceratohyal cartilage. (Reprinted with permission from Hormone and Behavior, copyright 2005 (Fig. 1; Wade, J. (2005). Current research on the behavioral neuroendocrinology of reptiles. Hormones and Behavior 48, 451–460).)

structures involve three pieces of bilateral cartilage in the throat and just under the skin overlaying the chest [reviewed in Wade (2005); Fig. 2]. Movement of the cartilage is controlled by the paired ceratohyoid muscles which lie in the throat; when these muscles contract, a lever-like action is induced among the cartilage components, and the 2nd ceratobranchials (which normally lie flat, running from under the chin to mid-chest) bow out, extending the colorful fan of skin, i.e., the dewlap (Font & Rome, 1990; Bels, 1990). Motoneurons innervate the ceratohyoid muscles ipsilaterally through the combined IX+X cranial nerve. These motoneurons are located in the caudal brainstem in the vagal component of the nucleus ambiguus (AmbX) and the region containing the glossopharyngeal portion of the nucleus ambiguus and the ventral motor nucleus of the facial nerve (AmbIX/VIIImv) (Font, 1991; Wade, 1998).

Dewlap structure and function are sexually dimorphic in anoles. The dewlaps of males are generally three to seven times the size of those in females (Jenssen et al., 2000), although the degree of this sexual dimorphism differs across species (Johnson & Wade, 2010). Green anole males extend these structures more often than females do, even when administered equivalent amounts of T after castration (Winkler & Wade, 1998; also see Adkins & Schlesinger, 1979; Mason & Adkins, 1976). In parallel, the length of the 2nd ceratobranchial cartilage and cross-sectional areas of motoneuron somas and the nerve containing their axons are larger in males (Wade, 1998; O'Bryant & Wade, 1999). However, dendritic arborization of dewlap motoneurons does not differ between breeding males and females (O'Bryant & Wade, 2000). Mass of the ceratohyoid muscles is greater in males than females, which is at least partially

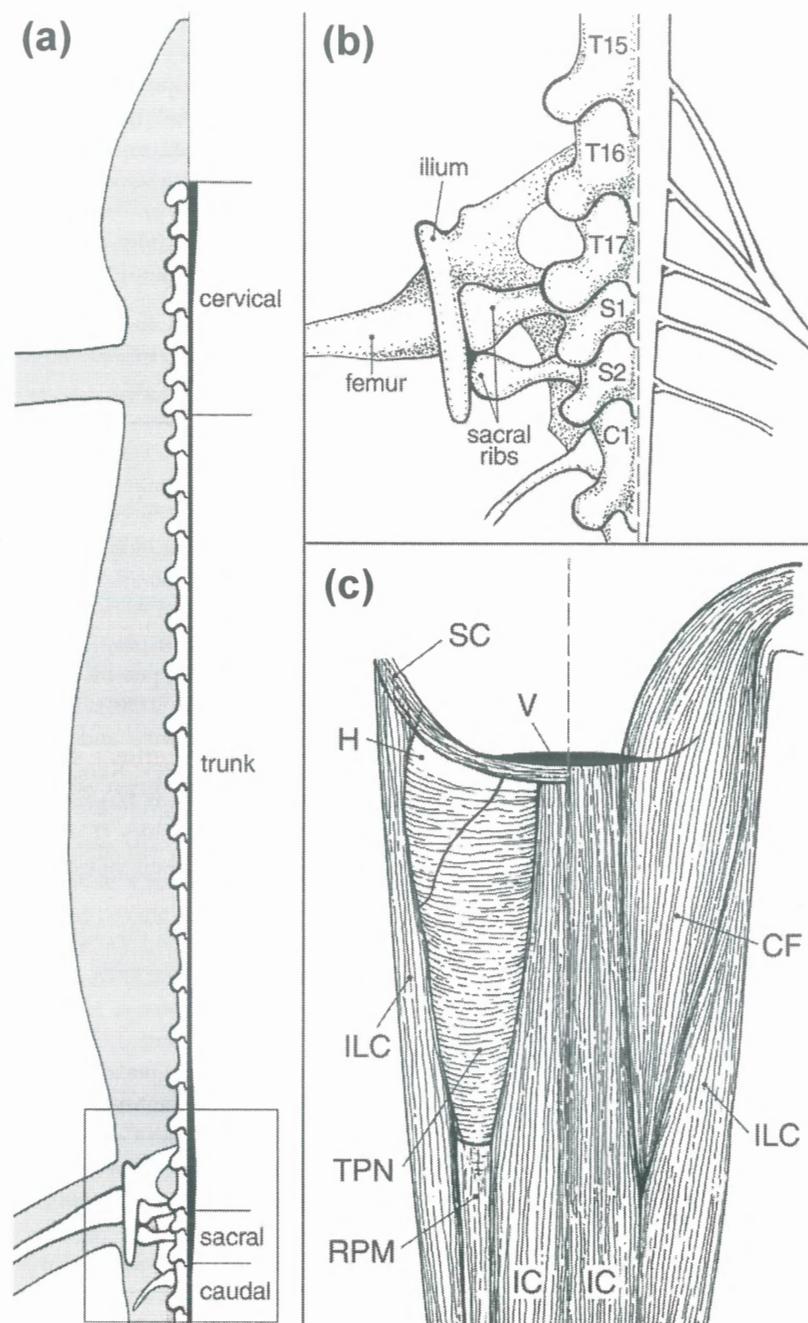
due to increased size of individual fibers (O'Bryant & Wade, 1999); neuromuscular junctions are also larger in males than females (O'Bryant & Wade, 1999, 2002a). In males, the size of these muscle fibers is correlated with the frequency in which dewlaps are extended in a sexual context (Neal & Wade, 2007b, but see Johnson et al., 2011). The composition of fiber types in the ceratohyoid muscle is similar between the sexes, with a majority of fast-twitch fibers (both glycolytic and oxidative-glycolytic). However, the percentage of tonic fibers is increased in males, whereas slow-oxidative fibers are enhanced in females (Rosen et al., 2004). These results are consistent with the fact that the basic features of dewlap extension are similar in the two sexes (Jenssen et al., 2000); however, more tonic fibers may help males to stabilize and/or maintain the extension of their much larger dewlaps.

In green anoles, sex differences in the neuromuscular system controlling hemipenis function during copulation are even more dramatic than in the dewlap system (Ruiz & Wade, 2002). Movement of each hemipenis is largely controlled by a set of ipsilateral muscles [reviewed in Wade (2005); Fig. 3]. The transversus penis (TPN) muscle wraps around the ventral surface of the hemipenis in a medial to lateral orientation; its contraction causes the organ to evert through the cloacal vent. The retractor penis magnus (RPM) muscle is attached to the caudal end of the hemipenis as it lies in the tail and controls its retraction following copulation (Arnold, 1984). TPN and RPM motoneurons are located in the last trunk and first sacral segments of the spinal cord (T17-S1; trunk segments are equivalent to the combined thoracic and lumbar regions of mammals). Within this region of the cord, just over half of the motoneurons project to the TPN and RPM; most of the rest innervate the caudifemoralis (a muscle that lies mostly in the tail and controls leg movement) and the sphincter cloacae (SC). Only males possess hemipenes and the associated TPN and RPM muscles (Ruiz & Wade, 2002), and T17-S1 motoneurons are larger and more numerous in males than females (Ruiz & Wade, 2002; Holmes & Wade, 2004a). The other two muscles are present in females as well as males (Ruiz & Wade, 2002; Holmes & Wade, 2004a), and are consistent across anole species (Johnson et al., 2014). Details on the copulatory motoneurons have rarely been described in reptilian species, but the general pattern of hemipenis and muscle anatomy is reasonably well conserved across squamates. Additional details and exceptions are described in Arnold (1984).

Androgen receptors (AR) are present in green anoles in the same general regions of the brain as in other vertebrates (e.g., high levels in limbic regions, such as the POA and AMY, and in some motor nuclei), as well as in one of the two motor nuclei for dewlap extension, AmbX (Rosen

et al., 2002). ARs are also expressed in copulatory motoneurons and both the dewlap and copulatory muscles, as well as the target structures for both systems (cartilages and hemipenes), with increased AR protein expression in breeding than nonbreeding hemipenes (Holmes & Wade, 2005a; Kerver & Wade, 2013). Expression of AR mRNA in whole brains is higher in males than females, and this effect is largely driven by an increase in AR mRNA during the NBS (Kerver & Wade, 2013). However, the number of cells expressing AR mRNA does not vary seasonally in the brain (Kerver & Wade, 2014), and in none of the reproductive tissues (brain regions, spinal cord, or muscles) does the number of nuclei expressing the AR protein relate to the frequency of behavioral displays in unmanipulated male green anoles (Neal & Wade, 2007b). However, T-treatment increases the expression of AR protein in the hemipenes and the percentage of nuclei in the copulatory muscles that express AR, an effect that is not detected in the ceratohyoid muscle, or in either copulatory or dewlap motoneurons (Holmes & Wade, 2005a; Kerver & Wade, 2013). In contrast, T treatment does not impact AR positive cells in the POA, AMY, or VMH, although gonadectomy results in an increased number of AR+ cells in the AMY of females compared to males (Kerver & Wade, 2014). Interestingly, expression of the AR protein in the POA, AMY, and dewlap and copulatory muscles and motoneurons is not related to the frequency of courtship or copulatory displays (Neal & Wade, 2007b). Yet in a comparative analysis of six anole species, AR was expressed in a greater proportion of myonuclei in the biceps in species that use the biceps more frequently in push-up displays and locomotion (Johnson, Kircher, & Castro, 2018), suggesting that the relationship between AR expression and behavior may differ among evolutionary scales.

Estrogen receptors alpha (ER $\alpha$ ) and beta (ER $\beta$ ) are both also expressed in the green anole in regions comparable to other vertebrates (Martinez-Vargas et al., 1978; Morrell et al., 1979; Beck & Wade, 2009a, 2009b; Cohen et al., 2012). Interestingly, in both sexes, ER $\alpha$  mRNA shows a two- to three-fold increase in the VMH (which is likely important for estrogen-dependent female receptivity) compared to the POA and AMY (which are critical for the display of male sexual behaviors). In unmanipulated animals, expression of ER $\alpha$  mRNA is sexually dimorphic in the POA and VMH; levels are higher in females than in males but do not differ between the BS and NBS (Beck & Wade, 2009a). Similarly, females have more ER $\beta$  mRNA-positive cells present in the AMY and VMH compared to males, with no sex difference in the POA (Cohen et al., 2012). In gonadectomized individuals, E<sub>2</sub> treatment down-regulates ER $\alpha$  mRNA in males but not females. In the VMH, females express greater levels of ER $\alpha$  mRNA compared to males (Beck & Wade, 2009b). At this time, it is unknown



**FIG. 3** Copulatory system anatomy in the green anole. (A) Vertebral morphology with relation to the body. (B) Enlargement of the boxed region in panel (A). Trunk 15 through caudal 1 spinal segments, the sacral ribs, ilium, and the femur are identified on the left side; the spinal cord and nerves are shown on the right. (C) Ventral view of the rostral tail muscles. On the left side, the hemipene (H) and associated transversus penis (TPN) and retractor penis magnus (RPM) muscles are depicted, structures, which are absent in females. The (more dorsal) muscles present in both sexes are identified on the right side, including a leg muscle called the caudifemoralis (CF), which is located primarily in the tail with the ischiocaudalis (IC) and the iliocaudalis (ILC) tail muscles. In the left portion of panel (C), the IC and ILC are depicted as slightly pulled apart to expose the RPM for the drawing. The sphincter cloacae (SC) muscle lies along the cloacal vent (V). (Reprinted with permission from Hormones and Behavior, copyright 2005 (Fig. 2; Wade, J. (2005). Current research on the behavioral neuroendocrinology of reptiles. Hormones and Behavior 48, 451–460.).)

whether E<sub>2</sub> treatment impacts ER $\beta$  expression patterns in this species.

Nuclear steroid hormone receptors primarily function as transcription factors regulating gene expression; coactivators are necessary for this process, potentially serving

as an additional level of steroid hormone regulation. Two major cofactors have been identified to increase transcriptional activity of steroid receptors: cAMP response element binding protein (CBP) and steroid receptor coactivator-1 (SRC-1) (Smith et al., 1996). In green anoles, *in situ*

hybridization revealed that CBP-positive cells are upregulated in females compared to males in the AMY, with no impact of T treatment on expression levels (Kerver & Wade, 2016). SRC-1 mRNA-positive cells were increased in the breeding male VMH compared to nonbreeding males or females from either season, and T treatment increased SRC-1 positive cells in the AMY and POA (Kerver & Wade, 2015). These data suggest some seasonal regulation of these coactivators, but more work is needed to determine what role, if any, these have in seasonal changes in behavior.

The pattern of neural steroidogenic enzyme expression in adult green anoles is also similar to other vertebrates (Balthazart, 1997; Lephart, 1996). Green anoles express many of the enzymes required for steroidogenesis in the brain at the mRNA level including the rate-limiter for steroidogenesis, steroidogenic acute regulatory protein (Peek & Cohen, 2018), although it is unknown whether de novo steroidogenesis occurs in the anole brain. The estrogen synthetic enzyme aromatase has relatively high activity in the hypothalamus and POA, and despite not being critical for male sexual behavior, activity of the enzyme in the brain is increased in males compared to females, greater in breeding than in nonbreeding males, and T treatment increases aromatase activity in breeding males (Rosen & Wade, 2001; Cohen & Wade, 2010b). In whole brains, aromatase mRNA is more highly expressed among nonbreeding than breeding green anoles (Peek & Cohen, 2018). Regionally, there are more aromatase mRNA-expressing cells in the POA of males compared to females, and T treatment does not alter the number of aromatase-expressing cells in the POA, AMY, or VMH (Cohen & Wade, 2011, 2012a). In contrast, 5 $\alpha$ -reductase activity, which seems important for the full production of male sexual behaviors (see above) is relatively high in the brainstem, which contains the dewlap motoneurons (Wade, 1997) and T treatment increases activity in males (Cohen & Wade, 2010b). Interestingly, unlike data from mammals, 5 $\alpha$ -reductase 2 mRNA is found in the adult green anole brain, whereas 5 $\alpha$ -reductase 1 mRNA is not (Cohen & Wade, 2010a). Yet, 5 $\alpha$ -reductase 1 mRNA is expressed in the hatchling anole brain, suggesting a role for this enzyme in development (Cohen & Wade, 2012b). Gonadectomy induced an increase in 5 $\alpha$ -reductase 2 mRNA-expressing cells in the VMH in males compared to females, but T treatment did not impact the number of cells expressing this enzyme (Cohen & Wade, 2012a).

Arginine vasotocin (AVT; the nonmammalian homolog of arginine vasopressin) is a neuropeptide implicated in regulating social behavior in vertebrates, including reptiles (Wilczynski et al., 2017). In green anoles, AVT is present in the POA and other regions of the hypothalamus (Hattori & Wilczynski, 2009). As in other vertebrates,

AVT plays a role in some social behaviors such as aggression, but it does not seem to be involved in courtship or other reproductive behaviors in this species (Dunham & Wilczynski, 2014; Campos et al., 2020; Kabelik et al., 2022). Other neurotransmitters, such as dopamine, serotonin, and norepinephrine have been examined in this species, but largely in the context of aggression (e.g., Korzan et al., 2021). Additional work is needed to determine how these compounds might impact reproductive behavior in green anoles.

## 4.2 Neural control of whiptail lizard reproduction

Consistent with the results from green anoles, lesion studies have documented the importance of the AH-POA in the control of male sexual behavior in whiptail lizards, in both male *A. inornatus* and the parthenogenetic *A. uniparens* (Kingston & Crews, 1994). Implantation of DHT in this region facilitates the display of male reproductive behaviors in both whiptail species (Mayo & Crews, 1987; Rozendaal & Crews, 1989). Lesions of the VMH inhibit the display of receptivity, and local implants of E<sub>2</sub> activate it (Wade & Crews, 1991; Kendrick et al., 1995). During the BS, the AH-POA of *i* is larger in volume in males, and the VMH is larger in females (Crews et al., 1990). These sex differences parallel their roles in the facilitation of sexual behaviors.

The most extensive work to date on the regulation of steroid receptor expression (mRNA for estrogen, androgen, and progesterone receptors) in reptiles has been conducted in whiptail lizards. In general, these studies have indicated that steroid receptors are present in regions similar to green anoles and other vertebrates, including the POA, VMH, and AMY [reviewed in Godwin and Crews (2002)], which are important for the control of reproductive behaviors. Overall, the parthenogen *A. uniparens* appears to express increased levels of steroid receptors compared to *A. inornatus*, perhaps because they are triploid, rather than diploid like the gonochoristic species. A variety of effects of sex, season, and hormone manipulation have been detected [reviewed in Godwin and Crews (2002) and O'Connell and Crews (2022)]. For example, consistent with its role in the control of sexual behavior, male *A. inornatus* express more AR mRNA in the medial POA than females of either species. These AR levels decrease with T treatment in males, but not in females. In contrast, this androgen (T) causes increases in estrogen and progesterone receptor (PR) mRNA in the VMH similarly in the two sexes. Aromatization of T to E<sub>2</sub> might be important in some cases, as cells positive for aromatase mRNA are present in the POA and VMH of *A. uniparens* (Dias et al., 2009). PR mRNA in the periventricular POA is increased in both males and

females by T, but not by the nonaromatizable androgen DHT. In *A. uniparens*, E<sub>2</sub> selectively increases the expression of estrogen receptors in brain regions including the VMH, while decreasing it in other areas, such as the lateral septum. The hormone also increases PRs in the VMH and POA of females of both species, but it is more effective in *A. uniparens*. In males (*A. inornatus*), these increases are not induced one week after castration, but after 6 weeks the response is similar to that of females.

P<sub>4</sub> is critical for the expression of male-like copulatory behavior in the parthenogen (described above), and males of the ancestral species (*A. inornatus*) display a polymorphism in behavior such that some males mount in response to P<sub>4</sub> treatment and other individuals do not. This difference in behavior is likely mediated through nitric oxide signaling, as neural nitric oxide synthase (nNOS) is upregulated in the POA of P<sub>4</sub>-sensitive males treated with P<sub>4</sub> (O'Connell et al., 2011). Similarly, postovulatory female *A. uniparens* have increased nNOS-positive cells in the POA compared to pre-ovulatory females, suggesting that differences in NO signaling might have been a driver for the evolution of male-like pseudosexual behavior in *A. uniparens*.

More recent research has also confirmed the importance of other neurotransmitters in the regulation of reproductive behaviors in whiptails. In the parthenogenetic *A. uniparens*, serotonin inhibits the display of male-like pseudosexual behaviors (Dias & Crews, 2006). In fact, it inhibits both male-typical and female-typical reproductive behaviors. Specifically, injecting serotonin into the POA of animals that were ovariectomized and treated with T decreases mounting, whereas injection into the VMH of gonadectomized, E<sub>2</sub>-treated *A. uniparens* suppresses receptivity (Dias & Crews, 2008). The level of serotonin is lower in postovulatory and ovariectomized, T-treated *A. uniparens* (both of which are likely to display male-typical sexual behaviors) compared to those that were pre-ovulatory or ovariectomized and administered E<sub>2</sub> (likely to be receptive) (Dias & Crews, 2008). Male-typical and female-typical behaviors in these parthenogens may be regulated via different serotonin receptors; systemic stimulation of the 1a receptor inhibits mounting, whereas activating the 2 $\alpha$  receptor facilitates receptivity (Dias & Crews, 2008). Dopamine may also be involved in this regulation, as treatment with a dopamine receptor agonist (D1) facilitates male-like pseudosexual behaviors in *A. uniparens* as well as sexual behaviors in P-sensitive male *A. inornatus*. Interestingly, a lower dose was active in the parthenogen, suggesting increased sensitivity (Woolley et al., 2001). Testosterone treatment has no effect on dopamine receptor (D1R and D2R) mRNA expression in the POA, VMH or AMY, although treatment does decrease both receptor mRNA expression in the nucleus accumbens (O'Connell et al., 2012).

### 4.3 Neural control of reproduction in other reptiles

In red-sided garter snakes, lesioning the POA abolishes courtship behavior in males (Friedman & Crews, 1985; Krohmer & Crews, 1987a). In contrast, destruction of the nucleus sphericus (NS; a homolog of a portion of the AMY, which receives vomeronasal input; Martínez-Marcos et al., 1999) facilitates courtship (Krohmer & Crews, 1987b), suggesting that the POA might be under inhibitory control by the NS. Interestingly, there is an increase in cell proliferation in both the POA and NS during the fall compared to spring (Maine et al., 2014), and cell proliferation in both of these areas is increased in male snakes when they are no longer courting females (Lutterschmidt et al., 2018). Additional work is needed to determine how these new cells might be involved in reproduction. Studies of neurotransmitters in this species have shown that melatonin and CORT both decrease courtship behavior in males, and their effects appear to be additive and may be modulated by the serotonin system (Lutterschmidt et al., 2004). Removal of the pineal gland in males blocks the onset of courtship following hibernation (Nelson et al., 1987), suggesting a potential role for melatonin in courtship behaviors.

Several regions of the forebrain have also been shown to play an important role in the social and sexual behavior of *U. ornatus*, and these nuclei are influenced by sex steroid hormones. The volume of brain nuclei including the POA, AMY, and VMH, are greater in males of this species than females (Kabelik et al., 2006). However, orange-blue and orange males were not found to differ in the volume of these brain regions (Kabelik et al., 2006) or in neural activity in the VMH (Kabelik, Crombie, & Moore, 2008).

The size of the POA and VMH vary in leopard geckos as well. However, the differences are due to incubation temperature, rather than gonadal sex (Coomber et al., 1997). Thus, the manner in which they are organized appears to differ from vertebrates with sex chromosomes, in which gonadal hormones frequently play a major role in organization of both brain and behavior (Adkins-Regan, 1981; De Vries & Simerly, 2002). In leopard geckos, all adults have hemipenes and copulatory muscles, although these structures are much smaller in females than in males (Holmes et al., 2005). In addition, sex differences do not appear to exist in the size or number of motoneurons in the region of the caudal spinal cord containing the associated motoneurons. Research on leopard gecko brain anatomy has also revealed that changes in plasma T concentrations combined with expression of AR in the female brain—including the POA, several regions of the hypothalamus, the AMY, and the lateral septum—are

associated with changes in the female reproductive cycle (Rhen et al., 2003).

Finally, generally, similar patterns of neural mechanisms of behavior may exist at higher taxonomic scales. In a comparison of two species of *Sceloporus*—*Sceloporus undulatus*, which exhibits high male (but not female) aggression, and *Sceloporus virgatus*, in which both sexes exhibit low levels of aggression—AR expression in the POA and VMH mirror these behavioral differences (Hews et al., 2012). Male *S. undulatus* had the highest AR-positive cells in both of these brain regions and males and female *S. virgatus* did not differ in AR expression in these regions of the social behavior network.

#### 4.4 Summary

Steroid hormones act at receptors within particular regions of the brain to regulate the display of sexual behaviors. In many species, these brain areas are sexually dimorphic. In a number of cases, sex differences in neural and other structures associated with reproduction arise during ontogeny, which is covered in the following section.

### 5 DEVELOPMENT OF REPRODUCTIVE TRAITS

#### 5.1 Development of sex differences in anoles and whiptail lizards

Sexual differentiation of the dewlap structures in green anoles is already evident when animals are juveniles. In an examination of 1- to 90-day-old green anoles, O'Bryant and Wade (2001) found that the 2nd ceratobranchial cartilage becomes longer in males than females between 30 and 60 days after hatching, and the muscle fibers are significantly larger in males by day 75. Sexual dimorphism of motoneuron soma size first appears later, between 90 days of age and adulthood (O'Bryant & Wade, 2001). Hatchlings of the congener *A. sagrei* also hatch with similar dewlap sizes, and during the juvenile period, their dewlaps grow in a sexually dimorphic manner, with growth enhanced by T (Cox et al., 2015).

Although the anole dewlap becomes sexually dimorphic during juvenile growth, Kircher (2021) found, surprisingly, that the mechanisms of skeletal growth that lead to this dimorphism differ in *A. sagrei* males and females far earlier in embryonic development. The length of the 2nd ceratobranchial cartilage that forms the perimeter of the extended dewlap is longer in males than females by embryonic stage 8, the earliest stage when cartilage can be measured. This is before the sex-specific gonads have differentiated (which

occurs in embryonic stages 9–11), and no sex differences in circulating T, or androgen or estrogen receptor expression in the cartilage, exist at this early stage (Kircher, 2021). Instead, a transcriptomic study suggests that genetic factors such as X-linked genes, thyroid hormone signaling, and ribosome biogenesis, in combination with differential cell proliferation, seem to determine the sexual dimorphism in early dewlap development (Kircher, 2021).

Although the earliest developmental differences in anole dewlap morphology appear to be independent of hormones, it is possible that the large increase in T associated with their first spring BS masculinizes the dewlap structures, or the dewlap increases in size due to greater use by adult males than females (Lailvaux et al., 2015). Male-typical growth of muscle and cartilage is controlled at least in part by T in juveniles 30–90 days after hatching (Lovern, Holmes, Fuller, & Wade, 2004), a period when plasma T is elevated in males compared to females (Lovern et al., 2001). T implants in females at this time significantly increase both the length of the 2nd ceratobranchials and the size of ceratohyoid muscle fibers, although it does not affect soma size of motoneurons that project to that muscle. In parallel, castration of males on posthatching day 30 decreases cartilage length. This manipulation does not modify muscle fiber size, but it is possible that conducting this surgery at a younger age would be more effective or that adrenal androgens, which were not eliminated, play some role.

Sexual differentiation of the peripheral male and female copulatory structures in green anoles occurs embryonically (Gredler, Sanger, & Cohn, 2015), in a pattern generally similar across tuatara (Sanger et al., 2015), snakes (Leal & Cohn, 2015), turtles (Larkins & Cohn, 2015), and alligators [Gredler, Seifert, & Cohn, 2015; reptilian genital evolution is also broadly reviewed in Raynaud and Pieau (1985) and Gredler et al. (2014)]. Hemipenes and primordial RPM fibers are initially present in both sexes, but they regress completely in females before hatching (Holmes & Wade, 2005b). Interestingly, both androgen and estrogen appear to play active roles in the sexual differentiation of these copulatory structures. Administration of T or DHT rescues male structures in females, whereas E<sub>2</sub> treatment feminizes males (Holmes & Wade, 2005b). Further, at embryonic day 13, when the tissues are still present in both sexes, males express higher levels of AR mRNA in their hemipenes and copulatory muscles than females, whereas females tend to express increased ER $\alpha$  mRNA (Beck & Wade, 2008). In contrast to the peripheral structures, the number of T17-S1 motoneurons is equivalent in males and females at hatching (Holmes & Wade, 2005b), suggesting a dissociation between muscle and motoneuron development similar to the dewlap system.

Patterns of yolk steroids are consistent with at least the possibility that T masculinizes copulatory structures. For example, T is higher in male than in female eggs sampled while still in the oviduct (Lovern & Wade, 2003a), as well as on the day the eggs are laid (Lovern et al., 2001). Intriguingly, these data suggest a maternal source for the sex difference, as well as an early time period when T may influence sexual differentiation. Maternal transfer to yolk is not simply passive, however. Plasma T in adult females is approximately 30% that of E<sub>2</sub>, whereas T in yolked follicles is over 600% that of E<sub>2</sub> (Lovern & Wade, 2003b). Further, exogenous radiolabeled T injected subcutaneously into gravid females was detected in all stages of developing eggs before oviposition (Cohen & Wade, 2010c). Embryos appear to start producing T, but perhaps not E<sub>2</sub>, in the latter half of incubation (Lovern & Wade, 2003b). The sex difference in yolk T is no longer detected one- to two-thirds of the way between laying and hatching (Lovern & Wade, 2001), but we do not know whether embryonic hormone levels differ in males and females. Molecular techniques are now available to determine the sex of *A. carolinensis* embryos (Gamble & Zarkower, 2014), which will advance our understanding of the specific roles of steroids during the prehatching developmental period. We can now determine, for example, relative levels of the production of androgens and estrogens by embryonic gonads.

Details regarding the development of sex differences in the forebrain of green anoles remain unclear. One study assessed morphological traits in the POA, VMH, and AMY around the time of hatching (which occurs after approximately 34 days of incubation at 27.5°C). A variety of changes occurred between embryonic day 26 and post-hatching day 5, including decreases in soma size, particularly in females, and increases in the density of cells in regions associated with male-typical behaviors (POA and AMY). However, only transient sex differences existed in any of these regions, suggesting that they arise at a later developmental stage (Beck & Wade, 2009c). As in adults, ER $\alpha$  expression in both sexes is higher in the VMH than the other two brain regions, which is consistent with the role E<sub>2</sub> plays in the display of female sexual behaviors (Beck & Wade, 2009c). Also similar to adults, aromatase is expressed in the POA, AMY, and VMH of hatchlings, although, unlike adults, 5 $\alpha$ R1 is expressed in the developing brain and 5 $\alpha$ R2 is not, suggesting different roles for these isozymes in development (Cohen & Wade, 2012b).

Sex differences do not exist in volume of the AH-POA or VMH in *A. inornatus* on the day of hatching. Treatment with T for approximately two weeks does not affect the sizes of these brain regions, but ovariectomy on the day of hatching decreases the size of the AH-POA in parthenogenetic *A. uniparens* measured 16 days later (Wade et al., 1993).

## 5.2 Development in reptiles with alternative reproductive morphs

In *U. ornatus*, T and P<sub>4</sub> are important factors in the organization of differences between the two male morphs (Hews et al., 1994; Hews & Moore, 1996; Moore et al., 1998). This conclusion is based on both measurements of endogenous hormones and studies in which hormones were manipulated. Castration of juvenile males produced more orange males than a control manipulation, whereas castrated juvenile males given T implants were more likely to be orange-blue than control males (Hews et al., 1994). This study also found the same pattern of results when 30-day-old intact males were given T implants or blank capsules, indicating that the critical period for the role of T or its metabolites in morph differentiation may continue beyond 30 days. Further work determined that T does not influence dewlap color after posthatching day 60 (Hews & Moore, 1996), suggesting that the critical period for this trait has ended by this developmental stage. Treatments with T or its metabolite DHT are equally effective for the development of male sexual traits, including dewlap color, waxy femoral pore secretions, and hemipenis-related tissues, whereas DHT specifically is required for full expression of the blue belly patches (Hews & Moore, 1995).

P<sub>4</sub> also plays an important role in the organization of morph phenotypes. Manipulations of P<sub>4</sub> or T at hatching result in differing morphs (Moore et al., 1998). P<sub>4</sub> may operate independently of T; or alternatively, it may be the initial trigger of differentiation, with T operating later in development (Moore et al., 1998). In vitro incubation demonstrated that during the period between embryonic day 30 through hatching, P<sub>4</sub> is released by the adrenal glands, and T from the testes (Jennings et al., 2004). This early production of T from *U. ornatus* gonads contrasts with results from turtles (Pieau et al., 1982; White & Thomas, 1992a) and crocodiles (Smith & Joss, 1994), in which levels of T are very low in early posthatching periods.

Galapagos marine iguanas provide an example of a species in which developmental and environmental conditions affect reproductive behavior via changes in circulating hormones. In this species, males can be successful territory holders only after achieving a certain body size, but this threshold is affected by climate conditions and population density (Wikelski & Trillmich, 1997), such that when territories are available, smaller males may adopt territorial behavior. The ontogenetic switches between sneaker and satellite, and satellite and territorial strategies can be imitated via manipulation of T. When exposed to increased T, satellite males begin to defend territories and sneaker males begin to openly court females (Wikelski et al., 2005), behaviors more typical of larger males. These changes do not increase the reproductive success of

manipulated males, however, as they experience more frequent fights with other, larger males and expend time and energy performing courtship displays that are largely ignored by females (Wikelski et al., 2005). Further, when T is blocked, territorial males decrease their defensive and courtship behaviors (Wikelski et al., 2005).

### 5.3 Development in reptiles with temperature-dependent sex determination

In addition to the diverse patterns of the neuroendocrine control of behavior described above, reptiles also offer a unique opportunity to compare taxa exhibiting different modes of sex determination. Sex is determined by genotype in many reptilian species; both XX/XY (in which males are the heterogametic sex) and ZZ/ZW (in which females are the heterogametic sex) chromosome systems exist. In other reptiles, sex is environmentally determined, as the temperature of egg incubation can determine sex in many crocodilians, chelonians, and some squamates [reviewed in Wibbels et al. (1994) and Warner (2011)]. One of the best-studied examples of temperature sex determination, or TSD, is the leopard gecko (*E. macularius*). Individuals of this species have no sex chromosomes, and an individual's sex is dependent on the temperatures experienced as an egg (Viets et al., 1993). At an incubation temperature of 26°C, all individuals become females, whereas at 32.5°C, mostly males are produced (Crews et al., 1996), although these effects can be overridden by the application of exogenous E<sub>2</sub> (Flores et al., 1994). Temperature appears to direct sex development in TSD species at least in part by influencing genes that code for steroidogenic enzymes and steroid hormone receptors (Crews et al., 1994).

In an experiment designed to determine whether incubation temperature and gonadal sex hormones produce irreversible effects on behavior in leopard geckos, Rhen and Crews (1999) found that some effects of temperature on male behaviors are permanently organized during development. When gonadectomized and then treated with androgens, adult males from male-biased incubation temperatures perform more scent-marking behaviors than males from female-biased temperatures, but males from female-biased temperatures perform more copulatory (mounting) behavior. In addition, they found that even when treated with androgens, adult females perform very little courtship or mounting behavior. Incubation temperature has also been found to affect the influence of T on aggressive behavior, suggesting that incubation temperature may have long-term effects on sensitivities to hormones (Flores et al., 1994; Flores & Crews, 1995; Rhen & Crews, 1999).

Incubation temperature also influences the volume and metabolic capacity of several brain nuclei in leopard geckos.

Experimental manipulations indicate that changes in androgen and estrogen levels do not affect POA or VMH volumes in females from all-female temperatures, or neural activity in the anterior hypothalamus, AMY, dorsal ventricular ridge, or septum, but hormones do alter the volume of these brain areas in individuals from both sexes who were incubated at male-biased temperatures (Crews et al., 1996). These manipulations showed that incubation temperature is acting through a mechanism other than the eventual production of differential levels of gonadal hormones to organize the brain (Crews et al., 1996).

Hormonal changes during development also affect leopard gecko reproductive morphology. Androgen (but not estrogen) levels increase during the course of male ontogeny, and hemipenis development tracks these changes. In particular, at 10 weeks of age, signs of hemipenes appear as androgens gradually increase, and at 25 weeks of age when androgen levels increase, hemipenes are fully developed (Rhen et al., 2005). In females, plasma levels of E<sub>2</sub> and DHT (but not T) vary by incubation temperature and change over the course of development (Rhen et al., 2005).

Most turtle embryos also experience a temperature-sensitive period [TSP; reviewed in Pieau and Dorizzi (2004)], when gonadal sex is influenced by both temperature and hormones (Wibbels et al., 1994). The synergistic relationship between temperature and hormones is exemplified in studies of red-eared slider turtles (*Trachemys scripta elegans*), in which treatments manipulating temperature and E<sub>2</sub> independently have the same effect on the sex of incubating eggs, and appear to act through the same developmental pathways (Wibbels et al., 1991).

During the TSP, the gonads are the main site of aromatase activity and estrogen synthesis, as well as a site of steroid action. One study that examined the relationship between ER and AR in the gonads during the TSP in the red-eared slider found that ER $\alpha$  and AR levels dramatically increase at female-producing temperatures during (but not before) this sensitive period and that expression of these receptors was reduced at male-producing temperatures (Ramsey & Crews, 2007). Also, treatment with E<sub>2</sub> at male-producing temperatures caused feminization of the gonads, showing that temperature and hormonal conditions can both influence the sex of the individual, although these effects can occur through differing steroid signaling pathways (Ramsey & Crews, 2007). Other work in red-eared sliders showed that P<sub>4</sub> secreted from adrenal and kidney tissues (White & Thomas, 1992a) also appears to be a factor in sex determination. During the temperature-sensitive embryonic period, plasma levels of P<sub>4</sub> are significantly higher in males than females (White & Thomas, 1992b).

## 5.4 Summary

The development of many morphological structures necessary for successful courtship and copulation depends on exposure to hormones (and/or temperature) or gene expression during embryogenesis or after hatching, and these organizational effects generally occur during a particularly critical period. While many adult reproductive traits are directly influenced by social and/or neuroendocrine traits during an animal's development, in a wide diversity of reptilian taxa, variation in reproductive behavior may be alternatively the result of adult plasticity due to changing external or internal environmental factors, as described below.

## 6 ADULT PLASTICITY

### 6.1 Effects of season and hormone manipulation in anoles

In the green anole, dewlap and copulatory system neuromuscular morphology differ in their responsiveness to adult T. Morphology of the dewlap neuromuscular system appears to not be plastic in adulthood. For example, despite large seasonal changes in circulating T, and the fact that castration removes the main source of the hormone, muscle fiber and motoneuron components are generally stable in size across the BS and NBS, and with castration and T replacement (O'Bryant & Wade, 1999; Neal & Wade, 2007a). Yet, the size and elasticity of the dewlap skin varies across seasons, and with behavioral use of the dewlap (Lailvaux et al., 2015). The dewlap muscle also appears to undergo changes in adulthood. Males have a higher percentage of fast oxidative glycolytic fibers during the BS compared to the NBS, and T treatment increases the percentage of fast oxidative glycolytic dewlap fibers. Thus, routine changes in this hormone may mediate fiber type in gonadally intact males (Holmes et al., 2007).

The pattern of hormone responsiveness for the copulatory system in green anoles is quite different, as morphology

of these structures is plastic in adulthood (Holmes & Wade, 2004b). Whereas the cross-sectional areas of hemipenes and RPM fibers do not differ between intact animals under breeding and nonbreeding environmental conditions, they increase dramatically in T-treated compared to control castrated males. Yet, these structures are not induced in females with adult T treatment (Holmes et al., 2007). The effect of T in males is greater in those housed with long days and warm temperatures (i.e., breeding conditions), suggesting that sensitivity to T in peripheral structures decreases as a result of nonbreeding environmental conditions (Neal & Wade, 2007a). It is possible that this change exists to prevent a decline in morphology due to a seasonal decrease in T. However, as males captured in the field after the end of the BS still have ~10 ng/mL of T (compared to ~20 ng/mL during the BS; Lovewell et al., 2001), they may have sufficient hormones available to maintain the structures. In addition, when males are gonadectomized, RPM fibers, and especially hemipenes, appear smaller than in intact animals. Thus, it seems more likely that the copulatory system of males shows an increased dynamic range in its sensitivity to T during breeding compared to nonbreeding environmental conditions. This process might facilitate the response of the copulatory system to even relatively small T fluctuations that may occur with particular experiences, possibly exposure to females. In contrast to effects on gross morphology, seasonal environmental conditions, and T manipulation have little to no effect on copulatory muscle fiber type (Holmes et al., 2007). Thus, this feature is not the primary mediator for seasonal changes in male copulatory behaviors. Finally, it is possible that a small decrease in T17-S1 motoneuron soma size occurs in the NBS. However, this effect is probably not specific to cells in that region of the spinal cord, and it is not mediated by plasma T levels (Holmes & Wade, 2004b).

In the forebrain of unmanipulated adult green anoles, volumes of the POA and VMH (but not AMY) are greater during the BS than in the NBS in both sexes (summarized in Table 1; Beck et al., 2008). However, mechanisms regulating the seasonal change in volume are not clear, as

**TABLE 1** Summary of sex and seasonal differences across three major brain areas controlling reproductive behavior in the unmanipulated green anole lizard (*A. carolinensis*). References throughout text.

Brain area	Volume	Cell number	AR	ERα/b	SRC1/CBP	ARO	5aR
POA	BS>NBS; M>F	ND	+	+	+	+	+
AMY	ND	NBS>BS; F>M	+	+	+	+	+
VMH	BS>NBS	F>M	+	+	+	+	+

+, mRNA and/or protein present in area; BS, breeding season; F, female; M, male; NBS, nonbreeding season; ND, no significant difference between seasons or sexes.

increased volume during the BS is not detected in the POA or VMH in gonadectomized animals, treated with E<sub>2</sub> or not (Beck & Wade, 2009b). Thus, the sexual dimorphism is present across diverse endocrine conditions and is probably not maintained by adult steroid hormones. The seasonal difference detected in intact animals could therefore involve gonadal secretions during adulthood other than E<sub>2</sub>, perhaps T, or nongonadal factors such as melatonin. T treatment of gonadectomized adult green anoles increases soma size in the POA and AMY, and in the AMY it does so to a greater extent in the BS than NBS (Neal & Wade, 2007a).

Although intact anoles show no sex difference in the volume of the AMY, this brain region is larger in gonadectomized males than gonadectomized females (Beck & Wade, 2009b). These results fit with the larger soma sizes detected in gonadectomized males than gonadectomized females in this region during the NBS, regardless of whether the animals are treated with T (O'Bryant & Wade, 2002b). E<sub>2</sub> treatment enhances the sexual dimorphism in AMY volume, suggesting it may support change in one or more of the characteristics that contribute to overall AMY volume other than total cell number (which is unaffected by this hormone), such as soma size or dendritic arborization. This issue has not been investigated, but T treatment does increase soma size in the AMY of males, and the same dose is more effective in the BS than NBS (Neal & Wade, 2007a). Perhaps even the relatively low levels of T in female anoles (~100 pg/mL in the NBS and ~750 pg/mL in the BS; Lovern & Wade, 2001) are sufficient to maximally increase brain region volume, thus masking an underlying sex difference in AMY size. The same may be true for the VMH, which is larger in gonadectomized males than females (regardless of whether they are treated with E<sub>2</sub>), but not in gonad-intact individuals (Beck & Wade, 2009b). Female green anoles also have a greater number of cells in the VMH and AMY than males (at least than gonadectomized males), suggesting that they may be smaller and/or more densely packed than in males (Beck & Wade, 2009b).

A greater total number of cells exists in the AMY during the NBS than BS, in both unmanipulated and gonadectomized animals, regardless of whether they were administered E<sub>2</sub> (Beck & Wade, 2009b; Beck et al., 2008). These results suggest that some feature(s) of nonbreeding environmental conditions facilitate the addition to or survival of cells in this region. Data on estimated total cell number in the VMH are a little more complicated, as an increase was detected in the NBS compared to BS in gonadectomized animals, some of which received E<sub>2</sub>-treatment, but not in intact green anoles (Beck & Wade, 2009b; Beck et al., 2008). It will be important in the future to determine the functional relevance of the increased number of cells in the AMY and VMH during the NBS, as well as whether they are mediated by differences in cell birth, incorporation, or survival.

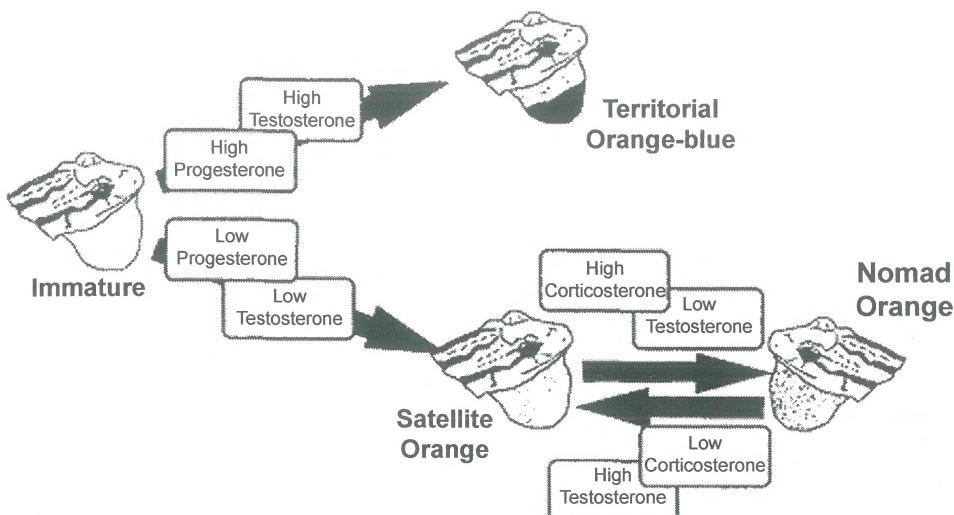
## 6.2 Effects of season and hormone manipulation in other reptiles

In male whiptail lizards, volumes of the AH-POA and VMH are plastic. During the NBS, these regions in male *A. inornatus* become female-like in size, an effect that is regulated by T. In contrast, the volume of these two brain regions appears stable in adult female *A. inornatus* and *A. uniparens* (Wade & Crews, 1991; Wade et al., 1993). In tree lizards, the POA and AMY are greater in BS than NBS males (Kabelik et al., 2006). The relationship among neural anatomy, hormone levels, and behavior in this species is still somewhat unclear. Although one study indicates that T and P<sub>4</sub> both cause an increase in the volume of the VMH and AMY (Weiss & Moore, 2004), another found that whereas T and P<sub>4</sub> increase aggression, only increased T affected brain nuclei volumes (Kabelik, Weiss, & Moore, 2008). Further, neural activity in the dorsolateral VMH, or VMHdl (as measured indirectly using the phosphorylated form of cyclicAMP response element binding protein (pCREB)), increases with greater aggressive behavior but is not affected by T (Kabelik, Crombie, & Moore, 2008). Therefore, although T influences both brain anatomy and aggressive behavior, these appear to be independent effects of the hormone (Kabelik et al., 2006; Kabelik, Weiss, & Moore, 2008), as no direct relationship between forebrain nuclei and aggression has been identified.

In leopard geckos, treating females with T dramatically increased the size of the hemipenes and the fiber size of the muscles that control hemipenis movement, but these structures do not reach the sizes of those in control males (Holmes & Wade, 2005a). Leopard gecko hemipenes contain AR (Endo & Park, 2003; Rhen & Crews, 2001), and so T likely acts directly on the hemipenes to cause their growth, but we do not yet know whether copulatory muscles contain AR. Compared to green anoles, it is clear that copulatory structures in adult leopard geckos are much more sensitive to hormonal changes. Although far more work must be done to address the idea, it would be fascinating if the lack of sex chromosomes in leopard geckos in some way lessens the degree to which these structures differentiate.

Sex and seasonal differences in the limbic forebrain were investigated in one study in red-sided garter snakes, and relatively few sexual dimorphisms were found. The POA was larger in males compared to females only during hibernation, and in the NS in animals not subjected to hibernation. The reproductive forebrain appears not to exhibit dramatic changes in morphology in males, but in females the POA and VMH were both smaller during hibernation than under other environmental conditions (Crews et al., 1993).

In side-blotched lizards (*U. stansburiana*) some males may change morphs during the reproductive season. In particular, late in the BS some yellow-throated males can begin



**FIG. 4** Dewlap polymorphism in the tree lizard (*Urosaurus ornatus*). This figure depicts a summary of the proposed endocrine mechanisms influencing dewlap coloration and territorial strategies in tree lizards. The permanent differences between orange and orange-blue males appear to result from organizational influences of progesterone and testosterone. Low levels of these hormones during early development result in orange males, while high levels produce orange-blue males. However, orange males may switch between satellite and nomadic tactics, a shift that appears to result from a two-step activational process that involves the stress increase in corticosterone and the consequent decrease in testosterone levels. (Reprinted with permission from American Zoologist, copyright 1998 (Fig. 12; Moore, M. C., Hews, D. K., & Knapp, R. (1998). Hormonal control and evolution of alternative male phenotypes: Generalizations of models for sexual differentiation. American Zoologist 38, 133–151.).)

to exhibit partial blue-throated morphology, with correlated increases in T levels and endurance (Sinervo, Miles, et al., 2000). In general, T appears to be a major factor in the physiological changes that all color morphs undergo during development (Sinervo, Miles, et al., 2000).

In tree lizards (*U. ornatus*), although male morph type is established early in development, reproductive tactics within the orange morph are determined by activational effects of hormones (Fig. 4) (Moore et al., 1998). Territorial orange-blue males are always territorial, but the nonterritorial orange morph adopts a satellite tactic in some years, and a nomadic tactic in others. This behavioral variation is correlated with climatic conditions, such that in drier years orange males are nomads, while they exhibit the more sedentary, satellite behavior in years with more rainfall (Knapp & Moore, 1997; Knapp et al., 2003). This difference appears to be driven by variation in sensitivity to T and CORT (Knapp & Moore, 1997). In harsher, dry conditions, males of both morphs have higher CORT levels, but territorial orange-blue males are much less sensitive to the effect of CORT on the suppression of T (Knapp & Moore, 1997; Knapp et al., 2003). These differences in CORT sensitivity may be due to differences between the morphs in binding capacity of androgen-glucocorticoid-binding globulin (AGBG), which lead to higher levels of circulating CORT in nonterritorial orange males than territorial orange-blue males, particularly when CORT is increased in response to stress (Jennings et al., 2000). Therefore, in harsher environmental conditions only orange males have lower T, which seems to drive plasticity in reproductive tactics.

### 6.3 Summary

In seasonally breeding animals, reproductive morphology often differs dramatically between BS and NBSs, and in a number of cases (although not always) in a manner that mirrors changes in the display of sexual behaviors. Further, adult neuroendocrine traits may vary as a function of the social environment, which may also produce plastic behaviors in adulthood.

## 7 CONCLUSIONS AND FUTURE DIRECTIONS

Research in the field of reptilian neuroendocrinology is thriving, yet there are many more exciting directions to pursue. It will be particularly useful to explore individual species in more depth, as more tools for this work become available. The genomes for two species of anoles, *A. carolinensis* (Alföldi et al., 2011; Eckelbar et al., 2013) and *A. sagrei* (Geneva et al., 2022), are now available and annotated, and draft genomes of a number of other reptilian species are rapidly becoming developed. These sequence data are greatly facilitating investigations of the molecular mechanisms regulating reproduction (e.g., Kircher, 2021; Hale et al., 2022), including those in neural and muscular structures that influence or are influenced by hormones (Kabelik et al., 2021). Further, molecular tools such as CRISPR now also allow us to manipulate the genome of study organisms, including brown anole lizards (Rasys et al., 2019).

Importantly, global climate change adds urgency to much of this work. The altered thermal regimes experienced by reptiles in our changing world may influence their reproductive biology in ways we do not yet understand. In addition to the dramatic impacts that species with temperature-dependent sex determination are likely to face (e.g., Valenzuela et al., 2019), changing temperatures may influence seasonal breeding (e.g., Deme et al., 2023), alter developmental processes of embryos in vulnerable eggs (e.g., Sanger et al., 2021), and may result in population or species extinction (Ihlow et al., 2012).

Work with the species described in this chapter has greatly informed our understanding of the relationships involving the neural and endocrine influences on reproductive behavior. However, our knowledge at this point is based on relatively few species, with the longest history of research in this area on the green anole. Increasing the diversity of study organisms, including more research with nonsquamate reptiles such as crocodilians and turtles, especially those with alternative reproductive strategies and sex determination, should yield valuable insights into critical mechanisms across life stages. Many of the neuroendocrine features and mechanisms described in this chapter share similarities with birds and mammals, suggesting that expanding these types of studies in reptiles may provide valuable insights across vertebrate groups with shared evolutionary histories.

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