Environmental sex determination in reptiles

Claudio Ciofi a,b,*, Ian R. Swingland b

a Institute of Zoology, Zoological Society of London, Regent’s Park, London, NW1 4RY, UK
b The Durrell Institute of Conservation and Ecology, University of Kent, Canterbury, CT2 7PD, UK

Abstract

In recent years, representatives of more than 40 families of reptiles have been studied to understand how environmental parameters affect sex determination. In this review, we summarise the distribution and taxonomic pattern of sex determining mechanisms, outline the main hypotheses of the adaptive significance of temperature sex determination (TSD), and of skewed population sex ratios. We also examine the competing hypotheses of the physiological and molecular mechanisms involved in TSD. © 1997 Elsevier Science B.V.

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1. Introduction

A remarkable diversity of sex determination systems is found among different animal taxa. In most cases, sex is determined chromosomally. The mechanisms involved have been under dispute since the first genetic system of sex determination was described in the fruit-fly Drosophila melanogaster (see Wachtel, 1993). In mammals, this debate has been resolved with the recent discovery of the SRY gene on the Y chromosome as the testis-determining factor (see Hawkins, 1993 for a review).

While genotypic sex determination (GSD) depends on genetic factors alone, environmental sex determination (ESD) depends on post-fertilisation environmental factors. In recent years, the sex of an increasing number of animals has been found to be influenced primarily by the environment, and ESD mechanisms have been examined in a variety of taxa (rotifers, nematodes, polychaetes and echiurids: Korpelainen, 1990; crustaceans: Rigaud et al., 1991; fishes: Conover et al., 1992; Beamish, 1993).
Reptiles exhibit different modes of both GSD and ESD, sometimes occurring together even within a single family (Ewert et al., 1990; Janzen and Paukstis, 1991a). Modes of GSD include at least three patterns of heterogamety in both sexes; XX/XY, XXX/XXY, and pseudo XO are forms of male heterogamety, while ZZ/ZW, ZZZ/ZZW, and \( ZZZ/\overline{ZWW} \) are types of female heterogamety. In addition, morphologically undifferentiated sex chromosomes have been found to occur in several families (Olmo, 1986).

Among environmental parameters, egg incubation temperature appears to be the most critical factor affecting gonadal differentiation in reptiles. Temperature sex determination (TSD) has been recorded in chelonians (turtles and tortoises), lizards, crocodilians (alligators and crocodiles) and, recently, tuataras. All species of snakes and amphisbaenians examined to date have been shown to have only GSD mechanisms (Janzen and Paukstis, 1991a).

Since Chamier (1966) first described the effect of temperature on sex ratio of the hatchlings in the lizard, *Agama agama*, there has been a series of papers describing the importance of temperature on the sex ratio of lizard and chelonian embryos (e.g. Pieau, 1971, 1972, 1974; Ynetema, 1976). Subsequently, several studies have focused on the ecological and evolutionary implications of TSD in reptiles and its effect on sex ratios in natural populations (e.g. Nichols and Chabreck, 1980; Ewert and Nelson, 1991; Burke, 1993), while an increasing amount of research has attempted to uncover the molecular and physiological mechanisms underlying TSD (Deeming and Ferguson, 1989; Etcheberger et al., 1991; Desvages et al., 1993; Crews et al., 1994; Pieau et al., 1994a; Pieau, 1996). Indeed, some of these studies have been carried out in zoological gardens (e.g. Hammack, 1989; Horn and Visser, 1989; Boyer et al., 1989; Hairston and Burchfield, 1992), where sex ratio manipulation by controlling incubation temperature is crucial in captive breeding programmes of threatened or endangered reptiles.

Potentially, zoos represent an important platform by which TSD theory and experimental designs on TSD can be tested in a practical setting. This review outlines the pattern of distribution of TSD in reptiles, its adaptive value and the relationships of sex determining mechanisms to the ecology of this taxa. Experiments investigating how temperature affects sexual differentiation are also described.

1.1. Distribution and taxonomic patterns of sex determining mechanisms

More than 70 species belonging to 43 families of reptiles have shown TSD either in laboratory experiments or in field studies. Chelonians and lizards exhibit large variation in sex determining mechanisms (Fig. 1). In chelonians, although Chelidae, Trionychidae and Staurotypidae show GSD only, TSD is prevalent in most other families studied. Within the Emididae, both Batagurinae and Emidinae show both GSD and TSD, though the latter is much more common (Janzen and Paukstis, 1991a). However, for some species, the sex determining mechanism is still uncertain. One species of the Chelididae, the alligator snapping turtle *Macrolemys temminckii*, has been reported to show TSD (Packard and Packard, 1988), although Ewert et al. (1994), report that females are produced even when the incubation temperature is shifted among male-producing values during development. Homomorphic sex chromosomes seem to be a necessary prerequisite for TSD (Bull, 1980), while GSD may occur under homo- or heteromorphy (Palmerallen et al., 1991).
In lizards, GSD occurs in both iguanids and scincids, where male heterogamety appears to be ubiquitous. Both GSD and TSD have been described in the Agamidae, Eublepharidae, Gekkonidae, and Lacertidae, while the coexistence of the two mechanisms is still uncertain for Iguanidae and Varanidae (Viets et al., 1994). The recurrence of GSD in these taxa may be partly due to the presence of viviparous and parthenogenetic species, whose reproductive mechanisms presumably require genetic input for sex determination (Bull, 1980). In Chamaeleontidae, *Chamaeleo pardalis* probably has GSD, while *Rhampholeon k. kerstenii* may have both GSD and TSD (Viets et al., 1994).

All 11 species of crocodilians examined to date show TSD (Deeming and Ferguson,
Three different patterns of TSD have been described in chelonians, crocodilians, and lizards, thus far.

1. Eggs incubated at constant, low temperatures produce only females, whereas those maintained at constant, higher temperatures produce only males. Intermediate temperatures produce both sexes. This pattern is found in most species of lizards (Fig. 2(a)), and has been reported in crocodilians by Ferguson and Joanen, 1982; Ferguson and Joanen, 1983; but see pattern 3 below). They describe three different types of nest site in the natural habitat of Alligator mississippiensis: wet marsh, dry marsh, and elevated firm ground. The latter are hot (34°C) and produce approximately 100% males. Wet marsh nests are cool (30°C) and produce approximately 100% females. Dry marsh nests have intermediate temperatures, producing males in hot sites, and females in cold ones.

2. Females are produced at high incubation temperatures and males at low temperatures in many chelonians (Fig. 2(b)). In Emys orbicularis all the animals are male below 27.5°C, whereas 100% are female above 29.5°C. Between 28°C and 29°C, both sexes and some intersexes appear (Pieau and Dorizzi, 1981; Pieau, 1982).

3. Females are produced at low and high temperatures, with males produced at intermediate temperatures. This occurs in some species of chelonians, lizards, and many species of crocodilians (Fig. 2(c)). In the leopard gecko Eublepharis macular-
ius, females are produced when the eggs are incubated at 26°C (100%), 30°C (70%), and 34°C (95%), whereas males (75%) are predominantly produced between 30 and 34°C (Deeming and Ferguson, 1988; Ewert and Nelson, 1991; Flores et al., 1994). In the snapping turtle Chelydra serpentina, incubation at 20 and 30°C results in 100% production of females, whereas a majority of males are produced between 22 and 28°C (e.g. Pieau, 1976; Bull, 1980). Additionally, Lang and Andrews (1994) report a similar pattern in crocodilians (Alligator mississippiensis, Caiman crocodilus, and seven species of the genus Crocodylus). For example, they incubated fertile eggs of Alligator mississippiensis at constant incubation temperatures, resulting in 100% production of females below 31°C and above 34°C, while 100% males were produced at 33°C (but see Deeming and Ferguson, 1989, 1991).

As we can see from these examples, selection for mode of sex determination varies extensively among reptilian groups. However, as GSD and TSD co-occur in some taxa, both modes seem to be viable evolutionary strategies. An interesting array of studies has been carried out concerning the origins of these two mechanisms, and has been reviewed by Janzen and Paukstis (1991a). TSD and GSD seem to occur with the same frequency among reptilians, and both are present in living members of families represented among the earliest known fossil chelonians. Thus far, it has been difficult to determine which character state (TSD or GSD) is ancestral, and there is the possibility of either multiple or independent origins of TSD and GSD within reptilians.

1.2. Adaptive significance of ESD

The prerequisite for the persistence of ESD in reptilians seems to be related to a thermally patchy environment, in which different incubation temperatures in different microhabitats allow natural populations to produce hatchlings of both sexes with potentially different physiological characteristics (e.g. different metabolic rates or potential growth rates).

A patchy distribution of the environmental factors (e.g. temperature) involved in embryo sex determination can also affect the lifetime fitness of hatchlings in different ways. To explain the adaptive significance of ESD, Charnov and Bull (1977) proposed a model where hatching fitness varies most among patches and between sexes, so that some patches provide optimum conditions for one sex but not the other. Following the Charnov-Bull model, Webb et al. (1987) proposed post-hatching growth rate and body size as the most important fitness traits correlating with incubation temperature and offspring sex (see also Dunham et al., 1988; Wilbur and Morin, 1988). According to this sexual dimorphism hypothesis, if the benefits of adult body size differ between sexes, the sex that benefits most from being large should be produced at the incubation temperature yielding large adults. As an example, in Alligator mississippiensis, larger males are produced at higher temperatures, and a selective advantage has been reported for those individuals; they control larger harems of females and produce more spermatozoa for a longer time than small males (Allstead and Lang, 1955; Deeming and Ferguson, 1989; Joanen and McNease, 1989). In this hypothesis, TAD would be favoured over GSD, as the latter would not guarantee that the eggs containing genotypically male embryos would be in optimum incubation conditions and therefore grow.
faster and into larger males. TAD, on the other hand, allows the association of sex with potential post-hatching growth rate, as both are determined by incubation conditions.

This relationship between incubation temperature and adult sexual dimorphism suggests a temperature-dependent differential fitness explanation for the adaptive significance of TSD. However, although this hypothesis could be regarded as a possible explanation for some taxa, such as crocodilians, it does not hold for the chelonians and lizards that have been examined (see below), and it fails to explain why some reptiles have sexual dimorphism and lack TSD (Ewert and Nelson, 1991).

In chelonians, incubation conditions can affect hatchling survival and growth (Janzen, 1993). Bobyn and Brooks (1994) found that low temperatures reduce hatching success, post-hatching survival and hatchlings' growth in the snapping turtle *Chelidura serpentina*. They suggest that incubation conditions may be the most important factor affecting the northern distribution of this species in Canada. However, no sex-linked selective advantage for growth rate or body size was recorded. In several species of chelonians, hatchlings do not show significant physical or developmental differences (e.g. Ewert and Nelson, 1991), and the relationships between sex-determining mechanisms and sexual dimorphism in adult body size seem to be inconsistent with the predictions of the sexual dimorphism hypothesis (Janzen and Paukstis, 1991b).

In lizards, a pattern similar to that of *Alligator mississippiensis* was found in the leopard gecko *Eublepharis macularius*. In this species, 95–100% females are produced at 26 and 34°C, while males (the larger sex) are predominantly produced at 32.5°C. Tousignant and Crews (1995) found that male-biased incubation temperatures produced larger female hatchlings than did female-biased incubation temperatures. In addition, females from higher incubation temperatures were found to produce larger offspring. Theoretically, this may result in better survival in natural situations. However, the authors found no evidence of incubation temperature directly affecting reproductive success.

Thus, if TSD is adaptive in these taxa, an explanation for its occurrence has to be found in alternative traits. For example, Gutzke and Crews (1988) and Flores et al. (1994) suggested behaviour and sexual receptivity as alternative parameters, producing strong evidence for the adaptive significance of TSD in the leopard gecko. Male *Eublepharis macularius* showed aggression in response to other individuals of the same sex, and females from a male-biased incubation temperature were found to be more aggressive towards males than embryos incubated at female-biased incubation temperatures. Moreover, females produced at low incubation temperatures were sexually receptive, while most females produced at warmer temperatures appeared to be functionally sterile.

Post-hatching physical performance has also been proposed as a fitness trait correlated with sex and incubation temperature. In the snapping turtle *C. serpentina* (where males and females are produced at low and high incubation temperatures, respectively), females from higher incubation temperatures and males from lower ones both had higher first-year survivorship than individuals incubated at intermediate temperatures (Janzen, 1995). A positive correlation was then found between hatchling survivorship and post-hatching ‘propensity to run’, considered as a temperature-dependent antipredator behaviour.
Thus, it seems that specific combinations of incubation temperature, gender and behavioural characteristics affecting fitness do provide contributions towards a functional explanation for the evolutionary significance of TSD.

1.3. Adaptive significance of biased sex ratios

Heavily biased sex ratios have been described in crocodilians (e.g. Ferguson and Joanen, 1982, 1983; Woodward and Murray, 1993), chelonians (e.g. Servan et al., 1989; Gibbons, 1990; Ewert and Nelson, 1991), and lizards (e.g. Auffenberg, 1981). Several different hypotheses have been proposed to explain these patterns in natural populations of reptiles (e.g. Ewert and Nelson, 1991), yet none to date provide a comprehensive explanation.

Ewert and Nelson (1991) reviewed field studies reporting female-biased hatchling sex ratios in natural populations of TSD reptiles. In their 'group-structured adaptation' hypothesis, they suggest that the observed pattern can only persist in small populations with minimal gene flow and periodic re-founding events. This is because if the population increases in size, the optimal sex ratio shifts toward 1:1 (Fisher, 1930). A female- or male-biased breeding system must be isolated to persist, because immigrants may have higher reproductive success than residents. Nevo et al. (1984) found further support for the group structure hypothesis in a number of taxa. Under these assumptions of isolation, inbreeding should be common and the average level of heterozygosity low, especially in comparison with GSD species. However, Burke (1993) found little evidence in chelonians of a correlation between a skewed offspring sex ratio and low heterozygosity, suggesting that there is no genetic evidence that inbreeding is more common in TSD species. Moreover, Smith and Scribner (1990) reported a high migration rate among breeding populations of Trachemys scripta, suggesting that isolated breeding units could hardly persist in this TSD species.

According to the 'sib-avoidance' hypothesis, females of TSD species produce unisexual clutches (e.g. Lang et al., 1989) in order to preclude sib-mating, and thus reduce inbreeding. In this model, TSD would be particularly advantageous in species with extremely low levels of dispersal. However, in chelonian species which do produce unisexual clutches, dispersal is common, and females are likely to produce clutches of different sexes when they lay eggs in nearby nests. Moreover, as female chelonians can store sperm for more than 1 year, eggs laid in sequential clutches may be full sibs. Moreover, Burke (1993) reported similar average heterozygosity values between TSD and GSD in 20 species of chelonians. He further showed that TSD species are not more likely to have unisex clutches.

The above hypotheses, based on eco-ethological factors associated with climatic variation, do not seem to match the observed pattern in natural populations very well. Servan et al. (1989) proposed the influence of genotypic mechanisms to explain the female bias observed in Emys orbicularis. Cold incubation temperatures produce males (see Fig. 2(b)); however, the authors found a surprising female bias in populations sampled from cold areas. In Emys orbicularis a male ZZ/female ZW genotypic sex determination pattern has been proposed (Zaborski et al., 1982, 1988). An increase of female progeny could be explained if the sex of a few genotypic females was inverted.
under the influence of temperature; that is, if a ZW (genotypic female) embryo was exposed to low incubation temperature during the thermosensitive period (see below), it would become a phenotypic male. When these ZW ‘neo-males’ mate with standard ZW females, a majority of genotypic females (ZW, WW) will result. Indeed, Servan et al. (1989) found that most individuals in natural populations of *Emys orbicularis* have sexual phenotypes matching their sexual genotypes, but that a low percentage of individuals are sexually inverted. Thus, unlike the group-structured adaptation and the sib-avoidance hypothesis, the influence of temperature on genotypic sex determination, resulting in populations with a few sexually inverted individuals, seems to provide a good explanation for the observed female-biased sex ratios, and could form the basis of an alternative hypothesis (see also Bull and Charnov, 1989).

1.4. TSD mechanisms

While the evolutionary significance of TSD remains to be clarified, physiological and molecular mechanisms are better understood. In the following sections we describe how a temperature sensitive period during embryogenesis has been defined, and what the main hypotheses regarding molecular mechanisms involved in TSD are.

2. Temperature sensitive stages

In several species of reptiles, temperature affects sex determination during particular stages in embryonic development when differentiation of the gonads occurs. Preliminary temperature-shift experiments, designed to assess the temperature sensitive period (TSP) of gonadal differentiation during incubation, were performed by Pieau and Dorizzi (1981) in *Emys orbicularis* (Fig. 2(b)). Eggs which had been first incubated at 24–26°C for either 27, 30 or 35 days (corresponding to different stages of testicular differentiation) were changed to an incubation temperature of 30°C. All three shifts resulted in both males and females, with some abnormalities, showing that in 27-day-old embryos, testicular differentiation had already begun, whereas in 35-day-old embryos, ovarian differentiation was still possible.

To investigate further the action of temperature on sexual differentiation in embryos, Pieau and Dorizzi (1981) examined the TSP for sexual differentiation by shifting the temperature between 25°C (100% male-producing temperature) and 30°C (100% female-producing temperature) at different stages of embryonic development. The TSP for both male and female differentiation was found to begin at a determinate stage in *Emys orbicularis*, defined as stage 16, in which gonads are still histologically undifferentiated. Eggs incubated at 25°C from stage 16 to 21 produce 100% males, while 100% females are obtained following a 30°C exposure from stage 16 to 22. Shifts occurring at different points during the TSP lead to varied proportions of males and females. In addition, during these experiments, the incubation temperature shifts applied before and after the TSP did not influence the sexual differentiation of the gonads.

Wibbels et al. (1991) reported a similar pattern in the alligator *Alligator mississippiensis*, where the sensitive period encompasses 23% of the total embryonic development...
period (Deeming and Ferguson, 1989; Lang and Andrews, 1994). In chelonians, this period represents 15–25% of development, while comparable data for the leopard gecko *Eublepharis macularius* indicate that the TSP extends for about 30% of the incubation period (Bull, 1987).

Thus, sex ratios seem to depend on the length of time eggs are exposed to a certain temperature regime. Moreover, temperature-shift experiments show that the duration of exposure at the temperature required for typical male or female differentiation depends on the stage within the TSP at which the temperature shift is initiated. The later the shift, the longer the exposure must be. This increase in time may reflect some modification of the gonads (male or female differentiation) due to the temperature applied between the beginning of the TSP and the shift.

### 3. Molecular mechanisms

Several different theories have been proposed to explain the molecular mechanisms behind TSD (Spotila et al., 1994). Studies highlighting the correlation between temperature and the expression of the heterogametic sex-linked H-Y histocompatibility antigen (e.g. Zaborski et al., 1982, 1988) gave quite contradictory results (Engel et al., 1981), and this hypothesis was eventually rejected (McLaren, 1988; Mardon et al., 1989). Similarly, theories underlining the effect of incubation temperature on hypothalamic control of secretion of luteinising hormone (Deeming and Ferguson, 1989), and on the involvement of the genes coding for zinc finger proteins in the sex determination process (Bull et al., 1988; Valleley et al., 1992) failed to provide a definitive explanation for the genetic basis of gonadal sex determination in reptiles (Wibbels et al., 1994).

After the demonstration by Pieau (1974) of the feminising effect of the administration of oestradiol to turtle eggs incubating at a male-producing temperature, a fourth theory, in which gonadal differentiation is determined by the ratio of androgenic to oestrogenic steroids, was suggested (Raynaud and Pieau, 1985; Bogart, 1987), and subsequently corroborated (Bull et al., 1988; Dorizzi et al., 1991).

This hypothesis contradicts the organisation/default system proposed by Jost (1961), where gender is determined by mechanisms operating on a pre-established condition. In fact, an important concept that can be inferred from the new model is that female and male sex determination are separate processes that are differentially affected by incubation temperatures (see below).

Several studies have produced supporting data to the steroid hormone-mediation theory and suggested possible pathways (see Raynaud and Pieau, 1985; Crews et al., 1989; Crews et al., 1994; Crews, 1994; Pieau et al., 1994a; Wibbels et al., 1994). The production of the androgenic steroid dihydro-testosterone (DHT) and oestradiol is regulated by two steroidogenic enzymes: reductase and aromatase, respectively; these enzymes might play a key role in sex determination as they are influenced in a different way by the egg incubation temperature (Pieau, 1974; Crews and Bergeron, 1994; Thomas et al., 1992). For example, high levels of aromatase were recorded by Desvages and Pieau (1992) in embryos incubated at female-producing temperatures, but not at male-producing incubation temperatures. Moreover, the administration of aromatase...
inhibitors produced males at intermediate and all female-producing incubation temperatures in turtles (Crews and Bergeron, 1994; Wibbels and Crews, 1994) and alligators (Lance and Bogart, 1994). The opposite pattern seems to characterise the effect of reductase on hatchling sex ratio at intermediate male-biased incubation temperature, where administration of reductase inhibitor produced a significant number of female hatchlings (Crews and Bergeron, 1994).

In addition, temperature affected aromatase activity during, but not after, the thermosensitive period of *Emys orbicularis* and *Dermochelis coriacea* (Desvages et al., 1993), indicating the importance of oestrogens in gonadal differentiation in TSD reptiles.

A key role in the activity of these steroidogenic enzymes is played by testosterone, which has been proposed as the precursor of oestradiol by aromatase and DHT by reductase. The fact that female hatchlings were produced when testosterone was administered to eggs incubated at male-biased or all male-producing incubation temperatures (Gutzke and Bull, 1986; Crews et al., 1989; Wibbels and Crews, 1992) suggested the conversion of testosterone to oestradiol through aromatase. In addition, this result was not observed using the non-aromatisable androgen DHT (Wibbels and Crews, 1992). Further evidence for this process is that the combined administration of testosterone and reductase inhibitor to eggs incubating at a male-producing temperature also resulted in the production of female hatchlings (Crews and Bergeron, 1994). Similar experiments using aromatase inhibitors (e.g. Wibbels and Crews, 1994) advocated the importance of both enzymes in the sexual differentiation pathway. In addition, it seems likely that temperature does not affect the aromatase enzyme activity, but directly or indirectly controls its synthesis (Desvages and Pieau, 1992; Smith et al., 1995).

Among the models proposed to account for the role of temperature in sex determination of reptiles, the most promising is based on the action of temperature on specific promoters associated with one or more genes encoding for steroidogenic enzymes (Crews and Bergeron, 1994; Pieau et al., 1994b). According to this hypothesis, at a female-producing temperature the genes encoding for aromatase would be activated and the genes encoding for reductase suppressed or simply remain at constitutive levels (see Pieau, 1996) resulting in increased oestradiol production. As further evidence for this model, Pieau et al. (1994b) recorded a positive feedback relationship between oestradiol secretion and aromatase production at a female-producing temperature. Even if no similar relationship has yet been found between DHT and reductase, a reverse process should lead to activation of reductase genes and inhibition of aromatase ones at male-producing incubation temperatures.

After the conversion of testosterone into either DHT or oestradiol, each hormone would bind to specific nuclear receptor proteins. Each of these complexes would in turn find a sex-specific site on the DNA, thus activating the transcription of genes associated with the sex-determining cascade of one sex, and repressing the genes responsible for the sex-determining cascade of the other.

Attempts to identify the genes involved in the sex-determining cascade are currently focusing on portions of the DNA similar to the sex-specific SRY genes of mammalian vertebrates. Tiersch et al. (1991) designed a molecular probe for the conserved region of the SRY gene, which gave a positive signal in the DNA of reptiles with either TSD or
GSD; however, no sex-specific pattern was revealed. A sex specific role for SRY-type protein in two species of reptiles with TSD has been investigated (Johnson et al., 1995), and the levels of similarity with those of mammals are being assessed (Coriat et al., 1993). Whether these proteins have similar roles in reptiles and mammals, and where this SRY-type gene may act in the sex-determining pathway, remain open to speculation.

In conclusion, a clear understanding of exactly how temperature determines the sex of reptiles remains elusive. Recent studies have produced data on an increasing number of species in chelonians and crocodilians, pointing out the remarkable level of variation in TSD patterns shown in these taxa. Much smaller proportions of lizard and snake species have been examined. Nonetheless, the evident evolutionary plasticity suggests that more variation in patterns of TSD, and possibly in sex-determining modes, will be discovered as the number of species studied increases.

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