PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Molecular Biology of Temperature-Dependent Sex Determination [and Discussion]

C. M. Johnston, M. Barnett, P. T. Sharpe, J. A. M. Graves, M. B. Renfree, B. Capel and D. Mireille

Phil. Trans. R. Soc. Lond. B 1995 **350**, 297-304
doi: 10.1098/rstb.1995.0165

The molecular biology of temperature-dependent sex determination

C. M. JOHNSTON, M. BARNETT AND P. T. SHARPE

Department of Craniofacial Development, UMDS Guy's Hospital, London Bridge, London, SE1 9RT, U.K.

SUMMARY

Many reptiles do not have heteromorphic sex chromosomes and for these species sex is determined during embryogenesis by the temperature of egg incubation rather than at conception. The phenomenon of temperature- dependent sex determination (TSD) was discovered almost thirty years ago, but few advances have been made towards the elucidation of its mechanism. In the past few years substantial progress has been made in the understanding of the molecular basis of XY chromosomal (genetic) sex determination (GSD) through the discovery of SRY. It is now possible to start comparing TSD with GSD. TSD is found in some evolutionarily ancient vertebrates and has been postulated to be the ancestral process from which GSD has evolved. If this is true then the two mechanisms may share a common molecular basis. This paper details the current knowledge of TSD, our progress on the investigation of the involvement of SRY-type proteins, and finally presents some of the problems that need to be resolved to gain an understanding of the molecular basis of TSD.

1. INTRODUCTION

One of the most fundamental decisions made by the developing embryo is that of sex determination. The survival of the species depends on the ability of embryos to differentiate into male and female individuals and therefore the mechanism by which this occurs must be robust. A variety of sex determining mechanisms exists in nature including chromosomal (genetic) sex determination (with either male or female heterogamy), environmental sex determination and parthenogenesis. Environmental sex determination is characterized by the determination of sexual fate in response to environmental cues and is independent of the genetic composition of the zygote. Many environmental features have the capacity to affect sex including: crowding, water potential, pH and most notably temperature. The most prominent feature of TSD is that a small change in egg incubation temperature can determine whether male or female offspring are produced.

The phenomenon of TSD is associated with species that lack heteromorphic sex chromosomes. TSD is commonly observed in reptilians and has been identified for all crocodilia studied to date. Some turtle and lizard species exhibit either temperature-dependent or chromosomal sex determination, whereas snakes have only been found to have genetic sex determination. TSD has been most extensively studied in the American alligator, *Alligator mississippiensis*. In the alligator, egg incubation temperatures of 30 °C and below produce 100% females; 33 °C produces 100% males; 35 °C produces approximately 90% females; and different sex ratios are produced at intermediate temperatures. This female-male-female pattern of sex determination is not unique to *A. mississippiensis* and is commonly seen in species with TSD. Remarkably, the temperature of an egg in an alligator nest is not constant but fluctuates over a 24-hour period; small changes in average incubation temperature can affect the ability of an individual to develop as a male or a female.

In species with TSD, intersex animals have not been observed to date; in alligators a temperature of 32 °C produces 50% males and 50% females. The results of early experiments showed that the temperature-sensitive period (TSP) during which temperature exerts its effect was different for male and female alligators (Ferguson & Joanen 1983; Deeming & Ferguson 1989) and that sex was determined earlier in females than in males. However, it now appears that the TSP may be the same for both sexes, between stages 20–22, corresponding to approximately 30–45 days of incubation at 32 °C (Lang & Andrews 1994), with most embryos becoming committed to male or female development by stage 23 (Smith & Joss 1994). The thermosensitive periods (TSP) of reptiles with TSD generally encompass the middle one third to one half of embryonic development (Wibbels *et al.* 1994 and references therein). In several species of reptiles thermosensitivity starts before gonadal differentiation (as determined histologically) and extends to a time when sex-specific changes are becoming evident in the gonads. An important question that is fundamental to the study of TSD is how a single temperature can operate to produce both sexes. The observation that intermediate incubation temperatures result in different ratios of males and females (i.e. that both sexes can develop at the same temperature) is difficult to reconcile with the idea that a single temperature-dependent 'switch' operates as the primary event in TSD. It implies that temperature affects a number of factors, the relative timing of which may be crucial to

Phil. Trans. R. Soc. Lond. B (1995) **350**, 297–304
Printed in Great Britain

male or female development (discussed in §4). This hypothesis fits with the observation that the temperature effect on sex determination appears to be cumulative over a period of time and is not a switch event occurring at a particular developmental stage (Deeming & Ferguson 1988, 1989).

In species with GSD, the sex of an individual is an inherited characteristic and tends to result in an approximately 50:50 sex ratio. In species with TSD, geographically separate breeding sites with slightly different mean temperatures may result in populations having varying sex ratios. For the American wild alligator, populations have been shown to have a skewed sex ratio with about five females to each male. This observation does not result from differential mortality, but appears to be a direct consequence of TSD. A skewed sex ratio is a common feature in species with TSD and allows the flexibility for rapid repopulation of the species following disaster (through the maintenance of a large breeding population) which would not be possible with a 50:50 sex ratio, as typically seen in species with genetic sex determination. TSD is clearly both an effective and robust mechanism as it is found in evolutionarily ancient vertebrates which have been in existence for some 250 Ma. TSD has been postulated to be ancestral to chromosomal sex determination which itself is thought to have evolved independently several times from homomorphic chromosomes: an assumption supported by the fact that homomorphism is ancestral in all orders.

The observation that closely related reptile species have either genetic sex determination or TSD, leads to the hypothesis that underlying mechanisms for these two processes may be similar. Our approach to understanding the mechanisms controlling TSD is based on the premise that some aspects of the genetic pathway leading to gonad differentiation are conserved between mammals and reptiles. Genes identified as being important in the control of mammalian gonad development may therefore have similar roles in reptiles and through the assembly of the molecular pathway of sex determination in mammals we may gain insight into the key molecules involved in the mechanism of TSD.

2. THE ALLIGATOR GENOME

The haploid alligator genome has 16 chromosomes: 12 metacentric, two submetacentric and two telomeric (Valleley *et al.* 1994). Karotypes of male and female hatchlings in 21 species studied including *A. mississippiensis* have repeatedly failed to identify heteromorphic sex chromosomes (Cohen & Gans 1970). The main features of the karyotype are five large and 11 small chromosome pairs with one pair possessing a secondary constriction on the short arm (Valleley *et al.* 1994). Constrictions are a common feature among crocodilian chromosomes and are thought to result from variation in copy number of rRNA genes between the two homologous chromosomes (King *et al.* 1986).

Because sex chromosomes are thought to have evolved from pairs of homomorphic chromosomes, it is of interest to trace any putative evolutionary origins of sex chromosomes in reptiles with TSD. The mammalian genes *ZFX* and *ZFY* are located on the X and Y chromosomes respectively, and were once thought to be involved in the determination of sex. We have mapped the chromosomal location of the alligator homologue of *ZFX/Y* (*Zfc*) to chromosome 3 (Valleley *et al.* 1994). Preliminary mapping studies of another alligator gene (*Znc6*) a homologue of the mammalian X-linked gene *ZNF6*, suggest that this gene is located on chromosome 3. Further mapping studies will be needed to establish whether the alligator homologues of mammalian sex-linked genes are clustered in the alligator genome. It is possible that alligator genes could be used as markers to trace chromosomal regions which, through evolution, have become part of the eutherian sex chromosomes.

3. ALLIGATOR *SRY*

The search for genes involved in the control of mammalian sex determination has resulted in the isolation of a Y chromosome gene from the mouse and human genome, the product of which was found to be restricted to gonadal tissue (Koopman *et al.* 1990). This gene was named *SRY* (human)/*Sry* (mouse) for sex determining region of the *Y* chromosome and its DNA binding domain has been found to be very highly conserved between eutherian mammals. Chromosomally female mice transgenic for *Sry* have been shown to develop as male mice (Koopman 1991). These experiments suggest that *Sry* is both sufficient and necessary for testis development.

The open reading frame of *SRY/Sry* encodes a putative 204 amino acid protein and includes a sequence-specific DNA-binding domain consistent with the suggestion that *SRY/Sry* encodes a transcription factor. This DNA-binding domain is encoded by an 80 amino acid motif which shares homology with several proteins with known DNA-binding properties. Although the exact mode of action of SRY/Sry is unknown, the SRY DNA-binding domain has recently been shown to recognize the proximal upstream elements (SRYe) in the promoters of both P450 aromatase and Mullerian inhibitor substance (MIS) which have roles in the secondary sexual development of embryos (Haqq *et al.* 1993). SRY/Sry has therefore been postulated to control male development in mammals through specific regulation of target genes.

In the search for common links between the sex-determining mechanisms of species with both chromosomal and environmental sex determination, we set out to isolate an alligator *SRY* homologue. Alligator genomic DNA was used as a target for polymerase chain reaction (PCR) amplification using degenerate PCR primers designed against conserved regions of human *SRY* and mouse *Sry*. Eight different sequences with homology to the DNA-binding domain of *SRY* were identified (Coriat *et al.* 1993), which were found to be most similar to the mouse *Sox* genes (*S*RY-b*ox* containing genes). The alligator *Sox* genes were seen to be 45–63% similar to *SRY* and up to 95% identical to mouse and human genes belonging to the *Sox*-1 class (*Sox-1*, *Sox-2* and *Sox-3*). A notable feature of *Sox* genes

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

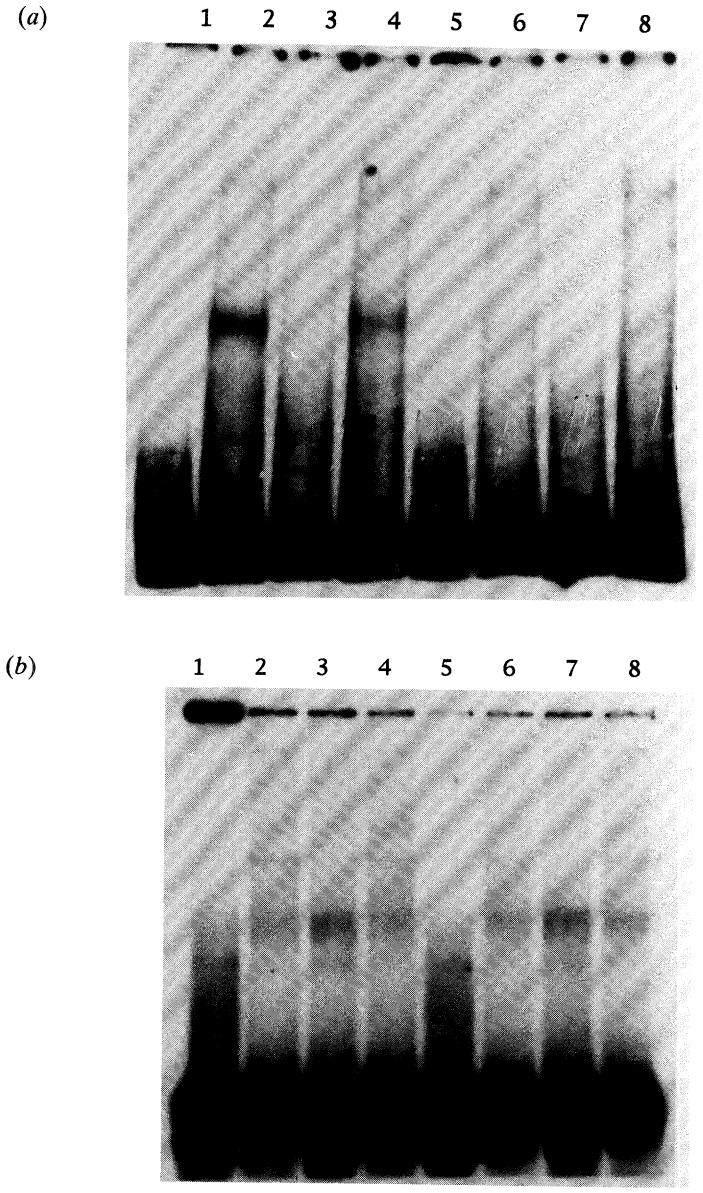


Figure 1. Gel retention assays showing a specific retarded band with protein extracts from stage 19 alligator urogenital tissue. (*a*) Stage 19 alligator embryo urogenital (UG) tissue proteins with an HMG-box binding sequence AACAAAG. Lane 1, free probe. Lane 2, 33 °C UG. Lane 3, 33 °C UG with excess competitor oligo (unlabelled HMG-box oligo). Lane 4, 33 °C UG with excess non-competitor oligo (COUP). Lane 5, free probe. Lane 6, 30 °C UG. Lane 7, 30 °C UG with excess competitor oligo. Lane 8, 30 °C UG with excess non-competitor oligo. Arrow indicates the position of the retarded band in lanes 2 and 4. + indicates the position of the common retarded band. (*b*) Stage 19 alligator embryo head proteins with the HMG-box consensus oligo. Lane 1, free probe. Lane 2, 33 °C. Lane 3, 33 °C with excess competitor oligo. Lane 4, 33 °C with excess non-competitor oligo. Lane 5, free probe. Lane 6, 30 °C. Lane 7, 30 °C with excess competitor oligo. Lane 8, 30 °C with excess non-competitor oligo.

is that the putative DNA-binding region shows very strong conservation between species, whereas regions outside the DNA-binding domain show very little (if any) conservation between different classes of gene in the same species or between species. However, individual genes are strongly conserved (both within and outside the DNA-binding region) across different species, so an alligator *Sox* gene can be recognized as the equivalent of a particular mammalian *Sox* gene. Because *SRY* is so divergent between different mammalian species (see pp. 205–214, this volume) and the PCR products generated represented only the SRY-box sequence, it was not possible to conclude that one of the PCR sequences was alligator *SRY*. The alligator clones did, however, show stronger identity to Sox genes than to *SRY*/*Sry*.

Sequence comparison of all SRY-boxes in human and mouse indicates that the SRY-box region of *Sox-3* is the most closely related to the DNA-binding domain of SRY. *Sox-3* is located on the mammalian X chromosome and is expressed in developing gonads (R. Lovell-Badge, personal comm.), which leads to the hypothesis that *Sox-3* may be the evolutionary ancestor of *SRY*: if this is the case *Sox-3* may be a potential candidate for a gene involved in the control of TSD. We have cloned alligator *Sox-3* (*Asox3*) by PCR of genomic

DNA and shown that *Asox3* is 92% homologous to murine *Sox-3* within the DNA-binding region. As with other *Sox* genes, conservation within the putative DNA-binding region between human, mouse, chick and alligator is very strong (92%) whereas sequences outside this region show only very weak similarity. The expression of *Asox3* in gonads developing at different temperatures is currently under investigation.

An alternative approach aimed at detecting SRY-like activity in gonadal tissue has also been pursued in our group. SRY/Sry protein and *Sox* gene products bind *in vitro* to a DNA consensus sequence of $^{A}/_{T}{}^{A}/_{T}$CAAAG (van de Wetering & Clevers 1992). If a protein with SRY-type activity is involved in TSD, an assay for specific DNA-binding activity should reveal its presence in developing gonads. Proteins were extracted from urogenital (UG) system (mesonephros and genital ridge) and brain tissue from alligator embryos incubated at all-male (33 °C) or all-female (30 °C) temperatures at several stages from before, during, and after the temperature-sensitive period. Proteins were hybridized to a radiolabelled DNA fragment containing the target DNA sequence and subsequently resolved by gel electrophoresis. A shifted band was observed for extracts from the urogenital system of 33 °C male embryos which was not found for similar extracts from 30 °C female embryos (see figure 1). Equivalent experiments using protein extracted from male and female brain tissue revealed no sex specific differences in the band pattern. This data implies that the specific band seen using extracts from male urogenital system was specific to developing male gonads. This experiment was repeated with protein extracts from lizard embryos (*Eublepharis macularius*, (leopard gecko)) which have a low-temperature female, intermediate-temperature male, high-temperature female pattern of TSD, similar to that of the alligator. In this case a sex-specific band shift pattern was also observed at the all-male producing temperature of 32 °C (data not shown). These experiments present the first evidence for a difference in male and female SRY-like activity in the developing urogenital system of a species with TSD. Furthermore, these data suggest that this band shift is associated specifically with the male sex and does not simply correlate with temperature, as the absolute male-producing temperature differs between the two species. Because the *Sox* gene products assayed to date have been shown to bind to identical or very similar target sequences to SRY/Sry, the band shift observed may be the result of binding by any SRY-box containing protein and not specifically by a reptilian equivalent of SRY. Identification of the protein involved would contribute significantly to our understanding of sex determination in species with TSD.

4. THE MOLECULAR MECHANISM OF TSD

(*a*) *Conformational change*

Several hypotheses may be postulated to explain the molecular mechanism of TSD. A simple explanation could be that temperature affects the expression or activity of an SRY-box protein which in turn either activates the male sexual differentiation pathway and/or represses the female pathway. There are well characterized examples of temperature effects on gene expression (heat shock proteins) and temperature effects on protein activity (conformational change). We hypothesise that the band-shift results seen in the alligator suggest that the higher temperature of male incubation induces a conformational change in an SRY-like protein allowing it to bind to DNA.

Two conceptual problems exist with the hypothesis that temperature induces protein conformational changes. First, for species with a female–male–female (FMF) pattern of sex determination such as the alligator, at least two conformational changes would need to occur; one to allow a (gain of function) 'switch' to male development (at around 31 °C), the other to allow a (loss of function) reversion to female development (at 35 °C), resulting in the same female-producing pathway that occurs at 30 °C. This may be possible because the FMF pattern of sex determination could be considered as an 'optimum' phenomenon where male development occurs at optimal temperatures and females develop when conditions are sub-optimal, i.e. when the putative 'sex-determining' protein has an alternative conformation. The second problem is that if the same protein is involved in all TSD species, the same temperature would be expected to result in the same protein conformation and hence the same pattern of downstream gene expression. Yet one temperature can produce different sex ratios in different species. This observation suggests that the active proteins in different species may not be identical: small differences in amino acid sequence and/or conformation of the reptile SRY proteins could be different in different TSD species, leading to variation in 'optimal' conditions. This is conceivable because the amino acid sequences of SRY proteins from different mammalian species are markedly different outside the conserved DNA-binding domain (Whitfield *et al.* 1993; Tucker & Lundrigan 1993). Temperature-related conformational change of similar (but non-identical) proteins could potentially result in expression of different target genes in different species, thus explaining different patterns of sex determination. If TSD is based upon conformational change then the protein must act over a period of some days as the temperature effect on sex appears to be cumulative over a period of time.

(*b*) *The 'threshold' hypothesis*

A hypothesis for TSD proposed by Deeming & Ferguson (1988, 1989) suggests that the development of the testis depends upon the production of a male-determining factor (MDF) during a critical period of embryonic development. Embryos incubated at 33 °C would be at an optimal temperature to accumulate sufficient MDF to reach a 'threshold' level to develop as males. Presumably the effect of temperature on the MDF gene would be such that at either side of the optimal temperature (i.e. 30 °C and 34 °C in alligators) subthreshold levels of MDF would accumulate and the embryos would develop as females. This theory may

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

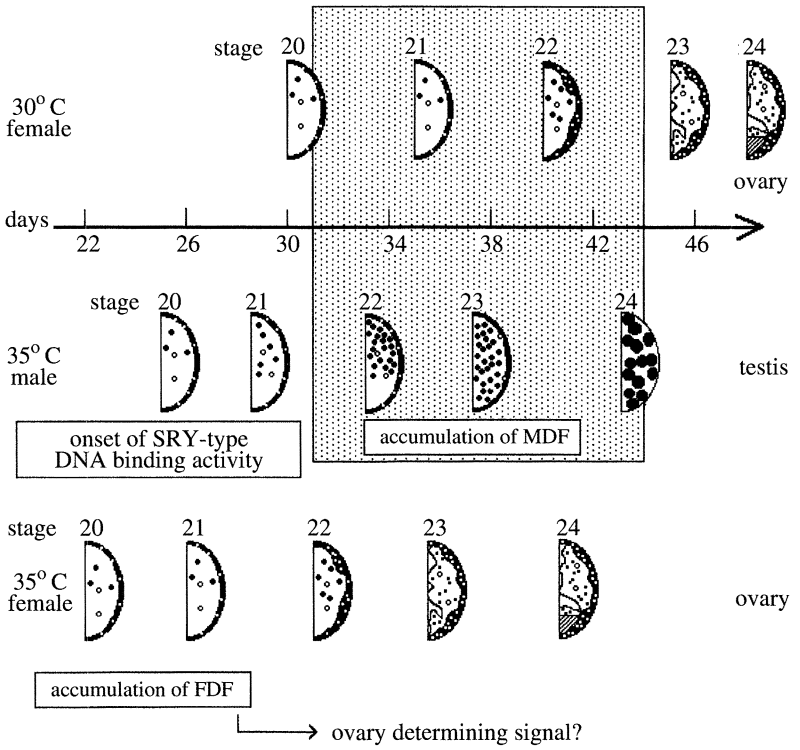


Figure 2. Hypothetical schemes of alligator sex determination at three different temperatures. In this model a possible mechanism of TSD is illustrated. Sex is determined by the accumulation of threshold levels of male determining factor (MDF) and/or female determining factor (FDF). If sufficient MDF is accumulated during the thermosensitive period, an embryo will follow the male sex determination pathway: this may be optimal at 33 °C. Alternatively if a threshold level of FDF is accumulated before the thermosensitive period, an embryo may become committed to the female pathway (and either lose the ability to accumulate MDF or the ability to respond to MDF). FDF may accumulate faster at 35 °C than at 33 °C or 30 °C. Females resulting from incubation temperatures of 30 °C may represent a 'default' pathway: insufficient FDF is accumulated before the thermosensitive period; the embryo then also fails to reach the threshold level of MDF by the appropriate stage. In this model, females have two different modes of determination whereas males have only one. A possible role of an SRY-type protein in this model is in pre-Sertoli cell proliferation and/or accumulation. Its activity may be optimal at 33 °C and sub-optimal at all other temperatures. See text for discussion. Adapted from Smith & Joss (1994).

account for the observation that at some temperatures embryos can develop either as males or as females: natural variation in the rate of production of MDF or in absolute threshold level could produce this effect. In different species the temperature at which MDF most quickly accumulates may vary, as may the range of temperatures over which it can reach its threshold, therefore producing different male:female ratios at a particular temperature. This hypothesis may also account for species with male–female or female–male patterns of sex determination (as well as species with bimodal patterns of sex determination), if the temperature at which the threshold level of MDF is reached is very close to a temperature which is lethal to the developing embryos. For example, a species with low-temperature females/high-temperature males may theoretically be able to produce females at an even higher temperature, except that these conditions would result in 100% mortality.

The mechanism of TSD may be more complex than the hypothesis outlined above and may involve accumulation of a female-determining factor (FDF) and an MDF, one or both of which may be influenced by temperature. At the time of egg laying, all embryos have the potential to develop as males or females. If a threshold level of the FDF has accumulated by the beginning of the TSP, the embryo will develop as a female and will not be affected by temperature after this period. If, however, the FDF threshold level is not reached, the embryo retains the potential to develop as either male or female. By the end of the TSP, if enough MDF has accumulated, an embryo will develop as male; if not it will follow a default pathway and develop as a female (see figure 2). The FDF may therefore act as a repressor or downregulator of MDF. It would be expected that MDF would accumulate faster at 33 °C than at 30 °C and that FDF would accumulate fastest at 34–35 °C. This hypothesis could be tested by temperature-shift experiments on embryos initially incubated at 34–35 °C: the expected outcome would be that embryos incubated at 34–35 °C between the time of oviposition and the start of the TSP would always develop as females, regardless of any subsequent shift in temperature. The 35 °C females, therefore, may be those which have accumulated the threshold level of FDF whereas 30 °C females are those which have failed to accumulate the required amount of MDF. The observation that mixed-sex clutches occur can be explained, as previously, by variation in absolute requirements between individuals.

Results obtained from single-shift and double-shift experiments have demonstrated that both the duration

(cumulative effect) and magnitude (potency effect) of incubation temperatures during the TSP affect the sex of alligator embryos (Lang & Andrews 1994). This observation is consistent with the hypothesis that temperature could be controlling the production of sex factors (e.g. growth factors, hormones) which if produced at a sufficient rate over a sufficient time period, initiate a sex determination cascade (Wibbels *et al.* 1991).

(*c*) *Gonad asynchrony*

Examination of embryonic alligator gonads by light microscopy of paraffin sections led Deeming & Ferguson (1988) to conclude that the testis differentiated earlier in 33 °C embryos than did the ovary in 30 °C embryos. Detailed analysis by Smith & Joss (1993, 1994) indicates that the onset of testis differentiation in *A. mississippiensis* (stage 21–22) occurs just before the onset of ovarian differentiation (stages 22–23). All stages refer to equivalent developmental stages defined by Ferguson (1985), and not to the overall size of embryos which may vary between the sexes and between embryos incubated at different temperatures. From these observations Smith & Joss (1993, 1994) hypothesised that TSD in *A. mississippiensis* may involve a temperature-dependent mismatch between the timing of pre-Sertoli/Sertoli cell differentiation and ovary determination. If a threshold number of pre-Sertoli cells is reached by a particular stage in development (the time of activation of the ovary determinant), the alligator develops as a male; if not the embryo develops as a female. The threshold number is achieved earlier at 33 °C than at 30 °C resulting in testis differentiation in 100 % of embryos at 33 °C (Smith & Joss 1994). The rate of accumulation of pre-Sertoli cells in embryos developing as females at 34–35 °C has not been established to date; pre-Sertoli cells may accumulate more slowly than at 33 °C (which may be an optimal temperature), or the ovary-determining signal may be advanced in development, preempting the testis-determining signal (Smith & Joss 1994). The ability to produce both males and females at a single temperature may be explained by differences in the absolute number of pre-Sertoli cells required for male development (threshold level) and rate of accumulation of pre-Sertoli cells between individuals. Pre-Sertoli cells may therefore represent the MDF postulated for alligator 33 °C embryos by Deeming & Ferguson (1989), and an as yet unknown ovary determinant may represent the FDF.

(*d*) *The effect of steroid hormones*

Treatment of developing reptile embryos with specific steroid hormones can alter the sexual differentiation of the gonad (Raynaud & Pieau 1985). The stimulation of ovarian differentiation by estradiol at male producing temperatures has been documented in a variety of reptiles with TSD (Gutzke & Bull 1986; Bull *et al.* 1988; Crews *et al.* 1989, 1991; Lance & Bogart 1991, 1992; Wibbels & Crews 1992). Exogenous estrogen can therefore override the effects of male-producing temperature. Studies on two species of turtle have shown that the period of steroid sensitivity roughly parallels the period of temperature sensitivity (Gutzke & Chymiy 1988; Wibbels *et al.* 1991) and furthermore temperature and estradiol have been shown to exert a synergistic effect on sex determination (Wibbels *et al.* 1991). These observations are consistent with the hypothesis that temperature and exogenous estradiol may act through the same physiological pathway (Wibbels *et al.* 1994). Testosterone treatments can also induce ovarian development at male producing temperatures (Pieau 1974; Gutzke & Bull 1986; Crews *et al.* 1991; Wibbels & Crews 1992), unlike treatment with a nonaromatizable androgen (dihydrotestosterone), supporting the hypothesis that testosterone-induced ovarian development results from the aromatization of testosterone to estradiol (Crews *et al.* 1989; Wibbels & Crews 1992). Nonaromatisable androgens can exert a masculinizing effect on gonadal differentiation, although the effect appears weak compared with the feminizing effects of estrogen (Wibbels & Crews 1992).

These experiments suggest that in reptiles with TSD, female development may be a 'default' state, on which certain conditions can act to induce male development. This is consistent with the concept of male development resulting from the accumulation of a specific factor under optimal conditions, and may also be supported by our data. The addition of steroid hormones may have two effects: first, the conditions may be no longer optimal for the rapid accumulation of MDF (embryos default to female), and second, that if temperature and estradiol share the same physiological pathway the addition of the hormone may developmentally advance the pathway of female development (embryos are committed to female development).

(*e*) *The potential role for SRY-type genes in* TSD

We have shown that an SRY-type protein may have a male-specific role in two species with different patterns of TSD. We do not know where in the sex-determining pathway this SRY-type gene may act, but there are a number of possibilities. For example, an SRY-type protein may act as a switch gene: in its presence the pathway of male development is initiated, in its absence female development occurs. The production of the protein would thus represent a developmental 'choice' and must be regulated directly or indirectly by temperature, perhaps by the differential binding of ubiquitous transcription factors at different temperatures. Alternatively, the conformation of the SRY-type protein itself may change in response to temperature. In this case it may act as a transcription factor that can bind differentially to the enhancer(s) of a gene or genes involved in the male sex determination pathway. SRY-type genes may also be considered as candidate genes for the MDF, as at least one is present in the male but not female urinogenital system during the TSP.

Although our experiments have demonstrated a potential role for an SRY-type protein in male sex determination, the function of such a protein remains

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

open to speculation. For example, it may act as a positive regulator of male development, or as a negative regulator of female development. It may act as a signal for pre-Sertoli cell differentiation, or may be required in the process of pre-Sertoli cell differentiation. Another possibility is that an SRY-type protein is not involved in the initiation of male sex determination but functions elsewhere in the male- determining pathway.

Once we have isolated the gene encoding the SRY-type protein, we can begin to investigate its expression pattern (and hence whether it is regulated at the level of transcription or translation), how it may respond to temperature and its potential role in TSD. If an SRY-like DNA-binding protein is involved in TSD, it will have significant implications for our understanding of evolution of vertebrate sex determination. The models proposed above are a very attractive view of TSD that can at least lend themselves to experimental investigation.

We thank Anne-Marie Coriat and Elizabeth Valleley for providing data, helpful discussions and input on this project. The invaluable contribution and cooperation of colleagues at the Rockefeller Wildlife Refuge in Louisiana is gratefully acknowledged. Thanks also to Robin Lovell-Badge for revealing information prior to publication.

REFERENCES

Bull, J. J., Gutzke, W. H. N. & Crews, D. 1988 Sex reversal by estradiol in three reptilian orders. *Gen. comp. Endocr.* **70**, 425–428.

Cohen, M. M. & Gans, C. 1970 The chromosomes of the order *Crocodilia*. *Cytogenetics* **9**, 81–105.

Coriat, A. M., Müller, U., Harry, J. L., Uwanogho, D. & Sharpe, P. T. 1993 PCR amplification of SRY-related gene sequences reveals evolutionary conservation of the SRY-box motif. PCR *Meth. Appl.* **2**, 218–222.

Crews, D., Bull, J. J. & Wibbels, T. 1991 Estrogen and sex reversal in turtles: a dose-dependent phenomenon. *Gen. comp. Endocr.* **81**, 357–364.

Crews, D., Wibbels, T. & Gutzke, W. H. N. 1989 Action of sex steroid hormones on temperature induced sex determination in the snapping turtle (*Chelydra serpentina*). *Gen. comp. Endocr.* **76**, 159–166.

Deeming, D. C. & Ferguson, M. W. J. 1988 Environmental regulation of sex determination in reptiles. *Phil. Trans. R. Soc. Lond.* B. **322**, 19–39.

Deeming, D. C. & Ferguson, M. W. J. 1989 The mechanism of temperature-dependent sex determination in crocdilians: a hypothesis. *Am. Zool.* **29**, 973–985.

Ferguson, M. W. J. & Joanen, T. 1983 Temperature-dependent sex determination in *Alligator mississippiensis*. *J. Zool.* **200**, 143–177.

Ferguson, M. W. J. 1985 The reproductive biology and embryology of crocodilians. In *Biology of the Reptilia*, vol. 14 (ed. C. Gans, F. Billet & P. F. A. Maderson), pp. 329–491. New York: John Wiley.

Gutzke, W. H. N. & Bull, J. J. 1986 Steroid hormones reverse sex in turtles. *Gen. comp. Endocr.* **64**, 368–372.

Gutzke, W. H. & Chymiy, D. B. 1988 Sensitive periods during embryogeny for hormonally induced sex determination in turtles. *Gen. comp. Endocr.* **71**, 265–7.

Haqq, C. M., King, C., Donahoe, P. K. & Weiss, M. A. 1993 *SRY* recognises conserved DNA sites in sex-specific promoters. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1097–1101.

King, M., Honeycutt, R. & Conteras, N. 1986 Chromosomal repatterning in crocodiles: C, G and N-banding and the *in situ* hybridisation of 18S and 23S rRNA cistrons. *Genetica* **70**, 191–201.

Koopman, P., Münsterberg, A., Capel, B., Vivian, N. & Lovell-Badge, R. 1990 Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature, Lond.* **348**, 450–452.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P. & Lovell-Badge, R. 1991 Male development of chromosomally female mice transgenic for *Sry*. *Nature, Lond.* **351**, 117–121.

Lang, J. W. & Andrews, H. V. 1994 Temperature-dependent sex determination in crocodilians. *J. exp. Biol.* **270**, 28–44.

Lance, V. & Bogart, M. 1991 Tamoxifen 'sex reverses' alligator embryos at male producing temperature but is an antiestrogen in female hatchlings. *Experimentia* **47**, 263–266.

Lance, V.A. & Bogart, M. H. 1992 Disruption of ovarian development in alligator embryos treated with an aromatase inhibitor. *Gen. comp. Endocr.* **86**, 59–71.

Pieau, C. 1974 Differenciation du sexe en fonction de la temperature chez les embryons d'*Emys orbicularis* L. (Chelonien); effets des hormones sexuelles. *Ann. Embryol. Morphog.* **7**, 365–394.

Raynaud, A. & Pieau, C. 1985 Embryonic development of the genital system. In *Biology of the Reptilia*, vol. 15 (ed. C. Gans, F. Billett & P. F. A. Maderson) pp. 149–300. New York: John Wiley & Sons.

Smith, C. A. & Joss, J. M. P. 1993 Gonadal sex differentiation in *Alligator mississippiensis*, a species with temperature-dependent sex determination. *Cell Tissue Res.* **273**, 149–162.

Smith, C. A & Joss, J. M. P. 1994 Sertoli cell differentiation and gonadogenesis in *Alligator mississippiensis*. *J. exp. Zool.* **270**, 57–70.

Tucker, P. K. & Lundrigan, B. L. 1993 Rapid evolution of the sex determining locus in Old World mice and rats. *Nature, Lond.* **364**, 715–717.

Valleley, E. M. A., Harrison, C. J., Cook, Y., Ferguson, M. W. J. & Sharpe, P. T. 1994 The karyotype of *Alligator mississippiensis*, and chromosomal mapping of the *ZFY/X* homologue, *Zfc*. *Chromosoma* **103**, 502–507.

van de Wetering, M. & Clevers, H. 1992 Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. *EMBO J.* **11**, 3039–3044.

Whitfield, L. S., Lovell-Badge, R. & Goodfellow, P. N. 1993 Rapid sequence evolution of the mammalian sex-determining gene *SRY*. *Nature, Lond.* **364**, 713–715.

Wibbels, T., Bull, J. J. & Crews, D. 1991 Chronology and morphology of temperature-dependent sex determination. *J. exp. Zool.* **260**, 371–381.

Wibbels, T. & Crews, D. 1992 Specificity of steroid hormone-induced sex determination in a turtle. *J. Endocrinol.* **133**, 121–129.

Wibbles, T., Bull, J. J. & Crews, D. 1994 Temperature-dependent sex determination: a mechanistic approach. *J. exp. Zool.* **270**, 71–78.

Discussion

J. A. M. GRAVES (*La Trobe University, Melbourne, Australia*). I don't think I'd agree that TSD systems are monophyletic, in fact. I would imagine that you could make a TSD system out of a GSD system very easily (that is, if you are not a homoeotherm!) just by making any one of a number of proteins in the pathway temperature-sensitive.

P. T. SHARPE. I do not believe it would be very easy to go from GSD to TSD by making any one protein in the pathway

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

temperature-sensitive. First, there is the question of why such temperature-sensitivity should evolve in the first place, and what would be the selective advantage for it to be maintained over GSD. Second, if a protein's function was simply to become temperature-sensitive, it would have to be able to behave in such a way as to allow the production of both sexes at one temperature, as well as directing female development at both low and high temperatures. Any conversion of a GSD system to a TSD system must therefore be more complex than a single temperature-related switch, and may well involve more than one protein. Third, if only one protein were to become temperature-sensitive, then it must be the first protein in the pathway, i.e. the 'testis determining' gene, to enable temperature to cause a 'switch' between male and female development. For example, suppose that the AMH (MIS) protein was to become temperature-sensitive. Activity of AMH alone is not sufficient to determine maleness i.e. AMH is not testis-determining. In addition to AMH becoming temperature-sensitive, the inherited genetic factor that initiates male/female development would also have to be lost. It is therefore hard to see how TSD could evolve from a system where only one sex inherits a gene that initiates a cascade of development.

M. B. RENFREE (*Department of Zoology, University of Melbourne, Parkville, Australia*). Would you speculate on the possible link between the sexually dimorphic expressions of the proteins, which are present at 33 °C and absent at 30 °C, and the key role of aromatase and estrogen in sex differentiation in TSD reptiles.

P. T. SHARPE. The simplest idea is that proteins involved in sex-steroid synthesis pathways such as aromatase are regulated directly or indirectly by the protein differentially expressed which is a transcriptional regulator. Thus in this situation we would expect the steroid synthesis pathways to lie downstream of the transcriptional regulators (similar to the situation found in GSD species) i.e. the sexually dimorphic protein expression may represent the determination of sex, whereas the downstream effects on steroid hormone synthesis may represent sexual differentiation of the alligator embryos.

B. CAPEL (*Duke University Medical Center, U.S.A.*). Cell migration is critical for testis cord formation, and testis cord formation is critical for sex determination. It occurs to me that cell migration is a mechanism which might easily be responsive to temperature shifts.

P. T. SHARPE. I agree with Dr Capel. I have been thinking along these lines for some time, with respect to TSD involving the coordinated timing of several different processes required for gonad development. As Dr Capel says, one of the obvious places to start looking is cell migration. However, to account for the observed male/female ratios, any effect of temperature on migration would have to be biphasic, for example, migration would be greater at 33 °C and decreased at both 30 °C and 35 °C (in alligators). This apparent paradox may not be a problem, if coordinated timing of different processes is involved, but it is certainly something we propose to investigate.

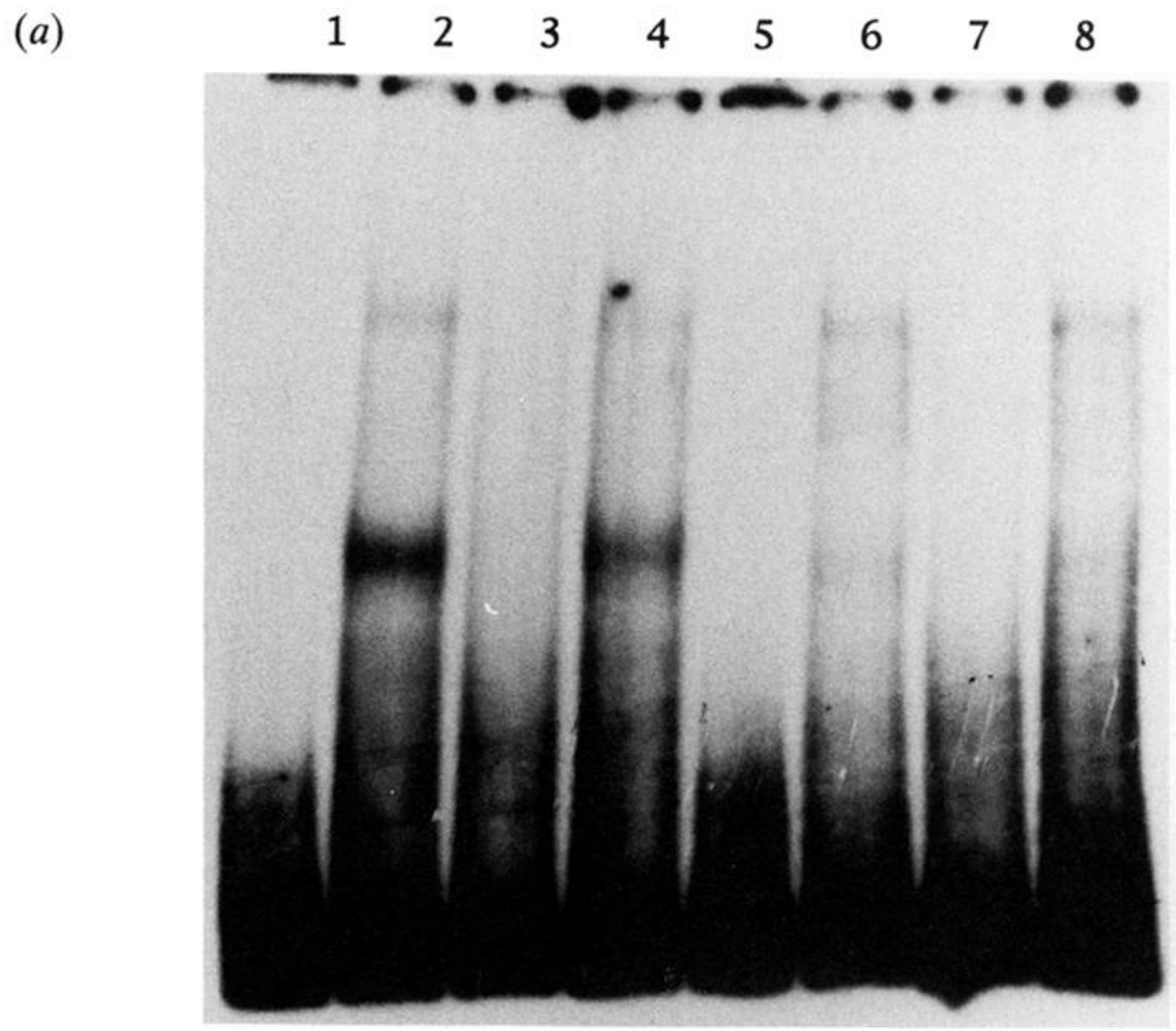
D. MIREILLE (*Institut Jacques Monod, CNRS et Université Paris VII, Tour 43, France*). In the European pond turtle which also exhibits TSD, we have shown that during the thermosensitive period, there is a significant difference in the levels of oestrogens between the gonads undergoing male-differentiation and those undergoing female-differentiation. Oestrogens are present in higher levels in the female gonads than in the male gonads. Likewise, gonadal aromatase activity, at the very early stages of gonadal differentiation, was found to be higher at female-producing temperatures than at male-producing temperatures. As was demonstrated by gonadal sex-reversal of embryos incubated at feminizing temperatures under the *in vivo* action of anti-oestrogens and aromatase inhibitors, oestrogens are involved in gonadal differentiation. The role of oestrogens in this process has also been shown in fishes, amphibians and birds, and in marsupials oestrogens have feminizing effects in males. Thus, we have proposed that in mammals, the different sexual expression of the aromatase gene could be regulated by SRY directly, or indirectly via another factor such as the anti-Müllerian hormone, which itself represses aromatase synthesis (Pieau *et al.* 1994).

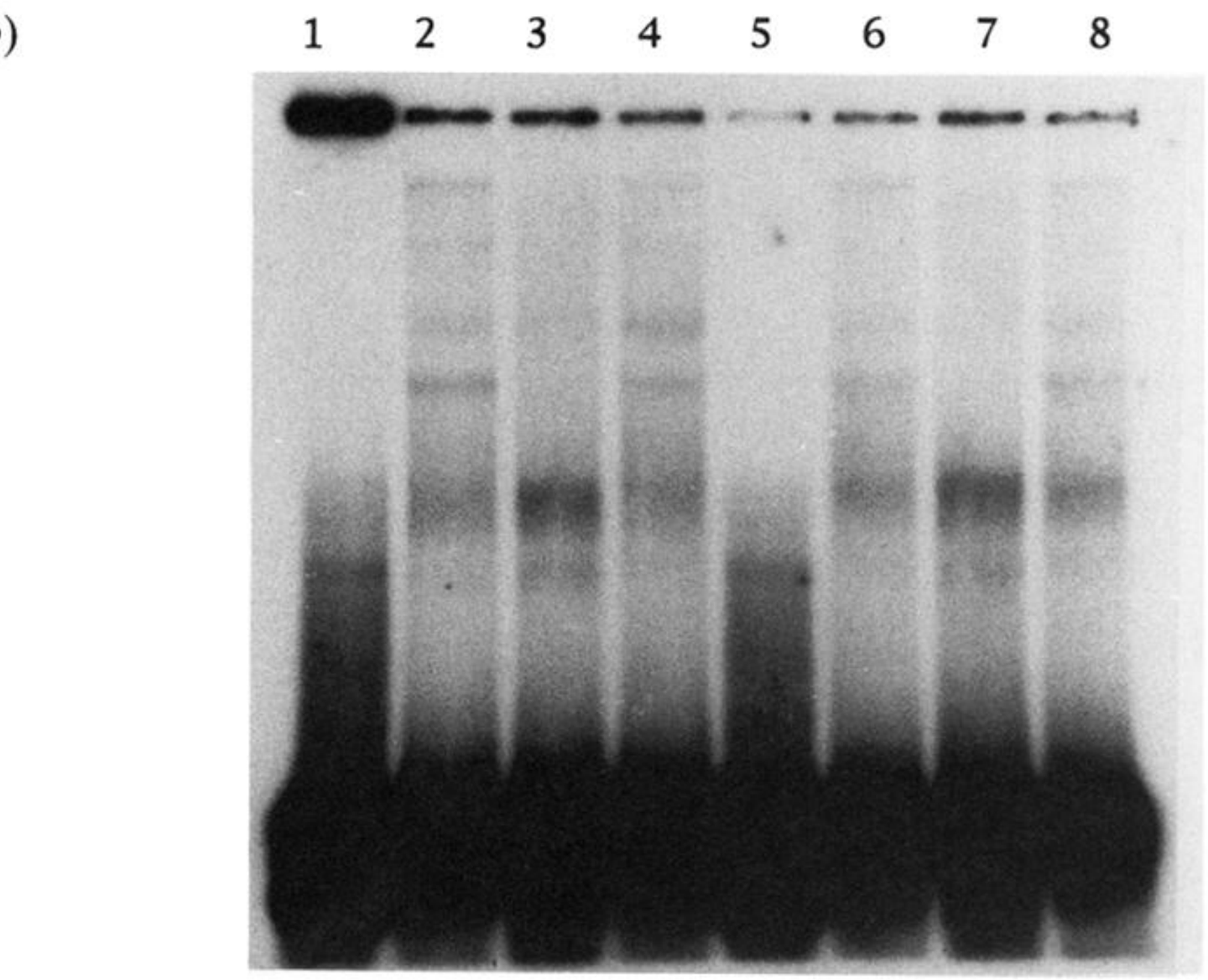
I would like to add a comment about the sex ratio in natural populations of turtles with temperature-sensitive gonadal differentiation. In the Brenne populations of the European pond turtle, the adult sex ratio is two females to one male. This skewed sex ratio cannot be explained by the sex ratio at hatching, which is 1 male: 1 female (Girondot *et al.* 1994). Better survival of the female turtles might explain this phenomenon (Girondot & Pieau 1993).

References

Girondot & Pieau 1993 *Evol. Ecol.* **7**, 645–650.
Girondot *et al.* 1994 *Genet. Res.* **63**, 117–127.
Pieau *et al.* 1994 *The differences between the sexes* (ed. R. V. Short & Balaban) pp. 433–448. Cambridge University Press.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES





igure 1. Gel retention assays showing a specific retarded band with protein extracts from stage 19 alligator urogenital ssue. (*a*) Stage 19 alligator embryo urogenital (UG) tissue proteins with an HMG-box binding sequence AACAAAG. ane 1, free probe. Lane 2, 33 °C UG. Lane 3, 33 °C UG with excess competitor oligo (unlabelled HMG-box oligo). ane 4, 33 °C UG with excess non-competitor oligo (COUP). Lane 5, free probe. Lane 6, 30 °C UG. Lane 7, 30 °C G with excess competitor oligo. Lane 8, 30 °C UG with excess non-competitor oligo. Arrow indicates the position of ie retarded band in lanes 2 and 4. + indicates the position of the common retarded band. (*b*) Stage 19 alligator mbryo head proteins with the HMG-box consensus oligo. Lane 1, free probe. Lane 2, 33 °C. Lane 3, 33 °C with xcess competitor oligo. Lane 4, 33 °C with excess non-competitor oligo. Lane 5, free probe. Lane 6, 30 °C. Lane 7, 0 °C with excess competitor oligo. Lane 8, 30 °C with excess non-competitor oligo.