# Pattern Does Not Equal Process: Exactly When Is Sex Environmentally Determined?

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Of prime importance in evolutionary biology are the description of pattern and explanations of process. Frequently, however, multiple processes can explain a given pattern. Such cases require experimental protocols or research criteria to distinguish among alternatives so pattern can be critically assigned to process. Noteworthy examples of this approach include evaluating adaptations and identifying character displacement (Gould and Lewontin 1979; Schluter and McPhail 1992). The field of vertebrate sex determination similarly requires such criteria.

The sex of organisms is determined by two distinct mechanisms. In genotypic sex determination (GSD), sex is determined at conception by genes usually contained in sex chromosomes. In environmental sex determination (ESD), sex is determined permanently after fertilization by environmental factors (Bull 1983). In ESD (unlike in GSD), there is little if any genetic difference between the sexes (Solari 1994), so sex cannot be predicted by zygotic genotype (Bull 1983). In many ESD vertebrates, sex is determined after fertilization by incubation temperature (TSD). Though ESD's biological significance seems clear for various taxa (Bull 1983; Conover 1984; Michaud et al. 1999), TSD evolution in vertebrates remains unexplained (Shine 1999). To complicate matters, temperature can influence sex ratios in a multitude of ways other than TSD. In order to understand the ecological and evolutionary significance of each of these alternative phenomena, discerning their commonalities and differences is important.

Charnier (1966) first reported TSD in *Agama agama* lizards. Temperature-dependent sex determination was later described in crocodilians, tuatara, and some turtles and lizards, while other reptiles exhibit GSD (snakes, other turtles and lizards; Ciofi and Swingland 1997 and references therein). The presence of TSD in fish (*Menidia menidia*; Conover and Kynard 1981) suggested it is more prevalent than originally thought. As more vertebrate taxa are studied and temperature-induced sex ratio biases found, an increasing number of TSD cases and intermediate forms of sex determination have been described (Robert and Thompson 2001; Devlin and Nagahama 2002 and references therein).

## The Problems

Despite numerous reports of TSD in vertebrates, research has failed to unveil a clear picture of its evolution (Shine 1999). This task is obstructed because not all purported instances correspond to the same phenomenon. In fact, a close look at all reports reveals that researchers widely agree on the definitions of TSD and GSD mentioned above, but no clear criteria exist to identify the presence of TSD. Data treated as "evidence" of TSD can sometimes be explained equally well by other phenomena. The use of the following terms as synonyms exemplifies this: thermolabile sex determination, thermally induced sex ratio biases, temperature effects on sex ratios, temperature-induced sex reversal, temperature-dependent sex differentiation, and temperature-dependent sex determination (e.g., Tokunaga 1985; Goto et al. 1999, 2000; Lee et al. 2001). Apparent TSD cases result from confusion between (1) pattern and process and (2) sex determination and sex differentiation. Additionally, the many reports of thermally biased sex ratios reveal a continuous pattern of sex-determining systems, ranging from strict genetic control to systems governed by temperature. An important question is whether or not this continuous pattern is the result of a continuous underlying process.

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Here we propose criteria (fig. 1) that are minimally required to infer TSD to the exclusion of alternative explanations. We address first whether any temperature effect on sex ratio is TSD and second, whether the process that generates the continuous phenotypic pattern of sex-determining systems is itself continuous. In both cases, we believe not. Our contention is that sometimes temperature modifies sex ratios under GSD, but these instances are not TSD, since sex determination remains under genotypic control (Bull 1983; Solari 1994). These instances of environmental susceptibility within GSD systems may represent important raw material for the evolution of TSD without constituting TSD per se, a distinction with important evolutionary implications.

Throughout this note, we use "sex determination" to indicate the initial instructions defining embryonic sex and producing the primary sex ratio and "sex differentiation" to indicate the developmental pathway leading to the production of testes or ovaries. We also use "primary sex ratio" to denote the sex ratio at conception and "secondary sex ratio" to denote the sex ratio at hatching/birth. We adhere to the definitions of TSD and GSD stated in the introduction.

## Sex-Determining Systems

In many vertebrates, sex is determined by major sex factors contained in sex chromosomes, which can differ morphologically from each other (heteromorphic) or not (homomorphic; Bull 1983; Solari 1994). In some species (XX/XY), males are heterogametic, while in other species (ZZ/ZW), females are heterogametic. Some heteromorphic sex chromosomes differ in shape and are easily observable, whereas others require more advanced cytological techniques (reviewed in Solari 1994). Heteromorphic sex chromosomes are present in amphibians, reptiles, fish, birds, and mammals (Bull 1983; Solari 1994 and references therein). Many amphibians and fish lack heteromorphic sex chromosomes, but progeny analysis, the analysis of sex-linked traits, and mating experiments demonstrate the presence of heterogametic sex determination, and thus, the existence of homomorphic sex chromosomes is inferred (Devlin and Nagahama 2002). In all vertebrate orders except birds, some species possess multiple sex chromosomes where more than one X or Y chromosome segregates per individual (Bull 1983; Solari 1994). In other species, three or more sex chromosomes segregate in a population (e.g., X, $X^*$, and Y in lemmings; and X, Y, and W in platyfish; Bull 1983; Basolo 1994). Few species have polyfactorial sex determination, where several minor sex-determining genes with additive effects define the sex of the organism depending on whether the sum of the effects at all sex loci surpasses a threshold value, as in swordtails (*Xiphophorus helleri*; reviewed in Bull 1983). Polyfactorial systems are rare, probably as a result of their evolutionary instability (Bull 1983; Rice 1986).

Mendelian segregation of sex chromosomes in heterogametic systems produces balanced (1 : 1) primary sex ratios. Sex ratios also tend to unity in most cases of multiple factor systems at equilibrium so long as fitness does not vary between the sexes (Bull 1983). However, sex ratios can be distorted by various means, as when environmental factors override sex chromosomes. Finally, some vertebrates lack sex chromosomes, have no consistent genetic differences between the sexes, and have sex determination that occurs after fertilization by incubation temperature (i.e., TSD; Bull 1983; Solari 1994). Because sex is not defined at conception, there is no primary sex ratio under TSD. Instead, primary sex ratio is delayed until after fertilization and can often be biased.

## Criteria for Identifying TSD

Our procedure emphasizes two essential observations (fig. 1): not all vertebrates lacking heteromorphic sex chromosomes have TSD, and not all thermal sex-ratio distorters acting after fertilization are TSD. Sex determination is dichotomous—an individual's sex is determined either by its genotype or not, in perfect accordance with GSD and TSD definitions. Therefore, testing for heteromorphic sex chromosomes is the first step in identifying the sex-determining mechanism (fig. 1*A*). If heteromorphic sex chromosomes are lacking, the existence of homomorphic sex chromosomes must be considered (Bull 1983; Dournon et al. 1990). For example, incubation experiments at a wide range of temperatures are used to test for TSD in such species, and resulting 1 : 1 secondary sex ratios independent of temperature would indicate the presence of homomorphic sex chromosomes (fig. 1*B*). Encompassing temperatures naturally experienced by the organism allow the assessment of ecologically significant effects. Homomorphic sex chromosomes can also be inferred by progeny analysis, sex-linked traits, or mating experiments.

Sometimes environmental factors override sex chromosomes directly or indirectly. Such systems are GSD modified by an environmental factor (GSD + EE in fig. 1) and must be ruled out before concluding a species has TSD. For instance, temperature-induced differential mortality (Burger and Zappalorti 1988) or embryo abortion or resorption (e.g., Krackow 1992; Blackburn et al. 1998) can skew balanced primary sex ratios (fig. 1*C*). However, these scenarios are not TSD (Burger and Zappalorti 1988) because temperature does not affect sex determination itself but simply prevents the development of zygotes of a given genetically determined sex.

Alternatively, differential fertilization, that is, skewed

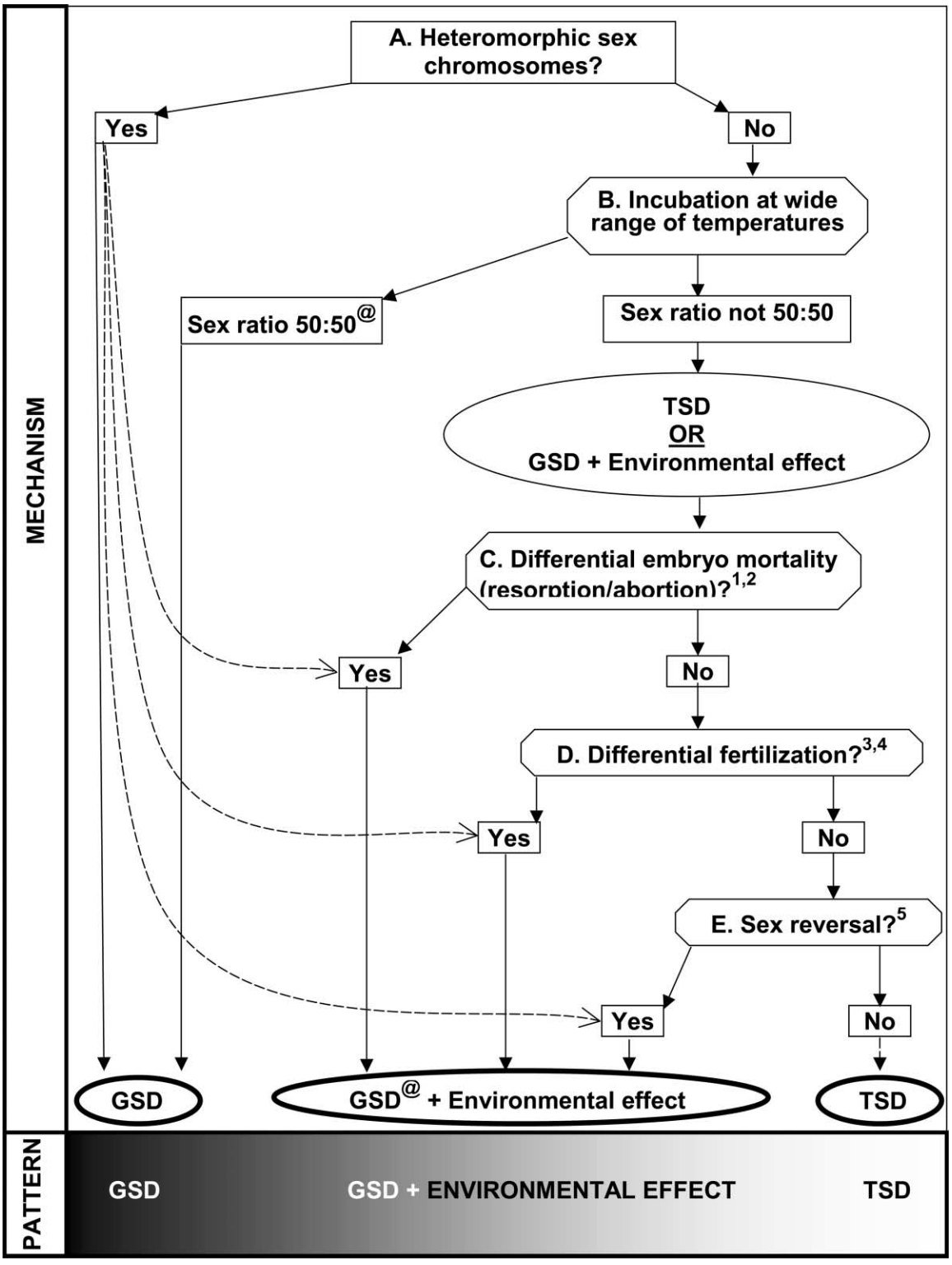



**Figure 1:** Protocol for identifying TSD, distinguishing between the continuous pattern of sex determination, and its underlying dichotomous process. The "@" symbol indicates that homomorphic sex chromosomes exist. Although some references do not report on effects induced by temperature per se, they are provided as methodological guidelines. The mechanisms of GSD plus an environmental effect (GSD + EE) are not rank ordered and are not intended to constitute an exhaustive list. 1 = Krackow 1992; 2 = Blackburn et al. 1998; 3 = Komdeur et al. 1997; 4 = Stockley 1999; 5 = Baroiller et al. 1995.

use/production of heterogametes favoring one sex (e.g., Komdeur et al. 1997; Stockley 1999) can bias primary sex ratios (fig. 1*D*). However, because sex is still determined genotypically at fertilization, this too is not TSD. Instead, temperature prevents the formation of zygotes of a given genetically determined sex. No temperature-induced differential fertilization has been reported to date, but that may be plausible since other types of selective gamete production/use can bias sex ratios (e.g., Ellegren et al. 1996; Komdeur et al. 1997; Stockley 1999).

Finally, temperature acting on sex differentiation can induce sex reversal in species possessing homomorphic or heteromorphic sex chromosomes (Dournon et al. 1990; Solari 1994; Baroiller et al. 1995), such that an individual develops into the opposite sex from the one coded by its genotype (i.e., discrepancy between phenotypic and genotypic sex). This is not TSD. Here sex determination remains genotypic because sex chromosomes exist that define the zygotic sex at fertilization (Dournon et al. 1990; Solari 1994; Baroiller et al. 1995). Instances of sex reversal can be identified by crossing reversed males with normal females (Devlin and Nagahama 2002). Primary sex ratios may be balanced in these species, but thermally induced sex reversal biases secondary sex ratios.

Sex change in sequential hermaphrodites was proposed as a form of ESD (Korpelainen 1990; Schultz 1993). However, by definition, sex under ESD is determined irreversibly. Using the term TSD to designate cases of thermal-differential mortality, differential fertilization, and sex reversal explicitly violates GSD and TSD definitions. Namely, TSD explicitly excludes mechanisms where temperature distorts the sex ratio but does not determine the primary sex ratio.

## Applicability of Our Model

Distinguishing between TSD and GSD systems as we propose is not merely a semantic question but a fundamental one to properly identify TSD, understand its evolution, and the evolution of related traits. Although many of the existing TSD cases come from reptiles (Ciofi and Swingland 1997 and references therein), not all reports are strongly supported, as explained below. Interestingly, most described TSD vertebrates as oviparous. The presence of TSD in viviparous species can be easily mistaken because other causes of sex ratio biases can pass unnoticed. Furthermore, TSD and viviparity seem incompatible because live-bearing parents thermally regulate their offspring's development (Bull 1980). Unexpected examples of this kind may be *Poeciliopsis lucida* (viviparous fish) and *Eulamprus tympanum* (viviparous lizard).

*Poeciliopsis lucida* was reported as having both TSD and GSD from experiments with highly inbred lines, one of which showed labile and the other consistent 1 : 1 offspring sex ratios across temperatures, suggesting Mendelian segregation of a major sex factor (i.e., GSD) in the latter (Sullivan and Schultz 1986). Additionally, certain labile-line females incubated at the same temperature produced variable offspring sex ratios due to unknown factors not expected under TSD (Schultz 1993). Further research is needed to discern whether *P. lucida* is GSD + EE or if GSD and TSD co-occur.

*Eulamprus tympanum* may lack heteromorphic sex chromosomes (no study has addressed this issue) and was recently reported as having TSD, since offspring sex ratios differed from 1 : 1 under various laboratory temperatures (Robert and Thompson 2001). Because sex ratios under GSD + EE and TSD can be identical, and although *E. tympanum* data are consistent with TSD, concluding it is a TSD species solely on the basis of these results is premature because alternative explanations were not empirically eliminated—that is, differential fertilization, and particularly differential embryo mortality and sex reversal.

A different example comes from *Gekko japonicus*, an oviparous lizard purportedly lacking sex chromosomes and reported as TSD because biased sex ratios were produced at several temperatures (Tokunaga 1985). However, *G. japonicus* possesses well-differentiated sex chromosomes (Yoshida and Itoh 1974; Moritz 1990), and therefore, is not a TSD but a GSD + EE species. Biased sex ratios are probably the result of temperature-induced sex reversal as found in amphibians, unless the population studied by Tokunaga (1985) lacks sex chromosomes altogether.

Unlike the previous examples, most other accounts of TSD in reptiles refer to oviparous species lacking sex chromosomes whose sex ratios can be biased from mostly males to mostly females across temperatures. Some of these conform to our criteria (several turtles, crocodilians, lizards, and tuataras) because sex-biasing temperatures are normally encountered in nature, and differential mortality does not account for sex ratio biases. If sex chromosomes are lacking, differential fertilization and sex reversal are not a concern.

Applying the criteria described in our protocol is straightforward for reptiles, amphibians, birds, and mammals. Fish, however, are more difficult to categorize because of their sex differentiation lability and multitude of chromosomal arrangements (reviewed in Francis 1992; Solari 1994; Devlin and Nagahama 2002). Nonetheless, a number of fish species have heteromorphic or homomorphic sex chromosomes identified by classical and modern cytological techniques or inferred by the observation of sex-linked traits or progeny analysis (Devlin and Nagahama 2002). Classifying these species as GSD or GSD + EE following our protocol should be direct. For

instance, homomorphic sex chromosomes (XX/XY) were identified in the tilapia (*Oreochromis niloticus*; Jalabert et al. 1974), but temperature can override them (Baroillier et al. 1995). Some authors have inappropriately referred to this temperature effect on sex differentiation (Baroillier et al. 1995) as TSD (e.g., Nakamura et al. 1998; Goto et al. 1999; D'Cotta et al. 2001). Likewise, existence of TSD has been claimed in other species with homomorphic and heteromorphic sex chromosomes such as the channel catfish *Ictalurus punctatus* (Patiño et al. 1996), sockeye salmon *Oncorhynchus nerka* (Craig et al. 1996), barfin flounder *Verasper moseri* (Goto et al. 1999), and marbled sole *Limanda yokohamae* (Goto et al. 2000). These are all examples of GSD + EE taxa (fig. 1).

Classifying fish species with polyfactorial sex determination (Bull 1983; Rice 1986; Francis 1992) may be difficult because such systems may be more prone to environmental influences (Bull 1983) without implying ESD. These effects would fall under GSD + EE from our protocol so long as the sum of the effects across sex loci defines the zygotic sex. In teleosts, genetic sex-determining factors may trigger a brain-mediated response rather than acting directly on the gonad as the developmental switch, thus explaining sex differentiation lability in this group, including environmental influences (Francis 1992). If this hypothesis is correct, teleost species could be readily classified as GSD, GSD + EE, or TSD.

We argue that the continuous phenotypic pattern of sex-determining systems is generated by a discrete, dichotomous underlying process. But are there counterexamples of species with intermediate sex-determining mechanisms that would be indicative of a continuous process? A few scenarios constitute apparent impasses for our protocol.

First, in *Menidia menidia*, sex is under the control of major sex-determining genes that segregate in Mendelian fashion (GSD by our protocol) in northern populations (TSD genotypes are rare [Lagomarsino and Conover 1993]) and under the control of incubation temperature and sex-determining genes in southern populations (Conover et al. 1992). At first glance, the presence of GSD and TSD genotypes within populations could be interpreted as a truly intermediate mechanism between GSD and TSD. However, the coexistence of GSD and TSD within populations does not invalidate the dichotomy of the underlying process that we propose. That is, at the individual level, the sex of a larva is determined either by the presence of the sex-determining genes (GSD) or by the incubation temperature (TSD). It is the proportion of TSD and GSD individuals that changes in *M. menidia* populations as they adapt to local conditions (Conover et al. 1992). Even in a species where sex is determined by the sum of polyfactorial genes plus a TSD gene, if a TSD gene puts the zygote above or below the necessary threshold, then that individual has TSD. Therefore, at the individual level, the process is dichotomous.

Second, distinguishing between ESD sensu stricto and environmentally mediated prematurational sex change in teleosts may be difficult (Francis 1992). Here, individuals start developing as one sex, but prior to maturation a portion of them undergo sex change, including an intersexual phase, and mature as the other sex. But importantly, our current technical incapacity to discern between alternatives does not make them the same phenomenon. Future research advances may prove valuable in disentangling some of those alternatives.

The third example is a thought experiment. Envision three systems (J. J. Bull, personal communication): (A) all males and females are XX and develop as each sex according to the environment (pure TSD by our protocol); (B) some individuals are XY and are always males, while others are $XX^{TSD}$ and develop as males or females depending on the environment (co-occurrence of GSD and TSD); (C) the environment changes such that $XX^{TSD}$ never encounters the conditions necessary to develop as males, and the population is composed of XY males and $XX^{TSD}$ females. How would scenario C be classified by our protocol, and how could it be distinguished from pure GSD? The answer is simple: scenario C is effectively GSD, because in the environments normally encountered in nature, XY individuals are always males and $XX^{TSD}$ are always females, so there is perfect one-to-one mapping of genotype to phenotype. Additionally, system C is evolutionarily unstable and the thermal sensitivity is likely lost, because $XX^{TSD}$ individuals have a disadvantage compared to XY males and $XX^{GSD}$ females. Namely, TSD individuals suffer an ontogenetic delay in the onset of the development of sexually dimorphic traits compared to GSD individuals because their sexual identity is undefined until gonadal differentiation begins (Bull 1983; Gutiérrez-Adán et al. 2000). Therefore, in the new environment there could be selection against temperature sensitivity and for evolving a $XX^{GSD}$ female system, where individuals will always develop as females irrespective of environmental conditions. In the best case scenario where $XX^{TSD}$ is neutral in the new environment, the temperature sensitivity could also be lost through drift. If the environment were to change again to an environment where TSD was selectively favored before $XX^{TSD}$ is completely lost, there will still be raw material available to return to a TSD system via the intermediate state of GSD/TSD co-occurrence with the eventual loss of the Y. In either case, scenario C would not exist for long, as it is evolutionarily unstable.

It is worth mentioning that some sex ratio biases reported in the literature are obtained at extreme temperatures that are rarely encountered in nature. Such temperature sensitivity not realized under field conditions

questions the ecological and evolutionary significance of these effects, as they may represent relicts or co-optable attributes in TSD evolution.

Fourth, the underlying genetic basis of TSD, as reflected in the genetic control of gonadal differentiation (Pieau et al. 2001) and the variation in temperature sensitivity within and among populations (Bull et al. 1982; Bull 1983) is not an argument against the existence of true TSD as defined here. Genes do indeed control sex differentiation in TSD species, and many are common to TSD and GSD systems (Lance 1997). However, the master switch that initiates the cascade of events of gonadal differentiation is genotypic in GSD, whereas it is environmental in TSD (Bull 1983; Lance 1997). In this respect too, we should distinguish between environmental influences in the expression of genes, sometimes as subtle as developmental instability under which the phenotypic outcome of otherwise identical genotypes in otherwise identical environments can vary, and TSD that is not a developmental instability or anomaly. Temperature-dependent sex determination is a mechanism by which sex is typically determined by temperatures commonly encountered in nature. Our argument cannot be characterized as resembling the shortcomings of genetic determinism. We obviously recognize that even in GSD there could be subtle environmental influences and that TSD also acts under some genetic influences; otherwise, sex ratio and TSD evolution will be unrealizable.

Finally, among-individual variation in temperature sensitivity within TSD species may also reflect genes controlling this trait (Bull et al. 1982) without implying GSD because an individual's sex is defined after fertilization by an environmental factor and not genotypically at conception. That is, genes controlling temperature sensitivity govern the phenotypic plasticity of sex differentiation, but because there are no consistent genetic differences between the sexes and no major segregating sex-determining factors such as those characterizing sex chromosomes, this process is not GSD. Variation in temperature sensitivity among individuals may be revealed, for example, during incubation experiments of oviparous reptiles at the pivotal temperature (population-wide constant temperature producing 1 : 1 sex ratios), where among-clutch differences in sex ratio are observed. Among-individual variation has been used as a measure of the heritability of temperature sensitivity (Conover and Kynard 1981; Bull et al. 1982; Janzen 1992), but epigenetic factors account for part of this variation, such as maternal hormones allocated to the eggs in turtles (Bowden et al. 2000). If genetically based, this variation may permit the microevolution of TSD systems among diverging taxa, with populations and species differing in their pivotal temperature, width, and shape of the transitional range (temperatures that produce mixed sex ratios) and TSD mode (male-female-male, male-female, or female-male with increasing temperature).

## Evolutionary Implications of Our Model

The correct identification of TSD taxa is not simply a semantic question, but rather an issue that influences the theoretical and empirical study of the evolution of sex-determining systems and related traits. First, it must be recognized that pattern does not equal process, and the continuous phenotypic pattern of sex-determining systems results from a dichotomous underlying process. Second, recombination rules differ between sex chromosomes and autosomes, the heterogametic sex is subject to constraints in the amount and location of recombinational events, and autosomal recombination can differ between the heterogametic and the homogametic sex (Solari 1994). Therefore, because this fundamental difference affects evolutionary potential, assigning a taxon as having TSD or GSD + EE impacts our understanding of the evolution of sex ratio, sex-linked traits, and autosomal traits. Traits carried on sex chromosomes will be subject to evolutionary forces such as Muller's ratchet and hitchhiking (Solari 1994), two phenomena that would not apply equally to TSD taxa. Sexually dimorphic phenotypes could develop earlier ontogenetically in GSD and GSD + EE compared to TSD species because the sex of the individual is defined at conception in the former and only after fertilization in the latter (Bull 1983; Gutiérrez-Adán et al. 2000). Sex ratio evolution also differs between TSD and GSD + EE, and differences in production of offspring sex ratios can drastically affect effective population sizes and the genetic dynamics of populations.

## Conclusion

Explaining the evolution and maintenance of TSD is difficult because biologists attempt to reconcile signals generated by different phenomena. Confusion between sex differentiation and sex determination, and between pattern and process, is the main factor contributing to this problem. The explicit criteria of our model (fig. 1) provides a means to distinguish among GSD, TSD, and other thermal effects on sex ratio and eventually to understand their evolution. By constructing a simplifying framework we do not imply that there is one general GSD mechanism or one general TSD mechanism, as indeed a multitude of GSD and TSD systems are found among taxa. Rather, we clarify how to discriminate between GSD and TSD mechanisms and, particularly, how to identify TSD to the exclusion of alternative phenomena. Our explicit definitions and criteria will aid future theoretical and empirical research in this fascinating and important field.

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