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Editorial

Mode and tempo in environmental sex determination in vertebrates

One of the more colorful areas for scientific research, bound to capture the imagination of audiences inside and outside of science alike, is sex. The difference between sexes defines much of human endeavor; although our society now strives for equality of the sexes, biology has never treated them as equivalent, and most biological research focuses on male and female differences. Sex determination is one the many foci in this broad field, but one that is especially well defined and easy to comprehend. Historically, before there was ever a field of genetics, sex 'determination' was a description of any and all factors influencing the sex of offspring—in essence, sex ratio determination. Some of the most colorful and inspiring models for how to conceive a son or daughter, or even how to predict sex of the unborn, are to be found in reviews of ideas dating back thousands of years [1].

We have clearly come a long way from the pre-science days in understanding sex 'determination.' Scientific progress is rarely smooth and steady, but usually in stutter steps separated sometimes by decades of seeming stasis [(cf, McLaren's essays [2,3] in *Nature* containing the first reports describing SRY similarly captures the episodic nature of scientific advance]. Advances can come in the form of new discoveries, or in syntheses codifying past discoveries into new perspectives. This volume contains 6 articles that summarize many advances in our understanding of the environmental control of sex determination and gonadal differentiation in vertebrate animals. Together they form the basis of a new perspective in this fascinating field and reveal a new way of looking not possible even a few years ago. Two major changes in perspective are evident.

The first "revelation" was actually discovered more than two decades ago [4] but has recently been resurrected, namely the coexistence within species of both strong environmental and genetic components to sex determination. There is in fact a continuum of possible mixes of genetic and environmental effects on sex. Uller and Badyaev [5] take a somewhat new perspective on this for birds, however. In birds, female heterogamety is universal, so it might seem that environmental effects on sex are nil. However, there are many documented cases in which the mother influences (biases) the sex ratio of her offspring, presumably by affecting the meiotic segregation and polar body retention of the sex chromosomes. Thus, whereas the Conover and Kynard study [4] found a mix of environmental and genetic effects on sex determination in the embryo (after fertilization), the bird case is a mix of environmental effects before fertilization with strict genetic effects after. Similarly, Navara and Nelson [6] describe the many levels at which the environment, defined broadly, can influence sex ratio in mammals. The second revelation has been that the developmental genetic basis of sex determination is not the linear cascade of gene actions at the top end posed by McLaren [7], but aside from the trigger, is a network of interacting genes through development.

A little historical perspective gives some useful background for these contributions. The genesis of understanding sex determination in the modern sense coincided with the dawn of the 1900s, when chromosomes were discovered and Mendel's work was rediscovered. Observations of chromosomes in animals revealed two indistinguishable sets of chromosomes, one set from each parent. Animals were diploid. But in some taxa, there was an odd chromosome with no partner. It was given the label 'X' for unknown. Furthermore, the X lacked a partner in only one sex, and the other sex had two copies of it. In addition, some species had equal numbers of chromosomes in both sexes, but one sex had an odd 'pair', in which the two chromosomes had different sizes and shapes. One of these odd-pair chromosomes was present in two copies in the other sex, but the other occurred only in a single copy and only in the one sex. Since X was already taken for the chromosome common to both sexes, 'Y' was assigned to the odd chromosome present in only one sex (which, at the time, was male). Thus, the discovery of sex chromosomes was in species with XO males and XX females, but it was quickly found that many species had XY males and XX females. Genetic evidence of a role of these 'sex' chromosomes in sex development awaited the famous study by Bridges [8] as the lead article in the first issue of the journal *Genetics*. Here he showed through the observation of aneuploid fruitflies (having too few or too many X chromosomes) that the number of X chromosomes relative to autosomes determines sex.

For half a century, most of the work on sex determination continued in this vein, with discoveries of the extent of sex chromosome systems and systems with similar inheritance, even if visibly distinct (heteromorphic) sex chromosomes were not evident. The male was found to be the XY sex in many species, often across broad taxa, but the female was the heterogametic sex in others, and the notation ZZ/ZW was reserved for it. Other mechanisms were discovered, such as haplo-diploidy in wasps and ants, and 'environmental' control in marine worms.

Aside from Bridges' work in *Drosophila*, some of the earliest work demonstrating actual genetic control of sex determination was done in amphibians and fish. In those taxa it was possible to override normal sex development with hormones and other techniques, creating 'sex reversed' individuals (XX males, XY females). By crossing sex reversed individuals with normal individuals, the existence of XX/XY and ZZ/ZW systems could be demonstrated by skewed sex ratios in the progeny, regardless of whether sex chromosomes could be observed in the microscope. For example, an XX individual turned into male by hormones and then bred to a

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normal (XX) female produces all daughters; XY bred with XY produces 3/4 sons if YY is viable, or 2/3 sons if not. At the time, the chief significance of this work in fish and amphibians was considered to be in adding support to the growing awareness of genetic control (inheritance) of sex determination throughout most of vertebrates. Hindsight reveals that these studies also yielded insights to the physiological basis of sex determination that may apply to most, if not all major groups of cold-blooded vertebrates.

In 1967, Ohno published a monograph summarizing the state of understanding of vertebrate sex determination [9]. The work, which included a broad synthesis amid daring genetic and evolutionary proposals, heralded a focus on vertebrate sex determination as a model system of genetics, evolution, and development. His proposal was that the evolutionary history of the vertebrates experienced a progression of ever-increasing genetic control of sex determination that was still evident today across the groups descended from those ancestors. Thus, fish exhibited the weakest genetic control of sex, with amphibians not far removed. Mammals and birds were at the pinnacle of this progression, with sex determination dominated by highly developed sex chromosomes. Reptiles represented intermediates in that progression, presumably all with genetic control of sex, but some exhibiting heteromorphic sex chromosomes, and others not.

The year before his book, however, an obscure publication heralded a radical alternative to genetic sex determination in vertebrates, the discovery in a lizard that incubation temperature determined hatching (secondary) sex ratio [10]. The finding of temperature-dependent sex determination (TSD) was extended to three turtles [11,12], and it was soon realized that TSD was present in at least three of the four major clades of classic reptiles (lizards and snakes, turtles, crocodilians). More importantly, these environmental mechanisms were not merely sloppy systems, but operated in natural nests, showed evidence of local adaptation in some cases, and exhibited profound temperature influences on embryonic sex, with most temperatures producing only a single sex [13]. The view that vertebrate evolution had been destined toward strict genetic control of sex was shattered.

Work up to the last quarter of the 20th century mostly resolved the inherited basis of sex, i.e., whether the sex of the embryo could be predicted from its genetic or environmental makeup. However, although the extent of environmental sex determination has been the focus of intensive work and considerable resolution in reptiles, such that we have a good understanding of which mechanisms and patterns apply to different groups and even the ecological consequences of the mechanisms, there has been much less of a systematic attempt to explore the extent of environmental effects on sex in fish and amphibians. Thus, two of the contributions are on fish [14,15], with much of the emphasis on the nature of environmental influences. Indeed, some fish change sex as adults, and the cues for this type of sex determination are interesting from an evolutionary perspective [16]. The chapter on flounder illustrates both the practical utility of being able to influence sex as well as the immense challenge to uncover taxonomic generalities in systems that are subject to moderate environmental effects. A single contribution on amphibians reveals the breadth of research on this group, but countless interesting observations that remain untouched [17].

The many elucidations of the nature of environmental and genetic effects on sex stand as the foundation for work today, which has shifted heavily to the molecular basis of sex development. Rather surprisingly, attention to this problem has a remote ancestry, in attempts by the earliest researchers to connect observations on inheritance of sex with the physiological basis of sex. Bridges' fruitflies were convenient for both genetic and cytological work, so he was able to study flies that not only had too many and too few entire X chromosomes, but also flies missing and extra pieces thereof. He observed that the sexual appearance of flies changed gradually with the total amount of X chromosome material (relative to the number of sets of autosomes), and he proposed a specific model of sex determination called 'genic balance' [18]. This proposal was the antithesis of a master regulatory gene, rather it was that loci throughout the genome affected sex development, and the final sex depended on the sum – balance – of those effects. Similar efforts to understand how genes collectively affected sex development were the focus of work on moths [19] and guppies [20]. With the benefit of hindsight, we can now see that it proved impossible to gain much insight about how the inheritance of sex translated into molecular mechanisms. Yet it is interesting that the underlying mechanisms of inheritance were the focus of so much work long before the advent of molecular genetics. Bridges proved to be correct that the X carried a large number of genes whose effects summed to determine sex development, but the nature of those genes has proved different than he proposed.

The push toward our current emphasis and paradigm in the genetics of vertebrate sex determination can probably be identified with a second monograph of Ohno [21]. The focus here was on sex development as a cascade of genes controlled by a master regulator. His candidate master was a gene encoding H-Y antigen, a difficult-to-measure phenotype specific to males, detectable only by antibodies. Despite being purely correlative, H-Y antigen acquired considerable momentum in theories of mammalian sex determination [22]. The next candidate for the human master gene was a gene actually cloned from the human Y that also proved to be erroneous [23,24]. The quest finally ended with the discovery of SRY (for sex-determining region of the Y chromosome), a gene from the human (and placental mammal) Y, whose ability to control or trigger male development was confirmed by transgenic mice [25,26].

The concept of a master regulatory gene has broad appeal—a master gene provides an immediate basis for an XX/XY or ZZ/ZW system, it being the gene on the Y or W (X or Z) whose presence triggers the developmental cascade toward one sex. It can even control sex determination in a purely 'environmental' mechanism, since expression of the master can in principle respond differently to different environments. Furthermore, the master need not be at the very top of the developmental cascade, instead operating downstream of (and being controlled by) genes that respond the environment. Thus, the varied mechanisms of inheritance in vertebrates can in principle have the same underlying developmental genetics, merely differing slightly at the top end. This basic understanding underlies the evolutionary models of Bull [27] and was captured in an elegant figure of McLaren [7], reprinted here (Fig. 1). Bull [27] showed that population changes in sex determination (from male heterogamety to female heterogamety, or from heterogamety to environmental sex determination, for example) could occur from single mutations that altered sex phenotype, such as in a regulatory gene. There was no intrinsic hurdle in evolving from one mechanism to another when the requisite regulatory muta-

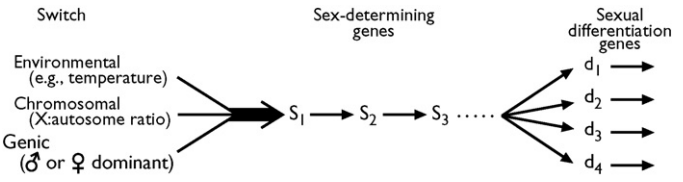

Fig. 1. The perspective since 1988 envisioned the diversity of sex determining mechanisms in animals as differing in the switch (left portion of figure) that directed gonadal development into the ovarian or testicular pathway via a succession or 'cascade' of sex determining genes (middle portion of figure). The nature of gene activation or inhibition was proposed to occur in a linear fashion with the final gene in the series engaging various loci involved in the differentiation of sexual organs and structures (right portion of figure). Redrawn from McLaren [7].

tion arose (and provided that any existing sex chromosomes did not block the transition, as would be caused by YY inviability). This perspective leads trivially to the result that taxa with different sex determining mechanisms should differ only in the master, i.e., at the top end of the developmental genetic cascade, while retaining a 'conserved core' (see also Wilkins [28]). Interestingly, SRY is apparently not the master gene even in marsupials, much less in non-mammalian vertebrates, so there has been some evolution in at least the top end of the cascade throughout vertebrates.

Recovering the gene at the top end of mammalian cascade has been a major milestone, but there are many equally compelling questions about the genetics of sex development. In addition to the master gene, many players immediately downstream of the mammalian master have been identified in mammals: *MIS*—Müllerian-inhibiting substance; *DMRT1*—doublesex mab3 related transcription factor 1; *SOX9*—SRY-like HMG-box containing transcription factor 9; *SOX8*—SRY-like HMG-box containing transcription factor 8; *FOXL2*—forkhead box protein L2; *WNT4*—wingless integration site family member 4; *DAX1*—DSS-AHC critical region on the X chromosome; *SF1*—steroidogenic factor 1; *WT1*—Wilms tumor 1; *FGF9*—fibroblast growth factor 9; *RSPO1*—R-spondin 1. Several decades of research with mammals has revealed at least some of the interactions among these genes during gonadal differentiation [29]. The most recent revelation of this sort is how the master regulator *Sry* triggers the male pathway by acting synergistically with the nuclear receptor *SF1* through an enhancer of *Sox9* to promote Sertoli cell differentiation [30]. Similar descriptions of gene interactions during gonadal differentiation are beginning to emerge with birds [31] and, as indicated in this volume, with reptiles [32,33] and amphibians [17]. Thus, a satisfying view of the molecular genetic cascade in mammalian sex determination has materialized, with spin-offs to other vertebrate groups.

Key to this success in mammals has been the isolation of mutant individuals deficient in the genes of interest (either as natural or transgenic constructs), as well as improvement in methods for manipulating proteins and gene expression. In particular, mice (for which there are now methods for creating transgenics) and spontaneous mutants of humans have been critical in working out many of these details. In contrast, there has been essentially no effort at developing model reptiles for genetic research, and although *Xenopus* and chickens are model amphibians and birds, they have not been pursued with the same intensity for genetic work as have humans and mice. Zebra fish are excellent model fish, but curiously, the basis of their sex determination is unknown (they do not have major effect genes, such as XX/XY), so they have not been as useful as they might otherwise.

As the reptiles are poor genetic models, virtually all efforts to elucidate the sex determining core genes have involved studying the sex-specific and temperature-specific expression of homologs of core genes identified in mammal sex determination, or of genes involved in steroid biosynthesis. The contributions focusing on amphibians [17] and reptiles [32,33] indicate that the mammalian core genes of gonad differentiation are conserved across amniotes (mammals, birds, and reptiles) and anamniotes (amphibians and fish), but expression patterns are not consistent even within a clade (e.g., among turtle species with TSD). No gene appears suggestive of a master control element, i.e., to have a clear on-off pattern associated with temperature, and although such a result would be convenient, it is not necessarily expected. Indeed, the results from reptiles and mammals collectively paint a picture that the nature of interactions among the core genes is evolutionarily plastic. Furthermore, given the evidence that reptilian and anamniote vertebrate systems can be influenced by the environment as well as by steroids and compounds that bind steroid receptors, one can easily imagine that the core genes concept may not fit any simple model of gene interactions leading to sex determination. Stepping back and looking at the evidence, we wonder if the Ohno–McLaren model of a master gene will even hold in these species. It is possible that the developmental decision of male versus female does not flow through a single gene but is instead determined by a 'parliamentary' system involving networks of genes (middle of Fig. 2) that have simultaneous inputs to several components of the downstream cascade (right side of Fig. 2). Systems with different balances of the inherited and environmental influences (left side of Fig. 2) could all operate this way (as they could also operate through a single master), merely by varying the inputs to the networks.

The implication here is that no single gene would be found whose expression alone was a predictor of sex. Perhaps the most compelling reason to contemplate such a complex system outside of mammals and birds is the near universal role of steroidogenic enzymes in sex determination of cold-blooded vertebrates. Although steroid hormones are present in the egg yolk, they do not appear to be involved in the determination of gonad type [34-36]. However, the ability to reverse sex with exogenous steroid hor-

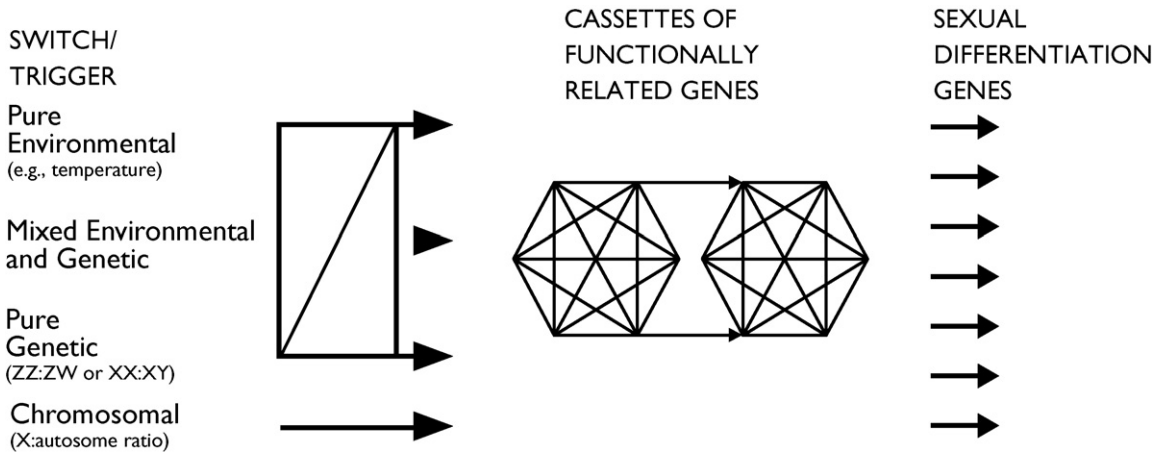

Fig. 2. Newly emerging perspective of sex determining mechanisms in animals. The switches once regarded as mutually exclusive (e.g., species with temperature-dependent sex determination lacking sex chromosomes), are now known to be present simultaneously in some species (e.g., sex determining mechanisms in some species as having a genetically predisposed response to temperature) (left portion of figure). The switch or trigger in turn engages gene "cassettes" of functionally related genes that interact (middle portion of figure, with each corner representing a gene in a network [46]). The nature of these interactions change through development and cassette can engage other cassettes of integrated gene assemblies. This parliament of interacting gene networks in turn have simultaneous inputs to several components of the downstream cascade represented by sex-typical morphological, physiological, and ultimately, behavioral, traits (right side of figure).

mones (principally estrogens and their mimics) across a breadth of developmental stages suggests a developmental lability to signaling. Levels of steroids or the enzymes that produce them in the embryo would themselves be downstream events of gene expression, not obvious candidates for the top-end regulator. If sex determination is subject to feedback effects, then many overrides could be possible as development progresses. When applied to the model presented in Fig. 2, it is evident that the physiological/molecular equivalents of environmental sensitivity are steroid hormones and the molecules that produce (steroidogenic enzymes) and sense (hormone receptors) them. If ESD is ancestral, with the 'trigger' or 'switch' initiating sex determination being external to the organism, and GSD is derived, with the trigger becoming internalized in the organism (i.e., heritable sex chromosomes), it is reasonable to make certain postulates. First, the function of the core gonad determining genes (the first cassette depicted in the middle of Fig. 2) was retained although the trigger initiating their action was shifted. Second, the genes involved in environmental sensitivity (the second cassette depicted in the middle of Fig. 2) became free to evolve other functions less directly linked to gonad determination, such as sexual differentiation of the phenotype. If this is the case, then it may explain why these genes (the steroid hormone receptor genes and steroidogenic enzyme genes) are involved in so many disorders of sexual development. This new perspective thus can contribute to our understanding of the role of environmental influences in ontogeny, providing key insights into the mechanisms by which environmental signals are translated into phenotypic change. This is a particularly important concern as the consequences of endocrine disrupting compounds in the environment appear to have their major effects on the gonadal differentiation in cold-blooded vertebrates, but on accessory and second sex structures in warm-blooded animals.

The great diversity in sex determining mechanisms across species also offers a possible test of the 'conserved-core' evolution model. Under this model, regardless of the master, the same downstream genes should be involved in sex determination of mammals as in birds, reptiles, amphibians, and fish (even if their interactions are not the same, as considered in the preceding two paragraphs). The key difference between the cold-blooded and warm-blooded vertebrates mentioned above, namely the effect of steroid hormones and their mimics on sex determination, raises the possibility that any of several genes encoding steroidogenic enzymes or steroid receptors may be part of the sex determining core: AR—androgen receptor; ERα—estrogen receptor α; ERβ—estrogen receptor β; CYP genes (aromatase); hydroxysteroid dehydrogenases; various *Sox* genes. If these genes prove to be part of the core for cold-blooded but not for warm-blooded animals, then the conserved-core model is rejected.

The downside of studying these alternative mechanisms is that it would be nigh impossible to elucidate the molecular basis of how sex was determined. The use of expression arrays to measure co-expression patterns throughout the critical developmental stages could at least shed light on such a possibility, although such an array would need to (i) include all the relevant genes, (ii) taken from the relevant tissues, and at the (iii) relevant developmental stage. Since most cold-blooded vertebrate species are not genetic model organisms, work on their sex determination is limited to candidate genes suggested from other systems (as well as genes coding for steroidogenic enzymes and steroid hormone receptors), so any sex determining genes unique to reptiles (for example) will be difficult to discover and include in gene expression studies.

In the past, the lack of developmental rigidity in 'lower' vertebrates was considered an imperfection toward highly regimented genetic control of sex. We may come to view it as a type of developmental superiority, allowing great flexibility and evolvability. It will certainly provide material for interesting research.

We might envision a volume on this topic in two decades and contemplate the research at that time. What questions will likely dominate the field? We can suggest one: the basis of gonadal development as testis versus ovary. The same primordium can develop either way, and it typically develops only one of those ways. There are many precedents for development one way versus another (e.g., segmental decisions), so perhaps the gonadal dichotomy will have parallels to pathways already known. But a unique dimension to this gonadal dichotomy is that the antagonism is actively maintained in some species even into the adult. Thus, removal of the ovary in adult birds results in testicular development of an otherwise quiescent rudiment, while removal of the testis in toads results in the Bidder's organ becoming an functional ovary.

However, it is noteworthy that in gonochoristic vertebrates ovotestes are observed in nature, sometimes at high frequency, and can be produced relatively easily experimentally. For example, administration of exogenous estradiol and dihydrotestosterone, or dihydrotestosterone and an aromatase inhibitor to the incubating egg of the slider turtle during the temperature-sensitive window, will produce ovotestes in the red-eared slider turtle [37]. Ovotestes can be produced by genetic manipulation in mammals and are not rare in humans, resulting from dysfunction in one or more of the genetic cassettes in the gonadal differentiation process [38-40]. The limited information available indicates that at least in the amniote vertebrates such individuals are infertile or at best subfertile.

This is not the case in some anamniote vertebrates, particularly fish. Thus, a question related to testis-ovary incompatibility in gonochoristic species is how the gonad is determined in hermaphroditic vertebrates. Here species differences are remarkable. In simultaneous hermaphrodites the gonad contains both testicular and ovarian tissue and produces both sperm and eggs, whereas in sequential hermaphrodites one gonad type is present for the first part of adult life, the other type for the remainder. In some sequential hermaphrodites, gonad sex changes as a function of social context in that the individual exists first as fertile females but then transform into functional males (protogyny) while in others individuals are male first and then transform into females (protandry). In both forms the gonad undergoes a complete transformation in a matter of days or weeks and is irreversible. Godwin [15] summarizes this literature, showing how in these fish changes in social context induce immediate change in behavior and neuroendocrinology (e.g., brain aromatase, neuropeptide hormones, monoamine neurotransmitters), while transformation of the gonad does not occur for days or weeks.

Recently a third form of hermaphroditism has been described, tentatively termed serial hermaphroditism. In this instance, and represented by the goby (*Trimma okinawae*), individuals exhibit serial hermaphroditism in which both testes and ovaries exist as discrete organs rather than the intermixed testicular and ovarian tissue evident in simultaneous hermaphrodites [41]. In this goby species only one gonad type (testis or ovary) is active at a time, and change in social context results in change in abundance of gonadotropin hormone receptor (GtHR) in the *inactive* gonad [42].

While the sex change evident in sequential and serial hermaphrodites may involve the activation or suppression of a testis- or ovary-determining pathway, the most interesting subjects are simultaneously hermaphroditic species in which individuals develop true ovotestes. During the breeding season, these individuals pair and trade roles in each spawning bout, first releasing eggs and then, in the subsequent bout, sperm [43,44]. How is it that both the testicular and ovarian pathways, in most species mutually exclusive, are both activated to produce a functional gonad? Further, how is the release of the appropriate gamete type assured? In this regard a particularly interesting study by Demski and Dulka [45] demonstrated that stimulating one brain area of the sea bass,

a simultaneous hermaphrodite, would cause sperm release while stimulation in another brain area resulted in egg release.

We of course do not pretend to know what interesting revelations will happen in the next two decades nor of what new questions can be explored due to methodological changes. We hope that an understanding of sex development gene actions will be attainable in amphibians, fish, and/or reptiles. As a greater understanding of development is acquired in the future, we look forward to an increased understanding of how the genetic program is able to produce such profound differences between the sexes from little, or in some cases, no genetic differences in the zygotes of both types.

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