

struggled for years with parasites that evolve resistance to commonly used drugs. Why should it be any different in animals? Yet as far as we know, no study has ever looked at whether parasites become resistant to the chemicals in the plants that hosts use for protection, though in the *Drosophila* example referenced above, ethanol did not negatively affect larvae of specialist wasps as strongly as larvae of generalist wasps. Exploring how parasites do or do not gain such resistance would be useful, both for people interested in animal behavior and in human medicine.

Finally, humans depend on both the ability to defend against parasites, especially in terms of human and agricultural health, as well as the *lack of current ability* to defend against parasites. The latter is critical in the use of biological control — the use of natural enemies to control pests, especially in agriculture. Many of these natural enemies are parasites or parasitoids. Biological control can be an effective and safe method of pest management, and may reduce or eliminate the need for chemical pesticides. However, understanding how pest species may evolve to resist parasitism is critical for successful implementation of biological control.

#### Where can I find out more?

- Castella, G., Chapuisat, M., and Christe, P. (2008). Prophylaxis with resin in wood ants. *Anim. Behav.* 75, 1591–1596.
- Clayton, D.H., and Wolfe N.D. (1993). The adaptive significance of self-medication. *Trends Ecol. Evol.* 8, 60–63.
- Daly, E.W. and Johnson, P.T.J. (2011). Beyond immunity: quantifying the effects of host antiparasite behavior on parasite transmission. *Oecologia* 165, 1043–1050.
- DeJoseph, M., Taylor, R.S.L., Baker, M., and Aregullin, M. (2002). Fur-rubbing behavior of capuchin monkeys. *J. Am. Acad. Dermatol.* 46, 924–925.
- Hart, B.L. (1990). Behavioral adaptations to pathogens and parasites: five strategies. *Neurosci. Biobehav. Rev.* 14, 273–294.
- Hughes, D.P., and Cremer, S. (2007). Plasticity in antiparasite behaviours and its suggested role in invasion biology. *Anim. Behav.* 74, 1593–1599.
- Milan, N.F, Kacsoh, B.Z., and Schlenke, T.A. (2012). Alcohol consumption as self-medication against blood-borne parasites in the fruit fly. *Curr. Biol.* 22, 488–493.
- Moore, J. (2002). *Parasites and the Behavior of Animals*, (Oxford: Oxford University Press).
- Schmid-Hempel, P. (2011). The natural history of defences. In *Evolutionary Parasitology*, P. Schmid-Hempel, ed. (Oxford: Oxford University Press), pp. 52–97.

Department of Biology, University of California, Riverside, 900 University Ave, Riverside, CA 92521, USA.  
E-mail: [briang@ucr.edu](mailto:briang@ucr.edu)

## Primer

# Sex determination

Tony Gamble and David Zarkower

Multicellular animals are a diverse lot, with widely varied body plans and lifestyles. One feature they share, however, is a nearly universal reliance on sexual reproduction for species propagation. Humans have long been fascinated by human sex differences and formal theories on how human sex is determined date at least to Aristotle (in *De Generatione Animalium*, ca. 335 BCE). However, it is only in the past couple of decades that the genetic and molecular programs responsible for generating the two sexes have been understood in any detail. Sex, it turns out, can be established by many very different and fast-evolving mechanisms, but often these involve a conserved class of transcriptional regulators, the DM domain proteins.

### Making sexes: determination and differentiation

Sexual reproduction in multicellular animals requires, at a minimum, male and female gametes. Indeed, these specialized haploid cells are how we define the sexes: in a given species individuals with big gametes are females and those with small gametes are males. Individuals that can make both kinds are hermaphrodites, and may be self-fertile or cross-fertile with other individuals. Gametes in most animal species are made in a specialized organ, the gonad. Before sexual reproduction can take place, sexual development must occur. That is, a mechanism is needed to decide which sex a given embryo will adopt — sex determination — as well as mechanisms to control subsequent development of those parts of the embryo that differ between sexes — sexual differentiation. The final result is individuals that can differ remarkably not just in their gametes and gonads but in many aspects of their anatomy, physiology, and behavior — think of the tail of the male peacock, milk production in female mammals, or the courtship rituals of the male bowerbird. Even though these sexual

dimorphisms are essential to the propagation of the species, they can be so extreme that in some cases it is difficult to recognize that their bearers are in fact members of the same species. Some sexually dimorphic traits are essential for reproduction or have obvious benefits to reproductive fitness. However, many sexually dimorphic characters seem antithetical to natural selection, which greatly troubled Darwin (“The sight of a feather in a peacock’s tail, whenever I gaze at it, makes me sick!”). The prevalence of these seemingly disadvantageous traits led to Darwin’s second great insight, the theory of sexual selection based on “the advantage which certain individuals have over other individuals of the same sex and species solely in respect of reproduction,” proposing that these traits provide a competitive advantage in mating. In thinking about the molecular basis of sexually dimorphic traits and how they evolve it helps to be mindful of the distinctive selection mechanisms shaping them.

### Many paths lead to sexual dimorphism

Despite its near universality, sex determination is controlled by quite different mechanisms in different species. Broadly speaking, sex can be determined two ways: genetically (genotypic sex determination or GSD), where the chromosomal composition determines an individual’s sex at fertilization; or environmentally (environmental sex determination or ESD), where conditions encountered during development determine an individual’s sex. These two categories can be further subdivided based on the precise mechanisms involved. In some GSD species, for example, the male is the heterogametic sex, that is, the gender with two different sex chromosomes. This includes the familiar XX/XY sex-determining mechanism in humans and other mammals where the presence of a Y chromosome initiates male development. Alternatively, as in birds, snakes and butterflies, the female can be the heterogametic sex; this is termed a ZZ/ZW system.

Just as GSD is composed of several distinct mechanisms, ESD can involve a variety of

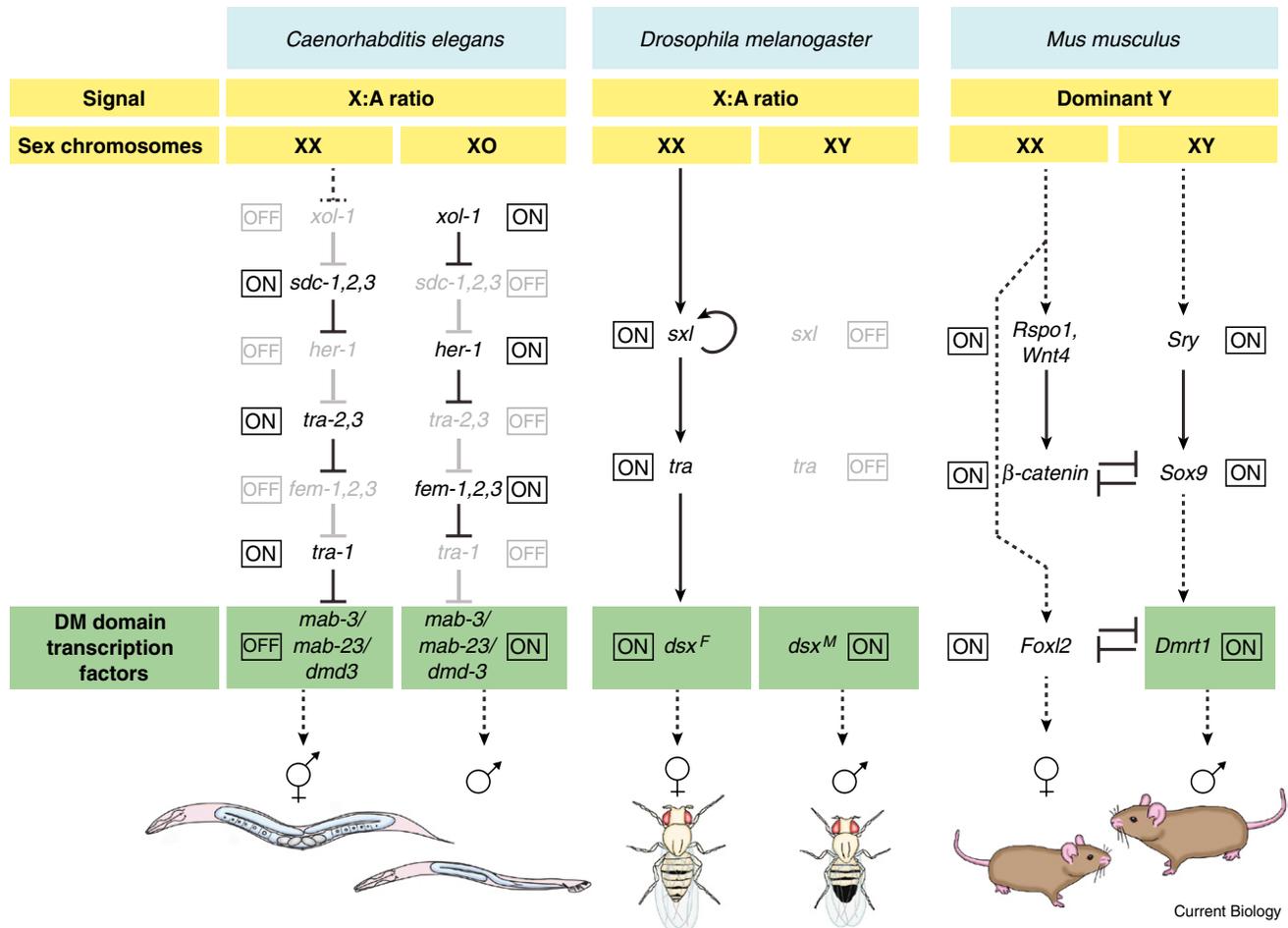


Figure 1. Sex determination pathways in diverse model organisms.

Variations in an XX/XY GSD system. Differences among species are evident throughout all stages of the sex determination hierarchy, although each of the pathways converge on conserved downstream regulators in the DM domain gene family that are essential for male development. For simplicity and clarity, in each gene network shown a number of peripheral, relatively minor, or sex non-specific regulators have been omitted. Regulatory interactions (solid lines) are meant to indicate the regulatory logic of each pathway but do not necessarily imply direct regulation. Dashed lines indicate temporal relationships.

environmental stimuli. Perhaps the best-known examples of ESD involve temperature-dependent sex determination, or TSD, where temperature during a critical window of embryonic development influences the sex of the offspring. A common TSD pattern, seen in alligators and crocodiles and some turtle and lizard species, for example, involves females developing at both low and high incubation temperatures while males are produced at intermediate temperatures. Other environmental variables that can act as sex-determining mechanisms include: the proximity of conspecifics, as is found in the echiurid marine worm, *Bonellia viridis*, where planktonic larvae that settle in isolation become females whereas larvae that settle near females become males; and

photoperiod, as seen in some populations of the brackish water shrimp, *Gammarus duebeni*, where males are produced on long days and females are produced on short days.

The traditional view that divides sex-determining mechanisms strictly into GSD and ESD is being challenged by evidence that both GSD and ESD can coexist in the same species. Sex determination in two lizard species exemplifies the false dichotomy between GSD and ESD. The bearded dragon *Pogona vitticeps* and the skink *Bassiana duperreyi* both possess sex chromosomes — ZZ/ZW and XX/XY systems, respectively — yet genotypic sex can be overridden at extreme incubation temperatures, resulting in individuals with a mismatch between genotype and

sexual phenotype (e.g., ZZ female bearded dragons and XX male skinks). Similarly, infection by the symbiotic bacterium *Wolbachia* can override GSD in a variety of insects, and depletion of oocytes can cause female-to-male sex reversal in some fish species (e.g., *Oryzias* and *Danio*). GSD and ESD may be better viewed, therefore, as extreme points along a continuum, with sex determination being more influenced by genetic factors in some species and by environmental factors in others.

Categorizing GSD species simply into male and female heterogamety obscures surprising variation in the underlying genetic mechanisms. A comparison of the male heterogametic systems of mice, roundworms and fruit flies illustrates this point (Figure 1). The

mammalian Y chromosome contains the dominant male determinant *sex-determining region on Y (Sry)*, a transcriptional regulator that antagonizes a *Wnt/β-catenin* pathway whose activity promotes female development. This permits expression of the related gene *Sry-box 9 (Sox9)*, whose activity is necessary to initiate male development. Sex determination in the fruit fly, *Drosophila melanogaster*, is determined by the ratio of X chromosomes to autosomes (X:A ratio) or possibly by the number of X chromosomes, currently a point of some controversy. An XX embryo shows transient early expression of the splicing regulator *Sex-lethal (Sxl)* and lifelong production of functional *Sxl* ultimately triggers production of the female-specific isoform of *doublesex (dsx)* via a cascade of sex-specific alternative mRNA splicing. An XY embryo has lower *Sxl* expression, resulting in the subsequent production of the male-determining splice form of *dsx*. The *Drosophila* Y chromosome is not involved in sex determination but contains genes required for male fertility; an XO fly, therefore, is male but sterile. Sex determination in the roundworm *Caenorhabditis elegans* is initiated by the X:A ratio, but unlike *Drosophila* there is no Y chromosome. An X:A ratio of 1 (XX) initiates an inhibitory signal transduction pathway resulting in elevated activity of the transcriptional regulator *transformer 1 (tra-1)* and development into a hermaphrodite (anatomically female but able to make sperm and oocytes). An X:A ratio of 0.5 (XO, the result of rare spontaneous chromosome nondisjunction during meiosis) results in low *tra-1* activity and male development. The *C. elegans* dose-sensing system is exquisitely sensitive: experiments using induced polyploids (worms with one or more extra sets of chromosomes) showed that embryos can reliably distinguish between X:A ratios of 0.67 and 0.75 to become males and hermaphrodites, respectively.

The great diversity in how sex is determined indicates that transitions among sex-determining mechanisms have occurred repeatedly across the tree of life. These transitions are clearly illustrated when sex-determining mechanisms are

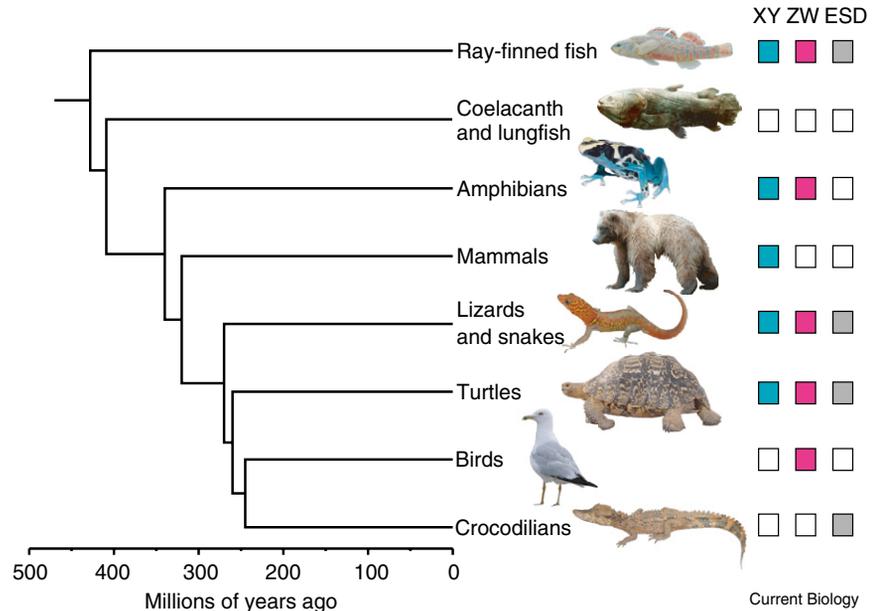


Figure 2. Frequent changes in sex-determining mechanisms among major clades of bony vertebrates.

Colored boxes indicate known sex-determining mechanisms for each clade. Lineages with more than one colored box possess multiple species with different sex-determining mechanisms. Some lineages, e.g. birds, mammals and crocodylians, show great stability of sex-determining mechanisms over long periods of time while other lineages, e.g. ray-finned fish, amphibians, lizards and snakes, and turtles, comprise species with diverse mechanisms, implying more frequent evolutionary changes within those groups. Sex-determining mechanisms are unknown in lungfish and coelacanths.

displayed in a phylogenetic context (Figure 2). Some transitions are ancient and have resulted in relatively stable sex determining mechanisms across all members of a clade. Thus, an XX/XY system regulated by *SRY* is found in virtually all therian mammals; a ZZ/ZW system is present in all birds, including ratites (e.g., ostriches and emus); and TSD occurs in all crocodylians. In other cases transitions have occurred among closely related species or even among populations within a single species. For example both ZZ/ZW and XX/XY systems are found among different populations of the Japanese frog *Rana rugosa*, with female heterogamety evolving at least twice independently within the species. Comparing divergent sex-determining systems among closely related species will be particularly useful for learning how these transitions can occur.

The extreme lability of sex-determining mechanisms was beautifully demonstrated in *C. elegans* by genetic experiments performed by Jonathan Hodgkin, who showed that simple loss- and gain-of-function mutations in seven of

the core nematode sex-determining genes could be used to create a wide variety of stable sex-determining mechanisms, including XX/XY, ZZ/ZW, and environmental mechanisms. Because the different genes that were altered are located on different chromosomes, it was possible to turn each autosome into a sex chromosome, and because these genes encode many different types of proteins, it appears that essentially any type of molecule should be able to control sex. Indeed, use of an amber nonsense allele (a UAG translational stop codon) introduced into the sex-determining gene *transformer 3 (tra-3)* allowed an amber-suppressor tRNA, which permits read-through of the stop codon, to serve as the primary determinant of sex. These laboratory manipulations strongly suggest that rapid and dramatic transitions in sex-determining mechanism should occur in nature and that they might result from relatively simple loss- or gain-of-function mutations in key sex-regulatory genes.

A picture emerges of pathways whose logic and individual components can exchange quickly

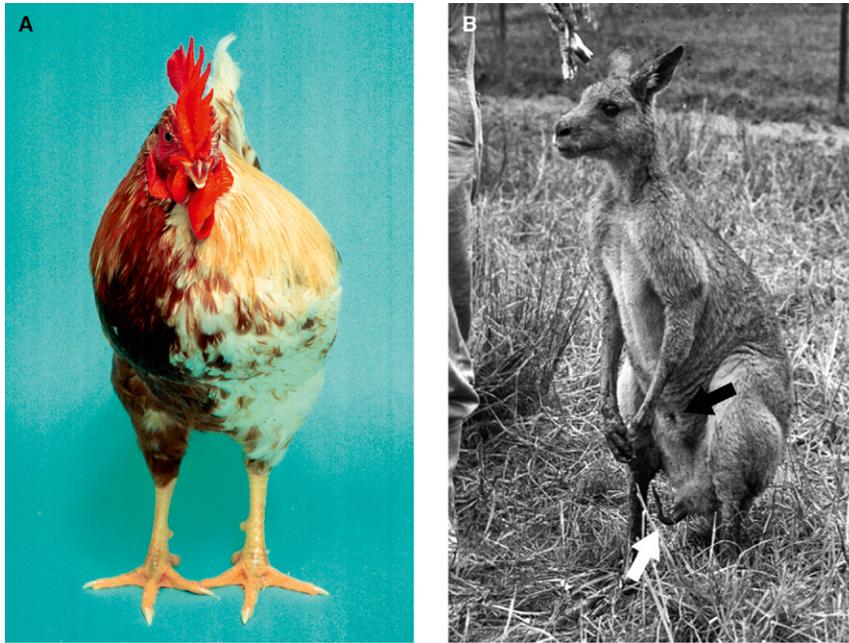


Figure 3. Animals of mixed-sex genotypes showing both male and female sexual traits. (A) Bilateral gynandromorph chicken. Right side has cells that are predominantly ZW, while left side cells are predominantly ZZ. Consequently, female characteristics, e.g. small wattle and small leg spur, are expressed on the bird's right side (brown plumage) while male characteristics, e.g. large wattle, large leg spur and greater muscle mass, are expressed on the bird's left side (gold and white plumage). Photo courtesy of Dr. Michael Clinton, The Roslin Institute, The University of Edinburgh. (B) "Vincent", an XXY gray kangaroo (*Macropus giganteus*) showing both male and female characteristics. The black arrow indicates the pouch, which is determined by the number of X chromosomes, while the white arrow indicates the penis, which along with the testes is determined by the presence of a Y chromosome. Photo courtesy of Professor D.W. Cooper, University of New South Wales.

during evolution, with a fluidity that is possible because the only essential function of a sex determination switch is to make different individuals develop as different sexes. Phylogenetic studies suggest that the downstream 'business' end of a sex-determination pathway does tend to be more stable than the triggering mechanism at the top; in insects for example, *tra* and *dsx* play widely conserved roles in sex determination while *Sxl* does not.

#### When and where sex is determined

The initial sex determination decision needs eventually to result in sexual dimorphism throughout the body, from brain to tail. There is, however, considerable diversity in where sex determination is initiated and how the decision is transmitted to the cells that require sex-specific development. A familiar example is that of mammals, where *Sry* acts exclusively in the fetal gonads and then gonadal sex hormones convey the sex determination decision to the rest of the body; this explains why

surgical removal of the male gonad during fetal development in mammals causes XY embryos to develop as females. Manipulations of gonadal sex hormones in other vertebrates also have confirmed the critical importance of the gonad in sexual development. However, animals such as roundworms and fruit flies lack gonadal sex hormones and in these creatures the sex determination decision is more spatially distributed, with the sex chromosome complement acting directly in cells throughout the body. Even these examples are generalizations, however: there is good evidence that the sex chromosomes also act directly on the fetal mammalian brain to cause sexually dimorphic gene expression, and secreted signaling molecules do play important roles in sexual dimorphism of both flies and roundworms.

In some species sex determination mechanisms seem to differ between the gonad and other tissues. In birds, for example, the Z-linked *doublesex* and *mab-3 related*

*transcription factor 1* (*Dmrt1*) genes are critical for gonadal sex, but the sex chromosomes act via a separate mechanism to control the sex of non-gonadal tissues. This is graphically illustrated by bilateral gynandromorphs, chimaeric birds in which one side is predominantly ZZ and the other ZW (Figure 3). In these birds one half appears physically male and the other female, a pattern that cannot involve *Dmrt1*, which is not expressed outside the urogenital system, and also cannot be explained solely by gonadal sex hormone levels, which should be similar in both sides. Similarly, in marsupials, like eutherian mammals, the Y-linked *Sry* gene controls gonadal sex, but the number of X chromosomes determines whether a pouch or scrotum develops. An XO kangaroo therefore is a female with ovaries but has a scrotum instead of a pouch. An XXY kangaroo, on the other hand, is a male with testes, but with a pouch — perhaps the prototypical 'man-purse' (Figure 3).

#### What are the genes that control sex?

Clearly a number of different mechanisms can trigger sex determination, but how similar are the downstream gene networks that respond to these triggers and actually do the work? Our current understanding of the genetic control of sex determination mainly derives from two intertwined strands of research: studies of human patients whose genetic and physical sex are discordant, and molecular genetic analysis in model organisms, mainly fruit flies, roundworms, and mice.

Sex chromosomes were identified in the early 1900s. However, it was not until the late 1950s that the mammalian Y chromosome was discovered to determine male sex, the late 1980s that the sex-determining region of the human Y chromosome was identified through cytogenetic analysis, and the early 1990s that *SRY* was discovered and confirmed, by human translocations of *SRY* and mice transgenic for *Sry*, to be the Y-linked gene that triggers testicular differentiation in males. *Sry* was found to act through the related gene *Sox9* and to oppose a female-promoting regulatory network involving *Wnt/β-catenin* signaling (Figure 1). At the same time, forward genetic studies in flies and worms

were identifying the major players controlling sex in these organisms. Surprisingly, there was initially no overlap among these model systems — though many of the genes controlling sex in flies or roundworms were conserved, their sex-determining roles were not. Thus, sex-determination pathways seemed to lack shared components, unlike many other major developmental regulatory pathways, a near heresy in the age of model organisms. Until the late 1990s, a molecular geneticist therefore could be excused for viewing sex determination much as Darwin initially viewed the peacock's tail.

A measure of resolution came when the downstream male regulator *male abnormal 3* (*mab-3*) was cloned from *C. elegans* and found to be related to the insect *dsx* gene. *dsx* and *mab-3* share a novel DNA-binding motif, the DM domain, which was subsequently found in many metazoan sexual regulators, most notably *Dmrt1* in vertebrates. *Dmrt1* is expressed in the embryonic gonad of all vertebrates examined and *Dmrt1* or a close paralog has been shown to be essential for testicular differentiation in mammals, birds, and fish. Thus, at least one family of regulators acting at the interface of sex determination and sexual differentiation does appear to be deeply conserved.

Studies of *Dmrt1* in vertebrates also have suggested that simple gene mutations can drive transitions between sex-determining mechanisms in nature, much like those created by Hodgkin in *C. elegans* in the laboratory. In three different vertebrate groups it appears that a new sex-determining mechanism resulted from a different mutational event affecting *Dmrt1*: the avian ZZ/ZW system likely arose from a recessive loss-of-function mutation in *Dmrt1*; in the medaka fish (*Oryzias latipes*) a new XX/XY system evolved due to a dominant gain-of-function mutation of *Dmrt1* in which the new *Dmrt1* allele — *Dmy* — functions analogously to *Sry* in mammals; and the African clawed frog (*Xenopus laevis*) ZZ/ZW system likely arose from a truncation that generated a dominant-negative ovary-determining *dmrt1* allele — *dm-w* — that blocks the masculinizing activity of the autosomal *dmrt1* gene. Therian mammals and stickleback fish are the only vertebrate groups so far

examined where it is clear that *Dmrt1* or a paralog does not act as a sex-linked sex-determining gene, although *Dmrt1* still plays a crucial role in male gonadal differentiation and maintenance in these species.

#### Acquiring and evolving sexual traits

Sexual selection and other selective pressures cause sexually dimorphic traits to evolve rapidly. Thus, even dramatic dimorphisms in one species may be very different or absent in related species. Recent work, mainly in fruit flies, has helped reveal how dimorphic traits arise and adapt during evolution. Two distinct mechanisms, both involving the activity of *dsx*, have been described. The first involves the ability of *dsx* to regulate downstream transcriptional targets and relies on variation in the presence or location of DSX binding sites in *cis*-regulatory elements. In abdominal pigmentation, which is present in males but reduced in females, changes in DSX binding sites have altered the ability of DSX to repress the two *bric a brac* (*bab*) genes, and thereby promote strong pigmentation in the male posterior abdomen. The second mechanism involves changes in the expression pattern of *dsx* itself. Although *dsx* controls most dimorphic traits, it is not actually expressed in all cells, so that in effect some cells 'know' their sex and some do not. This feature has been exploited in the evolution of sex combs, male-specific sensory bristles used in mating that are found on the first pair of legs in some *Drosophila* species. Sex combs develop as a result of the joint expression of the male-specific *dsx* isoform and the HOX gene sex combs reduced (*Scr*) and are present only in species that have evolved an appropriate *dsx* expression domain in the foreleg.

#### Staying committed

Sexual dimorphisms arise throughout much of development in most species and thus sex presumably is determined early. A wide range of experimental approaches have confirmed that this is so, including experiments involving surgical removal of the fetal gonad and conditional deletion of sex determination genes in mammals, temperature shifts in reptiles with TSD, and temperature shifts using conditional

alleles of sex-determining genes in worms. In each case sex reversal required intervention during embryonic development and did not alter phenotypic sex after that time (although in flies sexual phenotypes can be altered as late as the pupal stage). It came as a surprise, therefore, when it was shown recently that deleting either of two sex-specific mouse transcription factors — *forkhead box L2* (*Foxl2*) in females or *Dmrt1* in males — could cause gonadal cells to reprogram their sex, even in adults. Thus, sex is determined early but not irreversibly, and *Foxl2* and *Dmrt1* lie at the heart of two opposed maintenance networks that uphold the initial sex determination decision.

#### The future

Several decades of intensive genetic investigation have provided a detailed view of how sex is determined in several model organisms, including what triggers sex determination, what gene networks respond to the trigger, and how downstream genes integrate sex and pattern to promote sexual differentiation, and have begun to illustrate how sex determination and sexual dimorphism can evolve. New technologies, including inexpensive high-throughput DNA sequencing, RNAi, and other reverse-genetic strategies, herald rapid future progress in our understanding of sex determination and its evolution, making it easier to perform genetic mapping in non-model organisms, to identify and study the function of sex-biased regulatory networks, and to compare sex chromosomes across species. A detailed understanding of how transitions among sex-determining mechanisms can occur is currently lacking, although several theoretical models provide testable hypotheses about how such transitions might be accomplished. Fine-scale phylogeny-based studies will be one way forward and will identify many new models to study. Sex maintenance is another emerging field of study. While we now know that *Dmrt1* and *Foxl2* are required for maintaining the sex of postnatal gonads in mice, we know little of the gene networks in which they function nor whether sex maintenance occurs in other animals. Darwin's theories provided the

foundation for modern studies of how and why sexual dimorphisms arose and evolved; with the advent of new technologies the near future will certainly provide deeper insights into the molecular basis of these processes.

#### Further reading

- Bull, J.J. (1983). Evolution of Sex Determining Mechanisms. (Menlo Park, California: Benjamin Cummings Publishing Company, Inc.)
- Darwin, C. (1871). The Descent of Man and Selection in Relation to Sex. (London: John Murray.)
- Dewing, P., Shi, T., Horvath, S., and Vilain, E. (2003). Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. *Mol. Brain Res.* 118, 82–90.
- Graves, J.A.M. (2008). Weird animal genomes and the evolution of vertebrate sex and sex chromosomes. *Annu. Rev. Genet.* 42, 565–586.
- Herpin, A., and Schartl, M. (2011). *Dmrt1* genes at the crossroads: a widespread and central class of sexual development factors in fish. *FEBS J.* 278, 1010–1019.
- Hodgkin, J. (2002). Exploring the envelope: systematic alteration in the sex-determination system of the nematode *Caenorhabditis elegans*. *Genetics* 162, 767–780.
- Hosken, D.J., and House, C.M. (2011). Sexual selection. *Curr. Biol.* 21, R62–R65.
- Matson, C.K., Murphy, M.W., Sarver, A.L., Griswold, M.D., Bardwell, V.J., and Zarkower, D. (2011). DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature* 476, 101–104.
- Matson, C.K., and Zarkower, D. (2012). Sex and the singular DM domain: insights into sexual regulation, avolution and plasticity. *Nat. Rev. Genet.* 13, 163–174.
- Quinn, A.E., Georges, A., Sarre, S.D., Guarino, F., Ezaz, T., and Graves, J.A.M. (2007). Temperature sex reversal implies sex gene dosage in a reptile. *Science* 316, 411.
- Robinet, C.C., Vaughan, A.G., Knapp, J.M., and Baker, B.S. (2010). Sex and the single cell. II. There is a time and place for sex. *PLoS Biol.* 8, e1000365.
- Sekido, R., and Lovell-Badge, R. (2009). Sex determination and SRY: down to a wink and a nudge. *Trends Genet.* 25, 19–29.
- Tanaka, K., Barmina, O., Sanders, L.E., Arbeitman, M.N., and Kopp, A. (2011). Evolution of sex-specific traits through changes in HOX-dependent doublesex expression. *PLoS Biol.* 9, e1001131.
- Uhlenhaut, N.H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., Treier, A.-C., Klugmann, C., Klasen, C., Holter, N.I., et al. (2009). Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 139, 1130–1142.
- Veitia, R.A. (2010). FOXL2 versus SOX9: a lifelong “battle of the sexes”. *Bioessays* 32, 375–380.
- Williams, T.M., and Carroll, S.B. (2009). Genetic and molecular insights into the development and evolution of sexual dimorphism. *Nat. Rev. Genet.* 10, 797–804.
- Williams, T.M., Selegue, J.E., Werner, T., Gompel, N., Kopp, A., and Carroll, S.B. (2008). The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. *Cell* 134, 610–623.
- Zhao, D., McBride, D., Nandi, S., McQueen, H.A., McGrew, M.J., Hocking, P.M., Lewis, P.D., Sang, H.M., and Clinton, M. (2010). Somatic sex identity is cell autonomous in the chicken. *Nature* 464, 237–242.

Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN 55455, USA.  
E-mail: zarko001@umn.edu

## Correspondences

### Screening mammal biodiversity using DNA from leeches

Ida Bærholm Schnell<sup>1,2,†</sup>, Philip Francis Thomsen<sup>2,†</sup>, Nicholas Wilkinson<sup>3</sup>, Morten Rasmussen<sup>2</sup>, Lars R.D. Jensen<sup>1</sup>, Eske Willerslev<sup>2</sup>, Mads F. Bertelsen<sup>1</sup>, and M. Thomas P. Gilbert<sup>2,\*</sup>

With nearly one quarter of mammalian species threatened, an accurate description of their distribution and conservation status is needed [1]. For rare, shy or cryptic species, existing monitoring methods are often prohibitively expensive or unreliable. The problem is particularly acute in tropical forests, where a disproportionate number of species are listed by IUCN as ‘data deficient’ [2], due to the difficulty of monitoring with conventional approaches. This presents serious obstacles to conservation management. We, here, describe a new screening tool, the analysis of mammalian DNA extracted from haematophagous leeches. By demonstrating that PCR amplifiable mammalian blood DNA survives for at least four months post feeding in haematophagous *Hirudo* spp. leeches, we hypothesise that most wild caught adult leeches will contain DNA traces of their last blood meal. We subsequently demonstrate the efficacy of the method, by testing it *in situ* using terrestrial *Haemadipsa* spp. leeches caught in a tropical Vietnamese rainforest setting, and identify cryptic, rare and newly discovered mammalian species. We propose that DNA from leeches represents a quick, cost-effective and standardised way to obtain basic data on mammalian biodiversity and species occupancy, facilitating efficient use of limited conservation resources.

An emerging tool for assessing mammalian biodiversity is the profiling of DNA extracted from micropredators. In addition to ticks and mosquitoes [2], haematophagous leeches represent promising candidates as, following feeding, they store concentrated blood for several months [3]. Furthermore, several studies have demonstrated that

in the medical leech (*Hirudo medicinalis*) viruses remain detectable in the blood meal for up to 27 weeks, indicating viral nucleic acid survival [4,5]. To examine whether PCR amplifiable mammalian DNA persists in ingested blood, we fed 26 medical leeches (*Hirudo* spp.) freshly drawn goat (*Capra hircus*) blood (Supplemental information) then sequentially killed them over 141 days. Following extraction of total DNA, a goat-specific quantitative PCR assay demonstrated mitochondrial DNA (mtDNA) survival in all leeches, thus persistence of goat DNA, for at least 4 months (Figure 1A; Supplemental information).

We subsequently applied the method to monitor terrestrial mammal biodiversity in a challenging environment. *Haemadipsa* spp. leeches were collected in a densely forested biotope in the Central Annamite region of Vietnam (Figure 1B; Supplemental information), in which five new mammal species have recently been discovered. Despite the interest these discoveries have generated, including adoption of the saola (*Pseudoryx nghetinhensis*) as a regional flagship species by the World Wildlife Fund, all attempts to develop standardised survey and monitoring techniques for these species have failed.

Remarkably, 21 out of 25 leeches tested yielded mammalian mtDNA sequences, representing six species spanning three orders — Artiodactyla, Carnivora and Lagomorpha (Figure 1B; Supplemental information). We deliberately chose PCR assays that are unable to PCR amplify human DNA in order to prevent false positives derived from human contamination of the leeches at time of sampling (Supplemental information). Therefore, it is possible that human DNA is present in the four samples that failed to yield an amplicon; thus, the value is a conservative estimate of the blood-meal-derived mammalian DNA prevalence in the leeches. Although multiple clones were sequenced per amplicon, no leech yielded sequences from more than one species, suggesting that rapid decline in blood meal DNA concentration over time (Supplemental information) will render DNA levels derived from a new feeding to be greatly higher than those from previous feedings. Two of the detected species have been recently described, the Truong Son muntjac (*Muntiacus truongsonensis*, one leech) and