To "Bee or Not to Bee" Male or Female? An Educational Primer for Use with "The *Am-tra2* Gene Is an Essential Regulator of Female Splice Regulation at Two Levels of the Sex Determination Hierarchy of the Honeybee"

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SUMMARY An article by Nissen *et al.* in the November 2012 issue of *GENETICS* emphasizes the importance of alternative splicing in the sex determination cascade of the honeybee *Apis mellifera*. This study demonstrates the application of reverse transcriptase PCR and RNA interference screens as genetic tools to better understand the regulation of transcription and splicing. It also provides the opportunity to explore the evolutionary origins of genes by considering the functions of orthologs and paralogs in different species. This Primer article provides background information and explanations of the concepts and findings of Nissen *et al.* and offers discussion questions for use in the classroom.

Related article in *GENETICS*: Nissen, I., M. Müller, and M. Beye, 2012 The *Am-tra2* gene is an essential regulator of female splice regulation at two levels of the sex determination hierarchy of the honeybee. Genetics **192:** 1015–1026.

Background

THE origins of beekeeping and honey collecting in human civilizations can be traced back thousands of years. Honeybee colony management has thrived because of the important role played by honeybees in crop pollination and because human cultures worldwide consume hive products (honey, beeswax, and propolis). Importantly, bees also shape natural ecosystems by facilitating gene flow among plants.

Honeybees are eusocial insects that live in colonies of tens of thousands of individuals. A colony usually consists of three types of bees: queens, drones, and workers. The female queen (usually one per colony) is selected early in larval development and fed a diet of royal jelly. As an adult, the queen lays unfertilized and fertilized eggs. Male drones, whose main role is to mate with the queen, develop from unfertilized eggs. Female workers (the majority of bees in a colony) are the laborers that perform tasks required for colony growth and maintenance (*e.g.*, cleaning, defense, foraging for food). Workers will produce eggs only if the colony is queen-less. Because of this social structure, honeybees are important models for studying behavior, learning, and memory as well as the genetics of sex determination.

The signal transduction cascade that directs sex determination in honeybees (*i.e.*, development as a male drone or female queen/worker) directs developmental fate and, in turn, has a significant influence on the social structure of a colony. Over 150 years ago Johannes Dzierson hypothesized that male bees develop from unfertilized eggs and females from fertilized eggs (reviewed by Page *et al.* 2012). However, research to understand the genetics that controls male *vs.* female developmental fate is ongoing. The publication of the DNA sequence of the European honeybee (*Apis mellifera*) genome in 2006 (Honeybee Genome Sequencing Consortium 2006) provided important resources for studying bee genetics, including the molecular basis of sex determination. Martin Beye and his team of researchers at Heinrich Heine University in Dusseldorf, Germany, have made significant contributions in this field. In their most recent article, Beye and colleagues Inga Nissen and Miriam Müller examine the expression pattern of the gene *transformer-2* (*tra-2*) in *A. mellifera* and how it regulates the splicing and expression

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of other genes in the sex determination cascade. Their results enhance our understanding of the mechanisms of gene regulation that lead to either female or male development in the honeybee *A. mellifera*.

Sex determination in honeybees: the complementary sex determiner locus

What determines whether an animal develops as a male or female? There is no universal answer, but in many animals, sex determination is triggered very early in development by chromosomal differences, such as a specific complement of sex chromosomes. For example, in humans (and many animals) males are heterogametic, having two different sex chromosomes, *X* and *Y*, while females are homogametic, having two copies of *X*. The total number of sex chromosomes can also determine sex: female grasshoppers have two *X* chromosomes (*X/X*) while males have only one (*X/*O). In some animals, hermaphrodites (individuals with both male and female characteristics) are common; in the nematode *Caenorhabditis elegans*, males are *X/*O while hermaphrodites are *X/X*.

The haplodiploid sex determination system is the dominant mode in Hymenoptera, the insect order that includes bees, wasps, ants, and sawflies. Sex chromosomes are completely absent in Hymenoptera; instead, ploidy (the number of chromosome sets in an individual) seems to be linked to sex determination. Fertilized eggs are diploid (two chromosome sets) and develop as females, while unfertilized eggs are haploid (one chromosome set) and develop as males. In honeybees, however, it is the allelic state of the *complementary sex determiner* (*csd*) gene, rather than ploidy, that is the primary genetic signal of sex determination.

In honeybee populations, the *csd* locus is highly polymorphic; there are at least 15 different *csd* alleles among bee populations, so diploid individuals tend to be heterozygous for *csd*. Recall that a diploid organism inherits two alleles of each autosomal gene, one from each parent; an organism is homozygous for a particular gene if both alleles are identical and heterozygous if there are DNA sequence differences between the two alleles. Thus, in bees, it is zygosity (the degree of similarity between two alleles) of the *csd* locus, rather than ploidy, that is the primary determinant of sexual fate. Individuals heterozygous for *csd* are female (either a queen or a worker) while haploids develop as males (they are hemizygous for *csd*, having one copy) (Beye *et al.* 2003). Diploid bees that are homozygous for *csd* also develop as males, although workers eat these individuals soon after they emerge from the egg.

Transcription, alternative splicing, and sex determination

Is it that simple? Does a single gene determine honeybee sex? While *csd* is the primary signal of sex determination, the process actually involves a cascade of interactions among several other genes. In the honeybee, many genes involved in the sex determination pathway undergo sex-specific alternative splicing (*i.e.*, production of a variety of messenger RNA (mRNA) transcripts from a single gene; Figure 1), producing proteins that are specific to bees of each sex. During splicing, the spliceosome catalyzes the removal of introns and joins together exons to produce a mature mRNA that contains 5′ and 3′ untranslated regions (UTRs) and exons that code for an open reading frame. SR proteins are also involved in regulating splicing. Members of this family of RNA-binding proteins contain an RS domain rich in repeated arginine–serine dipeptides that regulates constitutive splicing and, importantly, alternative splicing. During alternative splicing, RNA recognition motifs in SR proteins bind to specific splice sites (*e.g.*, exonic splicing enhancers) within pre-mRNA.

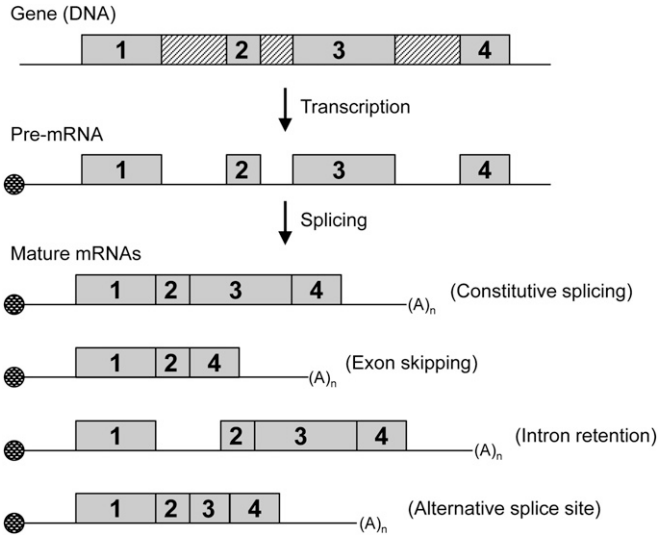

Figure 1 Alternative splicing can produce different mRNA transcripts from the same gene. In this figure, gray boxes with numbers are exons, and boxes with diagonal lines are introns. The circle represents the mRNA 5′ cap, and $(A)_n$ represents the poly(A) tail. DNA is transcribed into pre-mRNA that can then be spliced differently to produce transcripts of different lengths and sequence composition. Alternative splicing is indicated next to the different splice variants.

In female honeybees, heteroallelic Csd proteins direct female-specific splicing of the *feminizer* (*fem*) gene, which is then translated into a functional Fem (SR-type) protein. Fem proteins direct female-specific splicing of the *doublesex* gene (*dsx*), a transcription factor that mediates female development. Fem proteins are also necessary to direct female-specific splicing of their own pre-mRNA, thus creating an autoregulatory feedback loop (Gempe *et al.* 2009). In contrast, in male honeybees, homo- or hemiallelic Csd proteins lead to male-specific splicing of *fem*. However, this male-specific splice variant encodes a truncated nonfunctional Fem protein. Therefore, *dsx* is transcribed (without regulation by Fem proteins) and undergoes male-specific splicing to yield a Dsx protein that controls male development.

The buzz on important gene terminology

When a putative gene sequence is identified, understanding the evolutionary history of that gene can be informative for developing hypotheses about its function. Genes are homologs

if they are descended from a common ancestral sequence. Among types of homologs, orthologs are genes that evolved by speciation (*i.e.*, the same gene in different species) while paralogs are genes within the same genome that evolved by duplication. Many genes (*i.e.*, orthologs) in the *Apis* sex determination pathway are found in other insects. For example, despite their different names, the *fem* gene in *Apis* is an ortholog of the sex-determining gene *transformer* (*tra*) in the fruit fly *Drosophila melanogaster* (*i.e.*, *fem* and *tra* evolved from a common ancestral gene millions of years ago). However, *csd* and *fem* are paralogs specific to the honeybee lineage (Hasselmann *et al.* 2008) that have evolved and acquired distinct roles in the sex determination pathway. To alleviate confusion when discussing orthologs from different species, genes from the honeybee will be designated with an "*Am*-" prefix (for *A. mellifera*, as in Nissen *et al.* 2012). Gene names are written in lowercase italics (*e.g.*, *Am-dsx*) and protein names in non-italics with the first letter of the protein capitalized (*e.g.*, *Am*-Dsx).

The Objective of the Study

In the honeybee, expression of *csd* and *fem* paralogs, alternative splicing of their gene products, and the function of the Csd and Fem proteins in sex determination have previously been demonstrated (Hasselmann *et al.* 2008). In *D. melanogaster*, Tra works together with another protein called Transformer-2 (encoded by the gene *tra-2*) to promote female-specific splicing of *dsx*. A sequence homology search of the honeybee genome (Dearden *et al.* 2006) previously identified a *tra-2* ortholog; however, whether this gene is expressed or produces functional transcripts has not been demonstrated. Therefore, since *tra-2* and *Am-tra-2* are orthologs, the objective addressed by Nissen *et al.* (2012) was to determine whether Tra-2 and *Am*-Tra2 share the same function (*i.e.*, regulating sex-specific splicing of *dsx*) in the fruit fly and in the honeybee.

Using RACE and RT-PCR to characterize transcripts

To understand the function of *Am*-Tra2 in the honeybee, sequences of full-length *Am-tra2* transcripts (including the 5′ and 3′ UTRs) from male and female honeybees needed to be determined. RNA is single stranded and cannot be used as a template for polymerase chain reaction (PCR). Therefore, to characterize RNA sequences, mRNA is converted into complementary DNA (cDNA) using the enzyme reverse transcriptase. Together with cDNA synthesis, the RACE technique was used by Nissen *et al.* (2012) to determine the 5′ and 3′ sequences of a mature mRNA.

Recall that, during pre-mRNA processing, a 7-methylguanosine cap (5′ cap) is added to the 5′-end, the 3′-end is cleaved at the polyadenylation site, and a 3′ poly(A) tail (∼100–250 adenine residues) is added. Since the process of RNA isolation often partially degrades transcripts, it is necessary to select sequences that have the intact 5′ cap and 3′ poly(A) tail to characterize full-length sequences. In RACE, short oligonucleotide adapters (that contain RACE-specific primers) are added to the 5′ and 3′ transcript ends. For 5′ RACE, an adapter replaces the 5′ cap, and the transcript is then used for cDNA synthesis. In 3′ RACE, the adapter anneals to the poly(A) tail via a complementary oligo(dT) sequence that is extended by reverse transcriptase. The cDNA pools can then be used for PCR using a combination of gene-specific and RACE adapter-specific primers, and these PCR products can be isolated and sequenced to identify the first and last exons and the 5′ and 3′ UTRs for *Am-tra2* transcripts.

With these sequences in hand, Nissen *et al.* designed oligonucleotide primers to anneal to the 5′ and 3′ ends of *Am-tra2* transcripts. These primers were then used in RT-PCR to amplify full-length *Am-tra2* transcripts from male and female honeybee embryos and pupae. RT-PCR creates a pool of cDNA to use as a template for PCR, rather than genomic DNA. Six splice variants with minor sequence differences were identified in the pool of cDNAs created by Nissen *et al.* Despite the variation in the corresponding *Am*-Tra2 protein lengths, each isoform had the same domain structure as Tra2 orthologs in other species: a central RNA-binding domain (RBD) flanked by two RS domains. Within the RBD, two **r**ibo**n**ucleo**p**rotein consensus peptide elements (RNP-1 and -2) were identified. However, an amino acid substitution in RNP-1 in *Apis* at a position essential for female *dsx* splicing in *D. melanogaster* led Nissen *et al.* to ask whether *Am*-Tra2 has evolved a relatively different function.

Investigating the function of Am-Tra2

To investigate the role of *Am*-Tra2, Nissen *et al.* used semi-quantitative PCR to measure *Am-tra2* expression levels at different developmental stages in males and females. This method is similar to RT-PCR, except that it uses multiplex PCR to amplify transcripts of two different genes in the same reaction, providing an internal control in every reaction. Nissen *et al.* used primer pairs to amplify both *Am-tra2* and *elongation factor-1α* (*ef-1α*) mRNAs in each PCR reaction. *ef-1α* was included because it is a housekeeping gene (one that is required for translation), performs a basic cellular function, and is expected to be constitutively expressed at all stages of development. When the PCR products for each multiplex reaction are analyzed by gel electrophoresis, gel-imaging software can compare band intensities and calculate the fold change in expression between the gene of interest (*Am-tra2*) and the housekeeping gene (*ef-1α*). In this way, relative expression of *Am-tra2* is determined and used to measure relative transcription levels at different developmental stages. Nissen *et al.* found that *Am-tra2* transcripts in both males and females were most abundant during embryonic stages (before and after *csd* is transcribed) but that transcription significantly decreased at the pupal stage. Sex-specific splicing patterns were nonexistent.

What have we learned so far?

Consider the findings by the authors up to this point (refer to figures 1–3 in the article by Nissen *et al.*). Referring to

figure 1 in Nissen *et al.*, what did the RT-PCR data reveal about *Am-tra2* transcription and splicing patterns? How many splice variants are generated from the *Am-tra2* gene? What is the difference between the major and minor splice variants? In Nissen *et al.*'s figure 2, examine the amino acid alignments of the arthropod RBD domain and identify the amino acid substitution that led the authors to hypothesize that *Am*-Tra2 (and perhaps Tra2 in *Nasonia vitripennis*, another hymenopteran) might have a function distinct from that of Tra2 in *Drosophila* and other holometabolous (winged) insects. In figure 3 of Nissen *et al.*, are the splice variants equally abundant in embryos and pupae? Are they found in males and females? What does the figure demonstrate about transcription levels during embryonic and pupal development? Once the significance of the *Am-tra2* gene expression data is clear, the next question to consider is: what is the cellular function of *Am*-Tra2 protein?

Functional studies: RNA interference knockdowns

If Tra-2 in *Drosophila* and *Am*-Tra2 are indeed homologous proteins, one might assume that *Am*-Tra2 possesses the function of regulating male- and female-specific splicing of *Am-dsx* and *fem*. To test this hypothesis, Nissen *et al.* used a technique called RNA interference (RNAi) to determine whether sex-specific splicing patterns for *fem* and *Am-dsx* were affected when expression of *Am-tra2* was "silenced."

How does RNAi work? RNAi exploits the fact that double-stranded RNA (dsRNA) can be used to repress translation of a gene of interest. In many eukaryotes, small noncoding RNAs (such as small interfering RNA) can regulate gene expression post-transcriptionally. The enzyme Dicer cleaves these small dsRNAs into shorter pieces that associate with the multi-protein RNA-induced silencing complex (RISC). This RISC–RNA complex then binds to, and stimulates, the degradation of complementary cellular mRNA. RNAi can therefore be used to repress or "silence" a specific gene to examine the phenotypic effects. This is referred to as a "gene knockdown" since mRNA degradation is likely incomplete.

Nissen *et al.* used RNAi to knock down expression of *Am-tra2* by injecting honeybee embryos with dsRNA representing a segment of *Am-tra2*. This induced the RNAi response and subsequently targeted complementary *Am-tra2* transcripts for degradation. However, while the objective was to use RNAi to assess the role of *Am-tra2* in splicing regulation, the authors encountered an unexpected result. Injecting dsRNA copies of *Am-tra2* was actually lethal: male and female embryos failed to develop into larvae. And injecting lower concentrations of dsRNA only resulted in a very low frequency of larval development. If *Am-tra2* was specifically involved in regulating male- and female-specific splicing of *Am-tra2* and *dsx*, would this be the expected result (*i.e.*, lethality for both male and female larvae)? Is this result consistent with a specific role for *Am*-Tra2 in the sex determination pathway? Or does it suggest that *Am*-Tra2 might have broader functions in the honeybee?

To address their hypothesis about the relationship of *Am-tra2* and sex-specific splicing of *Am-dsx* and *fem*, Nissen *et al.* repeated the embryonic injections with *Am-tra2* dsRNA and isolated RNA from embryos (regardless of whether larvae hatched) after ~80 hr of development. The RNA was used in RT-PCR to amplify *Am-dsx* and *fem* transcripts and distinguish male and female splice variants.

What were their expectations? If *Am*-Tra2 promotes female-specific splicing of *Am-dsx* and *fem*, would male-specific or female-specific *Am-dsx* splice variants be expected in the *Am-tra2* knockdowns? Look at the data presented in figure 4 of Nissen *et al.* to learn what the authors found. For example, in the *Am-tra2* knockdown, only male-specific *Am-dsx* splice variants were produced while higher concentrations of injected *Am-tra2* dsRNA compromised female-specific splicing of *fem*. Based on these results, what do you conclude is the role of *Am-tra2*? What do the authors conclude? When *Am-tra2* was knocked down, male-specific *fem* transcripts were also absent. Does this mean that *Am*-Tra2 is also necessary for male-specific *fem* splicing? All together, these data suggest that *Am*-Tra2 is necessary to promote female-specific splicing of *Am-dsx* and *fem*, but it only regulates male-specific splicing for *fem*. *Am*-Tra2 does not appear to regulate male-specific splicing of *dsx* since male transcripts were produced in the *Am-tra2* knockdown.

Approach to Classroom Use

On the surface, the concept of haplodiploid sex determination appears quite straightforward: males are haploid and females are diploid. However, recent articles have started to unravel the genetics of sex determination in the honeybee. Reading a set of sequential articles from the Beye lab allows students to experience how research on this topic has progressed over several years. This teaching method, called CREATE (*c*onsider, *r*ead, *e*lucidate hypotheses, *a*nalyze and interpret the data and *t*hink of the next *e*xperiment), allows students to read and analyze primary literature and focus on a single line of scientific research (Hoskins *et al.* 2007). To apply this method, a suggested sequence of articles is the following: Beye *et al.* (2003) (the finding that *csd* heterozygosity is the primary sex-determining signal) followed by Hasselmann *et al.* (2008) (which characterizes the *Am-fem* and *csd* paralogs) and Gempe *et al.* (2009) (which explores the regulation and function of *Am-fem* and *csd*). After reading these articles, the methods and questions of Nissen *et al.* (2012) might seem more rational and logical to students. In addition, review articles on sex determination in Hymenoptera (Heimpel and de Boer 2008) and other insects (Gempe and Beye 2010; Verhulst *et al.* 2010a) are valuable reading. For a comparative approach, sex determination pathways in another Hymenopteran, the parasitoid wasp *N. vitripennis*, can be explored. While both *Apis* and *Nasonia* have haplodiploid sex determination, the genetics that regulates this mechanism in *Nasonia* is quite different (it is dependent upon the contribution of maternal transcripts in the embryo and some form of maternal imprinting) (Verhulst *et al.* 2010b).

The article by Nissen *et al.* (2012) can be used as a teaching aid to discuss laboratory techniques including RACE, RT-PCR, and RNAi as well as the concept and significance of alternative splicing in regulating gene expression. One approach might be to ask students to summarize the data that were produced using each molecular method: RACE, RT-PCR, semiquantitative PCR, and RNAi. For classroom or group discussion, considering the strengths and weaknesses of the authors' conclusions and contemplating follow-up experiments will provide a stimulating experience and a greater understanding of the material. Additional suggested questions and problems are summarized below:

1. Nissen *et al.* used semiquantitative PCR to quantify gene expression, but what other methods could have been employed? Does having a completed genome sequence for *A. mellifera* provide an advantage? What are some benefits and drawbacks to these other approaches?
2. Nissen *et al.* proposed a model for the role of *Am*-Tra2 in sex determination that implies interactions between Csd proteins with *Am*-Tra2 and/or Fem. What are some experiments that could be done to determine whether these protein–protein interactions take place *in vitro*? *In vivo*?
3. The concept of RNAi is explained in all modern genetics textbooks. However, delivery methods for dsRNA can vary between organisms. Discuss how dsRNA copies of *Am-tra2* were delivered into honeybee embryos. Are there other commonly used methods of dsRNA delivery?
4. The finding that *Am-tra2* appears to be essential for embryogenesis was intriguing and merits further investigation. What are some follow-up experiments that could determine the stages at which *Am-tra2* is required for development? Are there ways to visualize the production of *Am*-Tra2 proteins? To characterize the localization of these proteins or transcripts in embryos?
5. To understand how Nissen *et al.* designed PCR primers, find the *Am-tra2* gene sequence in an online database (*e.g.*, http://hymenopteragenome.org/beebase/). How many exons and introns are present? How long is the coding region?
6. The concept of gene homologs, orthologs, and paralogs is challenging. The Nissen *et al.* article provides examples of orthologs in *A. mellifera* and *D. melanogaster* and paralogs within *A. mellifera*. To reinforce these concepts, use a BLAST search with the gene sequences identified in the Nissen *et al.* article to determine the phylogenetic distribution of these genes in insects (and beyond) and find evidence for gene loss or gain by duplication. Nissen *et al.* provide sequence accession numbers that can be used to retrieve the *Am-tra2* mRNA sequence and the amino acid sequences for the six isoforms identified in their article from online sequence databases at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). For example, amino acid queries can be used to search for homologs within the protein databases (using the BLASTP program) or the nucleotide databases [such as Whole Genome Shotgun (WGS) or Expressed Sequence Tag (EST) databases] using the TBLASTN program.

Literature Cited

Beye, M., M. Hasselmann, M. K. Fondrk, R. E. Page, and S. W. Omholt, 2003 The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. Cell 114: 419–429.

Dearden, P. K., M. J. Wilson, L. Sablan, P. W. Osborne, M. Havler *et al.*, 2006 Patterns of conservation and change in honey bee developmental genetics. Genome Res. 16: 1376–1384.

Gempe, T., and M. Beye, 2010 Function and evolution of sex determination mechanisms, genes and pathways in insects. Bioessays 33: 52–60.

Gempe, T., M. Hasselmann, M. Schiøtt, G. Hause, M. Otte *et al.*, 2009 Sex determination in honeybees: two separate mechanisms induce and maintain the female pathway. PLoS Biol. 7 (10): e1000222.

Hasselmann, M., T. Gempe, M. Schiøtt, C. G. Nunes-Silva, M. Otte *et al.*, 2008 Evidence for the evolutionary nascence of a novel sex determination pathway in honeybees. Nature 454: 519–523.

Heimpel, G. E., and J. G. de Boer, 2008 Sex determination in the Hymenoptera. Annu. Rev. Entomol. 53: 209–230.

Honeybee Genome Sequencing Consortium, 2006 Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature 443: 931–949.

Hoskins, S. G., L. M. Stevens, and R. H. Nehm, 2007 Selective use of the primary literature transforms the classroom into a virtual laboratory. Genetics 176: 1381–1389.

Nissen, I., M. Müller, and M. Beye, 2012 The *Am-tra2* gene is an essential regulator of female splice regulation at two levels of the sex determination hierarchy of the honeybee. Genetics 192: 1015–1026.

Page, R. E., O. Rueppell, and G. V. Amdam, 2012 Genetics of reproduction and regulation of honeybee (*Apis mellifera* L.) social behavior. Annu. Rev. Genet. 46: 97–119.

Verhulst, E. C., L. van de Zande, and L. W. Beukeboom, 2010a Insect sex determination: it all revolves around *transformer*. Curr. Opin. Genet. Dev. 20: 376–383.

Verhulst, E. C., L. W. Beukeboom, and L. van de Zande, 2010b Maternal control of haplodiploid sex determination in the wasp *Nasonia*. Science 328: 620–623.

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