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SYMPOSIUM

Binary Outputs from Unitary Networks

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Synopsis When considering sex ratios, we have to first define the nature of the question. Are we referring to the gonads, secondary and accessory sex structures, physiology, brain, behavior, or to all of the above elements. If these elements are not concordant, the exceptions can prove illustrative of underlying processes at both the proximate and ultimate levels. At each of these levels, "sex" is the binary outcome resulting from the modulation of conserved networks of genes, proteins, cells, organs, and, in the case of the brain, discrete nuclei. These networks operate at multiple and sequential levels that usually are linear during the lifespan, but in some instances reversals are possible. For example, the gonads arise from a single "anlagen" and, in most instances, ovaries or testes result, although ovotestes are the norm in some species and gonadal reversal a property of other species. Other sexually dimorphic structures differentiate from multiple "anlaga" by reciprocal and sex-specific atrophy/hypertrophy, typically in an exaggerated manner, although the capacity to develop structures characteristic of the opposite gonadal sex remains inherent and intact. A perspective that integrates these different properties are presented here.

Introduction

Most animals begin as a single cell, develop into one of two sexes (which we call male and female) which, when mature, interacts with its complement to create the single cell that begins the generation to follow. This basic equation underlies all aspects of sexuality. It also suggests that, ultimately, all life forms were once bisexual, the remnants of which are common throughout all life processes. However, semantics can bind perception; in this case, male and female are defined in terms of the opposite sex. We tend to think of the sexes in terms of differences rather than of similarities.

Sex ratio (male to female or M/F) varies at different stages in the life history. However, it is important to note that each signpost refers to an evaluation at a particular time and not to the processes that underlie the change in sex ratio. In common parlance, "Primary Sex Ratio" refers to the number of ova successfully fertilized by sperm (conception); in rodents and humans, the species best studied, this is approximately 1.5:1. "Secondary Sex Ratio" is the second, and most common, evaluation and is taken at birth. Presently in humans, the secondary sex ratio is 1.06:1. "Tertiary Sex Ratio" refers to the number of males and females that live long enough to achieve sexual maturity. In most animals and in human societies, this is 1:1. However, in terms of evolution, it is the "Operational Sex Ratio" that matters as this signifies the number of mating individuals who actually reproduce. This varies enormously and depends upon the mating system.

Such life-history markers do not reflect the events that occur between the designated time points that bring about the change (Navarra and Nelson 2009; Navara et al. 2013, this issue). It is the interim periods that I discuss here, namely the mechanisms of Primary Adjustment (resulting in the Primary Sex Ratio), Secondary Adjustment (corresponding to Secondary Sex Ratio), Tertiary adjustment (corresponding to Tertiary Sex Ratio), and the Operational Sex Ratio. Before considering each of these, it is instructive to consider the origin of sex, sex determination, and sexual differentiation, all of which are central to the meaning of sex ratio.

Origin of sex

A sex ratio presumes the presence of sex, and so a short discussion of the origin of sex is valuable.

Evolution is predicated on reproductive success, often defined as the production of young that themselves reproduce. Since the first organisms were acellular or unicellular, this was a simple division of self (mitosis) or conjugation. Such organisms exist today and practice asexual reproduction with or without exchange of genetic material. With multicellularity came the development of specialized cells for reproduction (gametes). Initially, the gametes were of equal size (isogamy), but in most lineages, they began to diverge in size (anisogamy). The evolution of gametes as containers of heritable material coincided with the development of meiosis such that unlike gametes must combine to create a new organism (zygote).

It is widely accepted that this need for complementary haploid gametes (sometimes called biparental reproduction) developed early in the origin of life. That is, offspring were produced from the union of two gametes; those that produce small gametes (sperm) are called males whereas those that produce larger gametes (eggs) are called females. This semantic dichotomy, although necessary, has shaped how we view all things sexual; that is, as elements more separate than alike.

Most organisms are sexual but not necessarily gonochoristic (separate sexes in separate individuals). For example, hermaphrodites of various forms are capable of self-fertilization, but this is rare when there is an opportunity to join with other hermaphrodites. Gonochorism is the norm in animals. Although asexuality and parthenogenesis (cloning or ''virgin'' birth) exist, such modes of reproduction are secondarily derived. Indeed, only one kind of organism is believed to have always been asexual since its inception: cyanobacteria.

It is necessary to emphasize that it is inappropriate to refer to females as the ''default'' sex, whereas the male is the ''organized'' sex. This language originated from embryological, and subsequent behavioral studies that suggested that if testes, and male-typical behaviors, did not develop, then a feminine phenotype would be expressed. This simplistic view is being replaced as we come to understand that ovarian development (see below) and female-typical behaviors are actively organized just as in males. In light of this evidence, I have suggested that the more appropriate terminology is to regard the female as the ''original'' sex, whereas the male is the ''derived'' sex (Crews 1993, 2012).

Origin of sex determination

The discovery of sex chromosomes and their role in sex determination (in mealworm beetles) in 1905 by Nettie Stevens (Brush 1978) marked the beginning of the scientific era of research on sex determination. Although not immediately accepted by all, within a few decades, it was generally accepted that in animals, and certainly all vertebrates, sex is established at the moment of fertilization by complementary heritable units of genetic material. By mid-century, it was established that genetic control (inheritance) of sex determination led in turn to the differences between the sexes in morphology, physiology, and behavior. The 20th century ended with the discovery of the sex determining Y region of the Y chromosome (*Sry*) (Koop 2012). During this same period, it became evident that in mammals, birds, and snakes, gonadal sex is determined genetically either by a variant of the XX:XY (male heterogamety) system or the ZW:ZZ (female heterogamety) system. The discovery that in vertebrates, gonadal sex might be determined by the environment, rather than genotype, is relatively recent. Today we know that some amphibians, many fish and turtles, and all crocodilians depend upon external environmental cues to determine sex and that in some fish, this process can persist throughout life with animals switching sex in adulthood (Crews 1994; Wibbels et al. 1994).

Despite this enormous diversity in sex-determining mechanisms, we have learned that a constellation of evolutionarily conserved genes orchestrate whether testes or ovaries will be formed from the genital ridge (Crews and Bull 2009; Koop 2012). We have also learned that in taxa other than mammals, *Sry* does not play a sex-determining role. For example, members of another gene family, doublesex-/mab-3-related transcription factors (*Dmrt*), play a central role in gonad determination (Koop 2012). This has been supported by various studies of animals that lack sex chromosomes, yet develop into males and females as a consequence of their environment. Originally it was thought that such animals uniformly lacked sex chromosomes or a genetic predisposition to respond to environmental cues that dictated the sex of the offspring. Once regarded as mutually exclusive, recent research has revealed that environmental versus genomic triggers can be present simultaneously in some species.

Whatever the switch or trigger, this in turn engages a primary gene ''cassette'' of evolutionarily conserved, functionally related genes that interact to determine gonadal fate (Crews and Bull 2009). The nature of these interactions change through development and this cassette engages other cassettes of integrated gene assemblies, such as those responsible for sexual differentiation of secondary and accessory sex structures.

Thus, 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 that have simultaneous inputs to several components of the downstream cascade. Systems with different degrees of the inherited and environmental influences could all operate this way, merely by varying the inputs to the networks.

The binary nature of the product of sex determination in turn led to the evolution of distinct developmental trajectories resulting in complementary morphological, physiological, and behavioral differences between the sexes that facilitate their ultimate union for reproduction.

Origin of sexual differentiation

The origin of males and females generated an elaboration of traits that served to distinguish the sexes. In order for sexual differentiation to occur, there must be signaling systems that encourage alternative developmental pathways. The agents of these changes are hormones, principally the sex steroid hormones. Sex steroid hormones, the product of the differentiated gonads, are not involved in determination of gonad type in mammals and birds. However, in all ectothermic vertebrates, there is a near universal role of steroids and their enzymes in overcoming/overriding the primary cassette of sex-determining genes to re-direct the gonad's fate.

In the ancestral sex (female), the only requirement was a means of coordinating development of the ovarian follicle. This is accomplished by genetic a regulatory molecule (response element that engaged a specific DNA sequence) activated by an endogenous signal associated with follicular development (the estrogenic hormones). Determination of the ancestral hormone receptor at the root of the steroid receptor's evolutionary tree indicates that the ancestral steroid hormone was an estrogen receptor (ER) (Eick et al. 2012). Thus, the ER appears to have been the first steroid hormone receptor and co-opted estrogen as a signaling molecule. Since the synthesis of estrogen and the maturation of the egg are linked (for reasons as yet unknown), this complementary union served as a reliable indicator for coordinating the reproductive process.

In so doing, all of the intermediate steroid molecules in the steroidogenic pathway leading to estrogen became available as possible chemical signals for receptors. Subsequent receptors created by duplication of genes were shaped by the intermediates themselves. That is, the fixed molecular structure of the steroid intermediates shaped through selection the gene sequence that renders receptors having the greatest affinity/specificity for these intermediates. Progesterone receptor was the next steroid receptor to evolve, and its ancestral function may have been in the control of ovulation because of its close association with expulsion of the egg from the follicle. Only later did the androgen receptor (AR) and its role in the development of a sexually dimorphic phenotype appear in males. The fact that the ER is the most ancient sex steroid hormone receptor, while the AR is the most recent, is a pivotal point.

Origin of sex differences in the brain and its relation to sexual behavior

There is still a widespread belief that in animals with sex chromosomes, males and females are fundamentally different because of their different genetic constitutions. There is evidence that the presence of heterogametic sex chromosomes (mammals) and their dosage (birds) are represented in the brain. *Sry* and *Dmrt* and other genes (e.g., *TRP2*) are likely involved in sensory decoding of sexual stimuli critical for mate choice, but the extent to which they are important in an evolutionary sense is debatable.

Species having heritable sex chromosomes present two hurdles that must be recognized in any study of sex "differences" of the brain. Not only do the sexes differ in an elemental gene but also males and females develop and age in entirely different endocrine milieus and, as a consequence, have different life-history experiences.

It is accepted that the major sex differences in the brain are shaped by the sex hormones, and that in species having sex chromosomes, the structural and functional differences observed in the brain are related to behaviors that are different in their expression. It is also accepted that these major sex differences in the brain are shaped by the sex hormones. However, to say that these sex differences in behaviors are caused by sexual dimorphism in the brain and its chemistry is too much of an extrapolation.

This brings us back to the point that there is a basic conservation in the mechanisms that underlie sex determination and sexual differentiation. This is also reflected in the brain, where there is a conserved neural network of nuclei that regulate sexual behaviors.

Early in development (the "when" and "how" varies among species), genes and hormones interact to organize the functional neuroanatomy such that later, as adults, males and females will exhibit complementary behaviors necessary for successful

reproduction. It should be obvious that sexual behavior is the result of multiple brain nuclei acting in concert in addition to the external stimuli and the hormonal history of the participating individuals. Sarah Newman (1999) proposed an attractive formulation of this body of work as a Social Behavior Network. By shifting the focus of study from single nuclei (nodes) isolated from integrated networks, Newman predicted this would lead to new insights into brain–behavior relationships. Important for this discussion, this hypothetical model only focused on sex differences and did not consider its application to possible interactions within the network when animals display heterotypical sexual behaviors. Using her work as a platform, I have applied it to empirically derived data in several animal systems to show how both heterotypical and homotypical behaviors result from network activity and are ultimately determined by the reciprocally inhibitory interaction of two root nodes (POA and VMN).

The Common Network Model emphasizes how hormones act on a single neural network and result in two mutually exclusive outputs. This model reflects the increasing appreciation of how brain nuclei are networked by neurochemical and molecular interactions and how these neural systems are vital (in an evolutionary sense), particularly when the brain must alternate between mutually exclusive behavioral outputs. The Common Network Model suggests that sex-typical behavioral phenotypes are mirrored by specific neurotransmitter and molecular phenotypes in two functionally associated nuclei (as ''root nodes'' of a larger network of nuclei).

There is ample evidence that the POA and VMN are crucially involved in the control of male-typical and female-typical sex behaviors, respectively (Crews and Silver 1985; Morgentaler and Crews 1979; Wheeler and Crews 1978). What is less a part of the current orthodoxy is the possibility that the two centers operate simultaneously, but in mutually antagonistic ways. However, the involvement of each brain area in behaviors typical of the ''other sex'' is not lacking and there is considerable evidence of a reciprocal inhibition between the POA and the VMN. For example, implantation of testosterone into the VMN restores sexual motivation, but not copulatory behavior itself, in castrated male rats; administration of either AR antagonists or making lesions within the dorsomedial VMN impairs sexual motivation and copulatory behavior in male rats. Further, multiple lines of evidence indicate that POA and VMN are functionally related in an opposing fashion; the POA projects to, and receives projections from, the VMN. The POA and VMN also have opposing roles in the control of autonomic function and female reproductive behavior with the ''net effect of the outputs from the preoptic region is to reduce feminine-typical behavior and to increase male-typical behavior'' (Pfaff et al. 1994, 188). Neuronal activity increases in the VMN during sexual receptivity in the female rat and is reduced when there is increased activity in the POA. Effects of excitatory and inhibitory amino-acid neurotransmitters are opposite in the VMN and POA. Specifically, in the VMN, GABA is facilitatory to lordosis, whereas NMDA is inhibitory, and in the POA, GABA inhibits lordosis and NMDA facilitates it in hormone-primed females.

Nature's experiments and the common neural network underlying sexual behavior

Vertebrates lacking sex chromosomes, that is, those species having environmental sex determination (e.g., sequential and serial hermaphroditic species and species having temperature-dependent sex determination or TSD) are proof of the validity of the concept of a Common Neural Network underlying sexual behavior (Crews 1993). Indeed, only with such experiments of nature, it is possible to understand how the brain mechanisms that underlie the complex behaviors involved in sexuality interact.

Hermaphroditic species are another exception. Simultaneous hermaphrodites are species in which each individual produces both sperm and eggs, but curiously, never at the same time. When breeding, one individual assumes the ''male'' role and sheds sperm and its partner takes on the ''female'' role and sheds eggs. In the next spawning even the roles are completely reversed. Clearly, in both instances, the brain of each individual is bisexual in its organization and performance. In the only experiment that has been carried out to date on such animals, Leo Demski (1987) demonstrated that stimulating one area of the brain of the sea bass (a simultaneous hermaphrodite) caused release of sperm while stimulation in another area resulted in release of eggs. In sequentially hermaphroditic species, the individual begin as one sex, but as an adult it transform into the opposite sex if the appropriate social events present themselves. In sequentially hermaphroditic species, the individual begins as one sex, but transform into the opposite sex if the appropriate social events present themselves (Godwin 2009). The social stimuli are transduced, in turn, into signals that modulate neuropeptides and neurotransmitters, pituitary gonadotropins, and steroid

hormones. Thus, organisms that lack chromosomal sex determination and develop and age in entirely different endocrine milieus can, as adults, change sex depending upon environmental cues and can behave in a gamete-appropriate manner, further suggesting that a common neural network underlies such changes.

Many reptiles exhibit TSD in which incubation temperature during a narrow window during development causes the embryo to develop testes or ovaries. In most TSD species, extreme temperatures produce only one sex while intermediate temperatures produce a mixture of males and females. For example, in the leopard gecko, the temperature experienced during the mid-trimester of development shapes the morphological, physiological, behavioral, and neural phenotype of the individual, thereby accounting for the variation observed within a sex as well as between the sexes (Flores et al. 1994; Tousignant and Crews 1995; Sakata and Crews 2004). In the leopard gecko incubation at 32.5°C produces a male-biased sex ratio. If we look at the social and sexual behavior of males and females from this incubation temperature and analyze the metabolic activity in the Social Behavior Network, there is little difference other than in the POA and VMN nodes.

The ultimate proof of a common neural network with reciprocal inhibition between the POA and the VMN nodes comes from studies of the all-female lizard, *Cnemidophorus uniparens*, a species in which all individuals are morphologically female and reproduce by obligate cloning. The animal model system enables deconstructing the above-mentioned confounding properties of specificities of the genotype, sex hormones, and developmental processes inherent in conventional mammalian model systems.

The maternal ancestor of *C. uniparens* is *Cnemidophorus inornatus*, a typical gonochoristic species with males and females that look and behave in a sexually dimorphic manner; that is, males mount whereas females do not, and mounting in males is dependent on androgens acting on the POA. Female *C. inornatus* only exhibit receptivity when the follicles are large and estrogen levels are high. This estrogen acts on the VMN.

Remarkably, during the reproductive period, *C. uniparens* engage in behaviors physically identical to the mating behaviors of both males and females of their sexual congeners (albeit with the exception of intromission and insemination). I have termed such behaviors ''pseudosexual'' behavior and the union of females, ''pseudocopulation.'' When pairs of *C. uniparens* display these pseudosexual behaviors, they are always complementary, with a tight relationship between the behavior displayed and the ovarian state of the animal. Thus, the individual mounting and displaying other male-like copulatory behaviors is generally postovulatory and has elevated levels of progesterone, while the receptive individual is preovulatory, with high levels of estrogen. Any given individual will thus display either behavior but at different points in the ovarian cycle.

Examination of the POA and the VMN of *C. uniparens* indicate that these nuclei do not change in size or number of cells during these different behavioral phases, nor do these parameters respond to exogenous hormone treatment (Wade et al. 1993). However, they do differ in metabolic activity in predictable ways. During the male-like pseudocopulatory behavior, metabolic activity, as measured by 2-deoxyglucose uptake (2DG), is high in the POA but below baseline in the VMN (indicating suppression of activity); during female-like pseudoreceptive behavior, the opposite occurs, with 2DG suppressed in the POA and enhanced in the VMN (Rand and Crews 1994). Intracranial implantation of androgen (and progesterone) into the POA both of male *C. inornatus* as well as *C. uniparens* elicits mounting behavior, but fails to elicit either mounting or receptive behavior when placed in the VMN (Mayo and Crews 1987; Crews et al. 1996). On the other hand, implantation of estrogen into the VMN elicits receptive behavior in females of both species (Wade and Crews 1991).

Both the POA and the VMN are dimorphic in size, with the POA being larger and the VMN smaller, in sexually active male *C. inornatus* than in conspecific females or in the descendant parthenogenetic *C. uniparens*. Castration of male *C. inornatus* causes the POA to decrease and the VMN to increase to the size characteristic of females; androgen replacement restores the difference between the sexes. The overall change in nuclear volume is paralleled by the size of the soma of individual neurons in both areas, suggesting that the size of these neurons reflects their functional activity. However, once again, this difference between the sexes appears to be a correlate, rather than a necessary substrate of the expression of male-typical behavior, since *C. uniparens* exhibiting male-like pseudocopulatory behavior (either as intact postovulatory or ovariectomized, testosterone-treated animals) do not show an increase in region or somal area of the POA, and differences in the POA observed between the parthenogens displaying male-typical and female-typical behaviors have been subtle ones at the levels of gene expression and neurotransmitter levels. The parthenogenetic whiptails thus oblige us, while continuing to accept

the existence of differences between the sexes in brain morphology, to consider the possibility that such developmentally long-term differences in morphology are less important in determining behavior than is the short-term activity of the brain, which is determined by external stimuli as well as by immediate physiological state. However, in these animals, as in others studied, this sexual phenotype-determining ''activity'' can be studied profitably by focusing on the interaction between the POA and the VMN.

Conclusion

In his writings about the nature of the neuroendocrine control of sexual behavior in vertebrates, Frank Beach (1976, 1979) delineated four essential points: (i) both male and female individuals are capable of displaying the sexual behaviors of the opposite sex, (ii) the brain must have the neural circuitry sufficient to support these opposite behaviors, although (iii) each sex is predisposed to exhibit the behavior consistent with its sex, and (iv) the stimulus animal is essential in eliciting the complementary behavior. If one accepts these conclusions, it follows that male-typical and female-typical copulatory behaviors are mediated by structures in the brain that are present and (at least latently) fully functional in both sexes, i.e., not sexually dimorphic. Experimentally, one is then forced to examine how males and females can behave both in a sex-appropriate manner and in the manner appropriate to the opposite sex. It also begs the question of what the functions of the observed sexual dimorphisms in brain structure are if they do not mediate sex-typical copulatory behavior.

Taken together, this evidence suggests that at all levels of biological organization (genetic, morphological, physiological, and behavioral), modern vertebrates are fundamentally bisexual in nature and that the binary outcomes we observed are the product of modulation of a universal network. In amniote vertebrates, the genes involved in differentiation of the gonads are highly conserved, although the pattern of their expression in mammals and turtles vary from that of birds and crocodilians. At the level of the brain, the mechanisms mediating both male and female copulatory behavior are under tonic inhibition from a range of sources, and that activation constitutes relief from some of these inhibitory inputs. Major sexual dimorphisms in structure of the brain are seen mostly as sex-specific sources of additional inhibition so that, for example, the large POA typical of males is responsible not for mediating male-typical copulatory behavior, but for allowing a more sophisticated pattern of inhibition. In other words, ''differences'' between the sexes should not be seen as real differences, but as male-typical and female-typical features that enable males and females to do better the things they do, rather than enabling them to do something that the other sex cannot do. Either sex, the evidence shows, is intrinsically capable of doing either thing.

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