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Reproductive dysgenesis in wildlife: a comparative view

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Summary

Abnormal reproductive development in males has been linked to environmental contaminant exposure in a wide variety of vertebrates. These include humans, rodent models, and a large number of comparative wildlife species. In human males, abnormal reproductive development can manifest as a suite of symptoms, described collectively as testicular dysgenesis syndrome (TDS). TDS is also described as demasculinization or feminization of the male phenotype. The suite includes cryptorchidism, in situ germ cell carcinoma of the testis and overt testicular cancer, reduced semen quality, and hypospadias. In this paper, we review examples of TDS among comparative species. Wildlife exposed to environmental contaminants are susceptible to some of the same developmental abnormalities and subsequent symptoms as those seen in human males with TDS. There are additional end points, which are also discussed. In some cases, the symptoms are more severe than those normally seen in humans with TDS (i.e. oocytes developing within the testis) because some non-mammalian species exhibit greater innate reproductive plasticity, and are thus more easily feminized. Based on our review, we present an approach regarding the ontogeny of TDS. Namely, we suggest that male susceptibility to the androgynizing influences of environmental contaminants originates in the sexually undifferentiated embryo, which, in almost all species, including humans, consists of bipotential reproductive tissues. These tissues can develop as either male or female and their ultimate direction depends on the environment in which they develop.

Introduction

Skakkebaek *et al.* (2001) published a hypothesis suggesting that a suite of male reproductive abnormalities, observed with increasing frequency over recent decades, are in fact related components of a condition termed 'testicular dysgenesis syndrome' (TDS). Symptoms of human TDS include cryptorchidism (undescended testes), in situ germ cell carcinoma of the testis and overt testicular cancer, reduced semen quality, and hypospadias (incomplete fusion of the urethral folds that form the penis). Additional signs include presence of microliths in the testes, Sertoli-cell-only seminiferous tubules (without spermatogenic activity), or immature tubules with undifferentiated Sertoli cells (Damgaard *et al.*, 2002; Skakkebaek *et al.*, 2003). These symptoms can occur separately, or as a suite of characters and their severity can vary.

Causal mechanisms of TDS include genetic aberrations, such as deletions in the doublesex and mab3 related transcript (*DMRT*) gene cluster (Ottolenghi *et al.*, 2000; Stumm *et al.*, 2000), sex-chromosome mosaicism (Chemes *et al.*, 2003), chromosomal rearrangements affecting sex-determining genes sex determining region of the Y-chromosome (SRY) and *SOX9* (SRY-box containing gene 9) (Flejter *et al.*, 1998; Kadandale *et al.*, 2000), and X-chromosome duplication (Flejter *et al.*, 1998). However, Skakkebaek *et al.* (2001) noted that the majority of boys born with TDS lack the expected genetic defects. This observation suggests that environmental factors are possibly involved as causal agents. In fact, the number of human TDS cases has risen sharply over the past 50 years, concomitant with swift growth of the chemical industry and associated release of thousands of anthropogenic chemicals into the environment (Aitken *et al.*, 2004; Asklund *et al.*, 2004).

A growing number of animal studies show that environmental endocrine disrupting chemicals have the potential to derail reproductive development (Tyler *et al.*, 1998; Crain *et al.*, 2000; Boisen *et al.*, 2001). Wildlife studies are particularly informative because they sample

genetically diverse (usually) wild populations that live in direct contact with complex mixtures of anthropogenic environmental contaminants (pesticides, detergents, surfactants, fertilizers, petroleum derivatives, pharmaceuticals, hormones). As with the human literature, there has been a tendency to view various reproductive abnormalities in wildlife individually, rather than as components of a common syndrome.

Here, we review the literature for evidence of TDS in wildlife (Fig. 1) and discuss possible mechanisms by which symptoms of TDS may arise. Our review supports the hypothesis that TDS results from demasculinization or feminization of the male reproductive system. Studies from wildlife suggest that males are subject to androgynization because males and females share similar ontogenetic origins.

Definitions

In this paper, we will use the term *demasculinized* to describe male tissues that are abnormally developed, underdeveloped, or sub-functional. Hypospadias is an example of a demasculinized penis. *Feminized* refers to the unusual presence of female cells or tissues in a male. Ovotestes or gynecomastia are examples of feminization. The term *androgynized* is a more general term that describes a state of indeterminate sexual development or the presence of characteristics that are typically attributed to the opposite sex. We use *androgynization* as a more inclusive term when referring to both demasculinization and feminization.

Male testicular development and the origins of testicular dysgenesis syndrome

The symptoms of TDS are developmentally related. It is probable that they originate during embryogenesis and are dependent on whether or not the testis develops correctly (Boisen *et al.*, 2001). Proper male development in most vertebrates entails the same general sequence of events. Early in embryogenesis, paired indifferent gonads form at the genital ridge. The ridge epithelium proliferates to form the medullary and sex cords. Primordial germ cells migrate to the genital ridge from extragonadal regions near the hindgut. In mammals, testicular development occurs in response to a cascade of events initiated by *sry* gene expression in pre-Sertoli cells (Albrecht & Eicher, 2001). Sertoli cell differentiation begins in the gonadal medulla, along with progression of the medullary and sex cords to form the rete testis and seminiferous tubules, respectively. The developing Sertoli cells surround the pro-spermatogonial germ cells (gonocytes) within the seminiferous tubules (De Rooij, 1998). Outside the tubules, Leydig cells, the main androgen source in males, develop in the testicular stroma. In most vertebrates, Sertoli cells proliferate during both the fetal/neonatal period, and the peripubertal period, when they reach final maturity (Sharpe *et al.*, 2003).

In individuals with TDS, one or more of these general pathways is disrupted such that incomplete masculinization (or feminization) occurs (Klonisch *et al.*, 2004). Possible mechanisms include unsynchronized or delayed

Chondrichthyes 	No published data to date
Osteichthyes 	Sex reversal; skewed sex ratios; intersex gonads (ovotestis) and reproductive ducts; shortened gonopodium; decreased semen quality; abnormal steroidogenesis
Lissamphibia 	Sex reversal; skewed sex ratios; hermaphrodites; intersex gonads (ovotestis); disrupted spermatogenesis; altered testicular tubule morphology & gonadal development
Reptilia 	Sex reversal; skewed sex ratios; abnormal penis development; hypospadias; disrupted steroidogenesis and gene expression patterns; decreased precloacal length
Aves 	Abnormal gonadal differentiation; altered testicular tubule morphology; reduced testis size; decreased size of cloacal foam gland; decreased sperm quality
Mammalia 	Abnormal genital/gonadal development; disrupted steroidogenesis and gene expression; decreased anogenital distance; cryptorchidism; hypospadias; decreased semen quality; microlithiasis; altered testicular tubule morphology

Figure 1 Testicular dysgenesis and related conditions observed in comparative vertebrate groups.

timing of necessary signalling patterns or non-attainment of some developmental threshold that allows further masculinization (Palmer & Burgoyne, 1991; Klonisch *et al.*, 2004). For example, Sertoli cells are the first cells to differentiate in the indifferent fetal gonad. Their presence is required for proper testis formation and function (reviewed by Sharpe *et al.*, 2003). In male mammals, *sry* gene expression initiates signalling systems that work in an autocrine and paracrine fashion to recruit Sertoli cells (Brennan & Capel, 2004). The number of Sertoli cells appears to be directly related to the *sry* mRNA titre in the developing gonad (Nagamine *et al.*, 1999). Furthermore, it is thought that a threshold number of *sry*-expressing pre-Sertoli cells are needed to allow full testicular masculinization (Palmer & Burgoyne, 1991). Once formed, Sertoli cells facilitate formation of seminiferous cords and Leydig cells, induce Müllerian duct regression, and, following sexual maturation, support spermatogenesis (Sharpe *et al.*, 2003). In adulthood, the capacity for sperm production is directly related to Sertoli cell number as each Sertoli cell can support only a limited number of sperm cells (Sharpe *et al.*, 2003). If Sertoli cell maturation is delayed, then these other steps in testicular development are also delayed (Defranca *et al.*, 1995). However, as with most developmental processes, timing is critical. For normal testis development, *sry* must be expressed during the appropriate window of competence, which in mice occurs when the embryo has 13–18 tail somites (Nagamine *et al.*, 1999). Taken together, these observations suggest that if *sry* expression, production of downstream signals, and/or Sertoli cell number are inadequate, a demasculinized testis or ovary will result. This hypothesis was confirmed in chimeric mice with gonads composed of fewer than 30% XY cells. In these mice, the gonads developed as ovaries (Palmer & Burgoyne, 1991).

Comparative examples of testicular dysgenesis syndrome

Cryptorchidism

As a symptom of TDS, cryptorchidism, by definition, can only affect some mammalian wildlife species. In fishes, amphibians, reptiles and birds, the testes are maintained within the body wall and do not exhibit testicular descent. Further, some mammals (e.g. elephants, marine mammals) do not develop a scrotum and the testes are either held in an abdominal or inguinal location. Among wild mammals where cryptorchidism is possible, a few documented cases are known. These include the Florida panther (*Felis concolor coryi*) and black-tailed deer (*Odocoileus hemionus sitkensis*) of Kodiak Island, Alaska.

Between 1972 and 2001, the incidence of cryptorchidism (usually unilateral) among Florida panthers rose significantly, with a current occurrence rate of 54%, and delayed testicular descent observed in 23% of the juveniles studied (Buergelt *et al.*, 2002; Mansfield & Land, 2002). Mansfield & Land (2002) noted that testes were most often retained in the inguinal canal. Coincident with cryptorchidism, Florida panthers also exhibit reduced testicular volume, low sperm motility, density and semen volume, and higher numbers of morphologically abnormal sperm (flaws in the acrosome and mitochondrial sheaths) compared with other American *Felis concolor* populations, of which 3.9% are cryptorchid (Barone *et al.*, 1994). Due to its small size, the Florida panther population is reported to be severely inbred, and this lack of genetic diversity has been suggested to account for the high, possibly heritable, rate of cryptorchidism (O'Brien *et al.*, 1990). However, an analysis by Facemire *et al.* (1995) suggested that genetic composition does not fully explain the observed reproductive abnormalities. The number of polymorphic loci among Florida panthers is similar to that of several Asian and African populations of large felids (lions, cheetahs, leopards), and either similar or lower than some other populations of *F. concolor* (Miththapala *et al.*, 1991; Roelke *et al.*, 1993; Facemire *et al.*, 1995). Facemire *et al.* (1995) concluded that the cryptorchidism reported in the Florida panther could be the result of exposure to environmental contaminants known to disrupt endocrine function (Facemire *et al.*, 1995). These include elevated concentrations of p,p′-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene), mercury, and polychlorinated biphenyls (PCBs), found in raccoon prey, panther adipose tissue and environmental samples in south Florida (Facemire *et al.*, 1995).

Unilateral and bilateral cryptorchidism, along with many of the other symptoms of TDS, have also been reported in Alaskan black-tailed deer (Bubenik *et al.*, 2001). Cryptorchid testes obtained from black-tailed deer contained malformed or degenerated seminiferous tubules containing Sertoli cells but lacking spermatogenic activity (Bubenik & Jacobson, 2002). In bucks with unilateral cryptorchidism, the normal testis exhibited normal spermatogenesis. In addition, the seminiferous tubules contained concentric lamellae made of calcium salts, similar to microlithiasis, a condition observed in men with TDS (Skakkebaek, 2004).

Testicular cancer

Testicular cancer originating during development arises from carcinoma in situ (CIS) cells. These are germ cells that did not properly differentiate from gonocytes (transient cells derived from primordial germ cells) into spermatogonia (Skakkebaek *et al.*, 1998). This could occur if testis or germ cell development is delayed or arrested (Rajpert-De Meyts *et al.*, 1998). CIS cells appear to have stem cell potential, and, in humans, their proliferation is

particularly inducible postnatally and during puberty (Skakkebaek *et al.*, 1998). In fact, a recent study investigated expression patterns of Octamer-binding transcription factor (OCT)-3/4 (POU5F1), a transcription factor that supports the pluripotentency of embryonic stem cells (Rajpert-De Meyts *et al.*, 2004). In males, expression of OCT-3/4 was greatest during gonadal development, and then gradually decreased through postnatal age 3–4 months, when gonocytes normally complete differentiation. In patients exhibiting testicular dysgenesis or intersex, OCT-3/4 was expressed in gonocytes and CIS cells in older individuals, supporting the hypothesis that these cells remain totipotent.

Detection of testicular cancer in wildlife species is logistically difficult and, to the best of our knowledge, no comparative studies have detected testicular cancer arising from CIS cells. However, in frogs (*Rana esculenta*), primary spermatogonial proliferation can be induced using oestradiol (D'Istria *et al.*, 2003). This interesting observation suggests that frog spermatogonia retain some totipotency and that germ cell-related testicular cancer is an end point worth including in endocrine disruption studies focused on amphibians.

Reduced semen quality

Of the four symptoms arising from developmental abnormalities associated with TDS (hypospadias, cryptorchidism, testicular cancer and reduced semen quality), reduced semen quality is most often reported in wildlife species. Semen quality is a general term that refers to a number of different measurements of male fertility. These include sperm counts/density, sperm motility, sperm morphology, volume of ejaculate (called milt in fish) and sperm viability, which can refer to sperm cells being alive or dead, or alternatively, to the sperm's ability to fertilize an egg and produce a normal embryo. This last approach can be extended by evaluating the offspring produced by fathers with a history of exposure (Aitken *et al.*, 2004). In addition, semen quality, which is typically described for ejaculated sperm, depends on the condition of the reproductive ducts that deliver sperm from the testes to the outside of the body. For this reason, we have included descriptions of altered duct formation in this section on semen quality.

Because semen quality is defined by so many end points, there are numerous developmental causes of low quality in association with disrupted testicular development. For example, low sperm count, which is just one measure of reduced semen quality, can result from a reduction in the number of primordial germ cells, increases in germ cell apoptosis, altered Sertoli cell function, physical occlusion of the spermatic ducts, reductions in surface area of testicular tubules, and/or altered hormonal regulation of spermatogenesis through changes in hormone synthesis, degradation or sensitivity (i.e. receptor expression). Below, we describe examples that illustrate these hypotheses and that show the connection between contaminant exposure and reduced semen quality in comparative vertebrate species.

As noted above, Florida panthers, in association with exposure to elevated concentrations of p,p′-DDE, mercury and PCBs, exhibit reduced sperm density, motility and semen volume, and higher numbers of morphologically abnormal sperm compared with other panther populations (Barone *et al.*, 1994; Facemire *et al.*, 1995). Similarly, reduced spermatogenesis, low sperm counts, poor sperm motility and/or low milt volume have been observed in wild fishes captured from contaminated lakes and rivers. These include mosquitofish (Toft *et al.*, 2003), English flounder (Lye *et al.*, 1998) and English roach (Jobling *et al.*, 2002a,b;). The roach, which were collected from waterways polluted with treated sewage effluent, also exhibited reduced ability to fertilize eggs and produce viable offspring (Jobling *et al.*, 2002b). The males in these populations exhibited intersex, an abnormal condition in which a male's testes are characterized by a female-like ovarian cavity with oocytes and/or ovarian tissue embedded within the testicular tissue (Nolan *et al.*, 2001). The ovarian cavity is distinguished by its characteristic ciliated epithelial cell lining. Intersex individuals can lack fully formed sperm ducts (vas deferens), can possess oviducts or can possess both male and female reproductive ducts. Any sperm duct(s) that are present can be blind-ended (terminating before the opening of the genital pore), blocked or reduced, or they can form part of the ovarian cavity wall (Nolan *et al.*, 2001; Jobling *et al.*, 2002a). Intersex gonads, with primary oocytes scattered within testicular tissue, were also recently observed in South African sharptooth catfish (Barnhoorn *et al.*, 2004). In that study, water, sediment and serum samples from the fish all tested positive for p-nonylphenol, a xenooestrogen commonly found in treated sewage effluent. Other oestrogenic compounds found in sewage effluent include oestradiol-17β, oestrone, ethynyl-oestradiol (from birth control pills), and a number of alkyl phenolic chemicals, including 4-octylphenol, 4-nonylphenol, and nonylphenol mono- and di-ethoxylates (Rodgers-Gray *et al.*, 2001).

The causal link between contaminant exposure during development and reduced semen quality is supported by experimental studies that test the effects of exposure under controlled conditions. For example, feminized reproductive duct and ovarian cavity formation were induced experimentally in juvenile male roach treated with graded concentrations of sewage effluent. Oviduct development in place of the vas deferens, intersex, inhibited spermatogenesis and a reduction in the number of

primordial germ cells per gonadal section were reported in male carp exposed during sexual differentiation to 4-tert-pentylphenol or 17β-oestradiol (Gimeno *et al.*, 1998). In other studies, developing male Japanese medaka, exposed to octylphenol (oestrogen agonist) and oestradiol-17β, exhibited reduced fertilization success and increased incidence of intersex (Gray *et al.*, 1999; Knorr & Braunbeck, 2002). Hatching success was decreased in marine sheepshead minnow when the parents were exposed to 17-α-ethynyloestradiol during sexual maturation (Zillioux *et al.*, 2001). In this study, some exposed males also exhibited testicular fibrosis and/or testes that contained pre-vitellogenic (yolk protein) ovarian follicles, similar to the intersex roach described above. Similarly, the number of eyed embryos produced by male rainbow trout was reduced by 50% following exposure to 17-α-ethynyloestradiol during sexual maturation (Schultz *et al.*, 2003). In the exposed trout, plasma concentrations of 17α, 20β-dihydroxyprogesterone (17,20-DHP) were roughly twice the level of the controls, while 11-keto-testosterone (11-KT) concentrations were significantly reduced. In fishes, 17,20-DHP stimulates maturation of both oocytes and spermatozoa (reviewed by Tsubaki *et al.*, 1998), and 11-KT induces meiosis and the process of spermiogenesis (Miura & Miura, 2003). Finally, zebrafish, exposed during development to tributyltin (an aromatase inhibitor found in anti-fouling paints used on marine ship hulls) at very low concentrations (0.1–1 ng/L), exhibited a male-biased population with a high incidence of sperm lacking flagella and reduced sperm motility (McAllister & Kime, 2003). This finding is in agreement with impaired spermatogenesis found in aromatase knock out mice. In these adult male mice, the lack of aromatase results in grossly dysmorphic seminiferous tubules, the presence of degenerated round spermatids, lack of elongated spermatids and a reduction of motility (Murata *et al.*, 2002).

As in the literature on fish, several cases of disrupted sperm production and intersex (also described as ovotestes) have been observed in male amphibians. The testes of African clawed frogs exposed to PCBs during sexual differentiation were interspersed with oocytes (Qin *et al.*, 2003). They also presented with looser structure and fewer seminiferous tubes, spermatogonia and spermatozoa than controls. Similarly, intersex and altered testicular tubule morphology were observed in leopard frogs and wood frogs exposed as tadpoles to oestradiol, ethynyloestradiol or nonylphenol, in addition to a number of anti-oestrogens (MacKenzie *et al.*, 2003). Methoxychlor, an organochlorine pesticide, caused a skewed sex ratio (female biased) and reductions in testis weight and sperm cell counts in South African clawed frogs exposed during development (Fort *et al.*, 2004). Likewise, the herbicide atrazine, at very low doses of 0.1 p.p.b., caused retarded gonadal development and testicular oogenesis (intersex) in leopard frogs (Hayes *et al.*, 2003). Hayes *et al.* (2003) observed similar symptoms in frogs collected from atrazine-contaminated sites across the United States.

Birds exposed to environmental contaminants also exhibit symptoms of testicular dysgenesis. For example, the surface area of testicular tubules was reduced in leghorn chicks exposed to bisphenol A (oestrogenic component found in plastics) (Furuya *et al.*, 2003). In another study, multiple treatment levels of Aroclor 1254 (a PCB congener) injected into fertilized chicken eggs before incubation reduced testis size and seminiferous tubule diameter and retarded germ cell differentiation in hatchling chickens (Fang *et al.*, 2001). The highest dosages of PCBs resulted in tubule degeneration or absence. Treatment of fertilized quail eggs with diethylstilbestrol (DES, a synthetic oestrogen) decreased epididymis development and resulted in fewer, thinner seminiferous tubules in 100-day-old quail (Yoshimura & Kawai, 2002). Furthermore, the quantity of sperm attached to the epididymis epithelium was greatly reduced in the highest DES dosage group.

Hypospadias

In male mammals, the penis and scrotum, in response to androgens, develop from external genital primordia, which, like the gonads, are bipotential prior to sexual differentiation (Cohn, 2004). The urethral folds, which form the labia minor in females, fuse in a distal direction to enclose the urethra and create the penile shaft. The genital swelling, which forms the labia majora in females, fuses to form the scrotum; and the genital tubercle, which becomes the clitoris in females, expands to form the glans penis. Hypospadias results when fusion of the urethral folds is incomplete and the opening of the urethra locates somewhere along the ventral midline of the penis.

Reptiles, Chondrichthyans (sharks and their relatives), mammals, and some birds and fish all exhibit copulatory structures, which are maintained inside or outside the body cavity. Sharks, for example, possess claspers, paired intromittant organs formed from modified pelvic fins, while viviparous teleost fishes modify the anal fin to form a gonopodium (Helfman *et al.*, 1997). In those fish studied to date, gonopodium development is stimulated by androgen exposure, either endogenous or exogenous (Ogino *et al.*, 2004). Like mammals, the penile structures of reptiles and birds are derived from an embryonic genital tubercle (phallic anlage), a commonality that suggests these structures are homologous across these taxonomic groups (Raynaud & Pieau, 1985; Uchiyama & Mizuno, 1989). However, the condition of hypospadias, as defined above, has not been reported in any wildlife species to

date. In some cases, the condition may not apply. The urethra of the alligator penis, for example, does not normally fuse completely to the tip of the penis. It is instead characterized by a partially fused (proximately to the body wall) ventral groove. However, we have observed alligator phalli where the tip of the phallus presents as two completely separate halves (L. J. Guillette & T. M. Edwards, unpublished data). This could be considered extreme hypospadias.

Among wildlife species, a more common observation is that of reduced overall penis length. Relative to males from a reference alligator population, reduced penis size (average of 24% decrease) has been observed among juvenile male alligators collected from a lake contaminated with organochlorine pesticides and dichlorodiphenyltrichloroethane (DDT) derivatives (Guillette *et al.*, 1996). Similar observations have been reported for other populations of alligators living in lakes contaminated with agricultural run-off (Guillette *et al.*, 1999; Gunderson *et al.*, 2004). Similarly, in juvenile mink captured from the Columbia and Fraser Rivers in the north-western USA, the baculum (penile bone) length was negatively correlated with total PCB concentration (Harding *et al.*, 1999). Finally, shortened gonopodia (modified anal fin with dorsal groove; used in copulation) were observed among male mosquitofish collected downstream from a sewage treatment plant in Australia (Batty & Lim, 1999) and in a pesticide-contaminated lake (Toft *et al.*, 2003).

Additional end points associated with reproductive dysgenesis

While some components of TDS are difficult to analyse in wildlife species because they are hard to detect (testicular cancer) or often do not apply (cryptorchidism), there are also additional end points that can inform our overall understanding of reproductive dysgenesis. A sampling of those is presented here.

Anogenital distance and pre-cloacal length

Anogenital distance (AGD) is a sexually dimorphic feature that has been studied in rodents (Gray *et al.*, 2001) and in humans (Salazar-Martinez *et al.*, 2004; Swan *et al.*, 2005). In general, males display a greater AGD than females. In utero exposure of developing males to oestrogens or anti-androgens has been shown to feminize (reduce) AGD in male rodents. Tested chemicals include vinclozolin (Wolf *et al.*, 2000), butyl benzyl phthalate (Tyl *et al.*, 2004), DES (Gupta, 2000), methoxychlor, flutamide (McIntyre *et al.*, 2001) and oestradiol-17β (Amstislavsky *et al.*, 2004). Turtles possess a similar sexually dimorphic feature called the pre-cloacal length, the distance from the posterior lobe of the plastron (bottom shell) to the cloaca, which is longer in male than in female turtles. An elongated pre-cloacal length is functionally important to male turtles, allowing the tail to curl under the female's shell during mounting to facilitate intromission. Field observations indicate the ability of environmental contaminants to alter the development of the pre-cloacal distance in turtles. For example, male snapping turtles (*Chelydra serpentina*) from areas of the Great Lakes contaminated with oestrogenic and anti-androgenic compounds show a decrease in pre-cloacal distance compared with turtles from less polluted sites (de Solla *et al.*, 1998, 2002), indicative of feminization. This observation, like that of copulatory length and structure in other species, suggests that external genital geometry can be used as valuable, non-invasive investigative tools with wildlife populations.

The prostate–foam gland connection

Exposure of the developing mammalian prostate gland to oestrogens can result in impaired growth and differentiation during development and later diminished androgen activation and secretory function (Vom Saal *et al.*, 1997, 1998; Vom Saal & Timms, 1999; Prins *et al.*, 2001; Huang *et al.*, 2004). In mammals, these long-term effects have been called developmental oestrogenization or oestrogen imprinting of the prostate (Santti *et al.*, 1994). According to Santti *et al.*, developmental exposure to oestrogenic substances during this critical period upregulates the expression level of stromal oestrogen receptor alpha, progesterone receptor and retinoid receptor expression in the developing gland. Concomitantly, androgen receptor expression is downregulated. This changes a usually androgen-dominated developmental process to one regulated by alternate steroids, most notably oestrogens. Such a change leads to disruption of the coordinated expression of critical developmental genes and permanent differentiation defects of the prostate.

Analogous to the mammalian prostate gland, the cloacal foam gland of Japanese quail (*Coturnix japonica*) is an androgen-dependent, sexually dimorphic structure located at the dorsal cloaca (Balthazart & Schumacher, 1984). During copulation, foam produced by the gland is transferred to the female along with sperm, enhancing fertilization success (Mohan *et al.*, 2002; Marin & Satterlee, 2004). Cloacal glands exhibit seasonal cyclicity through regression and recrudescence with breeding seasons. Elevated androgens, either stimulated by long days or applied exogenously (Nagra *et al.*, 1959), cause seasonally regressed cloacal glands to return to active size and regain foam producing competence (Seiwert & Adkins-Regan, 1998). Gland size normally correlates with testicular weight (Siopes & Wilson, 1975), is dramatically reduced with castration (Mohan *et al.*, 2002) and is rescued with testosterone implants (Liang *et al.*, 2004). Experimentally,

the ability to impede seasonal gland development has been demonstrated through daily intramuscular injections with 10 mg of the anti-androgen flutamide (Liang *et al.*, 2004). Analogously, prostate cancer is treated with flutamide through inhibiting androgen receptors (Culig *et al.*, 2004).

In addition to seasonal inhibition of gland activation, development of the cloacal gland can be retarded organizationally during embryogenesis. In ovo treatment with oestrogenic compounds such as oestradiol (Adkins, 1979), DES (57 ng/egg) (Halldin *et al.*, 1999; Yoshimura & Kawai, 2002) and o,p′-DDT (2 mg/egg) (Halldin *et al.*, 2003) has been shown to reduce/demasculinize the size of the cloacal gland in its adult, active state. This change in glandular morphology suggests a parallel aetiology with developmental oestrogenization of prostate glands. Research has not addressed if in ovo oestrogenic exposure reduces foam production during reproduction; however, this seems parsimonious with the reduction of gland size. Therefore, reduction of the cloacal gland and oestrogenization of the prostate could both be related to reductions in reproductive success.

Feminization and demasculinization – insights from wildlife

Throughout this overview, we have examined cases of reproductive dysgenesis that might also be described as demasculinization or feminization of males. Similarly, androgynization of females has also been documented, although we have not addressed it here (for examples, see Arnold & Schlinger, 1993; Parks *et al.*, 2001; Wolf *et al.*, 2002). The fact that males and females are subject to androgynization during development by hormonally active, exogenous agents is easy to understand in the light of the ontogenetic similarities between males and females in all vertebrate taxa (reviewed in detail by Brennan & Capel, 2004). For example, as described above for mammals, if an individual has the *sry* gene, it will typically become male. However, if that individual lacks the *sry*, as is the case in normal females, ovaries develop, and the embryo follows the female pathway. That is, the medullary and sex cords degenerate, secondary sex cords form in the expanding gonadal cortex, primordial support cells differentiate to form granulosa cells and primordial steroid-producing cells become theca cells. As might be expected, granulosa and Sertoli cells share a common precursor (Albrecht & Eicher, 2001), and the same has been suggested for theca and Leydig cells (Capel, 2000).

Most mammals represent the gonochoristic (distinct male and female morphologies) end of the sexual plasticity continuum. A large number of vertebrates, however, exhibit surprising flexibility in sexual development and manifestation, such that an individual is in fact mostly female or male, rather that absolutely one sex or the other (Fig. 2). We refer to this flexibility as sexual plasticity. Some species carry this concept to an extreme. Like *Rivulus*, a tiny mangrove-dwelling fish, which has functional ovaries and testes in the same individual (Sato *et al.*, 2002). Female European moles (XX) also normally possess ovotestes, although the testicular region is non-functional (Jimenez *et al.*, 1993; Sanchez *et al.*, 1996). It contains seminiferous tubules, but no germ cells. Female moles also have epididymes (although poorly developed) and a masculinized clitoris that contains a urethral canal (Jimenez *et al.*, 1993; Sanchez *et al.*, 1996; Whitworth *et al.*, 1999). In this species, males have testes only (Whitworth *et al.*, 1999).

In addition to simultaneous hermaphrodites, there are a number of vertebrates that are sequential hermaphrodites, functioning first as one sex and then the other, following a brief period of sexual transition during adulthood. These include protogynous reef fishes like *Lythrypnus dalli*, the blue-banded goby, which fully converts from female to male in 5–14 days (Reavis & Grober,

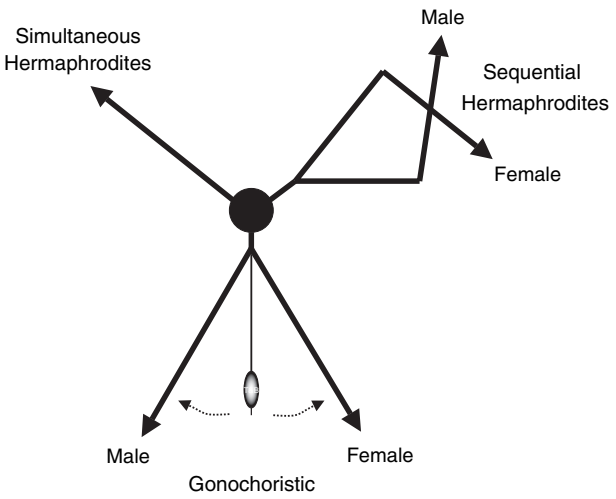

Figure 2 Three modes of sexual development observed among vertebrate taxa. The sexually undifferentiated embryo, represented by the black circle in the centre of the figure, can mature along one of three possible developmental pathways. Some species develop into simultaneous hermaphrodites, expressing functional adult male and female phenotypes at the same time. Other species, referred to as sequential hermaphrodites, mature first as one sex and then the other. The third option describes gonochoristic species, which typically mature as either male or female. The pendulum between gonochoristic males and females indicates that the masculine or feminine designation is not fixed; it is subject to genetic and environmental perturbation that can demasculinize or feminize a male embryo, or similarly defeminize or masculinize a female embryo. Thus the continuum of sexual plasticity we observe among hermaphroditic species is also subtly present among gonochores, and can explain many of the observed symptoms of reproductive dysgenesis.

1999). The change involves anatomical and physiological masculinization of the brain, gonad and phallus (Reavis & Grober, 1999; St Mary, 2000). Likewise, there are protandrous species, like clown fish and moray eels (Helfman *et al.*, 1997), which mature first as males, and secondarily as females. More in line with the human model are a variety of organisms that commit to the male or female phenotype, but do so relatively late in embryonic development and at the behest of some fairly labile environmental signal. Included in this group are some turtles, all crocodilians including caimans and alligators, and a variety of lizards and geckos (Bull, 1980, 1983; Crews, 2003). Sex in these species is primarily determined by temperature and the influential temperature windows are often narrow. For example, alligator eggs, incubated at 30 °C will hatch as females, at 33 °C will hatch as males and at 31–32 °C will hatch as a mix of both (Lang & Andrews, 1994). Therefore, the sex of the individual depends on incubation temperature and a given genotype has the potential to produce a male or female phenotype.

Thus, turtles, caimans and alligators, incubated at male-producing temperatures, have been shown to be sex-reversed – changed into females by the administration of oestradiol, oestrone, or environmental endocrine-disrupting contaminants like atrazine, bisphenol-A, PCBs, trans-nonachlor, cis-nonachlor, p,p′-DDE and chlordane (Dorizzi *et al.*, 1991; Crain *et al.*, 1999; Willingham & Crews, 2000; Stoker *et al.*, 2003; Willingham, 2005). In addition, abnormal sexual maturation has been observed in Florida alligators collected from Lake Apopka, a central Florida lake contaminated with several known endocrine disrupting contaminants (EDCs) (Guillette *et al.*, 1994). Symptoms included poorly organized seminiferous tubules, many of which were lined with a cuboidal epithelium or contained cells with bar-shaped nuclei. None of these characters were present in the testes of reference alligators.

In studying animals with marked sexual plasticity, we may begin to understand human development within the same flexible framework. In fact, vertebrate diversity in terms of sexual plasticity provides an evolutionary foundation on which to build our understanding of human bipotentiality. Human potential for sexual plasticity is greatest during our first 6 weeks of fetal life, when the development of the reproductive system is anatomically indistinguishable between males and females (Brennan & Capel, 2004). At this point, the embryo may develop normally as a male or female. It has all the cells, tissues and primordial organs needed for either sex. Furthermore, it may be that sexual plasticity during development explains the vulnerability of organisms to androgynizing influences (such as environmental oestrogens). This perspective may aid our understanding of complex and variable pathologies like TDS, in which male reproductive development may be viewed as incomplete, exhibiting aspects of the alternative female morphology.

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Discussion

Dr N Olea (Granada, Spain)
You have warned us for many years that we are not fundamentally different from alligators, and that our physiology is not too different from wildlife animals, therefore, we should be alarmed about what is happening to certain sentinel species because we are all part of the same environment. We have evidence about the environment effects on both individuals and populations. Clinicians primarily look at individuals but are now moving to populations examining birth cohort effects, whereas wildlife analysts have examined population numbers and statistics, and are now studying individual animals. What can we learn from environmentalists and clinicians?

Dr LJ Guillette (Gainesville, FL, USA)
When we study humans we worry about individuals. Most wildlife investigators are not so concerned with individuals because persistence of populations through time is more important at the policy level. Linear studies on individuals in wildlife are very different and necessitates capturing and marking animals. At best we take ''snapshots'' of populations over time. Modern epidemiological studies take a population-to-gene approach trying to understand mechanisms and how these relate to model systems. We try to find the most sensitive populations and assess if they are giving us warnings. Sometimes it is difficult to show that an effect is statistically significant. Individual and population studies are both important, and the advantage of wildlife studies is that we can perform experimental studies with real world populations and genetic variations.

Dr J McLachlan (New Orleans, LA, USA)
You describe the different genetics of sexual development in a variety of different vertebrate species, and how widespread oestrogenicity and oestrogen receptors (ER) are distributed. I am amazed how ubiquitous the single molecule oestradiol is which is a ligand found throughout the vertebrate animal kingdom. Can you make an evolutionary speculation about why that is such a conserved response?

Dr LJ Guillette
Oestrogens are certainly very important and ERs are ubiquitous. There are also oestrogen-like substances associated with gene expression in early caudates, and we must look at the role of other oestrogens apart from oestradiol. From an evolutionary approach we must examine how extensive role these conserved oestrogens play when trying to understand what is happening.

Dr D Page (Cambridge, MA, USA)
Your finding of partial sex reversal in developing alligators suggests that environmental contaminants and temperature changes are not targeting the same regulatory processes in sexual differentiation.

Dr LJ Guillette
We have found an interaction between these two factors. In a dose response experiment, raising the incubation temperature by half a degree causes an increase in sensitivity to the exogenous chemicals in favour of sex reversal towards the female. There are common pathways which impinge upon the genetic pathway but speculatively, these probably have two different origins.