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Mammalian sexual differentiation: lessons from the spotted hyena

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Female spotted hyenas (*Crocuta crocuta*) are the only female mammals that lack an external vaginal opening. Mating and birth take place through a urogenital canal that exits at the tip of a hypertrophied clitoris. This 'masculine' phenotype spurred a search for an alternate source of fetal androgens. Although androstenedione from the maternal ovary is readily metabolized to testosterone by the hyena placenta, formation of the penile clitoris and scrotum appear to be largely androgen independent. However, secretions from the fetal testes underlie sex differences in the genitalia and central nervous system that are essential for male reproduction. Naturally circulating androgens, acting prenatally, reduce reproductive success in adult female spotted hyenas. Effects on aggression and dominance might offset these reproductive 'costs' of female androgenization *in utero*.

Introduction

More than 2000 years ago, while writing his *History of Animals* on the island of Lesbos, Aristotle examined the urogenital system of the hyena. He was investigating a rumor, '...that every hyena is furnished with the organ both of the male and the female' [1]. After careful study, Aristotle concluded that the rumor was untrue and offered an explanation for the confusion – namely, that the pouches for the anal scent glands had been mistaken for an external vaginal opening in male hyenas. However, he noted that these were 'unprovided with duct' and therefore could not have served for fertilization. But there was a problem. Aristotle had dissected a striped hyena. We know this from both his description of the extended mane that runs 'all along the spine', and from the fact that only striped hyenas were found in that region [2]. There are four extant species in the family Hyaenidae: aardwolves (*Proteles cristatus*), striped hyenas (*Hyaena hyaena*), brown hyenas (*Parahyaena brunnea*) and spotted hyenas (*Crocuta crocuta*). If Aristotle had examined a female spotted hyena, although still not granting them hermaphroditic status, he would have understood the confusion because only female spotted hyenas display the suite of 'masculine' genital characteristics described below.

Urogenital anatomy of the female spotted hyena

In terms of their external genitalia, spotted hyenas are the most highly 'masculinized' extant female mammals. As can be seen in Figure 1, female spotted hyenas display a fused scrotum, rather than the labia majora of a typical female mammal. The hypertrophied clitoris is similar in form to the male penis, and both female and male spotted hyenas display erections – often in the nonsexual context of 'meeting ceremonies'. The clitoris is traversed by a central urogenital sinus exiting through a meatus at the tip of the glans clitoridis. In the absence of an external vaginal opening, female spotted hyenas urinate, receive the male during copulation and give birth (to one or two, 1–1.5 kg infants) through this organ. The oviducts, uterus and upper vagina of the female spotted hyena follow the essential configuration of other female carnivores (Figure 2).

Mating and birth place different demands on the penis and the clitoris. On close examination, the organs have obvious, functionally significant differences in external and internal morphology. As reviewed here, such sex differences are hormone dependent. Although Frank *et al.* [3] described differences in the external morphology of the clitoris and penis, and Neaves *et al.* [4] emphasized the functional significance of sex differences in internal morphology, when seeking to understand the formation and development of the spotted hyena phallus, the tendency has been to focus on the absence of an external vaginal opening, and the gross similarity of the clitoris and penis of the spotted hyena, ignoring an array of crucial sex differences in phallic anatomy. However, both the essential 'masculine' configuration of the external genitalia, and the sex differences described here, are present at birth, provoking a search for events during fetal life that could account for the unusual urogenital anatomy of this species.

In this review, our goals are threefold: (i) to examine the course of sexual differentiation in the female spotted hyena, against the background of contemporary understanding of such processes in traditional mammals; (ii) to illustrate how attempts to understand this unique

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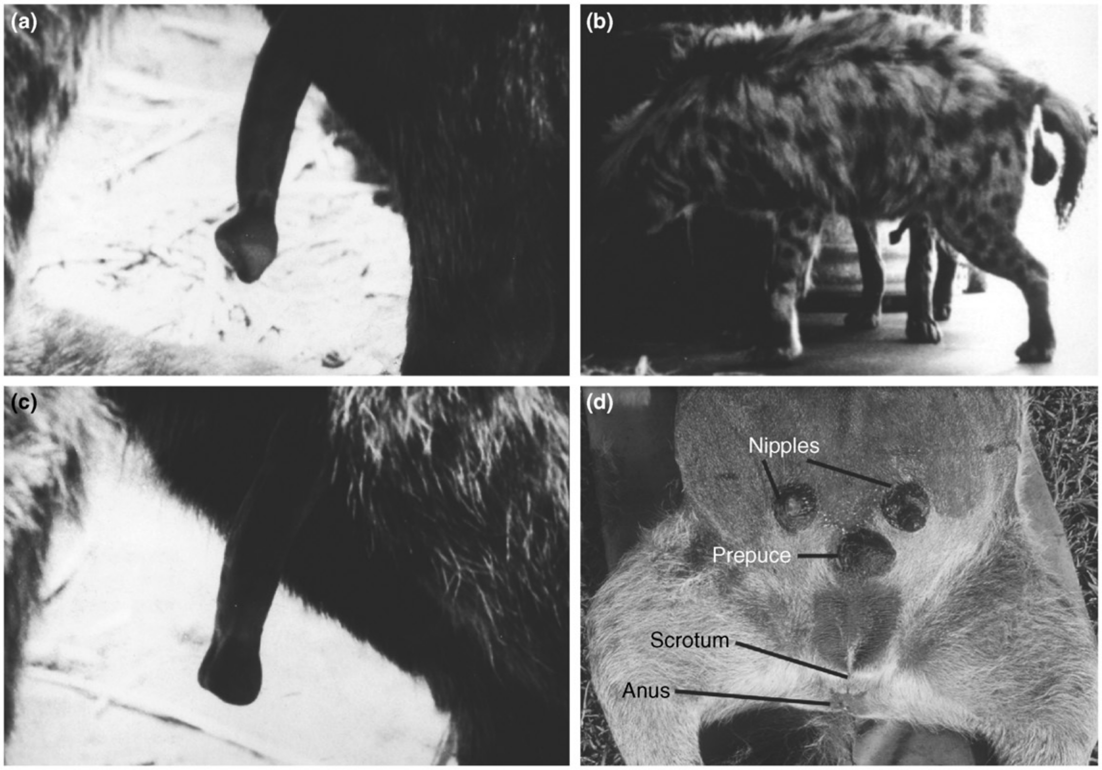

Figure 1. **(a)** The semierect penis of an adult male spotted hyena [6]. Note the angular glans. Figure 1a reproduced, with permission, from [3]. **(b)** Male spotted hyena, offering his erect penis for inspection by another hyena during a 'meeting ceremony' [40]. This animal was castrated between 6–7 months of age, nearly two years before puberty, and was 11 years of age when this photo was taken. His phallus was of normal size and he retained full erectile capability. Postnatal growth of the penis is largely androgen independent. Figure 1b reproduced, with permission, from [40]. **(c)** The erect clitoris of a female spotted hyena [3]. The clitoris is somewhat shorter and thicker than the penis, and has a rounded, rather than an angular, glans. Figure 1c reproduced, with permission, from [3]. **(d)** The abdomen of an adult female spotted hyena. Note the presence of a scrotal area instead of an external vaginal opening. The clitoris is retracted into the abdomen and only the prepuce is visible.

species call attention to mechanisms and processes that are often overlooked in common laboratory species; and (iii) to discuss how research on endocrine mechanisms in the spotted hyena directs attention to the costs and benefits of feminine 'androgenization' *in utero*.

Alfred Jost, mammalian sexual differentiation and the spotted hyena

The Jost model

Contemporary understanding of mammalian sexual differentiation dates from the work of Alfred Jost in the late 1940s and early 1950s [5,6]. He concluded that the development of the masculine urogenital phenotype resulted from secretion of androgens by the fetal testes at crucial stages of development, whereas secretion of anti-Müllerian hormone by the fetal testes was identified as the agent responsible for regression of the Müllerian duct system. Thus, the crucial initial event was development of the indifferent fetal gonad as a testis or an ovary. Emergence and development of the female urogenital system (UGS) occurred as the result of an unidentified process, in the absence of testicular androgens and/or anti-Müllerian hormone. This model, which was subsequently expanded to account for sex differences in brain and behavior [7], has received general acceptance as the route to formation of masculine genitalia in mammals, with subsequent recognition that conversion of testosterone to dihydrotestosterone is normally required for male genital development [8].

Extending Jost's model to the female spotted hyena

Following Jost's lead, administration of exogenous androgens, at crucial stages of development, often produced dramatic 'masculinization' of the external genitalia of female mammals. Comparing the gross morphology of the urogenital system of the normal female spotted hyena [9] with that of the androgenized female dog [10] (Figure 2) encouraged a search for naturally circulating androgens in the female spotted hyena. If androgens could be identified in the fetal female spotted hyena, perhaps they could account for the 'masculinized' external genitalia of this species?

A role for the fetal ovary?

In his classic monograph on spotted hyena urogenital morphology, Matthews [11] had noted the abundance of interstitial tissue and the relative paucity of follicles in the hyena ovary, speculating that the hyena ovary might have produced the androgens responsible for their masculinized genitalia. In a pioneering study, Lindeque and Skinner [12] reported that the fetal ovary indeed produced testosterone. But they were working with hyenas in the field and could not stipulate the gestational age of the fetuses through direct observation of mating. As portrayed in Figure 3, a large genital tubercle, with a central urethra extending to the tip of the organ, is in place at a time when the fetal ovary and the fetal adrenal are still in an undifferentiated state [13]. Evidently, the fetal ovary (or the fetal adrenal) could not be the source of

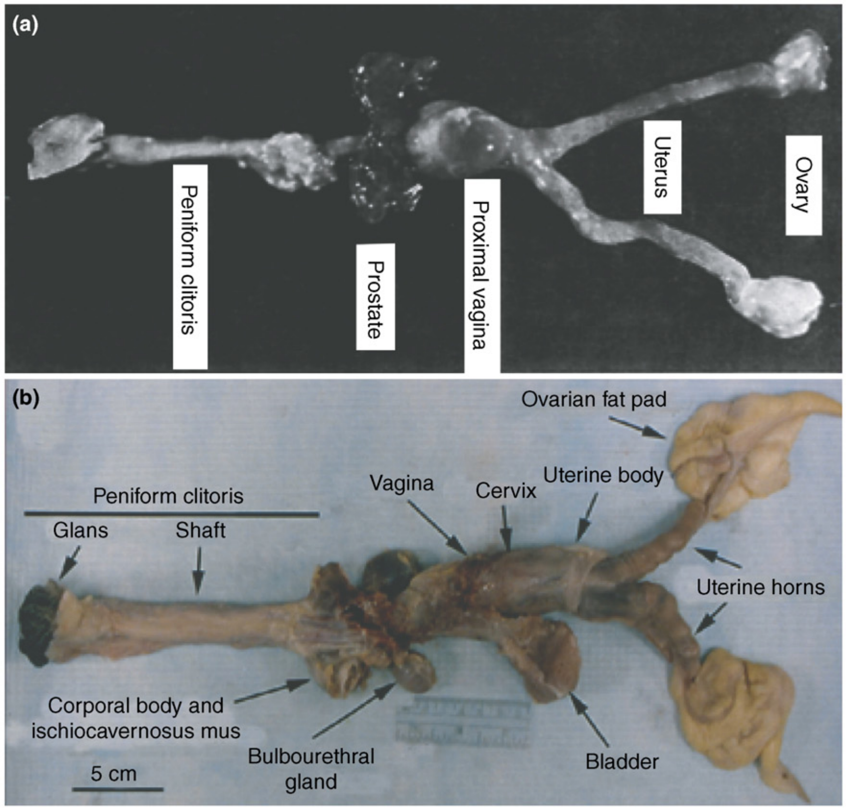

Figure 2. **(a)** The urogenital system of a female dog, exposed to androgens both pre- and post-natally [10]. Figure 2a reproduced, with permission, from [10]. **(b)** The urogenital system of a normal adult female spotted hyena [9]. The most obvious deviation from the normal feminine pattern involves the hypertrophied peniform clitoris, traversed in both subjects by a central urogenital canal. Both species also have an upper vagina, uterine horns and ovaries but only the androgenized female dog has a prostate gland. Figure 2b reproduced, with permission, from [9].

androgen supporting formation of the penile clitoris of the female spotted hyena. Browne *et al.* [14] have found that the fetal testes, but not the fetal ovaries, express the enzymes necessary for synthesizing androgens at 30 days of gestation.

Placental metabolism: an alternative source of fetal androgen in the spotted hyena

With the early gestation fetal ovary and adrenal removed as a source of androgen, responsible for formation of the phallus of the female spotted hyena, a

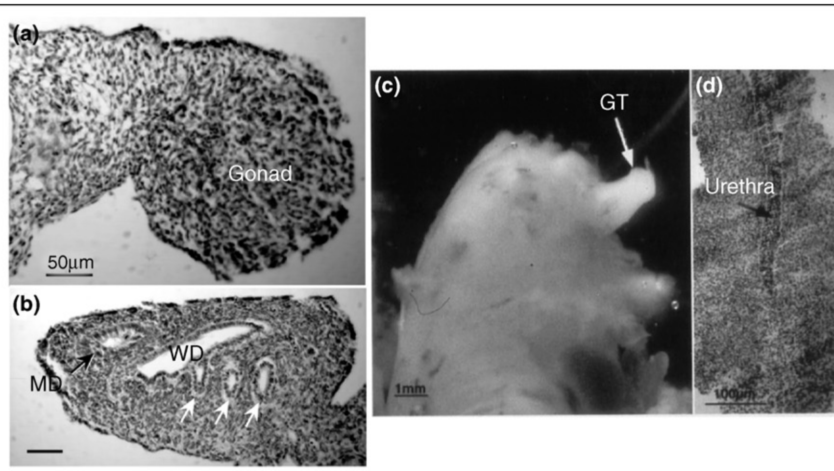

Figure 3. Urogenital structures from the female spotted hyena fetus at 33 days of (a 110-day) gestation [13]. **(a)** The Gonad was histologically undifferentiated. It was subsequently identified as an ovary, when the sex of the embryo was determined through the use of a molecular probe. **(b)** In this section, from the upper urogenital ridge, both the Mullerian duct (MD) and Wolffian Duct (WD) are visible. The white arrows indicate the location of the mesonephric tubules extending from the Wolffian duct to the mesonephros, which is out of the plane of the section. **(c)** A large genital tubercle (GT) and **(d)** a urethra, which penetrated the GT to the distal tip, are present before the differentiation of the fetal ovary, so development of those structures cannot be dependent on secretion of androgens by the fetal ovary. Figure 3 reproduced, with permission, from [13].

new hypothesis emerged. Androstenedione, largely of ovarian origin, had been identified as the primary androgen circulating in female spotted hyenas throughout postnatal life [15–17]. *In vitro* studies [18,19] established that, in contrast to the normal human placenta, the spotted hyena placenta has abundant 17β-hydroxysteroid dehydrogenase activity; thus, androstenedione is converted to testosterone. *In vivo* investigation supported these results [18] and established that testosterone derived from the maternal ovary and placenta is transferred to the developing fetus via the umbilical vein [13], and could account for *in utero* androgenization of the female fetus.

In addition, two clinical cases were described [20,21], in which women gave birth to genetic daughters with 'masculinized' external genitalia. The common observation of pseudohermaphroditism was tracked to a mutation that inactivated the production of aromatase by the human placenta. Instead of converting maternal androgens to estrogens, and binding those estrogens to proteins that could not pass the interface between the maternal and fetal circulation [22], androgens were now transferred to the developing fetus via the umbilical circulation. Conte *et al.* [21] referred to the role of the spotted hyena placenta as an endocrine transducer, supporting their interpretation, and it seemed that an explanation involving the androgenic formation of 'masculine' genitalia in the female spotted hyena was at hand.

Testing the theory: administration of anti-androgens in utero

To 'test' the theory that placental production of testosterone produced 'masculinization' of female offspring in spotted hyenas, a mixture of flutamide (an androgen receptor blocker) and finasteride (which prevents conversion of testosterone to dihydrotestosterone) was administered to pregnant female spotted hyenas [23]. Based on results obtained in other mammalian species [24,25], it was hypothesized that such treatment might produce the first female spotted hyena with an external vaginal opening and a 'typical' female clitoris, no longer penetrated to the tip by a central urogenital canal. Instead, a scrotum and prominent phallus were maintained in both female and male offspring of anti-androgen-treated dams [23]; however, there were profound, functionally significant effects of such treatment on genital morphology (Box 1) and endocrinology in both male and female spotted hyenas.

In males, exposure to anti-androgens *in utero* completely 'feminized' external and internal phallic morphology, as long as 'feminization' refers to the characteristics of the clitoris of the spotted hyena [23,26,27] (Figure 4a–d). The changes, including a penis that is too short to achieve and maintain entry into a clitoris retracted into the abdomen, and elimination of the angular glans that facilitates such entry, prevented successful mating by these animals [23,26]. In addition, anti-androgen treatment 'feminized' the normally robust bulbocavernosus muscles of neonatal males and eliminated the male-biased differences in the volume and number of motoneurons in Onuf's nucleus [28]. The hormonal response to gonadatropin-releasing hormone (GnRH) challenge was also modified in a feminine direction – that is, the magnitude of luteinizing hormone released in response to injection of GnRH was increased, whereas circulating concentrations of testosterone were reduced [29]. The 'masculine' configuration of the interior of the phallus, eliminated by anti-androgen treatment *in utero* (Figure 4 a–c), is formed between days 34 and 45 of gestation [27]. Because both sexes are receiving androgen from the placenta, this pattern suggests that an additional source of androgen is available in the male. As previously noted, using immunohistochemical techniques, Browne *et al.* [14] found that the steroidogenic enzymes responsible for production of androgen are present in the fetal testis, but not the fetal ovary, at 30 days of gestation. It seems likely that, as in other mammalian species, secretion of androgens by the fetal testes promotes the sex differences in phallic structure observed in the spotted hyena. A timeline, portraying developmental events during fetal life and the duration of anti-androgen treatments, is presented in Figure 5.

In female spotted hyenas, exposure to anti-androgens *in utero* enhanced 'feminine' characteristics of the clitoris [23,26]. That is, blocking the actions of naturally circulating androgens *in utero* increased the size and

Box 1. Androgens and sexual differentiation of the external genitalia in the spotted hyena

Evidence for androgen-independent creation of 'masculine' genitalia in the female spotted hyena

- 'Masculine' genitalia appear in the female fetus before differentiation of the fetal ovary and adrenal gland.
- Penile clitoris and scrotum survive anti-androgen treatment *in utero*, with treatments initiated as early as day 12 of a 110-day gestation.
- In terms of internal morphology, the spotted hyena clitoris is a unique structure, unlike any known mammalian penis, or androgenized Clitoris.
- Normal adult clitoral and penile length, in animals gonadectomized during the early months of life, is also compatible with some agent other than androgen modulating phallic development.

Evidence that sex differences between male and female spotted hyena external genitalia and prostate gland are dependent on secretions of the fetal testes *in utero*

- Pathways for androgen synthesis appear in fetal testes before fetal ovaries, and the timing is coincident with development of the masculine penile phenotype (between 34–45 days of gestation).
- Anti-androgen treatment in utero 'feminizes' the following traits in the male (as long as the reference point is the female spotted hyena):
  - The penis assumes the external morphological characteristics of the spotted hyena clitoris.
  - The internal structure of the penis assumes the unique morphological characteristics of the spotted hyena clitoris.
  - The prostate gland is eliminated.
  - The bulbocavernosus muscles assume the characteristic morphology of the female spotted hyena.
  - Onuf's nucleus in the spinal cord displays reduced volume and motoneuron number, similar to that in the female spotted hyena.

Evidence that female spotted hyena genitalia are 'androgenized' *in utero*

Anti-androgen treatment *in utero* modifies the clitoris in the 'feminine' direction, in terms of:

- Increased size and elasticity of the urogenital meatus.
- Decreased length.
- Increased glans diameter.

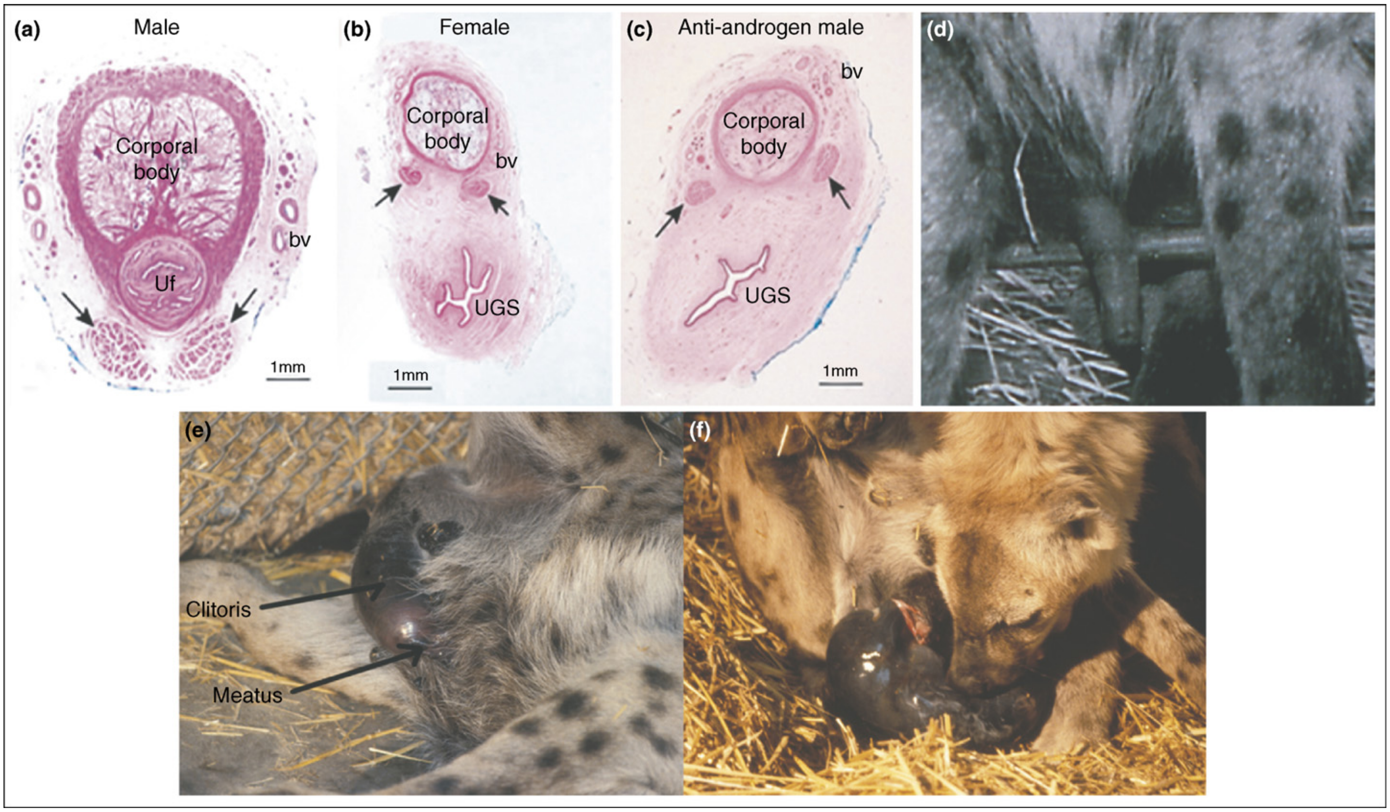

Figure 4. **(a)** A cross-section through the penile shaft of a neonatal male spotted hyena [27]. A tunica albuginea surrounds the corporal body and the corpus spongiosum, a small urethra (Ur) is embedded in the corpus spongiosum, and the retractor muscles (indicated by arrows) are found in an extreme ventral location. **(b)** A cross-section through the clitoral shaft of a neonatal female spotted hyena [27]. The tunica surrounds the corporal body (but not the ventral portion of the shaft). The large pleated urogenital sinus (UGS) is embedded in connective tissue, and the retractor muscles (indicated by arrows) occupy a dorsal location adjacent to the corporal body. The failure of the tunica to surround the tissue in which the UGS is embedded enables expansion of the UGS during mating and reproduction. This would not be possible in the male. **(c)** A cross-section through the penile shaft of an anti-androgen-treated neonatal male spotted hyena [27]. The interior structure of the phallus is now similar to that of the normal female, including the location of the tunica and retractor muscles, the absence of a corpus spongiosum and the presence of a large UGS embedded in connective tissue. Figure 4a–c reproduced, with permission, from [27]. **(d)** The erect penis of a male hyena treated with anti-androgens *in utero* [23]. Externally, it is similar to the clitoris of a normal female spotted hyena, having a shortened, thick phallus, with a rounded, rather than an angular, glans (see Figures 1a and 1b). Figure 4d reproduced, with permission, from [23]. **(e)** Large precocial fetus filling and stretching the clitoris of a normal nulliparous female [30]. Elasticity of clitoral tissue is facilitated by circulating estrogen [40] and, perhaps, by relaxin, levels of which increase markedly before parturition [54]. Figure 4e reproduced, with permission, from [30]. **(f)** The urogenital meatus has torn and the stillborn infant, still in its amniotic sac, is being licked by the mother [26]. As the anti-androgen-treated females matured, the size and elasticity of the urogenital meatus increased markedly, relative to control females, enabling much easier and more successful delivery, at the time of initial parturition. Abbreviation: bv, blood vessels. Figure 4f reproduced, with permission, from [26].

elasticity of the urogenital meatus, as well as decreasing clitoral length and increasing glans diameter in adult females. Anti-androgen-exposed females also displayed significantly higher concentrations of plasma estrogen, and an enhanced luteinizing hormone response to GnRH challenge [29].

'Costs' and benefits of feminine androgenization *in utero*

In captivity, ~60% of first births are stillborn, owing to prolonged retention within the tortuous birth canal. The meatus has to tear in primiparous females [30] (Figure 4d,e) to enable delivery. This is a time-consuming process, and retention of the fetus within the distended clitoris can result in severe anoxia because the short umbilical cord of the spotted hyena requires that the fetal–placental unit must detach from the uterus relatively early in the process of parturition. Consequent upon changes in clitoral morphology, first births are much easier in anti-androgen-treated females. In their initial pregnancies, five anti-androgen-treated females delivered eight cubs, and all survived [26]. This illustrates a serious reproductive 'cost' of natural feminine androgenization *in utero*.

However, adult female spotted hyenas and their subadult offspring totally dominate adult immigrant males when feeding on a carcass [31,32]. Several authors have suggested that the aggressiveness and dominance of female spotted hyenas over males is the result of natural feminine androgenization *in utero*, providing direct benefits during the highly competitive feeding which characterizes this species [33–35]. Such benefits could balance any 'costs' of female androgenization.

Recent data from the field offer the first evidence for a direct link between androgenization *in utero* and aggression in spotted hyenas [36]. Specifically, these researchers report that there are strong positive correlations between maternal rank in the hyena social group, maternal androgens during the late stages of pregnancy, and aggressiveness of juvenile offspring. Other data, from captive hyenas, are also compatible with a link between androgens and estrogens and female aggressiveness toward males in spotted hyenas. For example, prepubertal ovariectomy reduced the aggressiveness of female spotted

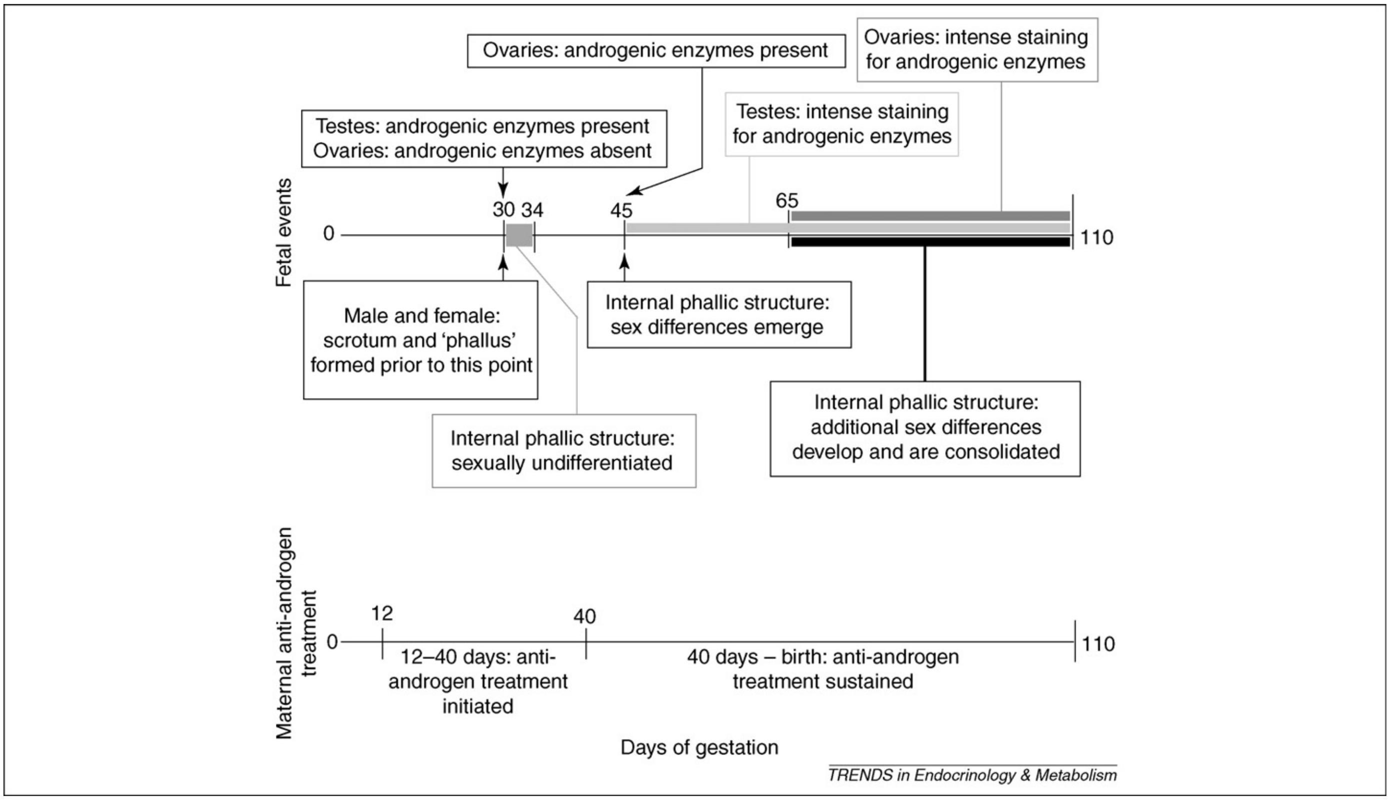

Figure 5. The upper line indicates endocrine events occurring during fetal gonadal development (based on data presented in [14]), and fetal urogenital development (based on data presented in [27]). The 'consolidation' of sex differences in the phallic shaft, during the period between 65 and 110 days, results in the pattern of sex differences portrayed in Figure 4a and 4b. The lower line indicates the range of gestational ages, at which anti-androgen treatment was initiated [23]. Typically, treatment was continued until birth, or 110 days post-mating.

hyenas toward males (Michael G. Baker, MA thesis, University of California, Berkeley, 1990). In addition, a male-biased sex difference in the neural circuitry underlying aggressive behavior, commonly observed in traditional laboratory rodents, does not appear in spotted hyenas, where substantial vasopressin innervation is found in the lateral septal area of the forebrain of the female of this species [37]. The preceding findings are compatible with the possibility that aggressive behavior organized during fetal life is further organized and/or activated by postnatal hormones, although, at this time, the effect cannot be ascribed to a particular androgen or estrogen.

Sexual differentiation of the spotted hyena: summary and implications

Jost's theory and the role of androgens in development of a phallus and scrotum

Contrary to Jost's formulation, the results discussed here strongly suggest that formation of the scrotum and penile clitoris of the spotted hyena is an androgen-independent event (Box 1a), although the morphology of the penile clitoris is modulated by naturally circulating androgens *in utero*. In accord with Jost's formulation, sex differences in the external genitalia result from the secretion of androgens by the fetal testes.

Until an alternative mechanism is revealed, however, the possibility of a formative role for androgens cannot be eliminated. Catalano *et al.* [38] have sequenced the spotted hyena androgen receptor and examined its response to various ligands in tissue culture. It is clear that the receptor is not constitutively active, a possibility that could have explained both the failure of our anti-androgen studies to block formation of the phallus and scrotum, and the apparent androgen-independent growth of the penis. Preliminary data suggest that estrogens modulate phallic growth during prenatal and postnatal life [39,40] but, as yet, there is no evidence indicating that phallic organogenesis is estrogen dependent.

Expanding our scope beyond the usual laboratory species calls attention to novel mechanisms of sexual differentiation that might be operating, albeit in less dramatic ways, in more traditional animal subjects [41]. For example, it is possible that, similarly to the situation in the wallaby [42], there are either direct genetic effects or a special role for nontraditional androgens. In the wallaby, the formation of the pouch of the female and the scrotum of the male are a result of genetic factors (XX versus XY), without sex steroid intervention. In addition, the formation of the penis is dependent on secretion of 5a-androstane-3a, 17b-diol by the testes, rather than testosterone [43]. Perhaps stimulated by the wallaby example, attention recently has been directed to the interaction between direct genetic effects and the effects of gonadal secretions in organizing structures in the brain and modulating behavioral correlates [44].

The active nature of feminine development and 'limits' of androgenization

The clitoris of the spotted hyena is a unique structure that does not mimic the penile clitoris produced by administration of exogenous androgens to pregnant female mammals.

Such treatments produce a penis-like structure for the species in question (e.g. in the rhesus monkey [45]). However, mating and birth through such a structure appear to be impossible, owing, in part, to the constraining effects of the corpus spongiosum and tunica that surround a masculine-phenotype urethra. This constraint would seem to place an absolute limit on the extent of permissible androgenization *in utero*.

Although not as 'complete' as the spotted hyena, there are many female mammals that display varying degrees of clitoral 'masculinization', and/or absence of traditional male-biased enhancement of anogenital distance (e.g. moles [46], fossa [47], elephants [41,48,49] and numerous prosimians [50]). The natural tendency has been to attribute such 'masculinization' to androgens. This might, or might not, be the case [51]. A clue might be found in detailed examination of the morphology of the hypertrophied clitoris. If the clitoris were similar to the penis of that species, then there would seem to be a *prima facie* case for androgenization. However, if not, other alternatives must be explored.

Placental metabolism and sexual differentiation

In the hundreds of studies in which androgens were injected into the maternal circulation to induce 'masculinization', there were few that considered the role of placental metabolism. However, there is wide variation in placental enzymatic activity [52] and, as described by Resko *et al.* [53] and here, the injected hormone is not necessarily the hormone that arrives at its target. The human clinical cases cited above emphasize the potentially crucial role of placental enzymes in mammalian sexual differentiation. In a less dramatic manner, individual variation in placental metabolism could account for some fraction of normal quantitative variation in sex-typical characteristics.

Studies of the spotted hyena have directed attention to neglected mechanisms of sexual differentiation, and reminded us that there are potential costs and benefits associated with all endocrine processes that naturally regulate sexual differentiation. It has also focused attention on the possible role of non-androgenic hormonal processes, and direct genetic mechanisms that could result in 'masculinization' of the external genitalia. These issues will be a focus of future studies with the spotted hyena and could result in novel insights relevant to more traditional species.

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