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REVIEW

Spotted hyaenas and the sexual spectrum: reproductive endocrinology and development

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Abstract

The spotted hyaena (*Crocuta crocuta*) is a unique species, even amongst the Hyaenidae. Extreme clitoral development in female spotted hyaenas challenges aspects of the accepted framework of sexual differentiation and reproductive function. They lack a vulva and instead urinate, copulate and give birth through a single, long urogenital canal that traverses a clitoris superficially resembling a penis. Recent and historical evidence is reviewed to describe our changing understanding of the biology of this species. Expanding upon observations from hyaenas in nature, much has been learned from studies utilising the captive colony at the University of California, Berkeley. The steroid environment of pregnancy is shaped by placental androgen and oestrogen secretion and a late gestational increase in sex hormone binding globulin, the regulated expression and steroid-binding characteristics of which are unique within the Hyaenidae. While initial external genital development is largely free of androgenic influence, the increase in testosterone concentrations in late gestation influences foetal development. Specifically, anti-androgen (AA) treatment of pregnant females reduced the developmental influence of androgens on their foetuses, resulting in reduced androstenedione concentrations in young females and easier birth through a 'feminised' clitoris, but precluded intromission and mating by 'feminised' male offspring, and altered social interactions. Insight into the costs and benefits of androgen exposure on spotted hyaena reproductive development, endocrinology and behaviour emphasises the delicate balance that sustains reproductive success, forces a re-evaluation of how we define masculine vs feminine sexual characteristics, and motivates reflection about the representative value of model species.

Introduction

Mammalian external genitalia are not always distinctly sexually dimorphic (Glickman *et al.* 2005) but the female spotted hyaena (*Crocuta crocuta*) is unique in the degree to which her genitalia resemble those of the male in form and even some functional aspects. Females of this species lack a vaginal opening in the perineal region, having instead a urogenital canal traversing a clitoris (similar in size to the penis) through which they urinate, copulate and give birth (Watson 1877, Matthews 1939). Female elephants also have a long urogenital canal (Perry 1964, Balke *et al.* 1988) and a well-developed clitoris which is difficult to differentiate grossly from the flaccid penis of the bull elephant (Perry 1964, Glickman *et al.* 2005). Especially when flaccid, the distinction between the clitoris and the penis in terms of size, shape and position (Fig. 1) is also minimal in spotted hyaenas (Matthews 1939, Neaves *et al.* 1980, Frank *et al.* 1990, Drea *et al.* 1998, Cunha *et al.* 2003, 2005, 2014, 2020). But, what makes the clitoris of the spotted hyaena so clearly unique is the degree of erectile tissue development, the functional significance of erection in both sexes when conspecifics greet one another (Hamilton III *et al.* 1986, East *et al.* 1993), and the difficulty (to an untrained eye) in distinguishing the clitoris from the penis in the erect state. Copulation is not an easy process in this species (Drea *et al.* 2018); the clitoris of the spotted hyaena must remain flaccid when the male is aroused and the penis is erect. Retractor clitoral muscles draw the clitoral meatus backwards to facilitate intromission made difficult by its rostral location (Neaves *et al.* 1980). Hence, the clitoris of the spotted hyaena has been variously described as 'peniform', 'phallic', 'masculinised' or 'virilised', the genitalia in general as 'hermaphroditic' (Glickman 1995), and the species has been seen to exemplify 'female deviance from gender norms' (Wilson 2003). Spotted hyaenas have thus fascinated biologists in the degree to which they deviate from a binary view of sexual dimorphism in mammals, exhibiting a behavioural repertoire (including pronounced aggression, social dominance, territorial leadership, glandular scent marking and rough play) that emphatically challenges the commonly perceived norms of male dominance. Accordingly, time-sensitive, androgen-dependent mechanisms that most commonly direct the development of a penis and scrotal sac (George & Wilson 1994, Griffin *et al.* 2001), as well as later, sexually differentiated behaviours (Phoenix *et al.* 1959), have been explored in efforts to explain the genesis of the external genitalia of the female spotted hyaena.
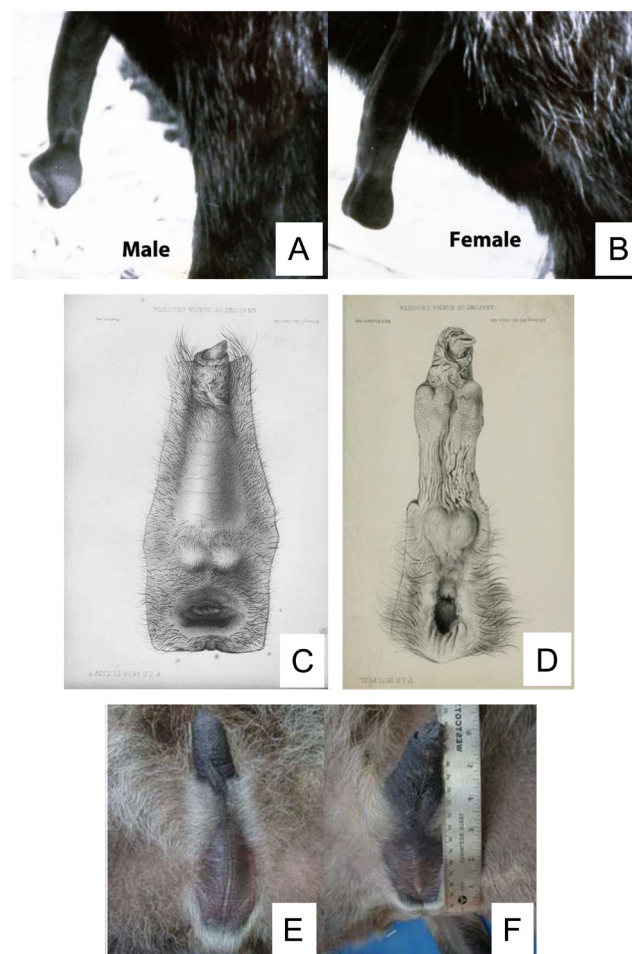
Figure 1

The gross anatomy of the male and female genitalia of the spotted hyaena (*Crocuta crocuta*). Shown are the penis (A) and clitoris (B) in the erect state. The external genitalia of a male (C) and female (D) as illustrated in the flaccid state by Watson (1877, 1878) showing the similarity in position and size of the clitoris compared with the penis, as well as the pseudoscrotum and lack of a vulvar opening in the female perineum. Also shown are photographic images of the male (E) and female (F) genitalia side by side. Note again, the lack of a vulva in the perineal region of the female.

Scientific and social context

Not surprisingly, the reproductive biology of the spotted hyaena has attracted attention from traditional biological (Glickman *et al.* 1992*a*) and evolutionary (Gould 1983) perspectives. However, researchers studying a species as unusual as the spotted hyaena can face significant challenges in obtaining federal funding which can require justification in terms of human relevance (Blaschko *et al.* 2012). This may have biased the way in which the biology of spotted hyaenas has sometimes been portrayed, used and perhaps even misused symbolically, drawing undue scrutiny in non-biological disciplines or social contexts

(Wilson 2003, Bewell 2014, Szczygielska 2017). Ironically, L Harrison Matthews, whose classic monograph (Matthews 1939) fuelled so much subsequent research on spotted hyaenas, 'objected strongly to anthropomorphism in any kind' (Matthews 1987). Funding imperatives might also explain discussions of the spotted hyaena as a 'model' for other species when in fact its real value perhaps lies in being the exception that tests/challenges so many biological rules, exposing the narrow vision that drives much 'model' research (Beach 1950). As proposed in an earlier review article, 'we hoped that through the use of an unusual female mammal we would challenge the adequacy of current understanding of the process of sexual differentiation' (Glickman *et al.* 1992a) which, indeed, it has. The following review will provide an update of the state of knowledge of the reproductive endocrinology in spotted hyaenas during foetal development as well as in post-natal life. References to other mammals have been utilised only to better appreciate what sets this species apart from all others, even those among the Hyaenidae (Drea *et al.* 2018), and to illustrate the breadth of variation in mechanisms and outcomes of mammalian sexual differentiation and reproductive function.

Sexual development and genital differentiation

A discussion of the reproductive endocrinology of the spotted hyaena must, by necessity, begin with a description of their sexual development which has drawn so much interest and attention over millennia (Glickman 1995). The predominant view of mammalian sexual differentiation is grounded largely on the pioneering experiments by Alfred Jost, who surgically removed and then transplanted gonads between foetal rabbits and observed the consequences with respect to subsequent development of the male and female reproductive tracts after the gonads had differentiated (Jost 1948, Jost *et al.* 1973). Based on these studies it was concluded that development of the male genitalia is directed by testicular hormonal secretions in early foetal life, and that the female habitus forms 'by default' in the absence of testicular endocrine influence (Jost *et al.* 1973, Jost 1991). This basic foundation was later extended to the neural structures underlying sexual differentiation of behaviour (Phoenix *et al.* 1959) and has been shaped and reinforced extensively by observed variations in human, macaque and rodent sexual development (Goy *et al.* 1988, Cunha *et al.* 2020), that have been traced to specific genes and

their products (Wilson *et al.* 1981, George & Wilson 1994, Gustafson & Donahoe 1994, Sobel *et al.* 2004, Rey & Grinspon 2011). The extent to which other species conform to this paradigm has received limited attention by comparison (Cunha *et al.* 2020).

The results of studies in pre- and post-natal spotted hyaenas indicated that 'masculinisation' of their genitalia is not as strictly androgen-dependent (Drea *et al.* 1998, Cunha *et al.* 2020) as it is in other mammalian species (Herman *et al.* 2000). In their recent review of sexual differentiation in the spotted hyaena, Cunha *et al.* noted that penile length at 2 years of age was similar in hyaenas castrated as cubs compared with those left intact, suggesting that post-natal penile development is largely androgen-independent (Glickman *et al.* 1992b, Cunha *et al.* 2014). Likewise, clitoral length at 2 years of age was minimally affected by ovariectomy of immature cubs. Moreover, prenatal penile and clitoral development also appears initially to be relatively androgen-independent, though androgens influence subsequent differentiation. For example, the embryonic genital tubercle is already prominent (Lindeque & Skinner 1982), and the basic form of the phallus is determined in both males and females (Cunha *et al.* 2014), long before the foetal gonads become capable of androgen synthesis (Browne *et al.* 2006).

As grossly similar as clitoral and penile morphology might be in spotted hyaenas, there are additional dimorphic features that differentiate in response to androgen stimulation in male foetuses. The penile urethra has a slit-like lumen and is surrounded by the corpus spongiosum and the tunica albuginea, whereas the clitoris has a large voluminous pleated 'urethra' the expansion of which is required for receipt of the penis during mating and to facilitate foetal delivery at parturition. Androgen-dependent developmental processes in male foetuses are necessary for generating the basic architecture required for successful reproduction and these include: (1) formation of the prostate, (2) ventral positioning of the retractor muscles relative to the urethra, (3) a small urethral lumen, (4) development of a thick tunica albuginea surrounding the corporal body, (5) formation of the corpus spongiosum surrounding the urethra, and (6) the form and position of erectile bodies in the glans that on erection determine the chisel-shaped tip required for successful mating (Cunha *et al.* 2014) (Supplementary Table 1, see section on supplementary materials given at the end of this article). Thus (and in contrast to the female), androgenic stimulation has an important influence on genital development in the male spotted hyaena.

Early evidence for androgens and female genital masculinisation

The idea that genital development in female spotted hyaenas was 'masculinised' by androgen exposure *in utero* derived, in part, from the perceived extreme masculine appearance of the female external genitalia (clitoral hypertrophy and labial fusion) and, in part, from the accompanying suite of additional traits traditionally viewed as 'masculine'. These include a reversal of sexual size dimorphism (i.e. female hyaenas are generally larger than males (Swanson *et al.* 2013, Drea *et al.* 2018)) as well as behavioural traits more routinely displayed by male mammals than by their female counterparts such as aggression and social dominance (Frank 1986), leadership in territorial defence (Boydston *et al.* 2001), pronounced scent marking (Henschel & Skinner 1991) and rough play (Pedersen *et al.* 1990), often associated with androgen influence. Given this composite picture, the search for a source of androgens in females initially directed much of the research.

The foundational endocrine studies relied on field specimens, using estimated ages (especially of foetuses), presumed maturity, and relatively basic analytical methods. Hence, Racey & Skinner (1979) used direct immunoassays to measure androgens (testosterone and androstenedione) and other steroids (progesterone, oestrone and oestradiol) in adult spotted hyaenas (parous, non-parous, lactating), as well as in adult brown (*Parahyaena brunnea*) and striped hyaenas (*Hyaena hyaena*). Initially, they suggested that males could not be discerned from female spotted hyaenas based on steroid concentrations in single serum samples, even though ovarian tissue contained less testosterone than did the testes or adrenal glands, the latter being considered another possible tissue source of androgens (Racey & Skinner 1979). In contrast, male brown and striped hyaenas had higher circulating testosterone concentrations than did females of those species. In addition, a single pregnant spotted hyaena female carrying twin female foetuses was investigated and testosterone concentrations were found to be within a similar range in maternal and foetal serum seemingly supporting the idea that female 'virilisation' in this species occurred *in utero* (Racey & Skinner 1979).

Androgen concentrations were explored further in another four pregnancies bearing mixed-sex twins that were sampled for direct immunoassay of androgens organically extracted from maternal or foetal plasma and tissue. Testosterone concentrations in maternal plasma at an estimated 31, 65, 75 and 80 days of gestation (based on hypothetical foetal growth velocity and duration of gestation) (Huggett & Widdas 1951, Frazer & Huggett 1974), were 0.98, 0.03, 0.63 and 3.60 ng/mL, respectively, the last value representing almost an order of magnitude increase (Lindeque & Skinner 1982). Maternal androstenedione concentrations were 2.6, 26.2 and 5.2 ng/mL on days 65, 75 and 80, respectively (Lindeque & Skinner 1982), consistently higher than had been previously reported (Racey & Skinner 1979). The highest plasma concentrations of testosterone were found in twin 31-day-old foetuses, at least twice as high as in any of the male or female foetuses at older ages. However, the defined sex of that foetus was based on 'phallic' size, which was assumed to be larger in males even at this early stage (Lindeque & Skinner 1982). Direct comparisons of foetal plasma androgen concentrations were made in mixed-sex twins at 65 and 80 days of gestation. Testosterone was slightly higher in the male than in the female co-twin at both gestational ages, but androstenedione concentrations were almost the same. Therefore, the authors concluded, '…that masculinisation of the genital tubercle of female spotted hyaena foetuses results, as in the male, from an episode of androgen secretion by the foetal gonad' (Lindeque & Skinner 1982).

A potential contribution of the ovaries and adrenal glands to circulating androgen concentrations was investigated in samples from juvenile, sub-adult and adult males and females (Lindeque *et al.* 1986). Females were classified by reproductive state as nulliparous or as parous and non-pregnant, pregnant and lactating, and gonadal response to human chorionic gonadotropin (hCG) and gonadotrophic releasing hormone (GnRH) was also assessed. Sub-adult males had lower androstenedione concentrations than females, and concentrations of both testosterone and androstenedione were higher in pregnant than non-pregnant females. Both testosterone and androstenedione were elevated after stimulation with hCG; however, a similar increase after adrenocorticotropic hormone stimulation suggested some adrenal contribution was possible (Lindeque *et al.* 1986). This finding was consistent with the earlier observation that androgen concentrations in adrenal tissue of adult females were considerably higher than those in the ovary (Racey & Skinner 1979). Ultimately, however, it was concluded that the ovary was the principal source of the unusually high, circulating concentrations of androstenedione observed in the non-pregnant female spotted hyaena (Lindeque *et al.* 1986).

Lastly, androgen concentrations in male and female spotted hyaenas were investigated once again, but in a

larger cohort of subjects of varying ages from cubs to adults stratified into social categories for each sex (van Jaarsveld & Skinner 1991). Non-parous females had higher androstenedione concentrations than mature and immature males, as did female cubs relative to male cubs; however parous females had higher testosterone concentrations than did immature males (van Jaarsveld & Skinner 1991). This was confirmed subsequently by faecal androgen analysis (Dloniak *et al.* 2006a) showing that socially central and integrated, immigrant males had higher plasma testosterone and faecal androgen concentrations than did adult natal males or pregnant females which did not differ from one another (Dloniak *et al.* 2004).

Collectively, the data from these early studies indicated that the differences in androgen concentrations between male and female spotted hyaenas were complex, dependent on age, residency, social status and female reproductive state. With respect to apparent female 'masculinisation', the broad conclusion from this early work was a tacit reaffirmation of Jost's dictum in terms of foetal gonadal androgens (androstenedione in this case) influencing genital differentiation. However, one observation also cast doubt on this interpretation. Lindeque and Skinner noted that despite apparent 'masculine' development of the external genitalia of the female foetuses, there was no stabilisation of the Wolffian ducts (Lindeque & Skinner 1982), which is another, well-recognised androgen response (Jost 1970, Schultz & Wilson 1974, George & Wilson 1994, Shaw & Renfree 2014). The Wolffian ducts had regressed in these female hyaena foetuses by what was estimated to be 80 days of gestation, despite apparent 'virilisation' of their external genitalia (Lindeque & Skinner 1982). Indeed, the possibility that the broader architecture of genital differentiation in the female spotted hyaena foetus might not rely solely on androgen exposure has been borne out in studies conducted since that time. For instance, it has become clear that the foetal ages in those early field studies were grossly underestimated. A foetus at 31 days of gestation cannot be sexed anatomically, too little blood can be recovered for analysis at this stage and the conclusions drawn with respect to androgenic effects on female development have proven to be incorrect (Browne *et al.* 2006, Cunha *et al.* 2014). More precise understanding of the timing of prenatal events has been key to advancing our understanding of spotted hyaena sexual differentiation.

Contributions from the Berkeley Hyaena Colony

In the mid-1980s, a captive colony of spotted hyaenas was established at University of California, Berkeley to better examine the premise of maternally acquired 'sexual mimicry' in genital anatomy, endocrinology and behaviour that eventually challenged aspects of Jost's framework (Cunha *et al.* 2014). This facility enabled protocols that were virtually impossible or otherwise lethal in the field and experiments that could be planned with more precisely timed, strategic sampling points, both pre- and post-natally (Glickman *et al.* 1987, 1992b, Frank *et al.* 1991). Captive animals were socially housed in mixed-sex groups, had access to outdoor enclosures and so experienced natural light cycles and temperatures, received an obligate carnivore diet that included unprocessed meats and bones, and could be observed at any time of the day or night, across the life span from the time of birth. This facilitated longitudinal blood sampling of live pregnant and neonatal animals, for example, showing that testosterone concentrations were very high in pregnant females (Licht *et al.* 1992) and were consistently higher in male than female neonates (Frank *et al.* 1991), but that androstenedione concentrations were similar in neonates of both sexes, as previously reported (Lindeque & Skinner 1982). The reversal of the sex difference in androgen concentrations was confirmed and extended by Glickman *et al.* (1987) who later included chromatography as part of assay validation (Glickman *et al.* 1992b). They demonstrated that the elevation of androstenedione was more pronounced in juvenile than in mature females and that the ovaries were the principal source (Glickman *et al.* 1987, 1992b). Tissues also became available for more extensive in vitro analysis. In fact, the capacity for androgen production by the ovary of the spotted hyaena is considerable. The rate of androgen synthesis by ovarian tissue, measured using a radiometric method (Moran *et al.* 2002), was higher than that of the testes or adrenal gland (13.4 ± 0.5, 11.2 ± 0.2 and 8.2 ± 1.0 nmol/mg/h in ovarian, testicular and adrenal tissue homogenates, respectively).

Access to a captive colony made larger-scale, comprehensive and longitudinal analysis of steroid concentrations in maternal plasma during pregnancy possible (Licht *et al.* 1992). Surgeries could also be planned in females known to be pregnant. Thereby, plasma was sampled from various maternal and foetal

blood vessels to investigate veno-arterial differences within and across the uterus at days 90 and 96 of gestation. Examination of progesterone, androstenedione, testosterone and oestrogen was conducted by immuno-assays validated in part by chromatography on pooled samples. Several important observations were made suggesting that progesterone, testosterone and oestrogen, the concentrations of which increased progressively during pregnancy (Licht *et al.* 1992), were synthesised by the placenta. First, progesterone concentrations were higher in pregnant than non-pregnant females. Second, progesterone concentrations progressively increased as pregnancy proceeded, as did testosterone and oestrogen, whereas androstenedione concentrations (>6 ng/mL), remained relatively constant. Third, progesterone and testosterone concentrations were higher in uterine venous than systemic plasma. Lastly, concentrations of progesterone, testosterone and oestrogen dropped within hours of placental removal at Caesarian section in two late-stage pregnancies. It was suggested that the placenta synthesised testosterone from circulating androstenedione although their concentrations were not correlated in these studies. The lack of clear difference in testosterone concentrations between male and female twin foetuses, together with higher concentrations in umbilical than foetal jugular vein, was also taken as evidence that maternal testosterone might even contribute to foetal concentrations to some degree (Licht *et al.* 1992). Subsequent reports of increased faecal and serum testosterone concentrations in pregnant vs non-pregnant females (Dloniak *et al.* 2004, 2006*b*), and of progressively increased faecal oestrogen across gestation (Van Meter *et al.* 2008), are consistent with the longitudinal data from pregnancies in the colony (Licht *et al.* 1992).

Despite the access provided by a breeding colony, mating was not routinely detected amongst spotted hyaenas (outside of timed pairings) and, therefore, estimates of the stage of gestation as reported often remained approximate (Licht *et al.* 1992). Still, earlier stages of foetal and placental development were investigated in a subsequent experiment involving Caesarian sections on seven pregnancies estimated to be 33, 48, 50, 58, 64, 78 and >100 days of gestation, yielding a total of 10 foetuses (Licht *et al.* 1998). Seminiferous cords were evident in the developing testes of a male foetus at day 50 of gestation, but the ovaries of a 48-day-old foetus were still undifferentiated. In these comparably aged foetuses, Müllerian and Wolffian ducts were present in both sexes, extending all the way to the urogenital sinus. Regression of the Müllerian ducts by anti-Müllerian

hormone (AMH) in the male had not begun or was not yet obvious at 50 days of gestation nor was there evidence of Wolffian duct regression in the female foetus. These observations contrasted those of Lindeque and Skinner who reported that Müllerian ducts had regressed at day 50 in a male foetus and the Wolffian ducts of a female foetus of the same age were detectably degenerating (Lindeque & Skinner 1982). The discrepancy likely reflects the inaccuracy in estimates of foetal age of the field specimens, but suggests that the window of time during which these events transpire might be relatively narrow, occurring sometime after day 50 of gestation.

Gonadal differentiation was examined in more detail in tissues recovered at Caesarian section from colony females from 30 to 95 days of gestation (Browne *et al.* 2006). Expression of AMH in Sertoli cells and enzymes required for androgen synthesis in Leydig cells were evident even at day 30 in foetal testes, helping to define early development of cords in the testes, a level of organisation not evident in ovaries of the female twin sibling. The ovaries remained generally unorganised anatomically and with little evidence of steroidogenic enzyme expression at gestation day 45 (Fig. 2B). In contrast, the foetal testes were well organised into seminiferous tubules and there was robust expression of enzymes enabling androgen synthesis in the interstitial space (Fig. 2A). By day 65 of gestation, however, the foetal ovaries were organised into distinct medullary and cortical compartments. Moreover, there was a strong expression of steroidogenic enzymes
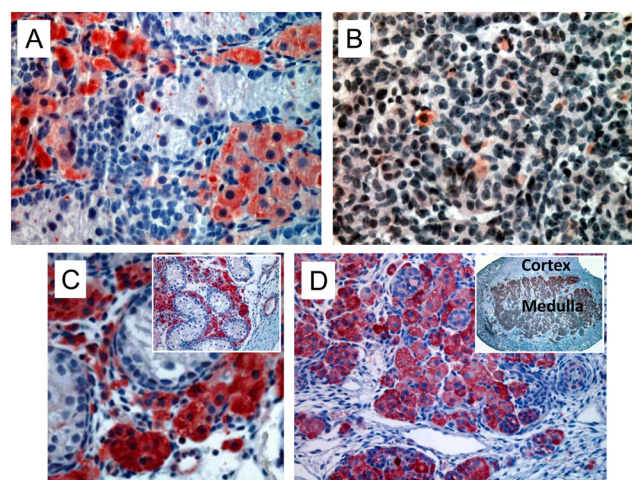
Figure 2
Histological development of testicular (A and C) and ovarian (B and D) organisation and steroidogenic differentiation, as indicated by immuno-histochemically detected the expression of 3β-hydroxysteroid dehydrogenase (red chromagen), in spotted hyaena foetuses at gestation days 45 (A and B) and 65 (C and D).

catalysing androgen synthesis in the ovarian medullary region (Fig. 2D) that was comparable in intensity to that seen in the Leydig cells in the testes of the male twin (Fig. 2C). The ovarian medullary expression of enzymes required for androgen synthesis was consistent with androstenedione concentrations in the plasma of female foetuses at days 65 and 95 that were similar to those in their male twins, but only the male foetuses had detectable testosterone concentrations (Browne *et al.* 2006).

Collectively, the evidence obtained suggested that male foetal gonads were capable of androgen secretion at a much earlier stage of development than had previously been thought. In addition, these data suggested that foetal ovarian development and steroidogenic differentiation was delayed relative to the foetal testis, not being evident until day 65. Thereafter, the apparent capacity for steroidogenesis in the ovarian medulla was robust, consistent with earlier speculations (Matthews 1939, Racey & Skinner 1979). The proposed time course of these events in the spotted hyaena illustrates the developmental delay in steroidogenic differentiation of foetal ovaries compared with the foetal testes, as summarised (Fig. 3), and argues against a role of foetal androgens in determining gross size with respect to female phallic development.

The steroidogenic potential of the spotted hyaena placenta was also investigated (Yalcinkaya *et al.* 1993,
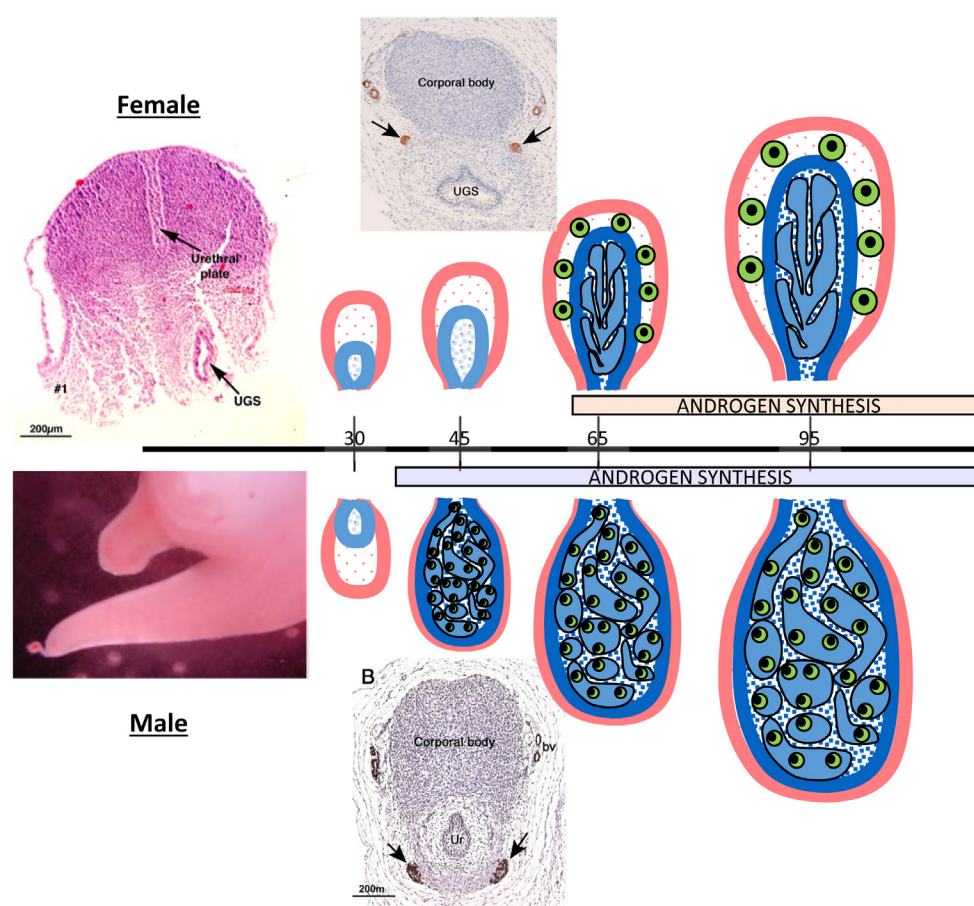
Figure 3

Schematic summary of developmental events involving the gonads and associated reproductive tissues in female (above the timeline) and male (below) spotted hyaena foetuses from days 30 to 95 of gestation (gestation averages 111 days from observed mating to parturition in this colony). The images (left) on the timeline show histology (upper) and gross appearance (lower, with tail beneath) of the genital tubercle in a day 30 foetus at which time there is no differential development in relation to sex. The schematic representation of gonadal development (right) depicts the gonadal cortex in pink, the medulla in blue and germ cells in green. The gonads of both sexes are relatively undifferentiated at day 30, though some primitive cords and expression of anti-Müllerian hormone were evident in foetal testes (not shown). The foetal testes are well differentiated by day 45 with robust expression of steroidogenic enzymes, but the ovaries were still unorganised and relatively undifferentiated. By day 65, the ovarian medulla is well organised and exhibits robust expression of steroidogenic enzymes comparable to the testes and consistent with androgen synthesis. Images of histological sections through the clitoris (above) and penis (below) showing the retractor muscles (immuno-positive for smooth muscle actin; arrows) dorsal to the urogenital canal (UGC) in the clitoris but ventral to the UGC in the penis, a response to foetal testicular androgen (see text).

Licht *et al.* 1998, Conley *et al.* 2007), complementing morphological studies clearly defining its hemochorial nature (Enders *et al.* 2006, 2007), a feature apparently also shared with the striped hyaena (A C Enders, C M Drea & A Conley, unpublished observations). Licht and colleagues provided evidence of testosterone synthesis by the spotted hyaena placenta based on a positive veno-arterial difference across the uterus coincident with a negative veno-arterial difference in androstenedione concentrations. In addition, they demonstrated the conversion of radio-labelled androstenedione to testosterone and oestradiol by the placenta *in situ* (Licht *et al.* 1998). These data confirmed that the spotted hyaena placenta expressed both robust 17β-hydroxysteroid dehydrogenase activity, enabling testosterone synthesis from androstenedione, and aromatase activity, as previously reported (Yalcinkaya *et al.* 1993). In the earlier studies on hyaena placentas collected at term, Yalcinkaya and colleagues found aromatase activity to be extremely low compared with activity in human term placentas, and proposed the provocative hypothesis that this might explain high androgen concentrations and 'virilisation' in female foetuses (Yalcinkaya *et al.* 1993). This idea seemed consistent with the fact that human placental aromatase deficiency was known to be associated with androgenisation of daughters at birth (Shozu *et al.* 1991, Conte *et al.* 1994). However, aromatase activity measured in spotted hyaena placentas from early gestation (days 48 and 58) were much higher than those from later in gestation (day 78) (Licht *et al.* 1998). This temporal pattern was confirmed in a subsequent study (Conley *et al.* 2007) in which it was shown that hyaena placental aromatase activity was highest at 45 days of gestation, around the time that foetal testicular differentiation and androgen synthesis is occurring. In fact, aromatase activity in hyaena placentas at this stage was nearly one-third of that seen in human placentas at an equivalent point in foetal development. Moreover, there was no obvious difference in aromatase enzyme activity between male and female placentas, though there were too few samples for a truly valid comparison by sex. In any event, the proposed species differential (relative to human placental aromatase activity) was not sufficient to explain 'virilisation' of female foetuses at the most crucial period for hyaena sexual differentiation (Conley *et al.* 2007). Collectively these data suggested that mechanisms other than placenta steroid synthesis or metabolism were more likely to be relevant in the development of the external genitalia of the female spotted hyaena.

Female genital development: the relative independence from androgen action

The most direct test of the hypothesis that the external genitalia of the spotted hyaena results from exposure to androgens *in utero* was tested in a series of experiments designed to minimise androgen synthesis while simultaneously antagonising the effects of androgens at the androgen receptor during foetal development (Drea *et al.* 1998). The so-called anti-androgen (AA) therapy was achieved by administering a cocktail of flutamide (an androgen receptor antagonist) and finasteride (a 5α-reductase inhibitor to minimise 5α-dihydrotestosterone synthesis) to females during gestation. It is notable that the androgen receptor of the spotted hyaena does not exhibit constitutive activity in the absence of ligand and ligand activation characteristics are as would be expected from studies in other mammals (Catalano *et al.* 2002). There was no evidence of constitutive or ligand-independent activity.

This AA treatment of pregnant dams did affect clitoral development of foetal and neonatal offspring from these pregnancies, specifically by minimising the unique sex differences normally observed in this species. In control females from untreated pregnancies, the clitoris is slightly shorter and thicker, the glans is less angular, and the opening of the meatus is much wider, than the male penis. Compared to control female hyaenas, those prenatally exposed to AA had an even shorter clitoris, with an even wider glans and a wider more elastic meatus (Drea *et al.* 1998). Likewise, AA-exposed male foetuses had penes that exhibited '…the essential morphological characteristics of the female hyaena clitoris (i.e. a shorter, thicker organ, with a large urogenital meatus and a glans with a rounded contour)'. In other words, exposure of foetuses to AA *in utero* impeded 'masculinisation' in both sexes, effectively 'feminising' the male phallus and exaggerating feminine characteristics of the female phallus (Drea *et al.* 1998). Anti-androgen treatment during female foetal development did not, however, result in the formation of a typical mammalian vagina and vulva, nor did it affect the formation of the pseudoscrotal pouches in females. Of interest from a comparative perspective, scrotal development in macropod marsupials is X-linked and not an androgen-driven event (Renfree *et al.* 2002).

There were additional effects on the organisation of the spotted hyaena phallus seen in response to AA exposure of male foetuses and to androgen exposure of female foetuses that were apparent on histological examination (recently reviewed (Cunha *et al.* 2014)). For instance, the

retractor penis muscles are located ventral to the urethra, whereas the female phallus has retractor clitoris muscles that are located dorsal to the urogenital canal (Cunha *et al.* 2005). This appears to be an androgen-sensitive feature and androgen receptor expression in the retractor penis and retractor clitoris muscles was found to be comparable (Cunha *et al.* 2014). Anti-androgen treatment of pregnant spotted hyaenas effectively relocated the retractor penis muscles of male cubs, shifting them to a dorsal position relative to a more voluminous urethra, mimicking the anatomy of the clitoris (Cunha *et al.* 2005). The development of the prostate and the tunica albuginea enclosing the urethra also appear to be androgen-dependent in males (Cunha *et al.* 2005). Consistent with the response to AA treatment, exposure of a female foetus to a synthetic androgen (mibolerone) resulted in retractor clitoris muscles situated ventral to a 'more compact' urogenital canal (Cunha *et al.* 2014). In aggregate, these observations indicate that the general architecture of the external genitalia of female and male spotted hyaenas develops in a largely androgen-independent fashion, but that androgens influence the subsequent size, shape of the glans and differentiation of the male penis (Glickman *et al.* 2006, Cunha *et al.* 2014) including volume of the urogenital canal. But once again, there is no evidence that the lack of a vulva in female spotted hyaenas derives in any way from exposure to androgens during foetal development as was initially postulated.

Re-evaluation of the endocrinology of the pregnant female

The repository of samples and tissue specimens from the Berkeley Hyena Project continues to generate novel findings, such as those of the possible evolution of the hemochorial placenta in this carnivoran (Funk *et al.* 2019), providing further opportunities to examine the unusual biology of this species. We have recently re-examined the endocrinology of pregnancy in spotted hyaenas using liquid chromatography tandem mass spectrometry (LC-MS/MS). The method was developed with an emphasis on progesterone and 5α-reduced pregnane metabolites (Legacki *et al.* 2016), such as 5α-dihydroprogesterone (DHP). But, it also includes testosterone and oestrone, and was modified to include cortisol and some unique, but potent, 11-oxygenated androgens (Rege *et al.* 2019), such as 11βOH-androstenedione. Serum samples from untreated, pregnant females and from AA-treated pregnancies were re-examined throughout gestation.

Steroid determinations using LC-MS/MS largely confirmed but also significantly extended what had been found previously in maternal blood in pregnant spotted hyaenas (Licht *et al.* 1992, Drea *et al.* 1998, Place *et al.* 2011). There was an increase in progesterone as pregnancy proceeded ($P < 0.01$) to concentrations exceeding 300 ng/mL in one case, averaging over 100 ng/mL overall (Fig. 4A). Testosterone concentrations also increased as pregnancy advanced (Fig. 4C), and androstenedione concentrations declined with time ($P < 0.05$; data not shown), but 5α-dihydrotestosterone was not detected in any samples. The principal oestrogen detected was oestrone (Fig. 4D), the concentrations of which increased ($P < 0.01$) in concert with testosterone ($r = +0.83$, $P < 0.01$) in the last 2 months of gestation. Cortisol (Fig. 4E) and corticosterone (data not shown) increased in the final month of gestation ($P < 0.01$), as did the 5α-reduced pregnanes,
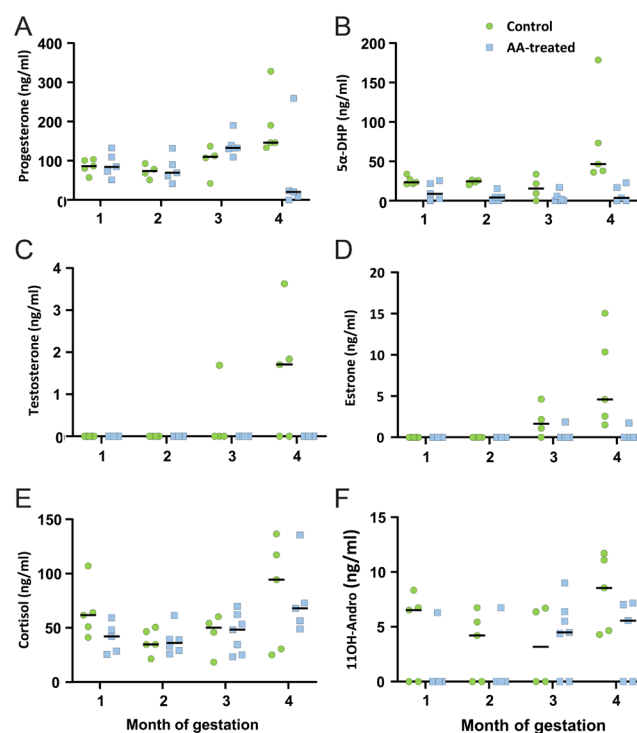
Figure 4

Steroid concentrations (ng/mL) in serum from pregnant spotted hyaenas sampled longitudinally throughout pregnancy and analysed by liquid chromatography tandem mass spectrometry in control females ($n = 4$–5, circles) and females receiving anti-androgen (AA) therapy ($n = 5$–6, squares) consisting of flutamide (androgen receptor antagonist) and finasteride (5α-reductase enzyme inhibitor). Samples were assigned to months 1–4, though the duration of gestation in spotted hyaenas in the colony is slightly less than 4 months (111 days, control; 114 days, anti-androgen-treated (AA)). A, progesterone; B, 5α-dihydroprogesterone (5α-DHP); C, testosterone; D, oestrone; E, cortisol; F, 11OH-androstenedione (11OH-Andro).

DHP (Fig. 4B, $P < 0.01$) and allopregnanolone (data not shown), the concentrations of which often exceeded 50 ng/mL. Adrenal androgens, 11βOH-androstenedione (Fig. 4F) and 11keto-testosterone (data not shown), were detected and at substantially higher concentrations than are found in most other mammals (Rege *et al.* 2019).

Consistent with the expected inhibition of 5α-reductase enzyme activity by finasteride in the treatment cocktail, DHP was decreased by AA treatment ($P < 0.01$, Fig. 4B). More surprisingly, AA treatment markedly depressed testosterone ($P < 0.05$, Fig. 4C) and oestrone ($P < 0.01$, Fig. 4D) concentrations in late gestation. In addition, a re-examination of the duration of gestation in control and AA-treated females, when mating had actually been observed, demonstrated that AA treatment significantly delayed parturition by 3 days on average (111.0 ± 0.4 vs 114.0 ± 1.1, control vs treated, respectively, $P < 0.01$). In fact, it is conceivable that gestation might have been even more extended if AA treatment had not been discontinued near term to avoid potentially adverse sequelae of prolonged gestation. It is curious that oestrone concentrations increase during pregnancy when placental aromatase activity is decreasing (Licht *et al.* 1998, Conley *et al.* 2007) to extremely low levels at parturition (Yalcinkaya *et al.* 1993). The apparent effects of AA treatment on testosterone and oestrone concentrations in late gestation are equally difficult to explain but insight might be provided by studies conducted on sex hormone binding globulin (SHBG) in the Hyaenidae and in samples from pregnant spotted hyaenas.

An unusual SHBG in spotted hyaenas: impact on androgen and oestrogen metabolism and action

Sex hormone-binding globulins are secreted by the liver and can be found circulating in most sexually mature mammals (Hammond 2016), including spotted hyaenas (van Jaarsveld *et al.* 1992), influencing the access of sex steroids to target tissues (Siiteri *et al.* 1982, Westphal 1986). Dependent on their concentrations and affinity for specific steroids, SHBGs can slow sex hormone clearance and decrease free concentrations, thereby affecting steroid access to target tissues. Recent studies on SHBG in all four extant hyaenid species have highlighted key differences in the properties of SHBG in spotted hyaenas compared with their relatives, brown hyaenas, striped hyaenas and aardwolves (*Proteles cristatus*) (Hammond *et al.* 2012). Although SHBG sequences are not highly conserved amongst mammals, even within the same taxonomic order (Lee *et al.* 1997), SHBG shares >98% identity amongst the four extant hyaenid species. Nevertheless, the SHBG precursor signal sequence that regulates secretion in spotted hyaenas is quite distinct. It lacks three, conserved leucine residues that limit its production, and likely explains the five- to ten-fold lower circulating SHBG concentrations seen in female spotted hyaenas when compared to those seen in the other three hyaenid species (Hammond *et al.* 2012). It is also notable that the SHBGs of all four hyaenids have a strikingly high affinity for dehydroepiandrosterone and oestrone, a characteristic not shared by other mammals. Furthermore, the apparent affinity of oestrone for the spotted hyaena SHBG is five-fold higher than that for the striped hyaena and the aardwolf (Hammond *et al.* 2012). This unusual steroid-binding specificity of hyaenid SHBGs cannot explain the reproductive and behavioural phenotypes found only in the spotted hyaena. However, the much lower SHBG concentrations seen in female spotted hyaenas means that oestrone and dehydroepiandrosterone can more effectively displace biologically active sex steroids from their SHBG steroid-binding sites, potentially increasing their free concentrations. The spotted hyaena *Shbg* gene also seems to be very sensitive to oestrogens because treatment of non-pregnant females with oestradiol valerate increased their plasma SHBG concentrations over a 2-week period by >20-fold from 35 ± 6 to 715 ± 31 nM. Of particular interest, the concentrations of SHBG in spotted hyaenas were 50-fold higher in late-gestation compared with those in non-pregnant females, increasing from 732 ± 325 to 2418 ± 80 nM between 65 and 95 days of gestation, coincident with increases in both oestrone and testosterone. This extraordinarily robust, pregnancy-related increase in plasma SHBG is not seen to a comparable degree in other species, and exceeds that seen in human pregnancy (Pearlman *et al.* 1967, Uriel *et al.* 1981) by almost an order of magnitude. We have also observed that treatment of pregnant females with the aromatase inhibitor letrozole (to inhibit oestrogen secretion at different stages of gestation) suppressed maternal SHBG concentrations (Supplementary figure 1). It is conceivable that SHBG secretion is also responsive to AA-treatment. In one pregnant female, SHBG concentrations increased >50-fold in late gestation but, in a subsequent pregnancy in the same female under AA treatment, no increase was observed (G L Hammond, S E Glickman & N J Place, unpublished observations). Thus, the lower oestrone and testosterone concentrations seen late in gestation in AA-treated females may be related to low SHBG

concentrations and higher rates of oestrone clearance. It seems likely that the AA effect on plasma SHBG levels in late gestation also accounts for the surprisingly lower testosterone concentrations mentioned above.

Post-natal development and androgen influences

Female spotted hyaenas are also interesting as social carnivorans because of their unusual behavioural repertoire, social dominance and size advantage. Aggression emerges unusually early, immediately after birth, in both sexes (Frank *et al.* 1991), and declines within the first week of life, as social play emerges and rank relations are rapidly established between siblings (Drea *et al.* 1996). Thereafter, maternal rank and gestational androgen concentrations positively correlate with aggression among juvenile offspring and their peers (Dloniak *et al.* 2006*b*). Clans are organised by a well-defined, matriarchal hierarchy, in which females routinely dominate adult, immigrant males (Frank 1986, Smale *et al.* 1993) and offspring of both sexes acquire a rank, initially just below that of their mother. Because daughters remain in their natal clan, early maternal rank determines their life-time trajectory, whereas sons generally disperse, lose their matrilineal status, and cue for their rank as adults after joining other clans (Smale *et al.* 1993, East & Hofer 2001) Still, even after dispersal, sons somehow gain fitness benefits from their mother's high rank (Honer *et al.* 2010).

In searching for endocrine correlates of social behaviour, it was found that androstenedione concentrations throughout juvenile development are higher in females than males, and relatively constant (Drea *et al.* 1998), although field studies have not always been in agreement (Goymann *et al.* 2001). A failure to detect differences under such circumstances might not be surprising given the difficulties posed by field conditions and the more constrained, albeit controlled, environment afforded by a captive colony. In contrast to androstenedione, oestrogen concentrations increase in females after a year of age and they achieve their first pregnancies around 2 years of age in captivity (Glickman *et al.* 1992*b*), as in the wild (Frank 1986). Testosterone concentrations increase progressively in males over the same time frame (Glickman *et al.* 1992*b*). Importantly, aggressively mediated dominance of females over males is reversed by ovariectomy at an early age (Baker 1990). Specifically, interactions were observed (18:00–22:00 h) between males and females (24–43 months in age) in

two separate cohorts (seven females/three males and five females/five males). In addition, two females in the second cohort were ovariectomised at 4–6 months of age. Dyadic aggression (bites, chasing/lunging attacks, displacement of one by another, threatening postures) and submission (avoidance, appeasement postures, crawling) was recorded. Of the mixed-sex interactions observed and averaged across both cohorts, females were 7.5 times more often the aggressors than were males ($P < 0.05$), and males displayed submission three times more often than females ($P < 0.06$). In contrast, ovariectomised females were less often aggressors than intact females in mixed-sex interactions ($P < 0.05$). Ovariectomised females also tended to experience more aggression from a male ($P < 0.1$) and males were significantly more submissive towards intact than ovariectomised females ($P < 0.01$). There was no observed effect of ovariectomy on female to female interactions (Baker 1990).

Contrary to the predominant mammalian paradigm, females are also larger and heavier than males at equivalent ages (Frank 1986, Swanson *et al.* 2013). These unusual physical characteristics were thought to diverge as the sexes reached the initiation of pubertal development. However, careful longitudinal studies in captivity have demonstrated that the sex disparity in size becomes evident earlier than this, at around a year of age, by which time (and thereafter) females are about 20% heavier than their male peers (Glickman *et al.* 1992*b*). Field studies are generally consistent with this finding, even if the differential is not as great (Swanson *et al.* 2013, Drea *et al.* 2018). The lack of a relationship to the onset of puberty suggests this phenomenon is not related to gonadal steroid secretion and growth factor concentrations have been investigated accordingly. Indeed, the female size advantage corresponds with elevated insulin-like growth factor type 1 (IGF-1) concentrations that are higher in females than males ($P < 0.05$) from 6 to 12 months (females: 833.3 ± 51.7; males: 613.3 ± 86.0 ng/mL), after which concentrations decline in both sexes from a peak of 723 ± 57 ng/mL when averaged together (E M Spencer, P Licht & S E Glickman, unpublished observations). It is interesting to note that the post-pubertal increase in testosterone in males does not overcome their reduced body weight relative to females (Glickman *et al.* 1992*b*).

Other physical traits of spotted hyaenas and their endocrine correlates also merit mention. Notably, there is considerable development of a single, inguinal pair of nipples during the neonatal period. Intact females have larger nipples than do males, from as early as a year of age, but if females are gonadectomised in the first year

of life, there is a lack of dimorphic nipple development (Glickman *et al.* 1992*b*). Similarly, females have a larger opening of the clitoral preputial meatus than males do in the penile preputial meatus, which is evident by 2 months of age (Drea *et al.* 1998). The sex difference is diminished by gonadectomy, but there is only a marginal effect of gonadectomy on meatal length in males (Glickman *et al.* 1992*b*, 1998). It appears likely that oestrogens promote both nipple and clitoral development (Glickman *et al.* 1998). Curiously, the glans of both sexes are also covered by spines and papillae, specialised male adornments in other mammals that are typically under post-natal androgenic control (Aronson & Cooper 1967, Drea & Weil 2008). AA treatment modified the appearance of these structures in males and females, implicating an organisational role for androgens in both sexes (Drea *et al.* 1998), but juvenile gonadectomy had minimal effects (Glickman *et al.* 1998).

Sexual dimorphisms in the brain have also been observed and documented in spotted hyaenas (Fenstemaker *et al.* 1999), as they have in spinal motoneurons (Forger *et al.* 1996). A sexually dimorphic nucleus exists in the medial pre-optic area and anterior hypothalamus of the spotted hyaena that is twice as large in males as in females. This is a relatively modest difference compared to many mammals, and other brain regions were less clearly dimorphic in spotted hyaenas than they are in most mammals (Rosen *et al.* 2006). Still, the dimorphisms that do exist may have functional relevance. Notably, gonadotropin secretion does exhibit clear, sexually divergent characteristics. Baseline concentrations of luteinising hormone (LH) were higher in female than male hyaenas and maximum concentrations of LH secreted in response to a GnRH challenge were higher in females than males (Place *et al.* 2002). Anti-androgen treatment during pregnancy had no observable effect on baseline concentrations of LH in offspring of either sex from those dams. Nevertheless, the LH response to GnRH stimulation in both males and females born from AA-treated pregnancies was increased compared with treatment-naïve contemporaries, and peak concentrations of LH were maintained 3–4 times longer in females from treated compared with untreated pregnancies. GnRH stimulation elicited significant increases in androstenedione and testosterone in both male and female hyaenas, whether born of naïve (control) or AA-treated dams (Place *et al.* 2002). While mechanisms remain to be defined, it is clear that reducing androgen exposure of hyaenas *in utero* influences both the differentiation of the genitalia and the development of the hypothalamic–pituitary axis long after birth.

It is equally as significant that the influence of AA exposure *in utero* on the genitalia of neonatal female and male spotted hyaenas persisted post-pubertally, until 30 months of age (adulthood) when profound consequences on reproductive success became apparent (Drea *et al.* 2002). Whereas the enduring feminising effects on the penis ultimately impeded intromission, preventing successful copulation, those on the clitoris facilitated live births in first-time mothers. As adults, those females exposed to AA treatment during development had a clitoral length that was proportionally reduced, the circumference of their clitoral glans was further increased and the clitoral meatus was even larger and more elastic than is typical for females from untreated pregnancies (Drea *et al.* 2002). Pregnancies were established in these experimental females, as in treatment-naïve contemporaries. Although androstenedione concentrations in the first few months of life were lower in female cubs from AA-treated vs untreated dams (Drea *et al.* 1998), no differences in steroid concentrations were noted between them as adults. These females, exposed to AA therapy during their development *in utero*, delivered their first cubs without a single stillbirth, whereas 60% of first cubs were stillborn when delivered by contemporary controls (Drea *et al.* 2002). Elasticity and dispensability of the clitoral meatus likely facilitated delivery, which is also likely promoted by placental relaxin secretion that increases in late gestation (Steinetz *et al.* 1997) as does oestrone. Collectively, the implication of these experiments is that natural exposure to androgen during normal foetal development influences both males and females throughout life, incurring a significant, initial reproductive cost in the case of females (Drea *et al.* 1998, 2002).

The aggressive and dominance behaviour of females from AA-treated dams was also affected as adults. As infants, aggressive interactions between siblings appeared less intense or less prolonged, such that the otherwise immutable dominance relationship between a set of twin sisters flipped repeatedly over the years (C M Drea, L K Greene, M L Weldele, and S E Glickman, unpublished observations). Again, although androstenedione concentrations in the first few months of life were lower in female cubs from AA-treated vs untreated dams (Drea *et al.* 1998), no differences in steroid concentrations were noted between them as adults, suggesting organisational vs activational effects. As adults presented with competitive feeding tests in mixed-sex dyads, an 'experimental' female exposed prenatally to AA tolerated a male eating at one of two feeding stations (each with an attached bone), but the contemporary control females aggressively

fended their male companion away from both stations (M L Weldele & S E Glickman, unpublished observations), as previously documented (Jenks *et al.* 1995). Both control and experimental females always won against males when there was a single bone available per pair. There appears to be strong selective pressure for female aggression: within the social hierarchy of the spotted hyaena, in which dominance acquisition is aggressively mediated (Frank 1986), low-ranking females incur a fitness cost relative to high-ranking females (Frank *et al.* 1995).

Summary and conclusions

The reproductive biology of the spotted hyaena, as reviewed above, illustrates how narrow our understanding of the biology of sex really is and how adaptable it can be while remaining evolutionarily successful. The spotted hyaena does not redefine sex in a generalisable sense, but emphasises that definitions are only relevant when constructed species by species. The spotted hyaena also illustrates how male-centric and inadequate our generalised definition of genitalia are. Lacking a perineal vulvar opening has imposed functions on the clitoris that defines the anatomical extremes this organ can attain in order to preserve fertility. The elaborate development of the spotted hyaena clitoris, so often defined as 'masculinised', is largely independent of, and notably protected from, androgenisation during its development when androgenisation is otherwise so important in other processes including neural organisation. Perhaps it is not too surprising then that the androgen receptor itself appears to be unexceptional in the spotted hyaena, functioning on a biochemical level much as it does in other species. The endocrinology of the pregnant spotted hyaena is unusual in terms of the increasing and high peak concentrations of progesterone, testosterone and oestrone. The properties of SHBG in spotted hyaenas is also notable in exhibiting characteristics not seen in other species, and appears to have a major influence on steroid concentrations during late gestation. A marked increase in circulating SHBG concentrations during pregnancy appears to promote high concentrations of both testosterone and oestrone, presumptively by decreasing clearance. At the same time, SHBG concentrations during pregnancy are clearly sensitive to oestrogens and perhaps even to androgens. The ultimate effects of this steroid environment influence behaviours post-natally which has an equally important impact on reproductive success. In a broader sense, exposure of pregnant dams and their

developing foetuses to AA treatment further illustrates, in a unique way, just how delicate an anatomical and physiological balance is struck in this species. Relieving female foetuses of the anatomical burden of androgens increased their reproductive success during birth (especially their first) but precluded males exposed as foetuses from successfully mating females after reaching sexual maturity. The spotted hyaena demonstrates how the more common anatomic and physiologic paradigms are not essential for evolutionary fitness and survival. Reproductive strategies do not have to make sense to us if they create viable, reproductively successful offspring. In fact, variations in reproductive biology provide confidence that adaptation and continued reproductive success are possible in ever-changing environments, and only those species that can adapt rapidly enough will be guaranteed a chance of survival.

The studies described above expose weaknesses in our narrow understanding of the mechanisms of sexual differentiation, particularly as regards establishment of the female form (Drea *et al.* 1998), and illustrate how variations in reproductive biology can be adaptable and evolutionarily successful, in this case exemplifying sexual selection operating in females. Despite displaying a suite of 'masculinised' traits, the female spotted hyaena does not defy any standard definition of sex (sex chromosome constitution, gametes, gonads, genitalia or phenotype). She has two X chromosomes, a functional ovary with a Müllerian ductal system, a uterus and a vaginal cavity even if not engaged with a penis during copulation, and she engages in female-typical mating behaviour, successfully develops a placenta, bears young, nurses them for an exceptionally long period and shows appropriate maternal behaviour. Moreover, she is certainly not alone in possessing a hypertrophied clitoris (e.g. proboscideans, elephant (Glickman *et al.* 2005); primates including platyrrhines (Campbell 2017, Carosi *et al.* 2020), strepsirrhines and others (Hill 1953, Dixson 1998, Drea & Weil 2008); viverrids (Hawkins *et al.* 2002); talpids (Sinclair *et al.* 2017)), suggesting that successful birth via this structure is what, ironically, derails notions about her femininity. These incongruities illustrate the inadequacy of a male-centric definition of reproductive biology.

The environment of the captive colony of spotted hyaenas was necessarily artificial, physically and socially. However, it provided a level of control over factors potentially influencing and possibly obscuring underlying biological differences that, therefore, may be difficult to observe otherwise. This may explain apparent discrepancies in data from colony and field studies.

Body size is perhaps a case in point. While many studies report that females are heavier than males, the differentials averaging perhaps 10% in the field (Drea *et al.* 2018) are much less than the 20% observed in the colony (Glickman *et al.* 1992*b*). Whether or not this reflects the controlled environment or a plentiful diet, it still suggests an underlying biological difference between the sexes. Moreover, no field data exist indicating that spotted hyaena males are heavier on average than females, which is in itself significant enough. Although the current review has focused primarily on reproductive endocrinology, the captive colony enabled studies on a broad range of topics in social behaviour, behavioural development and social psychology, including dominance and aggression (Zabel *et al.* 1992, Jenks *et al.* 1995, Wahaj *et al.* 2007), behavioural flexibility (Holekamp *et al.* 2013), prosociality and socialisation (Pedersen *et al.* 1990, Rifkin & Glickman 2004), vocal and olfactory communication (Woodmansee *et al.* 1991, Drea *et al.* 1996, Mathevon *et al.* 2010), feeding (Binder & Van Valkenburgh 2000), cognition (Yoerg 1991, Drea & Carter 2009) and personality (Gosling 1998), as well as other biological phenomena such as immune function (Flies *et al.* 2012). It is hoped these observations will find their way into future reviews, updating and expanding on those already published (Glickman 1995, Glickman *et al.* 1997, 2006, Drea & Frank 2003, Cunha *et al.* 2014). But it is also hoped that the lessons from these investigations convincingly underscore the value of comparative biology and the pressing need to study exotic species before they become extinct.

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