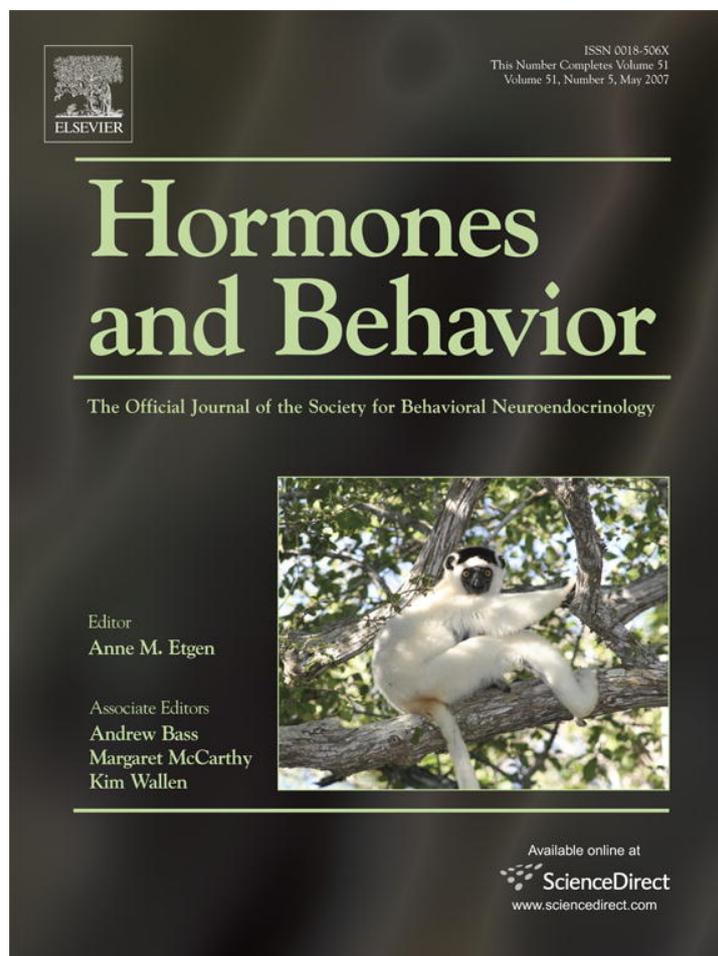


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Transient genital abnormalities in striped hyenas (*Hyaena hyaena*)

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Abstract

The highly masculinized genitalia of female spotted hyenas *Crocuta crocuta* is unique among mammals: *Crocuta* have no external vagina so urination, penile intromission and parturition take place through the clitoris, which mimics a fully erectile male penis. Among hyenids, virilization of external female genitalia has previously been observed only in *Crocuta*, so functional explanations of masculinization have focused on aspects of social ecology unique to the species. Here we first show that the striped hyena *Hyaena hyaena* exhibits both unusual similarity in male and female androgen concentrations and transient genital anomalies characterized by a convergence in genital appearance among young males and females. We then evaluate hypotheses regarding the evolution of genital masculinization in the Hyaenidae and other taxa. *Hyaena* are behaviorally solitary, so discovery of unusual genital development patterns in this species does not support any current evolutionary models for masculinization in *Crocuta*, which all rely on the trait originating within a highly social species. Some hypotheses can be modified so that masculinization in *Crocuta* represents an extreme elaboration of a preexisting trait, shared as a homology with *Hyaena*.

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Introduction

Although the three other extant hyenids are well studied, few studies have focused on striped hyenas *Hyaena hyaena*. These works indicate that *Hyaena* live in stable, polyandrous spatial groups with multiple adult males sharing a territory with a single reproductively mature female (Wagner, 2006; Wagner et al., submitted for publication). Within spatial groups, *Hyaena* are behaviorally solitary with low levels of direct interaction and solitary foraging. In contrast to *Hyaena*, the spotted hyena *Crocuta crocuta* is highly gregarious, living in large, matrilineal, territorial social groups and communally hunting or scavenging large mammals (Kruuk, 1972; Frank, 1986a,b).

Among carnivores, genital masculinization has been reported only in the spotted hyena and fossa *Cryptoprocta ferox* (Hawkins et al., 2002). In *Crocuta*, external female genitalia are completely virilized and the mechanistic and evolutionary

pathways underlying these traits have become, literally, a textbook example of mammalian genital masculinization. Models of female spotted hyena dominance and masculinization have assumed that unusual patterns of genital development and differentiation originated in *Crocuta* in response to selection pressures unique to the species. For example, Frank (1996; see Fig. 3) illustrates an evolutionary scenario in which masculinization arose in *Crocuta* from a normal ancestral female as a nonadaptive consequence of selection for female aggression associated with the evolution of communal hunting and feeding. Such nonadaptive hypotheses argue that masculinization is an unselected consequence of high fetal exposure to the androgens that promote aggression in adult females (Racey and Skinner, 1979) or siblicide in same sex litters (Frank et al., 1991; Frank, 1997). In the former, selection may have favored androgen-mediated mechanisms promoting adult female dominance and aggression that allow females to provide better access to food for their cubs. In the latter, female genital masculinization arose as an unselected consequence of androgen-mediated neonatal aggression and siblicide as a facultative adaptation to fluctuations in food resources. These

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dominant nonadaptive arguments both require that masculinization in spotted hyenas developed within a social system in which aggression carries fitness payoffs large enough to outweigh the reproductive costs associated with genital masculinization and the correlated modifications to the reproductive tract: in *Crocuta*, over 60% of cubs of first time mothers are stillborn and primiparous mothers commonly die while whelping (Frank et al., 1995). Adaptive hypotheses suggesting that the female phallus evolved as an important element in the *Crocuta* greeting ceremony (Kruuk, 1972) or that female genital masculinization prevents unwanted copulations (East et al., 1993) have been criticized for lack of adequate empirical support (e.g. Muller and Wrangham, 2002) and being unlikely to compensate for the associated risks of dystocia and anoxia during labor. However, sexual mimicry through masculinization as a defensive adaptation to infanticide (Muller and Wrangham, 2002) remains a viable hypothesis if high ranking females commonly kill the female offspring of lower ranking females (which has not been demonstrated).

In normal mammalian sexual differentiation, chromosomal sex determines gonadal sex by directing the embryonic development of ovaries or testes (Jost, 1972; Wilson et al., 1981). Sexual differentiation and the development of the male phenotype are then induced by the virilizing effects of androgens produced in the fetal testes. While exogenous estrogens and androgens are both known to cause masculinization, fetal ovaries do not normally produce estrogens until after the critical period for sexual differentiation of indifferent reproductive tissues (Breedlove and Hampson, 2002). Accordingly, genital masculinization in genotypic females would occur in response to maternal sex steroid production, and a fetal capacity to respond to them, or by atypical production of steroids by the female fetus. In *Crocuta*, male phenotypic development of female genitalia (i.e. masculinization) has been ascribed to unusually high levels of maternal ovarian androgen production and a placenta which, in contrast to other mammals, appears to promote enzymatic conversion of circulating androstenedione to testosterone via comparatively high 17- β hydroxysteroid dehydrogenase activity and low aromatase activity (Yalcinkaya et al., 1993; Licht et al., 1992; Glickman et al., 1987). The resulting fetal exposure to large amounts of potent maternal androgens became the leading mechanistic explanation for female masculinization in *Crocuta*.

Contrary to predictions under this model, experimental treatment of pregnant *Crocuta* females with anti-androgens, to block the actions of masculinizing hormones in utero, still produced female offspring with virilized genitalia, though genitalia were more “feminized” in both sexes (Glickman et al., 1998; Drea et al., 1998). These results suggest that female genitalia in *Crocuta* are not completely virilized by androgenic mechanisms and that these phenotypes may be mediated by direct genetic effects (Drea et al., 1998; Place and Glickman, 2005) independent of androgen levels, as in other models of sexual differentiation and development in birds (Wade et al., 2005), mice (Carruth et al., 2002) and wallabies (O et al., 1988; Renfree and Short, 1988).

All current evolutionary models of masculinization in spotted hyenas implicitly or explicitly assume that a sex-reversed hormonal profile developed in a species-specific social context, where high levels of aggression promote cub survival. Given the lack of support for a clear link between masculinization and high maternal androgen levels (prepartum), it is important to note that no data have directly rejected the hypothesis that masculinization in this and other species may arise simply through modifications in the timing and degree of androgen secretion by the gonads during development (pre- and/or postpartum) (Hawkins et al., 2002; Goymann et al., 2001; van Jaarsveld and Skinner, 1991). Consequently, androgenic mediation of genital masculinization cannot be ruled out as a mechanism of masculinization in response to such selection pressures.

Hyaena share few features of social ecology with the gregarious *Crocuta*. The selection pressures argued to drive masculinization in *Crocuta* are weak or absent in *Hyaena*. As solitary foragers, direct interactions when feeding are rare, so *Hyaena* experience little aggression in foraging situations, unlike *Crocuta*, there is no evidence of siblicide or neonatal aggression from captive studies (Rieger, 1978), and multi-female groups (which could promote female infanticide) have not been reported. Existing evolutionary models of genital development in *Crocuta* would uniformly predict normal genitalia in striped hyenas. Here we present data on unusual patterns of genital development and circulating androgen profiles in *Hyaena* and consider the implications of these findings on the contemporary understanding of the evolution of sexual mimicry in hyenids and the female genital masculinization, aggression and behavioral dominance characteristic of *Crocuta*.

Methods

In a field study of the striped hyena in Laikipia District, Kenya, we captured and immobilized 63 individuals in 79 captures (Wagner, 2006). We anesthetized hyenas with an IM combination of ketamine HCl (3.6 mg/kg) and medetomidine HCl (0.06 mg/kg). We sexed each *Hyaena* and assigned it to an age class based on known dates of birth or estimates from body measurements, weight and tooth wear (cub: < 6 months; juvenile: 6 months to 1 year; young adult: 1 to 3 years; adult: 3+ years). We drew blood from the jugular vein into evacuated tubes and isolated plasma by centrifugation, freezing all samples within 12 hours of collection (Packer et al., 1991). All animal handling procedures were approved by Montana State University's Institutional Animal Care and Use Committee and adhered to NIH standards.

We used a previously validated radioimmunoassay (RIA) to measure testosterone concentrations in plasma samples (cubs: females $n=2$; juveniles: females $n=3$, males $n=6$; young adults: females $n=11$, males $n=6$; adults: females $n=19$, males $n=32$), as detailed in Licht et al. (1982, 1992). Briefly, plasma was extracted in diethyl ether (Sigma-Aldrich, St. Louis, MO) and immediately dried under N_2 . The samples were resuspended in phosphate buffered saline with gelatin and separated into duplicate 50 μ l aliquots for RIA. After a one hour equilibration (at 37 °C) of the extract with tritiated testosterone (New England Nuclear, Boston, MA) and antiserum (T3-135, Endocrine Sciences, Calabasas Hills, CA), dextran-coated charcoal was added to remove unbound tracer. The supernatant recovered by centrifugation was counted by liquid scintillation. Cross reactivity of the testosterone antibody with dihydrotestosterone was 44%. The minimal detectable limit of testosterone was 0.13 ng/ml. Samples were assayed in five groups, all including both males and females. Testosterone measurements were repeatable across duplicate

assays run on a set of 27 samples ($r^2=0.988$, $F=2060.9$, $p<0.001$). We assayed samples collected at two separate captures for 12 individuals (1 female cub; 1 male and 2 female young adults; 5 male and 3 female adults) and at three capture events for 2 male and 1 female adults. For each of these individuals, we used the average testosterone concentration in statistical analyses to avoid pseudoreplication (imbalanced data did not allow us to use the alternative method of leaving values unpooled with individual identity as a random effect). All samples from the same individual were assayed in the same group. We analyzed log-transformed testosterone measurements by factorial ANOVA to test for effects of sex and age class.

Results

We found an unusual convergence in the appearance of genitalia in young male and female *Hyaena* (Fig. 1) between one and 18 months of age (females $n=4$, males $n=6$), with traits not reported for *Crocuta* or any other carnivore. Specifically, young females develop prominent swellings anterior to the urogenital opening that resemble a scrotum, despite their placement, and a pronounced conical protrusion with the urogenital opening on the posterior slope. Young males exhibit genital swellings between the scrotum and penis that resemble labial folds. For both sexes, these genital anomalies were consistently apparent in all sexually immature individuals older than a few weeks (*Hyaena* reach sexual maturity at 2–3 years of age; Rieger, 1981). These characteristics were transient; they did not appear in newborns (females $n=1$, males $n=3$), which had typical mammalian genitalia, or in adults (females $n=8$, males $n=7$). In adult females, the urogenital protrusion is far less prominent, the opening of the urogenital

canal is perpendicular to the body and the swelling of the labial folds diminishes completely, with only a patch of dark skin remaining. In adult males, the genital swellings are reduced and partially integrated into the raphae at the base of the testes, with a small, hairless, concave fold remaining. These minor residual effects were apparent in all sexually mature adults, which otherwise had typical mammalian genitalia.

Circulating testosterone levels (Fig. 2) were higher in males than in females ($F=21.03$, $p<0.001$, $df=1$), with no detectable effect of age class ($F=1.39$, $p=0.258$, $df=2$) and no detectable sex-age interaction ($F=1.14$, $p=0.329$, $df=2$). Back-transformed mean circulating testosterone in females was 0.29 ng/ml (95% CI=0.24–0.35, SE=1.09, $n=26$) and 0.77 ng/ml in males (95% CI=0.59–1.00, SE=1.14, $n=34$). Plasma was available for only one female cub from two capture events. Her testosterone concentrations were not included in the analyses but are included here for comparison: 0.21 and 0.15 ng/ml.

Discussion

In other studies of genital masculinization in mammals, authors have suggested, but not directly rejected, the hypothesis that mammalian genital masculinization in those species may be a consequence not of qualitatively unique mechanisms, but of simple modifications in the timing and degree of fetal androgen production and exposure during pre- and postpartum development. In this study, we did not anticipate the transient genital convergence found in striped hyenas and our work was not designed to collect the fine grained data needed to adequately test

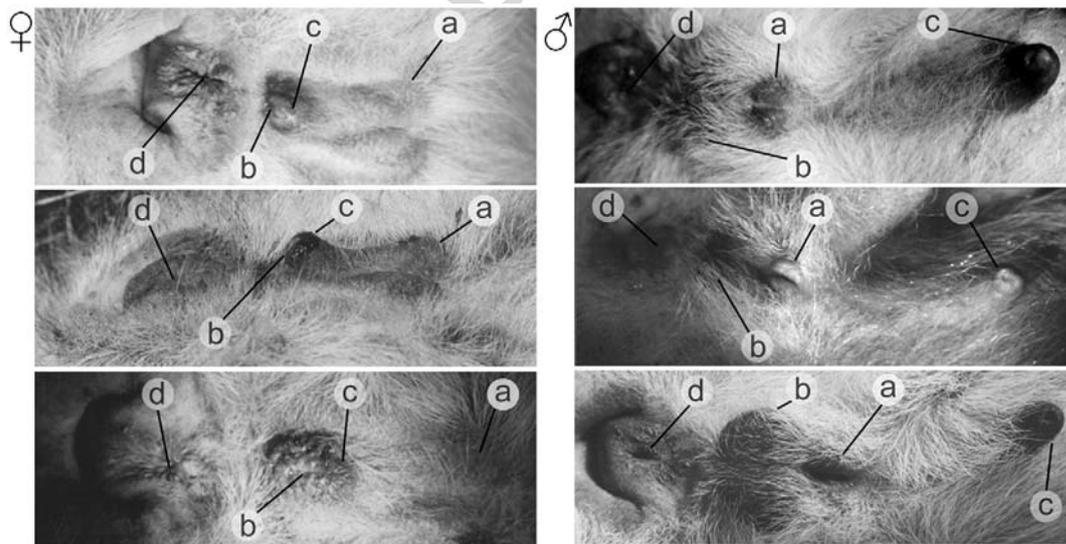


Fig. 1. Transient and convergent genital characteristics of female (left) and male (right) striped hyenas. Young striped hyenas between 1 and 18 months of age develop major genital structures that are ambiguous with respect to gender, compared to typical mammals. Convergent characteristics are apparent by 3 months of age (top figures) and most prominent at around 12 months (middle figures). Young females develop prominent, dark, hairless labia-like folds (a) anterior to the vagina (b) and a prominent genital protrusion just anterior to the urogenital opening (c); (d) anus. Young males develop smooth, hairless, pre-scrotal skin folds (a) lateral to the raphae at the junction between the scrotum (b) and penis (c); (d) anus. By 24 months of age (bottom figures), these transient and ambiguous genital structures are greatly diminished and genital appearance becomes more dimorphic. In mature females, only faint, dark skin patches (a) mark the previous position of the labia-like folds and the protrusion around the urogenital opening is no longer apparent (c). In mature males, the formerly prominent pre-scrotal skin folds are reduced to a small, concave skin patch (a) at the base of the testes. Images are oriented posterior to anterior, left to right. Photographic angles best illustrate the typical characteristics of each sex at each age.

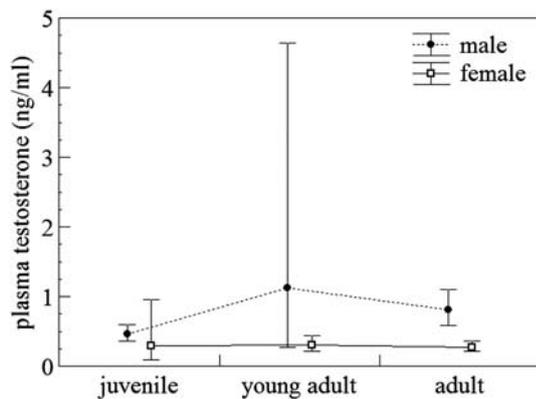


Fig. 2. Back-transformed mean plasma testosterone concentrations in *Hyaena* by sex and age class. Vertical bars denote 95% confidence intervals. Two females were found to be pregnant by ELISA of serum progesterone (11,054 and 22,019 pg/ml, compared to mean adult female progesterone levels of 5,835 ± 1,062 pg/ml), but corresponding testosterone levels were not outside the range for other females, so they were included. Inclusion or removal of these females did not affect inferences (with $\alpha < 0.05$) for any statistical test.

for correlated changes in genital morphology and androgen concentrations during juvenile development. This is an important limitation of our data. Nevertheless, because convergence in genital appearance in *Hyaena* occurs several weeks postpartum, our findings also suggest that maternal androgens may not be wholly responsible for the unusual patterns of genital development observed. Focusing purely on maternal androgens may then limit our understanding of the mechanisms underlying genital development in masculinized species.

Although *Hyaena* genital appearance (at any age) does not mirror the extraordinarily masculinized genitalia found throughout life in *Crocota*, the anomalous structures in young *Hyaena* clearly represent unusual patterns of reproductive development

in a second hyenid. These traits reflect the transient nature of female genital masculinization observed in the fossa (Hawkins et al., 2002), a closely related species. Fundamentally, masculinization of female genitalia in these species represents a deviation from mammalian norms. Accordingly, attempts to elucidate the underlying mechanism have sought to identify correlated ecological and endocrine deviations from mammalian norms (e.g. sex-role reversals in androgen profiles, sex-role reversals in behavior, unique selective pressures related to foraging ecology). Sexual development patterns in *Hyaena* clearly deviate from mammalian norms but, superficially, testosterone profiles appear in line with mammalian norms because circulating concentrations in males are higher than in females. However, testosterone levels in normal mammals (Table 1) are typically 5 to 100 times higher in males than in females. In *Hyaena*, the male to female ratio was only 2.66 (Fig. 2, Table 1), well below mammalian norms. The back-transformed 95% CI for the difference between male and female mean testosterone levels was 1.89–3.73 ($df=58$). That is, the ratio of testosterone levels in *Hyaena* males to females is 3.73 or less with 95% confidence, so the normal mammalian sex bias in testosterone levels is unusually muted in *Hyaena*. This reduced sex bias in testosterone in *Hyaena* (a nonseasonal breeder) is shared by other masculinized species outside of reproduction-related phases (i.e. Hawkins et al., 2002; Goymann et al., 2001; van Jaarsveld and Skinner, 1991; Whitworth et al., 1999). This pattern has not previously been recognized, so its significance has not been considered. However, it is striking that a muted sex bias in testosterone is shared by those species with muted sexual dimorphism. Testing exclusively for intersex differences of zero may not be uniformly appropriate and sensitive for finding biologically significant deviations from mammalian norms when those norms are actually far higher.

Table 1

Circulating testosterone concentrations in adult male and female mammals in species with genital masculinization of females and in normal species (lacking masculinization)

	Concentration (ng/ml)		M:F ratio (min–max)
	Male	Female	
<i>“Normal” species</i>			
Human (<i>Homo sapiens</i>)	4.62–6.46 ¹	0.209–0.60 ¹	7.709–32.30
Chimpanzee (<i>Pan troglodytes</i>)	4.95 ¹	0.419–0.71 ²	6.979–12.07
Rhesus macaque (<i>Macaca mulatta</i>)	4.579–17.76 ¹	0.209–0.84 ^{1,3}	5.449–25.12
Rat (<i>Rattus</i> sp.)	2.159–3.70 ¹	0.17 ¹	12.659–21.77
Natal rat (<i>Praomys natalensis</i>)	4.2 ³	0.4 ³	10.50
Rabbit (<i>Leporidae</i> sp.)	0.59–5 ³	0.059–0.1 ³	59–100
Brown hyena (<i>Parahyaena brunnea</i>)	15 ⁴	0.52 ⁴	28.85
<i>Masculinized species</i>			
European mole (<i>Talpa europaea</i>)	1.75/7.47 ⁵	0.799–1.44 ⁵	1.229–2.22/5.199–9.46
Fossa (<i>Cryptoprocta ferox</i>)	~1.8 ⁶	1.1 ⁶	1.64
Spotted hyena (<i>Crocota crocuta</i>)	0.51/3.99 ⁷	0.29 ⁷	1.76/13.76
Striped hyena (<i>Hyaena hyaena</i>)	1.5/10.5 ⁸	1.2 ⁸	1.25/8.75
Striped hyena (<i>Hyaena hyaena</i>)	0.77	0.29	2.66

Multiple entries for *Crocota* are for pre-dispersal adult males/post-dispersal adult males and non-pregnant adult females. Male *Talpa* values are from outside/inside the breeding season. In *Cryptoprocta* and *Hyaena*, only young females are masculinized, but there is no significant difference in testosterone concentrations between young and adult age classes. Male mean for *Cryptoprocta* was estimated from a published figure. Sources: ¹Overpeck et al., 1978; ²Nadler et al., 1985; ³Feder, 1985; ⁴Racey and Skinner, 1979; ⁵Whitworth et al., 1999; ⁶Hawkins et al., 2002; ⁷Goymann et al., 2001; ⁸van Jaarsveld and Skinner, 1991.

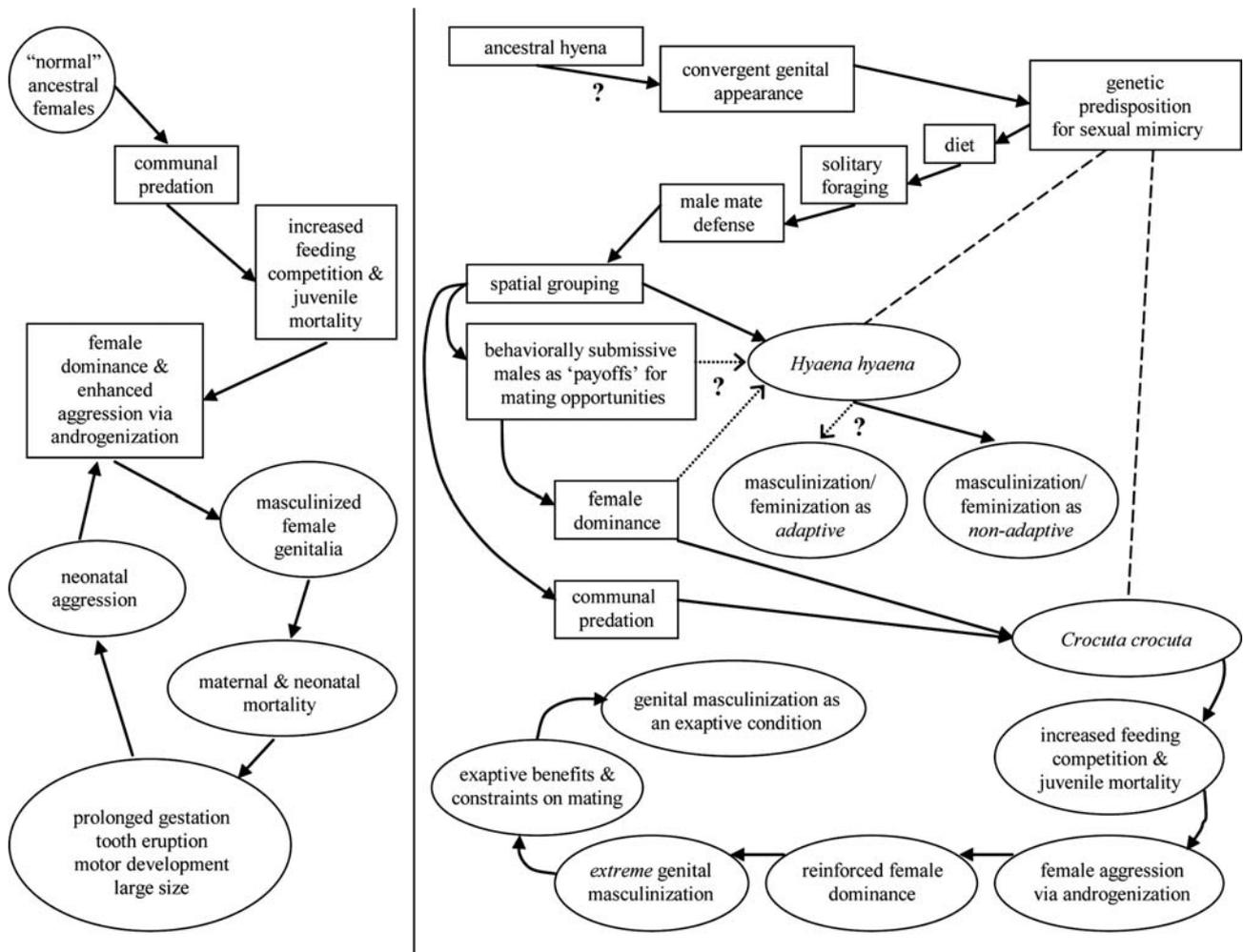


Fig. 3. Postulated evolutionary development of female masculinization and dominance as proposed by Frank (1996) (left panel) and after modifications suggested by findings from striped hyenas (right panel). The unusual genital characteristics of *Hyaena* and *Crocota* may represent expression of preadaptations for unusual genital development within the entire family (indicated by dashed lines). The ultimate cause of masculinization/feminization in the ancestral hyena, the final links from the ancestral hyena to *Hyaena* and the function, if any, of convergent juvenile genital appearance in striped hyenas are unclear (indicated by question marks). Evidence of behaviorally submissive males and/or female dominance in striped hyenas would clarify the ancestral links (indicated by dotted lines). Evidence of infanticide would clarify the function of convergent genital appearance in striped hyenas as adaptive (indicated by dotted line). In the absence of that evidence, currently supported links are indicated by solid lines. Consistent with the notation used in Frank (1996), ancestral developments are differentiated from development post-speciation by boxes and ovals, respectively (though there is no *C. crocuta* oval in Frank's model, it would lie at the transition from boxes to ovals). Note in particular the difference in the models of the placement of increased feeding competition and juvenile mortality, female dominance and aggression, and the origins of patterns of sexual mimicry.

Consequently, examining if normal sex biases are maintained, not just reversed or completely lost, may provide more meaningful tests for discovering biologically significant deviations from mammalian norms.

Development of evolutionary models of genital masculinization in the Hyenidae has historically been closely linked with development of mechanistic models. Existing evolutionary models of genital development in *Crocota* predict normal genitalia in *Hyaena* because those models rely on unique aspects of *Crocota* social ecology that are not shared with *Hyaena*. The discovery of genital anomalies in striped hyena reveals that, within the Hyenidae, unusual patterns of genital development are not unique to *Crocota* and suggests that these models are incomplete. Further, the "feminized" genital characteristics we have seen in young males are not directly addressed by any hypothesis developed for *Crocota*.

In combination with the low levels of direct interaction in *Hyaena*, the convergence in genital appearance makes identification of a clear adaptive function difficult. If mimicry were solely an adaptive trait to reduce female infanticide, convergent genital appearance would be costly in male *Hyaena*. However, the mimicry hypothesis was developed to explain masculinization as found in *Crocota* and its basic logic predicts maximum convergence in genital appearance when *Crocota* are most vulnerable to infanticide (Muller and Wrangham, 2002). This explanation fits well with what is known about *Crocota* ecology and, with some modification, sexual mimicry or ambiguity remains a viable explanation for the patterns observed in *Hyaena*. The convergence in genital appearance in *Hyaena* occurs several weeks after birth, whereas genitalia are 'normal' in neonates and adults. Although many specifics of *Hyaena* ecology are not yet well known, ambiguous genital

appearance might be advantageous for young males and females if both are more vulnerable to same-sex aggression at these ages. For example, increasing foraging movements could result in increased aggressive encounters with same-sex conspecifics from outside the natal group (or within the group, for males).

Explanations for the evolution of unusual patterns of genital development as a nonadaptive trait also cannot be ruled out: the transient genital anomalies observed in *Hyaena* suggest that the Hyaenidae may simply harbor inherited traits that predispose them to the unusual development patterns that have been elaborated in *Crocuta*. Under this scenario, an unusual pattern of genital development did not originate in response to unique aspects of *Crocuta* ecology. Instead, masculinization in *Crocuta* represents a highly developed exaptive trait (Gould and Vrba, 1982) that, once fixed, could not be lost. In addition to the new evidence from *Hyaena*, the high reproductive cost of masculinization in *Crocuta* (Frank et al., 1995), the inability of male offspring to breed if maternal androgens are blocked (Drea et al., 2002) and the secondary exaptive benefits identified within adaptive hypotheses (i.e. Hofer and East, 1995; Kruuk, 1972) support this nonadaptive concept. This logic also explains the lack of masculinization in other social carnivores with intense feeding competition, such as lions *Panthera leo* or African wild dogs *Lycaon pictus*.

Following the reasoning underlying Frank's (1996) depiction of the evolution of masculinization in *Crocuta*, we can modify and extend existing evolutionary hypotheses to develop a broader model that accounts for the emerging details of *Hyaena* biology (Fig. 3). In this model, an ancestral genetic predisposition for sexual mimicry is shared by both *Hyaena* and *Crocuta*. In the ancestral *Hyaena*, the species' diet constrains social development, but promotes development of multi-male mate defense and polyandrous spatial group formation (see Wagner, 2006; Wagner et al., submitted for publication). Female access to multiple resident males may then have selected for female mate choice and a 'food for sex' system in which females demand rewards or payoffs from resident males in exchange for tolerance and mating opportunities. Given an apparent lack of paternal care in *Hyaena*, an intriguing possible (and 'cheap') compensatory system is one in which males are behaviorally submissive to females in feeding situations. Published studies of *Hyaena* biology would have had little or no ability to detect such a system, so whether it exists remains unknown. However, if further research did find supporting evidence, it would indicate an origin to the female dominance system in *Crocuta* preexistent to the evolution of communal predation in the species. In the absence of evidence to this effect, for *Crocuta*, the model follows an alternative and more direct pathway broadly coinciding with that illustrated by Frank: the evolution of communal predation and increased feeding competition selects for female dominance, more heavily virilized female genital develop as nonadaptive consequence, and genital masculinization becomes a fixed exaptive trait. The alternative models for *Hyaena* simply bypass male behavioral submission and/or female dominance. Whether genital ambiguity in young *Hyaena* should be considered nonadaptive or

adaptive will depend on whether future research finds apparent adaptive or exaptive functions for these 'role reversed' traits.

Conclusion

The discovery of genital anomalies in *Hyaena* supports only those evolutionary and mechanistic models of masculinization in *Crocuta* that can be logically extended to *Hyaena*. Investigations into the proximal or functional pathways underlying these phenomena should therefore be broadened beyond *Crocuta*. Identifying aspects of the *Hyaena* and *Crocuta* ecology that differ from one another may help to explain the contrasts in the extent, appearance and function of these divergences from mammalian norms. Further, as indicated by the remaining uncertainties described above, identification of traits or combinations of traits that are common to these hyenids, but differ from other mammals, would help elucidate the evolutionary origins of unusual genital development. The most obvious commonality is that they are both hyenids, sharing a common ancestral history, supporting the long established comparative interpretation of these patterns as exaptive in *Crocuta* and now preaptive (Gould and Vrba, 1982) in *Hyaena*.

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