High maternal androstenedione levels during pregnancy in a small precocial mammal with female genital masculinisation

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Short title

Androgens and female genital masculinisation in a cavy

Abstract

Masculinisation of female genitalia is an intriguing phenomenon amongst some mammalian species and its endocrinological basis as well as its adaptive value is still heavily debated. We recently reported female genital masculinisation in *Cavia magna*. The closely related *C. aperea,* does not show such masculinisation providing an unique opportunity to investigate potential endocrinological mechanisms underlying this difference. For both species we determined plasma levels of androstenedione and testosterone in adults of both sexes, and in females during different stages of pregnancy. Consistent with the normal mammalian pattern males showed higher levels of both androgens than conspecific females. Androgen profiles during pregnancy differed significantly between *C. magna* and *C. aperea* females: during mid-pregnancy androstenedione levels were strongly elevated in the masculinised *C. magna*, but not in *C. aperea*, indicating that high levels of this androgen may be involved in the differentiation of masculinized genitalia in female *C. magna*, as has been suggested for the spotted hyena. In both *C. magna* and the spotted hyena the pups show a highly advanced state of maturation, but in contrast to the hyena female *C. magna* are not overly aggressive. We therefore propose that female genital masculinisation might be a side effect of early exposure to elevated levels of maternal androgens that might be selected for to speed up precocial development.

Keyword*s*

1. INTRODUCTION

The development of male-like traits in females represents an intriguing puzzle for both (medical) physiologists, behavioural biologists and evolutionary biologists alike. For human cases it is interpreted as a disturbance of normal development. However, female masculinisation is a normal phenomenon in several animal species. The female spotted hyena (*Crocuta crocuta*) with its fully erectile pseudopenis and a pseudoscrotum is the most prominent example of female genital masculinisation in mammals (Matthews 1939; Watson 1877). These morphological features along with the rare trait of female social dominance, high levels of aggression, and siblicide, have generated a large number of morphological, behavioural and endocrinological studies (reviewed e.g. in Frank 1997a; Glickman et al. 2006; Muller & Wrangham 2002). However, quite a number of less spectacular cases of female genital masculinisation spanning diverse mammalian taxa exist: moles, e.g. *Talpa spec* (Rubenstein et al. 2003; Sánchez et al. 1996), fossa, *Cryptoprocta ferox* (Hawkins et al. 2002), lemurs, e.g. *Lemur catta* (Drea & Weil in press; Petter-Rousseaux 1964), cebids, e.g. *Ateles spec*: (Hill 1953) and elephants *Loxodonta africana* (Short et al. 1967).

Recently, we reported an additional case of female genital masculinisation in the small precocial mammal *Cavia magna*, a close relative to *C. aperea*, the presumed wild ancestor of the domestic guinea-pig *C. porcellus* (Trillmich et al. 2004). Similar to the other cases listed above, female *C. magna* have an enlarged, penis-like clitoris which is traversed by the urethra, whereas female *C. aperea* lack this trait (figure 1). Together these two related species provide an excellent opportunity to study the endocrinology of masculinisation by using a closely related species as a control. Furthermore, the cavy species are interesting because females of both species, including the one that shows female genital masculinisation, lack the high

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aggressiveness and/or social dominance that has been described for masculinised females of the other species (Asher et al. in press; Kraus et al. 2003), suggesting that genital masculinisation is not necessarily linked to behavioural masculinisation.

----------*Figure 1 is here*----------

Evidence for the organizational effects of androgens mediating female genital masculinisation comes from experimental studies on species lacking female masculinisation. Injection of androgens such as testosterone propionate during female pregnancy leads to the formation of a penis or penis-like structure in female offspring (e.g. rat: Rhees et al. 1997; guinea-pig: Phoenix et al. 1959; dog: Beach et al. 1983; rhesus monkey: Goy et al. 1988). The situation for species that naturally show female masculinisation is, however, less clear. In the spotted hyena, the proximate mechanism of female genital masculinisation has been partly deciphered: During pregnancy, hyena ovaries produce large amounts of androstenedione (A4), which are metabolized in the placenta to testosterone (T). As a result, T enters the foetal circulation at levels found in adult males (Licht et al. 1992; Licht et al. 1998; Yalcinkaya et al. 1993). However, an additional non-androgenic mechanism of phallic growth and scrotal fusion must exist, since anti-androgens administered during pregnancy do not completely prevent the formation of the masculinised genitalia (Drea et al. 2002; Drea et al. 1998). There is also correlative evidence suggesting that high levels of A4 might be involved in masculinised features of *L. catta* (Drea 2007). However, elevated levels of plasma-T and/or –A4 during pregnancy have been described in several non-masculinised female mammals as well (e.g. marmoset monkey: Chambers & Hearn 1979; mouse: Soares & Talamantes 1983; guinea-pig:

Despres & Rigaudiere 1983; dog: Concannon & Castracane 1985). This renders it difficult to interpret the significance of elevated androgen levels for the phenomenon of interest und underlines the importance of using a closely related species lacking female masculinisation as a control when evaluating prenatal androgen profiles.

Based on the normal sex difference in aggression in both cavy species, we expected androgen profiles of non-reproducing adults to be consistent with the normal mammalian pattern, i.e., high T-levels in males compared to females. Sex differences in A4-levels are less consistent across mammals; however, in most rodents studied so far A4-levels are somewhat higher in males than in females and we had no a priori reason to expect cavies to deviate from this pattern. Because genitalia are already masculinised at birth in female *C. magna* organisational effects of androgens should be reflected in prenatal endocrine profiles. Based on the hyena case and the experimental studies described above, we hypothesized that in pregnant *C. magna* A4 concentrations should be more elevated than in *C. aperea*, in particular at the time of genital sexual differentiation, i.e., at mid-pregnancy (~ day 32 in *C. porcellus*: Rigaudiere 1979).

2. MATERIAL AND METHODS

(a) *Animals*

Animals were kept in environmentally controlled rooms on a photoperiod of 14:10 h light : dark at 20–23 ℃. Laboratory guinea pig chow (Höveler, Langenfeld, Germany), hay, and water (once per week supplemented with vitamin C) were provided ad libitum, supplemented with fresh carrots and beets. Groups of two females were housed in enclosures of 1 m² with wood shavings for bedding and two

huts for shelter. To investigate prenatal androgen levels, males were temporarily placed with females until pregnancy could be detected by palpation. Female reproductive state (oestrus, i.e. a perforated vaginal membrane; pregnancy) was checked daily. This information was used to compute when to take blood samples and to obtain exact parturition dates. Cavies have a postpartum oestrus, thus, pregnancy days when the blood samples were taken were re-calculated based on the parturition date and the average gestation length (*C. aperea*: 62 days: Trillmich 2000), *C. magna*: 64 days: Kraus et al. 2005).

(b) *Blood sampling*

Blood samples of non-reproducing (when housed in uni-sex conditions) male and female animals of both species (*n*=10 per sex-species category) were collected by puncturing the retro-orbital eye venous plexus after anaesthetising the animal for 4-5 minutes with isoflurane. Blood sampling of pregnant females (15 *C. aperea*, 13 *C. magna*) were taken once per trimester of pregnancy (early: ~ days 9/10, mid: ~ days 30/31, late: days 55-57). To avoid stress due to repeated anaesthesia, we used two alternative bleeding methods for both species. Blood was either drawn from the ear veins (*n* = 53, duration: 2 to 10 min) or, females were placed in a small cage together with a starved blood-sucking bug (*Dipetalogaster maxima*, Reduviidae, Heteroptera, larval state 3 or 4) for 15-30 min (*n* = 30). If by this time, the bug had completed its meal, the blood was extracted from the bug's abdomen with a syringe. For validation of this method in the context of steroid hormone analysis see Voigt et al. (2004). Blood samples were centrifuged at 9000 rpm for 10 min and the plasma was kept frozen at -20 °C until further analysis.

(c) *Hormone analyses*

We used radio-immuno assays (RIA) to determine blood plasma levels of T and A4. Samples were extracted with diethylether after adding tritium labelled hormone (incubation for 15 minutes at 37 °C) for determining recoveries (consistently high, average 90%). T-levels and A4-levels were determined using commercial kits (DSL-4000 ACTIVE Testosterone and DSL-3800 Androstenedione RIA kits from Diagnostic Systems Labaratories Inc. Webstrer, Texas, USA). Crossreactivity of the antibodies with other hormones was less than 3%. Parallelism was checked and confirmed. Due to low blood volumes, most samples could not be analysed in duplicates. We performed six assays, matches for samples of the two species and different reproductive stages. Inter- and intra-assay coefficient of variation were 9.66% and 16.25% respectively for T and 15.03 and 8.33. for A4.

(d) *Statistical analyses*

To meet standard general linear (mixed) model assumptions, all androgen concentrations were $\log_e$ or square-root transformed prior to analysis. We used a two-way ANOVA to investigate the effects of sex and species and their interaction on androgen levels of non-reproducing adult cavies. To analyse the effect of species and pregnancy phase on androgen levels, we employed general mixed models using REML (Restricted Maximum Likelihood, Pinheiro & Bates 2000). Species and pregnancy phase were included as fixed factors and allowed to interact. To account for repeated sampling of the same individual during pregnancy, subject was included as a random factor. We also included bleeding method (ear punction or bug) as fixed factor to control for potential biasing effects on androgen levels. Subsequent Bonferroni-adjusted two sample *t*-tests were conducted to test at which trimester of

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pregnancy the species' androgen levels differed significantly (corrected p-values, i.e., uncorrected p-value *3, are reported for the 3 pairwise comparisons).

3. RESULTS

As expected, in both species, T concentrations were substantially higher in non-reproducing males than in non-reproducing females (5-times in *C. aperea*; 25-times in *C. magna*; ANOVA, sex: $F_{1,35}=177.9$, $p<0.001$; figure 2a,b), consistent with the traditional mammalian pattern. *C. aperea* females showed almost 2-times higher plasma T concentrations than female *C. magna* (ANOVA, sex*species: $F_{1,35}=19.7$, $p<0.001$). A4 concentrations of males were roughly 2-times those of conspecific females (ANOVA, sex: $F_{1,36}=10.2$, $p=0.003$) and this androgen was generally higher in *C. magna* than in *C. aperea* (ANOVA, species: $F_{1,36}=8.2$, $p=0.007$).

----------Figure 2 is here----------

Changes in T concentration over the phases of pregnancy followed a similar course in both species: T concentrations in early pregnancy were only slightly higher than in non-reproducing females (figure 2a,c); they then increased strongly until mid-pregnancy and remained at the same level during late pregnancy (pregnancy phase: $F_{2,50}=27.40$, $p<0.001$). T concentrations increased somewhat more steeply in *C. magna* (3.0-times) than in *C aperea* (1.5-times) (species: $F_{1,26}=9.65$, $p=0.005$; species*pregnancy phase: $F_{2,50}=4.14$, $p=0.022$). T concentrations differed significantly between species only during the first trimester of pregnancy (post hoc t-tests for species effect at early pregnancy: $t_{25}=5.45$, corrected $p<0.001$; mid-

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pregnancy: $t_{26}=1.25$, corrected $p=0.669$; late pregnancy: $t_{25}=1.16$, corrected $p=0.771$). In contrast, and as expected, changes in A4 over the stages of pregnancy differed substantially between the species (species: $F_{1,26}=6.32$, $p=0.018$; pregnancy phase: $F_{2,49}=69.90$, $p<0.001$; species*pregnancy phase: $F_{2,49}=13.69$, $p<0.001$; figure 2d). In *C. aperea* A4 concentrations changed similar to T concentrations with a 2.5-fold increase until mid-pregnancy, reaching much higher levels than those in males. In female *C. magna* A4 concentrations in early and late pregnancy were comparable to those in *C. aperea* females, but at mid-pregnancy they were 9.4-fold higher than in early pregnancy due to a 4-fold stronger increase compared to *C. aperea* (post hoc *t*-tests for species effect at early pregnancy: $t_{25}=2.25$, corrected $p=0.10$; mid-pregnancy: $t_{25}=4.86$, corrected $p<0.001$; late pregnancy: $t_{25}=0.94$, corrected $p<1.0$). Bleeding method did not show a statistically significant effect on androgen levels (T concentration: $F_{1,50}=1.38$, $p=0.246$, A4 concentration: $F_{1,26}=0.25$, $p=0.619$).

4. DISCUSSION

During pregnancy concentrations of both T and A4 increased in the female circulation. Consistent with our main prediction, *C. magna*, the species that shows female masculinisation of genitalia, showed a much stronger increase in A4 concentrations than the congeneric, non-masculinised *C aperea*. As hypothesized, the pronounced peak of A4 in *C. magna* occurred during the second trimester of pregnancy, when sexual differentiation of genitalia takes place in the closely related guinea pig (Rigaudiere 1979). Thereafter, A4 concentrations decreased to levels of the other species, *C. aperea*. Although in the latter T concentrations were somewhat higher than in *C. magna*, this difference was much smaller than in A4, and did not

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specifically concern the period of genital sexual differentiation. This indicates a role for A4 in female masculinisation. The A4-levels reached in mid-pregnancy in female *C. magna* are well within those described for the spotted hyena (Licht et al. 1992), further supporting this conclusion.

A similar asymptotic increase in female androgen concentration during pregnancy has previously been described for maternal plasma levels of T in the guinea-pig (Rigaudiere et al. 1980), but other species show different prenatal androgen profiles (e.g. dog: peak in early pregnancy: Concannon & Castracane 1985; marmoset: peak in mid-pregnancy: Chambers & Hearn 1979). This high variability emphasizes the benefit and the importance of using *C. aperea* as a control before inferring a connection between temporarily elevated androgen levels in the maternal circulation and female genital masculinisation.

Masculinisation of different traits may take place at different time periods (e.g. Place & Glickman 2004).and the finding that the peak of A4 occurs only during the second trimester of pregnancy fits well with the phenotype of female *C. magna,* showing masculinised genitals but no masculinisation of aggression. The second trimester is the time of genital sex differentiation in the guinea pig (Rigaudiere 1979), while sexual differentiation of behaviour occurs later during foetal development (Goy et al. 1988; Goy et al. 1964). Levels of A4 in the hyena are highest during late gestation and this might be responsible for the highly aggressive behaviour in neonatal and adult females (Dloniak et al. 2006; Licht et al. 1992).

But why might this trait have been selected for in *C. magna,* either directly, or - more likely - indirectly as a by-product? A high level of aggressive behaviour seems a common denominator in many of the species with masculinised females and has often been proposed as the real target of selection in cases of female masculinisation.

In the spotted hyena hyper-aggressiveness has been causally connected to female reproductive fitness in a highly competitive hierarchical system (Dloniak et al. 2006; Frank 1997b). Additionally, even newborn hyena cubs are aggressive rather than playful and engage in facultative siblicide (East et al. 1993; Frank et al. 1991). Similarly, female ring-tailed lemurs are socially dominant over males and targeted aggression against subordinate females has been documented (Drea 2007). Finally, in the European mole, females are highly territorial and only allow males to enter their home range during the mating season when female testosterone levels are at their seasonal low (Whitworth et al. 1999). In contrast, the social system of *C. magna* is probably best described as colonial without stable social bonds and with widely overlapping home-ranges between individuals (Kraus et al. 2003). Even under space-limiting laboratory conditions neither juvenile nor adult female *C. magna* are especially aggressive and actually even less so than *C. aperea* (Kraus, pers. observation). The almost complete lack of overt aggression in females of both species was confirmed in experiments with staged dyads (Pfannkuche 2007). Hence, we can safely exclude the hypothesis that genital masculinisation in this species is a by-product of the selection for hyper-aggressive females.

Despite the difference in female aggression, the fierce spotted hyena and the timid cavy share important features regarding the developmental state of the offspring that may be linked to female masculinisation: both are more precocial than their non-masculinised close relatives. Frank et al. (1991) suggested precocial development of spotted hyena cubs as one of a suite of co-adapted traits causally connected to the increased level of prenatal androgens. At birth their eyes are already open, their incisors and canines are fully erupted and they show a high degree of coordinated movement. Likewise, *C. magna* is certainly one of the most extreme examples of

precociality in small mammals. They are born into small litters of 1 to 3 with their eyes open, fully furred and they are able to run about within an hour of their birth (Kraus et al. 2005). Although all caviomorph rodents are precocial, they are so to a widely varying degree. Whereas a newborn *C. magna* in the wild weighs on average 18% of its mother's body mass, other members of the Caviinae have a relative neonatal mass between only 8% in *Kerodon* (Nowak 1999) and 12.5% in *C. aperea* (Kraus et al. 2005). Maternal androgens deposited in eggs have been shown to speed up growth and development in birds (e.g. Groothuis et al. 2005; Schwabl 1996; Schwabl et al. 2007) and prenatal androgens are also known to influence postnatal growth in mammals (e.g. Gill & Hosking 1995). We have speculated before that the unpredictable habitat (temporary flooding and unstable food resources) might have exerted a strong selection pressure on high mobility of newborn *C. magna* (Kraus et al. 2003; Kraus et al. 2005). Here, we hypothesize that the partial genital masculinisation of female *C. magna* is a by-product of the selection for highly precocial offspring.

Finally, as expected, sex-specific androgen levels in non-reproducing animals of the two cavy species were totally inconspicuous, with males showing higher levels of both, T and A4 than conspecific females. These findings are well in accordance with the literature (e.g. see summary table in Drea 2007). Studies measuring androgens in non-reproducing individuals of species showing female genital masculinisation have usually found the typical mammalian pattern with higher levels of T in males than in females (ring-tailed lemur: Drea 2007; von Engelhardt et al. 2000; brown lemur: Ostner et al. 2003; spotted hyena: Goymann et al. 2001; Licht et al. 1992; fossa: Hawkins et al. 2002). One exception to this norm is the seasonally increasing T-level in non-reproducing female European moles at the time they

become extremely territorial (Whitworth et al. 1999). Sex differences in A4-levels are not as clear-cut (e.g. Drea 2007), and it is still debated whether female spotted hyenas generally have higher levels of this androgen than males (Glickman et al. 1992; Goymann et al. 2001).

In conclusion, high maternal levels of androstenedione during mid-pregnancy, i.e., at the time of sex differentiation of foetal genitalia are likely involved in genital masculinisation in female *C. magna* and other species showing female masculinisation. If indeed, maternal A4 proves to be responsible for the partially masculinised genitalia in *C. magna*, the cavy case of female masculinisation fits well within Jost's model of androgen-dependent sexual-differentiation (Jost 1970; Jost et al. 1973). The masculinised genitalia may be a by-product of selection for enhanced early growth and development

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Figure Legends

Figure 1. External female genitalia in (*a*) *Cavia aperea* and (*b*) *C. magna*.

Figure 2.   (a) Testosterone (T) and (b) androstenedione (A4) levels in adult non-reproducing females and males of *C. aperea* and *C. magna* (1 *C. aperea* male value was excluded as an extreme outlier: 16*SD). (c) T- and (d) A4-levels in female *C. aperea* and *C. magna* during pregnancy. Depicted are mean ± 95% CIs; sample sizes are given above upper CIs. Missing values (1 *C. magna* female, A4 & T, early pregnancy; 1 *C. aperea* female, A4, middle pregnancy) were due to blood samples being too small.
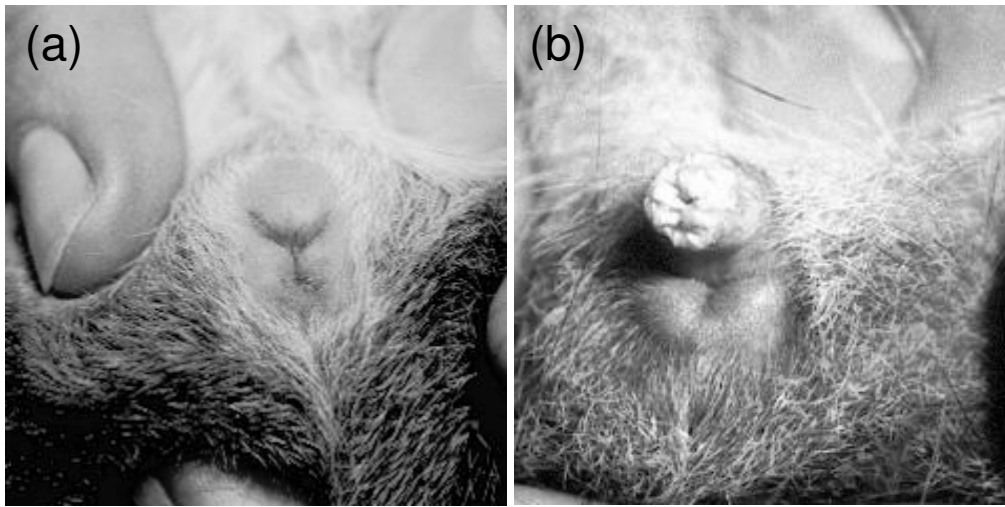
Figure 1

Fig. 2