



RESEARCH ARTICLE

Anogenital distance as a measure of male competitive ability in Rwenzori Angolan colobus

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Abstract

Anogenital distance (AGD) is positively correlated to fetal androgen exposure and developmental masculinization in mammals. Independent of overall body size, AGD shows a strong positive correlation with male fertility and in rodents, AGD is a good indicator of male competitive ability and is associated with female choice. We hypothesized that AGD will also predict male competitive ability in non-human primates. To test this, we measured AGD noninvasively with a parallel laser in a wild population of Angolan colobus monkeys (*Colobus angolensis ruwenzorii*) in Uganda and correlated it to their social structure. *C. angolensis ruwenzorii* form a multilevel society with both one-male/multifemale units (OMUs) and multimale/multifemale units (MMUs). We compared AGD in males from five OMUs and six MMUs and related it to their fecal androgen metabolite concentrations, dominance rank and body size, and to the number of females in their unit. Males in OMUs had greater access to females, so were predicted to have longer AGDs, but this was not found. AGD also did not correlate overall with mean fecal androgen metabolites in MMUs. However, AGD was correlated with dominance rank in MMUs, demonstrating that higher-ranking males in these multimale units had longer AGDs. Body size did not show the same relationship with dominance rank, suggesting that male rank was not just a reflection of absolute male size. Our findings indicate that AGD predicts male competitive ability in this species and that it may be a useful correlate throughout the non-human primates. These results also support the idea that prenatal androgen exposure increases the likelihood of the expression of behaviors that maintain high dominance rank.

KEYWORDS

badge of status, dominance rank, male quality, noninvasive measure, prenatal androgen exposure

1 | INTRODUCTION

Anogenital distance (AGD) is a sexually dimorphic trait in mammals (e.g., rodents: Gray, 2001; Gray et al., 1999; rhesus macaques: Goy & Robinson, 1982; Goy, Wolf, & Eisele, 1976; Hendrickx et al., 1987; Thornton, Zehr, & Loose, 2009; humans: Salazar-Martinez, Romano-Riquer, Yanez-Marquez, Longnecker, & Hernadex-Avila,

2004; Swan, 2008; Thankamony, Ong, Dunger, Acerini, & Hughes, 2009) associated with androgen exposure in utero, where males typically have AGD's that are about twice as long as females (Hsieh, Breyer, Eisenberg, & Baskin, 2008; Rhees, Kirk, Sephton, & Lephart, 1997). Experiments in rats indicate that AGD is a reliable signature of prenatal androgen exposure (hereafter PAE) and is not influenced by postnatal androgen exposure in the way that penis size

is (Van den Driesche et al., 2011). In rats, AGD is determined by androgen levels during a critical time period in fetal development called the “masculinization programming window” (Carruthers & Foster, 2005; Foster & Harris, 2005; Macleod et al., 2010; Welsh et al., 2008). Here, AGD is a stamp of PAE and only appears to grow in proportion to the overall growth of the rest of the body (Van den Driesche et al., 2011). However, because all male reproductive organs are programmed during the masculinization programming window, AGD is closely correlated with the size of the adult prostate, seminal vesicles, penis (length and girth), and testes (and therefore also sperm production) (Drake et al., 2009; Scott et al., 2008; Van den Driesche et al., 2011; Welsh, MacLeod, Walker, Smith, & Sharpe, 2010).

Within-sex variation in AGD can provide critical insights into our understanding of male behavior and fitness. In wild house mice (*Mus domesticus*), adult males with longer AGDs were more aggressive, had larger home ranges, and were more likely to disperse than males with shorter AGDs (Drickamer, 1996). Life-long pair-bonds are formed in prairie voles (*Microtus ochrogaster*), and here females preferred males with longer AGDs as partners, perhaps because this was a reliable cue to potency, correlating with testes size, seminal vesicle size, and the number of stored sperm (Ophir & DelBarco-Trillo, 2007). Longer AGD was a strong correlate of larger home range size for wood mice (*Apodemus sylvaticus*; Godsall, Coulson, & Malo, 2014). For females, shorter and thus more feminized AGDs correlate with greater fecundity and reproductive success in rodents (see Freeman, Sheehan, & Ophir, 2019 for a review). In litter-bearing animals, intrauterine position can affect the development of the AGD. For example, in mice, rats, and swine, females that were adjacent to male fetuses in utero are more masculinized, resulting in longer AGDs than those that were not near male fetuses (Clemens, 1974; Drickamer, Arthur, & Rosenthal, 1997; McDermott, Gandelman, & Reinisch, 1978; Ryan & Vandenberg, 2002).

Research findings in the human masculinization programming window echo those in rodents (Welsh et al., 2008). Men with shorter AGDs have been shown to have poorer semen quality (Mendiola, Stahlhut, Jørgensen, Liu, & Swan, 2011) and a higher likelihood of being infertile (Eisenberg, Hsieh, Walters, Krasnow, & Lipshultz, 2011). Eisenberg et al. (2011) found that for every 1 cm increase in a man's AGD, sperm density and motile sperm count went up significantly (sperm density increased by 4.3 million sperm/ml; motile sperm count increased by 6.0 million). In addition, intrauterine effects have been shown in humans, with women with a male cotwin having reduced reproductive success (Bütikofer, Figlio, Karbownik, Kuzawa, & Salvanes, 2019; Lummaa, Pettay, & Russell, 2007).

Given the resource holding correlates of AGD in rodents, our goal was to assess whether this also applied to wild non-human primates. We noninvasively measured AGD and related it to post-natal measures of masculinization and social status (i.e., androgen levels, body size, and dominance rank). To our knowledge, although the masculinization programming window also occurs in non-human primates (Herman, Jones, Mann, & Wallen, 2000; Prahallada

et al., 1997) and AGD is a reliable measure of PAE (Abbott, Colman, Tiefenthaler, Dumesic, & Abbott, 2012; Thornton et al., 2009), the correlates with adult behavioral traits in the wild have not been assessed. PAE and its masculinizing effects on the brain and subsequent behavior have been examined in captivity for play, mounting, and grooming behavior in prenatally androgenized female rhesus macaques (*Macaca mulatta*) that demonstrated more male-like behavior (Goy & Resko, 1972; Goy, Bercovitch, & McBriar, 1988). PAE and its effects on female dominance have been assessed in a few other non-human primates using the 2D:4D ratio (i.e., the ratio of length between the second and fourth digit on the hand) as a biomarker. 2D:4D ratios are sexually dimorphic, being smaller in males than females in many species (Baxter, Wood, Witczak, Bales, & Higley, 2019; Galis, Ten Broek, Van Dongen, & Wijnaendts, 2010; Manning, Bundred, Newton, & Flanigan, 2003; McIntyre et al., 2009; but see: Baxter et al., 2018; Roney et al., 2004). In captive (*Papio hamadryas*, *Papio ursinus*; Howlett, Marshall, & Hughes, 2012), wild (*P. ursinus*; Howlett, Setchell, Hill, & Barton, 2015), and semifree ranging (*M. mulatta*; Nelson, Hoffman, Gerald, & Shultz, 2010) non-human primates, masculinized 2D:4D ratios in females have been shown to be associated with higher female dominance rank. This effect of PAE on adult dominance is likely due to the organizational effects of androgens on the developing brain, which later increases the likelihood of aggressive behaviors that maintain dominance (e.g., non-human primates, Knickmeyer & Baron-Cohen, 2006; Nelson et al., 2010; Wallen, 1996, 2005; humans, Bailey & Hurd, 2005; Manning & Fink, 2008; Neave, Laing, Fink, & Manning, 2003; van der Meij, Almela, Buunk, Dubbs, & Salvador, 2012). However, 2D:4D ratio may be misleading as a biomarker for PAE since one hand may be more affected than the other (Abbott et al., 2012) and there are species differences in how the ratio is expressed in males versus females (Baxter et al., 2018; Roney et al., 2004).

Rwenzori Angolan colobus monkeys (*Colobus angolensis ruwenzorii*) are excellent study subjects for this analysis because adult males appear to be advertising the AGD region. Both males and females have a white perineal patch but in adult males these are larger and the white hair grows longer, overlapping in the center to create a bright white line in the AGD region, extending from the testicles to the ischial callosities that are adjacent to the anus. Adult male *C. angolensis ruwenzorii* also use penile displays frequently, where the pink, erect penis against the black body hair appears to extend this white line in the AGD region (Figure 1a). This subspecies forms extremely large groups (up to 500 individuals in montane areas, Miller, Uwingeneye, Kaplin, Judge, & Grueter, 2018) and the fission-fusion of social units can be frequent (Stead & Teichroeb, 2019). This is exactly the type of social organization that may favor the evolution of “badges of status” (Rohwer, 1982), which are defined as ornaments or signals that relate stable information about sender quality (Bergman & Sheehan, 2013). In very large groups, individual recognition as a way to assess rival males and quality mates is costly especially when association patterns also vary over time (Bergman & Sheehan, 2013; Grueter, Isler, & Dixson, 2015; Sheehan & Bergman, 2016).



FIGURE 1 Examples of photos used to assess the AGD region and body size of male *Colobus angolensis ruwenzorii*. (a) A male demonstrating a penile display and the way it elongates AGD. Total AGD was obtained by measuring (b) the distance from the posterior scrotum to the anterior edge of the ischial callosities and adding it to (c) the measurement from the anterior edge of the ischial callosities to the anus. (d) The anus was located directly posterior to the ischial callosities, indicating that measuring the pink of ischial callosities was a measurement to the anterior edge of the anus. Body size was assessed using the anterior part of the lower leg as shown in (e) measured from the bend of the knee to the bend of the ankle. Black lines on the photos show where measurements (pixel number) were assessed. AGD, anogenital distance

We have recently confirmed that the large groups formed by *C. angolensis ruwenzorii* are a unique multilevel society in the non-human primates. Cohesive core units that can be unimale/multifemale (i.e., a “one-male unit” [OMU]) or multimale/multifemale (i.e., a “multi-male unit” [MMU]) aggregate into at least three tiers of social organization—“core units” share a range in a “band” and show preferential patterns of association among one another, which we term “clans” (Stead & Teichroeb, 2019). It is the presence of many MMUs with socially integrated, reproductive males that make this social organization unique in primates. The number of females does not appear to vary between OMUs and MMUs, which suggests that males in OMUs have greater access to mates and thus are more competitive than males in MMUs. Our overall hypothesis is that males with longer AGDs have greater masculinization and competitive ability (i.e., dominance rank) than males with shorter AGDs. Thus, when comparing males in different unit-types, we predict that males in OMUs will have longer AGDs and higher endogenous baseline androgen levels than males in MMUs. We investigated levels of endogenous androgens because higher levels are often associated with high dominance rank (Muller, 2017). Additionally, although endogenous androgens should not influence relative AGD length after birth (Van den Driesche et al., 2011), greater

PAE has been associated with higher adult testosterone in primates (Manning, Scutt, Wilson, & Lewis-Jones, 1998; Roney et al., 2004). Furthermore, since male dominance rank is a good predictor of mating rates and rates of siring offspring in most primate species that form multimale groups (Cowlishaw & Dunbar, 1991), we predict that within MMUs, male dominance rank will be positively correlated with both AGD and baseline androgen levels. We further predict that longer AGD will correlate more strongly with higher male dominance rank and greater baseline androgen levels than larger body size.

2 | MATERIALS AND METHODS

2.1 | Study species

C. angolensis is one of five species of black-and-white colobus monkeys found in Sub-Saharan Africa. Black-and-white colobus are medium-sized, arboreal, folivores that typically occupy lowland moist forest. *C. angolensis* (weight range: males, 7.6–12.6 kg; females, 6.4–9.2 kg; Mittermeier, Rylands, Wilson, & Nash, 2013) is relatively widespread from Angola in the west to Kenya and Tanzania in the

east but the seven subspecies show discontinuous distributions. The subspecies we studied, Rwenzori Angolan colobus (AKA Adolf Friedrich's colobus, *C. angolensis ruwenzorii*) is an outlier within its clade forming extremely large group sizes (300–500 individuals in montane areas; Fimbel, Vedder, Dierenfeld, & Mulindahabi, 2001; Miller et al., 2018) and the only multilevel societies identified in an African colobine species (Stead & Teichroeb, 2019). In contrast, other black-and-white colobus typically show group sizes around 10–15 individuals (Fashing, 2011). In *C. angolensis ruwenzorii*, we have observed male dispersal ($N = 9$ males) to only occur within the band between core units and female dispersal to mostly occur to core units outside the band ($N = 3/4$ (75%); Stead & Teichroeb, 2019; unpublished data).

2.2 | Study site and study band

We studied a large band of 132 Rwenzori Angolan colobus in and around the Manwa Forest Reserve at Lake Nabugabo, Masaka District, Uganda (0°22′–12°S and 31°54′E). For details on the study site, see Teichroeb et al. (2019) and Arseneau-Robar et al. (2018). Our study band (TR) ranged over a forested area of approximately 1.5 km² and was organized into 12 cohesive core units, consisting of seven MMUs and five OMUs. These core units fission and fuse into (typically to ~ 20 m) and out of proximity with one another throughout the day, however core units usually remain spatially distinct (Stead & Teichroeb, 2019). We collected data on 11 of the 12 core units in TR band (six MMUs and five OMUs). We excluded one MMU because it contained seven adult males that were not yet individually identified.

2.3 | Behavioral data collection

Two of us (S. M. S. and F. L.) along with two skilled long-term field assistants (E. Mujjuzi and H. Kakeeto) followed TR band for 48 days from June 17th to August 10th, 2018. Each core unit was followed for several observation days in proportion to the number of adult males that it contained to get behavioral, morphological, and physiological data from several males. All individuals in core units were known individually and recognized using physiognomic features, such as broken fingers and tail shapes.

Male *C. angolensis ruwenzorii* compete for dominance rank within MMUs. The process of male dispersal between core units usually involves the two core units staying in close proximity over a few days with frequent displays (i.e., jump-displays and stiff-legs) between males ($N = 9$ males; J. A. T., unpublished data). After males immigrate into new MMUs ($N = 7$ males), there is typically a brief period of frequent displays and mild aggression between the males until a dominance hierarchy is settled.

To determine dominance hierarchies within MMUs during this study, the direction of dyadic dominance interactions observed during the 48-day study period were noted including aggression (e.g.,

displacements, stiff-leg threats, chases, contact aggression) and submission (e.g., supplants, cowers, presents). These interactions ($N = 14$) were not sufficient to create dominance hierarchies in all MMUs or determine linearity. We, therefore, asked field assistant E. Mujjuzi, who was blind to the outcome of all AGD measurements, to determine male dominance rankings in MMUs at the time. E. Mujjuzi has spent 18 days per month following one core unit per day for more than 2 years (since September 2016). These follows involve collecting data using 2-hr focal time samples on known individuals. The dominance ranks provided by E. Mujjuzi were consistent with the dyadic interactions observed during the 48-day study period. Photos and fecal samples were collected opportunistically from all adult males to assess morphological and physiological variables, respectively (detailed below).

2.4 | Measurement of AGD

We noninvasively measured the AGD of adult males using a camera (Canon EOS 1300D/Rebel T6 DSLR) equipped with a parallel laser device and a 300 mm zoom lens. We had the parallel laser device built in the Department of Mechanical Engineering, University of Toronto and followed previous work by using green lasers (model GM-CW02; Apinex, Montreal) for greater long-distance visibility (Bergeron, 2007; Rothman et al., 2008). Within our device, the lasers were oriented horizontally to circumvent the necessity of having to calculate parallax due to the angle of the lasers (Barrickman, Schreier, & Glander, 2015). The parallel lasers in our apparatus were positioned 4 cm apart and meant to project a scale bar of known length beside the adult male in each photo. The scale bar was subsequently used in a photo editing program to accurately measure male AGD. Though we calibrated the lasers at the field station to ensure they were parallel, they sometimes shifted in the field. Thus, after each set of photos was taken, we used a range-finder (Bushnell Trophy 800) to determine the distance from the laser apparatus to the male that was photographed. We then double-checked the distance between the laser points at the same distance that the male was located to ensure that the laser scale bar distance was known accurately.

Male AGD can be measured in a few different ways (Hsieh et al., 2008). In humans, the distance from the posterior scrotum to the anus correlates most strongly with measures of fertility (Mendiola et al., 2011). Hence, we also used this measure. To measure this distance accurately, we had to get two sets of measurements from each male colobus. The scale bar provided by the parallel lasers was only accurate if it was projected onto a parallel (flat) surface in same plane as the measured body part. The curvature of the body of Rwenzori Angolan colobus males has two different angled planes in the AGD region: a flat plane from the posterior scrotum to the anterior edge of the ischial callosities (Figure 1b) and another plane from the anterior edge of the ischial callosities to the anus (Figure 1c). The anus is located directly posterior to the ischial callosities, with the pink of this region leading right into the anus (Figure 1d). Thus, since it was impossible to see the anus in most

pictures, but very easy to see the pink of the ischial callosities against the black fur, we measured this plane as the width of the ischial callosities in the exact line of the AGD. We confirmed that for all males for which we had a clear photo of the anus ($N = 18$ of 22 males), the pink of the ischial callosities led directly into the anus.

During the long resting periods typically shown by colobus in the late morning and afternoon, we sought to obtain many replicate measurements of male AGD by taking as many pictures of males as possible when they were in a position where the white perineal patch or ischial callosities were in a parallel plane to the camera and the laser could be projected onto the branch that the male was seated on. Additionally, we sought to standardize the males' body positions in the photos to account for the ways that body position can affect the perceived length of the measured feature.

Pictures of males were analyzed in the program paint.net version 4.1.2 (dotPDN LLC). The number of pixels represented by the scale bar was assessed by drawing a line from the center of each laser point. This allowed a calculation of the length in cm denoted by each pixel within that specific photo. A line was then drawn on the photo in the AGD region pictured (either the posterior scrotum to the anterior edge of the ischial callosities, or the anterior to the posterior edge of the ischial callosities, Figure 1b,c) and the number of pixels was recorded, this was converted to a length using the previous assessment with the scale bar. Since there was some subjectivity on each measurement as to where to end the line drawn in the male AGD region, three of us (J. A. T., S. M. S., and F. L.) obtained measurements from each photograph. These measurements were then averaged for each male within one observer's data and then averaged over the three observers to obtain AGD for each male, or when possible, observer was controlled for in the models we used. Agreement between observers was high and the single score intraclass correlation of interrater reliability ($ICC = 0.701$) was significant ($F(21, 42) = 8.3$, $p < .0001$, 95% confidence interval: $0.507 < ICC < 0.854$). The mean difference in AGD between examiners on each subject was 0.6 cm (± 0.42), which was much less than between-subject variation (3.79 cm). In addition, all observers measuring photos were blind to the dominance rank of individual males. AGD was assessed for 22 males (five in OMUs and 17 in MMUs). Overall, 182 photos were assessed (mean = 8.27 photos/male) by three observers ($N = 546$ measurements, mean = 24.82 measurements/male).

Controlled measurements in the lab on items of known size (a book and stuffed animal) showed that the photos with the lasers had a mean error of 0.44 cm (± 0.29 , $N = 22$), and the error was always < 1 cm (range: 0.04–0.93 cm). There was no difference in the error for photos taken at different distances (range: 2–12 m; Pearson: $N = 22$, $r = -.03$, $p = .898$) or different angles (range: 0–50°; $N = 22$, $r = .198$, $p = .379$). There was also no difference in the overall error between measurements of the stuffed animal and the book (Mann-Whitney U : $N_{\text{animal}} = 12$, $N_{\text{book}} = 10$, $U = 42$, $p = .25$). There was a difference however in regard to where the lasers were projected. Errors were significantly minimized when the lasers were projected onto a flat surface beside the object (mean error: 0.17 cm), compared with when they

were projected onto the object (mean error: 0.54 cm; $N_{\text{surface}} = 6$, $N_{\text{object}} = 16$, $U = 87$, $p = .005$). This was especially the case with the stuffed animal (mean error: 0.61 cm) compared to the flat surface of the book (mean error: 0.42 cm).

2.5 | Body size assessment

For 21 of the 22 males for whom we achieved a measure of AGD, we also obtained photos that allowed us a measure of body size. Given the differing positions of males within the trees and the curvature of body parts, the best and most comparable measure of body size available to us was lower leg length. We were able to aim the lasers onto the branch below the male and we could usually see where the ankle and knee bent. Thus, we measured the anterior part of the leg from the bend of the knee to where the ankle bent into the foot (Figure 1e). Overall, 51 photos were assessed for lower leg length (mean = 2.32 photos/male) by three of us (J. A. T., S. M. S., and F. L.) for 153 measurements (mean = 6.95 measurements/male).

2.6 | Assessment of fecal androgen metabolites

To measure male fecal androgen metabolites, we noninvasively collected fecal samples from the forest floor immediately after defecation. We collected 82 fecal samples from 22 adult males (five from OMUs, and 17 from MMUs). Samples were placed in a cooler on ice upon defecation before transporting them back to the field station, where they were frozen until extraction. For extraction, we dried samples on low heat (50°C) over the day, while continually checking to make sure that temperature in the drying oven remained stable. Dried samples were then homogenized by crushing with a mortar and pestle. We weighed out 0.15 g of each sample and placed it in a 2-ml tube with 1.5 ml of 80% methanol. Samples were placed in an IKA Vibrax-VXR shaker mixer for 1 hr before centrifuging at 3,000 rpm for 10 min. A 0.5 ml aliquot of the supernatant containing the extracted hormones was transferred into a new 2-ml tube, which was left open to air dry. Dried extracts were transported to the University of Toronto Scarborough for further analyses. In the laboratory, the dried extracts were reconstituted in 0.5 ml of 80% methanol and then diluted to 1:250 in assay buffer.

A prior study of several primate species found that very little native testosterone was excreted in feces; it was primarily excreted as androgen metabolites (Möhle, Heistermann, Palme, & Hodges, 2002). Thus, we validated an enzyme immunoassay to measure fecal androgen metabolites (FAM) in Rwenzori Angolan colobus monkeys. This assay (originally described in Palme & Möstl, 1994) is immunoreactive with epiandrosterone, a metabolite of testosterone that is abundant in the feces, and has been validated in many other primates, including common marmosets (*Callithrix jacchus*), chimpanzees (*Pan troglodytes*), long-tailed macaques (*Macaca fascicularis*), rhesus macaques (*M. mulatta*), and orangutans (*Pongo pygmaeus* and *Pongo abelii*) (Girard-Buttoz, Heistermann, Krummel, & Engelhardt, 2009; Higham, Pfefferle,

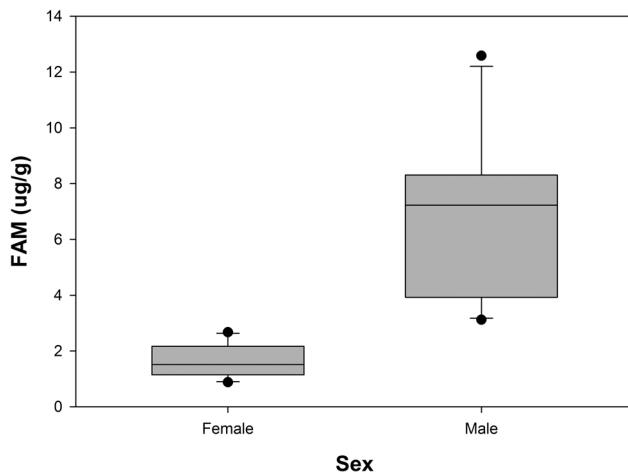


FIGURE 2 Comparison of adult male ($N = 10$) and adult female ($N = 10$) FAM. Males showed significantly higher levels than females ($p < .001$). FAM, fecal androgen metabolites

Heistermann, Maestriperi, & Stevens, 2013; Möhle et al., 2002; Weingrill, Willems, Zimmermann, Steinmetz, & Heistermann, 2011). To assess parallelism, a pooled sample was run in serial dilutions and compared with a standard curve. The slopes of the standard curve and serial dilution curve were compared by fitting, (a) a linear model with percent bound as the response variable and dilution fact and group (standard curve or pool) as effects and, (b) a linear model with the dilution fact and group as effects, and additionally the interaction effect between dilution factor and group. These two models were then compared with the F test, and no difference between the models was found ($F = 2.4$, $p = .17$) indicating that group (standard curve or poll) does not significantly affect the slope of percent bound. For the biological validation of the assay, a subset of random samples collected from adult males ($N = 10$) were compared with samples collected from adult females ($N = 10$) with the expectation that males should have significantly higher FAM levels. This was found to be the case ($p < .001$; Figure 2) with no overlap in levels between the sexes. This was the only analyses that included females in this study. All male hormone samples ($N = 82$) were then run in duplicate. Interassay coefficients of variation (CVs) were 5.8% for the low pool and 7.8% for the high pool ($N = 3$ plates). The average intra-assay CV was 14.6% for the low pool and 5.7% for the high pool.

This study was undertaken with the permission of the Uganda Wildlife Authority and the Uganda National Council for Science and Technology. All the methods were approved by the University of Toronto Animal Care Committee (Protocol #2011416) and were consistent with the American Society of Primatologists Principles for Ethical Treatment of Non-Human Primates.

2.7 | Data analyses

We first looked for a diurnal pattern in fecal androgen metabolites using a linear mixed model with mean log androgen concentration as

the independent variable and hour of the day when the sample was collected as the fixed factor, while including male ID as a random factor. No effect of time of day was found (Estimate = -0.0062 , $SE = 0.0143$, $t = -0.437$, Comparison to Null Model, $p = .65$) so we used the overall mean log androgen concentration for each male for comparisons. Next, we determined whether there were any associations between the physiological (mean levels of androgen metabolites) and morphological variables (mean AGD and mean body size) across our pool of males using Spearman correlations (using non-parametric tests since the sample size of males was small).

To analyze our predictions, we used Mann-Whitney U tests to compare adult males in OMUs versus those in MMUs on the number of adult females in the units, mean log androgen concentration, mean AGD, and mean body size. Similarly, for androgens, AGD, and body size we also compared the alpha male of MMUs to nonalpha males. To determine if mean log androgen concentration was associated with dominance rank in MMUs, we used a regression where we nested rank within core unit. However, to control for the potential effect of the observer on the assessment of male AGD and leg length from the photos, we used linear mixed models (using the lme4 package in R) to determine whether male dominance rank in MMUs was related to AGD or body size. In the first model, the independent variable was AGD and we were assessing its effect on male dominance rank so the fixed factor was rank nested within core unit, with the observer and male ID included as random factors. In the second model, the fixed and random factors were the same and the independent variable was lower leg length, since we were assessing the impact of body size on dominance rank. To determine the significance of these models, we used Likelihood Ratio tests to compare them to null models that included only the random factors.

To further validate the accuracy of AGD to predict dominance rank in our MMUs, we used a leave-one-out cross-validation technique. We left one unit at a time out of the data set and ran nested linear regressions on these “training data sets” to look at the relationship between rank nested within core unit and AGD. We then ran the same regressions separately on the units that were left out, which were the “test sets.” The predicted residuals were calculated as the differences between the standard errors of the regression models between training data sets that did not include a particular core unit and test sets that only included that core unit. The mean squared error between all of the data sets is the cross-validation value, with a low value demonstrating low error and a good predictive model (Arlot & Celisse, 2010).

All analyses were run in R version 3.5.1 (R core team, 2018) and an alpha level of 0.05 was set for significance, with the exception of the Mann-Whitney U tests comparing males in the two-unit types where we used a Bonferroni correction to control for multiple comparisons and the alpha level was 0.025. Linear mixed models were run with the package lme4 (Bates, Maechler, Bolker, & Walker, 2015) and inter-rater reliability tests were run with the package irr (Gamer, Lemon, Fellows, & Singh, 2019).

3 | RESULTS

3.1 | Associations between physiological variables

Male AGD ranged from 9.13 to 12.92 cm with a mean of 11.71 cm (± 96 , $N = 22$ males) and male lower leg measurements ranged from 14.58 to 19.71 cm (± 1.61 , $N = 21$ males). Overall, mean male AGD measurements did not correlate with mean log androgen concentration (Spearman: $N = 22$ males, $r_s = .094$, $p = .68$). Our measure of body size (lower leg length) also did not correlate with mean log androgen concentration (Spearman: $N = 21$, $r_s = -.047$, $p = .84$) but was positively associated with mean AGD measurements ($N = 21$, $r_s = .564$, $p = .008$).

3.2 | Males in one-male units compared with those in multimale units

The number of adult females in OMUs and MMUs was not different (Mann-Whitney U : $N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 6$, $U = 9$, $p = .32$). Thus, there were greater male/female ratios in MMUs compared with OMUs ($N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 6$, $U = 30$, $p = .008$) and presumably less access to females for males in MMUs. Despite this, the mean log androgen concentration in fecal samples did not vary between males in OMUs and MMUs ($N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 17$, $U = 22$, $p = .12$) or between alpha

males in the MMUs versus the sole male in OMUs ($N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 6$, $U = 8$, $p = .23$). Mean AGD measurements were also not different between males in OMUs and MMUs ($N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 17$, $U = 36$, $p = .64$) or between the alpha male in the MMUs versus the sole male in OMUs ($N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 5$, $U = 15$, $p = .67$). Similarly, our measure of body size (lower leg length) was not different between males in OMUs and MMUs ($N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 15$, $U = 54$, $p = .51$) or between alpha males of MMUs and sole males in OMUs ($N_{\text{OMU}} = 5$, $N_{\text{MMU}} = 5$, $U = 14$, $p = .83$).

3.3 | Influence of rank in multimale units

In the MMUs, mean log androgen concentration was not significantly explained by male dominance rank nested within core unit (Regression: $p = 1.245$, $df = 1,15$, adjusted $R^2 = 0.0151$, $p = .28$, Figure 3). However, our models showed that AGD within MMUs was associated with male dominance rank nested within core unit (Linear Mixed Model: estimate = -0.0463 , $SE = 0.0168$, $t = -2.765$, Comparison to Null Model, $p = .008$), in that males with high dominance rank had longer AGD (Figure 4). Our cross-validation procedure showed a low error value ($CV = 0.00098$), demonstrating excellent predictive power between AGD and dominance rank within MMUs. The body size of males in the MMUs was not significantly associated with dominance rank nested within unit (Linear Mixed Model:

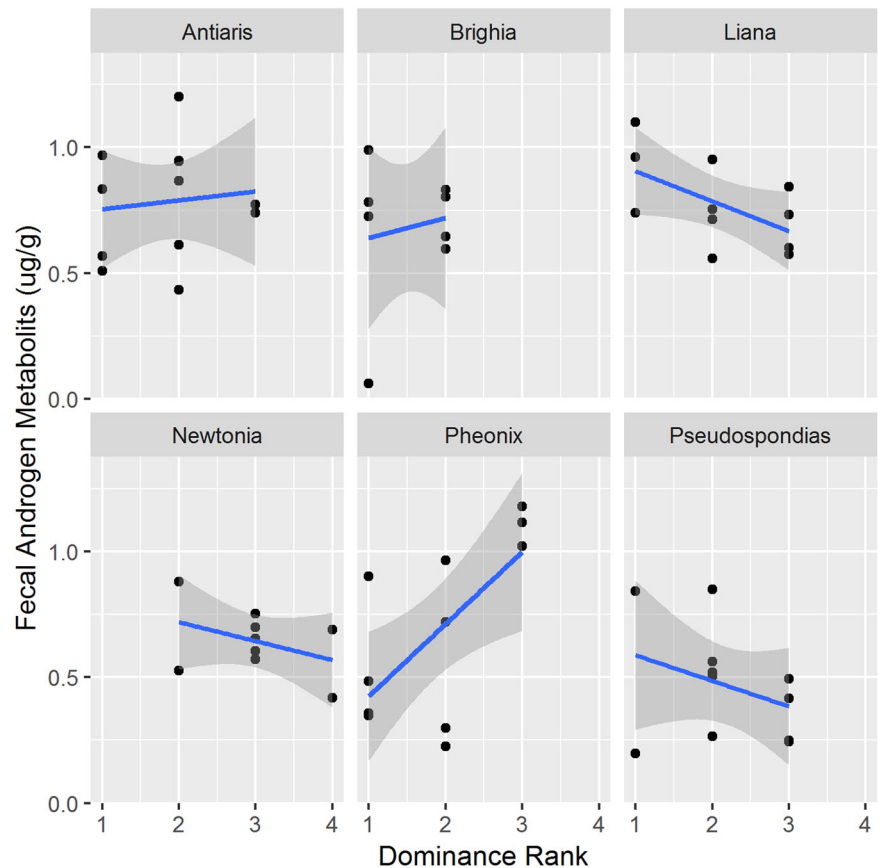


FIGURE 3 Fecal androgen metabolites in each multimale core unit relative to male dominance rank, including regression lines

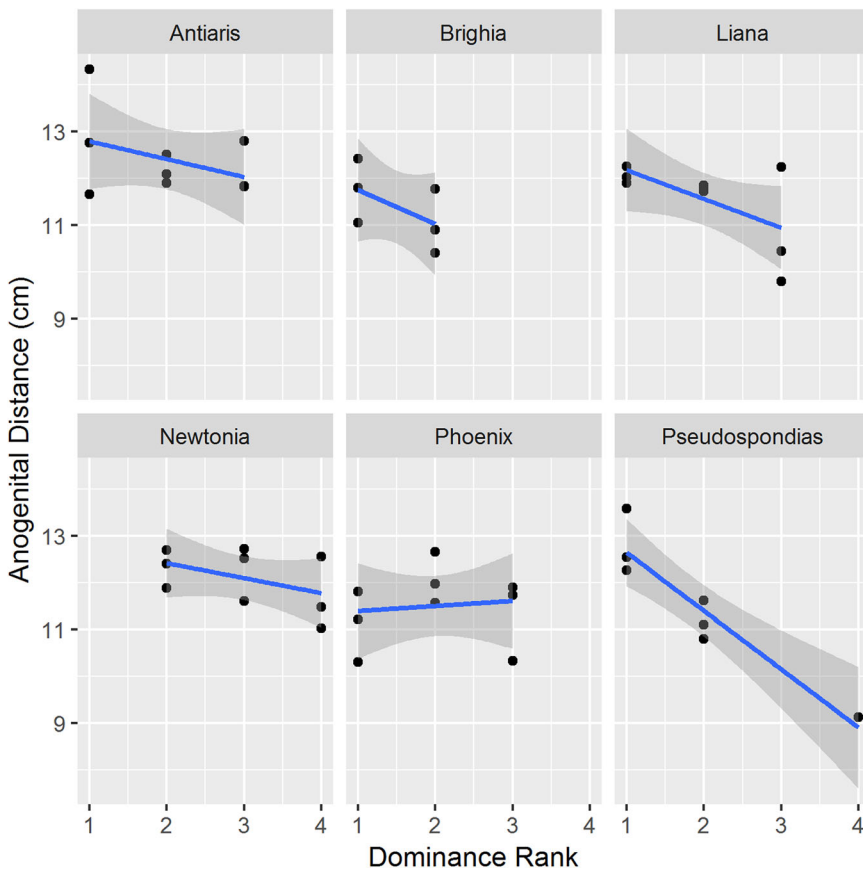


FIGURE 4 Measurements of anogenital distance in each multimale core unit relative to male dominance rank, including regression lines

estimate = -0.0419 , $SE = 0.0429$, $t = -0.977$, Comparison to Null Model, $p = .31$; Figure 5), indicating that there was no correlation overall with our measure of body size and rank in MMUs.

4 | DISCUSSION

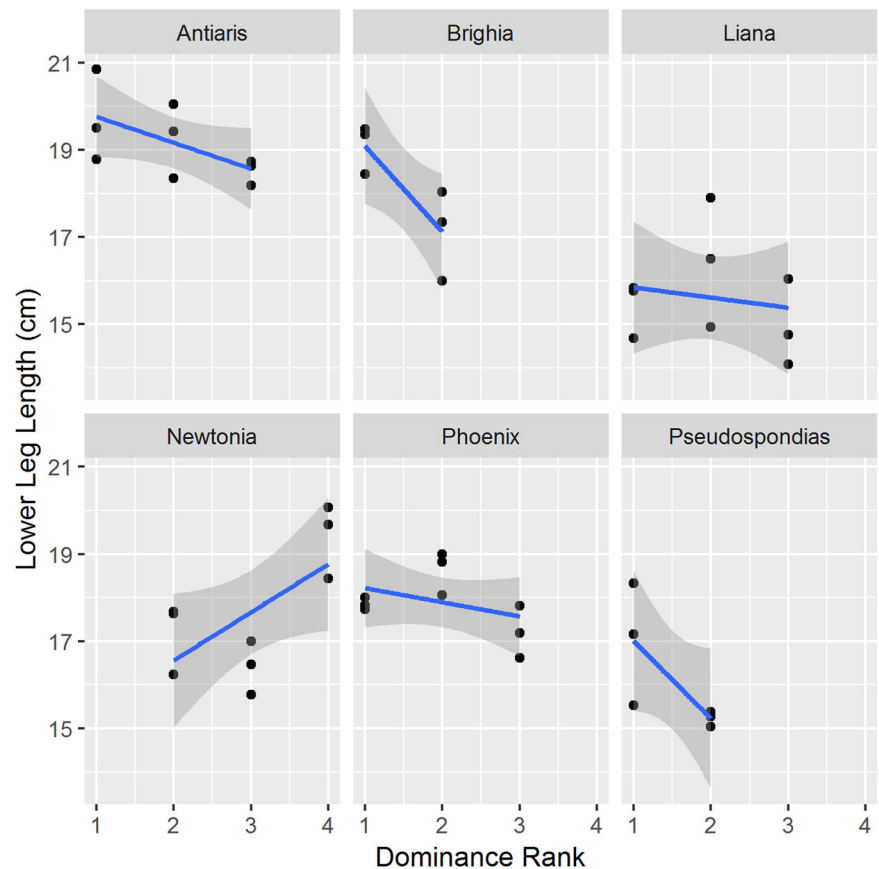
Our results show that in MMUs in Rwenzori Angolan colobus monkeys, high dominance rank is correlated with longer AGD. Given that males compete for dominance rank, this supports our second prediction and our hypothesis that longer AGDs in this species are indicative of greater masculinization and competitive ability. Phoenix was also the only MMU where AGD length did not correlate with dominance rank (Figure 4), and it was known that two males had just immigrated into this unit and challenges in the dominance hierarchy were occurring. These results suggest that the white perineal patch that advertises AGD in Rwenzori colobus is a “badge of status,” which are rare in primates, but appear to evolve more often in species with large, dynamic social groupings (Bergman & Sheehan, 2013; Grueter et al., 2015), as is seen in this subspecies (Stead & Teichroeb, 2019). Long AGD was an excellent predictor of high dominance rank in our MMUs but we did not find that our measure of body size (i.e., lower leg length) predicted dominance rank, even though AGD and body size were correlated. This suggests that AGD may be a more important measure than body size determining competitive ability in

this species. However, these results should be taken with caution. Lower leg length was the only measure of body size available to us but it may not be indicative of overall body mass (Jungers, Burr, & Cole, 1998).

Results from human (Manning et al., 1998) and guinea baboon males (*Papio papio*; Roney et al., 2004) have shown a positive correlation between endogenous testosterone levels and masculine 2D:4D digit ratio (i.e., higher PAE). However, when we assessed all of our MMUs together, we did not find that fecal androgen metabolites were significantly associated with either AGD length or dominance rank. These results are not surprising, since primate males often do not show rank related differences in androgens when their dominance hierarchies are stable (reviewed in: Teichroeb & Sicotte, 2008a).

We also did not find support for our first prediction that males in OMUs would have longer AGDs and higher baseline fecal androgen metabolites than those in MMUs. The results for the hormones echo those from the assessment of dominance rank in MMUs and suggest that challenges to the status of males in OMUs would likely have been required for them to have high androgen levels (Wingfield, Hegner, Dufty, & Ball, 1990). The AGD results between unit-types were unexpected though, since males in OMUs have access to proportionately more adult females in their units. However, we do not think that this result necessarily discounts our hypothesis. We are just beginning to understand the social organization and social structure of *C. angolensis ruwenzorii* (Stead & Teichroeb, 2019) and

FIGURE 5 Measurements of male lower leg length in each multimale core unit relative to male dominance rank, including regression lines



the form of male relationships is still unclear. We know that within their unique multilevel society, adult males and adult females groom most frequently within core units (Arseneau-Robar et al., 2018), which is supported by the bisexual dispersal we have observed from natal units (Stead & Teichroeb, 2019). However, the distance of dispersal between the sexes appears to differ. Female transfers have mostly occurred to core units outside of the band, while all male transfers observed thus far have been to other core units within the band (Stead & Teichroeb, 2019). Dispersal within the band suggests a higher degree of kinship between associated core units for males compared with females. Indeed, males do show affiliative behaviors that we have not yet quantified and male handling of infants occurs frequently, with males transferring infants between one another often (Stead et al., in prep). We have even observed several males transfer in parallel to a new MMU, leaving a single male back in their former core unit so that it became an OMU (S. M. S., J. A. T. unpublished data). This dispersal event appeared to be disadvantageous to the transferring males, considering that they had proportionately less access to within-unit females afterward. However, it may make sense if there are bonds among certain males that would be disadvantageous to break (e.g., white-faced capuchins, Jack, 2003; Jack & Fedigan, 2004). All of this suggests that there may be benefits to being in a MMU for males and potentially females but what these are is not yet clear, so it may not be the case that males in OMUs have better competitive ability than those in MMUs.

Given this, our results are most consistent with the idea that AGD in non-human primates is a noninvasive measure of competitive ability. When one considers temporal aspects of these traits, the correlation between AGD and dominance rank in this sample is intriguing. We measured these traits at one point in time, however male dominance rank in primates is typically variable throughout the lifespan, while AGD is a static indicator of PAE. Similar results have been shown for female dominance rank in non-human primates, with PAE assessed using digit ratios as a biomarker. For rhesus macaques (*M. mulatta*; Nelson et al., 2010), Hamadryas baboons (*P. hamadryas*; Howlett et al., 2012), and chacma baboons (*P. ursinus*; Howlett et al., 2012, 2015) high female dominance rank was correlated with what was thought to be a more masculinized 2D:4D ratio and therefore greater PAE (but see Baxter et al., 2018 for a different interpretation of digit ratios in *M. mulatta*). In rhesus macaques, PAE leading to higher dominance rank was most pronounced among related females within matriline, consistent with the fact that day-to-day competitive interactions occur most frequently between closely ranked kin (Nelson et al., 2010). Dominance rank for female macaques and chacma baboons is maternally inherited, thus the maternal gestational environment and PAE experienced by the fetus are likely related to the mother's experiences within the hierarchy (Baxter et al., 2018; Howlett et al., 2015; Nelson et al., 2010). Indeed, in some wild mammal species, high-ranking mothers have been shown to experience higher levels of testosterone than lower-ranking mothers,

which could influence PAE for offspring (e.g., *Papio* sp.: Beehner, Phillips-Conroy, & Whitten, 2005; *Crocuta crocuta*: Dloniak, French, & Holekamp, 2006). PAE have organizational effects on the brain, as well as the external genitalia, and can lead to hormonally influenced predispositions, increasing the likelihood of some behaviors over others (Knickmeyer & Baron-Cohen, 2006; Wallen, 1996, 2005), thus the effect of PAE on adult dominance is likely due to the responsiveness of individuals to rank-related challenges (Nelson et al., 2010). Individuals with greater PAE may be more likely to express dominance behaviors and respond aggressively in situations where their rank is challenged, leading to the tendency for them to be high ranking. For instance, in human males, a lower (i.e. more masculinized) 2D:4D ratio and thus greater PAE has been associated with greater endurance (Manning, Morris, & Caswell, 2007), sporting ability (Manning & Taylor, 2001), strength (Fink, Thanzami, Seydel, & Manning, 2006), aggressive dominance (Manning & Fink, 2008; Neave et al., 2003; van der Meij et al., 2012), and high trait aggressiveness (Bailey & Hurd, 2005). This suggests that for Rwenzori colobus males, it is unlikely that it is always the case that males assort by AGD length in the dominance hierarchy; however, if males with longer AGDs, and thus greater PAE, are more likely to respond to challenges aggressively and display behaviors associated with dominance, it may be that these males have longer tenures as alpha males, or are more likely to regain their status if they lose it. These are questions that can only be answered with continued research.

Our research suggests that AGD is an excellent feature for researchers to measure as an indicator of PAE and competitive ability, but the question remains as to whether conspecifics are paying attention to this feature in *C. angolensis ruwenzorii*? The white perineal patch and line in the AGD region are presumably static indicators of PAE in this species (i.e., a badge of status). We did not note any obvious differences in either the size of the patch or how bright the white hair was for different males that would suggest that this is a “current-state signal” (Bergman & Sheehan, 2013). Conspecifics could benefit from paying attention to this signal if males with longer AGDs are more likely to react aggressively when challenged (Knickmeyer & Baron-Cohen, 2006; Nelson et al., 2010; Wallen, 1996, 2005) or if, as in humans, these males are stronger and have greater endurance (Fink et al., 2006; Manning & Taylor, 2001). Indeed, the fact that males sit passively with penile displays for long periods of time, which seems to elongate the white AGD line (Figure 1a), suggests that AGD advertisement is important. Badge of status signals in other animals are often used to settle disputes between competitors without costly fighting (Rohwer, 1975, 1982; reviewed in: Johnstone & Norris, 1993). Indeed, the lead author (J. A. T.) also studied a close congener of *C. angolensis ruwenzorii* for several years (*Colobus vellerosus* in Ghana) and notes that male contests in *C. vellerosus*, who do not show the same line in the AGD region, are drawn out (sometimes lasting months; Sicotte et al., 2017) and involve more contact aggression and wounding that what is seen in *C. angolensis ruwenzorii*, where dominance rank becomes settled comparably faster and with less aggression. However, this difference could be due to the different social organizations in these two species, rather than the presence of a badge of status. *C. vellerosus* show small, cohesive groups with male-biased dispersal (Teichroeb, Wikberg, &

Sicotte, 2009; Teichroeb, Wikberg, & Sicotte, 2011) and frequent infanticide by males (Teichroeb & Sicotte, 2008b). Whereas, *C. angolensis ruwenzorii* males are much more familiar with one another given the close proximity of core units in the multilevel society and within-band male dispersal pattern (Stead & Teichroeb, 2019). Penile displays and emphasis of the AGD region in *C. angolensis ruwenzorii* could also be aimed at female conspecifics, given the very high correlation between AGD length and fertility in male mammals (e.g., Eisenberg et al., 2011; Mendiola et al., 2011; Ophir & DelBarco-Trillo, 2007; Van den Driesche et al., 2011).

A growing body of research utilizing 2D:4D digit ratios suggests that PAE are important in contributing to variation in many behavioral, cognitive, and psychological traits in humans (reviewed in Fink, Manning, & Neave, 2004; Teatero & Netley, 2013; Turanovic, Pratt, & Piquero, 2017; Valla & Ceci, 2011) and non-human primates (Howlett et al., 2012, 2015; Nelson & Shultz, 2010; Nelson et al., 2010). For most animal species however: (a) measurement of digit ratio is impossible (i.e., no digits) or impractical, (b) the associations between PAE and digit ratios have not been made, (c) some digits may grow postnatally (Hampson & Sankar, 2012; McIntyre, Cohn, & Ellison, 2006), (d) one hand may be more affected by PAE than the other (Abbott et al., 2012), and (e) the ratio may be opposite to expectations (i.e., Baxter et al., 2018; Roney et al., 2004). We, therefore, suggest that AGD is a better measure for PAE in wild animals and should be explored thoroughly in a range of species and taxa for its potential as a widely applicable metric to examine within- and between-sex variability.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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