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## RAPID COMMUNICATION

TESTOSTERONE IN ANCIENT HAIR FROM AN  
EXTINCT SPECIES

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**Abstract:** Testosterone is a key regulator in vertebrate development, physiology and behaviour. Whereas technology allows extraction of a wealth of genetic information from extant as well as extinct species, complementary information on steroid hormone levels may add a social, sexual and environmental context. Hair shafts have been previously used to sequence DNA from >50 000 <sup>14</sup>C years old Siberian woolly mammoths (*Mammuthus primigenius*). Hair-testing has also been used to measure endogenous steroids in multiple extant species. Here we use small quantities of woolly mammoth hair samples to measure testosterone, and a genomics-based

approach to determine sex, in permafrost-preserved mammoths dated to c. 10 000–60 000 <sup>14</sup>C years. Our validated method opens up exciting opportunities to measure multiple steroids in keratinized tissues from extinct populations of mammals. This may be specifically applied to investigating life histories, including the extinct Quaternary megafauna populations whose remains are preserved in the permafrost throughout the northern hemisphere.

**Key words:** testosterone, endogenous steroids, hair-testing, woolly mammoth.

STEROID hormones play a fundamental role in the regulation of development, physiology, reproduction and behaviour. For example, progesterone and testosterone can provide information on reproductive state, such as a pregnancy or an age class. Testosterone is also involved in social processes, including parental behaviour, social status formation, risk-taking behaviour, sexual displays and mating (Ketterson & Nolan 1999; Muller 2017). When elevated systemically, testosterone can have profound effects on fitness, often increasing reproductive success at the expense of survival and parental care (Dufty 1989; Paternostro 1994; Reed *et al.* 2006). Thus, testosterone levels may provide an insight on socio-environmental conditions that shape outcomes for individuals, groups and populations.

Traditionally, endogenous testosterone has been measured in the circulation through blood samples. However, this matrix is not available for extinct species. Hair, on the other hand, is more resilient in withstanding the wear and tear of time. Hair samples have been used for genetic

analysis, with DNA sequenced from >50 000 <sup>14</sup>C year old Siberian mammoths (*Mammuthus primigenius*) (Gilbert *et al.* 2007, 2008). In the same study, DNA was also extracted from hair of a specimen that had been kept in room temperature for over 200 years. Hair-testing for steroids has been used in extant species (e.g. Koren *et al.* 2002, 2008; Koren & Geffen 2009; Malcolm *et al.* 2013). Routes of entry into the hair may be the blood vessel that nourishes the hair as it grows, sweat, sebum, conspecifics, and local (i.e. follicular) synthesis (Cone 1996). Hair steroids have been shown to be associated with blood, saliva, urine and fecal steroids levels and their metabolites (e.g. Tian *et al.* 2015). Since hair integrates endogenous compounds as it grows, it reflects gross patterns, allowing inter-individual and population comparisons (Bryan *et al.* 2015). Ancient human cortisol has been measured in the hair of archaeological mummies (AD550–1532) (Webb *et al.* 2010) and ancient sterols have been detected in fecal samples >500 years old (up to 1000–2000 years old) (Lin

*et al.* 1978; Lin & Connor 2001). Here, we evaluated hair as a possible source of integrated steroids in ancient extinct species. We applied our published method for quantitation of steroids from hair (Koren *et al.* 2002, 2008; Koren & Geffen 2009; Di Francesco *et al.* 2017) on Siberian woolly mammoth hair shaft samples that had been previously utilized by Gilbert *et al.* (2007, 2008) for mitogenome sequencing. Three additional mammoth samples were added to the study for a total of 10 mammoths, and all samples were radiocarbon dated (for details see Gilbert *et al.* (2007, 2008). Our goal was to validate a method to measure long-term integrated testosterone levels in mammoth hair in order to establish means to gain insight on the life history of extinct species.

## MATERIAL AND METHOD

### Sexing

The sex of five mammoths was anatomically determined. However, only two of the mammoths from Gilbert *et al.* (2007, 2008) were sexed at sample collection (M1, M3; which were subsequently sexed both anatomically and genomically). Mammoths of unknown sex (Table 1) were analysed using whole-genome shotgun data from previously published studies (Gilbert *et al.* 2007, 2008; Lynch *et al.* 2015) following approaches that were previously described (Skoglund *et al.* 2015; Pečnerová *et al.* 2017). In order to avoid a bias in mapping due to the presence of nuclear mitochondrial DNA copies (numts), raw sequences were mapped against a merged nuclear-mitochondrial reference consisting of a nuclear genome of the African elephant (*Loxodonta africana*) generated by the Broad Institute (LoxAfr4) and a mitochondrial genome of a known woolly mammoth ('Krause', DQ188829) (Krause *et al.* 2006). Sample M25, which was sequenced on the Illumina platform, was mapped using the BWA-backtrack algorithm implemented in BWA 0.7.8 (Li & Durbin 2010), and the SAMtools 0.1.19 software package (Li *et al.* 2009) was used to align and generate mapping statistics. The other samples, sequenced on the 454 pyrosequencing platform, were analysed using the BWA-MEM algorithm in BWA 0.7.8 (Li & Durbin 2010) and SAMtools 1.3 (Li *et al.* 2009). Only reads passing a mapping quality filter of MQ = 30 were considered. The nuclear reference genome originated from a female elephant, thus Y chromosome reference was not available. Instead, the sex was determined by directly comparing the number of reads mapping to chromosome X and to chromosome 8, which are of comparable size. The number of mapped reads was normalized by the length of the chromosome sequence. Male mammoths are expected to have only half the

number of reads mapping to chromosome X compared to chromosome 8 (see Table 1), because males only have a single copy of chromosome X compared to two in the female elephant. Mammoth samples with higher percentages of reads mapping to chromosome X were designated females.

### Steroid extraction and quantitation

We extracted steroids from individual mammoth hair that was characterized in Gilbert *et al.* (2007), using our published protocol (Koren *et al.* 2002, 2008). Hair samples varied in weight and length (2 to >15 cm). To remove external contaminants from the hair surface, we washed each sample twice with water followed by isopropyl alcohol. Brief (i.e. 3 min) washing with isopropyl alcohol was validated over a decade ago by Davenport *et al.* (2006), who showed that it removes contaminants from the outside the hair but does not extract steroids from within the hair shaft. Hair samples were subsequently dried overnight, weighed and placed in glass vials. We used methanol to extract steroids from inside the hair shaft, sonicated and then incubated overnight at 50°C with shaking. Next, the methanol was collected and evaporated. Samples were reconstituted in assay diluent supplied with the commercial enzyme-linked immunosorbent assays (ELISA; Salimetrics, Ann Arbor, MI, USA). Testosterone immunoreactivity was quantitated in individual extracts following the manufacturer's recommendations.

To validate the kit, we tested whether serial dilutions of mammoth hair pool showed parallelism with the provided standards (univariate analysis of variance in SPSS;  $p = 0.11$ ). Linearity was demonstrated between 10–80 mg of hair, corresponding to 60–250 pg/mL of testosterone standard. The lowest concentration that we detected was 0.91 pg/mL, corresponding 10 mg hair. Salimetrics reported that antibody cross-reactivity was 36.4% with dihydrotestosterone, 21.02% with 19-nortestosterone, 1.9% with 11-hydroxytestosterone, 1.157% with androstenedione and <0.489% for other steroids. Intra-assay repeatability was 11.1%, determined using three replicates of the pool on the same plate.

We used a permutation test (JMP version 13; [https://www.jmp.com/en\\_gb/software/data-analysis-software.html](https://www.jmp.com/en_gb/software/data-analysis-software.html); SAS Inc.) to compare testosterone between sexes, and the correlation between geological age and testosterone (RT version 1.03, B.F.J. Manly, University of Otago, New Zealand).

### LC-MS/MS validation

The presence of testosterone in a pooled mammoth hair extract was confirmed with LC-MS/MS using a similar

**TABLE 1.** Testosterone levels and mapping statistics for sexing of mammoths of unknown sex using previously published whole-genome shotgun data.

Sample	SRA Accession no.	Reads processed	Reads mapped	% Mapped	Reads mapped			Sex/age	Testosterone (pg/mg hair)	
					Applying mapping quality filter (MQ = 30)	Chromosome X	Chromosome 8			Normalized X/8, %
M26	SRX001903	325 401	240 309	73.85	168 763	3002	6674	48.11	Male/a	1.86
M8	SRX001910	546 793	192 212	35.15	115 890	1893	4375	46.28	Male/b	2.13
M1	SRX001889	464 833	349 992	75.29	246 806	3993	8896	48.01	Male*/a	4.54
M4	SRX001905	385 143	325 113	84.41	223 583	3967	8573	49.50	Male/u	3.16
M3	SRX001904	453 661	236 369	52.10	164 717	2847	6241	48.79	Male*/a	4.57
M15	SRX001894	538 422	151 177	28.08	96 216	2951	3608	87.49	Female/u	3.48
M25	SRR2017948	105 906 176	54 745 400	51.69	46 875 136	1 246 276	1 803 890	73.90	Female/u	0.77
Genome data not available:										
Yuribei									Female*/b	0.93
Berezovka									Male*/a	2.83
Yuka									Female*/j	2.13

Data from Gilbert *et al.* (2007, 2008); Lynch *et al.* (2015).

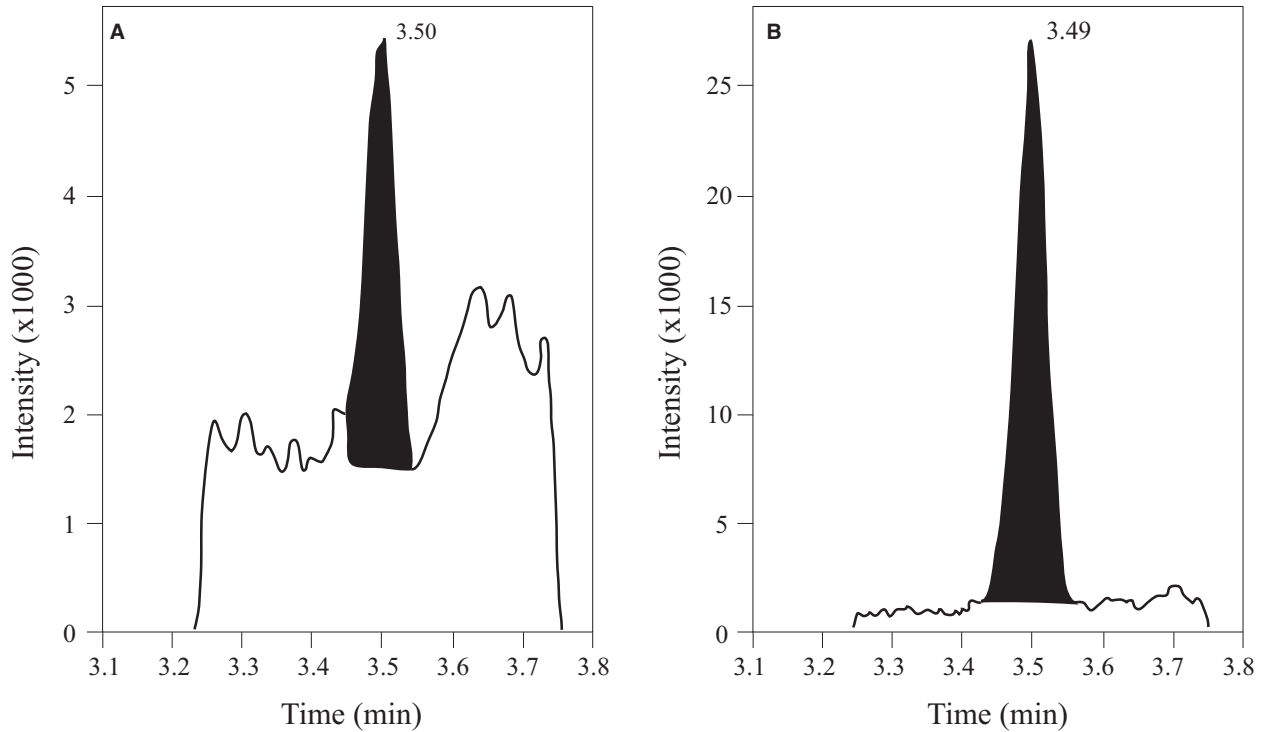
\*Mammoths that were anatomically sexed. Age classes are: a, adult; b, baby; j, juvenile *c.* 10 years old; u, unknown.

sample preparation method as a recent study of cortisol in muskox qiviut (Di Francesco *et al.* 2017). The hair pool was gently washed by water for 3 min, rinsed with HPLC grade isopropyl alcohol, dried by paper towel and placed in a fume hood overnight. The sample was then transferred into a 13 × 100 mm culture test tube. As an additional validation, another test tube contained 200 mg of the burial matrix that surrounded the individual hair samples. To both test tubes, 100 µL of deuterium labeled internal standard solution and 9 mL cold methanol were added. The test tubes were capped and stored in a 4°C fridge for 20 h. After the sample was removed from the test tube, the extract was evaporated to dryness under N<sub>2</sub> at 40°C using a Techne Sample Concentrator and reconstituted with 150 µL H<sub>2</sub>O/MeOH (50/50, v/v). The solution was centrifuged at 14 000 rpm (Legend micro-21R; Thermo Scientific) for 20 min and 120 µL of supernatant was submitted to LC-MS.

All samples were analysed using an Agilent 1200 binary liquid chromatography (LC) system connected to an AB SCIEX QTRAP® 5500 tandem mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. LC separation was performed on an Agilent Poroshell 120 C18 column (50 × 3 mm, 2.7 µm particle size) at 45°C. The mobile phase A was H<sub>2</sub>O/MeOH (75/25, v/v) and the mobile phase B was MeOH/IPA (90/10, v/v). The 8.5 min gradient was 20–40% B (0–1.0 min), 40–60% B (1.0–5.0 min), 60–100% B (5.0–5.5 min), 100% B (5.5–6.5 min), 100–20% B (6.5–7.0 min), and held at 20% B (7.0–8.5 min). The flow rate was 0.6 mL/min and the injection volume was 20 µL. The analytes were ionized under positive APCI mode and data were acquired via multiple reaction monitoring (MRM). More details about the quantification protocol are provided in the Koren *et al.* (2018).

## RESULTS AND DISCUSSION

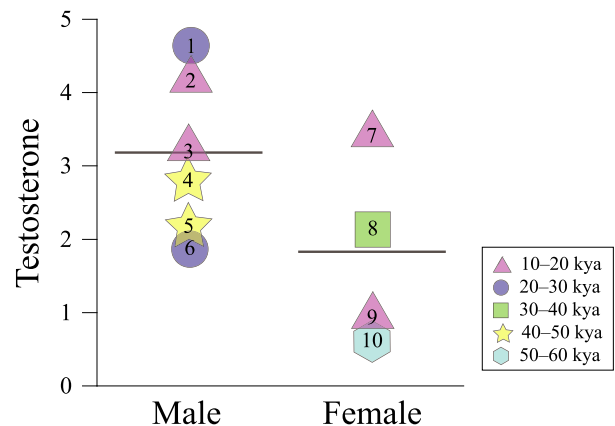
Using a testosterone-specific antibody in a commercial EIA kit we measured testosterone immunoreactivity in mammoth hair samples that are 10 000–60 000 <sup>14</sup>C years old. The presence of testosterone in a mammoth hair pool extract was unambiguously verified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS; Fig. 1). The LC-MS/MS analysis is highly specific, authenticating the presence of testosterone in mammoth hair pool extract via the time of elution, the deuterated internal standard, and two sequential mass-to-charge (*m/z*) transitions. The pooled sample contained hair from unknown individuals from different geographical locations. However, the amount of testosterone that was measured in the pooled sample (0.38 pg/mg) via LC-MS/MS fell within the same order of magnitude as the



**FIG. 1.** Chromatograms showing the presence of testosterone (A) and deuterated internal standard (B), in a pooled mammoth hair sample analysed via LC-MS/MS.

testosterone immunoreactivity measured in an individual female's hair sample (0.77 pg/mg). No testosterone was found in the burial matrix surrounding the hair samples via LC-MS/MS. In addition, the LC-MS/MS detected quantifiable amounts of 17-OH-progesterone, progesterone, DHEA, and androstenedione in the pooled hair sample. Although we have too few cases for statistical tests, we measured testosterone immunoreactivity in all mammoth hair, and sex differences (although not significant; permutation test,  $p = 0.095$ ) in immunoreactive hair testosterone. Mean male ( $n = 6$ ) hair testosterone levels were 3.18 pg/mg hair, whereas female ( $n = 4$ ) were 1.83 pg/mg hair (Fig. 2). Standard deviations were similar between the two sexes (1.26 in females vs 1.16 in males). Male minimum levels were more than twice those of females' (1.86 pg/mg hair in males compared with 0.77 pg/mg hair in females) while maximal levels detected for a male was 4.57 pg/mg hair (vs 3.48 pg/mg hair for a female). High testosterone is typical of the third trimester pregnancy, and it is thus possible that females with high testosterone were pregnant.

To the best of our knowledge, this study is the first to report testosterone quantitation in a prehistoric extinct species. Although steroids are stable compounds, and the specimens that we used were collected from permafrost deposits in northern Siberia where the environmental conditions support the preservation of steroids in the samples, we observed a declining trend in testosterone over time.



**FIG. 2.** Hair testosterone (pg/mg hair) in male ( $n = 6$ ) and female ( $n = 4$ ) woolly mammoths. Colour and shape denotes  $^{14}\text{C}$  age. Numbers inside shape denote individual mammoths: 1, Fishhook (M3); 2, Yukagir (M1); 3, Stephan (M4); 4, Berezovka; 5, Magadan baby (M8); 6, M26; 7, Lacumiu (M15); 8, Yuka; 9, Yuribei; 10, Vanous (M25). Black midlines denote mean testosterone for each sex.

However, the decline was not significant ( $r^2 = 0.2$ ,  $\beta = -0.00004$ , permutation test,  $p = 0.174$ ), possibly due to the lack of power (least significant number = 22). Controlling for the effect of geological time by the regression residuals showed that the differences in testosterone between the sexes is approaching significance (permutation test,  $p = 0.074$ ). In the African elephant, male

(0.38–0.68 ng/mL) and female (0.14–0.46 ng/mL) circulating testosterone concentrations overlap outside the mating period (Rasmussen *et al.* 1984). In elephant hair that we ran alongside this study (N = 5; two males, three females), we found similar levels of testosterone to those measured in mammoths (range 0.98–1.94 pg/mg hair; comparable with mammoths in Table 1), suggesting that perhaps testosterone accumulated in a similar manner in both elephant and mammoth hair. In extant Elephantidae, testosterone rises in males to a peak during the musth (Brown *et al.* 2007; Sukumar 2003), but no seasonal variation is otherwise found in either sex (Brown *et al.* 2007; McNeilly *et al.* 1983). Unfortunately, the age classes of three mammoths in our study was unknown. In modern day elephants, testosterone levels are higher in adults than in pre-pubertal males, but no age effects are detected in non-pregnant females (McNeilly *et al.* 1983). Nonetheless, it is possible that mammoth age is associated with testosterone levels. Clearly, the time period that is reflected in the mammoth hair samples is unknown, and dynamics in testosterone levels in mammoths remain obscure. However, measurements probably represent the last months to a year of the mammoth's life, tens of thousands of years ago, offering novel insight into their lives and possibly their death.

Our results introduce innovative opportunities to quantify multiple steroids in remains of extinct populations of megafauna. These may provide fresh understanding of the ecological conditions that extinct species were subject to. Our method may be extended to other type of keratinized tissue (e.g. feathers, nails, claws, horns, hooves; Koren *et al.* 2012; Matas *et al.* 2016). Many remains have already been radiocarbon dated for genetic analysis and may be also used as a source for steroid studies. Hypothetically, measuring glucocorticoid concentrations of a large number of individuals (Di Francesco *et al.* 2017), over tens of thousands of years, may link changes in physiological processes with major climatic or other environmental changes, and changes in the mean level of population progesterone over time could suggest trends in reproductive output. Moreover, in keratinized tissues with seasonal growth patterns, such as woolly rhinoceros' horns, steroid hormones could even be used to examine seasonal changes in the reproductive biology of extinct species. Despite the challenges involved in this analysis, massive datasets that span tens of thousands of years may yield important information on past periods, through detection of trends in steroid concentrations.

## CONCLUSIONS

The palaeontological assemblages of megafauna from the Quaternary era are suitable for ancient steroid studies due to their pristine preservation and the large number of

individuals. Here, we illustrate the feasibility of studying ancient steroids in hair preserved from the permafrost, and the potential for more detailed future studies in this field.

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*Author contributions.* LK designed the study, coordinated the study, carried out the analysis, and helped draft the manuscript; DM carried out the molecular lab work, and drafted the manuscript; PP sexed the mammoths; LD, AT, and MTPG collected field data; KEWE carried out the LC-MS/MS analysis and helped to interpret the data, EG conceived of the study and carried out the statistical analyses. All authors gave final approval for publication.

## DATA ARCHIVING STATEMENT

Data for this study are available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.q951rb9>

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