Allelic variation of the \textit{COMT} gene in a despotic primate society: A haplotype is related to cortisol excretion in \textit{Macaca fuscata}

Lena S. Pflüger\textsuperscript{a,b,}, Daria R. Gutleb\textsuperscript{a,b}, Martin Hofer\textsuperscript{b}, Martin Fieder\textsuperscript{a}, Bernard Wallner\textsuperscript{a,c,1}, Ralf Steinborn\textsuperscript{b,1}

\textsuperscript{a} Department of Anthropology, Faculty of Life Sciences, University of Vienna, A-1090 Vienna, Austria
\textsuperscript{b} Genomics Core Facility, VetCore, University of Veterinary Medicine, A-1210 Vienna, Austria
\textsuperscript{c} Department of Behavioural Biology, Faculty of Life Sciences, University of Vienna, A-1090 Vienna, Austria

**A R T I C L E   I N F O**

Article history:
Received 23 February 2015
Revised 26 November 2015
Accepted 28 November 2015
Available online 2 December 2015

Keywords:
\textit{Macaca fuscata}
Catechol-O-methyltransferase
Single nucleotide polymorphism
Cortisol excretion
Aggression
Social rank
Non-invasive genotyping

**A B S T R A C T**

Sequence variations in genes of the monoamine neurotransmitter system and their common function in human and non-human primate species are an ongoing issue of investigation. However, the \textit{COMT} gene, coding for the catechol-O-methyltransferase, has not yet attracted much scientific attention regarding its functional role in non-human primates. Considering that a polymorphism of the human \textit{COMT} gene affects the enzyme activity and cortisol level in response to a social stressor, this study investigated the impact of \textit{COMT} on endocrine stress and behavioural parameters in Japanese macaques (\textit{Macaca fuscata}). The species exemplifies a despotic hierarchy in which males’ social rank positions require an adaptation of behaviour strategies. During the mating period, steroid secretion and the frequency of aggressive encounters between males increase. We addressed i) whether this species exhibits potential functional \textit{COMT} variants, ii) whether these variants are associated with faecal cortisol excretion of males, iii) how they are distributed among different social rank positions and iv) whether they are associated with behavioural strategies during times of mate competition. By genotyping 26 males we identified three \textit{COMT} haplotypes (HT), including a putative splice mutant (HT3). This variant was associated with increased cortisol excretion. Given the observed inverse correlation between cortisol and physical aggression, we assume that different \textit{COMT} haplotypes may predispose individuals to pursue more or less aggressive strategies. How these gene-stress effects might favour a specific social role is discussed. Our study of non-invasive genotyping in combination with behavioural and endocrine parameters represents an important step towards understanding the relationship of gene-stress effects in a hierarchically organised primate society.

\textcopyright 2015 Elsevier Inc. All rights reserved.

**Introduction**

Social interactions of group-living animals entail a variety of stressful situations such as aggressive encounters, displacement from resources and the challenge of adapting to dynamic social hierarchies (Summers, 2002). The impact of a stressor can be temporarily counteracted by a repertoire of physiological and behavioural adaptive mechanisms common to most vertebrates. This is accomplished by a complex stress system that extends throughout the central nervous system and the periphery. The hypothalamic-pituitary-adrenal (HPA) axis is responsible for the secretion of glucocorticoids (GCs; Chrousos, 2009; Kyrou and Tsigos, 2008). Its chronic activation impairs an organism’s homeostasis, causing long-term physiological stress reactions related to elevated cortisol secretion (Chrousos, 1998). Catecholamines such as norepinephrine and dopamine (DA) play a major role in regulating HPA axis-induced GC release as well as in central coping mechanisms in response to acute or perceived stressors (Belujon and Grace, 2015; Cabib and Puglisi-Allegra, 2012; Cunningham et al., 1990; Plotsky et al., 1989; for review see Herman and Cullinan, 1997). Concerning cortisol secretion mechanisms, approximately 50% of inter-individual variability is assumed to involve genetic predisposition (Linkowski et al., 1993). Genes of the HPA axis and those responsible for clearing catecholamines are of interest in this regard (Allen et al., 2014; Jabbi et al., 2007). The enzyme catechol-O-methyltransferase (COMT) degrades catecholamines, thereby acting as a key modulator of dopaminergic and noradrenergic neurotransmission (Cumming et al., 1992; Gogos et al., 1998; Li et al., 1984). A non-synonymous single nucleotide polymorphism (SNP) located in the exon 4 (Val\textsuperscript{158}Met; rs4680) of the human \textit{COMT} gene (\textit{COMT}) affects the stability of mRNA secondary structure. In case of the Met allele, this results in an up to 3-fold reduced enzyme activity (Lotta et al., 1995; Nackley et al., 2006). Consequently, the low-active \textit{COMT} allele (Met variant) is suggested to alter HPA function and therefore to modulate stress sensitivity (Oswald et al., 2004). First, it is associated with higher catecholaminergic activity (Karoum et al., 1994), which is expected to increase hypothalamic corticotropin-releasing hormone release. Second, the over-activity of the dopaminergic system induced by the low-active \textit{COMT} allele (Met/Met) alters opioid

http://dx.doi.org/10.1016/j.yhbeh.2015.11.012
0018-506X/© 2015 Elsevier Inc. All rights reserved.
neurotransmission. This results in a lowered capacity to suppress responses to pain and other stressors (Zubieta et al., 2003). Social stress experiments in humans revealed that carriers of the low-active Met allele exhibit an increased cortisol response compared to Val carriers (Armbruster et al., 2012; Bouma et al., 2012; Mueller et al., 2012; Walder et al., 2010). Furthermore, the COMT genotype has been associated with conflict behaviour, central processing of fear, and pain perception (Nackley et al., 2006; Papaleo et al., 2008; Rujescu et al., 2003; Shehzad et al., 2012; Williams et al., 2010). To summarize, in humans, different COMT alleles apparently provide different behavioural adaptation to challenging situations. Whereas the Val allele is assumed to be advantageous in stress resilience (warrior strategy), the low-active Met allele rather increases anxiety and cognitive performance (worrier strategy; Palmer and Dulawa, 2010).

Until now, little is known about functional COMT variants and their roles in closely related primates. Considering the conservative function of the stress system and its key regulators among vertebrates (Uchoa et al., 2014), it can be assumed that functional mutations in the COMT gene would affect stress-related phenotypes not only in humans but also in non-human primates. Previous research on non-human primates explained individual differences in the endocrine stress response by using social interactions in relation to the hierarchical organisation. For example, a despotic social style, which is also known for Japanese macaques, is characterized by steep dominance gradients and a high degree of asymmetry in agonistic and affiliative social interactions (de Waal and Luttrell, 1989; Flack and de Waal, 2004). According to the social rank position, subordinates are thought to experience higher levels of physiological stress reactions in relation to aggression and intimidation by dominant peers. Such a reaction was associated with decreased social support and fewer affiliated partners (Abbott et al., 2003; Sapolsky, 2005).

Other studies (e.g., on long-tailed and Japanese macaques) reported that alpha males are more physiologically stressed than other males, particularly during times of social instability. This was associated with dominant reproductive strategies to compete over the access to mates which involves increased mate-guarding activities, the maintenance of increased courtships, and the engagement in frequent aggressive encounters (Barrett et al., 2002; Girard-Buttoz et al., 2014; Higham et al., 2013; Shimizu, 2005). Repeatedly exposures to elevated levels of behavioural stress result in physiological costs, which may weaken a male’s ability to mate-guard (Girard-Buttoz et al., 2014) or to compete with opponents. Therefore, a genetic predisposition for physiologically stress resistance would be of advantage to maintain costly social strategies without expressing chronically increased glucocorticoid levels. Sequence variations in behaviour-relevant genes, that are orthologous to those in humans (e.g., MAOA, 5-HTT, DAT), have been previously discovered in non-human primates and discussed in the light of their functionality for individuals living under strict hierarchically conditions (Brent et al., 2013; Chakraborty et al., 2010; Lesch et al., 1997; Miller-Butterworth et al., 2008; Barr and Driscoll, 2014; Wendland et al., 2006). The impact of stress-related gene variants, which may modulate individual differences in the endocrine stress response has received only little attention so far.

The present study asked i) whether males of despotically organised Japanese macaques exhibit COMT variants with putative functionality, ii) whether these variants are associated with males’ cortisol excretion during the mating season, iii) how they are distributed among males of different social rank positions and iv) whether they are associated with behavioural strategies during times of mate competition. Japanese macaques (Macaca fuscata) are an appropriate model for this investigation because this species represents a despotic society with a distinct dominance asymmetry, high amounts of unidirectional aggression and low reconciliation rates (Chaffin et al., 1995). Furthermore, they are seasonal breeders and during the mating season males compete over access to females (Huffman, 1987). Throughout this period gonadal and adrenocortical steroid secretion is increased in both sexes and severe aggressive encounters are frequent (Eaton, 1974; Muroyama et al., 2007).

Material and methods

Study site and animals

The investigated population of Japanese macaques (M. fuscata) at the Affenberg Landskron in Carinthia, Austria (GPS: 46°37’60 N and 13°52’60 E), was kept under semi-free conditions. The population derived from 39 monkeys relocated from a wild troop from Osaka prefecture (Japan) in 1996. During data acquisition in winter 2012, the park housed 134 macaques (36 adult males, 54 adult females, 44 immature individuals). All individuals were visually identifiable and lived unseparated on a habitat area of 40,000 m² (Fig. S1). The animals were able to roam freely and no contraceptives were administered. The Affenberg Landskron is a touristic enterprise (Affenberg Zoobetriebsgesellschaft mbH, Landskron Austria) open for guided tours from April to October. During guided tours only one third of the enclosure is accessible to visitors via demarcated pathways. Feeding, touching or any other interactions with the monkeys are prohibited. The other two thirds of the enclosure offer the monkeys the possibility to evade visitors (Fig. S1). The vegetation of the park (coniferous and deciduous trees) enables the animals to forage by themselves (e.g., leaves, herbs, roots, fallen seeds, insects). Food was provided on a daily basis at 10 a.m. and at 3 p.m. by the animal care staff. This food supply (in total 130 kg a day) consisted of different kinds of vegetables, fruits, and pellets, including trace elements and fibres.

Male Japanese macaques reach sexual maturity between 4.5 and 5.5 years (Wolfe, 1978). For the present study 26 focal males (mean age in years ± SD: 12.4 ± 4.2) were selected that were unambiguously physiologically matured (aged ≥5.5 years) and provided us with a sufficient amount of faecal material required for our hormone and DNA analyses (see Sections ‘Hormone assay’ and ‘DNA isolation’). For the population at the Affenberg Landskron, paternity was recorded in 2011 at the Institute of Zoology, University of Graz (Radler, 2014). The analysis of eight short tandem repeat (STR) loci identified six fathers and two paternal half-brothers among the 26 focal males of the present study. Information on four pairs of maternal half-brothers was available from mother–child records provided by the animal management at the study site. For six focal males, paternity testing was precluded as they were born in the wild. For the remaining animals, no paternal lineage information could be specified based on the eight STR loci.

The present study was non-invasive, involving only behavioural observations and collection of individual faecal samples. All research protocols minimized distress and were approved and authorized by the animal care appointee of the Affenberg Zoobetriebsgesellschaft mbH, Landskron, Austria.

Behavioural observation and analyses

The behaviour of males was recorded from late October to early December on a daily basis between 9 a.m. and 4 p.m. The observation spots were chosen according to the main gathering sites of the group spread along the pathway of the guided tour (Fig. S1). At each spot, interactions between males were recorded ad libitum (Martin and Bateson, 1993) by a single observer, yielding 200 observation hours. The following agonistic and socio-positive behaviours were recorded in males: chase, lunge, grab, hit, bite, threat (O-shaped mouth), ground slap, (symbolic) mount, presenting, displacement, silent bare-teeth display, cower, flee, mock leave, lip smack, leave (avoidance), groom, and spatial proximity (i.e., sit next to or pass by). All variables were recorded according to common definitions (Takahata, 1980). Categories of aggressive interactions between males were determined by variables on overt physical aggression. The behaviour category initiation of physical aggression implied the variables chase, lunge and physical attacks such as grab, hit and bite. Receivers of these behaviours were assigned as target of physical aggression insofar as they showed an immediate submissive response to the received
aggression or responded aggressively. Subsequently, for each individual the amounts of being a target of aggression (tAgr) and initiating aggression (iAgr) were first defined as two single categories and secondly combined to the category involvement in physical aggressive interactions (Agr). Additionally, the variables grooming and spatial proximity were used for the analysis of socio-positive (i.e. non-aggressive) behaviours that were observed among males (Socio). The methodological approach of recording the behaviour ad libitum enabled the observer to scan all possible dyadic interactions between males that occurred during the time of observation. This approach enabled controlling for an individual’s presence in the group and thus for that individual’s general tendency to engage with other group members. We therefore applied a standardized comparison of individual involvement in the above-mentioned behaviour categories. This was accomplished by setting the categories (tAgr, iAgr, Agr, Socio) in relation to all other remaining intra-sexual interactions recorded for this individual. From this procedure, the analysed values reflect the proportion of the four categories in relation to all other received or initiated behaviours, i.e. a value of Agr = 0.3 equals 30% of interactions observed for this individual were of aggressive nature.

Dominance rank

Pflüger et al. (2014) analysed the linear social rank relationships of males according to Singh et al. (2003), who provided a method to assess these relationships in Japanese macaques. During winter 2012, the authors recorded the behaviour of 30 adult males including the focal males of the current study. In order to create a hierarchy matrix, male dyadic interactions between all possible pairs were recorded and calculated into a proportion of wins, i.e. being dominant over another male. Note that the analyses include not only aggressive encounters but also variables of no physical contact such as avoidance or spatial displacement. For details on dominant and submissive behaviour variables see Pflüger et al. 2014. The resulting P2 value referred to the proportion of encounters won by an animal against another in a pairwise interaction. The final d5 score of each individual equalled the total of his entire P2 values received from all his encounters with other males (for methodological details see Singh et al., 2003). The d5 scores of our focal animals, ranging from d5 = 2.09 (lowest rank position) to d5 = 27.71 (highest rank position), entered our subsequent analyses.

Faeces sampling

Throughout the observation period all available faecal samples were collected per focal animal, yielding in a distribution of three (N = 2), four (N = 22) or five (N = 2) samples per male. Directly after defecation about 10 g of faecal matter without urinary contamination were collected into plastic tubes (Kartell S.P.A., Noviglio, Italy). Afterwards the samples were homogenized to avoid bacterial or fungal contamination and then immediately frozen at −20 °C. During shipment from the study site at Landskron to the laboratories in Vienna, samples were kept frozen with cool bags for only a few hours.

Hormone assay

The hormone analyses were conducted at the endocrinology lab of the Department of Behavioural Biology (University of Vienna, Vienna, Austria). As cortisol is metabolized in the liver before excretion, an 11-o xoetiocholanolone immunnoassay (Wallner et al., 1999) was applied to measure 3α-OH-11-one cortisol metabolites in faeces (Moestl et al., 2002). Cortisol excretion was quantified using a microtiter plate enzyme immunoassay (EIA, Palme and Moestl, 1997). Faecal samples were dried at 60 °C for 72 h. A 0.5 g aliquot was extracted with 1.5 ml double-distilled water and 3 ml methanol, vortexed for 30 min and centrifuged at 1942 × g for 15 min. Ten microliters of the supernatant was 50-fold diluted with assay buffer consisting of 20 mM Tris (hydroxymethyl)aminomethane (pH 7.5; Merck Millipore Darmstadt, Germany), 0.3 M NaCl (Merck Millipore), 0.1% (w/v) bovine serum albumin (Sigma-Aldrich, Steinheim, Germany) and 0.1% (v/v) Tween 80 (Merck Millipore) and subjected to the EIA. Each experimental sample was measured in duplicate. Samples and controls were processed at the same time. Standard fluids and 96-well microtiter plates were obtained from the Institute of Medical Biochemistry (University of Veterinary Medicine, Vienna, Austria). Plates were incubated with the 11-o xoetiocholanolone-17-CMO:BSA antibody raised in rabbit and the enzyme label 11-o xoetiocholanolone-17-CMO-biotin-3,6,9-trioxauandecanediamin (the biotinyl label was synthesized using a mixed anhydride reaction; Møstl et al., 2002; Palme and Møstl, 1993) followed by four washing steps with a 0.02% (v/v) Tween 20 solution (Merck Millipore). Subsequently, 30 ml assay buffer (see above) supplemented with 1 μl streptavidin-POD-conjugate (Labsystems Multidrop, Cambridge Scientific, USA) was added to the wells. The wells were further treated with 250 μl substrate solution prepared by mixing 30 ml substrate buffer with 500 μl 0.4% 3,3′, 5,5′-tetramethylbenzidine solution and 100 μl 0.6% H2O2 (Merck Millipore). After incubation at 4 °C, the reaction was stopped by adding 50 μl of 2 M sulfuric acid (Merck Millipore). The absorbance was measured on an ELx808 Absorbance Microplate Reader (Biotek, Winooski, USA) using the 2.0.18 version of the Gen5™ software and equipped with measuring and reference filters of 450 and 620 nm, respectively.

The samples (N = 104) were measured in the same assay on four microtiter plates. The intra-assay coefficient of variation was 8.3%. The sensitivity of the assay at 90% binding was 2.3 pg/well. The inter-assay variation was 18.26%. For each individual, the resulting values of cortisol excretion obtained from single faecal samples were averaged and the means entered the subsequent statistical analyses.

Faecal samples used for the hormone assay were collected throughout the day. We therefore controlled for the time of sampling to ensure that our hormone measures are not affected by any daily fluctuations (Beechner and Whitten, 2004). This was accomplished by investigating the association between time point of sampling and the amount of cortisol metabolites measured in all samples collected (N = 104). Furthermore, mean amounts of cortisol metabolites obtained from morning (9.30 to 11 a.m., N = 18) and afternoon samples (3 to 6 p.m., N = 18) were compared.

DNA isolation and determination of genome equivalents

DNA was extracted from frozen faecal samples using the ZR Fecal DNA MiniPrep™ kit (Zymo Research Europe GmbH, Freiburg, Germany) following the manufacturer's instruction. For homogenisation with the Lysis Solution (steps 1–2 of the Zymo Research protocol), samples were vortexed for five minutes at full speed (3000 rpm). DNA concentration and its purity (A260/A280 ratio) were measured on the NanoDrop ND-2000c spectrophotometer (Peqlab Biotechnology GmbH, Erlangen, Germany). Values of experimental samples ranged between 16 to 32 ng/μl and 1.5 to 2.0, respectively. Genotyping of samples with a low target amount would be susceptible to stochastic sampling effects during extraction of nucleic acids or more likely during PCR amplification due to the exponential nature of the method (Budowle et al., 2009; Gill et al., 2005). This may cause an imbalance of heterozygous alleles and allelic or complete locus dropout, complicating correct interpretation of the genotyping data. The minor contribution of host DNA to faecal DNA, which is mainly composed of genetic material derived from the intestinal microbiome and digested plant material, was quantified by quantitative PCR (qPCR). The assay targeted the single-copy gene MYC (Morin et al., 2001). Complete homology of the primer and probe sequences used in this qPCR assay to our model species was concluded from sequences of the related macaques Macaca mulatta (GenBank: NM_001142873.1, DQ976874.1) and Macaca nemestrina (DQ977093.1). The 20-μl reaction consisted of 1 × B2 buffer (Solis Biodyne, Tartu, Estonia), 3.5 mM MgCl2, 0.2 mM of each dNTP (Solis
Biodyne), 200 nM of each primer and probe (Sigma-Aldrich, Vienna, Austria), 5 ng/μl non-acetylated bovine serum albumin (Sigma-Aldrich), 1 unit hot-start Taq DNA polymerase (HOT FIREPol® DNA Polymerase, Solis Biodyne) and 2 μl DNA. Duplicates, triplicates or duplicates/ quadruplicates were measured in experimental samples. Random faecal DNAs were used to estimate the average amplification efficiency and dilutions of the mass standard (see below). Three no-template controls (NTCs) were randomly distributed across the 96-well plate. A mass standard with a DNA concentration of 7 ng/μl was isolated with the Tissue & Cell Genomic DNA Purification Kit (GMbiolab Co. Ltd., Taiwan) from postmortem muscle of a deceased animal. The quantitative dynamic range of qPCR was determined based on three serial sixteen-fold dilutions (438 to 1.7 pg/μl) of the standard. qPCR was run on the StepOnePlus™ Real-Time PCR System (Life Technologies, Foster City, USA; software version 2.3) for 15 min at 95 °C followed by 50 cycles at 95 °C for 10 s and 61 °C for 50 s. A normalized reporter signal minus background (ΔRn) value of 3000 was used to determine Cq values across plates. A qPCR replicates was classified as an outlier and omitted when it deviated from mean Cq by more than two standard deviations.

A standard curve was generated by linear regression analysis of the \( \log_{10} \) of the DNA mass contained in the standard dilutions plotted against Cq values. Amplification efficiency (E) was calculated from the slope of this curve (E = \( 10^{-(1/\text{slope})} - 1 \)) and averaged based on the three dilution series, each prepared from a different faecal DNA. Cq values generated at an average E < 1 were corrected by the term \( C_q = \log_{10}(E + 1)/\log_{10}(2) \) (Kubista et al., 2007; Robledo et al., 2014). Samples containing less than the critical value of 200 pg host DNA per input, corresponding to 30 diploid cells, were rejected and a new DNA extraction was performed. The number of host cell equivalents was calculated in agreement with the value for the haploid DNA content of 3.14–3.59 pg per cell given for the rhesus monkey by the Animal Genome Size Database (Release 2.0; http://www.genomesize.com/index.php).

The qPCR data comply with the Minimum Information for Publication of Quantitative Digital PC Experiments (Bustin et al., 2009).

**Genotyping the exon 4 of COMT and its intronic surrounding**

Variation at the target site — the orthologous site of Val108→159Met of human COMT — was analysed by Sanger sequencing. In detail, primers covering exon 4 and parts of its neighbouring introns were placed into highly conserved positions in the orthologous genes of Homo sapiens, Macaca fascicularis and M. mulatta (GenBank accessions NC_000002.11, NC_002281.1 and NC_007867.5). Primers for amplification of 236 bp and 288 bp products overlapping each other by 136 bp were designed by Primer Express 2.0 software of Life Technologies (sequences: 5′-CGTTGCTGGTGGGAGAGGTAG and 5′-GGTTGATCTC "SplicePort (Dogan et al., 2007), the Human Splicing Finder (version 2.0.2) of NCBI, the Exonuclease Nucleic Acid Stain (Biotium, Hayward, USA) and visualized on an AlphaImager HP gel documentation system (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) equipped with a blue light screen (example in Fig. S2c).

**Verification of Sanger sequencing electropherograms**

In the case of heterozygosity the resulting peak pair migrates at the same position as a mixed trace. The signal strength of each component is approximately half of the homozygous counterpart (example in Fig. S2a). In two animals the Sanger electropherogram showed peak imbalance at the SNP site of intron 4 (example in Fig. S2b) regardless of the strand sequenced and even though the amount of host DNA passed the critical threshold (see above). It has been reported that reducing amplicon size down to less than 100 bp significantly improved PCR-based genotyping of degraded DNA (Hellmann et al., 2001). This would be of special value for genotyping faecal samples known to contain degraded nuclear host DNA and to suffer from co-puri fi cation of PCR inhibitors. Hence, we amplified a shorter product, 80 bp instead of 236/288 bp (primers: 5′-GGATCACCACGGCGGAT and 5′-CTTCCAGGCGGACGCCGACGGCGCGG) for another analysis by Sanger sequencing and restriction endonuclease digestion in parallel.

The 80 bp amplicon was digested with the enzyme Hin6I (Thermo Fisher Scientific Inc., Waltham, USA) using 1 × Tango Buffer (Thermo Fisher Scientific Inc.) and incubated at 37 °C for 16 h. To exclude inhibition by faecal contaminants, a PCR product yielding fragments of 140 and 223 bp by restriction with Hin6I was spiked into each sample before digestion (details not shown). Heterozygous and homozygous samples yielding unambiguous sequencing electropherograms were used for control. Digested and undigested amplicons were electrophoresed on a 2% agarose gel stained with GelGreen™ Nucleic Acid Stain (Biotium, Hayward, USA) and visualized on an Alphalager HP gel documentation system (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) equipped with a blue light screen (example in Fig. S2c).

**Number of COMT haplotypes**

Two SNPs — in our case the C/T and G/T polymorphisms of exon 4 and intron 4 of COMT (see Result Section) — could form a maximum of four haplotypes (HItS). To resolve the linkage of the two SNPs and to determine the number of haplotypes constituted, we preformed restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) and Sanger sequencing for one of the resulting fragments. Briefly, the 258 bp product generated with the primers 5′-CACGCTTCCATGCTCGT and 5′-ACATGGTTGTTTTAATGACGTG was digested with Hin6I (Thermo Fisher Scientific Inc.) as described above. The success of digestion in each sample was controlled by a 53 bp fragment produced by a successful Hin6I digestion irrespective of haplotype. A guanine base at the intronic SNP site produced restriction fragments of 91 and 114 bp. The transversion to thymine destroyed the Hin6I site and yielded a 205 bp fragment. The base at the exonic SNP site that is linked to the thymine was finally determined by Sanger sequencing of the restriction fragment.

**Splice site prediction and bioinformatics**

The nucleotide sequence of exon 4 was translated into protein using the translate tool of the bioinformatics resource portal ExPaSy (web.expasy.org/translate). Protein identity was confirmed by protein–protein BLAST (blastp) of NCBI (blast.ncbi.nlm.nih.gov).

Three core splicing sequences, namely the 5′ splice site beginning with an invariant GU dinucleotide, the 3′ splice site ending with an invariant AG dinucleotide and the branch site sequence, are essential for splicing eukaryotic exons (Ward and Cooper, 2010). To determine whether the SNPs identified in M. fascata affect splicing, acceptor and donor splicing sequences were predicted using four freely available online network tools: the Berkeley Drosophila Genome Project (BDGP splice site prediction; NNSPLICE version 0.9; Reese et al., 1997), the SplicePort (Dogan et al., 2007), the Human Splicing Finder (version 2.4.1: Desmet et al., 2009) and the MaxEntScan (MaxEntScan:score 3ss and MaxEntScan:score 5ss; Yeo and Burge, 2004). Exonic splicing
enhancers (ESEs) are regulatory sequences located within exons that are required for exon recognition and that modulate splice site selection of alternatively used splice sites (Nielsen et al., 2007; Ward and Cooper, 2010). The Human Splicing Finder and the ESEFinder (version 3.0; Cartegni et al., 2003; Smith et al., 2006) were applied to analyse whether the exonic SNP identified in M. fascata change the ESE sequence located in the exon 4 of COMT. Sequences available at NCBI were used to predict homologous splice sites and ESEs of Homo sapiens (NC_000022.11), Pan troglodytes (NC_006489.3), Pan paniscus (NW_003870398.1), Gorilla gorilla gorilla (NC_018446.1), Pongo abelii (NC_012613.1), Papio anubis (NC_018161.1), M. mulatta (NC_007867.1) and M. fascicularis (NC_022281.1).

Exon 4 sequences of COMT of M. fascata and of eight human and non-human primates (see above) were aligned by the CLC Main Workbench version 7.5 (Qiagen). The sequence logo alignment was created with the WebLogo online tool (version 2.8.2; Crooks et al., 2004). The proportional display of exons, introns and untranslated regions (UTRs) of the COMT gene was created based on sequences available from the closest relative, M. mulatta (NC_007867.1; NM_001261012.2, DR767585.1), using the Exon-Intron Graphic Maker (version 4; wormweb.org/ exonintron).

Statistical analyses

For the following statistical analyses the IBM SPSS Statistics 19.0 was applied with α set at 0.05: the correlation between time of faeces sampling and resulting cortisol metabolites was calculated with Spearman’s-Rho (rs). Mean rank of cortisol metabolites in morning and afternoon samples was compared by the Mann–Whitney U test. Males were categorized as carriers (in homo- or homozygous state) or non-carriers of the C-T haplotype (HT3, see Result Section ‘Identified COMT haplotypes’). The Mann–Whitney U test was applied to compare the mean cortisol ranks of the two categorical groups. Correlation between behaviour categories on physical aggression (Agr, iAgr and tAgr), cortisol excretion and linear social rank was calculated with Spearman’s-Rho (rs).

For the following statistical analyses the R version 3.0.2 (R Core Team, 2014) was applied with α set at 0.05: a LD’ (linkage disequilibrium prime) between the two identified COMT SNPs was calculated using the R library ‘genetics’, a well-established toolset in statistical genetics (Foulkes, 2009). Linkage disequilibrium (LD) is defined as the non-random association between two or more gene variants (or alleles) such that certain combinations are more likely to occur together, e.g. the presence of an association between two variants at different loci (here exonic and intronic SNPs) that is different from what would be expected if those were independent.

General linear models (GLMs) were calculated for the regression of each identified haplotype (HT1-HT3, see Result Section ‘Identified COMT haplotypes’) on males’ cortisol excretion: first, the association between the presence of each haplotype in individuals (carriers in either homo- or heterozygous status, respectively non-carriers) and cortisol excretion was assessed (model 1). Second, three models were calculated, each including one additional variable, i.e. males’ linear social rank (model 2), Agr (model 3) and tAgr (model 4). These variables were included in separate models as they appeared to be correlated according to Spearman’s-Rho (Spearman’s rank correlations, see Result Section). Moreover, in models 2, 3 and 4 we calculated the interactions of each haplotype and the additional variable (linear social rank, Agr and tAgr). For instance, in model 3 the following interactions were assessed: HT1:iAgr, HT2:iAgr and HT3:iAgr. Subsequently, models 2, 3 and 4 were stepwise reduced according to the Akaike information criterion by using the ‘step algorithm’ implemented in R. Finally, we performed three separate GLMs for the regression of COMT haplotypes on the linear social rank (model 5), displayed aggression (iAgr, model 6) and socio-positive behaviour (Socio, model 7).

Results

Quantification of genome equivalents by qPCR

The number of genome equivalents in the macaque host contained in the faecal DNA samples was determined by qPCR (Table S1; detection limit: one haploid macaque genome equivalent). The number of genome equivalents per amplification reaction isolated from the experimental samples ranged from 200 to 11,300 pg with a mean of 1800 pg (Table S2 and Fig. S3, see also Table S3 and Fig. S4 for more details on the assay). None of the samples fell below the threshold of 200 pg DNA input (DNA content of 30 diploid cells) introduced to avoid stochastic effects during sample processing and DNA amplification (Budowle et al., 2009; Gill et al., 2005). According to the control samples included in each run, no significant inconsistency between the different runs was observed (Table S1).

SNPs in exon 4 and in the upstream part of intron 4 of COMT

Before starting the genotyping analysis, we compared exon 4 sequences of COMT publically available from eight different primate species including humans. Both alleles present at the orthologous site of human Val108/158Met were distributed among species of the genus Macaca (NCBI: XM_005567960, NM_001261012, Fig. S5). Next, we investigated intraspecific genetic variations of COMT in Japanese macaques by targeting exon 4 and parts of the neighbouring introns. At the orthologous SNP site of Val108/158Met, all 26 investigated males carried the codon coding for the amino acid methionine (COMT Met allele). In close vicinity, however, two novel polymorphisms — an exonic and an intronic SNP — were identified (Fig. 1). The exonic SNP, a synonymous C to T transition, was present at frequencies of 61.5%, 30.8% and 7.7% determined for the C/C, C/T and T/T genotypes, respectively. According to in silico analysis the C to T transition in exon 4 did not affect the sequence of the ESE motif, which was conserved among all investigated human and non-human primates including M. fascata (Fig. S6, Table S4). The intronic SNP, a G to T transversion, altered the splicing donor site of intron 4 (Fig. S6). It was detected at frequencies of 50%, 42.3% and 7.7% for the C/G, G/T and T/T genotypes, respectively. Both SNPs were in high linkage disequilibrium (LD = 0.999).

Identified COMT haplotypes

Theoretically, the two identified SNPs (one in exon 4 and one in intron 4) could lead to four haplotypes via different nucleotide combinations, i.e. C-G, T-G, C-T and T-T. To determine the actual number of haplotypes in the experimental cohort, we applied PCR-RFLP analysis followed by Sanger sequencing. Here, only three haplotypes termed HT1 (C-G), HT2 (T-G) and HT3 (C-T) were found (Fig. 1). The T-T haplotype was not present in the 26 investigated males. All identified haplotypes (HT1-HT3) were detected in either homozygous or heterozygous state (Table 1). We refer to ‘carrier’ of one specific haplotype if the individual carries at least one copy of the respective allele. For instance, a homozygous carrier of HT3 carries two copies of the C-T allele. A heterozygous carrier of HT3 carries one copy of the C-T allele and one copy of either the C-G (HT1) or the T-G (HT2) allele. Detailed information on the distribution of the identified COMT haplotypes in the experimental cohort is presented in Table 1. Sequences of COMT haplotypes were submitted to the NCBI GenBank with the accession numbers KM820841, KM820842 and KM820843.

In silico analyses predicted canonical splice donor and acceptor sites for COMT HT1 and HT2 sequences of the investigated Japanese macaques. These splice sites were also predicted for orthologous COMT sequences of eight primate species including humans (Table S5). However, impaired splicing of the donor splice site was predicted in case of HT3. In detail, no donor splice site was predicted by the BDGP
splice predictor, and lowered and/or altered scores were reported by SplicePort, Human Splicing Finder and MaxEntScan (Table S5).

Hormone data

The EIA revealed mean amounts of cortisol metabolites ± SD of 471 ± 234 ng/g with a range of 116.53 to 834.87 ng/g of dry faeces (N = 26). We found no correlation between sampling time and cortisol metabolite values (rs = 0.06, N = 104, P = 0.533) and no difference in the amount of cortisol metabolites between morning and afternoon samples was observed (Mann–Whitney U test: U = 0.4, N = 18/18, P = 0.501).

Association of COMT haplotype with cortisol excretion and behaviour

The social rank of males was positively correlated with the involvement in physically aggressive interactions (Agr; rs = 0.65, N = 26, P < 0.001, Fig. S7). This association was based on the amount of initiated overt physical attacks (iAgr; rs = 0.68, N = 26, P < 0.001). The linear social rank of males did not correlate with the mean cortisol excretion during the time of observation (rs = −0.2, N = 26, P = 0.317, Fig. S8). However, male cortisol excretion was negatively correlated with the involvement in aggressive encounters (Agr; rs = −0.45, N = 26, P = 0.02, Fig. 2). More precisely, males who displayed high levels of aggression had lower cortisol excretion than those who showed lower levels of aggression (iAgr; rs = −0.4, N = 26, P = 0.045). No association was found between received aggression and cortisol excretion (tAgr; rs = −0.09, N = 26, P = 0.646).

The GLM revealed that out of the three haplotypes identified, only HT3 regressed positively on male cortisol excretion (model 1, Table 2). Model 2 was applied to control for any confounding effect of social rank position on the above-mentioned regression. Again, only HT3 showed a significantly positive effect on cortisol excretion (Table 2). Also the implementation of behaviour categories on physical aggression (iAgr and tAgr) in models 3 and 4 did not reduce the significantly positive effect of HT3 on cortisol excretion (Table 2). The assignment of males according to the presence of the HT3 allele revealed higher cortisol excretion in carriers compared to non-carriers (Mann–Whitney U test: U = −2.74, N = 13/13, P = 0.005; Fig. 3). The regression of COMT haplotypes on males’ social rank (model 5) or displayed behaviour (iAgr, Socio, model 6 and 7) revealed no association (see supplement Tables S6–S8).

Discussion

The present study asked whether a non-human primate species, namely Japanese macaques, exhibits COMT variants that are associated with cortisol excretion of males during the mating season. We further addressed the distribution of identified COMT haplotypes among different social ranks and whether specific COMT alleles are associated with individuals’ behaviour. In humans and rodent model organisms, catecholaminergic gene variants were extensively investigated. Allelic variations in the COMT gene were found to modulate physiological stress reactions in response to social challenges (Armbruster et al., 2012; Bouma et al., 2012; Mueller et al., 2012), pain sensitivity (Diatchenko et al., 2005; Nackley et al., 2006) and behavioural adaption to a perceived stressor (Papaleo et al., 2008; Rujescu et al., 2003; Shehzad et al., 2012; Volavka et al., 2004). In studies on non-human primates, however, COMT received only little attention. For Japanese macaques the mating season represents a period of high social stress. During this time, males compete over access to females, steroid excretion increases and severe aggressive encounters occur at high frequencies (Barrett et al., 2002; Eaton, 1974; Muroyama et al., 2007). In our non-invasive study, we used the measure of faecal cortisol metabolites as an estimate of the endocrine stress response of adult male macaques. Since the liver metabolizes circulating steroids before they enter the gut via the bile (MacDonald et al., 1983), the measure of faecal hormone metabolites reflects a delayed production rate of a target steroid over time. In contrast to blood or saliva samples this provides an estimate of the overall hormone status of an animal rather than mirroring daily fluctuations or an acute stressor (Palme et al., 2005). By comparing results received

Table 1

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Nucleotide compositiona</th>
<th>Number of homozygous males</th>
<th>Number of heterozygous males</th>
<th>Percentage of carriersb</th>
<th>Allele frequencyc</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1</td>
<td>C-G</td>
<td>5</td>
<td>15</td>
<td>77</td>
<td>0.48</td>
</tr>
<tr>
<td>HT2</td>
<td>T-G</td>
<td>2</td>
<td>8</td>
<td>38</td>
<td>0.23</td>
</tr>
<tr>
<td>HT3</td>
<td>C-T</td>
<td>2</td>
<td>11</td>
<td>50</td>
<td>0.29</td>
</tr>
</tbody>
</table>

a Combination of nucleotides found at the SNP sites in the exon 4 and the intron 4 of COMT.
b Percentage of carriers in both homo- and heterozygous status.
c Relative proportion of a particular allele in a specific population, refers here to the number of a particular COMT allele divided by the total number of alleles present in the experimental cohort (N = 52 alleles).
from samples collected at different day times, we also ensured statistically that our measure of cortisol metabolites was not affected by any daily fluctuations of hormone secretion. Furthermore, we used faecal DNA to investigate COMT gene variants in focal males. The application of several pre- and post-measures ensured the reliability of our genotype data, which were based on low amount of host DNA. Our analyses targeted the exon 4 of the COMT gene because the orthologous site has been extensively investigated in the context of human psychological and physiological stress responses (Armbruster et al., 2012; Oswald et al., 2004; Ursini et al., 2011; van Winkel et al., 2008; Walder et al., 2010). Two SNPs surrounding the orthologous human Val108/158Met polymorphism were identified in the experimental cohort—a (synonymous) SNP in the adjacent codon and a SNP at the splice donor site of intron 4. According to in silico analyses the exonic SNP does not affect the ESE sequence, which is a director and enhancer of accurate splicing positioned near exon ends (Fairbrother et al., 2004). However, the analyses yielded impaired splicing of the haplotype HT3 that involved a thymine at the SNP position in intron 4. More precisely, the donor splice site of intron 4 was absent or altered in comparison to HT1 and HT2 of Japanese macaques as well as to all other primate sequences involved in our splice site analyses. Single base pair changes occurring within the 5ʹ and 3ʹ splice sites of introns have been previously reported to affect splicing in silico and in vitro and typically result in exon skipping, activation of a cryptic splice site, or intron retention (Carmel et al., 2004; Faustino and Cooper, 2003; Tazi et al., 2009; Wang et al., 2008; Wang and Cooper, 2007; Ward and Cooper, 2010). Recently, SNP-effects were categorized according to their functionality. Here, mutations affecting splice sites, stop- or start codons are assumed to have the highest impacts (Rašić et al., 2014). Based on this classification, a main effect can be assumed for HT3 according to in silico splice site analysis conducted

Table 2
Linear regression analyses on mean cortisol excretion (ng/g faeces).

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coefficients</td>
<td>Haplotypes (HTs)</td>
<td>HTs and social rankb</td>
<td>HTs and iAgrc</td>
<td>HTs and tAgrd</td>
</tr>
<tr>
<td>(Intercept)</td>
<td>625.81 (126.08)</td>
<td>726.21 (162.76)</td>
<td>683.43 (126.31)</td>
<td>623.94 (127.89)</td>
</tr>
<tr>
<td></td>
<td>t = 4.96***</td>
<td>t = 4.46***</td>
<td>t = 5.41***</td>
<td>t = 4.88***</td>
</tr>
<tr>
<td>HT1 carriersa</td>
<td>21.31 (111.97)</td>
<td>39.72 (113.66)</td>
<td>15.32 (107.87)</td>
<td>-4.17 (120.58)</td>
</tr>
<tr>
<td>(ref. HT1 non-carriers)</td>
<td>t = 0.19</td>
<td>t = 0.35</td>
<td>t = 0.14</td>
<td>t = -0.04</td>
</tr>
<tr>
<td>HT2 carriersa</td>
<td>27.08 (108.3)</td>
<td>48.56 (110.62)</td>
<td>43.34 (104.74)</td>
<td>-8.09 (123.29)</td>
</tr>
<tr>
<td>(ref. HT2 non-carriers)</td>
<td>t = 0.25</td>
<td>t = 0.44</td>
<td>t = 0.41</td>
<td>t = -0.07</td>
</tr>
<tr>
<td>HT3 carriersa</td>
<td>265.99 (101.63)</td>
<td>256.56 (102.19)</td>
<td>229.23 (100.35)</td>
<td>244.53 (108.58)</td>
</tr>
<tr>
<td>(ref. HT3 non-carriers)</td>
<td>t = 2.62*</td>
<td>t = 2.51*</td>
<td>t = 2.28*</td>
<td>t = 2.25*</td>
</tr>
<tr>
<td>Social rankb</td>
<td></td>
<td>-6.07 (6.22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>t = -0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iAgrc</td>
<td></td>
<td>-612.48 (370.66)</td>
<td></td>
<td>-566.47 (902.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t = -1.65</td>
<td></td>
<td>t = -0.63</td>
</tr>
<tr>
<td>tAgrd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Given are estimates (standard error) and t-values. N = 26. Degree of freedom = 25.

* 0.01 ≤ P < 0.05.
** P < 0.001.

a Carriers in either homo- or heterozygous status.
b da score.
c Initiation of physical aggression in relation to all other received or performed behaviours observed.
d Target of physical aggression in relation to all other received or performed behaviours observed.

Fig. 2. Correlation between cortisol excretion and the involvement in physically aggressive interactions recorded for each male (r = −0.45, N = 26, P = 0.02). Individuals are marked as carriers (full circles) and non-carriers (open circles) of HT3. Numbers depict individuals according to their social rank position (1 = highest rank position, da score, 26 = lowest rank position). Note: Agr-values refer to the involvement in physically aggressive interactions of one male in relation to all other received or initiated behaviours observed in this individual.
with four algorithms. These tools have previously been proven to reliably predict effects on human RNA splicing. Particularly, the prediction of whether or not changes in the intronic 5' and 3' splice site region affect RNA splicing seems to be relatively straightforward and highly reliable (Houdayer et al., 2012; Théry et al., 2011; Vreeswijk et al., 2009).

The association of COMT haplotypes and cortisol excretion observed in this study further supports the existence of the predicted transcript isoform. We found elevated cortisol excretion during the mating season in carriers compared to non-carriers of HT3, pointing to a modified physiological stress response.

As for other despotic species, the influence of social rank on males' cortisol excretion has been an issue in previous studies on male Japanese macaques (e.g., Barrett et al., 2002; Shimizu, 2005). Hence, social rank position as well as the amount of physical aggressive attacks each individual received or performed were included as explanatory variables in three independent models. In each case, the underlying genotype remained the main predictor for cortisol excretion. A comparable phenotype has been previously described for the human COMT Val108/158Met: carriers of the low active Met allele showed increased cortisol responses in social stress experiments compared to those carrying the Val allele (Armbuster et al., 2012; Bouma et al., 2012; Mueller et al., 2012). Different genetic changes causing similar enzymatic effects and physiological phenotypes among mammals have been already described (e.g., Tellam et al., 2012). Hence, the results of the present study are valuable with regard to the mentioned gene-stress effects, although the investigated primate species does not carry the COMT Val/Met variation described in humans. Future experiments are required to characterize the transcriptional isoform putatively produced by HT3 of COMT, including the analysis of enzyme abundance and activity in Japanese macaques. Genome-wide sequencing data could decipher whether other COMT variants and genes participate in linkage disequilibrium or epistasis. Faecal DNA, however, represents a limited source for such experiments because of the low concentration, integrity and purity of host cell DNA. This calls for using other tissue samples, which would unfortunately be incompatible with non-invasive research in free-ranging animals.

In humans and rodents, functional variants of the COMT gene have been associated with aggression-related behaviour (Kulikova et al., 2008; Rujescu et al., 2003; Volavka et al., 2004). Our analysis on male Japanese macaques does not support a direct link of identified COMT variants and initiated physical attacks. As mentioned above, however, non-carriers of HT3 seem to have lowered cortisol reactions compared to carriers of this haplotype. In humans this phenotype itself is thought to modulate aggression levels: studies revealed a relationship between low levels of cortisol and heightened trait aggression due to interactions with testosterone (for review see Rosell and Siever, 2015). Furthermore, low basal levels of cortisol apparently reduce anxiety and stressful feelings towards threat, helping individuals to not avoid stressful or negative encounters (Shoal et al., 2003). Using a non-parametric correlation test, we found excreted cortisol metabolites to be inversely related to the amount of conflict involvement and displayed physical aggression in male Japanese macaques. This inverse correlation between cortisol and aggressive behaviour has also been reported in other despotic macaques (Miller et al., 2004), humans (Böhnke et al., 2010; McBurnett et al., 2000; Schulz et al., 1997) and lower vertebrates (Överli et al., 2002). Nonetheless, in our data this association was detectable only when excluding the effect of COMT HT2. This points to a confounding effect of genotype that might mediate the causal relation between cortisol and physical aggression. The concept of how stress-related haplotypes contribute to individual variability in aggression via the endocrine pathway has been previously shown using the OPRM1 gene of the rhesus macaque (Miller et al., 2004).

In accordance with our data, a high-ranking male is supposed to have a low threshold for initiating attacks, a high tolerance for pain and less fear of punishment (Eaton, 1974). Pursuing the assumption that high-ranks are more stress- and/or more pain-resistant, it might be advantageous to examine stress-related genetic predispositions in relation to dominant behaviour strategies. Our linear regression models, however, did not provide evidence for a direct link between underlying COMT haplotypes and linear social rank. Nonetheless, we can assume that a non-carrier of HT2 is better equipped to cope with physiological and social challenges. The contribution of more or less stress-resistant COMT alleles, distributed among the investigated males, could furthermore explain the lack of association between cortisol excretion and linear social rank relationships. Yet, more research is needed to verify our assumption in other populations of Japanese macaques and/or other despotic primate species. Furthermore, there is a need to pursue the question whether haplotypes associated to different cortisol reactivity contribute to the acquisition and maintenance of a specific social rank position. This, however, can only be answered by longitudinal data on free-ranging primate groups by taking the stability of dominance conditions and social bonds into account (Higham et al., 2013; Ostner et al., 2008).

In conclusion, we provide a carefully designed non-invasive approach for genotyping free-ranging animals that avoids interfering with the investigated social stress responses. Our analysis of genotype in combination with physiological and behavioural parameters represents an important step towards the understanding of gene-stress effects and their possible function in a despotic primate society. Our findings emphasize the contribution of COMT in modulating physiological stress response in a non-human primate model that deserves greater attention in future studies. Further steps are required to draw a more comprehensive picture of variable COMT alleles and related stress physiology in non-human primates, although these would have to overcome the limitations given by the non-invasive sampling approach of the present study.
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2015.11.012.

Author contributions
First author (L.S. Pflüger)
– research design;
– data collection in the field;
– molecular gene analyses;
– hormone analyses;
– statistical analyses;
– behaviour analyses; and
– manuscript writing.

Second author (D.R. Gutleb)
– data collection in the field;
– molecular gene analyses;
– hormone analyses;
– behaviour analyses; and
– commented on and corrected the manuscript.

Third author (M. Hofer)
– molecular gene analyses; and
– commented on and approved the final manuscript.

Fourth author (M. Fieder)
– statistical analyses (GLMs);
– commented on and approved the final manuscript; and
– contributed new reagents and analytical tools.

Last author (B. Wallner*)
– commented on research design;
– contributed personal funding;
– commented on and approved the final manuscript; and
– supervised the hormones and behaviour part.

Last author (R. Steinborn*)
– research design;
– manuscript writing;
– contributed personal funding;
– provided protocols; and
– supervised the molecular genetic part.

*These authors contributed equally to this work as senior authors.

Acknowledgements and funding information
We thank the anonymous referees for reviewing our manuscript and providing us with constructive recommendations and helpful comments. We thank all members of the Affenberg Zoobetriebsgesellschaft mbH and especially Svenja and Peter Gaubatz, Markus Dorner and Alexandra Werdenig for their support at the study site. We acknowledge Elisabeth Pschernig for the support in the endocrinology lab, and Marion Janschitz, Daniela Drin and Celine König for the help with DNA genotyping. We also thank Michael Stachowitsch and Timon Salar Gutleb for valuable comments on the manuscript. This investigation was funded by the University of Vienna (IP number: 547011).

References


