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Cover: Grey langur (*Trachypithecus crepusculus*), female. Photo: T. Nadler

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Evolutionary history and phylogenetic position of the Indochinese grey langur (*Trachypithecus crepusculus*)

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Key words: *Trachypithecus crepusculus*, mitochondrial DNA, nuclear DNA, introgression, hybridization

Summary

Although traditionally classified as a subspecies of *Trachypithecus phayrei*, recent genetic studies using mitochondrial sequence data have shown that the Indochinese grey langur, *T. crepusculus*, represents a distant relative of the *T. francoisi* species group and not of the *T. obscurus* group or specifically of *T. phayrei*. To further elucidate the phylogenetic position of *T. crepusculus* and to uncover possible hybridization events in the evolutionary history of the species, we expanded earlier mitochondrial studies by sequencing complete mitochondrial genomes, and generated sequence data from five autosomal, one X chromosomal and six Y chromosomal loci. According to the depicted mitochondrial phylogeny, *T. crepusculus* is indeed closely related to the *T. francoisi* group. In contrast, nuclear sequence data, although providing only limited information due to the low number of polymorphic sites, support a sister grouping of *T. crepusculus* to the *T. obscurus* group. Hence, nuclear sequence data are in agreement with the traditional classification of *T. crepusculus* as member of the *T. obscurus* group. We explain the discordance between mitochondrial and nuclear phylogenies with ancient male introgression events from *T. phayrei* into a basal member of the *T. francoisi* group, which led after repeated introgression to a complete replacement of the original nuclear genome of the ancestral *T. crepusculus* form and thus to a phenotype similar to that of *T. phayrei*.

Lịch sử tiến hóa và vị trí chủng loại phát sinh của loài voọc xám đông dương (*Trachypithecus crepusculus*)

Tóm tắt

Mặc dù lâu nay vẫn được xem là loài phụ của *Trachypithecus phayrei*, nhưng các nghiên cứu di truyền sử dụng trật tự gene ty thể đã cho thấy voọc xám đông dương (*Trachypithecus crepusculus*) có quan hệ họ hạng xa với loài voọc đen má trắng (*T. francoisi*) và không có quan hệ với voọc *T. obscurus* hoặc *T. phayrei*. Để làm rõ vị trí chủng loại phát sinh của *T. crepusculus* và xác định sự lai tạp có thể xảy ra trong quá trình tiến hóa của loài này, chúng tôi đã mở rộng nghiên cứu ty thể trước đây bằng cách giải

mã trình tự toàn bộ bộ gene ty thể và tạo lập các số liệu trật tự gene của 5 locus thể nhiễm sắc thể thân, một locus nhiễm sắc thể X và 6 locus nhiễm sắc thể Y. Dựa vào cây phát sinh được tạo ra cho thấy *T. crepusculus* thực sự có mối quan hệ chặt chẽ với nhóm *T. francoisi*. Điều này tương phản với số liệu về trật tự gene nhân, mặc dù có ít thông tin do có số lượng các vị trí đa hình thấp, nhưng lại ủng hộ *T. crepusculus* là nhóm chị em với nhóm *T. obscurus*. Do các số liệu trật tự gene nhân phù hợp với sự phận loại truyền thống rằng *T. crepusculus* là thành viên của nhóm *T. obscurus*. Chúng tôi giải thích sự không phù hợp giữa cây phát sinh ty thể và cây phát sinh nhân là do có sự nhập gene của các cá thể cái xa xưa vào thành viên cơ sở của nhóm *T. francoisi*, sau đó qua nhiều lần lập lại của sự nhập gene đã dẫn đến thay thế hoàn toàn bộ gene nhân ban đầu (gốc) của dạng *T. crepusculus* cổ xưa và do vậy nó có phenptyp tương tự như phenotyp của *T. phayrei*.

Introduction

Within the Asian leaf monkey genus *Trachypithecus*, traditionally five species groups (*T. pileatus*, *T. vetulus*, *T. francoisi*, *T. cristatus* and *T. obscurus*) are recognized, mainly due to differences in fur colouration, behaviour, ecology and distribution (Groves, 2001). However, recent genetic investigations have shown that the *T. vetulus* group is actually a member of the genus *Semnopithecus* and that the *T. pileatus* group might be the product of ancestral hybridization between *Semnopithecus* and *Trachypithecus* (Geissmann et al., 2004; Karanth et al., 2008; Osterholz et al., 2008). Thus, only three species groups, *T. francoisi*, *T. obscurus* and *T. cristatus*, remain as true members of the genus *Trachypithecus* (Osterholz et al., 2008).

Each of these three species groups include taxa that are genetically closely related to each other (Geissmann et al., 2004; Osterholz et al., 2008; Roos, 2003; 2004; Roos et al., 2007; 2008), and which are also similar in fur colouration, behaviour and ecology (Brandon-Jones et al., 2004; Groves, 2001; Nadler et al., 2003). Accordingly, *T. francoisi*, *T. poliocephalus*, *T. delacouri* and *T. laotum* are combined in the *T. francoisi* group (Osterholz et al., 2008; Roos, 2003; 2004; Roos et al. 2007), *T. obscurus*, *T. phayrei* and *T. barbei* in the *T. obscurus* group (Geissmann et al., 2004; Osterholz et al., 2008; Roos et al., 2007), and *T. cristatus*, *T. auratus*, *T. mauritius*, *T. margarita* and *T. germaini* in the *T. cristatus* group (Nadler et al., 2005; Roos et al., 2008).

An additional taxon, the Indochinese grey langur (*T. crepusculus*), is distributed from central to north-east Thailand and south Yunnan, east to south-west Laos and northern Vietnam, and west of the Bay of Bengal, south of the range of *T. phayrei phayrei* (Groves, 2001). Although originally described as a distinct species, *Pithecus crepuscula* by Elliot (1909), this taxon is traditionally recognized as a subspecies of *T. phayrei* because of similar colouration (Corbet & Hill, 1992; Groves, 2001; Napier & Napier, 1967). Ignoring whether it is subspecies or a distinct species, based on general appearance the taxon is obviously a member of the *T. obscurus* group. Most prominent in this respect are the light eyerings and depigmented lips, which are present in all members of the *T. obscurus* group and also in *T. crepusculus* (Groves, 2001). Contradicting this view, however, are recent genetic studies, which depict *T. crepusculus* as a distant relative of the *T. francoisi* group and not as member of the *T. obscurus* group (Geissmann et al., 2004; Roos, 2003; 2004; Roos et al., 2007).

Discordance between phenotype and genetic data or even between phylogenies derived from different genes occurs relatively frequently (Avise, 2000). In addition to other reasons such as incomplete lineage sorting or insufficient data, hybridization has been gaining increasing acceptance as an explanation for such discordances (Avise, 2000; Funk & Omland 2003; Seehausen, 2004). However, for primates, information about hybridization is still scare compared to

that for fishes, birds or other mammals, but recent investigations have uncovered natural hybridization events for primates (for review see Arnold & Meyer, 2006; Arnold, 2008). These occurred mainly between species of the same genus (e.g., *Lepilemur* sp., Rumpler et al., 2008; *Alouatta* sp., Cortés-Ortiz et al., 2007; *Macaca* sp., Tosi et al., 2000; *Papio* sp., Zinner et al., 2009a; *Gorilla* sp., Thalmann et al., 2007) but also between genera (e.g., *Trachypithecus* x *Semnopithecus*, Osterholz et al., 2008; *Rungwecebus* x *Papio*, Zinner et al., 2009b), and even led to the formation of new species (e.g., *Macaca arctoides*, Tosi et al., 2000; *Macaca munzala*, Chakraborty et al., 2007; *Trachypithecus pileatus*, Osterholz et al., 2008). Even for the human lineage, hybridization was suggested as important evolutionary mechanism (Pääbo, 2003).

To uncover possible hybridization events in the evolutionary history of the Indochinese grey langur and to further settle its phylogenetic position, we analysed complete mitochondrial genome data as well as sequences from five autosomal (Alb3, IRBP3, TP2, TTR1, vWF11), one X chromosomal (Xq13.3) and six Y chromosomal (DBY5, SMCY7, SMCY11, SRY, UTY18, ZFY_LI) loci from one representative of each of the three *Trachypithecus* species groups as well as from *T. crepusculus*.

Material and Methods

Blood samples were obtained from *T. crepusculus* and *T. delacouri* (both from the Endangered Primate Rescue Center, Vietnam), *T. auratus* (Wilhelma, Germany), *T. obscurus* (Wuppertal Zoo, Germany) and *Presbytis melalophos fluviatilis* (Howletts Wild Animal Park, UK). *T. delacouri*, *T. auratus* and *T. obscurus* were chosen because they represent members of the three species groups *T. francoisi*, *T. cristatus* and *T. obscurus*, respectively.

Genomic DNA was extracted with the DNeasy Blood & Tissue Kit from Qiagen. To exclude the miss-amplification of nuclear pseudogenes, the complete mitochondrial genome (ca. 16,500 bp) was amplified via two overlapping fragments with a size of ca. 6,500 and 13,800 bp, respectively. Using these templates, PCR fragments with ca. 1,000-1,200 bp in length were amplified via nested PCRs. Nuclear loci were amplified with already published primers (DBY5, SMCY7, SMCY11: Hellborg & Ellegren, 2003; SRY: Tosi et al., 2000; vWF11: Chaves et al., 1999; Xq13.3: Tosi et al., 2005) or newly designed primers (Alb3, IRBP3, TTR1, TP2, UTY18, ZFY_LI). Primers and PCR programs are available from the authors. All PCR reactions were checked on 1% agarose gels, excised from the gel and purified with a standard silica method (Sambrook et al., 1989). Afterwards, PCR products were sequenced on an ABI 3730xl sequencer using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Coding regions of the mitochondrial genome and the SRY gene were translated into amino acid sequences to check for unexpected stop codons. All other loci represent non-coding intronic sequences.

For phylogenetic analyses, sequences were aligned with ClustalW v1.7 (Thompson et al., 1994) and manually checked by eye. In all statistical analyses, *Presbytis melalophos fluviatilis* served as outgroup. Due to the low number of informative sites, nuclear sequence data were only inspected by eye and not subjected to complex phylogenetic reconstructions. For the mitochondrial genome data, we performed neighbor-joining (NJ), maximum-likelihood (ML) and Bayesian reconstructions. Before analysing the data, indels and poorly aligned positions were removed with G-blocks v0.91b (Castresana, 2000). As optimal nucleotide substitution model, the GTR + I + G model was selected under the Akaike Information Criterion by MODELTEST v3.7 (Posada & Crandall, 1998). NJ and ML reconstructions were performed in PAUP* v4.0b10 (Swofford, 2002) and GARLI v0.951 (Zwickl, 2006). For both calculations, only the model specification settings were adjusted according to the

data set; all other settings were left at their default value. NJ and ML bootstrap percentages were estimated by performing 10,000 and 500 replications, respectively. To calculate a majority-rule consensus tree to obtain ML bootstrap percentages, PAUP was used. Bayesian analyses were conducted with MrBayes v3.1.2 (Huelsenbeck et al., 2001; Ronquist & Huelsenbeck, 2003) using four Monte Carlo Markov Chains with the default temperature of 0.1. Four repetitions were run for 10,000,000 generations with tree and parameter sampling occurring every 100 generations. The first 25% of samples were discarded as burnin, leaving 75,001 trees per run. Posterior probabilities for each split were calculated from the posterior density of trees.

To evaluate the reliability of the depicted phylogenetic position of *T. crepusculus*, alternative tree topologies with *T. crepusculus* being closer related to either *T. obscurus* or *T. auratus* instead to *T. delacouri* were evaluated with the Kishino-Hasegawa (Kishino & Hasegawa, 1989) and Shimodaira-Hasegawa (Shimodaira & Hasegawa, 1999) tests with full optimization and 1,000 bootstrap replications in PAUP.

Results

We successfully amplified and sequenced the complete mitochondrial genome from *T. crepusculus*, *T. delacouri*, *T. auratus*, *T. obscurus* and *Presbytis melalophos fluviatilis*. The alignment comprised 16,590 bp, which was reduced to 16,452 bp after gaps and poorly aligned positions were removed. Among them, 2,353 sites were variable and 954 parsimony-informative. Phylogenetic reconstructions were performed with NJ, ML and Bayesian algorithms. For all of them, identical and strongly supported tree topologies were obtained. Accordingly, *T. crepusculus* clusters with *T. delacouri*, and *T. auratus* forms a clade together with *T. obscurus* (Fig. 1a). To test for the reliability of the depicted relationships we evaluated alternative relationships. However, trees, in which *T. crepusculus* clusters with either *T. obscurus* or *T. auratus* instead with *T. delacouri*, were significantly rejected ($P<0.001$).

The concatenated nuclear data set including sequence data from 12 loci generated from *T. crepusculus*, *T. delacouri*, *T. auratus*, *T. obscurus* and *Presbytis melalophos fluviatilis* comprised 13,994 bp. After the removal of gab positions, the alignment was 13,551 bp in length. Among them, 301 sites were polymorphic and only nine parsimony-informative. Hence, mutations were visually inspected and no detailed phylogenetic reconstructions were performed. All in all, we detected five mutations (SMCY11: position 150, TP2: position 875, TTR1: position 436, Xq13.3: positions 4886 and 4979), which supported a grouping of *T. crepusculus* and *T. obscurus* (Fig. 1b). No mutations were found which supported a close affiliation between *T. crepusculus* with either *T. delacouri* or *T. auratus*.

Discussion

The present study shows that nuclear data support a close relationship between *T. crepusculus* and the *T. obscurus* group, which is in agreement with phenotypical characteristics and the traditional classification of *T. crepusculus* as subspecies of *T. phayrei* (Corbet & Hill, 1992; Groves, 2001; Napier & Napier, 1967). However, our mitochondrial phylogeny which supports earlier studies (Geissmann et al., 2004; Roos, 2003; 2004; Roos et al., 2007) suggests a close affiliation of *T. crepusculus* to the *T. francoisi* group. Thus, discordance between mitochondrial and nuclear/phenotypical phylogeny concerning the phylogenetic position of *T. crepusculus* becomes obvious.

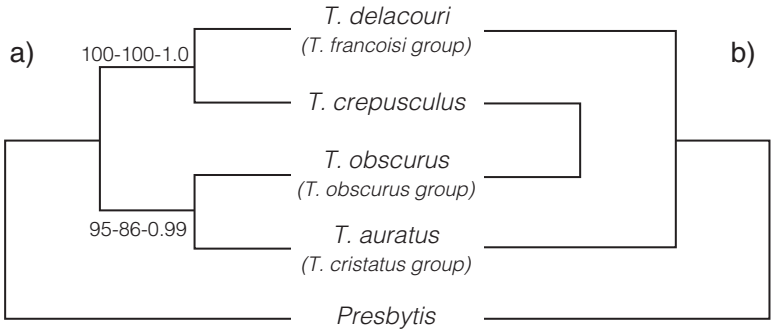

Fig.1. Phylogenetic relationships among *Trachypithecus* species groups and *T. crepusculus* as obtained from a) mitochondrial genome data and b) nuclear sequence data. Numbers on nodes represent bootstrap or posterior probability values (first: NJ, second: ML, third: Bayesian). Note that the phylogeny depicted in b) is in agreement with the phenotypical classification of *T. crepusculus* as member of the *T. obscurus* group.

The observed discordance might be explained by introgressive hybridization or incomplete lineage sorting, since both can result in similar phylogenetic patterns, and hence, complicate the interpretation of phylogenetic reconstructions (Avise & Ball, 1990; Morando et al., 2004). Although we can not rule out completely that incomplete lineage sorting may have had an effect, the geographical pattern, i.e. sympatric occurrence of *T. crepusculus* and the *T. francoisi* group members, provides some evidence against incomplete lineage sorting, because lineage sorting is a random process and the paraphyletic relationships that result from the failure of haplotypes to sort during speciation events should be random with respect to geography (Avise, 2004). In contrast, in our mitochondrial phylogeny sympatric populations cluster together, which is a strong indication of reticulation (Funk & Omland, 2003). Thus, we conclude that introgressive hybridization rather than incomplete lineage sorting has resulted in the discordance between mitochondrial and nuclear/phenotype phylogeny in the case of *T. crepusculus*.

Male dispersal and female philopatry are the norm in langurs (Davies & Oates, 1994; Fleagle, 1999) and one can assume that this is the ancestral state. Therefore, male introgression would be the most likely introgression scenario, where males from one taxon, i.e. *T. phayrei*, invaded groups of a basal relative of the *T. francoisi* group and reproduced successfully. Extensive backcrossing of the hybrid offspring over generations with more invading males of *T. phayrei* would have resulted in nuclear swamping. This process finally led to the extinction of the distantly related *T. francoisi* group member and only its mitochondrial genome remained as the only vestige of its former existence in a population, which is phenotypically similar to the introgressing *T. phayrei* (Fig. 2,3).

The classification of hybrid taxa is highly disputed and no consensus has been reached yet. In the case of *T. crepusculus*, we find a nuclear genome and a phenotype, which is similar to that of the *T. obscurus* group. Hence, a classification of *T. crepusculus* as species of the *T. obscurus* group or even as subspecies of *T. phayrei* would be appropriate. However, *T. crepusculus* carries mitochondria of a species, which is extinct now and was originally closely related to the *T. francoisi* group. Hence, *T. crepusculus* can not be regarded as true member of the *T. obscurus* group. Due to this incongruence, we propose to recognize *T. crepusculus* besides *T. cristatus*, *T. obscurus* and *T. francoisi* as a fourth distinct species group in the genus *Trachypithecus*.

Fig. 2. Grey langur (*Trachypithecus crespusculus*), adult female with long whiskers. Photo: T. Nadler.

Fig. 3. Grey langur (*Trachypithecus crespusculus*), young female. Photo: T. Nadler.

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Observations of Lao langurs (*Trachypithecus* [*laotum*] *laotum*) and black langurs (*Trachypithecus* [*laotum*] *hatinhensis* morph *ebenus*) in Khammouane Province, Laos and remarks to their systematic position

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Key words: Lao langur, black langur, records, systematics, Laos

Summary

In March 2009 observations of Lao langurs were made on several days for many hours in the northern part of Phou Hin Boun National Biodiversity Conservation Area (NBCA). Single males and a group with 14 adult and subadult animals, and three yellow juveniles were observed.

Currently there is no agreement about the systematic position of the taxon. Molecular genetic differences between Lao langur and Hatinh langur places the taxa on subspecies level.

In April 2009 three black langurs were observed in the southern part of Phou Hin Boun National Biodiversity Conservation Area. The taxonomic status of the black langur which occurs in northern central Vietnam and central Laos is still unclear.

Molecular genetic studies of one individual kept at the EPRC correspond with the type specimen in Smithsonian Museum, and also with the Hatinh langur. It seems that these all-black individuals are a melanistic morph of the Hatinh langur.

The white headed Laos langur and the black langur occur both in the southern part of Phou Hin Boun NBCA. Based on the current state of knowledge and information from the recent surveys, both forms are separated and should also use different habitats. The limestone range in Hin Nam No NBCA - with records of black langurs - has a spur towards the west until the Mahaxai limestone range, south of Phou Hin Boun NBCA. The large valley of the Bangfai river between the Mahaxai limestone range and Phou Hin Boun NBCA do not currently act as zoogeographical barriers, but the nature of this large valley and alluvial land indicate a possible historical separation between the northern Phou Hin Boun limestone range and the southern limestone range east of Mahaxai. A connection between the two taxa – the black morph of the Hatinh langur south of the Bangfai river, and the Lao langur north of the Bangfai river – likely exists only since recent palaeoglacial times.

It would be interesting to study the possibility of different niches between the taxa and likelihood of interbreeding actions.

Một số quan sát về loài Voọc Lào (*Trachypithecus* [*laotum*] *laotum*) và loài Voọc đen (*Trachypithecus* [*laotum*] *hatinhensis* morph *ebenus*) ở Tỉnh Khammaouane, Lào và một số nhận xét về vị trí phân loại của chúng

Tóm tắt

Những quan sát về loài Voọc Lào được thực hiện một số ngày trong nhiều giờ vào tháng 3 năm 2009 tại khu vực phía Bắc Khu bảo tồn đa dạng sinh học Quốc gia Phou Hin Boun (NBCA). Các cá thể đực đơn lẻ và một nhóm gồm 14 cá thể trưởng thành và bán trưởng thành, và 3 con non màu vàng được quan sát.

Hiện tại vẫn chưa có sự thống nhất về vị trí phân loại của nhóm phân loại này (taxon). Những khác biệt về di truyền phân tử giữa loài Voọc Lào và Voọc Hà Tĩnh đặt nhóm phân loại này vào mức loài phụ.

3 cá thể Voọc đen được quan sát tại khu vực phía Bắc Khu bảo tồn đa dạng sinh học quốc gia Phou Hin Boun vào tháng 4 năm 2009. Tình trạng phân loại của Voọc đen phân bố ở Bắc trung bộ Việt Nam và Trung Lào vẫn chưa rõ.

Các nghiên cứu về di truyền phân tử của một cá thể nuôi dưỡng tại EPRC tương xứng với kiểu mẫu ở bảo tàng Smithsonian, và cũng tương xứng với Voọc Hà Tĩnh. Dường như tất cả các cá thể Voọc đen này là một sự thay đổi về sắc tố của Voọc Hà Tĩnh.

Loài Voọc Lào đầu trắng và Voọc đen phân bố ở cả khu vực phía Bắc Khu bảo tồn đa dạng sinh học quốc gia Phou Hin Boun NBCA. Dựa trên những hiểu biết hiện tại và những thông tin từ các cuộc điều tra gần đây, cả 2 loài là riêng rẽ và chắc cũng sử dụng các sinh cảnh khác nhau. Dãy núi đá ở Hin Nam No NBCA với sự ghi nhận của loài Voọc đen có một mỏm hướng về phía Tây cho tới tận dãy núi đá Mahaxai, phía Nam của Phou Hin Boun NBCA. Thung lũng rộng lớn của sông Bangfai giữa dãy núi đá Mahaxai và Phou Hin Boun NBCA hiện tại không được xem như là rào cản địa lý động vật, nhưng bản chất của thung lũng rộng lớn này và đất phù xa chỉ ra một sự chia cắt lịch sử có thể giữa dãy núi đá phía Bắc Phou Hin Boun và dãy núi đá phía Nam, Đông của Mahaxai. Sự liên hệ giữa hai nhóm phân loại, đen sự biến thái của Voọc Hà Tĩnh, Nam của sông Bangfai và Voọc Lào, Bắc của sông Bangfai có thể tồn tại chỉ từ khi các thời điểm cổ đóng băng.

Sẽ là hay khi nghiên cứu khả năng về các ổ sinh thái khác nhau giữa các nhóm phân loại và có thể về các ảnh hưởng của giao phối cận huyết.

Observations of Lao langurs (*Trachypithecus* [*laotum*] *laotum*)

Two short surveys conducted in 2007 and 2008 with the intention of sighting and data collection failed despite several hikes in difficult terrain with experienced local guides. Only calls of animals were heard.

In March 2009 observations of Lao langurs were made on several days for many hours in the northern part of Phou Hin Boun National Biodiversity Conservation Area (NBCA). Single males and a group with 14 adult and subadult animals, and three yellow juveniles were observed (Fig. 1-4). Such large groups in other “karst langur” species are also known but not common. The average group size is nine to ten individuals. Larger groups often split after a while.

The Lao langur is one of the least know primate taxa in Indochina. The distribution is limited to the steep karst range of Khammouane Province in Central Laos. The area is extremely difficult to access, which protects the langurs from extensive poaching but also biological research.

Fig. 1. A single male Lao langur in the extreme and bizarre karst landscape of Phou Hin Boun NBCA. All "karst langur" species have acrobatic skills to climb on vertical rock walls which sometimes have knife-like edges. Fig. 1 to 4 are the first published images of the Lao langurs in the wild. Photo: T. Nadler.

Fig. 2. Single males stay occasionally in the margins of the home range of a group, with the intention to take over a group. Photo: T. Nadler.

Fig. 3. The alpha male of the group often appeared on an exposed position to overview an area before the group approached. Photo: T. Nadler.

Fig. 4. A large group of Lao langurs with 14 adult and subadult animals and three yellow juveniles arrived minutes after the alpha male on the way to the sleeping place. Photo: T. Nadler.

Fig. 5. Lao langur at the Endangered Primate Rescue Center. Photo: T. Nadler.

Fig. 6. Subadult black langur at the Endangered Primate Rescue Center. Photo: T. Nadler.

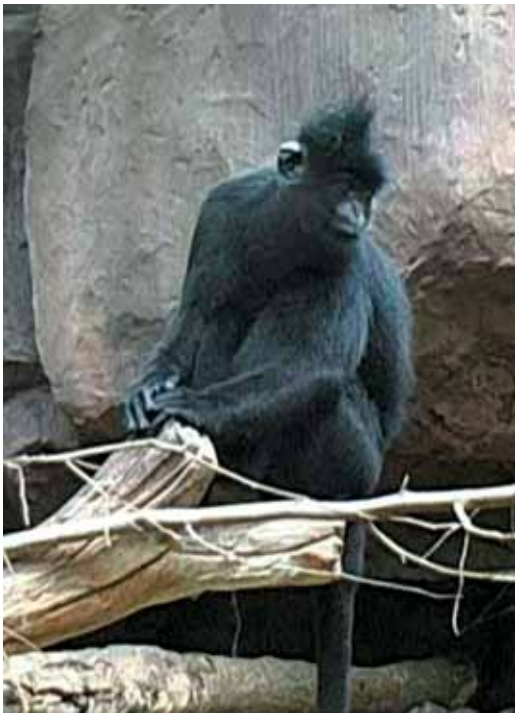

Fig. 7. Adult black langur at Korat Zoo, Thailand. Photo: R. Männel.

Fig. 8. Adult black langur at Korat Zoo, Thailand. The adult langurs show a smoky-grey highlight in the same region where the Hatinh langurs show their white beard. Photo: R. Männel.

The systematic position of the species remains unclear and controversial despite a long history of discussion. Molecular genetic studies – as part of the Vietnam Primate Conservation Program of Frankfurt Zoological Society in cooperation with the German Primate Center – supports the validity of the taxon as one of the so-called "karst langurs" Indochinas. These taxa have a high affinity to steep karst outcrops and occur only in Indochina east of the Mekong. All of this species are highly threatened and most of them occur in restricted areas.

Currently there is no agreement about the systematic position of the taxon. Molecular genetic differences between the Lao langur and Hatinh langur is only 1,9-2,3% (Roos, 2004) which places the taxa on subspecies level. However, according to aspects of allopatric distribution and different phylogenetic development they are also recognized as valid species (Groves, 2001).

Observations of black langurs
(*Trachypithecus* [*laotum*] *hatinhensis* morph *ebenus*)

On April 13, 2009 three black langurs – most probably males - were observed in the southern part of Phou Hin Boun NBCA, close to road no. 12 at a distance of about 100 m. No sign of white on the head was visible.

The taxonomic status of the black langur which occurs in northern central Vietnam and central Laos is still unclear. There are only a few animals and records known. This taxon has also an unusual history. In 1924, F.R. Wulsin (Thomas, 1928) collected the skin and skull of an all-black langur, now in Smithsonian Museum, USA (USNM 240489). The locality on the label "French Indo-China" does not indicate its true provenance. Brandon-Jones (1995) speculates that the animal originated from Fan Si Pan area in North Vietnam and allocates it as a subspecies to the Javan langur *Semnopithecus auratus* as a new subspecies *S. auratus ebenus*.

In January 1998 the Endangered Primate Rescue Center, Vietnam received a young all-black langur which came presumably from the Hin Nam No NBCA in Lao (Nadler, 1998), and was approximately two years old. Anatomical features, behaviour and vocalization of this animal show strong affinities to the Hatinh langur (*Trachypithecus* [*laotum*] *hatinhensis*) (Nadler et al., 2003) (Fig. 6). Currently at an age of about 12 years, the beard which was completely black as a young animal now shows a smoky-grey highlight in the same region where the Hatinh langurs show their white beard. Another adult male black-langur kept in Korat Zoo, Nakhon Ratchasima, Thailand of unknown age also shows a beard with some grey hairs (Fig. 7, 8).

Molecular genetic studies of the EPRC individual correspond with the type specimen in Smithsonian Museum, and also with the Hatinh langur. It seems that these all-black individuals are a melanistic morph of the Hatinh langur (Roos, 2003). This is also supported by field observations, in that both morphs are present in the same area in Hin Nam No NBCA, Laos (Ruggieri & Timmins, 1995; Timmins & Khounboline, 1996). Le Khac Quyet (2004) reported sightings of groups of black-langurs from Nui Giang Man Nature Reserve, Vietnam.

There are also reports about black langurs from the southern extremity of the Phou Hin Boun NBCA, Khammouane Province, Laos but in the past all sightings have only confirmed the occurrence of Lao langurs (Duckworth et al., 1999).

Hunters and villagers in the southern part of Phou Hin Boun NBCA mentioned the occurrence of two langurs, the white headed Laos langur and an all-black form. Based on the current state of knowledge and information from the recent surveys, both forms are separated and should also use different habitats; the Laos langur in high, naked and steep limestone outcrops, at the northern part

of the Phou Hin Boun NBCA, and the black langurs at lower altitude with more rich vegetation, and only around the southern extremity of the Phou Hin Boun NBCA (Nadler, pers. comm.).

It remains questionable whether black morphs of the Hatinh langurs, found in Hin Nam No area, stretch along the limestone range to the west and contact the distribution of the Lao langur, or if there similarly exists a melanistic morphe in the Lao langur in the southern part of Phou Hin Boun NBCA.

The limestone range in Hin Nam No NBCA has a spur towards the west until Mahaxai. The northern boundary and interruption to Phou Hin Boun NBCA is the valley of the Bangfai river. The Bangfai river and the valley do not currently act as zoogeographical barriers. However, the condition and nature of this large valley and alluvial land indicate a possible historical separation between the northern Phou Hin Boun limestone range and the southern limestone range east of Mahaxai. The Bangfai is the largest river with an east-west direction in central Laos.

A connection between the two taxa – the black morphe of the Hatinh langur south of the Bangfai river, and the Lao langur north of the Bangfai river – likely exists only since recent palaeoglacial times.

Hatinh and Lao langurs are very similar taxa. There is most probably no reproductive barrier. It would be interesting to study the possibility of different niches between the taxa and likelihood of interbreeding actions.

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Observations on the Hatinh langur (*Trachypithecus hatinhensis*) during point and line transect sampling in the Phong Nha – Ke Bang National Park, Central Vietnam

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Key words: Hatinh langur, transect sampling, karst forest

Summary

Hatinh langurs (*Trachypithecus hatinhensis*) are endemic to central Vietnam and southern Laos, and in Vietnam the distribution is restricted to Quang Binh and Quang Tri Provinces. This endangered langur inhabits the dense primary forests in the limestone areas of the Annamite Mountains. The difficult-to-access habitat may have led to only little knowledge of its ecology and behaviour in the past. From April to August 2007 we conducted point (PTS) and line (LTS) transect sampling in the Phong Nha – Ke Bang National Park (PNKB NP) in Quang Binh Province and recorded ecological and behavioural data. We could confirm Hatinh langurs in all survey areas of LTS and at nine of 16 different points. We recorded a more reliable mean group size with PTS and our analyses revealed more than three times higher efficiency of PTS than of LTS. Hatinh langurs use limestone cliffs as sleeping sites. We did not detect preferences in the choice of cliff aspect and size. A Hatinh langur group seems to occupy more than one cliff and to use them alternately. Loud calls (whoops) of male Hatinh langurs were produced mainly early in the morning before sunrise as well as late in the afternoon, and we suggest that these long distance calls of the males mainly serve as territorial markers and spacing mechanisms. The PNKB NP is the most important protected area for the Hatinh langur in Vietnam and we recommend further surveys to improve the knowledge of this rare langur in its natural habitat.

Quan sát Voọc Hà Tĩnh (*Trachypithecus hatinhensis*) bằng phương pháp đường cắt và điểm cố định tại Vườn Quốc gia Phong Nha – Kẻ Bàng, miền Trung Việt Nam

Tóm tắt

Voọc Hà Tĩnh (*Trachypithecus hatinhensis*) là loài đặc hữu cho khu vực miền Trung Việt Nam và Nam Lào. Ở Việt Nam, vùng phân bố của loài giới hạn ở hai tỉnh Quảng Bình và Quảng Trị. Loài voọc đặc hữu này sinh sống trong kiểu rừng kín nguyên sinh phát triển trên núi đá vôi của dãy Trường Sơn. Điều kiện địa hình hiểm trở có thể đã hạn chế những hiểu biết về sinh thái và tập tính của loài trong

quá khứ. Từ tháng 4 đến tháng 8 năm 2007, chúng tôi đã tiến hành khảo sát loài này bằng phương pháp đường cắt và điểm cố định tại VQG Phong Nha – Kẻ Bàng thuộc tỉnh Quảng Bình và ghi nhận các số liệu về sinh thái và tập tính của loài. Kết quả khảo sát đã xác định Voọc Hà Tĩnh ở tất cả các đường cắt và 9 trong số 16 điểm cố định. Kết quả khảo sát cho thấy phương pháp điểm cố định cho kết quả về kích thước trung bình của bầy đáng tin cậy hơn và hiệu quả hơn 3 lần so với phương pháp đường cắt. Voọc Hà Tĩnh sử dụng các mõm đá vôi làm chỗ ngủ đêm. Chúng tôi chưa thấy có ưu tiên chọn lựa theo kích thước và hướng phơi của các mõm đá. Một bầy Voọc Hà Tĩnh dường như chiếm nhiều hơn một mõm đá và luân phiên sử dụng chúng. Các tiếng hú lớn (whoops) của Voọc đực thường phát ra vào buổi sáng trước khi mặt trời mọc cũng như lúc sắp tối. Chúng tôi cho rằng những tiếng hú dài này của các con đực đóng vai trò đánh dấu vùng lãnh thổ và những cơ chế không gian. Vườn Quốc gia Phong Nha – Kẻ Bàng là khu vực bảo vệ quan trọng nhất đối với Voọc Hà Tĩnh ở Việt Nam và chúng tôi kiến nghị rằng những khảo sát khác cần được tiến hành nhằm nâng cao hiểu biết của chúng ta về loài vọc quý hiếm này và sinh cảnh tự nhiên của chúng.

Introduction

The Hatinh langur (*Trachypithecus hatinhensis*) represents one of Vietnam's eleven colobine species and inhabits the limestone forests of Central Vietnam and Southern Laos. In Vietnam the distribution is restricted to Quang Binh and Quang Tri Provinces (Nadler et al., 2003; Nguyen Manh Ha, 2006). In the IUCN Red List of Threatened Species and in the Red Data Book of Vietnam the Hatinh langur is listed as Endangered (Le Xuan Canh et al., 2008; Ministry of Science and Technology & Vietnamese Academy of Science and Technology, 2007). Similar to other langurs in Vietnam the main threat is hunting for traditional medicine, meat and wildlife trade, and it is also threatened because of habitat loss (Le Xuan Canh et al., 2008; Nadler et al., 2003; Nguyen Manh Ha, 2006). Hatinh langurs are members of the [*francoisi*] group including the taxa *T. hatinhensis*, *T. francoisi*, *T. poliocephalus*, *T. laotum*, *T. delacouri* and *T. ebenus* (Groves, 2005). The taxonomy is still disputed. Groves (2001; 2005) listed it as full species; however, based on molecular genetics Roos (2003; 2004) included it as subspecies of *T. laotum*, which was followed by several authors (Nadler et al., 2003; Nadler & Streicher, 2004; Nguyen Manh Ha, 2006; Vogt & Forster, 2008; Vogt et al., 2008).

Since the rediscovery of the Hatinh langur in 1992 in Phong Nha (Le Xuan Canh, 1993), there have been several surveys which contributed to the knowledge of the distribution of Hatinh langurs in Vietnam (Pham Nhat et al., 1996; Le Xuan Canh et al., 1997; Timmins et al., 1999; Nguyen Manh Ha 2004, 2006). Information on the population status were published by Pham Nhat et al. (1996) and Le Xuan Canh et al. (1997), who estimated 520-750 individuals in the Phong Nha – Ke Bang area in central Vietnam. Population density estimates of our recent study in 2007 resulted in 2,143 (±467) individuals in the whole PNKB NP (Haus et al., 2009). Pham Nhat et al. (1996) and Nguyen Manh Ha (2006) published the first ecological and behavioural information on Hatinh langurs, but there is still little known. The natural habitat of Hatinh langurs is characterized by steep limestone areas covered by dense primary forests. It is difficult to approach and to follow Hatinh langur groups at steep limestone slopes to get more detailed data (Nguyen Manh Ha, 2006). Like most other taxa of the [*francoisi*] group, Hatinh langurs use limestone caves and cliffs for sleeping. At these sites they are not only easy to hunt (Nadler et al., 2003; Ngo Xuan Phong, pers. comm.) but also much easier to detect and to observe than in the dense canopy of the karst forests during foraging and travelling (Nguyen Manh Ha, 2006).

In 2005 the Frankfurt Zoological Society (FZS) in cooperation with Cologne Zoo initiated a primate reintroduction program in the Phong Nha – Ke Bang National Park. This program - as part of the Primate Conservation Programme Vietnam of FZS - aims to release groups of captive-born Hatinh and red shanked douc langurs from the Endangered Primate Rescue Center in Cuc Phuong NP into the PNKB NP (Nadler & Streicher, 2003; Vogt & Forster, 2008; Vogt et al., 2008). Therefore more information about the status, distribution and ecology on these langurs is necessary to find appropriate sites for the final release.

From April to August 2007 we conducted point and line transect sampling to study the distribution and population densities of the primates in the PNKB NP. Here we present our observations on Hatinh langurs and compare the efficiency and the output of both methods in the difficult habitat of this endangered langur.

Methods

Study area

PNKB NP is located in Quang Binh Province in Central Vietnam at the border to Laos and covers more than 85,000 ha (BirdLife International & Forest Inventory and Planning Institute, 2001; Vogt et al., 2006). As part of the Annamite Mountains it is largely at altitudes between 50 and 1000 m above sea level and is characterized by steep limestone hills and dense primary forests. The karst forests of the PNKB NP with its numerous limestone cliffs and caves constitute a suitable habitat for the Hatinh langur (Nadler & Streicher, 2003; Vogt & Forster, 2008). In the past there was a high activity of loggers and hunters, which disturbed not only the habitat of the primates but also diminished its populations (Nadler et al., 2003; Nguyen Man Ha, 2006). Even though illegal logging and hunting have decreased in recent years, such activities are still present in the PNKB NP affecting primate distribution and densities. Nevertheless, the PNKB NP is the most important protected area within the distribution of the Hatinh langur in Vietnam (Haus et al., 2009).

Point transect sampling (PTS)

We conducted PTS from May to August in 2007 in the PNKB NP. We surveyed 16 different points along Ho Chi Minh (HCM) Road and Road 20 that cross the National Park (Fig. 1). We located the points randomly along the roads, but within view of at least one potential sleeping cliff within the survey area. Mostly, distances between points were at least 570 m. Due to large limestone escarpments and a multitude of potential sleeping sites, the first five points in the northern part of the HCM Road were 360-465 m apart. We surveyed adjacent points simultaneously, but survey areas of different points never overlapped.

We measured exposures and distances of the cliffs from the observation points with a compass and a range finder (Bushnell, Yardage Pro Legend), and we took digital photographs of all cliffs. Most points were surveyed at least at three days, but three points could be surveyed only one time due to weather conditions. The data were recorded by four observers, who were trained before the study began. We started the surveys around 4:15 p.m. and continued to dusk so that each survey took around two hours. During this time Hatinh langurs are frequenting their sleeping sites. We observed the survey areas using binoculars and a spotting scope (Bushnell, D = 63 mm, model 787363). For all surveys we recorded date, time, observer, point identity, and weather conditions. At each detection event we measured the radial distance to the first sighted individual using a range finder and a compass (Buckland et al., 2001; Ross & Reeve, 2003). In addition we recorded

time, group size and structure, cue, substrate and activity. We documented all sightings independent of the distance from the observer. If we observed a Hatinh langur group, we recorded ad libitum data of all behavioural patterns.

Line transect sampling (LTS)

We conducted LTS in four different areas within the PNKB NP: Hung Lau, Hang E, Cha Noi and Ban Doong (Fig. 1). In each area we designed three different line transects. We intended to survey each transect ten times, but two transects in Ban Doong were surveyed only eight and nine times respectively due to weather conditions (Haus et al., 2009). We recorded the same data as in PTS, but we measured the perpendicular distance or the radial distance and the angle relative to the transect line and, if possible, the sighting height in the tree at each detection event (Buckland et al., 2001; Ross & Reeve, 2003; Haus et al., 2009). We collected the data during two survey phases from April to June and July to August, staying in each area two times for 5-8 days. During these periods we recorded all loud calls of Hatinh langurs heard at any time.

We described basic vegetation structures along transect lines (Haus et al., 2009) resulting in an average canopy cover of 78.90% (45.38-100%) and canopy height of 18.48 m (9.28-25 m). The density of understory was 78.25% (28.59-100%). We estimated the growth of liana on a scale from one (few) to three (plenty) (Haus et al., 2009). Liana growth averaged 2.27 m (1.56-3 m).

Data analyses

We recorded all sightings with GPS (Garmin GPS 60 with an external antenna: Gilsson Technologies, High Gain GPS Antenna, MCX, and Garmin GPS 12). We produced a distribution map with MapInfo Professional 7.8 SCP importing the GPS data with the program GarFile 1.5.2. For statistical analyses we conducted Mann-Whitney U and regression tests (linear correlation (r) and Spearman rank correlation (rs)) on a level of significance with $\alpha = 0.05$, using the statistical software PAST (Hammer et al., 2001). We analysed some behavioural data in relation to sunrise and sunset times (Gerding, 2008) by calculating differences between time of data record and respective times of sunrise and sunset for each day.

Results

Point and line transect sampling

Comparing the efficiency of both sampling methods there are differences in PTS and LTS (Table 1). In relation to the survey effort we recorded more Hatinh langurs during PTS than during LTS resulting in a more than three times higher efficiency for PTS than for LTS.

Table. 1. Hatinh langur sightings (N), survey effort and efficiency of point (PTS) and line (LTS) transect sampling in the Phong Nha – Ke Bang National Park in 2007.

Method	N	Survey effort [h]	Efficiency [n/h]
PTS	23	115.2	0.2
LTS	27	419.3	0.06

We recorded Hatinh langurs in all survey areas of LTS, most in Hang E and fewest in Hung Lau (Fig. 2). With PTS we confirmed groups at nine of 16 survey points alongside the roads. We recorded a significant higher group size with PTS (mean 5.09, range 1-11) than with LTS (mean 3.54, range 1-10; $N_{LTS} = 27$, $N_{PTS} = 23$, $p = 0.025$; Fig. 3). During LTS we observed a single individual 13 times, ten of which have been confirmed to be single males sitting

Fig. 1. Survey areas of point and line transect sampling in the Phong Nha – Ke Bang National Park in 2007.

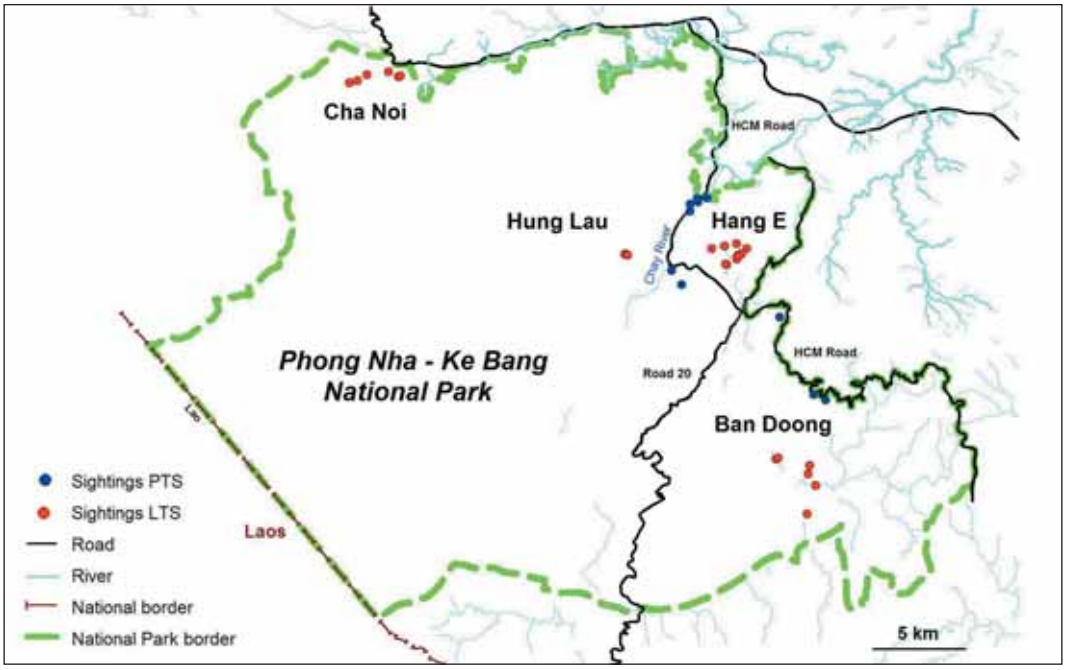

Fig. 2. Hatinh langur sightings during point and line transect sampling in the Phong Nha – Ke Bang National Park in 2007.

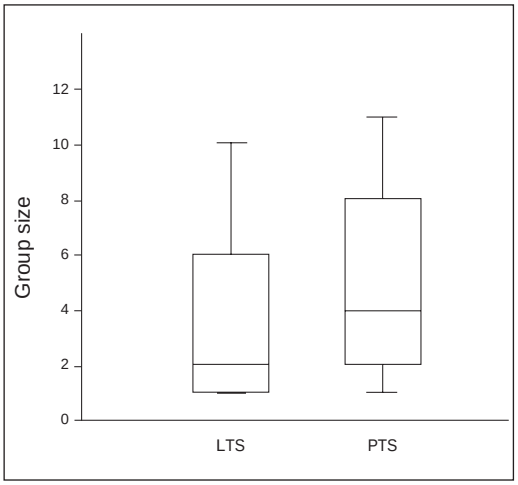

Fig. 3. Box plot comparing group sizes between line (LTS) and point (PTS) transect sampling.

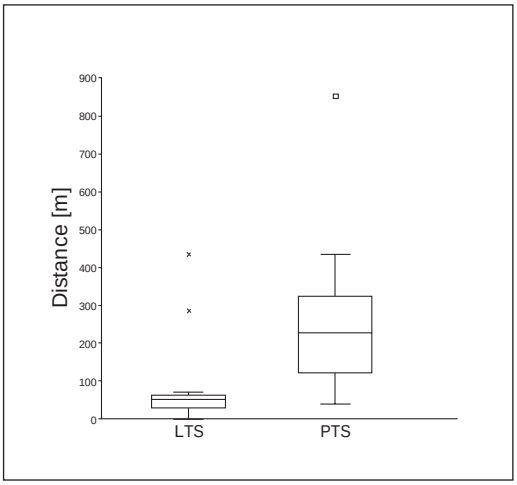

Fig. 4. Box plot comparing detection distances between line (LTS) and point (PTS) transect sampling.

in the upper canopy of trees. During PTS, we detected only four single individuals, two times a male. Mean group sizes are neither correlated with perpendicular or radial distance of LTS (N = 22, r = -0.22, p = 0.33) nor with radial distance of PTS (N = 23, r = -0.35, p = 0.1).

Radial distances of PTS were significantly higher than sighting distances of LTS (N_{LTS} = 22, N_{PTS} = 23, p ≤ 0.001; Fig. 4). We detected more Hatinh langurs by acoustical cues during LTS and more by visual cues during PTS. Most acoustical cues were caused by movement and feeding in the trees. In LTS 31% of acoustical cues were loud calls. We never detected Hatinh langurs by loud calls in PTS (Fig. 5).

Ecological and behavioural observations

We detected Hatinh langurs in trees every time during LTS and in 87% of the sightings in PTS. Both during LTS and PTS we observed them in trees growing at steep limestone cliffs in 37% and 52% respectively. We recorded Hatinh langurs directly on cliffs in 13% of all sightings of PTS, but we never detected them on the ground during both PTS and LTS. Combining all data of PTS and LTS, mostly we observed Hatinh langurs in trees (94%), 50% of which grew on limestone cliffs, and in 6% of all sightings we detected them on limestone cliffs between 6:00 a.m. and 6:50 p.m. (Fig. 6).

Analyses of habitat structure revealed no correlations in Hatinh langur abundance and canopy cover (N = 34, r_s = 0.02, p = 0.93), canopy height (N = 34, r_s = -0.19, p = 0.27), density of understory (N = 34, r_s = -0.28, p = 0.12) and growth of liana (N = 34, rs = 0.03, p = 0.86). We recorded Hatinh langurs along transects in primary forests with an average canopy cover of 79.49% (50-100%) and an average canopy height of 17.92 m (12.65-25 m). The density of understory was 73.83% (33.96-100%) and growth of liana 2.32 m (1.73-3 m) on average. In a valley in Hang E area, we twice detected Hatinh langurs in edges of a regenerated secondary forest, which is surrounded by large limestone escarpments covered by primary forests. On average we saw Hatinh langurs at a height of 10.43 m (5-20 m) in the trees during detection.

With PTS we could confirm seven limestone cliffs as sleeping sites. The limestone cliffs surveyed were faced to almost all directions. Hatinh langur groups occupied cliffs faced to 0-89° (N NE), 135-179° (S SE), 225-269° (W SW) and 315-359° (N NW). We could not detect any preference for cliffs (Fig. 7). Furthermore they occupied small as well as large limestone cliffs for sleeping. We detected a high abundance of Hatinh langur sleeping sites at large limestone escarpments alongside the Chay River (Fig. 2). From May to August the groups returned to the vicinity of their sleeping sites 65 minutes before sunset (4:35 p.m. - 6:16 p.m.) on average, foraging and playing until they entered the sleeping places at the cliff on average 14 minutes after sunset (6:10 p.m. – 6:45 p.m.). We observed a group of Hatinh langurs and eastern Assamese macaques (*Macaca assamensis assamensis*) at the same limestone escarpment at HCM Road. The macaques occupied trees close to the Hatinh langur sleeping places on the cliff for three consecutive days; they always appeared before the Hatinh langurs and we did not recognize any interactions between the groups.

To collect LTS data we spent a total of 59 days in the forest. During this time we could record a total of 51 loud calls (whoops) of Hatinh langurs (Fig. 8). Most whoops (16) were heard between 5:00 a.m. and 6:00 a.m. before sunrise. We observed a second peak of loud calls between 4:00 p.m. and 5:00 p.m. We did not hear whoops before 4:54 a.m. and after 5:43 p.m. as well as between 11:25 a.m. and 1:25 p.m.. If we detected males producing whoop calls, we could observe typical jumping displays in the crowns of trees during the call.

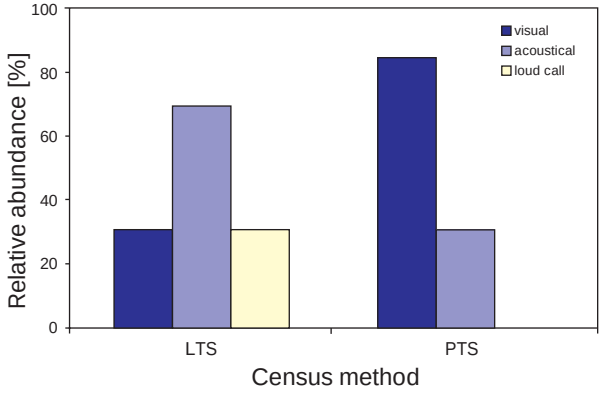

Fig. 5. Detection cues in line (LTS) and point (PTS) transect sampling. Loud calls represent percentage of acoustical cues.

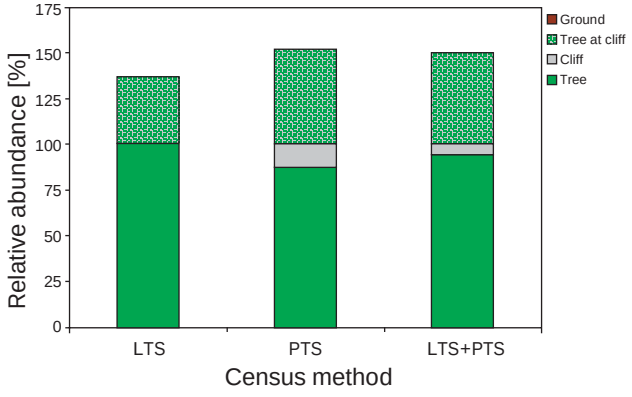

Fig. 6. Substrates used by Hatinh langurs (*Trachypithecus hatinhensis*) during detection events in line (LTS) and point (PTS) transect sampling.

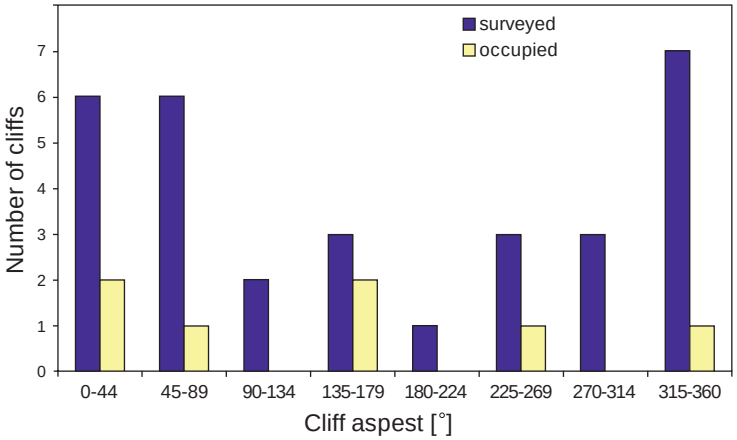

Fig. 7. Cliff aspects of surveyed limestone cliffs and cliffs occupied by Hatinh langur groups.

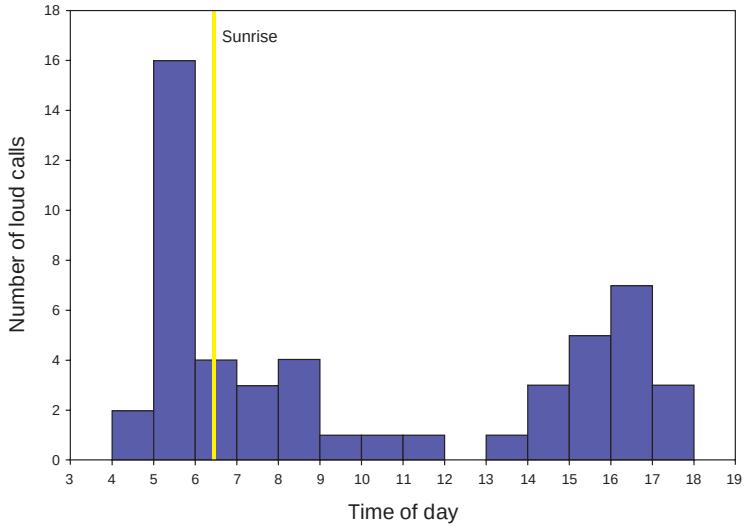

Fig. 8. Frequency distribution of loud calls (whoops) of male Hatinh langurs during line transect sampling.

Discussion

Point and line transect sampling

During PTS we detected Hatinh langurs in longer distances and more often by visual cues, whereas sightings of LTS were closer to the observers and hence more often detected by acoustical cues. Therefore we suggest a higher detection probability of Hatinh langurs applying LTS. However, in dense karst forests the observers are sometimes distracted by uneven ground and climbing in the karst, which can be minimized by frequent stops and walking as slowly as possible.

The mean group size of LTS was relatively low compared to mean group sizes of PTS and previous results of Pham Nhat et al. (1996; mean 7.3) and Nguyen Manh Ha (2006; mean 8.2). We

often recorded single males in the canopy during LTS and we suggest the possibility that some group members were undetected in the dense vegetation beneath (Haus et al., 2009).

In the forest the survey areas of points are limited by dense canopy and understory. Therefore we chose areas along the roads with wide visibility ranges. The points were easy to access by car or motorcycle. To collect LTS data we walked along the transect routes which was very time-consuming and required high physical effort. We could detect more Hatinh langurs with lower effort during PTS by standing at the viewpoints and recording all data from these locations. Due to wide visibility ranges of PTS and the mountainous terrain of karst forests, we were not able to measure applicable survey areas of the points to compare the efficiency of both methods in terms of sightings per hectare. However, according to the long sighting distances recorded during PTS, we suggest that we also could survey a wider area in relation to time expenditure and physical effort.

In respect to survey design and effort we would prefer PTS to LTS for censuses of Hatinh langurs in karst forests, because points are easier to locate randomly and to survey than transect lines (Ross & Reeve, 2001). Furthermore PTS with views to potential sleeping sites allowed us to record more detailed information about ecology and behaviour of Hatinh langurs and to obtain more reliable group sizes at exposed cliff sites. However, it should be noted that there are difficulties in estimating the survey area of points which may lead to an overestimation and underestimation of survey area and primate abundance respectively. In contrast to LTS (Haus et al., 2009) the sample size of PTS was too small to estimate the density of Hatinh langurs in the PNKB NP. To compare density estimates of both methods, further studies are required to increase the sample size of PTS.

Ecology and Behaviour

Nguyen Manh Ha (2006) observed Hatinh langurs more often at cliffs facing west or southwest and he described those cliffs as warmest in the late afternoon. In contrast we did not detect any preference for cliffs, and Hatinh langurs occupied cliffs that faced almost all directions.

The longest time we surveyed one limestone cliff was four consecutive days. Therefore our data do not provide enough information to suggest how many sleeping sites are occupied by one group and for how many consecutive days they are frequented. Nevertheless, on three occasions we observed Hatinh langur groups at the same limestone cliff for four consecutive days. Furthermore during surveys at HCM Road km 33.16 we did not record Hatinh langurs occupying the limestone cliff for two days. Due to weather conditions we surveyed the point two weeks and observed a group occupying the limestone cliff for three consecutive days. Therefore we assume that Hatinh langur groups occupy more than one cliff for sleeping and that these cliffs are used alternately.

Loud calls of Hatinh langurs can be divided into two types: whoops and grunts. Whereas the whoops are produced by exhalation, the grunts are produced by a long inhalation-exhalation interval (Stünkel, 2003). In contrast to other taxa of the genus *Trachypithecus* (Stünkel, 2003), we more often recognized whoops of the Hatinh langur during the time spent in the forest. We heard grunts only few times during detection events and they seemed to be produced as warning calls in direct response to the observers. We did not hear grunts from far distances. We found the greatest peak of the whoops early in the morning before sunrise and a second peak in the afternoon. During these periods, the groups depart from and travel to their sleeping sites, respectively. Most times we were unaware of the stimuli of the whoop calls. Due to the concentration of loud calls early in the morning and late in the afternoon and the observed jumping displays during the call, we assume

that these long distance calls of the males are mainly produced as territorial markers and spacing mechanisms to keep adjacent groups apart (Bates, 1970; Vogt, 2003). However, we twice recognized whoops by several males in response to thunder, indicating that the whoops of Hatinh langurs may also be produced as alarm calls in response to disturbance and threat (Stünkel, 2003; Vogt, 2003).

Conclusions

Our data provide fundamental information on Hatinh langurs in the PNKB NP. Further surveys are necessary to improve the knowledge of status, distribution, behaviour and ecology, and to find appropriate sites for the final release of the Hatinh langur groups of the primate reintroduction program of the Frankfurt Zoological Society and Cologne Zoo.

In terms of future studies in the PNKB NP, PTS along roads provides a low-effort method to observe changes of status, group structure and size, and occupation of sleeping sites.

Acknowledgements

We thank the People's Committee of Quang Binh, the Phong Nha - Ke Bang National Park directorate and Science Research Centre and the Centre for Natural Resources and Environmental Studies, Hanoi, for issuing necessary permits and their good cooperation. The Cologne Zoo and the Frankfurt Zoological Society provided financial support for our studies. Special thanks go to Thomas Ziegler, curator and project coordinator of the Nature Conservation Project Vietnam, Cologne Zoo, for his great support in many respects. We also thank Nguyen Hoang Dung, assistant of the Cologne Zoo Nature Conservation Project Vietnam and Frankfurt Zoological Society, for his kindness and cooperation. Some data were collected by Björn Behlert and Judith Riedel and we are grateful for their support. We thank all staff of the Phong Nha - Ke Bang National Park who joined our surveys for their assistance. We also thank Vu Ngoc Thanh, Zoological Museum, Vietnam National University, Hanoi, for his advice and good cooperation. Finally, T. Haus thanks Professor Wolfgang Böhme of the Zoologisches Forschungsmuseum Alexander Koenig, Bonn, for his kind supervision of her diploma thesis at Bonn University.

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The chemistry of eaten and uneaten leaves by Delacour's langurs (*Trachypithecus delacouri*) in Van Long Nature Reserve, Vietnam

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Key words: Delaour's langur, food choice, Vietnam, limestone langurs, soils, leaf chemistry

Summary

Several morphological, physiological, and ecological factors influence colobine leaf selection, but nutritional factors, especially leaf protein and digestibility, are among the most powerful. In particular, the protein/fiber ratio of leaves has repeatedly been supported as a strong indicator of leaf selection across for African and Asian colobines. However, the influence of leaf chemistry on food choice has not yet been analyzed for the limestone langurs of Southeast Asia, six taxa found in close association with limestone karst. Vegetation on limestone differs greatly in species composition and structure relative to forests with other xeric and edaphic conditions, and therefore we wanted to determine if protein/fiber ratio holds as an important determinant of food choice in limestone habitats.

We collected instantaneous focal animal data on Delacour's langurs (*Trachypithecus delacouri*) on a 265 hectare limestone karst block in Van Long Nature Reserve, Vietnam from August 2007-July 2008. We collected leaf samples that were eaten (n=50) and uneaten (n=49) by focal animals. Samples were analyzed for protein, fiber, condensed tannins, phenolics, water, and ash content. Among eaten leaves, we also made chemical comparisons between young vs. mature leaves. The protein/fiber ratio was higher in eaten versus uneaten leaves (p = 0.0003), but not significantly different for any other comparison.

The Delacour's langur population at Van Long does not seem to be nutritionally stressed and the population is increasing, and therefore Van Long Nature Reserve offers the best chance for long-term survival of this species

Thành phần hóa học của các lá cây là thức ăn và không là thức ăn của voọc mông trắng (*Trachypithecus delacouri*) ở KBTTN Vân Long, Việt Nam

Tóm tắt

Có nhiều yếu tố ảnh hưởng đến sự lựa chọn lá cây làm thức ăn của các loài voọc như các yếu tố hình thái, sinh lý và các yếu tố sinh thái, nhưng các yếu tố dinh dưỡng, đặc biệt là chất protein và khả năng tiêu hóa của lá cây là những yếu tố tác động mạnh mẽ nhất. Đặc biệt, tỷ lệ giữa hàm lượng protein và chất xơ chứa trong lá thường được xem là chỉ thị mạnh của sự lựa chọn lá cây của các loài voọc châu Á và châu Phi. Tuy nhiên, chưa có nghiên cứu về ảnh hưởng thành phần hóa học của lá cây đến sự lựa chon thức ăn của các loài voọc núi đá ở Đông Nam Á, 6 bậc phân loại có mối quan hệ chặt chẽ

với sinh cảnh núi đá caxtơ. Thành phần loài và cấu trúc của thảm thực vật núi đá khác nhiều so với các khu rừng có các điều kiện khô hạn và thổ nhưỡng khác. Vì vậy, chúng tôi muốn xác định xem liệu tỷ lệ protein/chất xơ có là yếu tố quan trọng quyết định sự lựa chọn thức ăn ở các sinh cảnh núi đá.

Chúng tôi đã thu thập các số liệu của voọc mông trắng (*Trachypithecus delacouri*) bằng phương pháp quan sát ngẫu nhiên con vật trọng tâm tại khu vực núi đá 265 ha ở KBTTN Vân Long, Việt Nam, từ 8/2007 đến 7/2008. Chúng tôi thu thập các mẫu lá cây mà các thể voọc quan sát ăn (n=50) và không ăn (n=49) để phân tích hàm lượng các chất protein, chất xơ, tannin đậm đặc, phenolics, nước và tro. Trong số các là cây vo ọc ăn, chúng tôi so sánh thành phần hóa hoạc của các lá non với các lá trưởng thành. Tỷ lệ protein/chất xơ là cao hơn ở các lá voọc ăn so với các lá voọc không ăn (p=0,0003), nhưng không có sự khác biệt đáng kể đối với các chỉ số so sánh khác.

Quần thể voọc mông trắng ở Vân Long có vẻ không bị ức chế về dinh dưỡng và đang gia tăng, vì vậy, KBTTN Vân Long là cơ hội tốt cho sự tồn tại lâu dài của loài này.

Introduction

For three decades, protein/fiber ratio has been recognized as a good predictor of leaf choice for relatively small mammalian herbivores, including primates (Milton, 1979). While an optimal level of fiber is needed to regulate the emptying of the colobine forestomach, fiber is inversely related to digestibility (Waterman & Kool, 1994). Chapman et al. (2002) list several studies that have supported the importance of protein and fiber in colobine leaf selection (McKey et al., 1981; Davies et al., 1988) and others that support colobine selection for easily digestible material (Oates et al., 1980; Waterman & Choo, 1981). As leaves age, they contain less protein and more fiber and lignin, and therefore young leaves are generally more digestible than mature ones (Baranga, 1986). The importance of protein and fiber in colobine leaf choice is further emphasized by the robust link between mature leaf protein/fiber ratio and colobine biomass across Africa and Asia (Waterman & Kool, 1994).

While protein and fiber levels are of paramount importance in leaf selection, various secondary compounds may also influence selection. Phenolics are the parent group of tannins, hydrophilic polymeric phenols that precipitate starch and proteins, lower nitrogen availability, lower nutrient quality, and reduce digestion (Rhoades & Cates, 1976). Tannins sometimes have beneficial effects in the diet by decreasing bloat (a foaming of digesta in the forestomach) and binding to, precipitating, and detoxifying alkaloids (Cork & Foley, 1991; Glander, 1994), but condensed tannins bind proteins, and there by negatively influence food choice, (Feeny, 1976; Coley & Barone, 1996).

Despite extensive studies of nutritional dietary ecology among colobines, few data exist for the six limestone langur taxa of Southeast Asia. Detailed studies of feeding ecology in the wild have only been conducted on *Trachypithecus leucocephalus* (Huang Chengming et al., 2000; Li Zhaoyuan et al., 2003; Li Zhaoyuan & Rogers, 2006) and *Trachypithecus delacouri* (Workman, in review), but the relationship between plant chemistry and food selection has not yet been considered for any of these species. This omission has important implications given that vegetation on limestone differs greatly in species composition and structure relative to forests with other xeric and edaphic conditions (Sterling et al., 2006). Vegetation on karst is notoriously stunted, with many grasses, lithophytic plants, shrubs and small trees (Li Zhaoyuan et al., 2003; Day & Chenoweth, 2004; Liu Zaihua et al., 2004). This stunted vegetation, coupled with the presumed soil conditions (thin, highly alkaline, sandy, dry, low in mineral nutrients) has been assumed to reflect plants whose leaves are well-defended by defensive compounds because they are growth-limited (Sterling et al., 2006).

The largest population of wild Delacour's langurs lives at Van Long Nature Reserve (VLNR) in northern Vietnam. A recent study at VLNR (Workman, in review) recorded VLNR Delacour's langurs as highly folivorous, eating 78% foliage annually: 59.3% young leaves and leaf buds, 20.4% mature leaves, 9.2% unripe fruit, 5.1% flowers and flower buds, 0.6% seeds, 0.3% stems, 0.1% ripe fruit, and 5% unclassified items. Young leaves contributed the greatest percentage to the diet during all months and seasons. Having a radiation of leaf-eating monkeys that are so highly folivorous and found in close association with limestone habitat creates a new opportunity to reassess the degree of variation in colobine diets and food selection. In this study, we wanted to determine how leaves eaten by Delacour's langurs differ in protein, fiber, and phenolics (especially tannins) with uneaten leaves. We then compare the chemistry of leaves eaten and uneaten by Delacour's langurs with those eaten and uneaten by *Colobus guereza* in Kakamega Forest, Kenya (Fashing et al., 2007).

Methods

Study site

Research was conducted at the Dong Quyen karst mountain of Van Long Nature Reserve (20°20'55"N, 105°48'20"E) in Ninh Binh Province, northern Vietnam. This habitat is characterized by limestone massifs fragmented by shallow wetlands. Dong Quyen Mountain is a 265 hectare massif that rises from 1 m to 328 m elevation (Fig. 1, 2). There is no dominant plant family or species on Dong Quyen and vegetation is comprised of woody trees and shrubs (43.5%), herbs (25.4%), climbers (29.7%), and grasses (1.4%) (Workman & Nguyen The Cuong, unpublished data). During the study period, the mean maximum temperature was 31°C, and the mean minimum was 13°C (n=394, range=9-37°C). Total annual rainfall was 1375.62 mm, with 89% falling between May-October.

Plant sample collection and plant chemistry

Both eaten and uneaten plant samples were collected for chemical analysis. We collected samples from plants fed on by focal individuals that CW observed. We collected additional feeding samples on non-focal individuals that LVD observed. Instantaneous focal animal sampling data were collected from August 2007-July 2008 (n=21,012 minutes; 200 days). We observed Delacour's langur adult males, adult females, females with dependent young, and subadults in seven groups, but we focused on three groups which were most easily observed (n=28). We made a concerted effort to rotate age/sex groups daily. Details of the behavioral sampling methods have been described previously (Workman, in review).

Plant feeding samples were collected at the end of a morning or evening observation session, when groups had moved out of view. Most plants were short enough that tree-climbing was usually not necessary. A sample from the plant that was eaten (e.g. the young leaves of a small tree) was collected as well as a matching phenophase sample from a plant that was next to the eaten plant, but not consumed. If there was a plant of the same species close by, a sample from that plant was collected. While the species consumed is reported here, the focus was less on the plant species eaten than on the plant individual that was eaten.

Intraspecific variability in the nutritional content of primate foods has been well documented (Chapman et al., 2003), but the aim of this study was not to determine the nutritional content of certain species on which Delacour's langurs fed, but rather on the differences between plant individuals at a given time. The steep topography of the karst habitat precluded us from accessing

Fig. 1. The Dong Quyen karst mountain of Van Long Nature Reserve, the locality of the study. Photo: T. Nadler.

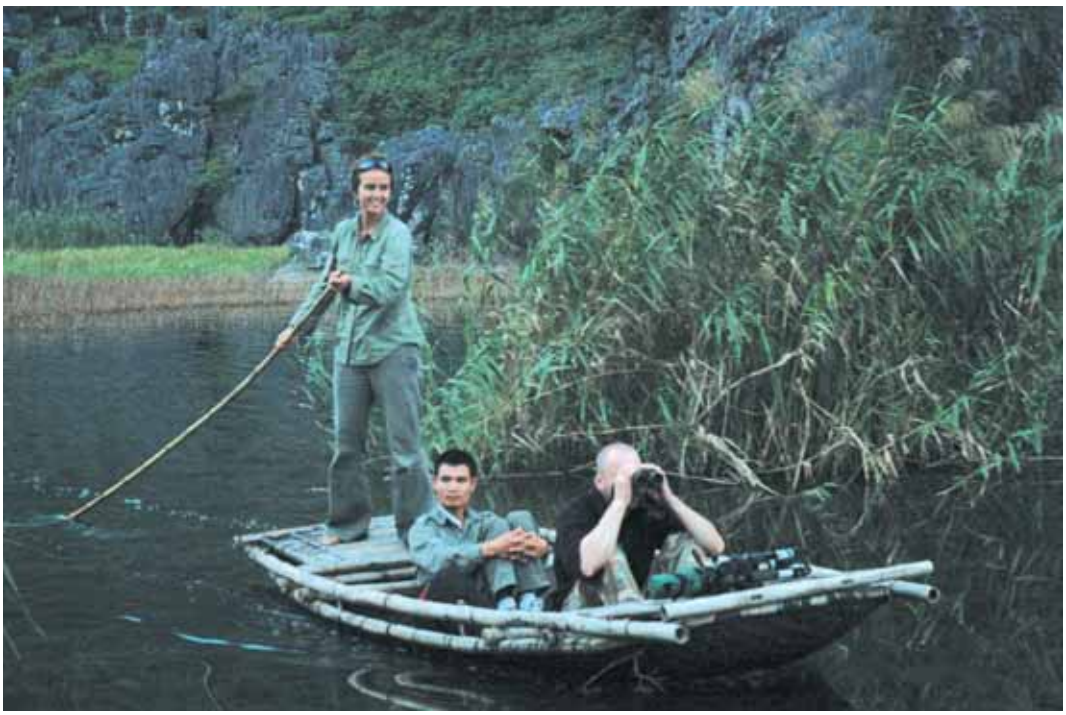

Fig. 2. The area of the study is only accessible by boat. Photo: T. Nadler.

certain areas and therefore we realize that our sample is limited to those plants which we could safely access. Eaten and uneaten samples had fresh masses between 80 g and 1047 g, with a mean of 535 g and SD of 116 (*n*=50 eaten samples (40 young leaves, 10 mature leaves; *n*=49 uneaten samples (39 young leaves, 10 mature leaves).

Samples were transported in bags to the Van Long Nature Reserve Headquarter and weighed within two hours of collection, dried in the shade over a period of days or weeks (depending on weather), and then kept at room temperature until analysis. Dried samples were taken to Ms. Lan Anh at the Hanoi University of Science for transport to one of two testing facilities: 1) Food and Chemical Microbiology and Food Testing Laboratory of Quality Assurance and Testing Center Number, Hanoi; 2) National Institute of Animal Husbandry, Hanoi. Eaten and uneaten samples were analyzed for crude protein, neutral detergent fiber (NDF), condensed tannin, total phenolics, crude ash, and water content. Samples were ground dry in a Wiley laboratory mill and passed through a 1 mm wire screen (Chapman et al., 2002). All samples were dried to a constant weight of 100ºC and all results are provided on a dry matter basis.

Crude protein content was assessed using the Kjeldahl method (Horowitz, 1970). Total nitrogen content was first measured and then used to estimate the crude protein level (protein content = nitrogen * 6.25: Maynard & Loosli, 1969). Fiber (Neutral detergent fiber (NDF)) was measured by following the methods described in van Soest (1963). NDF is a measure of the structural components in plant cells (i.e. lignin, hemicellulose and cellulose), but not pectin. NDF was measured because it is a more reliable measure of the fibrous component in the diet (compared to ADF) but we realize that not having ADF makes our results difficult to compare with other studies. Total phenolics were analyzed by the Folin-Denis method (Swain & Hillis, 1959). Tannins were determined using the KMnO4 titration method (Tempel, 1982).

Statistical analyses

Differences in plant chemistry (crude protein, NDF, total phenolics, condensed tannins, water, ash) were analyzed between two groups. First, we compared eaten leaves (*n*=50) vs. uneaten leaves (*n*=49). Second, from August 2007-July 2008, young leaves dominated the Delacour's langurs' diet monthly, seasonally, and annually. We therefore compared young leaves (n = 40) vs. mature leaves (*n*=10). All differences were analyzed using a non-parametric Wilcoxon test in R 2.7.1 software (the equivalent of the non-parametric Mann-Whitney U test).

Results

Eaten versus uneaten leaves

Leaves eaten by Delacour's langurs (*n*=50) had a higher protein/fiber ratio than leaves not selected (*n*=49) (mean = 0.42 +/- 0.18 vs. mean = 0.31 +/- 0.16; $p < 0.0004$) (Table 1). Leaves eaten and uneaten were not statistically different in protein (mean = 12.04 +/- 6.88 vs. mean = 10.58 +/- 5.82; $p = 0.13$), fiber (mean = 32.47 +/- 18.62 vs. mean = 38.18 +/- 21.09; $p = 0.06$), total phenolics (mean = 2.13 +/- 2.17 vs. mean = 1.52 +/- 1.68; p = 0.09), tannins (mean= 6.36 +/- 4.81

Table. 1. Mean values of eaten (*n* = 50) and uneaten leaves (*n* = 49). Standard deviations in parentheses, * significant $p < 0.0004$.

	Eaten leaves	**Uneaten leaves**
CP/F*	0.42 (0.18)	0.31 (0.16)
Protein	12.04 (6.88)	10.58 (5.82)
Fiber	32.47 (18.62)	38.18 (21.09)
Tannins	6.36 (4.81)	4.81 (3.92)
Phenolics	2.13 (2.17)	1.52 (1.68)
Water	80.88 (5.74)	78.92 (8.53)
Ash	16 (6.97)	19.53 (9.18)

vs. mean = 4.81 +/- 3.92; p = 0.08), water (mean = 80.88 +/- 5.74 vs. mean = 78.92 +/- 8.53; p = 0.36), or ash content (mean = 16 +/- 6.97 vs. mean = 19.53 +/- 9.18; p = 0.11).

Leaf stage differences in plant chemistry

Samples of young (n=40) and mature leaves (n=10) eaten by Delacour's langurs did not differ in their content for any of the analyzed constituents: protein (mean = 12.13 =/- 6.92 vs. mean = 11.94 +/- 6.74; p = 0.91), fiber (mean = 34 +/- 19.53 vs. mean = 26.17 +/- 13.28; p = 0.22), total phenolics (mean = 2.25 +/- 2.38 vs. mean = 1.55 +/- 0.87; p = 0.75), condensed tannins (mean = 6.01 +/- 4.74 vs. mean = 7.79 +/- 5.1; p = 0.28), water (mean = 81.19 +/- 5.51 vs. mean = 79.61 +/- 6.75; p = 0.57), ash (mean = 16.24 +/- 6.89 vs. mean = 16.04 +/- 7.82; p = 0.88), protein/fiber ratio (mean = 0.41 +/- 0.19 vs. mean = 0.46 +/- 0.16; p = 0.53).

Discussion

The leaves eaten by Delacour's langurs at Van Long contain less than half the protein of leaves eaten by guerezas at Kakamega, Kenya (Table 2) (Fashing et al., 2007). Fiber content of leaves eaten by Delacour's langurs is also less than that of guerezas. At Kakamega, protein content was the primary factor determining whether or not guerezas consumed specific leaf items, with eaten leaves at or above a protein threshold of 14% dry matter (Fashing et al., 2007). At Van Long, protein content did not differ between eaten and uneaten leaf items. Further, protein content of eaten leaves averaged 12% dry matter, with several leaf items containing protein levels far below that.

Table. 2. Neutral detergent fiber (NDF) and protein levels for young leaves (YL) and mature leaves (ML) consumed by *Colobus guereza* and *Trachypithecus delacouri*.

Species	NDF	Protein	Plant part	Citation
Colobus guereza	48.0	23	mean- leaves 3.5	Fashing et al. (2007)
Trachypithecus delacouri	38.0	12.2	mean- YL	this study
	33.4	10.4	mean- ML	

While low compared to guerezas at Kakamega, Delacour's langurs are meeting the 7-11% protein (of dry matter) that primates need for maintenance and growth (Oftedal, 1991). Delacour's langurs at VLNR are also eating leaf items which are above the critical protein needed for ruminants to maintain positive nitrogen balance (4-8% dry weight) (Milton, 1979). Oftedal (1991) states that primate populations need to consume protein at 14% dry matter to sustain reproduction. Delacour's langurs are not quite meeting this threshold; however, the population on Dong Quyen Mountain at VLNR has doubled in 9 years (~35 langurs in 2000, ~ 70 langurs in 2009). It appears, therefore, that langurs are not limited in sustaining reproduction and are possibly receiving additional protein from food sources that we did not sample. Further, because Van Long has both evergreen and deciduous species, it is possible that - as at Kibale National Park, Uganda - the forest never reaches a very low nutrient value and colobines are not nutritionally stressed, allowing for quick population rebound (Baranga, 1986).

At Kibale, *Colobus guereza* chose young leaves that had more protein and higher protein/fiber ratios than mature leaves, although the two leaf stages did not differ in secondary compound content (Chapman et al., 2004). The preference for leaves with higher protein/fiber ratios also held at Van Long and young leaves had slightly higher protein content than mature leaves, although the

difference was not significant. Further, our study supports the contention that tannin concentrations may be of minor significance to primates (Oates et al., 1980).

Future studies should collect community-level plant data at Van Long to facilitate colobine biomass comparisons across Africa and Asia. However, explaining primate biomass using the protein/fiber ratio will be misleading if populations are not at carrying capacity (Chapman et al., 2004). For the monophyletic limestone langur species of northern Vietnam, southern China, and eastern Laos, intense hunting pressure precludes a solely ecological explanation of their distribution and abundance on karst habitats. A similar ecological conundrum for African primate communities has been addressed by Struhsaker (1999). He notes that the present-day distribution of many species may be the artifact of recent hunting, rather than the result of resource base selective pressures. Given this consideration a focus on proximate factors influencing langur food choice seems appropriate.

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Diet and feeding behaviour of pygmy lorises (*Nycticebus pygmaeus*) in Vietnam

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Key words: Diet, feeding behaviour, pygmy loris

Summary

Little is known about the diet and feeding behaviour of the pygmy loris. Within the Lorisidae there are faunivorous and frugivorous species represented and this study aimed to characterize where the pygmy loris (*Nycticebus pygmaeus*) ranges on this scale. Feeding behaviour was observed in adult animals which had been confiscated from the illegal wildlife trade and housed at the Endangered Primate Rescue Center at Cuc Phuong National Park for some time before they were radio collared and released into Cuc Phuong National Park.

The lorises were located in daytime by methods of radio tracking and in the evenings they were directly observed with the help of red-light torches. The observed lorises exploited a large variety of different food sources, consuming insects as well as gum and other plant exudates, thus appearing to be truly omnivorous. Seasonal variations in food preferences were observed.

Omnivory can be an adaptive strategy, helping to overcome difficulties in times of food shortage. The pygmy loris' feeding behaviour enables it to rely on other food sources like gum in times when other feeding resource become rare. Gum as an alternative food sources has the advantage of being readily available all year round. However it does not permit the same energetic benefits and consequently the same lifestyle as other food sources. But it is an important part of the pygmy loris' multifaceted strategy to survive times of hostile environmental conditions.

Thức ăn và tập tính kiếm ăn của Cu li nhỏ (*Nycticebus pygmaeus*) ở Việt Nam

Tóm tắt

Những hiểu biết về thức ăn và tập tính kiếm ăn của cu li nhỏ còn rất hạn chế. Trong họ cu li có một số loài ăn quả và một số loài ăn động vật và nghiên cứu này nhằm xác định thức ăn của loài cu li nhỏ nằm ở đâu trong phạm vi này. Tập tính kiếm ăn được quan sát trên các cá thể đực trưởng thành, đây là những cá thể được thu giữ từ các vụ buôn bán động vật hoang dã trái phép và nuôi dưỡng tại Trạm Cứu hộ Linh trưởng Nguy cấp tại Vườn Quốc gia Cúc Phương một thời gian trước khi chúng được gắn thiết bị radio ở cổ và thả vào Vườn Quốc gia Cúc Phương.

Vị trí của các con Cu li nhỏ ban ngày được xác định bằng phương pháp theo dõi sóng vô tuyến (radio tracking) và vào ban đêm chúng được quan sát trực tiếp bằng đèn pin ánh sáng đỏ. Những cá thể cu li được quan sát ăn rất nhiều nguồn thức ăn khác nhau, ăn côn trùng cũng như nhựa cây và mủ thực vật, vì vậy chúng dường như là loài ăn tạp. Sự biến đổi theo mùa về các loại thức ăn ưa thích cũng đã quan sát được.

Ăn tạp có thể là một chiến lược thích nghi, giúp vượt qua khó khăn trong thời gian khan hiếm thức ăn. Tập tính kiếm ăn của cu li nhỏ cho phép nó phụ thuộc vào các nguồn thức ăn khác như mủ trong những thời điểm các nguồn thức ăn khác trở nên khan hiếm. Mủ (Gum) là một nguồn thức ăn thay thế có thuận lợi là sẵn có quanh năm. Tuy nhiên, nó không cho phép cùng lợi ích về năng lượng và bởi vậy cùng phương thức sống giống như các nguồn thức ăn khác. Tuy nhiên nó là một phần quan trọng trong chiến lược nhiều mặt của cu li nhỏ để tồn tại trong những thời điểm điều kiện môi trường khắc nghiệt.

Introduction

Little is known about the diet and feeding behaviour of the pygmy loris. Within the lorises a variety of different feeding ecologies are represented. Usually the slow loris *Nycticebus coucang* is counted among the frugivorous and the slender loris *Loris tardigradus* among the insectivorous species. In a recent field study in Malaysia, the slow loris was found to feed preferably on plant exudates, gum and fruit (Wiens, 1995; 2002) and a field study in India proved that the slender loris is indeed almost exclusively faunivorous with termites and ants being an important component of the diet (Nekaris & Rasmussen, 2003). On this background it seems interesting to investigate the pygmy loris' dietary habits. Where does it range on the scale of faunivory, frugivory and gummivory?

Observations on wild pygmy lorises feeding are very rare and only two reports are available. On one occasion pygmy lorises were found in a tree (Tan, 1994), which showed typical gnaw marks ("gouges") and it was suggested that the animals had been feeding on gum there. On another occasion a pygmy loris was observed in a tree feeding on an unidentified fruit (Duckworth, 1994).

For a long time the pygmy loris was considered a subspecies of *N. coucang* (Hill, 1953; Petter & Petter-Rousseaux, 1979). Based on external similarities to the slow loris the pygmy loris was assumed to differ from the larger species only in size, but have similar dietary habits. Like *coucang pygmaeus* was assumed to be a species that inhabits the main canopy and lives on a frugivorous diet (Fleagle, 1978).

In captivity, pygmy lorises are usually maintained on a mixed diet, with the majority of the offered food items being fruit and vegetables and insects comprising the rest of the diet (Fitch-Snyder et al., 2001). At the Endangered Primate Rescue Center, Vietnam pygmy lorises are fed on a diet consisting of fruit, vegetable, boiled eggs, milk powder and seasonally varying insects.

Newly confiscated animals at the rescue centre show a strong preference for invertebrates. Insects are mostly the first food item accepted, whilst recently confiscated animals mostly reject fruit, boiled eggs or vegetables. Obviously, wild pygmy lorises are more familiar with insects than with the other food items offered in particular cultivated fruit. Another hint to potential wild feeding habits is the gouging on fresh branches, which nearly all the confiscated pygmy lorises exhibit (Fig. 1).

Fig. 1. Typical gauge hole in a piece of furnishing. Photo: U. Streicher.

Methods

Data have been collected from four reintroduced individuals. The animals had all been captured as adults and had only been in captivity for several months. Thus all of them must have had previous experiences with wild food sources. The animals were genetically identical, with individuals originating from Cuc Phuong, but the exact locality of their origin and the habitat type they originated from was unknown.

The release site comprised forested limestone hills surrounded by old plantations and scrub and was located in the Cuc Phuong National Park in northern Vietnam (Vo Quy et al., 1996). The released animals had been equipped with transmitters and were located during the daytime in their sleeping sites by radio-telemetric methods. Before dusk, an observer returned to the loris' sleeping site and observed it during the beginning of its active period. Observations lasted from a few minutes to more than two hours. Head torches with redlight filters were used for observation and the animals could be fairly well-observed from a distance between 5 and 15 meters; only when the canopy was extremely dense visibility was sometimes limited. The animals showed different degrees of habituation. Whereas three animals got used to the observer's presence within a short time, one proved extremely reluctant to accept the presence of a researcher and was continuously hiding when an observer was around, exhibiting minimal activity. If the observer lost direct contact with the observed loris, observations were discontinued. In addition, the animals were not followed by telemetry at night due to the treacherous character of the terrain.

Each animal was observed for four to six weeks from the date of release onward. Data was collected *ad libitum* (Altman, 1974) on prepared data sheets. Feeding trees were identified the following day by collecting a branch sample and having it identified at the scientific department of the National Park.

Results

Feeding could be observed from the first day after the release onwards. A total number of 27 feeding bouts were observed. Several different types of feeding behaviour and food items were observed. In eleven cases, the food item was identified or suspected to be an insect, in eight cases it was gum, and in eight cases it was not exactly identified plant exudates.

Animal prey

Pygmy lorises searched for animal prey by moving slowly along branches with the nose close to the substrate. On nine occasions the pygmy lorises caught insects. Insects were caught either using one or using both hands and then put in the mouth. If both hands were used, the animals clung with both legs to a branch or stood bipedal. In one case the captured insect was a moth (Hymenoptera) attracted by the head torch of the observer. The most detailed observation of insect feeding was after one animal captured a very large cricket (Hemiptera). The cricket was held with both hands and slowly eaten starting from the head. The hard skin was broken using the molars and by pushing the prey into the mouth with the hands. The skin was partly bitten off and the loris frequently got rid of access parts of skin by fiercely shaking the head. The wings of the insects were bitten off and "disposed." Towards the end of this feeding session, the animal changed the position two times, moving to another branch, whilst holding the remains of the insect in one hand. The seemingly very sticky inner contents of the insect finally covered the surrounding area of the mouth and the hands of the loris and the animal spent several minutes grooming, concentrating on the

hands by licking them intensively. On one occasion the animal was observed licking on a branch of *Dracontomelum duperreamum* (Anacardiaceae). Whereas licking on branches in most cases was associated with feeding on plant items, this case was different because the animal frequently interrupted feeding to fiercely shake its head. Similar behaviour has been found to be associated with feeding on ants (Nekaris, 2001) and therefore it was assumed that the animal was feeding on ants, which attacked the intruding loris. On another occasion the animal was observed feeding for an extended time in low scrub with climbing weeds. The food source was not identified, but later inspection of the scrub found all young shoots showed marks of an insect foraging. Feeding on insects was usually a short event. Only when the loris found a number of insects in the same location or if an insect was exceedingly large, it spent several minutes feeding. For example, the devouring of the large cricket required over twenty minutes. All feeding on insects occurred at heights less than ten meters.

Gum and other plant exudates

Feeding on gum or other plant exudates comprised the majority of observed feeding events. The common feature was intense licking on branches without locomotion. Feeding on plant substrates comprised short sessions only lasting one minute and extended sessions lasting up to twenty minutes in the same location (Fig. 2). One of the tree species where the animals showed extensive licking behaviour was *Saraca dives* trees (Fabaceae). In full blossom these trees carried large bundles of big orange flowers that were inspected intensively on at least one occasion. However, it could not be ascertained if the animal actually found something to eat in the flowers. In this tree species the animals were licking intensively on the branches but this was not accompanied by audible scratching and bark-breaking sounds. The food sources must have been rather on the surface and easily accessible. The behaviour was not observed when the trees were not flourishing. Of the few observations of wild pygmy lorises at Cuc Phuong National Park, two were made in the same tree species carrying blossoms (Roberton, pers. com.). Obviously these trees were particularly attractive, when flourishing.

Fig. 2. Pygmy loris scraping for gum on the trunk of a *Spondias axillaries* tree. Photo: U. Streicher.

Similar licking on branches was observed as well in a *Sapindus* sp. tree (Sapindaceae), a *Vernicia montana* tree (Euphorbiaceae) and at least two other non-identified tree species.

Another tree species exploited for its exudate were *Spondias axillaris* (Anacardiaceae). Here the food source could clearly be identified as gum: the tree had an old injury and was visibly shedding gum (Fig. 3). The scraping for gum was accompanied in most cases by sounds of scratching and breaking bark. The animal fed with the body orthograde, clinging with all four legs to the bark (Fig. 2). Feeding sessions on this tree were the most extended ones observed. A remarkable observation was that one animal returned to the same feeding site every time it had slept in the near vicinity.

After it became active, it always first passed the "gum bar," when it was nearby. Scratching and bark-breaking sounds were very intense.

All but one feeding event related to gum or other food exudates were observed at heights over eight metres.

Fig. 3. The same site in daytime. The shedding of gum is clearly visible. Photo: U. Streicher.

Questionable food items

A possibly but not clearly feeding-related behaviour was observed in dense scrub areas where no plants were covering the soil and where the foliage was not very dense. From tree heights below one meter, the lorises frequently visited the ground for up to thirty seconds without actually covering any distance on the ground. Before going to the ground the animals always carefully observed the area where they intended to go. They usually went to the ground along the same tree, which they climbed up again after finishing the ground visit. It seems likely that these ground trips served a feeding purpose. But because these events were not clearly identified as feeding-related behaviour, they were not counted amongst the feeding bouts.

Feeding on fruit described by Duckworth (1994) was never observed.

Solitary feeders

In our observations, pygmy lorises were never observed feeding together or even in close proximity, but the observations are too scarce to evaluate if pygmy lorises actually prefer solitary feeding. They did exploit the same food sources on the same tree but not simultaneously.

Seasonal variations in food exploitation

Animals released in spring fed preferably on different tree species than animals that were released later in the year. In spring, the animals preferred *Saraca dives* as a food tree. At that time of year the trees are in full blossom. Later on, when the tree does not have any flowers, lorises did not show any specific preference for this tree species. The animals released from September onward showed a strong feeding preference for *Spondias axillaris* trees.

Discussion

Omnivory as a strategy to overcome times of food shortage.

Feeding behaviour of the pygmy loris has previously been assumed to be largely similar to the slow loris (Fleagle, 1978). Based on the large areas of sympatric occurrence, Ratajszczak (1998) suspected different feeding preferences and suggested the pygmy loris to be the more insectivorous species. Indeed the pygmy loris shares many characteristics of feeding behaviour with the insectivorous slender loris. Details of the feeding behaviour are identical (Nekaris & Rasmussen, 2003) and both animals capture insects single-handedly or bimanually with

stereotyped movements typical for prosimians and specifically adapted to catch small rapidly moving or flying insects (Hladik, 1979). However, ants which make up a large percentage of the prey of the slender loris (Nekaris & Rasmussen, 2003), seem to play no important role for the pygmy loris' diet. The feeding on gregarious insects has been assumed to relate to the gregariousness amongst the slender lorises themselves (Nekaris & Rasmussen, 2003); slender lorises in captivity devote more time to social interactions than any other loris species (Rasmussen, 1986; Schulze & Meier, 1995). Correspondingly, the insects preferably devoured by the pygmy lorises would facilitate a solitary way of foraging not risking intraspecific competition.

The pygmy loris also shares feeding characteristics with its larger relative, the slow loris. Previously considered predominantly frugivorous (Chivers & Hladik, 1980, Barret, 1983), the slow loris was recently found to spend a large percentage of its foraging time feeding on plant exudates (Wiens, 1995; 2002). In pygmy lorises, gum and plant exudates also make up an important food source. Active stimulation of exudate flow by gouging trees has previously been documented for some callitrichids *Cebuella* and *Callithrix* (Coimbra-Filho & Mittermeier, 1978) and the fork-marked lemur *Phaner furcifer* (Petter et al., 1971) and a similar behaviour has been suggested for the pygmy loris as well (Tan & Drake, 2001). According to our observations, pygmy lorises indeed actively stimulate the exudate flow and possibly maintain a steady food source by scraping gum at the same location every night, thus inducing additional gum shedding. Licking plant exudates off the branches in flourishing trees is also a behaviour which the pygmy and the slow loris have in common, and both species show the behaviour in trees of the same family (Fabaceae). Nectarivory had been a suggested explanation (Wiens, 1995) and it is likely that the pygmy lorises were also feeding on nectar, since this behaviour was not observed when the trees were not carrying blossoms.

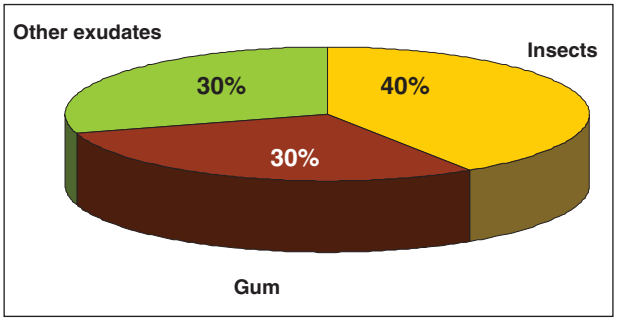

Fig. 4. Observed feeding bouts.

According to our observations, the pygmy loris is more of a generalist than the other Asian loris species and includes animal prey as well as gum and plant exudates in its diet (Fig. 4). Being a generalist could be a mere result of physiological requirements. With a body size of around 350 g, the pygmy loris is among the larger forms of prosimians. Hladik (1979) postulated that prosimians of this size have to utilize a variety of different food sources, since they are too large to be able to maintain themselves merely on insects because they simply do not find enough prey in a given habitat in one night.

But being a generalist could also be a potential advantage to overcoming difficult environmental conditions.

The winter in northern Vietnam is characterized not only by water shortage (dry season), but also by low temperatures unfavourable for many tropical plants (Nguyen Khanh Van et al., 2000). The number of insects during these winter months is much lower than during the rest of the year. There are no flourishing trees for several months and plant growth rates are at their minimum. There appears to be a seasonal variation in the majority of the loris' food sources like insects and nectar. For several months these resources are extremely rare.

During periods of low resource availability, primates may switch to alternative, poorer quality food sources and incorporate them into the diet in greater than usual quantities (Hladik, 1979; Gursky 2000). The insectivorous spectral tarsier *Tarsius spectrum* reacts to seasonal fluctuations in food availability by including higher percentages of lower quality insects in its diet (Gursky, 2000). However, the tarsier remains fully insectivorous even if this means depending on a diminished resource. But a tarsier is small, requires less insects for maintenance and can travel fast and cover a larger area in order to find sufficient food if necessary. Indeed, in times of food shortage tarsiers increase their travelling distances while foraging (Gursky, 2000). Being limited to quadrupedal locomotion without the ability to leap, the pygmy loris can't greatly increase its daily travel path length and must use a different strategy to overcome times of food shortage.

Gum has been found to be part of the pygmy loris' diet and gum is available all year round and thus a reliable food source. Consequently, gum could be an ideal food source to overcome periodical food shortage. Gum contains high concentrations of carbohydrates (Bearder & Martin, 1980) and some prosimians, such as the lesser bushbaby *Galago senegalensis* and thick-tailed bushbaby *Galago crassicaudatus* are able to persist on gum alone when other food sources are scarce (Bearder, 1987). In the bushbabies, gum is digested in the enlarged caecum through the action of symbiotic bacteria (Charles-Dominique, 1977; Hladik, 1979). In contrast, for mammals that lack microbial fermentation, gum is largely indigestible (Waterman, 1984) and the pygmy loris has no chambered site for microbial fermentation in its digestive tract (Hill, 1953). Moreover, gum has been suspected to be a self-limiting food source since detoxifying toxic components in gum might be energetically expensive and is considered responsible for a low metabolism and reduced pace of life (Wiens, 2002).

Reduced activity and extensive resting of the pygmy lorises during the winter period thus are not only measures to reduce energetic expenses but also a way to respond to the available energy sources.

The pygmy loris feeding behaviour enables it to switch to other food sources in times when its main feeding sources are rare and exploit gum as a steady reliable food source. Gum is a low quality food source and only allows living at a low energy level with a reduced metabolism. However, it is an important part of the pygmy loris multifaceted strategy to survive times of hostile environmental conditions.

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Conservation of douc langurs in Vietnam: An assessment of Agent Orange exposure in douc langurs (*Pygathrix*) at the Endangered Primate Rescue Center, Cuc Phuong National Park, Vietnam

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Key words: TCDD, dioxin, faecal enzyme immunoassay, douc langurs, Endangered Primate Rescue Center

Summary

Humans residing in "dioxin hotspots" of Vietnam have markedly elevated serum dioxin (TCDD) levels, but no comparable data exist on the other mammals in those areas. The long-term goal of this research is to assess the role of TCDD (Tetrachlorodibenzo-p-dioxin) in the health of endangered primates inhabiting "dioxin hotspots," in southern Vietnam. This study examined the utility of faecal dioxin (fTCDD) enzyme immunoassays for quantifying TCDD levels in douc langurs (*Pygathrix nigripes*, *P. nemaeus*, *P. cinerea*) housed at the Endangered Primate Rescue Center, Cuc Phuong National Park, Vietnam. Analyses were based on a total of 22 faecal samples collected from douc langurs from known capture locations north and south of the demilitarized zone (DMZ), Vietnam, 4 from the north and 18 from the south. A fTCDD enzyme immunoassay (EIA) developed by CAPE Technologies, Inc. (South Portland, Maine) was previously validated and found to reliably detect 2,3,7,8-TCDD in *Pygathrix* feces. Results of TCDD EIA procedures showed that fTCCD levels in this population were variable, ranging from non-detectable to 21 pg/g (wet wt). Results from multiple linear regression analysis showed that fTCDD levels were unrelated to geographic location, species, residence time or sex. Fecal dioxin levels were, however, linked to age, juveniles exhibiting significantly elevated mean fTCDD levels over those observed in adults from central and southern Vietnam. Age-related patterns of TCDD excretion are interpreted in the context of possible developmental biomarkers of dioxin toxicity in some adult members of this population. The importance of this research for conservation of primates in Vietnam resides in identifying TCDD-exposed primate populations/habitats for immediate protection and possible remediation through the establishment of "Species Conservation Areas."

Bảo tồn các loài Chà vá ở Việt Nam: Đánh giá sự phơi nhiễm Chất Da cam ở các loài chà vá (*Pygathrix*) tại Trung tâm Cứu hộ Linh trưởng, Vườn Quốc gia Cúc Phương, Việt Nam

Tóm tắt

Con người sống ở những "điểm nóng dioxin" của Việt Nam có mức độ dioxin (TCDD) huyết thanh cao rõ rệt; tuy nhiên ở các loài động vật có vú khác chưa có dữ liệu nào có thể so sánh. Mục tiêu dài hạn của nghiên cứu này là đánh giá vai trò của TCDD đối với sức khỏe của các loài linh trưởng bị đe dọa tuyệt chủng đang sống ở những "điểm nóng dioxin" ở miền nam Việt Nam. Nghiên cứu này kiểm tra tính hiệu quả của xét nghiệm miễn dịch enzyme phát hiện dioxin trong phân (fTCDD) nhằm lượng hoá mức độ TCDD ở các loài chà vá (*Pygathrix nigripes*, *P. nemaeus*, *P. cinerea*) được nuôi tại Trung tâm cứu hộ Linh trưởng, Vườn Quốc gia Cúc Phương, Việt Nam. Các phân tích dựa trên tổng cộng 22 mẫu phân của chà vá từ các địa điểm đã biết nằm ở phía bắc và phía nam vùng phi quân sự (DMZ), Việt Nam gồm 4 mẫu phía bắc và 18 mẫu phía nam. Xét nghiệm miễn dịch enzyme phát hiện dioxin trong phân được Công ty CAPE Technologies, Inc (South Portland, Maine) phát triển đã được đánh giá trước đây và cho thấy khả năng phát hiện đáng tin cậy chất 2,3,7,8-TCDD trong phân của các loài chà vá. Các kết quả phân tích cho thấy các mức độ dioxin trong phân trong quần thể này thay đổi, từ không phát hiện đến 21 pg/g (trọng lượng tươi). Các kết quả phân tích hồi quy đa tuyến tính cho thấy mức độ fTCDD không liên quan đến vị trí địa lý, loài, thời gian cư trú và giới tính. Tuy nhiên, mức độ dioxin trong phân liên quan đến độ tuổi; những con chưa trưởng thành có mức trung bình fTCDD cao có ý nghĩa so với những con trưởng thành từ vùng miền trung và nam Việt Nam. Các kiểu đào thải TCDD liên quan với tuổi cho thấy rằng có thể đã có các dấu hiệu sinh học mang tính phát triển về sự ngộ độc dioxin ở một số cá thể trưởng thành trong quần thể. Tầm quan trọng của nghiên cứu này đối với việc bảo tồn các loài linh trưởng ở Việt Nam tập trung vào xác định các quần thể hoặc sinh cảnh của linh trưởng phơi nhiễm TCDD nhằm bảo vệ kịp thời và điều trị thông qua việc thành lập các "Khu bảo tồn loài".

Introduction

In addition to its remarkable biodiversity, Vietnam is distinctive for being home to 20% of the world's 25 most endangered primates, including the critically endangered *Trachypithecus delacouri* (~200-250 individuals), *Trachypithecus p. poliocephalus* (~65 individuals), *Pygathrix cinerea* (~600-700 individuals); *Rhinopithecus avunculus* (~150-200 individuals), and *Nomascus nasutus* (~110 individuals) Mittermeier et al., 2007). It has long been recognized and documented that subsistence hunting and increasing habitat fragmentation continue to threaten Vietnamese primates (Mittermeier et al., 2007), but few data are available on the potential toxic effects of environmental herbicides (e.g. Agent Orange) on the health and well-being of human and wildlife populations. While there is widespread recognition that since the mid-1940's diverse endocrine-disrupting chemicals, including dioxins, have been released into the environment worldwide (reviewed in Colborn et al., 1996), the dosage and geographic extent of dioxin exposure in Vietnam has only recently been precisely documented. Between 1965 and 1971 the US government sprayed more than 45 million litres (~11,623,570 gallons) of dioxin-containing herbicides on the forests and hamlets of central and southern Vietnam destroying approximately 10% of the forests in that region (Stellman et al., 2003, Fig. 1, 2). U.S. military tactical zones Corps Areas I and III received the vast majority of the herbicide (Table 1) while use of the defoliant north of the 17th parallel (demilitarized zone, DMZ) was negligible (Westing, 2002).

Fig. 1. Map of administrative divisions in Vietnam (ca. 1967).

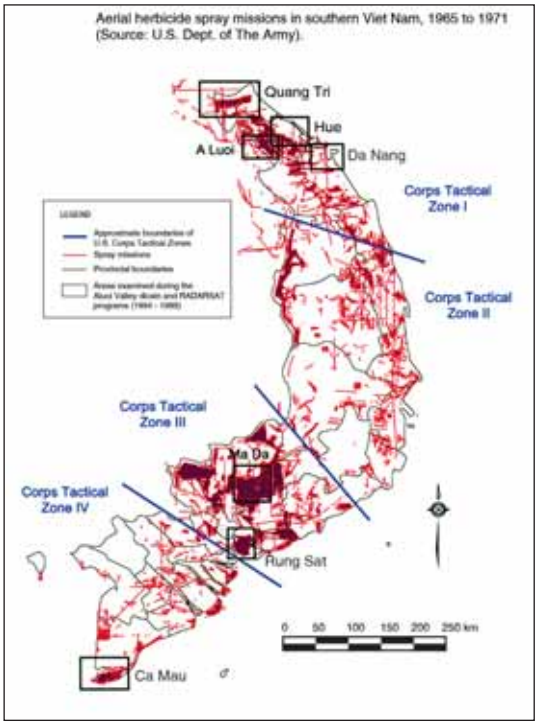

Fig. 2. Agent Orange spray missions in central/south Vietnam (Web Images: CIA and U.S. Department of Defense public domain).

The most toxic of the polyhalogenated aromatic hydrocarbons, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a lipophilic chemical that is known to bioaccumulate in the fat of vertebrates through the food chain, although the largest fraction of dioxin is excreted in faeces (Fries, 1995). The half-life of dioxin has been shown to be dose-dependent, excretion being slower at lower concentrations, and also varies by body mass (and sex) so that higher levels of body fat results in increased persistence of dioxin in the body (Schecter et al., 2006). Laboratory studies of vertebrates show that species vary widely in their sensitivity to lethal doses of dioxin: LD50 (i.e. 50% lethal dose) for guinea pigs is reported to be ~1µg/kg body weight (bw) whereas it is ~1000 µg/kg bw for hamsters, and no data exist on LD50 for humans (Schecter et al., 2006). Because dioxins in soil pose lingering threats to human health via food, the World Health Organization recommends that tolerable daily intakes (TDI) in foods not exceed 4 pg TEQ per kg bw/per day (WHO/EURO, 1998a,b in Dwernychuk et al., 2002) based on new toxicological data on the neurodevelopmental and endocrinological effects of TCDD.

Table 1. TCDD (i.e. Agent Orange) sprayed south of the 17th parallel during Operations Trail Dust and Rand Hand, 10 August 1961 – 31 October 1971 (data from fixed-wing aircraft only; US Department of Defense, HERBS file).

Tactical Zone*	Total TCDD (gal.)	Mean TCDD ± SD (gal.)	Number of Missions	TCDD Range (gal.)
I Corps	1,874,360	48,060.51 ± 38,071.39	39	150,145.00 – 4,490.00
II Corps	879,338	5,173.52 ± 28,847.49	25	98,220.00 – 110.00
III Corps	2,103,608	63,745.70 ± 97,472.14	33	484,383.00 – 320.00
IV Corps	456,380	25,354.44 ± 37,587.67	18	150,345.00 – 1875.00

*Mann Whitney U: P=0.028

Known environmental sources result principally from incineration and chemicals (i.e. TCDD), aerial transport of dioxins in the former being deposited in soils, plants, and water through combustion, incineration, and industrial processes (Fries, 1995). Chemical sources of TCDD have been identified as the principal source for the introduction of 2,3,7,8-TCDD into the Vietnamese environment (Hatfield & 10-80 Committee, 2006), having been used primarily for defoliation and crop destruction during the Vietnam War. Dioxins are resistant to chemical or biological breakdown (Diliberto *et al.*, 2001) and have a half-life ranging from 6 to 20 years (Portier et al., 1999 in Fenton et al., 2002), although they can persist in subsurface soils to a depth of greater than 10 cm (Hatfield Consultants & 10-80 Committee, 1998) and thus available for plant uptake for as long as 100 years (Paustenbach et al., 1992).

Dioxins exhibit low water solubility and are resistant to rapid degradation and tend to persist for decades in soil as noted above, making this medium a TCDD reservoir (Hatfield Consultants & 10-80 Committee, 2006) having the capacity to remobilize and transport TCDD as dust through soil disturbance, including logging, agricultural activities, natural erosion, and road construction (Dwernychuk et al., 2001). Schecter et al.'s (2003) research shows that human populations have been directly impacted by toxic herbicide concentrations in the environment, and includes evidence that dioxin-contaminated food (e.g. fish, toads, ducks, chickens) is the source of the markedly elevated serum dioxin levels (e.g. 413 parts per trillion) observed in the residents living in and around Bien Hoa City (Dong Nai Province), a recently designated "dioxin hotspot" located ~30 km northeast of Ho Chi Minh City, Vietnam. Endangered primates also live in areas surrounding Bien Hoa City (e.g. Cat Tien National Park), including the black-shanked douc langur, *Pygathrix nigripes* (Fig. 3), but no comparable data exist on TCDD exposure in these or any other primates.

The long-term goal of this research is to investigate the role of Agent Orange (e.g. dioxin or TCDD) in the developmental and reproductive health of Vietnamese primates inhabiting "dioxin hotspots" in central and southern Vietnam. Vietnam is encompassed within the Indo-Burma Biodiversity Hotspot region, one of the top 25 hotspots identified for urgent conservation action; it is also among the nine leading hotspots in terms of endemics (Myers et al., 2000). Historically, habitat loss/degradation has occurred via the use of chemical defoliants [TCDD], logging, and clearing of land for agriculture, this threat being rivalled only by subsistence/trophy hunting for body parts in its devastating impact on primate populations (Nadler & Streicher, 2004).

The objective of this research was to examine the utility of faecal dioxin (fTCDD) assays for non-invasively quantifying dioxin levels in 22 wild-caught douc langurs (*Pygathrix nigripes, P. nemaeus*, *P. cinerea*, Fig. 4, 5, 6) housed at the Endangered Primate Rescue Center (EPRC), Cuc Phuong National Park, Vietnam. EPRC maintains and breeds some of Vietnam's most endangered primates, including the critically endangered grey-shanked douc langur, *Pygathrix cinerea* (Mittermeier et al., 2007). The last confirmed sightings of this species (1995-2005) indicate that remnant populations are currently limited to the provinces of Quang Nam, Quang Ngai, Kon Tum, Binh Dinh, and Gia Lai in the Central Highlands of Vietnam (Nadler et al., 2003). The presence of one of the largest populations of grey-shanked doucs (~139 individuals) in Gai Lai Province was genetically confirmed (Ha Thang Long, 2007) thereby establishing the southern distribution of the species to latitude 14º13'N. Based upon previous studies of TCDD concentrations in river sediment, rodents, food, wildlife, and human populations from northern and southern Vietnam (Olie et al., 1989; Schecter *et al.*, 1989a; 1989b) we predicted that fTCCD levels would 1) be higher in central and southern Vietnamese subjects than in northern Vietnamese subjects, 2) vary by species, 3) be higher in non-lactating females than in males, 4) be higher in subjects residing for longer periods of time in the wild than those residing in the wild for shorter periods of time, and 5) be higher in immature subjects than in adults.

Materials and Methods

Subjects

Subjects included individuals of three species of douc langurs, *P. nemaeus* (red-shanked douc, 4 males, 6 females), *P. cinerea* (grey-shanked douc, 6 males, 5 females), and *P. nigripes* (black-shanked douc, 1 male). Douc langurs are large (7-12 kg, in Nadler et al., 2003), diurnal, arboreal and largely folivorous primates which inhabit the primary and secondary forests of Vietnam, Laos

Fig. 3. Black-shanked douc langur in Cat Tien National Park. Photo: Phan Duy Thuc

Fig. 4. Adult red-shanked douc langur male *Pygathrix nemaeus* at the EPRC. Photo: T. Nadler.

Fig. 5. Adult black-shanked douc langur male *Pygathrix nigripes* at the EPRC. Photo: T. Nadler.

Fig. 6. Adult grey-shanked douc langur male *Pygathrix cinerea* at the EPRC. Photo: T. Nadler.

PDR, and Cambodia, east of the Mekong River (Corbet & Hill, 1992; Fooden, 1996 in Nadler et al., 2003). With few exceptions, quantitative data on the social structure, day range, and behavioural ecology of douc langurs is limited (reviewed in Hoang Minh Duc, 2007). Social groups in the wild are reported to range in size from 3 to 50 individuals and typically contain multiple males and females (reviewed in Nadler et al., 2003). Results of a recent 13-month study of *P. nigripes* at Nui Chua and Phuoc Binh National Parks (Ninh Thuan Province) show that this species exhibits seasonal variation in activity budget, diet, group composition, and reproduction (reviewed in Hoang Minh Duc, 2007). *P. nigripes* here are distributed in a range of vegetation types ranging from mixed coniferous/broadleaf forests, to sclerophyll evergreen forests and semi-deciduous forests, to scrubs and thorny woodland, and occur from sea level to 1500 m in elevation (Hoang Minh Duc, 2007). Diet varies seasonally and is composed primarily of leaves, with fruit (whole fruit, pulp and seeds) and flowers being consumed less often during the wet season (Hoang Minh Duc & Baxter, 2006). Black-shanked doucs have been observed feeding on the ground, especially in the dry season, and occasionally consuming mud at small pools in the forest (Hoang Minh Duc, 2007), and populations at Hon Heo, Khan Hoa Province have been observed spending as much as ~20% of their daily time budget on the ground where they can access terrestrial water sources (Nadler, 2008). Group sizes and composition also vary by season and average 10-13 individuals (range: 1-45), and are either composed of one-male groups or mixed-sex or all male bands. Mating and births occur during the April – September hot/wet season and the October – March cold/dry seasons respectively (Hoang Minh Duc, 2007).

Little is known of the social behaviour of *Pygathrix* (but see Brockman, 1976; Kavanagh, 1972; 1973) and virtually no life history data exist on wild populations. Studies of captive *P. neameus*, however, suggest that sexual maturity in males and females is attained at ~5-8 yrs. and ~5-7 yrs., respectively (Ruempler 1998 in Nadler et al., 2003). Data from EPRC records indicate that male and female douc langurs are sexually dimorphic in body mass, *P. nemaeus* males exhibiting significantly greater body mass than females (male mean wt: 9.75 kg, n=8; female mean wt: 7.03 kg, n=6, $P = <0.001$). Likewise, male *P. cinerea* are heavier than females, but not significantly so (male mean wt: 9.63 kg, n=9; female mean wt: 7.3 kg, n=5; Brockman, unpub. data), and black-shanked males are twice as heavy a females (male wt: 10.5 kg, n=1; female mean wt: 5.7 kg, n=2). Red-shanked douc langurs also exhibit phenotypic variation in secondary sex characteristics: adult males have small tufts of white hair extending above the superior lateral edges of the triangular rump patch (Lippold & Brockman, 1974) and also have larger facial ruffs than females.

The subjects in this study ranged in age from 1.67 to 14 yrs, age estimates having been assigned by EPRC staff during quarantine based upon patterns of dental eruption and tooth wear (Nadler, unpubl. data). Subjects in this study had been confiscated through Vietnamese Forest Protection Authorities from illegal wildlife trade in various provinces in north central, south central, and southern Vietnam and had been residing in the wild from 0.17 to 10.87 yrs before being brought to EPRC (Tables 2, 3, 4). Permission to conduct this research was granted by T.N. on June 29, 2004 and University of North Carolina, Charlotte IACUC Protocol # 06-016 was approved for this research on 21 November 2006.

Assessment of biomarkers of TCDD exposure

In preparation for this research, data on the biomarkers of TCDD exposure in primates housed at EPRC were collected in December 2006. The primates at EPRC were ideal subjects for this

research because: 1) the species represented there come from all regions of Vietnam (n= 123+ individuals; 14 species/subspecies), including some areas in the south that have experienced heavy dioxin exposure; 2) five species have successfully reproduced at the facility, yielding opportunities to examine potential developmental effects of dioxin exposure; and 3) complete health records on the population, spanning seven years, have been collected by U. Streicher, the staff veterinarian of EPRC, providing endpoints (Table 5) that were examined for evidence of dioxin exposure in that primate population.

Table 2. Red-shanked douc langurs (*P. nemaeus*) from known capture locations. (4 males, 6 females).

ID	Sex	Est. Age (Yrs. as of 12/06)	Arrival Date	Resident in Wild (Yrs)	Capture Location (District, Province)	Fecal TCDD (pg/g wet weight)	Notes
*6-02	F	14	3/17/96	4	Unknown	9.0	Stunted; total agalactia; thickened eyelids, male vocal behavior; 11 handraised offspring
6-28	M	10	8/18/00	4	Da Nang	3.0	
6-34	F	4	3/23/04	2	Tam Ky, Quang Nam	5.0	
6-36	M	9	6/28/04	3.5	Hue, Thua Thien-Hue	3.0	
6-38	F	9	12/13/04	7	Phong Nha, Quang Binh	3.0	From north of DMZ
6-39	M	10	4/13/05	8.25	Ha Tinh	8.0	From north of DMZ
6-42	M	1.67	6/11/05	0.17	Minh Hoa, Quang Binh	6.0	From north of DMZ
6-45	F	1	5/31/06	0.42	Phong Nha, Quang Binh	0.0	From north of DMZ
6-46	F	5	8/17/06	4.67	Dong Giang, Quang Nam	7.0	
6-47	F	9	8/17/06	8.67	Da Nang	3.0	

* Shows possible morphological/behavioural indicators of TCDD effects.

Table 3. Grey-shanked douc langurs (*P. cinerea*) from known capture locations. (6 males, 5 females).

ID	Sex	Est. Age (Yrs. as of 12/06)	Arrival Date	Resident in Wild (Yrs)	Capture Location (District, Province)	Fecal TCDD (pg/g wet weight)	Notes
7-04	M	1994	8/4/97	3	Tam Ky, Quang Nam	5	
7-13	F	Adult	7/12/02	4.5	Ba To, Quang Ngai	10	
7-14	M	1997	8/18/02	4.5	An Lao, Binh Dinh	4	
7-16	M	Adult	12/11/02	5	An Lao	4	
7-19	M	1998	3/13/03	7	An Lao	5	
*7-25	M	2000	11/9/04	4.17	An Lao	3	Feminized (facial ruff, small biomass and testes)
7-29	F	2004	8/14/05	5	An Lao	19	
7-30	F	Adult	11/9/05	3.75	An Lao	3	
7-34	F	2000	10/19/06	5.33	Phu Cat, Binh Dinh	5	US Airbase Located here
7-35	F	Adult	11/3/06	10.87	Phu Cat	4	US Airbase Located here
7-37	M	~3	12/24/06	3	Ho Chi Minh Region ?	21	

* Shows possible morphological/behavioural indicators of TCDD effects.

Table 4. Black-shanked douc langur (*P. nigripes*) from known capture location.

ID	Sex	Est. Age (Yrs. as of 12/06)	Arrival Date	Resident in Wild (Yrs)	Capture Location (District, Province)	Fecal TCDD (pg/g wet weight)	Notes
13-05	M	1996	3/15/01	5	Nui Chua, Ninh Thuan	3	

Table 5. Biomarkers of TCDD exposure and toxicity (Rice et al., 2003).

Acute TCDD Toxicity	Chronic TCDD Toxicity	Reproductive, Developmental and Behavioural Biomarkers of TCDD Exposure
Wasting and edema; alopecia; swollen eyelids	Chloracne; thickened eyelids	Males: penis and testicular abnormalities; feminization of male traits; cryptorchidism; sperm abnormalities; decrease in anogenital distance, accessory sex organ weights and spermatogenesis; cleft penis; delayed puberty; lower birth weight; aberrant sexual behaviour (feminization of male behaviour). Females: abnormalities of ovarian cycle (irregular cycles, endometriosis, reduced ovarian weight, etc.); masculinization of female traits; decreased fertility; birth rate abnormalities (abnormally long inter-birth intervals); aberrant parental behaviour; cleft clitoris; incomplete vaginal opening; hypospadia; delayed onset of menarche. Both Sexes: cleft palate; low birth weights; delayed growth; lowered core body temperature.

Data collection

Faecal samples were collected from all individuals from known capture locations, including four *Pygathrix* from north of and 18 from south of the DMZ. Faecal samples (5-10 g) were collected immediately after voiding and placed in 2 x 3 inch Zip-loc bags which were labelled as to individual, date and time of collection, placed in an ultra-cold freezer, and later shipped frozen to CAPE Technologies, Inc. (South Portland, MA) where they were stored at -20°C until they were extracted and enzyme immunoassayed by R. O. Harrison (Director CAPE Technologies, Inc.). Potential body burden (e.g. the total amount of dioxin in the body at any given time due to storage in fat/blood/tissues) could not be determined as no data are available on levels of dioxin exposure (if any) in this population and husbandry protocols understandably prohibit collection of biological samples unless warranted for health assessments. Given the endangered status of douc langurs and substantial ethical concerns, pharmacokinetic studies assessing relationships between dioxin intake, rates of tissue/fat absorption, and excretion rates are equally prohibitive.

Validation of faecal dioxin enzyme immunoassay

Initial fTCDD validations of a novel enzyme immunoassay (EIA) method for detection of 2,3,7,8-TCDD in primate faecal samples were carried out by R. O. Harrison on two control samples obtained from *P. nemaeus* residing at the Philadelphia Zoo. Results showed that this fTCDD EIA procedure reliably detected fTCDD in feces at levels of 10 pg/g or less (Harrison, unpubl. data).

Faecal dioxin extraction and enzyme immunoassay

Solvents were HPLC grade (Fisher Scientific), except for toluene, which was residue analysis grade (Burdick & Jackson). Acids were ACS grade (Fisher Scientific). Analytical standard grade 2,3,7,8-TCDD was obtained from Ultra Scientific. All cleanup columns and immunoassay kit

materials were manufactured by CAPE Technologies. All glassware was rinsed with toluene and dried before use. All faecal assay procedures were performed at 20-25°C unless noted otherwise. Faecal sample bags were weighed to 1 mg before thawing, then again after the sample was removed to determine the weight of sample delivered to the cleanup procedure.

Samples were removed from their bags by dispersing the sample in concentrated HCl and pouring bottle for hydrolysis. Sample bags were rinsed with additional concentrated HCl to remove as much residue as possible. Total volume of HCl used was 50 ml per sample. The entire sample and rinsate were poured into a 250 ml borosilicate glass bottle with a teflon lined cap. Solvent (50 ml 3:1 hexane:dichloromethane) was added and the 2,3,7,8-TCDD spike, if any, was added at this point (spike levels were 10 pg/g). Bottles were capped and rotated end over end at 30 rpm for 12-15 hrs. Bottles were then centrifuged 15 min @ 1000 x g and the supernatant solvent was removed and passed through a column of 5 g NaHCO3. The treated solvent was then oxidized by mixing with acid silica (activated chromatographic silica with conc. H_2SO_4 adsorbed) until the solvent was clear.

The dioxin in the oxidized supernatant was captured for analysis using the CAPE Technologies coupled column cleanup system (ref AN-008). The oxidized supernatant was passed through a column of the same acid silica as used previously, then directly onto a column of activated carbon. Hexane washes of the acid silica oxidation bottle (2 x 50 ml) were added sequentially to the acid silica column to maximize sample recovery. After washing, the carbon column containing the captured dioxin was removed and attached to a clean empty reservoir. The column was washed in the forward direction with 6 ml of 1:1 hexane:toluene, then eluted in the reverse direction with 12 ml toluene (ref TN-005) and captured in a 16 x 125 mm glass tube. A keeper solution of methanol containing 20% polyethylene glycol and 100 ppm of Triton X-100 was added and the toluene was evaporated at 70-80°C under a stream of filtered dry compressed air.

The samples were reconstituted by centrifuging the evaporation tubes 5 min @ 1000 x g, then replacing the evaporated methanol. Dioxin levels in the prepared samples were then analyzed by immunoassay according to the kit insert (ref IN-DF1).

Standards in keeper and samples prepared as described above were added to sample diluent in tubes coated with anti-dioxin antibody. After incubation for 14-17 hours, the sample was removed and the tube washed 4 times with 1 ml of distilled water plus 0.01% Tween 20 detergent. Enzyme conjugate was added and tubes were incubated 15 min., and then washed with distilled water. Enzyme substrate was added and colour was allowed to develop for 30 min. Stop solution containing 1 ml NHCl was added and the optical density (OD) of each tube was read at 450 nm using a portable differential photometer (Artel).

OD values were used to construct a standard curve of dioxin concentration. A non-linear least squares method based on a 4 parameter logistic equation was used for curve fitting (CAPE Technologies Calculation Module C). The individual sample OD values were converted to raw concentration values (as pg per EIA tube), then divided by the sample size to obtain dioxin concentration in the original sample.

Statistical tests and assessments of fTCDD-variable interactions

Statistical analyses were performed using SIGMASTAT 3.5 (Systat Software, Inc. Point Richmond, CA). Multivariate and univariate statistical tests were used to identify which variable(s) best predicted variation in fTCDD concentrations. The effects of geographic location (north vs. south of the DMZ), species, residence time (time spent in the wild), sex and age on fTCDD were

tested using multivariate (multiple linear regression) and univariate (Student's t-test, Mann-Whitney U test) analyses. Variables with departures from normality were adjusted by removal of outliers (samples more than two standard deviations from the mean) or were long transformed. Results are reported as means ± SD with significance set at $P \leq 0.05$.

Results

Assessment of biomarkers of TCDD exposure

An examination of the health and necropsy records of 130 wild-caught Vietnamese primates in December 2006 showed that two individuals, a female *P. nemaeus* (6-02) and a male *P. cinerea* (7-25), appeared to exhibit developmental consequences of possible TCDD exposure (Tables 2, 3). Female 6-02 ("Lola", Fig. 7, 8, 9) was extremely small (i.e. stunted; ¾ the size/wt of average adult females: 5.2 kg vs. female mean 7.03 kg), had thickened eyelids, total agalactia (the absence of mammary glands; Kloeden & Streicher, 2000), and exhibited male vocal behaviour (Brockman, pers. observ.). Male 7-25 ("Sung", Fig. 10) appeared to be morphologically and behaviourally feminized as shown by the absence of the typical male facial ruff, small body size, diminutive testes, and female vocal behaviour (Brockman, pers. observ.).

Extraction and enzyme immunoassay

Quality assurance samples were included with each batch to monitor performance of the analytical method. Split method blanks and faecal samples were spiked with 100 pg of 2,3,7,8-TCDD, added as 10 µL of toluene solution. Differences between spiked/unspiked pairs were evaluated to determine spike recovery and to demonstrate the ability of the analytical method to detect low levels of dioxin in faecal samples. This evaluation was performed using raw concentration data (pg/EIA tube), before the introduction of sample mass into the calculation chain (Tables 6, 7).

Nominal spike recovery for undiluted samples was 80 pg/EIA tube and for diluted samples was 16 pg/EIA tube (1/5 dilution) because only 80% of the final prepared sample volume is recovered in the EIA procedure. Percent recovery values were calculated for each of 11 QA sample pairs. The overall (n = 11) mean recovery was 39%, while for 4 method blank pairs the mean was 34%, and for 7 sample pairs the mean was 42%. While this value is lower than typical for instrumental methods of dioxin analysis, it shows clearly that low pg/g levels of dioxin can be recovered from faecal samples and measured by EIA.

It should be noted that spike recovery can be and often is used to correct the values determined for unspiked unknowns. This was not done here because the application of a uniform correction factor would not change the results of the statistical analysis that was performed on the results. The maximum expected change in absolute concentration would be approximately threefold, based on mean spike recovery values. The most accurate absolute quantification would require additional optimization of the sample preparation method and confirmation by the reference method for dioxin, based on high resolution mass spectrometry.

Faecal TCDD concentrations

Results of EIA analysis showed that TCDD levels averaged 6.05 pg/g (wet wt) ± 5.1 SD and ranged from 0.0 – 21.00 pg/g (n=22 samples). Total dioxin equivalency (TEQ) value expresses the level of toxicity as if the mixture was pure TCDD (Fries, 1995). TEQ (pg/tube TEQ at $1/5^{th}$ dilution) values in this study averaged 3.62 pg/tube ± 1.86 SD (range: 1.0 – 9.0 pg/tube TEQ).

Fig. 7. Adult red-shanked douc langur female *Pygathrix nemaeus* "Lola" (6-02; left) and a "normal" adult female *Pygathrix nemaeus* (right) to compare body size and tail length. Photo: T. Nadler.

Fig. 8. Adult red-shanked douc langur female *Pygathrix nemaeus* "Lola" (6-02; right) and adult male "Macho" (6-06) to compare body size. Photo: T. Nadler.

Fig. 9. Adult grey-shanked douc langur male *Pygathrix cinerea* "Sung" (7-25). Photo: D.K. Brockman.

Fig. 10. Adult red-shanked douc langur female *Pygathrix nemaeus* "Lola" (6-02; right) and adult male "Macho" (6-06). Photo: D.K. Brockman.

Table 6. Summary of fTCDD QA results. CAPE Technologies EPA Method 4025m (pg/tube data [yellow] shown only if used in final summary [green]).[1] MB-: Method blank unspiked; MB+: Method blank spiked. [2] "-" Sample unspiked; "+": Sample spiked (see Results, Extraction and Enzyme Immunoassay).

Batch	Sample ID	Undiluted %NC	Undiluted pg/tube TEQ	1/5 Dilution %NC	1/5 Dilution pg/tube TEQ	Pair Pg/tube diff	% Spike Recovery
2	MB-[1]			93	2		
	MB+			81	6	2	22
	6-38-[2]			97	1		
	6-38+			81	6	5	29
	6-39-			84	5		
	6-39+			80	6	1	8
3	MB-			84	4		
	MB+			67	11	7	43
	6-02-			80	5		
	6-02+			67	11	6	36
	7-14-			92	2		
	7-14+			68	11	8	53
4a	MB-	71	11				
	MB+	55	27			15	19
	6-34-	70	12				
	6-34+	31	#NUM!			#NUM!	100 set = to 100
	6-46-	62	17				
	6-46+	46	51			33	42
4b	MB-	55	17				
	MB+	39	59			42	53
	6-47-			90	2		
	6-47+			73	7	4	28

Overall Stats
Mean
39
SD
25
%CV
63
N
11

MB Only
Mean
34
SD
16
%CV
48
N
4

Samples Only
Mean
42
SD
29
%CV
69

Faecal TCDD, geographic location, species, residence time, sex and age

Multiple linear regression analysis showed that contrary to expectations, none of the independent variables predicted fTCDD levels in this population. FTCDD levels were not related to geographic location, species, residence time, or sex. Results of t-tests and Mann-Whitney U tests showed that fTCDD levels varied little in subjects regardless of whether they originated in north or central/southern Vietnam, whether they derived from different species, how long they resided in the wild before being brought to the EPRC, or whether they were males or females (Table 8). Results of Pearson Product Moment Correlation tests showed, however, a significant negative correction between fTCDD levels and age ($r = -0.50$, $P = 0.03$, $n = 21$). Results of Mann-Whitney Rank U tests indicated that fTCCD levels were significantly higher in immatures than in adults south of the 17th parallel ($P = 0.025$, Table 8). Mean fTCCD levels in *P. cinerea* subjects 2- to 3-years of age (female 7-29; male 7-37, Table 3) obtained from Binh Dinh and Ho Chi Minh Provinces were three-fold higher than they were in exposed adults from central and southern Vietnam. Although EPRC staff took legal possession of male 7-37 in the region of Ho Chi Minh City, he was likely transported there from an unknown locality in the Central Highlands as this species does not occur south of latitude 14°13'N (Ha Thang Long, 2007).

Table 7. Summary of fTCDD QA results (continued). CAPE Technologies EPA Method 4025m.

		undiluted		1/5 dilution		1/25 dilution							calc pg/g				
	sample ID	%NC	pg/tube TEQ	%NC	pg/tube TEQ	%NC	pg/tube TEQ	gross	tare	net	g Into cleanup	g/EIA tube	undil	1/5 dil	1/25 dil	sample ID	pg/g
batch 2	MB-	31	#NUM!	93	2											MB-	
	MB+	30	#NUM!	81	6											MB+	
	6-38-	65	33	97	1			9.046	3.108	5.9	3.0	2.4	14	3	0	6-38-	3
	6-38+	29	#NUM!	81	6			9.046	3.108	5.9	3.0	2.4	#NUM!	13	0	6-38+	13
	6-39-	24	#NUM!	84	5			10.977	3.092	7.9	3.9	3.2	#NUM!	8	0	6-39-	8
	6-39+	34	#NUM!	80	6			10.977	3.092	7.9	3.9	3.2	#NUM!	10	0	6-39+	10
	6-42	51	#NUM!	89	4			6.800	3.080	3.7	3.7	3.0	#NUM!	6	0	6-42	6
	6-45	29	#NUM!	109	#NUM!			5.114	3.149	2.0	2.0	1.6	#NUM!	#NUM!	0	6-45	0
	13-05	31	#NUM!	89	4			10.213	3.126	7.1	7.1	5.7	#NUM!	3	0	13-05	3
batch 3	MB-	36	170	84	4	99	0									MB-	
	MB+	35	244	67	11	93	2									MB+	
	6-02-	51	28	80	5	95	1	10.230	3.104	7.1	3.6	2.9	10	9	12	6-02-	9
	6-02+	23	#NUM!	67	11	101	#NUM!	10.230	3.104	7.1	3.6	2.9	#NUM!	19	#NUM!	6-02+	19
	7-14-	61	17	92	2	100	0	10.480	3.055	7.4	3.7	3.0	6	4	2	7-14-	4
	7-14+	31	#NUM!	68	11	85	4	10.480	3.055	7.4	3.7	3.0	#NUM!	18	32	7-14+	18
	6-28	46	39	90	2	92	2	7.468	3.007	4.5	4.5	3.6	11	3	15	6-28	3
	7-04	61	17	86	3	97	1	7.640	3.046	4.6	4.6	3.7	5	5	6	7-04	5
	7-13	20	#NUM!	78	5	90	2	6.730	3.045	3.7	3.7	2.9	#NUM!	10	21	7-13	10
	7-16	33	#NUM!	86	4	98	1	8.674	3.009	5.7	5.7	4.5	#NUM!	4	4	7-16	4
	7-19	30	#NUM!	81	5	95	1	8.918	3.059	5.9	5.9	4.7	#NUM!	5	7	7-19	5

	undiluted		1/5 dilution		1/25 dilution							calc pg/g				
	sample ID	%NC	pg/tube TEQ	%NC	pg/tube TEQ	%NC	pg/tube TEQ	gross	tare	net	g Into cleanup	g/EIA tube	undil	1/5 dil	1/25 dil	sample ID	pg/g
batch 4a	MB-	71	11													MB-	
	MB+	55	27													MB+	
	6-34-	70	12					8.958	3.088	5.9	2.9	2.3	5	0	0	6-34-	5
	6-34+	31	#NUM!					8.958	3.088	5.9	2.9	2.3	#NUM!	0	0	6-34+	>25
	6-46-	62	17					9.196	3.024	6.2	3.1	2.5	7	0	0	6-46-	7
	6-46+	46	51					9.196	3.024	6.2	3.1	2.5	21	0	0	6-46+	21
	7-29	52	31					5.138	3.045	2.1	2.1	1.7	19	0	0	7-29	19
	7-35	69	12					7.158	3.061	4.1	4.1	3.3	4	0	0	7-35	4
	7-37	55	26					4.653	3.102	1.6	1.6	1.2	21	0	0	7-37	21
batch 4b	MB-	55	17	91	2	119	#NUM!									MB-	
	MB+	39	59	102	#NUM!	92	2									MB+	
	6-36-	66	9	87	3	94	1	14.254	2.982	11.3	5.6	4.5	2	3	8	6-36-	3
	6-36+							14.254	2.982	11.3	5.6	4.5				6-36+	NA
	6-47-	70	8	90	2	97	1	13.483	3.036	10.4	5.2	4.2	2	3	5	6-47-	3
	6-47+	40	50	73	7	93	2	13.483	3.036	10.4	5.2	4.2	12	8	11	6-47+	8
	7-25	66	10	87	3	93	2	8.623	3.103	5.5	5.5	4.4	2	3	9	7-25	3
	7-30	73	7	88	3	93	2	9.345	3.106	6.2	6.2	5.0	1	3	8	7-30	3
	7-34	52	20	68	9	82	4	7.272	3.053	4.2	4.2	3.4	6	13	31	7-34	13

Table 8. Faecal TCDD levels and associated variables.

Variable	Mean fTCDD ± SD (pg/g wet weight)	fTCDD Range (pg/g wet weight)	Numbers of Subjects	P Value
Geographic Location				
North of DMZ	4.25 ± 3.50	8.0 – 0.0	4	0.63
South of DMZ	6.44 ± 5.35	21.00 – 3.00	18	
Species				
P. nemaeus	5.22 ± 2.39	9.0 – 3.0	10	0.38
P. cinerea	7.55 ± 6.46	21.0 – 3.0	11	
P. nigripes	3.00	0.0	1	
Sex/Species				
P. nemaeus				
Males	5.0 ± 2.45	8.0 – 3.0	4	0.80
Females	4.5 ± 3.21	9.00 – 0.00	6	
P. cinerea				
Males	7.0 ± 6.90	21.0 – 3.0	6	0.79
Females	8.2 ± 6.61	19.0 – 3.0	5	
Pooled				
Males	6.2 ± 5.43	21.0 – 3.0	10	1.00
Females	6.18 ± 5.13	19.0 – 0.0	11	
Residence (wild)				
0-4 yrs	7.0 ± 6.81	21.0 – 0.00	11	1.00
>4 yrs	5.1 ± 2.30	10.0 – 3.0	11	
Age*				
Immature (< 3yrs)	20.0 ± 1.41	21.0 – 19.0	2	P=0.025
Adult	4.75 ± 2.18	10.0 – 3.0	16	

* Subjects from south of the 17th parallel (i.e. DMZ).

Discussion

This study demonstrates the utility of faecal dioxin (fTCDD) enzyme immunoassays for non-invasively quantifying dioxin levels in members of the genus *Pygathrix*. Although the population of douc langurs in this study exhibited relatively low levels of fTCDD, averaging ~6 pg/g (mean: 3.62 pg/TEQ), three individuals exhibited higher faecal concentrations of TCDD, ranging from 10 - 21 pg/g fTCDD. The biological significance of these results, if any, vis-à-vis the health and well-being of this population are unknown; however these values can be interpreted to reflect possible background concentrations of environmental TCDD and/or exposure pathways associated with a primarily folivorous diet and arboreal lifestyle, the latter limiting dermal exposure to dioxin in soils. Background concentrations of fTCDD have not been determined for any primate, including humans, in Vietnam, but pooled blood concentrations of TCDD in the general Vietnamese population is reported to be 2.2 ng/g TCDD (13 ng/g TEQ; Schecter et al., 2006). The specific source of dioxin exposure in the members of this douc population from central/southern Vietnam is also currently unknown, but likely sources are potentially contaminated food (e.g. leaves, fruits, flowers) and perhaps dirt/mud, that obligate folivores such as *Pygathrix* consume.

Known pathways of dioxin/dioxin-like compounds into vertebrates include 1) deposition of dioxin vapours and particles on plants and consumption of plants by animals; 2) deposition of

dioxin on soils, subsequent transfer from soils to plants via root uptake and translocation or volatization (i.e. transformation of liquid dioxin to a gas/vapour) and consumption of plants by animals; and 3) ingestion of contaminated soil by animals (Fries, 1995). Previous studies indicate that soil-to-plant transport of lipophlic compounds such as dioxin occur primarily via volatization from the soil surface/disturbed soils and subsequent atmospheric deposition on plants (Fries, 1995) and are thus the likely source of exposure in this population of douc langurs. Previous research on TCDD concentrations in soils and sediments in Vietnam have led Hatfield Consultants & Committee 10-80 (2006) to conclude that TCDD soil contamination is insignificant other than in designated dioxin hotspots and environs, such as former US military airbases where herbicides were stored, dispensed, and spilled (e.g. A So in Aluoi Valley, Thua Thien Hue Province; Bien Hoa, Dong Nai Province), but no data are available on TCDD concentrations in soils from primate habitats in close proximity to US military airbases and known to have been chemically sprayed with TCDD.

Inter-individual patterning of fTCDD excretion was variable, ranging from 0.0 – 21 pg/g, and appeared unrelated to geographic location, species, residence time, or sex (Table 8). However, mean fTCDD concentrations in two very young, and apparently healthy, grey-shanked doucs were three-fold higher than those observed in adults from central and southern Vietnam, indicating the apparent absence of a relationship between exposure (i.e. dose) and effect (i.e. response) and consequently the absence of an obvious clinical or biological effect. Nevertheless, these results suggest that immature individuals potentially have higher dioxin body burdens than adults (depending upon the length of dioxin exposure) and therefore may be at increased risk for functional reproductive and developmental defects, including possibly agalactia in females. *P. nemaeus* "Lola" was diagnosed with total agalactia for reasons that are unknown, but one possibility is that she was exposed *in utero* to increased levels of environmental TCDD. Impairment of mammary gland differentiation is known to occur in female offspring of pregnant Holtsman rats exposed to 2,3,7,8-T on gestation day 15 (Lewis et al., 2001) and similar mammary gland abnormalities (and stunting) have been reported for female Long-Evans rats whose dams were exposed *in utero* and lactationally to TCDD (Fenton et al., 2002). Other than agalactia, Lola has exhibited no other apparent reproductive abnormalities, and has produced ~11 offspring (as of 31 December 2007; Nadler, 2008) since arriving at EPRC in 1996, including three daughters, 6-21, 6-31, 6-32 who appears to be developmentally and reproductively normal. Red-shanked female 6-21 has produced two female offspring in May 2005 (6-41) and 2007 (6-51), one of which survives (6-41), female 6-31 one in March 2009 (6-62), and female 6-32 one in March 2008 (6-58).

Although the mean level of fTCDD in this population was fairly low (e.g. ~6 ng/g), the 3-fold elevations observed in the immature grey-shanked doucs, female 7-29 and male 7-37, are of potential concern (Table 3), and warrant further investigation into the length and possible source of dioxin exposure. Confiscation location data for male 7-37 provide no insights into his geographic origins, but female 7-29 was confiscated within the known distribution for this species, EPRC records indicating that she originated in An Loa District, Binh Dinh Province (Nadler, unpubl. data). Grey-shanked doucs are known to occur in small fragments of unprotected secondary forests here and are heavily hunted by local farmers (Nadler *et al.*, 2003). Between September 1965 and June 1970, 289 US military missions were flown in Binh Dinh Province, including the An Loa District, spraying about 2 million litres (553,311 gallons) of Agent Orange; 76% of these missions were flown for the express purpose of defoliating forests and destroying crops (calculated from HERPS Tape, 2000). The levels of potential residual TCDD concentrations remaining in soils here are unknown.

Conclusions and Significance

The results of this research indicate that the non-invasive fTCDD enzyme immunoassay developed here has great utility for quantifying relative TCDD concentrations in wild douc langurs residing in regions of central and southern Vietnam previously exposed to high levels of dioxin and dioxin-like chemicals during the Vietnam War. Inter-individual patterning of fTCDD excretion in this study was variable, ranging from 0.0 – 21 pg/g, and was found to be unrelated to geographic location, species, residence time, or sex. However, significantly higher levels of fTCDD were detected in a small sample of very young grey-shanked doucs than were observed in adult *Pygathrix* from south of the 17th parallel, although the specific source of TCDD exposure in these subjects is unknown. Future studies will focus on elucidating fTCDD concentrations in a larger sample of immature doucs at EPRC to further document the effect (or not) of age on fTCDD levels in the genus *Pygathrix*, and identifying potential field sites in close proximity to "dioxin hotspots" (e.g. Cat Tien National Park, ~60 km northeast of the Ben Hoa "dioxin hotspot") for a broader investigation of fTCDD in free-ranging populations of studies of *P. nigripes*.

The importance of this research derives from the potential insights gained regarding the health and reproductive effects of environmental pollutants on wildlife populations (Colborn & Clements, 1992). Concordance of wildlife observations with the effects observed in animal experiments strongly suggests that endocrine disruptors are responsible for significant impairments in wildlife health. TCDD is of particular concern due to the low doses required for action and its ability to bioaccumulate in higher vertebrates (Portier et al., 1999). Moreover, a significant portion of the body burden in mammals is transferred to infants in breast milk, resulting in extremely high exposures for infants (Pantadin et al., 1999) and the potential for trans-generational exposures. As noted above, soil half-life estimates have been reported to range from 10 to 100 years, depending upon the depth of TCDD contamination (Nauman & Schaum, 1987), suggesting that present vertebrate populations continue to experience potentially high levels of exposure. Biomarkers of individual health and chemical exposure provide early warnings of population-level insults that can threaten the survival of wildlife (Fox, 2001). Moreover, because the effects of TDCC in wildlife parallel those observed in humans, wildlife populations can act as crucial sentinels for potential impairments in human populations worldwide (Fox, 2001).

The anticipated conservation implications of this research reside in identifying, in close collaboration with Vietnamese colleagues, endangered populations and habitats for increased protection and possible remediation through the establishment of "Species Conservation Areas" (Nadler & Streicher, 2004). This cutting edge technology has the potential to broaden our understanding of the effects of TCDD exposure in endangered primates and ecosystems, adding to our arsenal of compelling arguments for increased *effective* protection of Vietnam's biodiversity.

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Variation in fecal glucocorticoid concentrations in captive red-shanked douc langurs (*Pygathrix nemaeus*)

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Key words: douc langur, glucocorticoid, captivity

Summary

The goal of the current study was to gather baseline glucocorticoid data from red-shanked douc langurs (*Pygathrix nemaeus*) housed at the Endangered Primate Rescue Center (EPRC), Cuc Phuong National Park, Vietnam. We quantified fecal glucocorticoid concentrations in both males and females, and examined variation in levels in relationship to environmental variables (temperature, weather, housing condition). Samples were collected from four animals, two male and two female, over a three-month period. The results of this study suggest significant inter-individual differences in glucocorticoid levels, and while patterns of fecal glucocorticoids among individual animals showed varying degrees of fluctuation, the significance or underlying cause of these patterns remains unclear.

Thay đổi hàm lượng glucocorticoid trong phân của Chà vá chân nâu (*Pygathrix nemaeus*) trong điều kiện nuôi nốt

Tóm tắt

Mục đích của nghiên cứu này nhằm thu nhận các dữ liệu cơ bản về glucocorticoid ở loài chà vá chân nâu (*Pygathrix nemaeus*) được nuôi tại Trung tâm cứu hộ Linh trưởng Cúc Phương, Việt Nam. Chúng tôi đã tiến hành đo hàm lượng glucocorticoid trong phân của cả hai giới tính và kiểm tra mức độ thay đổi trong mối tương quan với các thông số về môi trường (nhiệt độ, thời tiết, điều kiện chuồng trại). Mẫu phân tích được thu từ 4 cá thể, hai đực và hai cái trong khoảng thời gian 3 tháng. Kết quả nghiên cứu cho thấy có những sự khác biệt có ý nghĩa về mức độ glucocorticoid giữa các cá thể, và trong khi lượng glucocorticoid trong phân thay đổi khác nhau giữa các cá thể thì nguyên nhân của những khác biệt này vẫn còn chưa rõ.

Introduction

Primates living in captive conditions often exhibit signs of stress, including stereotypic movement, hair-plucking, huddling, pacing, and rocking (Boinski et al., 1999). The physiological conditions underlying these responses are complex and are not fully understood, but they are

known to involve increases in the amounts of glucocorticoid hormones, including cortisol and corticosterone (Axelrod & Reisman, 1984). Increased levels of these hormones can have both short-term benefits, and long-term costs. In the short-term, increases in these hormones prepare the body for the 'fight or flight' response, by increasing oxygen intake, and increasing immediate availability of energy. In the long-term, prolonged high glucocorticoid levels results in the pathological effects of stress (Sapolsky, 1994). While not all primates living in captivity show outward signs of stress, sustained elevated glucocorticoid concentrations may nevertheless result in detrimental physiological effects.

These issues are important for the management of captive animals in general, and of primates in particular. Past studies have successfully monitored the concentrations of glucocorticoids to assess relative stress levels in captive animal populations with regard to enclosure characteristics, husbandry techniques, and stimuli from other animals, and human interaction (e.g., Davis et al., 2005; Wielebnowski et al., 2002). The results of these studies have been used to recommend changes in the captive habitats of these animals as well as exhibition practices, usually with success. Lowering pathologically high and sustained glucocorticoid levels increases immune function and reproductive capacity, both important factors in the conservation and management of endangered species.

The goal of the current study was to gather baseline glucocorticoid data from red-shanked douc langurs (*Pygathrix nemaeus*) housed at the Endangered Primate Rescue Center (EPRC), Cuc Phuong, Vietnam. By gathering data on glucocorticoids from this population, future comparisons can be made with conspecific individuals housed at zoological institutions in the United States – where it has proven challenging to maintain this species (Edwards & Killmar, 2004).

Red-shanked douc langurs (*Pygathrix nemaeus*) are Old World monkeys that are found only in Vietnam and Laos, and occupy primary and secondary forest habitats, at both medium and high altitudes (Fooden, 1996). They are diurnal primates, and spend at least 50% of their waking hours feeding on a variety of leaves (Pham Nhat et al., 1994). They live in multimale-multifemale groups of variable size (from 3-50 individuals), from which both males and females emigrate. With a life span of up to 30 years, their life history is divided into infant (birth-24 months), juvenile (24 months to 5 years), and adult (post-sexual maturity: 4-6 years in females, 4-9 years in males) phases (Ruempler, 1998). Unfortunately, these animals are endangered. One of the main reasons for their declining population numbers is habitat destruction, with hunting and the lasting effects of environmental disruption by the military during the Vietnam war also playing a role (Lippold, 1995).

Contributing to the difficulty in conserving this species are the problems associated with successful captive maintenance. Due to their highly specialized dietary needs and habitat requirements, zoological institutions have encountered serious challenges (Ensley et al., 1982). For example, of 28 offspring born at the San Diego Zoo, 8 died between the ages of 1.5-3.5 years (Lippold, 1989). Currently, there are only a low number captive individuals alive in zoological institutions worldwide. Only four are found in the United States (at the San Diego Zoo and the Philadelphia Zoo). However, there is a relatively substantial population housed at the EPRC. This rescue and rehabilitation center is home to over 150 animals from 16 taxa, including 29 red-shanked douc langurs. In contrast to the situation faced by the zoological institutions in the US, the population at the EPRC is thriving and reproducing.

A number of factors may contribute to the differences between the Vietnam and US groups, including ambient temperature, humidity, shade cover, and plant species available for consumption. It is possible that these factors, as well as other aspects of the captive environment, such as

enclosure size, social groupings, and exposure to sights/sounds of other species (including humans), may play a role, through the physiological effects of stress, in the differential survivorship between these captive red-shanked douc langur populations. Monitoring of glucocorticoid concentrations (e.g., cortisol, corticosterone) can be used to gauge physiological stress, by detecting fluctuations in adrenal activity. The fact that glucocorticoids are secreted in a pulsatile manner in many mammals (Wasser et al., 2000), coupled with the confounding effects of darting an animal (in order to obtain blood samples) on the hypothalamic-pituitary axis, argues for the use of noninvasive sampling and measurement of glucocorticoid metabolites in studies that wish to explore the relationship between hormones and stress (Whitten, 1998; Wielebnowski et al., 2002).

One previous study measured fecal glucocorticoids in red-shanked douc langurs, in order to assess the physiological consequences of social changes on ovarian function in captive females (Heistermann et al., 2004). These researchers noted elevations in cortisol metabolites associated with changes in housing conditions and group compositions, some significant with much variation across different groups. That study did not employ an ACTH challenge and instead used the stress of anaesthesia associated with a dental operation for validation of their assay. The current study examines glucocorticoid metabolite excretion in relationship to differing social conditions, and employs an ACTH challenge in the development and validation of the cortisol assay.

Materials

Study animals

The animals at the EPRC are housed in outdoor enclosures constructed of wire mesh. The size of the cages is 10m x 5,5m x 3,5m. The cages are furnished with bamboo poles and horizontal bamboo construction. Animals are housed in mixed- and same-species groups, same- and mixed-sex groups. The animals included in this study were housed in one of two housing conditions: 1) same-sex, mixed-species and 2) mixed-sex, same-species. Fecal samples were collected from four individuals occupying two different enclosures (Table 1).

Table 1. Animals sampled in this study.

Individual	Sex	ID	Age (y)	Enclosure	# of fecal samples
Bruno	Male	#14	11	13A – Housed with two adult Hatinh langurs	45
Binh	Male	#28	11	5A – Housed with Jarra and Halffeet	59
Jarra	Female	#31	5	5A	59
Halffeet	Female	#46	5	5A	49

Sample collection

Sample collection began March 11, 2007. Daily fecal samples were collected between 9:00 and 11:30 a.m., in order to control for differences in diurnal fluctuation in hormone levels. At the time of collection, information about the animals' activities was recorded on data sheets (e.g., resting, grooming, feeding, sleeping), along with information about weather conditions (e.g., temperature, precipitation, amount of sunshine). Approximately 0.5 g of fecal material was transferred from the enclosure floor to a

container marked with the animals' name and date. Samples were frozen at -20ºC until shipment to the United States. Samples were shipped frozen, by air courier and arrived in good condition.

Methods

ACTH challenge

Adrenocorticotropic hormone (ACTH) is the pituitary peptide hormone that regulates glucocorticoid release from the adrenal cortex. In order to show that the glucocorticoid metabolites measured in fecal samples are a reliable indicator of physiological stress, the "ACTH challenge" is used. This quantifies the relationship between behavioral or environmental variables and stress hormones. Fecal and urine samples were collected starting one month prior to the challenge in order to determine baseline hormone concentrations. Then, ~2 IU/kg ACTH (Synacthen Depot, 100 IU/ml, Novartis Pharma SA, Vilvoorde, Belgium) was administered via blowpipe (Table 2). The ACTH challenge was performed on April 16 for all study animals. Fecal and urine samples continued to be collected for two months following the challenge.

Table 2. ACTH administration for each individual.

Individual	Body weigh	Time of injection	Amount of ACTH administered
Bruno	12 kg	8:00 a.m.	20 IU
Binh	12 kg	10:00 a.m.	20 IU
Jarra	5 kg	9:35 a.m.	10 IU
Halffeet	6.5 kg	9:35 a.m.	12 IU

Extraction of steroids from feces

Frozen faecal samples were lyophilized, pulverized using a rubber mallet and processed as described by Young et al. (2004), except that a shaking extraction method was used instead of boiling. Briefly, add 0.5 ml distilled water and 4.5 ml ethanol to ~0.1 g well-mixed dried feces, cap tightly and place on a multi-tube vortexer and vortex for 30 min. After centrifugation (500 g, 20 min), the supernatant was transferred into a glass tube and the pellet resuspended in an additional 5 ml 90% ethanol, vortexed for 1 min and recentrifuged for 20 min at 500 g. Combined ethanol supernatants were dried under air and resuspended in 1 ml 100% methanol. Methanol extractants were vortexed (1 min), sonicated (15 min) and revortexed (30 sec) prior to decanting into a plastic tube for storage at -20°C until assayed. The efficiency of steroid extraction from feces of each species was evaluated by adding ^{3}H-cortisol (~4,000 dpm) to faecal samples before extraction. Mean extraction efficiency was 90.3 ± 0.7%.

Faecal and urinary glucocorticoid metabolite analyses

Cortisol enzyme immunoassay

A cortisol EIA was used to analyze extracted feces by a modification of methods (Young et al., 2004) developed by Munro & Lasely (1988). The assay employed a cortisol-horseradish peroxidase ligand and antiserum (No. R4866; C.J. Munro, University of California, Davis, CA) and cortisol standards (hydrocortisone; Sigma-Aldrich Inc., St. Louis, MO). The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin and cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5% and <1% with

corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone and 11-desoxycortisol (C.J. Munro, pers. comm.). Faecal extracts were evaporated to dryness and diluted 1:16-1:50 in steroid buffer (0.1 M $NaPO_4$, 0.149 M NaCl, pH 7.0). All samples were assayed in duplicate. The EIA was performed in 96-well microtiter plates (Nunc-Immuno™, Maxisorp™ Surface; Fisher Scientific, Pittsburgh, PA) coated 14-18 h previously with cortisol antiserum (50 µl per well; diluted 1:20,000 in coating buffer; 0.05 M $NaHCO_3$, pH 9.6). Cortisol standards (50 µl, range 3.9-1000 pg/well, diluted in assay buffer, 0.1 M NaPO4, 0.149 M NaCl, 0.1% bovine serum albumin, pH 7.0) and sample (50 µl) were combined with cortisol-horseradish peroxidase (50 µl, 1:8,500 dilution in assay buffer). Following incubation at room temperature for 1 h, plates were washed five times before 100 µl substrate buffer [0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mM H2O2, 0.05 M citrate, pH 4.0] was added to each well. After incubation for 10-15 min, the absorbance was measured at 405 nm. Parallel displacement curves were obtained by comparing serial dilutions of pooled fecal extracts (1:8 - 1:256) with the cortisol standard preparation. Intra- and interassay coefficients of variation were <10% and 15%, respectively. Assay sensitivity was 3.9 pg/well at 90% binding. Glucocorticoid metabolite concentrations are expressed as nanograms per gram dry fecal weight (ng/g).

Corticosterone radioimmunassay

Faecal extracts were also analyzed using a double-antibody 125I corticosterone RIA (MP Biomedicals, Costa Mesa, CA) shown effective in quantifying faecal glucocorticoids in diverse species (Wasser et al., 2000; Young et al., 2004). The polyclonal antiserum was raised in rabbits against corticosterone-3-carboxymethyloxime coupled to bovine serum albumin and cross-reacts with corticosterone 100%, desoxycorticosterone 0.34%, testosterone 0.1%, cortisol 0.05%, aldosterone 0.03%, progesterone 0.02%, androstenedione 0.01%, 5Δ-dihydrotestosterone 0.01% and <0.01% with all other steroids tested (manufacturer's data). Sensitivity of the assay at 90% binding was 12.5 ng/ml. There was no parallelism between serial dilutions of fecal extracts (neat - 1:64) and the corticosterone standard preparation. All dilutions bound at 60 - 80%.

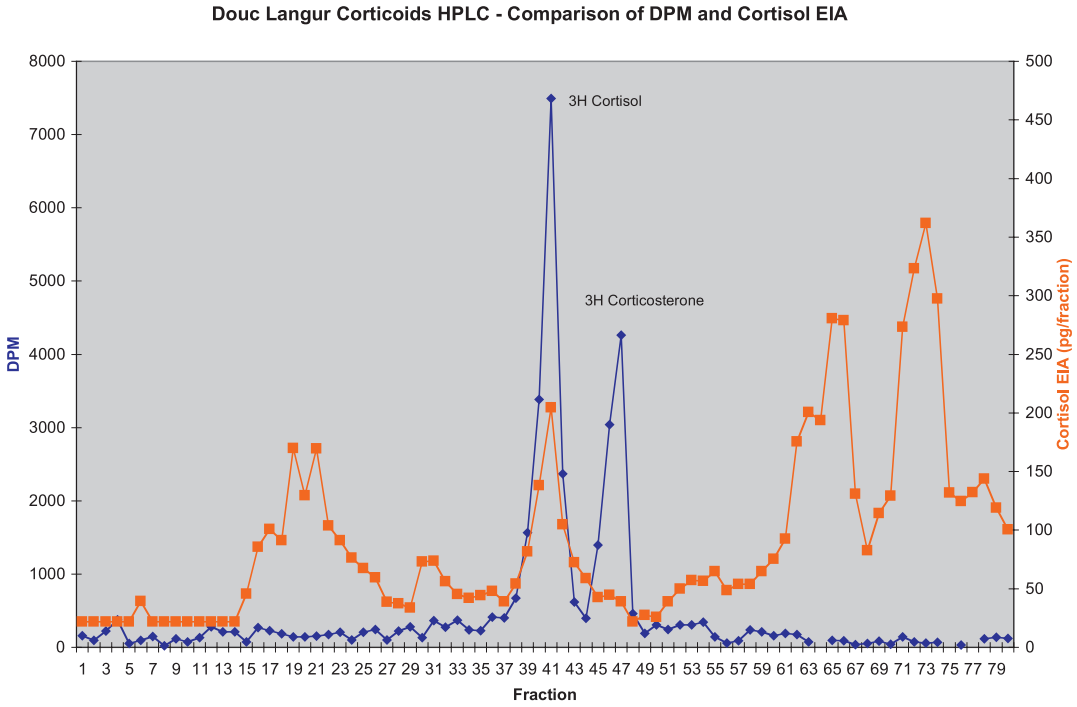
High-performance liquid chromatography (HPLC)

The number and relative proportions of immunoreactive glucocorticoid metabolites in feces were determined by reverse-phase HPLC as previously described (Young et al., 2004). Six extracts were from post-ACTH faecal samples were pooled, evaporated to dryness and reconstituted in 0.5 ml phosphate-buffered saline (0.01 M NaPO4, 0.14 M NaCl, 0.5% bovine serum albumin, pH 5.0) before loading the total volume on a pre-conditioned C-18 matrix cartridge (Spice™ Cartridge; Analtech Inc., Newark, DE). The cartridge was washed with 5 ml distilled water and the total steroids eluted with 5 ml 100% methanol, evaporated to dryness, then reconstituted in 300 µl 100% methanol containing 3H-cortisol and 3H-corticosterone (~4,000-8,000 dpm for each radiolabeled glucocorticoid). Filtered faecal extracts (55 µl) were separated on a Microsorb C-18 column (Reverse Phase Microsorb™ MV 100 C18, 5 µm diameter particle size; Varian Inc., Woburn, MA) using a linear gradient of 20-100% methanol in water over 80 min (1 ml/min flow rate, 1 ml fractions). A subsample of each fraction (100 µl) was assayed for radioactivity to determine the retention times for the radiolabeled reference tracers. The remainder of each fraction (900 µl) was evaporated to dryness, reconstituted in 125 µl l steroid buffer and an aliquot (50 µl) assayed singly in the cortisol EIA and corticosterone RIA as described above.

Results

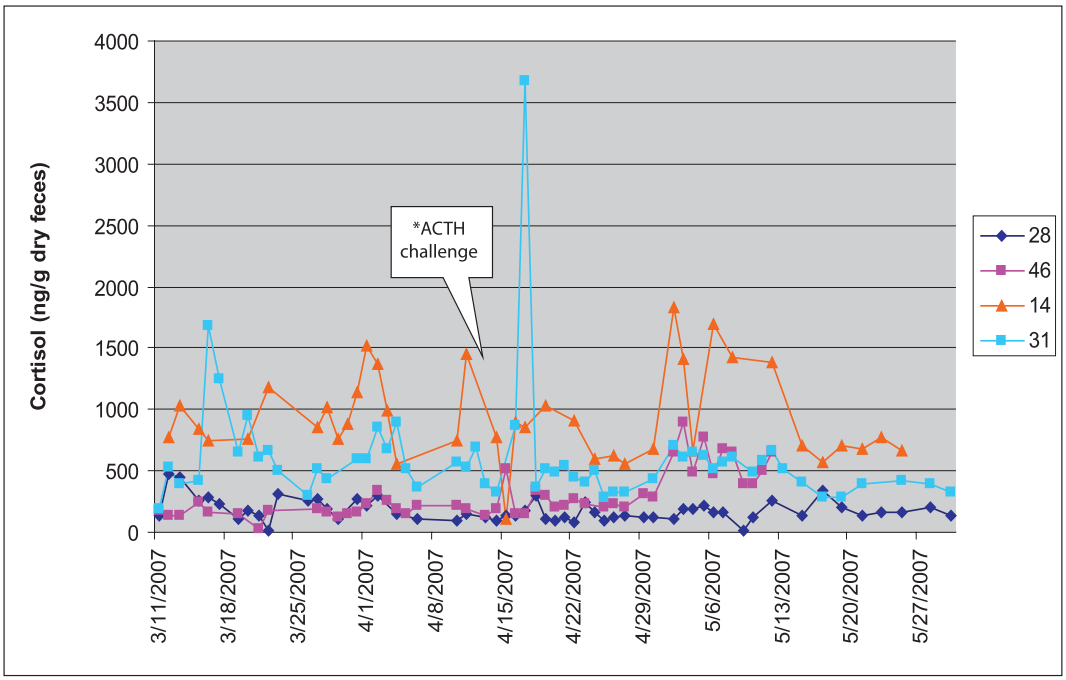
There was no immunoactivity in faecal extracts purified by HPLC using the corticosterone RIA. By contrast, analysis of HPLC fractions using the cortisol EIA detected several fecal metabolites, one of which corresponded with the ^{3}H-cortisol tracer (fractions 40-44) (Fig. 1). Three additional immunoreactive peaks were observed, one of which was less polar (fractions 18-29) and two that were more polar (fractions 63-69 and 73-77) than cortisol. No immunoactivity was associated with the ^{3}H-corticosterone reference tracer (fractions 46-48).

Fig. 1. Cortisol EIA immunoactivity of HPLC-separated fecal extracts.



Plotting individual fecal data by date illustrates differences in both concentrations of and patterns of glucocorticoid secretion (Fig. 2). Most notably, all animals except Jarra (#31) lacked a post-ACTH increase in glucocorticoid concentrations. Within a day of ACTH, there was a marked elevation in concentrations in that female. The lack in response in the other individuals might have been due to differences in efficacy of ACTH administration via blowpipe. Both Bruno (#14) and Jarra (#31) exhibited considerable fluctuations in glucocorticoid concentrations in the first half of the study period, and both also showed moderate glucocorticoid increases at the end of April, lasting roughly two weeks. Analyzing average glucocorticoid values across the study period, there was a nonsignificant trend towards increasing values in the third month of the study (Fig. 3). When individual trends were examined, two opposing patterns were noted: an increase in average glucocorticoid concentrations from the first to third month in #46 and #14, and a decrease in levels from the first to the third month in #28 and #31 (data not shown). There also was marked inter-individual variation in average fecal glucocorticoid concentrations (Fig. 4). Specifically, average

Fig. 2. Fecal glucocorticoid concentrations in study animals.



values for Bruno (#14) and Jarra (#31) were significantly higher than those for Binh (#28) and Halffeet (#46). In addition, Bruno's average glucocorticoid concentrations were significantly higher than those in the other animals ($P < 0.001$, Kruskal-Wallis). When analyzed according to housing condition (Fig. 5), Bruno (housed with two adult Hatinh langurs) had significantly higher glucocorticoid concentrations than the other three animals (housed with individuals of the same species) ($P<0.001$, Mann-Whitney U). Analyses of other variables (e.g. weather, temperature) in relationship to glucocorticoid concentrations did not reveal any significant relationships (Fig. 6, 7). Finally, there were no significant sex differences in glucocorticoid concentrations (Fig. 8).

Fig. 3. Average monthly glucocorticoid concentrations throughout the study period (1=March, 2=April, 3=May).

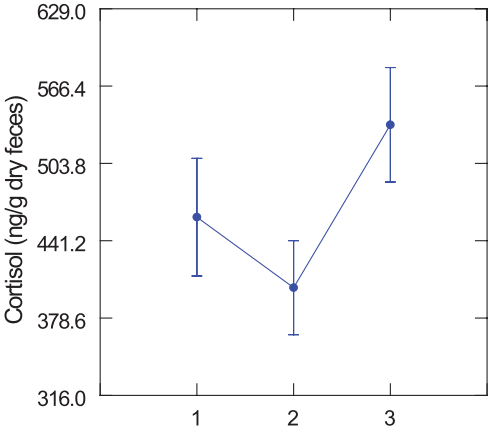


Fig. 4. Average glucocorticoid concentrations by individual.

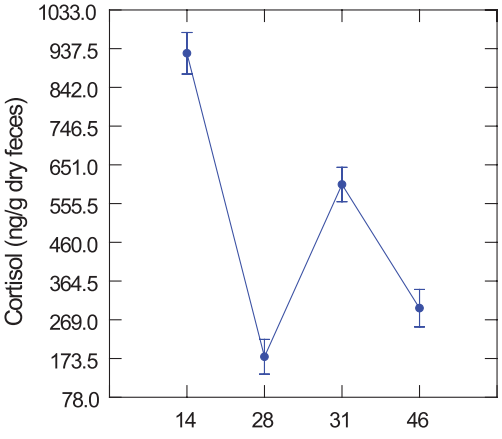


Fig. 5. Average glucocorticoid concentrations by housing condition (DS=different species (Hatinh langur), SS=same species).

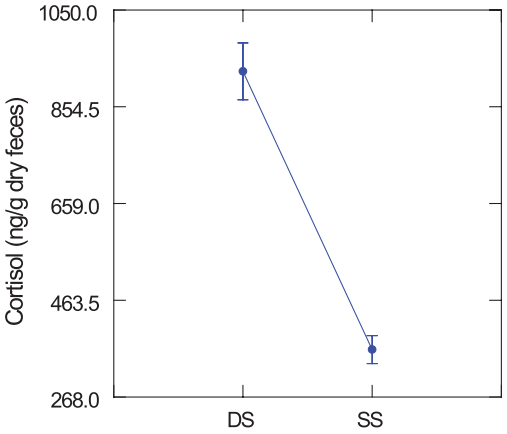


Fig. 6. Average glucocorticoid concentrations by weather; first letter indicates weather in first half of day, second letter indicates weather in second half of day (C=cloudy, R=rain, S=sunshine).

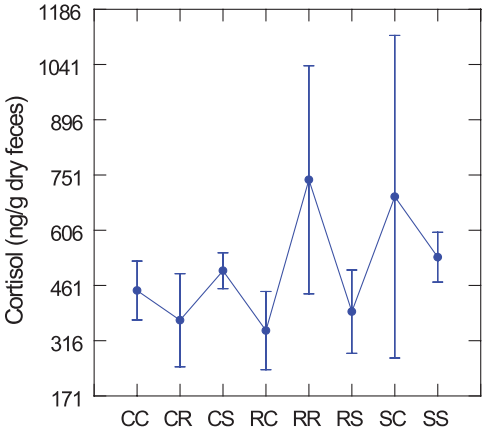


Fig. 7. Glucocorticoid concentrations plotted against average daily temperature, in degrees Celsius (average of morning, noon, and night temperatures).

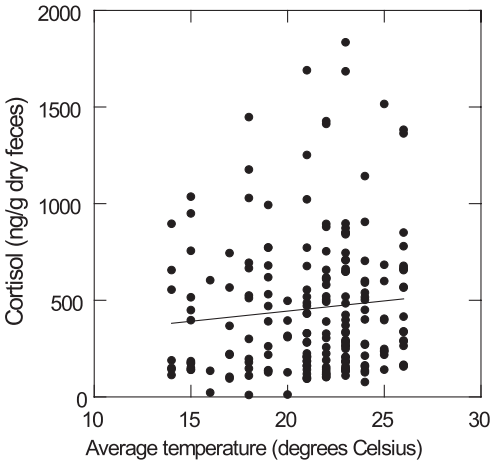
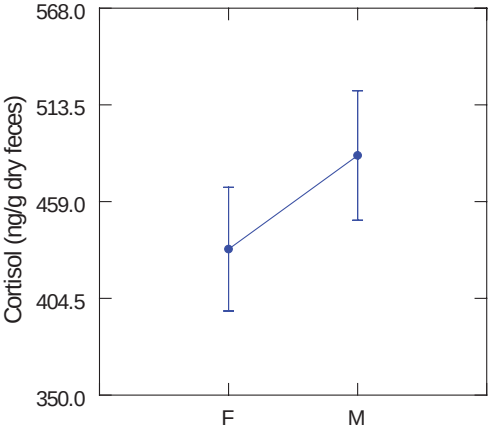


Fig. 8. Average glucocorticoid concentrations for females (F) and males (M).



Conclusions

The current study contributes data on fecal glucocorticoid levels in *Pygathrix nemeaus*, and adds to the one existing study on the subject (Heistermann et al., 2004) by examining concentrations in both males and females, and by looking at variation in relationship to environmental variables (temperature, weather, housing condition). The results of this study suggest significant inter-individual differences in glucocorticoid concentrations, and while patterns of fecal glucocorticoids among individual animals showed varying degrees of fluctuation, the significance or underlying causes of these patterns is unclear. Group housing with conspecifics versus members of a different species, particularly adult members of the same sex, may modulate glucocorticoid secretion. These results, while potentially indicative of "real" patterns, require further sampling over extended time periods to be substantiated. In particular, the lack of a rise in fecal glucocorticoids following ACTH administration in three of four individuals suggests that this component of the study may need to be repeated, perhaps utilizing a different route of

administration or higher ACTH dose. Further studies should examine, in more detail, the relationship between seasonality (e.g., Fichtel et al., 2007), reproductive status (e.g., Lynch et al., 2002), and social and environmental factors (e.g., Weingrill et al., 2004) on fecal glucocorticoids in this species.

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A case of an *Echinococcus ortleppi* infestation in a red-shanked douc langur (*Pygathrix nemaeus*) in northern Vietnam

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Key words: *Pygathrix*, species identification, illegal trade, mtDNA, PCR-based sex-typing

Summary

An *Echinococcus ortleppi* infestation was demonstrated in a red-shanked douc langur (*Pygathix nemaeus*) at the Endangered Primate Rescue Center, Cuc Phuong National Park, Vietnam. In pathology, four parasitic cysts were found within both lungs. In parasitology, *Echinococcus ortleppi* was identified by polymerase chain reaction and mitochondrial gene sequencing. This is the first record of *E. ortleppi* from a non-human primate, and, to the authors' knowledge, the first ever isolate of the *E. granulosus*-assemblage from a monkey that has been molecularly characterized using strain-specific methods. The presence of *E. ortlepp*i, in particular, is rather unexpected, as the nearest region where that species has been recorded is the Indian subcontinent.

Một trường hợp nhiễm *Echinococcus ortleppi* ở Chà vá chân nâu (*Pygathix nemaeus*) ở Bắc Việt Nam

Tóm tắt

Một trường hợp chà vá chân nâu (*Pygathix nemaeus*) tại Trung tâm Cứu hộ Linh trưởng, VQG Cúc Phương, Việt Nam được phát hiện nhiễm *Echinococcus ortleppi*.

Trong bệnh lý học, bốn nang bào ký sinh đã được phát hiện ở cả hai lá phổi. Đối với ngành ký sinh trùng, *Echinococcus ortleppi* được phát hiện bằng phản ứng khuếch đại gen (PCR) và kỹ thuật phân tích gen trong ty thể. Ngoại trừ được phát hiện ở người, đây là ghi nhận đầu tiên loài *Echinococcus ortleppi* ký sinh ở một loài linh trưởng khác, và đối với sự hiểu biết của các tác giả, đây là loài thuộc nhóm *E. granulosus* đầu tiên ký sinh trên một loài khỉ được xác định về mặt phân tử sử dụng các phương pháp chủng-đặc hiệu. Cách riêng, sự hiện diện của loài *E. ortleppi* nằm ngoài dự kiến bởi khu vực gần nhất mà loài này được phát hiện là tiểu lục địa Ấn Độ.

Introduction

Cystic echinococcosis

Cystic echinococcosis (CE), or hydatidosis, is a parasitic disease caused by various taxa of the cestode genus *Echinococcus*. It is typically transmitted between domestic dogs or wild canids as definitive hosts, harboring tapeworms in their intestines, and domestic or wild ungulates as

intermediate hosts, where the cystic metacestodes grow in various organs and may cause severe symptoms or death due to the occupating space in the organs. Apart from ungulates, a wide range of other mammals, including humans and non-human primates, are known to be susceptible as, mainly aberrant, intermediate hosts.

CE was previously ascribed to one diverse species, the dog tapeworm (*E. granulosus*). By now it is apparent that a number of independent species with distinct genetic, morphological and biological features were hidden under that name, and the '*E. granulosus*'-assemblage have recently been split into *E. granulosus* sensu stricto, *E. equinus*, *E. ortleppi*, *E. canadensis* and *E. felidis* (Nakao et al., 2007; Hüttner et al., 2008; Saarma et al., 2009). Diagnostic morphological characters are not known for the cyst stage for any of these species, and the various molecular tools for differentiation of these taxa have only recently been developed. Therefore, relatively few data are available on the geographical distribution and host range of these forms (Jenkins et al., 2004), although a large body of epidemiological information was collected in the past on the *E. granulosus* assemblage as a whole (Eckert et al., 2001).

E. ortleppi has been known as the 'cattle strain' (G5) of *E. granulosus*, because the transmission of this parasite seems to occur preferentially between dogs and cattle (Eckert and Thompson, 1988; Thompson and McManus, 2002). Originally described from South Africa, it is (or was) widespread in Europe, and was also recorded from South America, sub-Saharan Africa and some Asian countries (India, Sri Lanka and Nepal) (Thompson & McManus, 2002; Dinkel et al., 2004).

Endangered Primate Rescue Center

The Endangered Primate Rescue Center was established in 1993 to house highly endangered primates confiscated from the illegal wildlife trade. Vietnamese endemic species are a major focus of the center's work. Over the years the center has developed as a breeding facility for several species. The final stage of this program will be the release of captive bred offspring into protected areas to strengthen dramatically declining wild populations. Currently the centre keeps more than 150 animals of 15 taxa, six of which are only successfully kept in captivity at the EPRC (Nadler, 2008).

Material

Animal and keeping conditions

This paper reports on a female red-shanked douc langur (*Pygathix nemaeus*) from the Endangered Primate Rescue Center, Cuc Phuong National Park, Vietnam. The animal was born on 17 April 2004 and died on 29 September 2008. It was caged since its birth in a group with its mother, a wild born female which died in 2007, a wild born adult male and another adult female, born at the EPRC in 2001.

The group of monkeys lived in an open cage (10m x 5.5m x 3.2m) made of wire mesh, metal pillars a concrete floor. The cage is furnished with bamboo poles. About forty similar cages are situated in a park-like area surrounded by a fence.

Only the animal keepers have access to the cages. Visitors are required to stay on a foot path about 3.5m distance from the wire mesh of the animal cages.

The EPRC keeps a disciplined watch dog which does not approach the cages very closely. Wild mammals which occasionally have closer contact with the cages include squirrels (*Callosciurus* sp.) and mongoose (*Herpestes javanicus*).

Clinical history

The langur became apathetic only two days before it died, but continued to ingest foods. On the morning of 29 September the animal ate but was found dead at 1 pm. The body was frozen at -20°C for further pathological investigations to determine the cause of death.

Methods

Pathology

Necropsy was performed immediately after the thawing of the carcass. Photos were taken and tissues of interest were fixed in 70 % ethanol.

Parasitology

Parasitological identification of the parasite was performed by polymerase chain reaction and subsequent sequencing of parts of the mitochondrial 12S rRNA, *nad*1 and *cox* 1 genes.

DNA extraction

DNA was isolated from ethanol fixed cyst material as described by Dinkel et al. (1998): About 0.5 g of the cyst wall was cut into small pieces and digested in the presence of 2 mg/ml proteinase K in 500 µl of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 2% sodium dodecyl sulfate and 20 mM dithiothreitol. DNA was extracted with phenol chloroform isoamyl alcohol (25:24:1) and ethanol precipitation. After drying, the DNA was suspended in 200 µl TE-buffer (pH 7.6).

Polymerase chain reaction

A cestode specific PCR (cs PCR) was done as described previously (Dinkel et al.,1998; Dinkel et al., 2004) in 50 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 20 pmol of each primer and 1.25 units of Ampli-Taq Polymerase (Applied Biosystems). Amplification was done for 40 cycles (denaturation for 30 s at 94°C, annealing for 1 min at 55°C and elongation for 30 s at 72°C). For identification of genotypes and species of *Echinococcus* a semi-nested PCR assay specific for *E. canadensis* G6/7 and *E. ortleppi* as described in Dinkel et al. (2004) was performed. For the first PCR (g5/6/7), the 50 µl reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM of $MgCl_2$, 200 µM of each dNTP, 25 pmol of each primer, and 1.25 units of Ampli-Taq Polymerase (Applied Biosystems) for 40 cycles (denaturation for 30 s at 94°C, annealing for 1 min at 53°C and elongation for 40 s at 72°C). To discriminate between *E. ortleppi* and *E. canadensis* G6/7, the semi-nested PCRs for *E. ortleppi* (g5 PCR) and *E. canadensis* G6/7 (g6/7 PCR) were used in a second step, both in a 50µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM of $MgCl_2$, 200 µM of each dNTP, 25 pmol of each primer, and 1.25 units of Ampli-Taq Polymerase (Applied Biosystems) for 40 cycles (denaturation for 30 s at 94°C, annealing for 1 min at 60°C and elongation for 30 s at 72°C). For all PCRs, target sequence for amplification is a part of the mitochondrial 12S rRNA gene.

For subsequent gene sequencing, two additional PCRs were performed as described in Bowles et al. (1992) and Bowles & McManus (1993) with the target sequences of a part of the mitochondrial cox 1 and nad 1 genes.

All amplification products were resolved on a 1.5% ethidium bromide stained agarose gel.

Mitochondrial gene sequencing

Amplification products of the cox 1, nad 1 and cs PCR were purified over QIAquick™ columns and cycle sequencing was performed on the Gene Amp 9700 (Applied Biosystems) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) for 25 cycles (denaturation for 10 s at 94°C and annealing for 4 min at 60°C). Electrophoresis was carried out on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) and nucleotide sequence analysis was made using the National Center for Biotechnology Information BLAST programs and databases.

Results

Necropsy

At necropsy, four parasitic cysts approximately the size of table tennis balls were found within both lungs occupying at least 70 % of the lung tissue (Fig. 1). The cysts were filled with clear watery fluid and the walls of the cysts were yellowish-white and smooth. No protoscolices were found. The wall of each cyst contained a small ovoid thickened area the size of about 2mm.

In the left thoracic cavity, one cyst had been disrupted resulting in a severe fibrinous-exsudative pleuritis with some yellowish watery fluid (Fig. 2).

Residual lung tissue was severely atelectatic due to compression of the parasitic cysts.

Parasitology

Using the specific PCR system, DNA isolated from the cyst material was found to belong to *E. ortleppi*. This result was confirmed by gene sequencing of parts of the mitochondrial 12S rRNA, cox1 and nad1 genes. The sequences obtained showed 99% identity with published sequences of *E. ortleppi* on GenBank™.

Discussion

This is the first record of *E. ortleppi* from a non-human primate, and, to the authors' knowledge, the first isolate of the *E. granulosus*-assemblage from a monkey that has ever been molecularly characterized using strain-specific methods. Non-human primates are known to be susceptible to cystic echinococcosis, but are usually accidental intermediate hosts which are not substantially involved in maintaining the transmission of the parasites. Natural infections are known from baboons (*Papio* sp.) in Kenya and Mozambique (Macpherson & Wachira, 1997), probably as a spill-over from domestic or wildlife cycles involving canids and ungulates. Accidental infections of primates in captivity were apparently common in the past, although detailed reports are few (reviewed in: Toft, 1986). Rhesus monkeys and baboons had been successfully used for experimental infections with CE (Hutchison, 1966; Macpherson et al., 1986).

From a geographical view, this is an interesting record from a region where only spurious information on echinococcosis (in general) is available: CE was recorded sporadically in Southeast Asia, but there are no details known on life cycles, species, or frequency (Eckert et al., 2001; Schantz et al., 1995; Segal & Humphrey, 1968). The presence of *E. ortleppi*, in particular, is rather unexpected, as the nearest region where that species has been recorded is the Indian subcontinent (Thompson & McManus, 2002). There is no doubt that the animal acquired the infection locally. However, it would be a matter of interest if a transmission cycle of *E. ortleppi* is autochthonous in the area, or whether the parasite was introduced from elsewhere, e.g. via the cattle trade. As to the

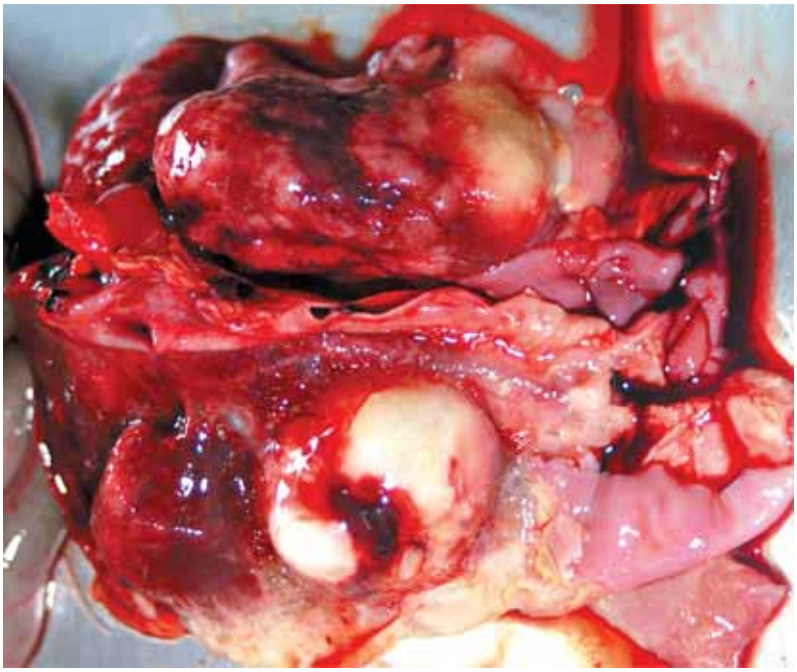

Fig. 1. Lungs of a red-shanked douc langur (*Pygathrix nemaeus*) with yellowish-white cysts of *Echinococcus ortleppi*. Photo: R. Plesker.

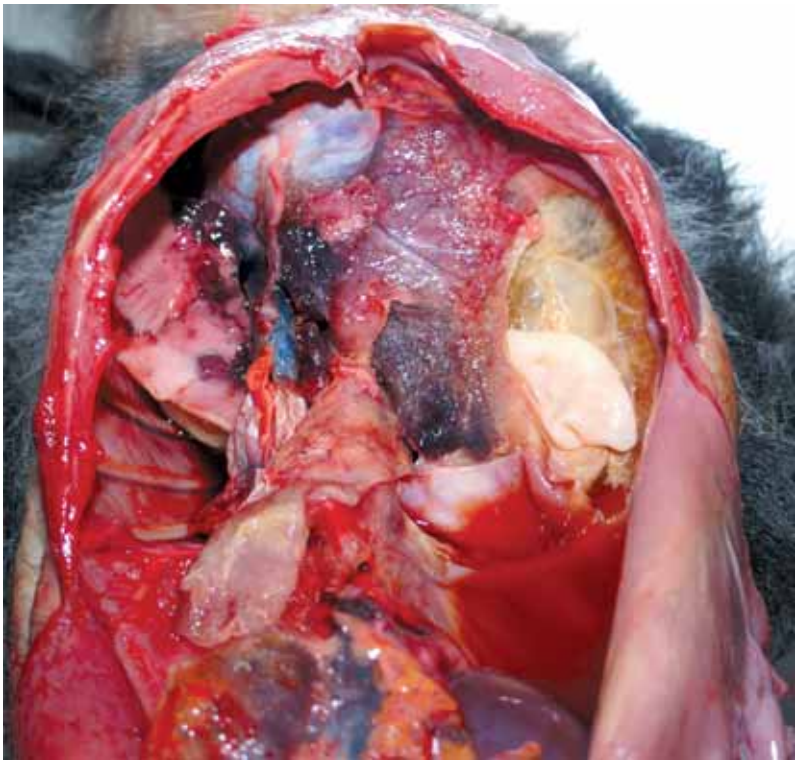

Fig. 2. Caudal view of the thorax of a red-shanked douc langur (*Pygathrix nemaeus*) infested with *Echinococcus ortleppi*: The right side shows a disrupted parsitic cyst (white) and a fibrinous pleuritis with yellowish frozen fluid in the thorax. Photo: R. Plesker.

immediate infection route to the captive monkey, faeces from a domestic dog are certainly the source of the parasite eggs, as wild canids are not known to be present in the immediate vicinity of the animal enclosure. Apart from faeces deposited immediately outside the cage, the contamination of vegetative matter (branches, leaves, fruits) brought as food or environmental enrichment from outside is a distinct possibility. The latter route seems to be important for the infection of primates in European and Japanese zoos with *E. multilocularis* (Sato et al., 2005; Tappe et al., 2007).

From the data available, *E. ortleppi* shows a strong predilection for cattle as intermediate hosts, where it produces large cysts predominantly in the lungs. In Switzerland, 95% of these cysts were fertile (Eckert & Thompson, 1988). Infection in other animals, e.g. sheep, goats, water buffaloes, domestic pigs and even zebra are known (Zhang et al., 2000; Dinkel et al., 2004; Obwaller et al., 2004), but the epidemiological role of these hosts is unclear. Only a few human cases are on record from the Netherlands, Argentina and Mexico (Bowles et al., 1992; Kamenetzky et al., 2002; Maravilla et al., 2004). Whether the small number of human cases is due to low exposure or indicates a certain degree of resistance to this species is not known. In the absence of such data, the presence of this parasite in the area should be a matter of concern for those involved in public health issues.

Cyst location in the lungs appears to be a typical feature of *E. ortleppi* in ruminants. The case referenced here suggests that this might also be the case in primates. In contrast, a massive zoo-borne infection of a colobus monkey and baboons in the USA with unspecified '*E. granulosus*' produced masses of cysts in all organs posterior to the diaphragm, but did not involve the lungs (Myers et al., 1965), and a more recent case of unspecified CE in a captive pig-tailed macaque also showed cysts only in the liver (Plesker et al., 2001).

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Gastrointestinal parasites of the Delacour's langur (*Trachypithecus delacouri*): Comparison between caged, semi-wild, and free-ranging individuals

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Key words: Gastrointestinal parasites, *Trachypithecus delacouri*, Colobinae

Summary

From March through May 2009, fecal samples were collected from caged, semi-wild, and free-ranging individuals of the Delacour's langur (*Trachypithecus delacouri*) to quantify the prevalence of gastrointestinal parasites. Helminth eggs and larvae were isolated by various diagnostic techniques. Helminth parasites were identified, and infection prevalence was determined for all individuals. Six nematode species (*Trichuris* sp., *Trichostrongylus* sp., *Oesophagostomum* sp., *Strongyloides stercoralis*, *Ancylostoma* sp., and *Physaloptera* sp.) were detected. The most prevalent nematodes in this study were *Trichuris* sp. and *Oesophagostomum* sp. Only one type of nematode was found in the free-ranging animals, this finding seems to support the theory that small isolated host populations harbor fewer parasite species.

Vật ký sinh trong hệ tiêu hóa của voọc mông trắng (*Trachypitecus delacouri*) sống nuôi nhốt, nuôi nửa hoang, và trong rừng

Tóm tắt

Từ tháng 3 tới tháng 5 trong năm 2009, mẫu phân của voọc mông trắng (*Trachypithecus delacouri*) sống nuôi nhốt, nuôi nửa hoang và trong rừng đã được thu lượm để xác định số lượng sự phổ biến của vật ký sinh trong hệ tiêu hóa. Trứng và ấu trùng của giun ký sinh đã được tách ra bằng nhiều phương pháp chẩn đoán khác nhau. Giun ký sinh đã dược nhận ra và sự phổ biến lây nhiễm được xác định tro voọc sống nuôi nhốt, nuôi nửa hoang và trong rừng. Sáu loài giun tròn (*Trichuris* sp., *Trichostrongylus* sp., *Oesophagostomum* sp., *Strongyloides stercoralis*, *Ancylostoma* sp., and *Physaloptera* sp.) đã được phát hiện. Loài giun tròn thường thấy nhất trong cuộc nghiên cứu này là *Trichuris* sp. và *Oesophagostomum* sp. Chỉ có một loài giun tròn đã được tìm thấy trong voọc sống trong rừng, cái khám phá này dường như ủng hộ cái lý thuyết là vật chủ sống cách ly với dân số nhỏ sẽ chứa chấp ít loài vật ký sinh.

Introduction

Vietnam is home to 22 primate species and of those; four are endemic to Vietnam and are listed as "Critically Endangered" by the IUCN "Red List of Threatened Species" (Southeast Asian Mammal Databank, 2006). Delacour's langur, *Trachypithecus delacouri*, which was first discovered in 1930 in Northern Vietnam (Osgood, 1932), is one of the "Critically Endangered" species. This species is only found in a restricted area in the Pu Luong – Cuc Phuong limestone range in Northern Vietnam. Survey indicated that populations of Delacour's langur have declined by 50-55% since 1992 (Nadler et al., 2002). Isolation of remaining subpopulations and intense hunting pressure pose the most severe short-term threat to their survival in the wild. Sixty percent of all known Delacour's langurs occur in isolated subpopulations, with a maximum of 20 animals per subpopulation. These small subpopulations are at severe risk of local extinction. The two largest subpopulations consist of only about 30 and 100 animals (Nadler et al., 2004).

Hopkins and Nunn (2007) global gap analysis of infectious agents in wild primates found that despite a growing importance of geo-referenced data for reducing disease risk, information on parasite threats are globally limited. In their study they utilized gap analysis to investigate the global distribution of parasite sampling in non-human primates and found that Southeast Asia as one of three regions in the world with the most deficient sampling.

Currently, there are no studies that look at the parasites in the critically endangered Delacour's langur. The most recent review of the Delacour's langur indicated that only 281-317 individuals remain in 19 small isolated subpopulations (Nadler, 2004). These small subpopulations are extremely vulnerable to environmental and human disturbance. A study on gastrointestinal parasites is important for the management of critically endangered primates and the safety of animal keepers in centers such as the Endangered Primate Rescue Center (EPRC) because many of these parasites are potentially zoonotic.

The study reported here was conducted with the aim of documenting some of the different gastrointestinal parasites found in the Delacour's langur and to compare the prevalence and types of parasites in caged, semi-wild and free-ranging animals.

Materials and Methods

Study Animals

Fecal samples from caged and semi-wild animals were collected at the EPRC, Cuc Phuong National Park, Vietnam. There are a total of 15 animals kept in cages and four animals in the semi-wild enclosure. Five of the confiscated animals are founders for the captive population at the EPRC (Table 1).

The semi-wild enclosure is two hectares and has various vegetation types that are eaten by the langur. Four individuals have been in the semi-wild enclosure for over five years, no food supplements are given except for one feeding of sweet potatoes every day in the morning to check the health of the animals.

Ten fecal samples from Van Long Nature Reserve (VLNR) were collected from two different groups in the reserve, each group was composed of about 10-18 individuals.

Sample Collection

Fifteen fecal samples were collected from 14 caged animals, and four fecal samples were

Table 1. Demographic data of caged and semi-wild animals.

Name	No.	Sex	Date Born	Sire	Dam	Source	Type
Short Tail	1-01	M	1990	Wild	Wild	Confiscated	Caged
M. Delacouri	1-03	F	Unknown	Wild	Wild	Confiscated	Caged
Marco	1-04	M	1993	Wild	Wild	Confiscated	Caged
Jonathan	1-07	M	21.02.98	1-01	1-03	Born EPRC	Caged
Franz	1-08	F	16.08.99	1-01	1-03	Born EPRC	Caged
Ella	1-09	F	03.04.01	1-01	1-03	Born EPRC	Caged
Hai	1-10	M	04.06.01	1-02	1-05	Born EPRC	Caged
Scott	1-12	M	07.12.02	1-01	1-03	Born EPRC	Caged
Johanna	1-13	F	09.07.03	1-02	1-06	Born EPRC	Caged
Joris	1-15	M	14.07.04	1-01	1-03	Born EPRC	Caged
Sascha	1-16	M	01.06.05	1-04	1-08	Born EPRC	Caged
Fritz	1-18	M	14.04.07	1-04	1-08	Born EPRC	Caged
Unnamed	1-20	M	30.01.08	1-10	1-09	Born EPRC	Caged
Jojo	1-21	F	29.07.08	1-07	1-13	Born EPRC	Caged
Longtail	1-02	M	1990	Wild	Wild	Confiscated	Semi-wild
Manu	1-06	F	28.07.96	1-01	1-03	Born EPRC	Semi-wild
Buschi	1-17	F	27.10.05	1-02	1-06	Born EPRC	Semi-wild
Gil	1-19	M	08.01.08	1-02	1-06	Born EPRC	Semi-wild

collected from the four animals in the semi-wild enclosure at the EPRC. Ten fecal samples from free ranging animals were collected at VLNR. Fecal samples were collected soon after defecation and examined macroscopically for adult nematodes and tapeworm proglottids. Samples collected from the EPRC were examined within an hour after collection. Samples collected at VLNR were divided into two parts: one part was preserved in a 25 ml vial in 10% formalin solution and the second part was refrigerated at 4^{o}C. Refrigerated and preserved samples were examined within two days after collection.

Parasitological Techniques

Several diagnostic techniques were used to identify different parasites. Fecal smear, simple flotation, centrifugal flotation, and fecal sedimentation procedures were followed (Zajac & Conboy, 2006). Parasites were identified on the basis of egg color, shape, contents, and size (Foreyt, 2001; Roberts & Janovy, 2005; Zajac & Conboy, 2006). Measurements were made to the nearest 0.1 µm, using an ocular micrometer fitted to a compound microscope, and representatives were photographed (Fig. 1).

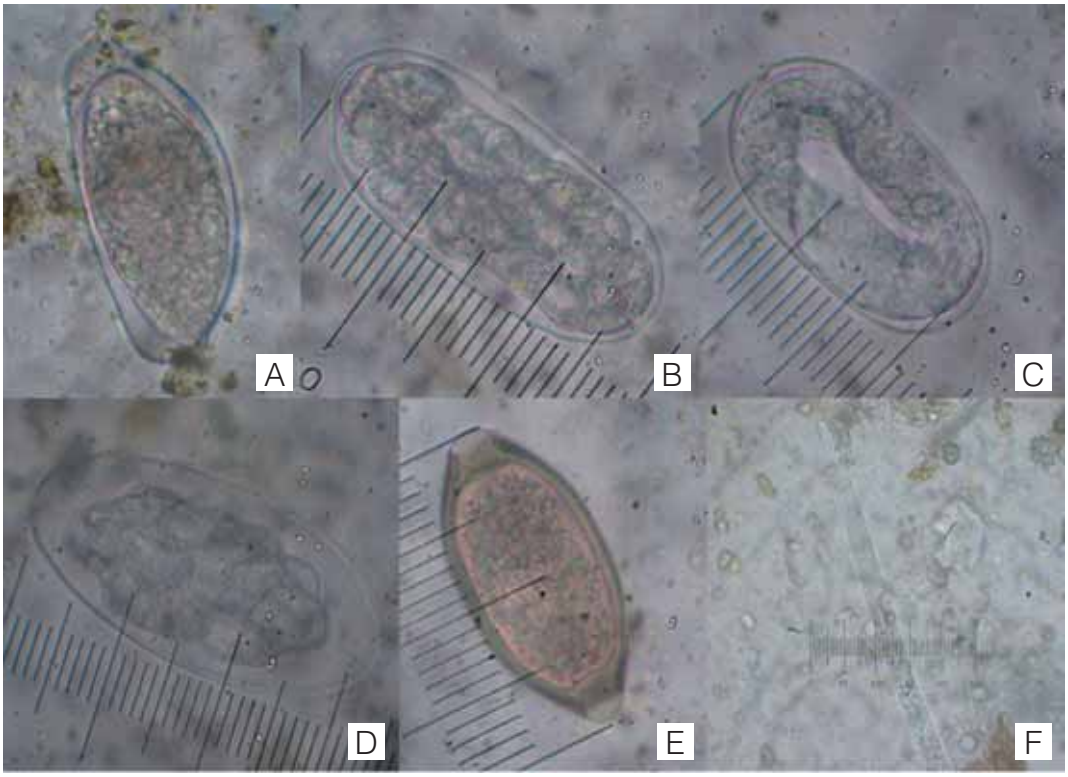

Fig. 1. A: *Ancylostoma* spp. egg (57.2 X 30 µm); B: *Oesophagostomum* spp. egg (73.6 X 31.6 µm); C: *Physaloptera* spp. egg (55.2 X 28.9 µm); D: *Trichostrongylus* spp. egg (63.2 X 42.1 µm); E: *Trichuris* spp. egg (55.3 X 28.3 µm); F: *Strongyloides stercoralis* larvae.

Results

Nematoda

Trichuroidea: *Trichuris* spp. were identified on the basis of egg size, barrel-shaped yellow-orange eggs, and bipolar plugs. Eggs were only found in feces of caged and semi-wild animals and measured 55.3 X 28.3 µm. The prevalence of *Trichuris* sp. was 100% in caged and 75% in semi-wild animals.

Strongyloidea: *Oesophagostomum* spp. were identified on the basis of egg size, elliptical eggs, a large dark cell in the morula, and non-larvated. Eggs were only found in feces of caged and semi-wild animals and measured 73.6 X 31.6 µm. The prevalence of *Oesophagostomum* sp. was 14% in caged and 24% in semi-wild animals.

Rhabditoidea: *Strongyloides stercoralis* was identified based on larvae size, presence of a rhabditiform esophagus, prominent genital primordium, and short buccal cavity. Larvae of S. stercoralis were only found in feces of caged animals and measured 473 µm in length. The prevalence of *Strongyloides stercoralis* was 7% in caged animals.

Spirurida: *Physaloptera* spp. were identified on the basis of egg size, elliptical eggs with a smooth cell wall, and coiled larva. Eggs were only found in feces of free-ranging animals and measured 55.2 X 28.9 µm. The prevalence of *Physaloptera* spp. was 10% in free-ranging animals.

Strongyloidea: *Ancylostoma* spp. was identified on the basis of egg size, elliptical eggs with a smooth cell wall, and grapelike cluster of cells. Eggs were only found in feces of semi-wild animals

and measured 57.2 X 30 μm. The prevalence of *Ancylostoma* spp. was 25% in semi-wild animals.

Trichostrongyioidea: *Trichostrongylus* spp. was identified on the basis of egg size, elliptical eggs with a smooth cell wall, and non-larvated. Eggs were only found in feces of caged animals and measured 63.2 X 42.1 μm. The prevalence of *Trichostrongylu*s spp. was 43% in caged animals.

Discussion

This is the first study of gastrointestinal helminth parasite infections in caged, semi-wild and free-ranging individuals of the Delacour's langur. The similarities of parasites found in both caged and semi-wild animals suggest that the parasite infections could be due to the close proximity of the semi-wild enclosure to the caged animals (Table 2). The five confiscated Delacour's langurs produced 15 animals in captivity. There are 19 in total kept by the EPRC, four in the semi-wild enclosure and fifteen in cages. It is very likely that the animals got the parasite infections before they were transferred to the semi-wild enclosure. *Ancylostoma* spp. was the only parasite found in the semi-wild animals and not the caged animals suggesting a new infection. The animals in cages also harbor two species (*Trichostrongylus* spp. and *Strongyloides stercoralis*) that were not found in the semi-wild animals. The 15 animals in cages are housed in five different enclosures with other species housed next to the Delacour's langur cages. The new parasite infections could transfer through the animal keepers.

Interestingly, only one type of parasite (*Physaloptera* spp.) was found from free-ranging animals.

Table 2. The prevalence (%) of gastrointestinal Helminth parasite infection in caged, semi-wild, and free-ranging animals. (Sample size is in parentheses.).

	Prevalence		
Parasite	Caged (15)	Semi-wild (4)	Wild (10)
*Ancylostom*a spp.	0	25	0
Oesophagostomum spp.	14	25	0
Physaloptera spp.	0	0	10
Trichostrongylus spp.	43	0	0
Trichuris spp.	100	75	0
Strongyloides stercoralis	7	0	0

A total of 10 samples from free-ranging animals were examined and only one sample contained *Physaloptera* spp. eggs. Common knowledge suggests that since the animals live in a natural environment the animals are exposed to more infectious agents and thus should be infested with parasites, but this was not the case with the free-ranging Delacour's langurs. Altizer et al. (2007) tested the theory of whether species richness and prevalence of parasites differed between threatened and non-threatened host species. Their results showed that total parasite species richness was lower among threatened primates. This small isolated population of Delacour's langur hosts few parasite species.

This is the first baseline study that looked at the gastrointestinal parasites of the "Critically Endangered" Delacour's langur and compared the prevalence between caged, semi-wild, and free-ranging animals. The sample sizes in this study are small, statistics on prevalence should be

looked at with caution. Future studies should sample all of the Delacour's langur groups at Van Long Nature Reserve and other regions to have a better understanding of the effects of parasites on small isolated populations.

Acknowledgement

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Vietnamese Journal of Primatology (2009) 3, 89-91

Frankfurt Zoological Society: "Vietnam Primate Conservation Program" and the Endangered Primate Rescue Center, Vietnam – Report 2008

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Key words: primates, Vietnam, Vietnam Primate Conservation Program, Endangered Primate Rescue Center

Summary

The *Vietnam Primate Conservation Program* of Frankfurt Zoological Society focuses on highly endangered and particularly Vietnamese endemic primates. The main activities of the program include the protection of habitats. A part of this project is the Endangered Primate Rescue Center which provides housing for confiscated individual endangered primates and has established breeding programs to support decreased wild populations with the reintroduction of captive bred animals.

FZS continued supporting Van Long Nature Reserve which holds the largest and most probably the only viable population of the "Critically Endangered" Delacour's langur.

Research and scientific work continued in the field and at the EPRC. Research and studies on the grey-shanked douc langur in Kon Ka Kinh National Park continued. In September started a Vietnamese master student studies on the black-shanked douc langur population on Hon Heo Peninsula, Khanh Hoa Province. The work on the primate database continued with a German biologist from University Trier and a PhD student from University Colorado, USA. One German master student of University Mainz, studied water consumption of langurs at the EPRC.

A scientific highlight was the symposium "Conservation of Primates in Indochina" held in Cuc Phuong National Park 27–30 November, organized by Frankfurt Zoological Society, Conservation International and Cuc Phuong National Park. 130 participants from 10 countries attended the event and more than 35 scientific presentations were given.

Surveys and population monitoring continued for two "Critically Endangered" primates. In August began surveys in several areas with Delacour's langur populations. The survey activities should continue in 2009. The focus of these surveys is collecting data which allows for an assessment of necessary and possible translocation for extremely decreased subpopulations. To gather a complete overview about the distribution of the grey-shanked douc langur, at the end of the year 2008 surveys were carried out in several protected areas.

The Endangered Primate Rescue Center has been continuously working with a staff of 20 Vietnamese employees and one experienced foreign animal keeper as trainer and supervisor. At the end of 2008 the EPRC housed 145 primates of 15 taxa. During the year six confiscated animals arrived at the EPRC, 14 individuals were born, including two stillbirth and 14 individuals died. A highlight was the birth of three Delacour's langurs which all survived.

The captive bred and reintroduced animals to Phong Nha-Ke Bang National Park adapted very well to the natural conditions. The Reintroduction project should continue for Hatinh and douc langurs.

Several education activities were supported by the *Vietnam Primate Conservation Program* and a number of scientific papers published resulting from the Program and the work of the Endangered Primate Rescue Center. The Program supported reports in VTV2 about "Critically Endangered" and Vietnamese endemic primates.

Hội Động vật học Frankfurt: "Chương trình Bảo tồn Linh trưởng Việt Nam" và Trạm Cứu hộ Linh trưởng Nguy cấp, Việt Nam – Báo cáo 2008

Tóm tắt

Chương trình Bảo tồn Linh trưởng Việt Nam của Hội Động vật học Frankfurt (FZS) quan tâm chính tới những loài thú linh trưởng đang có nguy cơ đe doạ bị tuyệt chủng cao và đặc biệt là những loài đặc hữu của Việt Nam. Một trong những mục tiêu quan trọng của chương trình là bảo vệ môi trường sống tự nhiên. Một phần của dự án là Trạm Cứu hộ Linh trưởng Nguy cấp (EPRC), đây là cơ sở cứu hộ, nuôi dưỡng và cho sinh nhằm tăng viện số lượng cho động vật quý hiếm cho tự nhiên bằng chương trình tái hoà nhập động vật hoang dã được sinh sản trong điều kiện nuôi nhốt.

FZS vẫn tiếp tục quan tâm đến Khu bảo tồn đất ngập nước Vân Long, đây là nơi sống của một quần thể lớn nhất loài voọc mông trắng (*Trachypithecus delacouri*) - cấp độ đe doạ là "Cực kỳ Nguy cấp".

Nghiên cứu điều tra trên thực địa và ở EPRC vẫn được tiếp tục thực hiện. Nghiên cứu và điều tra thực địa về loài voọc chà vá chân xám (*Pygathrix cinerea*) tại Vườn Quốc gia Kon Ka Kinh. Trong tháng 9, một cán bộ sinh học Việt Nam đã triển khai đề tài Thạc sỹ về Điều tra nghiên cứu và bảo tồn loài voọc chà vá chân đen (*Pygathrix nigripes*) tại bán đảo Hòn Hèo, tỉnh Khánh Hoà. Công tác cập nhật dữ liệu linh trưởng vẫn tiếp tục được thực hiện bởi sinh viên sinh học tình nguyện đến từ trường Đại học Trier, CHLB Đức và sinh viên Tiến sỹ sinh học đến từ Trường Đại học Colorado, Hoa Kỳ. Một sinh viên sinh học từ Trường Đại học Mainz, CHLB Đức đã thực hiện đề tài Thạc sỹ về dung lượng tiêu thụ nước của một số loài voọc tại EPRC.

Một hoạt động khoa học tiêu biểu là sự thành công của Hội thảo khoa học "Bảo tồn Linh trưởng Đông Dương" tại Vườn Quốc gia Cúc Phương từ ngày 27-30/11 với sự hợp tác và tài trợ của Vườn Quốc gia Cúc Phương, Tổ chức Bảo tồn Quốc tế (CI) và Hội Động vật học Frankfurt. Đã có hơn 130 đại biểu đến từ 10 quốc gia. 35 bài báo cáo khoa học đã được thuyết trình thành công.

Điều tra thực địa và theo dõi hai loài thú linh trưởng "Cực kỳ Nguy cấp" đã được thực hiện. Trong tháng 8 đã tái điều tra thực địa trên một số khu vực phân bố của loài voọc mông trắng, điều tra về loài này vẫn được tiếp tục thực hiện trong năm 2009. Mục tiêu của các cuộc tái điều tra này nhằm đánh giá hiện trạng quần thể, địa hình, địa bàn cư ngụ của chúng nhằm đưa ra các giải pháp di dời một số quần thể đang gặp nhiều nguy cơ cao. Kế tiếp là hoàn thành điều tra toàn bộ các khu vực phân bố của loài voọc chà vá chân xám, tới cuối năm 2008 vẫn thực hiện điều tra tại một số khu bảo tồn là những khu vực phân bố của loài này.

Trạm Cứu hộ Linh trưởng Nguy cấp (EPRC) vẫn duy trì hoạt động bởi 20 nhân viên Việt Nam và một chuyên gia chăn nuôi thú người Đức với nhiệm vụ giám sát công tác chăn nuôi và đào tạo nhân viên Việt Nam. Tới cuối năm 2008, EPRC đã cứu hộ và nuôi dưỡng 145 cá thể của 15 loài và phân loài thú linh trưởng nguy cấp. Trong năm đã có 6 ca thể được tiếp nhận cứu hộ, 14 cá thể được sinh sản, bao gồm 2 cá thể chết sau khi sinh và 14 cá thể bị tử vong. Đặc biệt đã có 3 cá thể loài voọc mông trắng được sinh sản thành công và phát triển rất tốt.

Những cá thể được sinh sản trong điều kiện nuôi nhốt được tham gia chương trình tái hoà nhập thú linh trưởng tại Vườn Quốc gia Phong Nha Kẻ Bàng đã hoà nhập rất tốt với môi trường sống mới

trong khu bán hoang dã. Chương trình này sẽ vẫn tiếp tục thực hiện và từng bước tiến tới đưa động vật hoà nhập hoàn toàn vào tự nhiên cho cả hai loài voọc Hà Tĩnh (*Trachypithecus hatinhensi*) và chà vá chân nâu (*Pygathrix nemaeus*).

Nhiều hoạt động và chương trình giáo dục đã được thực hiện và nhận được sự hỗ trợ của Chương trình Bảo tồn Linh trưởng Việt Nam/FZS. Nhiều bài báo khoa học đã được đăng tải về các hoạt động của Chương trình và EPRC. Hợp tác và cố vấn khoa học cho Đài truyền hình Việt Nam VTV2 thực hiện một số phim khoa học về các loài thú linh trưởng đặc hữu và nguy cấp cao của Việt Nam.

Introduction

The *Vietnam Primate Conservation Program* of Frankfurt Zoological Society focuses on highly endangered and particularly Vietnamese endemic primates. The main activities of the program include the protection of habitats through the support of direct protection work and through the study of wild populations with the intention of collecting background information to provide for better protection and conservation activities.

A part of this project is the Endangered Primate Rescue Center which provides housing for confiscated individual endangered primates and has established breeding programs to support decreased wild populations with the reintroduction of captive bred animals. The temporarily keeping of rare and endangered primates is also a source for studies to gather detailed biological information on these species.

Habitat protection at Van Long Nature Reserve

FZS continued supporting the Nature Reserve which holds the largest and most probably the only viable population of the "Critically Endangered" Delacour's langur. Since establishment of the nature reserve in 2001 the society continued the payment of salaries for 20 local guards.

Intensified patrol activities inside the nature reserve with rangers, guards, commune leaders and local police have been organized in cooperation with the management board and financed by FZS.

Regularly meetings with guards, government employed rangers and commune leaders, and training courses for guards have been organized in cooperation with the management board of the nature reserve. The guards were equipped with sets of new uniforms.

Poaching of the Delacour's langur, the flagship species of the nature reserve could be eliminated. This is most probably the sole population of the existing 17 isolated subpopulations of this primate without poaching. Since establishment of the nature reserve the population increased to double the number of individuals.

Scientific work

Under the leadership of Ha Thang Long four Vietnamese biologists Ho Tien Minh, Nguyen Ai Tam, Nguyen Thi Tinh, and Tran Huu Vy, continued research and studies on the grey-shanked douc langur until August in Kon Ka Kinh National Park. The data will be used for the PhD of Ha Thang Long at Cambridge University, and should provide background information for the improvement of protection for this "Critically Endangered" species.

Catherine Workman, PhD student of Duke University continued her studies and data collection on the ecology of Delacour's langur in Van Long Nature Reserve with support of the FZS biologist Le Van Dung until June.

Nguyen Ai Tam, biologist of Frankfurt Zoological Society started in September his studies on the black-shanked douc langur for his master thesis on Hon Heo Peninsula, Khanh Hoa Province.

Linus Günther, Student at University Trier, Germany continued work on the primate database for six month from April to September.

Hanno Kullik, master student of University Mainz, studied water consumption of langurs at the EPRC from June to October.

Larry Ulibarri, PhD student of University Colorado, Boulder continued work on the primate database from September to December and was also involved in the preparation of scientific publications and the organization of the primate symposium.

A scientific highlight was the symposium "Conservation of Primates in Indochina" held in Cuc Phuong National Park 27–30 November, organized by Frankfurt Zoological Society, Conservation International, and Cuc Phuong National Park. 130 participants from 10 countries attended the event and more than 35 scientific presentations were given (Fig. 1). The exchange of experiences, discussions about conservation strategies, and personal contacts between the primatologists in the region has been a valuable effect besides the scientific presentations. A discussion about the possibilities to host the International Primatological Congress in 2014 in Vietnam was the starting point for initiating the bidding process. A photo exhibition about primates in the region, good weather and nice sunshine, and delicious Vietnamese food formed a pleasant frame for the event (Fig. 2, 3, 4).

Fig. 1. Participants of the International Symposium "Conservation of Primates in Indochina" held in Cuc Phuong National Park 27–30 November. Photo: N. Rowe.

The second issue of the *Vietnamese Journal of Primatology* was published middle of the year. Already with this second issue the journal is recognized as a high standard scientific publication entered on the website of the IUCN Primate Specialist Group, and accepted in "Scopus", the largest abstract and citation database of research literature and quality web sources.

Surveys

In September, FZS biologist Le Van Dung began surveys in several areas with Delacour's langur populations. The survey activities should continue in 2009. The focus of these surveys is collecting data which allows for an assessment of necessary and possible translocation for extremely decreased subpopulations.

Fig. 2. The Museum at Cuc Phuong National Park provided the locality for the symposium. Photo: T. Nadler.

Fig. 3. The hall of the museum with the photo exhibition and possibilities for discussions and personal contacts. Photo: T. Nadler.

Fig. 4. The participants of the symposium check in to a boot trip in Van Long Nature Reserve. Photo: T. Nadler.

Information about the status of Delacour's langurs in Cuc Phuong National Park was gathered during a survey period from August to October carried out by national park staff, the biologist Le Trong Dat and the ranger Luong Van Hao. The survey shows a dramatic decline of the Delacour's langur – the flagship species of the park – to about 8 to 11 individuals. The data documents that the extinction of the species is foreseeable in this area.

After a primate training course at Danang University in 2007, FZS continued supporting students to carry out surveys and studies on the red-shanked douc langurs on Son Tra Peninsula, Danang until September 2008.

To gather a complete overview about the distribution of the "Critically Endangered" grey-shanked douc langur, at the end of the year 2008 biologists Ho Tien Minh and Tran Huu Vy carried out surveys in several protected areas: Chu Mom Ray National Park, Chu Prong and Ayun Pa Nature Reserves. The results of the survey document the occurrence of the species in these areas, but also a very high hunting pressure and a low or absent awareness about protection among the people's surroundings these protected areas.

Endangered Primate Rescue Center

Animals

At the end of 2008 the EPRC housed 145 primates of 15 taxa. During the year 14 individuals were

born, including two stillbirth and 14 individuals died. A highlight was the birth of three Delacour's langurs which all survived.

In cooperation with Forest Protection Departments in several provinces 6 primates were confiscated (see appendix).

Staff at the EPRC

The center has been continuously working with a staff of 20 Vietnamese employees. Since April 2007 Falk Wicker from Leipzig Zoo, Germany worked as supervisor for animal keeping. Leipzig Zoo extended his stay until August 2008 and generously covered his travel and salary. For the continuation of the head keeper position at the EPRC Kelly Blakemore from California started her work in April. Kelly worked in several animal keeping facilities, Zoos and rescue centers where she obtained some experience in primate keeping.

Nguyen Thi Thu Hien, now more than ten years in the position of Vietnamese project assistant continued in project management.

Reintroduction project in Phong Nha-Ke Bang National Park

The reintroduction of Hatinh langurs in Phong Nha-Ke Bang National Park started with the transfer of eight captive bred animals in September 2007 into a semi-wild area of 20 ha. The animals adapted very well to the natural conditions (see Vogt et al., 2008). The release to the wild and transfer of another group from the EPRC to Phong Nha-Ke Bang should be the next steps.

Education

In April the biologist Ha Thang Long started in Cambridge University the evaluation of nearly two years field work on grey-shanked douc langurs in Kon Ka Kinh National park for his PhD.

The guidebook "Protected Animals of Vietnam" was finished and printed in a high number of copies (4000) as a tool for ranger work in the whole country (Fig. 5). With high efforts, FZS organized the distribution of this book to all provincial and district Forest Protection Departments all over the country.

Several education materials were printed to support awareness raising for primate conservation, such as the poster "Save the Vietnamese Primates" with an overview about all Vietnamese primate species, a poster about FZS's primate work in Vietnam, primate post cards, and T-shirts.

Fig. 5. The guidebook "Protected Animals of Vietnam" provides a tool to the forest protection authorities, rangers, police, and customs throughout the country.

Publications, reports, and presentations resulting from the "Vietnam Primate Conservation Program" and the Endangered Primate Rescue Center

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Roos C, **Osterholz M**, **Yang M**, **Walter L** & **Nadler T** (2008): Taxonomy and evolution history of odd-nosed monkeys. Primate Eye 96 – Special Issue. Abstracts of the XXII Congress of the International Primatological Society. Abstract 798.

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TV reports

- With support from FZS biologists in Kon Ka Kinh National Park, Vietnam TV (VTV2) produced an excellent documentation about the grey-shanked douc langur. The report is the first to shows this primate – only discovered in 1997 – in the wild.
- A similar documentary, also supported through the primate work of FZS, introduced the Delacour's langur. The report showed fascinating sequences of this primate at Van Long Nature Reserve – a project area of FZS.
- The German TV channel "Bavaria TV" produced two reports about Vietnamese primates, with focused on the reintroduction project for Hatinh langurs in Phong Nha-Ke Bang National Park and about douc langurs.

Appendix

Register of primates by the EPRC 2008 (up to date 31.12. 2008)

(* species or subspecies only held in EPRC anywhere in the world)

No.	Date of arrival	Sex	Date born or estimated	Sire	Dam	Source	Current status
Delacour's langur *Trachypithecus delacouri* (*)							
1-01	Jan.93	M	1990	wild	wild	confiscated	EPRC
1-02	Jan.93	M	1990	wild	wild	confiscated	EPRC
1-03	17.5.94	F	ad.	wild	wild	confiscated	EPRC
1-04	17.5.94	M	1993	wild	wild	confiscated	EPRC
1-06	28.7.96	F	28.7.96	1-01	1-03	born EPRC	EPRC
1-07	21.2.98	M	21.2.98	1-01	1-03	born EPRC	EPRC
1-08	16.8.99	F	16.8.99	1-01	1-03	born EPRC	EPRC
1-09	3.4.01	F	3.4.01	1-01	1-03	born EPRC	EPRC
1-10	4.6.01	M	4.6.01	1-02	1-05	born EPRC	EPRC
1-12	7.12.02	M	7.12.02	1-01	1-03	born EPRC	EPRC
1-13	9.7.03	F	9.7.03	1-02	1-06	born EPRC	EPRC
1-15	14.7.04	M	14.7.04	1-01	1-03	born EPRC	EPRC
1-16	1.6.05	M	1.6.05	1-04	1-08	born EPRC	EPRC
1-17	27.10.05	F	27.10.05	1-02	1-06	born EPRC	EPRC
1-18	19.4.07	M	19.4.07	1-04	1-08	born EPRC	EPRC
1-19	8.1.08	M	8.1.08	1-02	1-06	born EPRC	EPRC
1-20	30.1.08	M	30.1.08	1-10	1-09	born EPRC	EPRC
1-21	29.7.08	F	29.7.08	1-07	1-13	born EPRC	EPRC
Hatinh langur *Trachypithecus laotum hatinhensis* (*)							
2-01	11.5.93	M	1990	wild	wild	confiscated	EPRC
2-03	13.1.94	F	1993	wild	2-02	confiscated	EPRC
2-05	9.4.94	F	1994	wild	2-04	confiscated	EPRC
2-09	14.1.96	F	ad.	wild	wild	confiscated	EPRC
2-10	6.2.96	M	6.2.96	2-01	2-08	born EPRC	EPRC
2-11	27.4.96	F	27.4.96	2-01	2-04	born EPRC	EPRC
2-12	27.11.96	M	1995	wild	wild	from private	EPRC
2-13	28.3.97	M	28.3.97	2-01	2-09	born EPRC	EPRC
2-14	22.5.97	F	22.5.97	2-01	2-08	born EPRC	EPRC
2-15	15.10.97	M	1995	wild	wild	from tourists	EPRC
2-17	11.12.97	F	1994	wild	wild	from tourists	EPRC
2-20	11.3.98	F	1995	wild	wild	from tourists	EPRC
2-21	11.3.98	M	11.3.98	2-01	2-04	born EPRC	EPRC
2-22	24.2.99	M	24.2.99	2-01	2-08	born EPRC	EPRC
2-23	9.4.99	M	9.4.99	2-01	2-09	born EPRC	EPRC
2-24	25.3.00	M	25.3.00	2-15	2-17	born EPRC	EPRC
2-26	20.11.00	M	20.11.00	2-15	2-11	born EPRC	EPRC
2-27	7.1.01	F	7.1.01	2-15	2-20	born EPRC	EPRC
2-32	4.4.02	F	4.4.02	2-15	2-17	born EPRC	EPRC
2-36	14.11.03	F	14.11.03	2-12	2-05	born EPRC	EPRC
2-41	28.11.04	M	28.11.04	2-01	2-09	born EPRC	EPRC
2-46	1.8.05	F	ca. 2004	wild	wild	confiscated	EPRC
2-47	27.11.05	M	27.11.05	2-12	2-05	born EPRC	EPRC
2-48	14.2.06	F	14.2.06	2-15	2-11	born EPRC	EPRC
2-49	29.6.06	F	29.6.06	14-01	2-14	born EPRC	EPRC

2-50	28.9.06	M	28.9.06	2-12	2-03?	born EPRC	EPRC
2-51	20.10.06	M	20.10.06	2-10	2-27	born EPRC	EPRC
2-52	31.10.06	F	31.10.06	2-10	2-32	born EPRC	EPRC
2-53	10.12.06	M	10.12.06	2-15	2-20	born EPRC	EPRC
2-54	30.3.07	M	30.3.07	2-15	2-17	born EPRC	EPRC
2-55	17.5.07	M	17.5.07	2-01	2-09	born EPRC	†28.3.08
2-56	??.9.07	M	??.9.07	2-12	2-36	born EPRC	EPRC
2-57	18.3.08	F	18.3.08	14-01	2-14	born EPRC	EPRC
2-58	19.5.08	F	19.5.08	2-15	2-11	born EPRC	EPRC
2-59	15.10.08	F	ad.	wild	wild	confiscated	†27.10.08

Black langur *Trachypithecus laotum hatinhensis* morph "*ebenus*" (*)

14-01	12.1.98	M	1996	wild	wild	from tourists	EPRC

Laos langur *Trachypithecus laotum laotum* (*)

3-01	26.9.95	M	1995	wild	wild	confiscated	EPRC

Grey langur *Trachypithecus crepusculus*

4-04	22.1.97	F	1996	wild	wild	from private	EPRC
4-05	14.4.00	F	1999	wild	wild	confiscated	EPRC
4-07	24.1.02	F	24.1.02	4-06	4-04	born EPRC	EPRC

Cat Ba langur (Golden-headed langur) *Trachypithecus p. poliocephalus* (*)

15-01	8.11.98	F	1998	wild	wild	confiscated	EPRC
15-04	2.6.03	M	2.6.03	15-02	15-01	born EPRC	EPRC

Francois' langur *Trachypithecus francoisi*

17-01	8.1.02	F	1997	wild	wild	confiscated	EPRC
17-02	30.9.05	M	2003	wild	wild	confiscated	EPRC

Red-shanked douc langur x Hatinh langur *P. nemaeus* x *T. laotum hatinhensis* (*)

18-01	14.10.03	F	14.10.03	6-9/12?	2-03	born EPRC	EPRC

Red-shanked douc langur *Pygathrix nemaeus*

6-02	17.3.96	F	1992	wild	wild	confiscated	EPRC
6-05	8.5.97	M	ad.	wild	wild	confiscated	EPRC
6-06	24.5.97	M	1994	wild	wild	from tourists	EPRC
6-09	10.7.97	M	1997	wild	wild	confiscated	EPRC
6-12	28.11.97	M	1997	wild	wild	from tourists	EPRC
6-14	12.1.98	M	1996	wild	wild	from tourists	EPRC
6-16	2.4.98	M	1994	wild	wild	from tourists	EPRC
6-21	30.12.98	F	30.12.98	6-05	6-02	born EPRC	EPRC
6-26	6.5.00	M	6.5.00	6-05	6-17	born EPRC	†3.1.08
6-28	19.8.00	M	1996	wild	wild	confiscated	EPRC
6-29	25.4.01	M	25.4.01	6-05	6-13	born EPRC	EPRC
6-30	6.6.01	F	6.6.01	6-06	6-02	born EPRC	EPRC
6-31	21.4.02	F	21.4.02	6-06	6-02	born EPRC	EPRC
6-32	24.2.03	F	24.2.03	6-06	6-02	born EPRC	EPRC
6-34	26.3.04	F	2001	wild	wild	confiscated	†17.2.08
6-35	17.4.04	W	17.4.04	6-05	6-13	born EPRC	†29.9.08
6-36	28.6.04	M	ad.	wild	wild	confiscated	EPRC
6-37	25.8.04	M	25.8.04	6-06	6-02	born EPRC	EPRC
6-38	13.12.04	F	ad.	wild	wild	confiscated	EPRC

6-39	13.4.05	M	ad.	wild	wild	confiscated	EPRC
6-41	9.5.05	F	9.5.05	6-12	6-21	born EPRC	EPRC
6-42	11.6.05	M	April 05	wild	wild	confiscated	EPRC
6-46	17.8.06	F	2001	wild	wild	confiscated	EPRC
6-50	29.4.07	M	29.4.07	6-12	6-34	born EPRC	†20.2.08
6-52	14.9.07	M	2003	wild	wild	confiscated	EPRC
6-53	17.10.07	F	2003	wild	wild	confiscated	EPRC
6-54	21.1.08	M	21.1.08	6-06	6-02	Stillbirth EPRC	†21.1.08
6-55	2.2.08	F	2.2.08	6-28	6-46	born EPRC	EPRC
6-56	6.2.08	F	6.2.08	6-08	6-30	born EPRC	EPRC
6-57	7.3.08	?	7.3.08	6-28	6-31	Stillbirth EPRC †7.3.08	
6-58	27.3.08	F	27.3.08	6-16	6-32	born EPRC	EPRC
6-59	1.4.08	M	1.4.08	6-16	6-38	born EPRC	EPRC
6-60	20.7.08	M	2007	wild	wild	confiscated	EPRC

Grey-shanked douc langur *Pygathrix cinerea* (*)

7-01	31.8.95	M	1992	wild	wild	confiscated	EPRC
7-04	4.8.97	M	1994	wild	wild	confiscated	EPRC
7-09	13.2.01	M	ca.1996	wild	wild	confiscated	EPRC
7-11	15.12.01	F	ca. 1997	wild	wild	confiscated	EPRC
7-13	12.7.02	F	ad.	wild	wild	confiscated	EPRC
7-14	18.8.02	M	1998	wild	wild	confiscated	EPRC
7-16	11.12.02	M	ad.	wild	wild	confiscated	EPRC
7-19	13.3.03	M	subad.(1998)	wild	wild	confiscated	EPRC
7-24	15.1.04	F	15.1.04	7-04	7-13	born EPRC	EPRC
7-25	9.11.04	M	2000	wild	wild	confiscated	EPRC
7-28	6.6.05	F	6.6.05	7-01	7-11	born EPRC	EPRC
7-29	14.8.05	F	ca. 2005	wild	wild	confiscated	EPRC
7-30	9.11.05	F	ad.	wild	wild	confiscated	EPRC
7-31	5.3.06	M	5.3.06	7-04	7-13	born EPRC	EPRC
7-34	19.10.06	F	2000	wild	wild	confiscated	EPRC
7-35	3.11.06	F	ad.	wild	wild	confiscated	EPRC
7-37	24.12.06	M	2003	wild	wild	confiscated	EPRC
7-39	17.3.07	M	2003	wild	wild	confiscated	EPRC
7-40	10.10.07	M	10.10.07	7-09	7-34	born EPRC	EPRC
7-41	5.2.08	F	5.2.08	7-04	7-08	born EPRC	EPRC
7-42	1.3.08	M	1.3.08	7-19	7-30	born EPRC	†15.6.08
7-43	5.5.08	M	5.5.08	7-01	7-11	born EPRC	EPRC
7-44	24.8.08	F	2007	wild	wild	confiscated	EPRC

Black-shanked douc langur *Pygathrix nigripes*

13-05	15.3.01	M	1996	wild	wild	confiscated	EPRC

Red-shanked douc langur x Black-shanked douc langur *Pygathrix nemaeus* x *P. nigripes* (*)

16-01	1.1.00	M	1.1.00	6-06	13-02	born EPRC	†12.4.08

White-cheeked gibbon *Nomascus leucogenys leucogenys*

8-01	30.9.94	F	1993	wild	wild	from foreigner	EPRC
8-02	30.9.94	F	1994	wild	wild	from foreigner	EPRC
8-03	28.5.02	M	1999	wild	wild	confiscated	EPRC
8-08	19.11.04	F	2001	wild	wild	confiscated	EPRC

Southern white-cheeked gibbon *Nomascus leucogenys siki*

9-02	18.9.93	F	1993	wild	wild	from foreigner	EPRC
9-05	10.11.94	M	1992	wild	wild	from foreigner	EPRC
9-06	24.2.95	F	1993	wild	wild	from tourists	EPRC
9-07	30.10.96	M	1996	wild	wild	from tourists	EPRC
9-08	1.12.98	F	1998	wild	wild	from tourists	EPRC
9-09	23.6.99	M	23.6.99	9-05	9-02	born EPRC	EPRC
9-10	10.3.00	M	1999	wild	wild	confiscated	EPRC
9-11	25.7.02	F	25.7.02	9-03	9-06	born EPRC	EPRC
9-12	17.12.02	M	17.12.02	9-05	9-02	born EPRC	EPRC
9-13	21.11.06	F	21.11.06	9-05	9-02	born EPRC	EPRC
9-14	30.12.07	M	30.12.07	9-07	9-06	born EPRC	EPRC

Yellow-cheeked crested gibbon *Nomascus gabriellae*

10-01	26.2.95	F	1994	wild	wild	from tourists	†15.8.08
10-02	6.2.97	F	1994	wild	wild	confiscated	EPRC
10-04	3.6.01	F	1997	wild	wild	confiscated	EPRC
10-05	11.6.04	F	2001	wild	wild	confiscated	EPRC
10-06	21.5.04	F	2003	wild	wild	confiscated	EPRC
10-07	7.10.06	F	2005	wild	wild	confiscated	EPRC
10-08	7.10.06	F	2005	wild	wild	confiscated	EPRC

Slow loris *Nycticebus bengalensis*

11-09	20.11.07	F	ad.	wild	wild	confiscated	EPRC
11-10	22.6.08	F	ad.	wild	wild	confiscated	EPRC

Pygmy loris *Nycticebus pygmaeus*

12-36	22.2.01	F	22.2.01	12-09	12-04	born EPRC	EPRC
12-67	24.2.06	M	ad.	wild	wild	confiscated	†8.10.08
12-68	24.2.06	M	ad.	wild	wild	confiscated	†3.2.08
12-69	24.2.06	F	2/2005	wild	wild	confiscated	EPRC
12-70	24.2.06	F	ad.	wild	wild	confiscated	EPRC
12-72	24.2.06	F	ad.	wild	wild	confiscated	EPRC
12-78	15.3.06	F	ad.	wild	wild	confiscated	EPRC
12-83	7.10.06	M	ad.	wild	wild	confiscated	†11.5.08
12-84	3.11.06	M	ad.	wild	wild	confiscated	†31.1.08
12-86	17.5.07	F	ad.	wild	wild	confiscated	EPRC
12-87	17.5.07	M	ad.	wild	wild	confiscated	†17.3.08
12-88	17.5.07	F	ad.	wild	wild	confiscated	EPRC
12-90	17.5.07	M	ad.	wild	wild	confiscated	†11.4.08
12-94	28.10.07	M	ad.	wild	wild	confiscated	EPRC
12-96	11.1.08	M	ad.	wild	wild	confiscated	EPRC
12-97	12.4.08	M	ad.	wild	wild	confiscated	EPRC
12-98	5.12.08	F	ad.	wild	wild	confiscated	EPRC

Creation of the "ENDANGERED PRIMATE CONSERVATION FUND"

In 1991 Frankfurt Zoological Society started conservation activities for endangered primates in Vietnam with a first survey for the Delacaour's langur (*Trachypithecus delacouri*) after its surprised rediscovery in Cuc Phuong National Park. This has given the cause to start the *Vietnam Primate Conservation Program* in 1993, in the beginning on a small scale and with the focus on the protection and conservation of the Delacour's langur in Cuc Phuong National Park.

During the years the Program developed, contains multifaceted activities, and contributed to the conservation of several highly endangered primate species through direct protection work, education and scientific research as well.

The results of the program are appreciated by the Vietnamese Government and also by international operating conservation bodies.

The project leader - since beginning of the project - Tilo Nadler received awards, in 2006 the Bruno-H.-Schubert-Preis in Germany and in 2008 the Margot Marsh Award for Excellence in Primate Conservation.

With the financial support of these awards he decided to create the

"ENDANGERED PRIMATE CONSERVATION FUND".

The fund should support research and conservation activities on Vietnam's highly endangered primate species. Preferred are applications from students or young primatologists.

The criteria for supporting are:

- the primate species in focus should occur in Indochina, preferred in Vietnam.
- the primate species is listed as "Critically Endangered" or "Endangered" by the "IUCN Red Data Book of Threatened Species."
- the subject of the application should support directly the conservation of the species or the research should contribute to conservation.

Proposals can be supported up to US$2,000. Three quarters of the requested budget are paid by acceptance of the proposal in advance, one quarter after delivery of a final report (in English).

Proposals are requested (in English) with a clear concept, time frame and detailed budget line.

Proposals are to send to:

"ENDANGERED PRIMATE CONSERVATION FUND"
ENDANGERED PRIMATE RESCUE CENTER
Cuc Phuong National Park
Nho Quan District / Ninh Binh Province
Vietnam

or by e-mail to t.nadler@mail.hut.edu.vn

INSTRUCTIONS FOR CONTRIBUTORS

The **Vietnamese Journal of Primatology** welcomes manuscripts from all areas related to the conservation and research of non-human primate taxa which occur in Vietnam and the neighbouring countries of Cambodia, China and Laos. The journal publishes both original research papers and review articles. Original papers may be published as standard research articles or as short communications.

Submission: Submit English manuscripts electronically (as unformatted Microsoft Word file attachments) to Tilo Nadler or Christian Roos:

Tilo Nadler
Frankfurt Zoological Society
Endangered Primate Rescue Center
Vietnam
Email: t.nadler@mail.hut.edu.vn

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Manuscript Preparation: Manuscripts should be divided into the major divisions given below in the order indicated.

Title Page

The first page of the manuscript should include the complete title of the paper, the authors' names, a summary and key words. The complete postal addresses, e-mails and affiliated institutions of the authors must be given.

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Each paper must include a summary of no more than 300 words, which clearly summarizes the contents of the paper. Summary will also be presented in Vietnamese and English.

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Key words in English (maximum 10 words) should be included for indexing purposes.

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Research articles and short communications must be organized into the following sections: Introduction, Materials and Methods, Results, Discussion, Conclusions, Acknowledgements and References. Acknowledgements may include funding sources such as agency and grant numbers, and the names of those who contributed.

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Tables and illustrations should be sent as separate files (in JPG format). Tables require a heading and figures require a legend. All tables and illustrations must be cited in the text. For the reproduction of illustrations, only high quality drawings and photos will be accepted. Color illustrations are welcome. Photographer or artist name must accompany all illustrations. Submit the figures as a separate file.

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Papers published in periodicals

Dao Van Tien (1989): On the trends of the evolutionary radiation on the Tonkin Leafmonkey (*Presbytis francoisi*) (Primates: Cercopithecidae). J. of Human Evolution 4, 501-507.

Fooden J (1996): Zoogeography of Vietnamese Primates. Int. J. Primatol. 17, 845-899.

Books and Monographs

Groves CP (2001): Primate Taxonomy. Smithsonian Institution Press, Washington DC.

Edited books and book chapters

Groves CP 2004: Taxonomy and Biogeography of Primates in Vietnam and Neighbouring Regions. In: Nadler, Streicher & Ha Thang Long (eds.): Conservation of Primates in Vietnam; p 15-22. Frankfurt Zoological Society, Hanoi.

Dissertations

Otto C (2005): Food intake, nutrient intake, and food selection in captive and semi-free Douc langurs. PhD dissertation, University Cologne.

GPXB số: .

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