Vietnamese Journal of Primatology (2013) vol. 2(2), 49-64

Agent Orange exposure in black-shanked douc langurs (*Pygathrix nigripes*) at Nam Cat Tien National Park, Vietnam

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Key words: dioxin, fecal enzyme immunoassay, douc langurs, Nam Cat Tien National Park

Summary

Vietnam is home to some of the world's most critically endangered primates, and while hunting and habitat loss/fragmentation continue to threaten many taxa, few data exist on the impact of environmental herbicides, such as dioxins (Agent Orange, TCDD) and related compounds (DRC), on the health of Vietnamese wildlife. We previously demonstrated the utility of a novel enzyme immunoassay (EIA) procedure to quantify faecal dioxin (fTCDD) concentrations in douc langurs (*Pygathrix*) housed at the Endangered Primate Rescue Center (EPRC), Cuc Phuong National Park, Vietnam and found significant fTCDD-age effects, juveniles exhibiting levels of fTCDD substantially above those observed in adults. In this study we field-tested the utility of fTCDD EIAs for quantifying dioxin in wild black-shanked douc langurs (*Pygathrix nigripes*) residing in the Nam Cat Tien National Park (NCTNP), and confirmed fTCDD-age effects in a larger sample of juvenile *Pygathrix* at EPRC. EIA analyses were based on faecal samples obtained from 22 *P. nigripes* at NCTNP, 6 *P. cinerea* and 3 *P. nemaeus* housed at EPRC. NCTNP *P. nigripes* and EPRC *Pygathrix* exhibited similar equivalent fTCDD concentrations, averaging 8.62 pg/g +/- 5.6 SD and 11.1 pg/g +/- 7.4, respectively. Age continued to be a strong predictor of fTCDD levels in individuals originating from south of the 17th Parallel, but in divergent ways: EPRC juveniles exhibited nearly 3-fold higher fTCCD levels than those observed in adults, whereas CTNP adults exhibited nearly 2-fold higher dioxin concentrations than those observed in juveniles. The significance of these results resides in the development of a novel method to quantify dioxin levels in wild *P. nigripes* populations which has broad utility for assessing dioxin exposure in other *Pygathrix* populations inhabiting "dioxin hotspots" in southern Vietnam.

Nghiên cứu phơi nhiễm chất phát quang da cam ở quần thể voọc chà vá chân đen (*Pygathrix nigripes*) Vườn quốc gia Nam Cát Tiên, Việt Nam

Tóm tắt

Việt Nam là ngôi nhà của nhiều loài linh trưởng quý hiếm và đang đứng trước nguy cơ tiệt chủng. Trong khi những mối đe dọa chính như việc săn bắn, môi trường sống bị chia cắt và phá hủy được nói đến nhiều thì những số liệu về ảnh hưởng của chất phát quang, diệt cỏ chẳng hạn như dioxin (Agent Orange, TCDD) và các hợp chất liên quan (DRC) lên sức khỏe của động vật hoang dã lại ít được báo cáo. Trước đây chúng tôi đã chứng minh được việc hiệu quả của việc sử dụng enzyme

miễn dịch (EIA) trong việc đánh giá sự nhiễm chất dioxin (fTCDD) tập trung ở các cá thể chà vá (*Pygathrix*) được nuôi nhốt tại Trung tâm cứu hộ thú linh trưởng (EPRC) Vườn quốc gia Việt Nam. Kết quả khi đó cho thấy việc nhiễm chất fTCDD ở các cá thể bán trưởng thành luôn cao hơn những cá thể trưởng thành. Trong nghiên cứu này, chúng tôi sử dụng enzym EIAs để đánh giá mức nhiễm fTCDD ở quần thể chà vá chân đen (*Pygathrix nigripes*) tại Vườn quốc gia Nam Cát Tiên và tái khẳng định ảnh hưởng của độ tuổi đến mức nhiễm fTCDD tại trung tâm cứu hộ linh trưởng EPRC. Chất EIA được phân tích dựa trên việc thu thập các mẫu phân từ 22 cá thể chà vá chân đen tại VQG Nam Cát Tiên, 6 cá thể chà vá chân xám và 3 cá thể chà vá chân nâu tại EPRC. Kết quả, những cá mức nhiễm fTCDD của những cá thể chà vá chân đen tại Nam Cát Tiên và chà vá tại EPRC là tương đối như nhau, trung bình 8.62 pg/g+/- 5.6 SD và 11.1 pg/g +/- 7.4 SD. Độ tuổi có ảnh hưởng rõ rệt đến mức nhiễm fTCDD ở những cá thể có nguồn gốc ở phía nam vĩ tuyến 17 với hai khuynh hướng khác nhau. Tại trung tâm cứu hộ, những cá thể bán trưởng thành mức nhiễm fTCDD cao gấp ba lần con trưởng thành. Tại Nam Cát Tiên, mức nhiễm ở con trưởng thành lại cao gấp hai lần con bán trưởng thành. Kết quả trên cho thấy việc sử dụng phương pháp đánh giá mức nhiễm dioxin trong quần thể chà vá chân đen *P. nigripes* là hiệu quả và mở ra hướng nghiên cứu rộng hơn trên những quần thể khác ở miền Nam Việt Nam, nơi mà chất dioxin được sử dụng nhiều.

Introduction

Vietnam is home to some of the world's most critically endangered primates, including the Delacour's langur (*Trachypithecus delacouri*, <250 individuals) and Cat Ba langur (*Trachypithecus poliocephalus*, ca. 60 individuals) (Mittermeier et al., 2012). Encompassed within the Indo-Burma Biodiversity Hotspot region, Vietnam is one of the top 25 hotspots identified for urgent conservation action and is also among the nine leading hotspots in terms of endemics (Myers et al., 2000). The principle threat to biodiversity in this region continues to be habitat loss/degradation via the use of chemical defoliants [TCDD], logging, and clearing of land for agriculture, but when coupled with subsistence/trophy hunting for body parts, these combined threats can have a devastating impact on primate populations (Nadler & Streicher, 2004).

To date, there are few data on the impact of environmental herbicides, such as dioxins (i.e., Agent Orange, TCDD) and dioxin-related compounds (DRC), on the health of Vietnamese wildlife. Considered the most toxic of the organohalgen compounds, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a lipophilic chemical that bioaccumulates in the fat of vertebrates through the food chain (Fries, 1995) and persists in the environment for decades, thereby making it extremely hazardous to both wildlife and humans who are subject to long-term exposure. Anthropogenic sources of DRCs include by-products from the manufacture of chlorinated phenols used in pesticides in the 1930's, incineration, forest fires (Field & Sierra-Alvarez, 2008) and chemicals (i.e. TCDD), aerial transport of dioxins in the former being deposited in soils, plants, and water through combustion, industrialization, and e-waste recycling processes (Tanabe & Tu Binh Minh, 2010). While it is well known that dioxins are subject to photodegradation, it has been only recently demonstrated that TCDD can be degraded through the actions of aerobic bacteria from the genera *Sphingomonas*, *Pseudomonas* and *Burkholderia* (reviewed in Field & Sierra-Alvarez, 2008). Chemicals are the primary vehicle for the introduction of dioxin into the Vietnamese environment (Hatfield & 10-80 Committee, 2006), Agent Orange having been used extensively to defoliate forests and destroy crops during the Vietnam War. The United States government sprayed more than 45 million liters of TCDD on the forests and villages of Vietnam between 1965 and 1971, U.S. military CORPS Tactical Zones I and III receiving the vast majority of the herbicide (Stellman et al., 2003 in Brockman et al.,

2009). Humans residing in "dioxin hotspots" such as Bien Hoa City (Dong Nai Province), have been shown to exhibit markedly elevated serum dioxin levels (Schecter et al., 2003), but until recently, few data have been available on the extent of dioxin exposure in Vietnamese primates (Brockman et al., 2009; Vu Ngoc Thanh et al., 2010).

In previous research (Brockman et al., 2009), we demonstrated the utility of a novel enzyme immunoassay (EIA) procedure to quantify faecal dioxin (fTCDD) concentrations in *Pygathrix* housed at the Endangered Primate Rescue Center (EPRC), Cuc Phuong National Park (Ninh Binh Province), Vietnam and found significant fTCDD-age effects, juveniles exhibiting substantially elevated fTCDD levels above those observed in adults, suggesting that immature individuals *may* be at risk of functional developmental effects. We identified two adult individuals, a female *P. nemaeus* (6-02) and a male *P. cinerea* (7-25), which appeared to exhibit developmental consequences of possible TCDD exposure (Brockman et al., 2009), but we have found no definitive clinical links between TCDD exposure and reproduction or frequency of stillbirths in the *Pygathrix* population at EPRC.

The objectives of this research were 1) to field-test the utility of fTCDD EIA procedures to quantify dioxin in the faeces of a wild population of endangered black-shanked douc langurs (*Pygathrix nigripes*) (Fig. 1), residing in the Nam Cat Tien National Park, an area located in CORPS Tactical Zone III, which is known to have been extensively sprayed with TCDD during the Vietnam War (see below), and 2) to confirm fTCDD-age effects in a larger sample of red-shanked douc langurs, (*P. nemaeus*) (Fig. 2), and grey-shanked douc langurs (*P. cinerea*), residing at EPRC (Fig. 3). Based upon the results of our previous studies of fTCDD-age effects in *Pygathrix* housed at the EPRC (Brockman et al., 2009), we predicted that fTCDD concentrations in juvenile *P. nigripes* at NCTNP would be significantly elevated above those observed in adults.

Fig.1. Black-shanked douc langur (*Pygathrix nigripes*) at Nan Cat Tien National Park. Photo: Jonathan A. O'Brien.

Fig.2. Red-shanked douc langur (*Pygathrix nemaeus*) at the Endangered Primate Rescue Center. Photo: Tilo Nadler.

Fig.3. Grey-shanked douc langur (*Pygathrix cinerea*) at the Endangered Primate Rescue. Photo: Tilo Nadler.

Material and Methods

Study site

Cat Tien National Park is located ~160 km northwest of Ho Chi Minh City and covers an area of approximately 740 km^2 situated within the three Provinces of Dong Nai, Lam Dong, and Binh Phuoc,

Fig.4. Map of Cat Tien National Park Complex, indicating locations of Tay Cat Tien and Nam Cat Tien.

11o21' – 11o48'N, 107o 10' – 107o 34'E. The CTNP complex is divided into two large sections, Cat Loc in the north and Cat Tien in the south, the latter being further subdivided into Tay Cat Tien and Nam Cat Tien (Fig. 4) which is home to nine species of primates (Polet et al., 2004), including *P. nigripes*. Militarily strategic districts located in the Provinces of Dong Nai and Binh Phuoc were targeted by the U.S. military for extensive aerial spraying with Agent Orange during the Vietnam War, including Bien Hoa (837,755 L), Long Khanh (6,017,499 L), Phuoc Long (7,570,823,568 L) and Binh Long (148,762 L). 367,090 L of Agent Orange was sprayed on the entire Province of Lam Dong (calculated from HERPS Tape, 2000). CTNP is highly seasonal, a five-month dry season from December to April alternating with a seven-month wet season from May to December, when rainfall averages 2175-2975 mm annually (Vidya et al., 2007). Habitats within the NP are diverse and include not only one of the last remaining lowland evergreen/semi-evergreen forests in Vietnam, but also large expanses of deciduous and secondary forests, dominated by bamboo forests, grassland, and wetlands bordering the Dong Nai River (Polet et al., 2004). This research was conducted at NCTNP where a substantial population of black-shanked douc langurs is known to reside, i.e. 18 groups, ~109 animals, three of which have been reported to contain habituated adults and immatures which are regularly encountered and easily observed (Polet et al., 2004).

Subjects

Subjects in this study included individuals from three species of douc langurs: *P. nigripes* (black-shanked douc langurs, 14 adults, 8 juveniles) residing at NCTNP, and *P. nemaeus* (red-shanked douc langurs, 2 adults, 1 juvenile) and *P. cinerea* (grey-shanked douc langurs, 4 adults, 2 juveniles) residing at EPRC, the latter having been donated from tourists or confiscated from known capture locations south of the 17th Parallel (demilitarized zone, DMZ). FTCDD results from 17 subjects from our previous study conducted at EPRC in 2006 (Brockman et al., 2009) were included in our analyses here in order to increase sample sizes of individuals in the adult and immature age classes. As reviewed previously in Brockman et al. (2009) with the exception of Hong Minh Duc's studies (2007) of *P. nigripes* at Nui Chua and Phuoc Binh National Parks (Ninh Thuan Province), quantitative data on the social structure, day range, mating behaviour and behavioural ecology of douc langurs is extremely limited, and data on life history parameters are virtually nonexistent, the likely consequence of the scarcity of habituated populations of douc langurs

available for long-term demographic study. Recent studies of ontogenetic cranial development and dental eruption patterns in captive *Pygathrix* at EPRC suggest, however, that the adult age-class is attained at 5-6 years of age (Stephen & Nadler, 2012). Previous studies by Ruempler (1998 in Nadler et al., 2003) indicate that male and female douc langurs become sexually mature at ~5-8 years and ~5-7 years, respectively. Age estimates for wild *P. nigripes* in this study were based on observations of body size differences, adults being approximately twice the size (or more) than juveniles. Sexes of the wild subjects could not be reliably determined. EPRC subjects ranged in age from 1 to 14 years, age estimates having been assigned by EPRC staff during quarantine based upon patterns of dental eruption and tooth wear (Nadler, unpubl. data). I received permission to conduct research at CTNP and EPRC from Mr. Tranh Van Thanh, Park Director, Nam Cat Tien National Park and from Tilo Nadler (TN), Director EPRC (June 2004), respectively. This research was approved by the University of North Carolina-Charlotte Institutional Animal Care and Use Committees (UNCC protocol no. 09-404).

Data collection

Nam Cat Tien National Park

A total of 102.5 hours were spent in NCTNP surveying the population of ~100 resident largely unhabituated black-shanked douc langurs (Fig. 1) located ~.5-1.5 km from the Ranger Station. Four different groups were subsequently located (Table 1). Visual contact occurred most frequently at dawn and terminated at noon when groups moved into inaccessible regions of the park dominated by thick bamboo forests. During the total 2.78 hrs of animal contact, groups were in view for 5-20 min during which 22 (~10 g) faecal samples were collected immediately after voiding (or soon thereafter) from adult and immature individuals (Fig. 5 and 6; Table 1) and placed in 2 x 3 inch Ziploc bags which were labelled as to individual, date and time of collection (Brockman et al., 2009). At the end of each daily field session, the faecal samples were vacuum sealed in individual zip-loc bags and then repackaged again in quart-sized Zip-loc bags (labeled adult and immature) until they could be transported to an ultra-cold freezer at the Institute of Tropical Biology in Ho Chi Minh City 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 Robert Harrison, Director (CAPE Technologies, Inc.). Vietnamese and U.S. permits were obtained to ship the faecal samples to CAPE Technology laboratory, South Portland, ME where EIA procedures were employed to quantify fTCDD concentrations as described below.

Fig.5. Adult faecal sample from black-shanked douc langur (*Pygathrix nigripes*) at Nan Cat Tien National Park. Photo Diane K. Brockman

Fig.6. Juvenile faecal sample from Black-shanked douc langur (*Pygathrix nigripes*) at Nan Cat Tien National Park. Photo Diane K. Brockman

Table. 1. Faecal samples collected from wild *Pygathrix nigripes* at Nam Cat Tien National Park (n=22 samples).

Sample ID	Group	Adult/Immature	g sample/tube	pg/gm TCDD	Notes
B1-1	Gang of 5	Adult	9.7	5.8	conclusive; negative spike[1]
B1-2	Gang of 5	Adult	6.2	2.8	EPA Method 4025m (soil)
B1-3	Gang of 5	Immature	7.8	7.0	
B2-1	Gang of 11	Adult	6.6	16.5	Mean TCDD of 2 runs (8.3, 24.0)
B2-2	Gang of 11	Adult	8.1	4.4	
B2-3 & 10-3	Gang of 11	Immature pooled sample	7.1	1.9	EPA Method 4025m (soil)
B3-1	Gang of 35	Adult	9.2	7.8	
B3-2	Gang of 35	Adult	7.8	0.0	Inconclusive; negative spike[1]
B4-1	Gang of 35	Adult	5.4	10.3	EPA Method 4025m (soil)
B4-2	Gang of 35	Adult	6.6	15.2	
B4-3	Gang of 35	Adult	5.5	8.7	
B4-4	Gang of 35	Adult	4.0	19.9	
B5-1/2	Yellow Trail Group	Immature pooled sample	5.1	2.9	EPA Method 4025m (soil)
B5:2/18-2	Yellow Trail Group	Adult	9.8	20.0	
B5:2/19-1	Yellow Trail Group	Adult	6.6	6.0	EPA Method 4025m (soil)
B6-1/3/4	Gang of 5	Immature pooled sample	6.3	5.0	Mean of 2 unspiked/dupes
B6-2	Gang of 5	Adult	4.0	8.2	EPA Method 4025m (soil)
B7-1/2/3/4	Gang of 35	Immature pooled sample	7.9	18.2	Inconclusive; negative spike[1]
B8-1/2/3/4	Gang of 35	Immature pooled sample	6.7	11.1	Mean of 2 unspiked/dupes
B9-1/2/3/4	Gang of 35	Immature pooled sample	5.9	5.4	
B11-5/6/7/8	Gang of 35	Immature pooled sample	4.9	5.9	EPA Method 4025m (soil)
B11-9	Gang of 35	Adult	9.7	4.4	EPA Method 4025m (soil)
Adult mean TCDD				**10.35 +/- 6.1 SD**	**1.8-fold higher**
Immature mean TCDD				**5.64 +/- 3.1 SD**	**p=0.08**

[1]: Not included in analysis of results.

Cuc Phuong National Park

Tilo Nadler, Director of EPRC, identified three juvenile and six adult douc langurs from southern Vietnam which had come into the population since my previous research there in December 2006 and were thus candidates for faecal collections (Table 2). Single (10 g) faecal samples were collected from the nine subjects and placed in individually labelled 2 x 3 inch Ziploc bags and placed in EPRC's ultra-cold freezer until they could be shipped to the U.S. for analysis. Vietnamese and U.S. permits were obtained to ship the fecal samples to the CAPE Technology laboratory, South Portland, ME where EIA procedures were used to quantify fTCDD levels as described previously (Brockman et al., 2009).

Table. 2. Faecal samples collected from gray-shanked (*P. cinerea*) and red-shanked (*P. nemaeus*) douc langurs at the Endangered Primate Rescue Center, Cuc Phuong National Park (n=9 samples).

Animal ID	Species	Sex	Birth Date	Capture Location	Age	g sample/tube	pg/gm TCDD	Notes
7-49	*P. cinerea*	Male	2009	Quang Nam	~ 1 yrs	2.84	27.0	
7-45	*P. cinerea*	Female	2007	A Lao	~ 3 yrs	4.45	11.8	
6-60	*P. nemaeus*	Female	2007	?	~ 3 yrs	5.07	3.9	See Note[1]
7-46	*P. cinerea*	Male	2005	Ba To	~ 5 yrs	4.41	9.8	
7-52	*P. cinerea*	Male	2005	?	~ 5 yrs	8.25	6.6	
7-47	*P. cinerea*	Male	2004	An Lao	~ 6 yrs	6.10	6.1	
7-39	*P. cinerea*	Male	2003	An Lao	~ 7 yrs	5.25	11.0	
6-53	*P. nemaeus*	Female	2003	Binh Dinh	~ 7 yrs	6.19	7.7	
6-63	*P. nemaeus*	Male	2002	Hue	~ 8 yrs	4.43	6.6	
Adult mean TCDD							**7.0 +/- 2.6 SD**	**p=0.07**
Immature mean TCDD							**19.4 +/- 10.7 SD**	**2.8-fold higher**

[1]: Not included in analysis of results; could not confirm origin south of the 17th Parallel.

Faecal dioxin extraction and EIA

Nam Cat Tien NP Phase I Faecal Dioxin Extraction and EIA

Dr. Robert H. Harrison (Director, CAPE Technologies) received 22 frozen *P. nigripes* faecal samples in April 2010 which were immediately placed in his laboratory's ultra-cold freezer at -20^{o} C. Fourteen faecal samples were subsequently hydrolyzed and extracted prior to being enzyme immunoassayed

using EPA Method 4025m which had been previously validated for *Pygathrix* (Brockman et al., 2009). EIAs of the remaining 8 faecal samples were postponed until issues relating to poor spike recoveries and incomplete hydrolysis (n=3 samples) could be resolved (see Phase II below).

Processed sample appearance and method suitability

Sample hydrolysates appeared grossly different from those reported in Brockman et al. (2009) and different than any other sample matrix previously analyzed at CAPE Technologies using this protocol. After hydrolysis and extraction the centrifuged samples were well separated (Fig. 7) and the extracts appeared "normal", but the hydrolysates did not. "Normal" hydrolysates are entirely liquid and have a distinct purple color. These hydrolysates were dark brown to black with a very slight purplish cast and contained a large amount of solid residue, including a significant amount of material adhering to the glass above the hydrolysate, something not seen with other samples.

Fig.7. 125 ml hydrolysis/extraction bottles after hydrolysis, extraction, and centrifugation. The acid hydrolysate is dark material on bottom and the solvent extract of that hydrolysate is the clear liquid on top. This liquid was removed by suctioning and processed through the cleanup described in the materials and method section.

After the extracts were removed for cleanup and analysis, the hydrolysates were examined further. Several hydrolysates were treated with additional conc. HCl, approximately doubling to tripling the ratio of acid to sample. After shaking, solvent extraction, centrifugation, and extract removal, these hydrolysates remained largely insoluble. Heating of the hydrolysate to roughly 50°C had no visible effect on the hydrolysate.

Other hydrolysates were treated by filtering the solids (which often had a weight approaching that of the original sample), washing with water, then treatment with 6 N NaOH. The remaining processing was otherwise identical to the repeat acid treatments, including heat treatment. After treatment, these samples appeared nearly identical to the acid treated samples.

The possibility of effect of sample size on the process was also considered. Sample sizes were variable, but within a fairly narrow range (mean ± SD = 7.2 ± 1.6 g). The maximum sample size analyzed in the current group (9.8 g) was smaller than the largest sample analyzed previously (11.3 g; Brockman et al., 2009). Additionally, the protocol was also modified slightly from Brockman et al. (2009) to include a larger amount of conc. HCl (60 ml here vs. 50 previously). Since the resulting hydrolysates appeared uniform across the range of sample sizes, neither acid:sample ratio nor sample size appeared to explain these divergent outcomes.

There is no currently obvious explanation for this difference between EPRC samples (Brockman et al., 2009) and the current CTNP samples other than perhaps species-specific diet differences and/or gut microflora composition.

QA data

Standard curve data (and their QA parameters), used as the basis for concentration calculations, were within recent norms. Method blanks, unspiked and spiked, gave results within

normal ranges with one exception (flagged as possible invalid due to protocol deviation). Three sets of intra-batch duplicates gave % cv values of 19, 5, and 17. However, sample spike recoveries ranged from 160% to -62%, with no obvious pattern.

Only two original samples were large enough to split into 4 subsamples. One of these, B1-3, had all four subsamples (two unspiked and two spiked) analyzed in different sub-batches of Run A. The calculated pg/g TEQ values ranged from 2.1 to 7.6, all below the unspiked method blank for Run A. The spike recovery values for the two unspiked/spiked pairs were 10 and -62%. The other large sample, B2-1, was split between Run A and Run B. Spike recoveries were 57% and 76%, both within acceptable ranges. Unspiked sample values, uncorrected for spike recovery, were 8.3 and 24.0 pg/g. While there is no obvious explanation for this discrepancy, it should be noted that this sample was different than all the other samples in one potentially important respect. The amount of color in the extracts was significantly greater than all the other samples (Fig. 8); for repeat processing of original sample hydrolysates- see legend for complete explanation).

Fig.8. Solvent extracts removed from repeat acid hydrolysis treatments of original hydrolysates. Variations in volume primarily reflect the effect of different amounts of solid residue in the original hydrolysate. Extract color and variations thereof were parallel to what was seen for the original extracts (no photos taken at that point). Note color difference in pairs 2-1, 3-2, and 7-P (triplicates).

While color by itself does not imply sample preparation issues, outlier samples often pose challenges which are difficult to assess and overcome with limited sample quantities such as in this study. The only way to guarantee sample homogeneity during preparation is by complete liquefaction during acid hydrolysis. Since this was not observed for any samples, it is reasonable to assume that none of the samples were adequately homogenized after splitting.

Because of the unusual nature of the hydrolysates noted above and the variable spike recovery values, the repeat treatments noted in the Method Suitability section above included some spikes, of both previously spiked and previously unspiked samples. The resulting data showed a wide range of spike recovery values similar to that seen with the original samples.

Interpretation of data for unspiked unknown samples

Quality assurance data for some samples were acceptable, yet it remains difficult to justify specific conclusions about any individual sample concentrations. Since all samples presented the same difference in physical appearance after hydrolysis, it is reasonable to assume they all showed whatever differences exist in sample chemistry between these samples and the previous EPRC samples. It is possible that dietary components such as foliar lignin vary greatly due to both species and geography. Such a difference might explain both poor spike recoveries and incomplete hydrolysis.

Regardless, significant solid residue in the acid hydrolysate indicated that the entire sample was not available to the solvent for extraction. In contrast, a fully liquid acid hydrolysate can interact completely with the extraction solvent and thus the entire sample is available for extraction and subsequent analysis.

Revised sample preparation protocol

The above data and observations lead to the possibility of an alternative sample preparation protocol being superior (see Phase II below). The justification for the current protocol was that vegetation samples previously analyzed at CAPE Technologies gave adequate results in terms of appearance and QA data. Hence, faecal samples from CTNP folivores should have been amenable to the same sample preparation protocol. Such a protocol allows the complete removal of even intracellular and membrane integrated dioxin, which may not be removable with a simple solvent extraction. This was borne out in the earlier work reported in Brockman et al. (2009), to the maximum degree possible within the structure of the QA samples analyzed.

In this case however, it was more reasonable to use a solvent extraction coupled with vigorous mechanical agitation, such as used for soils analyzed by Method 4025m. Samples are agitated vigorously in a glass vial containing sample, solvent, sodium sulfate for drying, stainless steel BBs for gross scale pulverization, and sand for fine scale pulverization. The resulting pellet after extraction and centrifugation is generally extremely fine-grained (like silt) and entirely homogeneous. However, since this method is not routinely used by CAPE Technologies for biological samples, validation for fecal samples was highly desirable (see below).

Nam Cat Tien Phase II faecal dioxin extraction and EIA

In Phase II, the remaining eight (of 22) NCTNP faecal samples were extracted using an acetone:hexane soil method of extraction and subsequently enzyme immunoassayed to quantify dioxin concentrations.

Soil method extraction and EIA

All glassware to be in contact with method blanks or samples was first rinsed with toluene, and then air dried before use. Samples were weighed using an identical tare bag to determine net weights. Sodium sulfate was added directly to samples in their original Ziploc bags, the bags were then closed and the sample was kneaded by hand until the mixture was uniform and free flowing. Pooled samples were combined and mixed further, and the samples were then split by weight (as appropriate), and transferred to 125 ml glass bottles for extraction. Sand (10 g) and BBs (n=5) were added to each bottle. Spikes of 100 pg 2378-TCDD (10 µl of 10 ppb stock ppb in toluene) were added to selected samples for quality assurance and the bottles were shaken until no clumps were visible.

Acetone:hexane (50 ml of 1:1) was added to each bottle and all were shaken vigorously for 10 hours. Bottles were centrifuged for 20 min and the supernatants were poured off into 60 ml vials and weighed. The sample pellets were washed with 30 ml hexane, hand shaken, and spun and the supernatants recovered and weighed as before. A second wash was performed in the same manner using 20 ml hexane. These washes were added to the evaporation vials, 1 ml of tetradecane was added to each vial, and the pooled supernatants were evaporated under a stream of clean compressed air at approx. 45° to 65°C. After evaporation of the acetone and hexane, the tetradecane residue was diluted with 5 ml hexane. Fine acid silica (FAS) was added to each hexane sample and swirled until the supernatant was clear and colorless.

Carbon columns (CAPE Technologies) were attached to 15 mm acid silica columns (CAPE Technologies) and 8 ml hexane was added to each column. Interstitial air was removed by vacuum depressurization, followed by pressurization to purge residual air from the carbon column. Samples were added to the acid silica columns, including both supernatant hexane and acid silica from sample

pretreatment. Columns were pressurized to push the sample into the columns, followed by a hexane wash. Hexane (3 x 10 ml per column) was added and pushed through the coupled column system. When air penetrated the lower portion of the acid silica columns, flow was stopped and the carbon columns were moved to clean 15 mm reservoirs. Carbon columns were washed in the forward direction with 6 ml 1:1 hexane:toluene, which was discarded. Carbon columns were reversed on the same reservoir and eluted with 12 ml toluene, which was captured in 16x125 mm tubes. Keeper solution (62.5 µl/tube) was added and the toluene was evaporated under a stream of clean compressed air at ≤85°C. Evaporation tubes were centrifuged 15 min to concentrate the keeper residue at the bottom. Methanol was added to reconstitute the samples, which were added to the immunoassay tubes and incubated 16 hrs. The EIA was finished according to the kit insert (IN-DF1).

EPRC fecal dioxin extraction and EIA

Nine (10 g) faecal samples were collected from wild-born douc langurs arriving at EPRC since December 2006 and subsequently extracted and enzyme immunoassayed as described previously (Brockman et al., 2009). The procedure is briefly described as follows: solvents utilized were HPLC grade (Fisher Scientific), except for toluene, which was residue analysis grade (Burdick & Jackson). Acids were ACS grade (Fisher). 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.

Faecal samples were collected in 2 x 3 inch Ziploc bags, shipped frozen, then stored at -20°C until analysis. All subsequent procedures were performed at 20-25°C unless noted otherwise. Ziploc bags were weighed to 1 mg before thawing and then weighed again after the sample was removed to determine the weight of the sample delivered to the cleanup procedure.

Samples were removed from their bags by dispersing the sample in conc. HCl and pouring bottle for hydrolysis. Sample bags were rinsed with additional conc. 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 2378-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 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. H2SO4 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 INDF1).

Standards in keeper and samples prepared as described above were added to sample diluent in tubes coated with antidioxin 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 color was allowed to develop for 30 min. Stop solution containing 1 N HCl was added and the optical density (OD) of each tube was read at 450 nm using a portable differential photometer (Artel).

Statistical Tests and Assessments of fTCDD-Population and-Age Interactions

Statistical analyses were performed using SIGMAPLOT 12 (Systat Software, Inc. Point Richmond, CA). Univariate statistical tests (Student's t-test, Mann-Whitney U test) were used to examine variation in fTCDD levels in NCTNP and EPRC douc langurs. The effect of age on fTCDD concentrations in adult and juvenile douc langurs was tested using multivariate (multiple linear regression) and univariate analyses. Variables which departed from normality were adjusted by removal of outliers (samples more than two standard deviations from the mean) or were log transformed. Results are reported as means ± SD with significance set at $P < 0.05$.

Results

Sample preparation in phase I vs phase II

Results of faecal extraction/EIA procedures of 3 of 22 Phase I faecal samples were eliminated from further analysis due to negative spike recoveries and incomplete hydrolysis. Employing the soil method of faecal extraction in Phase II analyses completely eliminated the problematic issues encountered in the Phase I extraction procedures.

FTCDD concentrations in *Pygathrix* from South of the 17th Parallel

Results from a reanalysis of FTCDD concentrations obtained from the 17 *Pygathrix* in 2006 (Brockman et al., 2009) pooled with those from this study, showed that *Pygathrix* originating from south of the 17th Parallel exhibit fairly low fTCDD levels, averaging 8.11 pg/g fTCDD ± 5.8 SD (n=44). Assessments of the effect of age on fTCDD levels showed that dioxin concentrations in juveniles were ~69% higher than those observed in adults (juvenile: 10.73 pg/g fTCDD ± 8.2, n=11; adult: 7.39 pg/g fTCDD ± 4.7 SD, n=33, P=0.31, Table 3).

Table. 3. Faecal TCDD concentrations in *Pygathrix*.

Location/ species	Total Mean fTCDD +/- SD (pg/g wet weight)	P Value	Adult Mean fTCDD +/- SD (pg/g wet weight)	Juvenile Mean fTCDD +/- SD (pg/g wet weight)	P Value
Pygathrix: south of the DMZ	8.11 +/- 5.8 (range: 1.9 – 27.0) (n=44)		7.39 +/- 4.7 (range: 2.8 – 20.0) (n=33)	10.73 +/- 8.2 (range: 1.9 – 27.0) (n=11)	0.31
NCTNP: *P. nigripes*	8.62 +/- 5.6 (range: 1.9 – 20.0) (n=19)		10.35 +/- 6.1 (range: 1.9 – 20.0) (n=12)	5.60 +/- 3.0 (range: 1.9 – 11.1) (n=7)	0.07
EPRC: *Pygathrix*	7.94 +/- 6.1 (range: 3.0 – 27.0) (n=25)	0.58	5.7 +/- 2.5 (range: 3.0 – 11.0) (n=21)	19.7 +/- 6.3 (range: 11.8 – 27.0) (n=4)	0.001

FTCDD and the effect of age in *P. nigripes* at NCTNP

At the population level, *P. nigripes* exhibited low total fTCC levels, averaging 8.62 pg/g fTCDD ± 5.6 SD, n=19. Results of Student's t-tests showed that adult *P. nigripes* exhibited nearly 2-fold higher fTCDD levels than those observed in juveniles (adult: 10.35 pg/g ± 6.1 SD, n=12; juvenile: 5.64 pg/g ± 3.1 SD, n=7) though not significantly so (P=0.07, Table 3, Fig. 9).

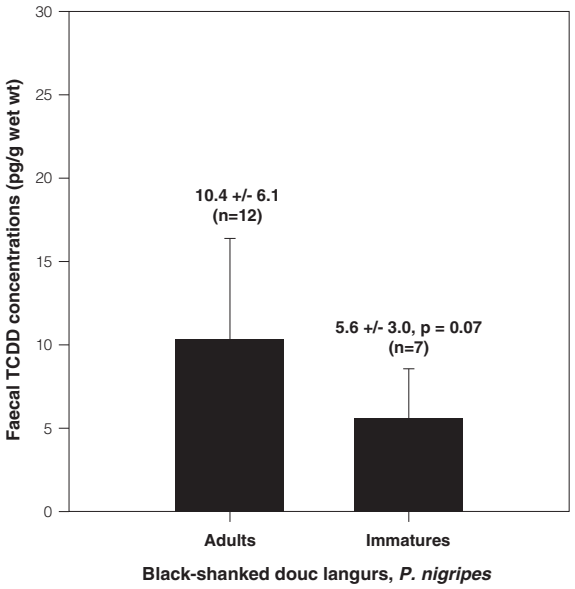

Fig.9. Age related variation in fTCDD concentrations in black-shanked douc langurs (*Pygathrix nigripes*) at Nam Cat Tien National Park.

FTCDD concentrations and the effect of age in *P. nemaeus* and *P. cinerea* at EPRC

The small sample size of *P. nemaeus* originating from south of the 17th Parallel (n=2) precluded an examination of species differences in fTCDD levels; previous studies demonstrate, however, no effect of species on fTCDD concentrations (Brockman et al., 2009). At the population level, EPRC douc langurs in this study exhibited low total fTCDD concentrations, averaging 11.1 pg/g TCDD ± 7.4 SD, n=9. Results from Mann-Whitney U tests indicate that juvenile douc langurs exhibited nearly 3-fold higher fTCDD levels than those observed in adults (juvenile: 19.4 pg/g fTCDD ± 10.7 SD, n=2; adult: 7.0 pg/g fTCDD ± 2.6 SD, n=7) though not significantly so (P=0.07, Table 2). Results from pooling the fTCDD values obtained previously from the 17 *Pygathrix* at EPRC (Brockman et al., 2009) with those obtained here indicate that total mean fTCDD concentrations in this EPRC population were somewhat lower than the subpopulation above, averaging 7.94 pg/g fTCDD ± 6.1 (n=25), being broadly equivalent to those obtained from *P. nigripes* at NCTNP (i.e. 8.62 pg/g fTCDD, Table 3). Pearson Product Moment Correlation tests showed a significant negative correlation between fTCDD levels and age (r = - 0.72, P = < 0.0001, n = 25). Mann-Whitney Rank U tests indicated that fTCCD levels in immatures were significantly higher than those in adults south of the 17th parallel, juveniles exhibiting nearly four-fold higher fTCDD levels that those observed in adults (juvenile: 19.7 pg/g fTCDD, ± 6.3 SD, n=4; adult: 5.7 pg/g fTCDD ± 2.5 SD, n=21, P=0.001, Table 3, Fig. 10).

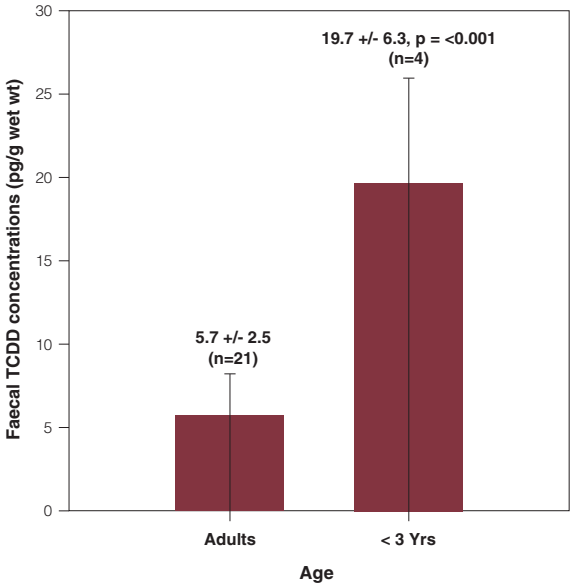

Fig.10. Age-related variations in fTDD concentrations in douc langurs (*Pygathrix*) at the Endangered Primate Rescue Center.

Discussion

The results of this study demonstrate that the extraction procedures employed in our previous study (Brockman et al., 2009) were ineffective in extracting TCDD from faeces obtained from wild *P. nigripes*. However, by employing a modified soil method (EPA Method 4025m) of fTCDD extraction, we were able to obtain a fully liquid acid hydrolysate which could interact completely with the extraction solvent, thereby enabling the entire *P. nigripes* faecal sample to be available for extraction and subsequent analysis. This study further demonstrates that our previously validated novel EIA procedures reliably quantified relative fTCDD concentrations in wild black-shanked douc langurs residing at NCTNP, an area that was exposed to high levels of dioxin and dioxin-like chemicals during the Vietnam War. Although total fTCDD concentrations in this population were fairly low (mean: 8.6 pg/g fTCDD), they were broadly equivalent to those observed in wild-caught red-shanked and grey-shanked douc langurs residing at EPRC (~7.94 pg/g fTCDD, Table 3), though not as high as might be expected for a site exposed to high levels of dioxin. As noted previously (Brockman et al., 2009), these low fTCDD levels likely reflect background levels of environmental dioxin and/or exposure pathways associated with a folivorous diet and arboreal lifestyle, the latter limiting dermal exposure to dioxin in soils.

Potential sources of dioxin in *P. nigripes* include ingestion of contaminated plants and soils at NCTNP as this species has been known to feed on the ground, particularly in the dry season, and occasionally consume mud at small pools in the forest (Hoang Minh Duc, 2007). Black-shanked douc langurs at Hon Heo (Khanh 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), but no data are available on how much time (if any) *P. nigripes* spend resting/moving on the ground at NCTNP. As noted previously (Brockman et al., 2009), one of the known pathways of dioxin/dioxin-like compounds into vertebrates includes ingestion of contaminated soil by animals (Fries, 1995). Previous studies have shown that soil-to-plant transport of organohalgen compounds such as dioxin occur principally via volatization from the soil surface/disturbed soils and subsequent atmospheric deposition on plants (Fries, 1995), and are thus a likely source of exposure in this population of *P. nigripes* at NCTNP.

Concordant with our previous findings (Brockman et al., 2009), age was a reliable predictor of fTCDD concentrations in *Pygathrix* originating from south of the 17th parallel, fTCDD levels among juveniles being moderately elevated above those observed in adults (Table 3). Age was also a strong predictor of fTCDD concentrations in douc langur populations residing at NCTNP and EPRC, but in divergent ways. FTCDD levels in juvenile *P. cinerea* at EPRC were nearly 3-fold higher than those observed in adults, whereas dioxin concentrations in adult *P. nigripes* at NCTNP were nearly 2-fold higher than those observed in juveniles for reasons that are unclear. Overall, fTCDD concentrations in *Pygathrix* at EPRC were found to decline significantly with age (Fig. 11).The higher concentrations of fTCDD observed in the two juvenile *P. cinerea* at EPRC may derive from having originated from areas which were heavily sprayed with TCDD during the Vietnam War. Confiscation location data from EPRC quarantine records indicate that male 7-49 (1 year) and female 7-45 (3 years) originated from Quang Nam and An Lao District, Binh Dinh Provence, respectively (Nadler, unpubl. data), these regions having been intensively spayed with dioxin during the Vietnam War. Between September 1965 and June 1970, 289 and 194 US military missions were flown into Binh Dinh and Quang Nam Provinces, spraying 2,094,510 l and 1832953 l of Agent Orange in those provinces, respectively (calculated from HERPS Tape, 2000). The levels of likely residual TCDD concentrations remaining in soils in these provinces are unknown.

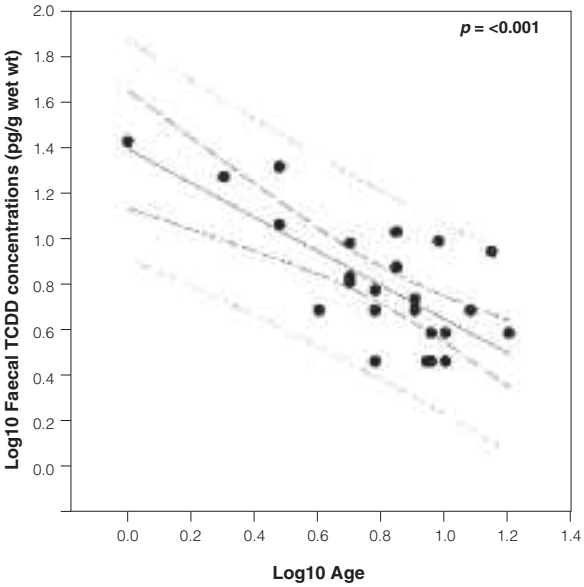

Fig.11. FTCDD concentrations decline with age in douc langurs at the Endangered Primate Rescue Center.

The divergent fTCDD-age results obtained from *P. nigripes* at NCTNP are difficult to explain, but they may be related to the potentially contaminated herbivorous diet consumed by obligate folivores. As noted previously (Brockman et al., 2009), dioxin has the capacity to persist in soils to a depth of greater than 10 cm for decades (Hatfield & 10-80 Committee, 1998), and has been estimated to be available for plant uptake for as long as 100 years (Paustenbach et al., 1992). Residual TCDD in soils at NCTNP (if any) can therefore function as a TCDD reservoir, having the capacity to remobilize and transport TCDD as dust which is deposited on the leaves and buds of *Afzelia xylocarpa* known to be consumed by adult *P. nigripes* at NCTNP.

Alternatively, divergent age-related variations in TCDD concentrations observed in NCTNP *P. nigripes* and *Pygathrix* at EPRC may be a consequence of species-specific and age-related variations in microbial communities that inhabit the gastrointestinal tract (Ochman et al., 2010). The array of microorganisms that coexist peacefully with their hosts is collectively referred to as microbiota or microflora, those inhabiting the gut being composed of strict anaerobes, over 50 bacterial phyla of which have been described to date (reviewed in Sekirov et al., 2010). The composition of microfloral communities is known to be host specific, changing continually throughout an individual's lifetime and susceptible to external (i.e. diet, geography) and internal (i.e. disease state, stress) modifications. Studies in humans show that infants are inoculated with maternal microbes at birth as they transverse the birth canal and that during the first year of life these communities are relatively simple, but then diversifies and stabilizes after weaning, resembling the microbiota communities of young adults/adults. Microbial communities maintain homeostasis of the intestinal mucosa and are crucially important for the development of systemic immune systems and for modulating nutritional and metabolic responses (Sekirov et al., 2010). Recent studies of fecal community composition in mammals show that phylogeny and diet influence bacterial diversity, bacterial phyla being observed to be partitioned among hosts according to diet, herbivore microbiota containing the most phyla, followed by omnivores and carnivores (Ley et al., 2008). An investigation of microbial communities in great apes (chimpanzees, gorillas) and humans

reveals clear species-specific differences in microbial community structure (Yildirim et al., 2010), and while gut microbial communities of *Pygathrix* are unknown, one might reasonably posit that the divergent fTCDD-age effects observed between the NCTNP and EPRC populations of douc langurs may be related to species-specific differences in microbial communities and the likely influence of geography and diet on the microbiota in these respective populations. While it has been previously demonstrated that aerobic bacteria effectively degrade 2,3,7,8-TCDD in sediments (Field & Sierra-Alvarez, 2008), a similar role of anaerobic/gut microflora for dioxin degradation in vertebrates, including primates, has yet to be demonstrated.

Conclusion

The results of this research show that by employing a modified soil method (EPA Method 4025m) of fTCDD extraction in conjunction with a novel EIA procedure, we could reliably quantify fTCDD concentrations in wild *P. nigripes* at NCTNP. We also confirmed fTCDD-age effects in a larger sample of *P. nemaeus* and *P. cinerea* residing at EPRC and showed that juveniles exhibited substantially elevated fTCDD levels above those observed in adults whereas fTCDD concentrations in adult *P. nigripes* were substantially elevated above those observed in juveniles for reasons that are unclear, but may be related to diet and species-specific variation in gut microflora.

Acknowledgements

We are grateful to Tranh Van Thanh, Park Director, and Nguyen Van Thanh, Manager of Science and Technical Development, for granting DKB permission to conduct research at NCTNP. We are especially grateful to Bert Covert for his unflagging encouragement and support of this research and to Tilo Nadler, Director, and his staff for their continued enthusiastic support of our research at EPRC. We are also indebted to an anonymous reviewer who provided comments that substantially improved the manuscript. Funding was provided by the Schwartz Family Foundation Trust and by a Senior Faculty Grant from the College of Liberal Arts and Science, University of North Carolina Charlotte.

References

Brockman DK, **Harrison RO** & **Nadler T** (2009): Conservation of douc langurs in Vietnam: An assessment of biomarkers and Agent Orange exposure in *Pygathrix* at the Endangered Primate Rescue Center, Cuc Phuong National Park, Vietnam. Vietnamese J. Primatol. vol.1(3), 45-64.

Data Management Agency, **USMACV** (1970): Herbicide Report System (HERBS). Document No. DARU07 (US Military Assistance Command) Vietnam, San Francisco.

Field JA & **Sierra-Alvarez R** (2008): Microbial degradation of chlorinated dioxins. Chemosphere 71, 1005-1018.

Fries GF (1995): A review of the significance of animal food products as potential pathways of human exposures to dioxins. J. Anim. Sci. 73, 1639-1650.

Hatfield Consultants & **10-80 Committee** (2006): Identification of a new Agent Orange/dioxin contamination hot spots in southern Vietnam. 10-80 Division, Ministry of Health, Viet Nam and Hatfield Consultants Ltd., West Vancouver, BC, Canada.

Hoang Minh Duc (2007): Ecology and conservation status of the black-shanked douc (*Pygathrix nigripes*) in Nui Chua and Phuoc Binh National Parks, Ninh Thuan Province, Vietnam. PhD thesis, University of Queensland, Australia.

Ley RE, **Hamady M**, **Lozupone C**, **Turnbaugh PJ**, **Ramey RR**, **Bircher JS**, **Schlegel ML**, **Tucker TA**, **Schrenzel MD**, **Knight R** & **Gordon JI** (2008): Evolution of mammals and their gut microbes. Science 320, 1647-1651.

Mittermeier RA, **Rylands AB**, **Schwitzer C**, **Taylor LA**, **Chiozza F** & **Williamson EA (eds.)** (2012): Primates in Peril: The World's 25 Most Endangered Primates 2010–2012. IUCN/SSC Primate Specialist Group, International Primatological Society and Conservation International, Arlington.

Myers N, **Mittermeier RA**, **Mittermeier CG**, **da Fonseca GAB** & **Kent J** (2000): Biodiversity hotspots for conservation priority. Nature 403, 853-858.

Nadler T & **Streicher U** (2004): The Primates of Vietnam – An Overview. In: Nadler T, Streicher U & Ha Tang Long (eds.): Conservation of Primates in Vietnam; pp. 5-11. Frankfurt Zoological Society. Hanoi.

Nadler T, **Momberg F**, **Ngugen Xuan Dong** & **Lormee N** (2003): Vietnam Primate Conservation Status Review 2002-part 2 leaf monkeys. Fauna & Flora International-Vietnam Program and Frankfurt Zoological Society. Hanoi.

Ochman H, **Worobey M**, **Chih-Horng Kuo**, **Ndjango JBN**, **Peeters M**, **Hahn BH** & **Hugenholtz P** (2010): Evolutionary relationships of wild hominids recapitulated by gut microbial communities. PLoS Biology 8 (11), 1-11.

Paustenbach DJ, **Wenning RJ**, **Lau U**, **Harrington NW**, **Rennix DK** & **Parsons AH** (1992): Recent developments on the hazards posed by 2,3,7,8,-Tetrachlorodibenso-p-dioxin in soil: Implications for setting risk-based cleanup levels at residential and industrial sites. J. Toxicol. Environ. Health 36, 103-149.

Polet D, **Murphy DJ**, **Becker I** & **Phan Duy Thuc** (2004): Notes on the primates of Cat Tien National Park. In: Nadler T, Streicher U, Ha Tang Long (eds.): Conservation of Primates in Vietnam; pp. 78-84. Frankfurt Zoological Society. Hanoi.

Ruempler U (1998). Husbandry and breeding of douc langurs (*Pygathrix nemaeus*) at Cologne Zoo. Inter. Zoo Yearb. 36, 73-81.

Schecter A, **Quynh HT**, **Pavuk M**, **Papke O**, **Malisch R** & **Constable JD** (2003): Food as a source of dioxin exposure in the residents of Bien Hoa City, Vietnam. J. Occupa. Environ. Med. 45, 781-788.

Sekirov I, **Russell SL**, **Antunes CM** & **Finlay BB** (2010): Gut microbiota in health and disease. Physiol. Rev. 90, 859-904.

Stefen C & **Nadler T** (2012) Comparative cranial morphology of douc langurs (*Pygathrix cinerea*, *P. nemaeus*, *P. nigripes*). Vietnamese J. Primatol. 2(1), 7-24.

Stellman JM, **Stellman SD**, **Christian R**, **Weber T** & **Tomasallo C** (2003): The extent and patterns of usage of Agent Orange and other herbicides in Vietnam. Nature 422, 681-687.

Szekely RA, **Singh J**, **Marsh TL**, **Hagedorn C**, **Were ST** & **Kaur T** (2010): Fecal bacterial diversity of human-habituated wild chimpanzees (*Pan troglodytes schweinfurthii*) at Mahale Mountains National Park, Western Tanzania. Am. J. Primatol. 72, 566-574.

Tanabe S & **Tu Binh Minh** (2010) Dioxins and organohalogen contaminants in the Asia-Pacific region. Ecotoxicology 19, 463-478.

U.S. Air Force Communications Service (2000): HERBS Tape: Defoliation missions in south Vietnam, 1965-1971. Data by Province. U.S. Data Services Center, record 00108.

Vidya TNC, **Varma S**, **Nguyen Xuan Dang**, **Tranh Van Thanh** & **Sukumar R** (2007): Minimum population size, genetic diversity, and social structure of the Asian elephant in Cat Tien National Park and its adjoining areas, Vietnam, based on molecular genetic analyses. Conserv. Genet. 8, 1471-1478.

Vu Ngoc Thanh, **Le Khac Quyet**, **Nguyen Thien Tao** & **Pham The Cuong** (2010): A preliminary field survey of the effects of chemical toxin (dioxin) on primates in Ma Da area, Dong Nai province. VNU Journal of Sciences, Natural Sciences and Technology 26 (15), 57-62.

Yildirim S, **Yoeman CJ**, **Sipos M**, **Torralba M**, **Wilson BA**, **Goldbery TL**, **Stumpf RM**, **Leigh SR**, **White BA** & **Nelson KE** (2010): Characterization of the fecal microbiome from non-human wild primates reveals species specific microbial communities. PLoS ONE 5(11), 1-11.