Is malaria the cause for decline in the wild population of the Indian White-backed vulture (*Gyps bengalensis*)?

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The populations of three species of *Gyps* vultures have shown a decline of more than 95% between 1988 and 1999 in the Indian subcontinent and are now classified as 'critically endangered'. The indiscriminate and widespread veterinary use of diclofenac has been implicated for the decline of the White-backed vulture (*Gyps bengalensis*) in Pakistan, India and Nepal. Similar trends in population decline as seen in the northern regions have also been recorded in Central and South India, but the cause for the decline was not investigated. Here we report a study carried out in a densely forested and sparsely populated region in Central India. An intracellular malarial parasite was identified from the tissues of both live and dead White-backed vultures. Further, amplification and sequence analysis of the consensus sequence of the mitochondrial small and large sub-unit rRNA genes indicated a 95–96% similarity with the mitochondrial sequence of *Plasmodium falciparum* (DQ642845) and other *Plasmodium* species. In addition, amplification and sequencing of a 502 bp fragment of the mitochondrial *cyt b* gene identified the haemoprotozoan with *Plasmodium* sp. AP70, an avian malarial parasite. During the course of this study we also rescued two terminally ill vultures with symptoms of malaria, and treatment with anti-malarials led to their recovery. None of the affected vultures had diclofenac residues, thus implying that malaria could be an additional cause for the decline for the White-backed vulture population.

Keywords: *Gyps bengalensis*, malaria, visceral gout, population decline.

A sudden and inexplicable population decline of more than 95% in both *Gyps bengalensis* and *Gyps indicus* was recorded between 1988 and 1999 in the Keoladeo National Park, Bharatpur, Rajasthan, India, while the populations of other genera of vultures remained virtually unchanged[1]. Both these species, from robust populations in the early 90s, are now classified as 'critically endangered'[2]. Such a rapid decline triggered several investigations to determine the mechanism/s driving the *Gyps* vulture populations towards extinction. Within a few years, almost irrefutable evidence was presented that the indiscriminate and widespread veterinary use of the anti-inflammatory drug, diclofenac, is responsible for the decline of *G. bengalensis* in Pakistan[3]. Subsequent studies in India and Nepal[4,5] further strengthened this evidence. All the surveys and studies reported on the vultures in India so far have been restricted to the northern and northwestern regions, where the vulture populations were always high[1]. But there are no reports on the causes of death in the vultures from Central and South India, although a similar trend in population decline has been recorded in these areas.

With this background, the present study was undertaken over a period of two years (since September 2004) in Gadchiroli District, Maharashtra (19.57°N and 80.29°E), which is a densely forested region in Central India (Figure 1 *a*). This district comprises of several tiny villages, each separated by a 30–40 km stretch of dense forest. It is in these hamlets that the White-backed vultures have been breeding annually, especially on tamarind trees (*Tamarindus indica*). Over the last decade, the vulture population in Gadchiroli has also declined drastically (unpublished). This was surprising since according to the available information, the farmers in these regions were extremely poor and had no resources to purchase and use diclofenac. Therefore, it appeared that this breeding site for the White-backed vulture could provide valuable information on other causes for the decline in the vulture population, in addition to diclofenac. Here we report the detection of a malarial parasite in the blood smears, and brain and liver sections of 12 dead birds and two live birds. In addition, using the genomic DNA of these vultures a 502 bp fragment of the mitochondrial *cyt b* gene was amplified which following sequencing identified with the haemo-

Figure 1. *a*, Map showing the location of Gadchiroli District (solid black) in Maharashtra (19.57°N and 80.29°E), Central India (map not to scale). *b*, Post-mortem microscopic examination of a White-backed vulture showing extensive chalky-white deposition over the pericardium (arrow).

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protozoan *Plasmodium* sp. AP70, an avian malarial parasite. Further, it is also demonstrated that the two live but sick birds with signs of malaria recovered following anti-malarial therapy.

During the study period from September 2004 to September 2006, a total of 48 active nests spread over 11 villages with 100-odd vultures were constantly monitored in the Gadchiroli District (Figure 1 *a*) with the help of the local forest officials and volunteers. The aim was to observe the nesting and feeding behaviour of the vultures, and to carry out detailed pathological examiations of the dead vultures to ascertain the cause of death. The dead birds were categorized as adults and juveniles, and their sex was also recorded. Attempts were also made to rescue and treat sick birds as and when possible. In addition to screening the dead birds for diclofenac residues, attempts were also made to identify intracellular pathogens and other parasites.

Blood for microscopic examination was collected from all the fresh carcasses by puncturing the heart. Further, each carcass was subjected to a necropsy using a standard protocol, which included external followed by an internal, detailed visual examination. The organs were observed *in situ* prior to removal (Figure 1 *b*). Samples were frozen for DNA isolation and diclofenac detection, and were fixed in neutral buffered 10% formalin for histopathological examination. Tissues taken for histopathological examination included liver, kidney, heart, small intestine, rectum and brain. These tissues were processed using routine methods, embedded in paraffin wax, sectioned (5 μm) and stained with haematoxylin and eosin. Any macro-parasites found at post-mortem examination were collected for identification.

In addition to the dead birds, two live but sick birds were also found on 12th of April 2005 and 16th of March 2006 in the villages Nimgaon and Dhanora respectively, in Gadchiroli District. Both the birds showed signs of malaria, and therefore, after an initial treatment with oral fluids (100 ml of 5% dextrose administered orally and intermittently), they were treated with anti-malarial drugs – 20 mg chloroquine (Chloroquine syrup I.P., Medicamen Biotech Ltd, India; 50 mg/5 ml) and 2.5 mg of primaquine (Primaquine I.P., ALPA Laboratories, India; 15 mg tablet) orally for 5 days and fed minced meat daily with *ad libidum* water. The water was supplemented with B-complex vitamins (Beplex Forte syrup, Anglo-French Drugs and Industries, India; 2.5 ml added to 500 ml drinking water).

The method described by Oaks *et al.*[3] was followed to detect diclofenac in fresh kidney samples of three vultures which were found dead in Gadchiroli District. About 0.5 g of kidney tissue (three samples) was homogenized in 4 ml acetonitrile and centrifuged. The supernatants were passed through solid-phase extraction cartridges (Waters Sep-Pak Plus TC-18) at 1 ml/min. The cartridges were washed using 4 ml of acetonitrile, and all

elutes were pooled and concentrated to a final volume of 1 ml. Diclofenac was detected by HPLC (Agilent 1100) following analysis on a C-18 reverse phase column (Bio-Rad, USA) with the detector set at 280 nm. Diclofenac sodium standard (Sigma, D6899) was prepared in 1 : 1 acetonitrile : water, to a final concentration of 10.4 μg/ml. Further, serial dilutions of diclofenac were prepared (0.104, 0.052, 0.026, 0.0104 and 0.0052 μg/ml) to generate a linear calibration curve to estimate unknown concentrations of diclofenac. Standards and samples were subjected to a binary gradient elution profile with solutions A (0.1% acetic acid in water) and B (100% acetonitrile). The starting condition was 75% A/25% B for 0.1 min followed by a 15 min linear gradient from 75% A/25% B to 5% A/95% B at 0.7 ml/min. The column temperature was maintained at 40°C.

Total genomic DNA was isolated from blood of 14 (12 dead and two live) vultures by standard phenol–chloroform method[6]. This was used as a template for PCR amplification of the consensus sequence of the mitochondrial small and large sub-unit rRNA genes using the primers 343F (GCTCACGCATCGCTTC) and 496R (CAAAGAAAAT-GACCGGTC), which were designed by Fallon *et al.*[7] based on five complete sequences of *Plasmodium* mtDNA. PCR was carried out in a 20 μl volume containing 50–100 ng total genomic DNA, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl$_2$, 5 pmol each primer and 0.5 U Ampli *Taq* Gold (Ampli Taq Gold™, Applied Biosystems). The cycling conditions were as follows – an initial denaturation of 95°C for 10 min followed by 35 cycles of 30 s at 95°C, 30 s at 47°C and 30 s at 72°C, and a final elongation of 72°C for 5 min. Bands were observed following electrophoresis on 2% agarose gels.

A fragment of the *cyt b* gene of the malarial parasite was amplified using HaemF (ATGGTGCTTTCGATA-TATGCATG) and HaemR2 (GCATTATCTGGATGT-GATAATGGT) primers. These primers were used earlier to study the host specificity and phylogeny of the *Plasmodium* and *Haemoproteus* spp. infecting birds[8]. The PCR mixture of 20 μl was the same as above, except that specific primers and 1.25 U of Ampli *Taq* Gold (Ampli Taq Gold™, Applied Biosystems) were used. The PCR conditions were as follows – an initial denaturation of 95°C for 10 min followed by 39 cycles of 30 s at 95°C, 30 s at 55°C and 45 s at 72°C, and a final elongation of 72°C for 5 min. The PCR products were run on a 2% agarose gel and evaluated for positive amplification.

The amplified products of the mitochondrial LSU and SSU rRNA genes and the *cyt b* gene were sequenced with Big dye Terminator and ABI 3700 automated sequencer. The sequences obtained were compared with the GenBank database (National Centre for Biotechnology Information) using BLASTN 2.2.3 software. The *cyt b* sequence obtained from our samples (*Plasmodium* sp. LaC1) was edited and aligned along with those of other

Plasmodium species infecting birds using CLUSTALX[9]. A neighbour-joining tree based on Kimura 2-parameter distances[10] with gamma correction as implemented in MEGA 3.1 was created to provide a graphic representation of the phylogenetic relationships among the various species. The tree was rooted with mammalian *Plasmodium* sequences (GenBank accession numbers AF069610, AY588280, AF069624, AB182496).

During the course of this study, the White-backed vultures were observed to nest on tall trees, especially on tamarind trees, from October to April. The birds depend heavily on the carcasses of domestic animals for their sustenance and have often been seen to compete with dogs for their food. A total of 31 birds died during the study period, i.e. from September 2004 to September 2006, with 18 and 13 deaths respectively, in the first (September 2004 to September 2005) and the second year (September 2005 to September 2006). Out of the 18 birds which died in 2004–05, 17 were females (14 adults and three juveniles) and one was an adult male. In 2005–06, out of the 13 dead birds, 12 were males (nine adults and three juveniles) and one was an adult female.

Freshly dead vultures were difficult to obtain from the field owing to a combination of factors, like low number of birds spread over wide areas of dense forests and high ambient temperatures causing rapid decomposition. Out of the 31 carcasses, only 12 (four in the first year and eight in the second year) were suitable for post-mortem examinations, and the remaining 19 carcasses had putrefied to an extent that the internal organs had liquefied beyond recognition.

During the course of the study two vultures with symptoms of malaria were found, which were taken for treatment. At the time of capture the bird from Nimgaon was unable to fly, showed visible signs of respiratory distress and greenish diarrhoea, was anaemic and had a body temperature of 104°F. Blood smear examination of the rescued bird showed the presence of schizonts, indicating that the bird was suffering from malaria. However, following treatment with anti-malarial drugs within a couple of days the bird visibly improved. After two weeks of captivity and therapy, the blood of the bird was free of schizonts, and it was released on 24th of April 2005 near a wild nesting colony at Dhanora, with a weight gain of 0.5 kg.

Similarly, the second sick vulture from Dhanora also had all the symptoms of malaria as mentioned earlier. In addition, it also exhibited circling movements of the neck, was unable to move its legs or wings (partial paralysis) and had ruffled feathers. This bird was also diagnosed positive for malaria based on the presence of intra-erythrocytic schizonts in the peripheral blood. Immediate veterinary care and anti-malarial treatment revived the bird, which showed rapid and progressive recovery. Ten days after treatment, the bird's blood was negative for the presence of the parasite. On 14th of April 2006, the healthy bird with a weight gain of 1.5 kg was released close to the location where it was found.

The 12 fresh bird carcasses appeared normal externally. The conjunctiva of all the birds were extremely pale, suggestive of anaemia. Several ectoparasites including ticks, fleas and lice were found on the bodies.

Visceral gout, characterized by the deposition of uric acid crystals on all the organs, was a predominant finding in all the birds (Figure 1 *b*). In all the cases, gout appeared to be acute. Occasional moderate congestion was seen in the brain, lungs, digestive tract, pancreas, spleen and gonads. The entire digestive tract, including the small and the large intestines, was devoid of food. A few round worms and tape worms were seen in the small intestine. In all the cases, liver was considerably swollen and was also pale, except in two birds where it was extremely congested. Occasionally there were haemorrhagic lesions within the parenchyma of the liver. The liver was always covered with white, pasty deposits. Gout was most pronounced in the kidneys. The kidneys were extremely fragile, brittle and filled with white crystalline material. The interlobular blood vessels were congested. The bursa of Fabricius was absent in all the birds, including the six juveniles.

Microscopic examination of blood smears from the 12 fresh carcasses and two live, infected birds stained with Giemsa stain revealed the presence of intra-erythrocytic *Plasmodium* schizonts.

Most of the tissues had depositions of uric acid crystals. In all the cases gout was acute, but without any surrounding inflammatory reactions. In some tissues there were ulcerations or necrotic changes around the uric acid deposits. The brain tissue revealed diffuse gliosis. The endothelial cells were swollen and some of them contained exoerythrocytic schizonts. The neurons were swollen. The liver section revealed generalized congestion. The sinusoidal spaces were widened and filled with erythrocytes (Figure 2 *a*). The linings of the Kupffer's cells were hypertrophied. The kidney sections revealed generalized haemorrhages. The tubular epithelial cells were swollen, showed coagulative necrosis and were detached from the basement membrane. There were focal areas of lymphoid aggregates (Figure 2 *b*). The degree of inflammation seen in the different tissues cannot be considered to be severe, but may indicate a chronic or resolving inflammatory disorder. Congestion and inflammation of the liver and spleen are routine findings in avian malaria in poultry.

Diclofenac estimation was done in the tissue samples of 16 dead birds from the northern and northwestern states of India and three dead birds from the present study site. Diclofenac was detected in all the vulture samples. This is not surprising since it has indeed been reported earlier that the cause of the decline in the vulture population in these regions is due to the indiscriminate use of diclofenac[1]. However, diclofenac was not detected in the
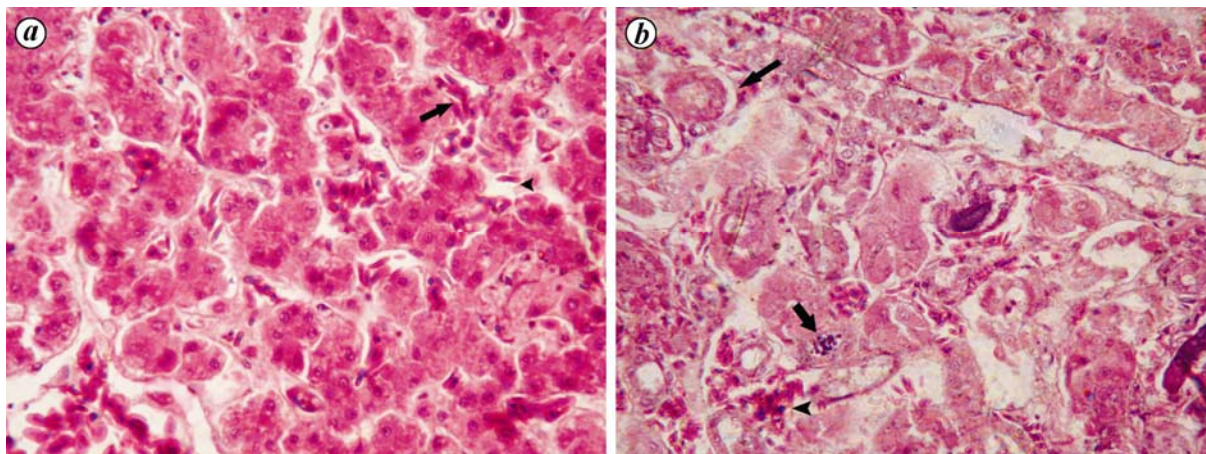
Figure 2. *a*, Section of the liver showing widened sinusoidal spaces (arrowhead) with erythrocytes indicating inflammation (small arrow) (200×). *b*, Section of the kidney showing intertubular congestion with occasional haemorrhage (arrowhead). Some tubules show coagulative necrosis (arrow) and focal areas of lymphoid aggregates are visible (small arrow; 200×).

three birds sampled in this study. In the present study, we could analyse the kidney and liver tissues of only three dead birds because they were fresh deaths and the samples were promptly transported to CCMB, Hyderabad under proper storage conditions. In case of the remaining nine carcasses, although they were suitable for post-mortem examinations and DNA extraction, the tissues could not be transported immediately to CCMB for HPLC analysis.

Using primers specific to a consensus sequence of the small sub-unit and large sub-unit rRNA genes of the mito-chondria of *Plasmodium* and *Haemoproteus* sp.[7], it was possible to amplify an 180 bp fragment in all the 12 dead birds. Sequencing of the amplicons followed by BLASTN indicated a 96% similarity with the mitochondrial sequence of *Plasmodium falciparum* (DQ642845). The similarity with other *Plasmodium* species, namely *P. juxtanucleare*, *P. relictum*, *P. reichenowi*, *P. gallinaceum* and *P. vivax* was 95–96%.

Further, amplification of the *cyt b* gene from the 12 dead vultures yielded a PCR product of 502 bp (*Plasmodium* sp. LaC1, GenBank accession no. EF552403), which when sequenced showed that all the 12 sequences were identical and exhibited a 100% match with the sequence of *Plasmodium* sp. AP70 (AY714203), which was earlier identified as a strain infecting the mountain thornbill (*Acanthiza katherina*) in tropical Australia and Papua New Guinea[11]. Further, the similarity of *Plasmodium* sp. LaC1 with *P. gallinaceum*, *P. relictum*, *P. cathemerium*, *P. elongatum*, *Plasmodium* spp. haplotype ORW1G278, *Plasmodium* spp. ORW1, *Plasmodium* sp. AP71 etc., which are known to infect birds ranged from 93 to 99%. Phylogenetic analysis further confirmed that *Plasmodium* sp. LaC1 (EF552403) is closely related to *Plasmodium* sp. AP70 (AY714203) (Figure 3) and all the avian malarial parasites, including *Plasmodium* sp. LaC1 formed a monophyletic clade. The primate malarial para-

sites which were used as out groups did not form a part of the avian malarial parasite clade.

In addition, when DNA from the blood of the two live, sick birds was used as a template, the amplicons corresponding to 12S and 16S rRNA genes and *cyt b* gene, following sequencing, identified with the respective genes of *P. falciparum* (DQ642845) and *Plasmodium* sp. AP70 (AY714203). These diseased birds were treated with anti-malarial drugs (chloroquine and primaquine) and then checked for the presence of *Plasmodium* by PCR using the above primers. The amplicons corresponding to 12S and 16S rRNA genes and *cyt b* gene were no longer visible. In fact, these birds recovered completely following treatment, showed no signs of lethargy or anorexia, and took to the air on their own when released. Blood DNA samples of four White-backed vultures from the Nehru Zoological Park, Hyderabad, did not yield amplicons corresponding to 12S and 16S rRNA genes and *cyt b* gene of either *P. falciparum* or *Plasmodium* sp. AP70.

Gadchiroli District is a dense forest region, with the local population living mainly on the forest by-products. By and large the tribal people here, who account for roughly 80% of the population of Gadchiroli, are poor. The dependence on livestock as a source of food is minimal, and nondescript cattle are usually used only for some primitive farming activity. The medical and veterinary facilities are extremely rudimentary and people generally do not take their livestock for treatment. Our preliminary questionnaire survey among the livestock owners and veterinary doctors revealed no usage of diclofenac as an anti-inflammatory drug (data not shown). Also as a recent development, in most of the cases, animals which have crossed their productive age are driven all the way to the nearest big city, i.e. Hyderabad or Nagpur, to be sold to the abattoirs. The vultures which have always depended heavily on the carcasses of domestic animals for their sustenance have to now cover larger areas
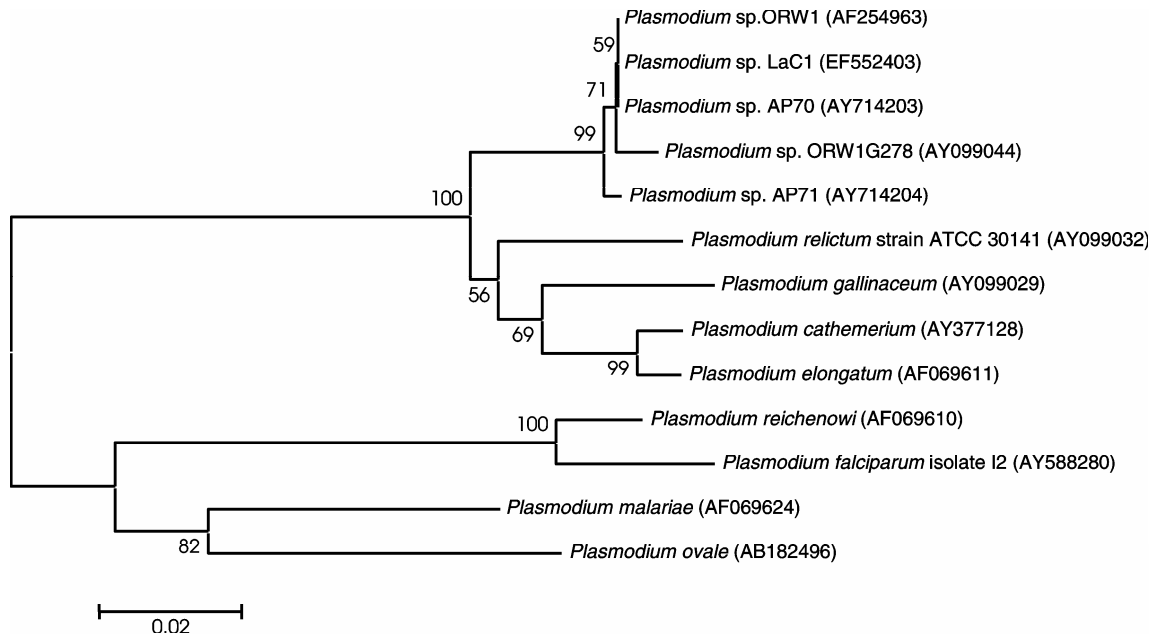
Figure 3. Neighbour joining phylogenetic tree based on the *cyt b* sequence of *Plasmodium* sp. from infected vultures (*Plasmodium* sp. LaC1, accession no. EF552403) and of other *Plasmodium* species known to infect birds. Species of *Plasmodium* known to infect mammals were taken as outgroups. Bootstrap values (expressed relative to 1000 replications) are given at the respective nodes. Bar represents two substitutions per 100 nucleotides. Reference sequences were retrieved from GenBank under the accession numbers indicated in parentheses.

each day and compete with dogs and other scavengers for the available feed. This also increases the time interval between two consecutive feedings for the nestlings. Although visceral gout was a predominant necropsy finding in our study as in all the previous reports[3,5,12], this is more likely due to starvation than due to a primary kidney failure. Kidney and liver tissues of only three birds could reach the laboratory in conditions suitable for HPLC, and all these tissues were devoid of diclofenac residues.

During the course of our study, a large number of mosquitoes were observed swarming around the brooding White-backed vultures and in the nests during the nesting season, i.e. from October to March, which also corresponds to the outbreaks of cerebral malaria and Japanese encephalitis in the resident humans (District Malaria Officer, Gadchiroli; pers. commun.). Subsequently, during the various associated diagnostic investigations we noticed the presence of intra-erythrocytic *Plasmodium* schizonts. Amplifications of the consensus sequence of the 12S and 16S rRNA genes and *cyt b* gene similar to *Plasmodium* sp., further confirmed our findings. Two extremely sick birds were successfully treated with anti-malarials and released back to the wild. Whether these recoveries were due to the anti-malarial drugs or simply due to the availability of sufficient food and water is debatable.

A segment of the *cyt b* gene (502 bp) was amplified from the two diseased and 12 dead vultures to identify the *Plasmodium* species. BLASTN analysis of the *cyt b* gene indicated that the 14 sequences were identical and showed 100% similarity with the sequence of *Plasmodium* sp. AP70 (AY714203), which infects a subset of bird species from tropical Australia and Papua New Guinea[11]. Phylogenetic analysis confirmed the close relationship between the *cyt b* gene sequence obtained from the vulture (*Plasmodium* sp. LaC1, accession no. EF552403) and *Plasmodium* sp. AP70 (AY714203). The phylogenetic tree also indicates that the avian malarial parasites are closely related and form a monphyletic clade (Figure 3). Earlier studies have suggested that compared to *Haemoproteus* sp., *Plasmodium* sp. appear to be less constrained by the phylogenetic relationships of their hosts and show no evidence of host-specificity at any depth within the parasite phylogeny[8,11,13,14].

Preliminary PCR tests indicate a prevalence of ~45% in the wild population of the White-backed vultures in Gadchiroli District. However these data do not indicate that this malarial parasite is an obligate pathogen. Although inflammation was noted in the brain, liver and spleen, there is not sufficient evidence for the cause of death. It is most likely that the *Plasmodium* sp. is a commensal organism of the White-backed vulture and possibly an opportunistic pathogen. The potential of this organism as a mortality factor in the existing free-ranging vulture populations needs to be further explored.

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