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Biochemical and environmental insights of declining vulture population in some Asian countries

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Abstract

Traces of diclofenac and its derivative compounds have been found in the carcasses of vultures across India and its neighboring countries, and it is known that the biomagnification of diclofenac from the consumption of infected domestic animal carcasses contributes to vulture mortality. However, reports also indicate that problems associated with their habits and habitats, food and feeding behavior, nesting and breeding, reproduction, epidemic and endemic diseases, and environmental factors, such as high temperatures and cyclones, might also be contributing factors. Adequate information is not available to confirm whether only diclofenac is the primary cause of vulture mortality versus their susceptibility to microbial pathogens, diseases or physiological conditions, such as oxidative stress due to diclofenac biomagnification. Death due to other contaminants or pollutants has also been not adequately studied. So future research may be able to determine whether the biomagnification of diclofenac and other organic/ inorganic pollutants or some other factors are responsible for vulture mortalities. Further investigations into the health issues related to life cycles and pathology need to be performed to restore the sharply declining vulture populations in India and across the globe. In this context, India is ahead of other countries in adopting recovery plans for vultures, for which the rate of decline of long-billed vultures is now 16% per year compared to that of oriental white-backed vultures which is 44% per year.

Key words: Diclofenac, biomagnification, remediation, eco-pharmacological effects, pollution, vulture extinction

1. Introduction

Vultures are highly significant in food webs as they play the key ecological role of consuming the carcasses of dead animals, which prevents the spread of diseases to livestock (1). Vulture populations at national and global scales are declining and are on the threshold of extinction. This has prompted researchers to restore their populations through laboratory and field interventions. An attempt is made in this article to identify the possible causes of and remedies for the decline of vulture populations with special consideration of the biomagnification of diclofenac from infected domestic animal carcasses. Emphasis is placed on the fact that diclofenac contamination cannot be the only cause behind declining vulture populations, and it is likely that several factors cause vulture mortality.

Different trophic levels stabilize ecosystems, and if one is lost, its entire ecosystem will be pushed into an unbalanced state. Threats to organisms at different trophic levels, such as producers, consumers or scavengers, eventually lead to disturbances in the food chain as well as imbalances in the food web (2). It is noteworthy that scavengers perform several crucial roles in a food chain without which the ecosystem's maintenance of dead carcasses will either stop or be delayed (3), which disturbs the relationships between prey and predators and between producers and consumers (4, 5). Therefore, the absence of scavengers can lead to a serious crisis in an ecosystem (6), so the conservation of these species is especially important (7).

Many vultures such as *Gyps bengalensis* and *Gyps indicus* are now classified as critically endangered by the IUCN (12), and many vulture populations are either severely declining or already locally extinct, which is not only true for the Indian subcontinent but for many places across the globe (8-14, Fig. 1). Vultures usually do not kill animals but rather eat dead ones thereby keeping the environment clean (Fig. 2). Domestic animal carcasses are disposed of openly in the absence of safe alternatives, which not only leads to an increased risk of diseases, such as rabies, but also provides a platform for other livestock borne diseases, such as anthrax. Thus, it can be inferred that the scavenging role of vultures prevents the spread of dangerous diseases that could threaten wildlife, livestock and human beings (15). Therefore, it is clear that vultures are very important to terrestrial ecosystems (6). However, despite their role in maintaining the "balance of terrestrial ecosystems", a current topic of debate among ecologists and environmentalists, not much work has been performed to protect these species. For example, *Gyps* vulture populations across the Indian subcontinent began to decline in the 1990s and the process continues (15). Repeated demographic surveys have shown that the rate of decline was so rapid that elevated mortality of adult birds must be a key factor (16). Different nations have adopted several field and laboratory interventions and formulated different conservation strategies to protect vultures (17), but recovery efforts have not had the success anticipated, although the rate of population decline has slowed in countries such as India and Nepal (18). While India is ahead of other nations in the adoption of specific strategies for vulture conservation, the recovery rate has not reached the expected level.

Fig. 1. Vultures of India, reproduced after Taigor (15).

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Fig. 2. Vultures feeding on carcasses in the Moyar Valley, Sathyamangalam Forest Division, Tamil Nadu (**A**) (after Ramakrishnani et al. (32) and in Nepal (B) (www.birdlife.org).

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Multiple reasons have been ascribed to the mortality of vultures worldwide. In India and Nepal, the biomagnification of diclofenac from the carcasses of domestic animals to vultures is considered to be the main cause of vulture mortality (17-31). However, in the absence of specific studies or observations, this claim is difficult to accept (32). Other factors, such as problems with vulture habits and habitats, food, diseases, breeding, and environmental extremities may also contribute to mortality. These external and internal factors affect the normal physiology of animals and can lead to metabolic depression and eventually to death (33). One of the important cellular responses that creates metabolic depression in animals is oxidative stress (OS), which is resulted due to the oxidation of proteins, lipids and nucleic acids by the overproduced free oxygen radicals (34). OS is always positively correlated with the magnitude of any external and internal stress, so the susceptibility of vultures to OS due to infection by microbial pathogens, diseases or physiological disorders cannot be ignored.

2. The status of vulture populations in some Asian countries : In the protected areas of India, vultures numbers are few, i.e., from 13 to 65 individuals (15). Approximately four decades ago, two vulture species, namely, the Indian white-backed (*G. bengalensis*) and long-billed (*G. indicus*) vultures, were abundant (9, 15, 17), but, according to the reports of the Bombay Natural History Society (BNHS), these species are now on the verge of extinction (35). Beginning in 1985, Prakash et al. worked in Rajasthan's Keoladeo National Park, where white-backed vultures nested and long-billed vultures came to forage from nearby breeding sites. They observed the number of the park's white-backed go from a peak of 1,800 in 1985-86 to only 86 in 1998-99 while long-bills declined from 816 to 25 (36, Fig. 3). In 1999, data published from a BNHS study in Keoladeo National Park (KNP), a world heritage site, showed a 96% decline in the Indian white-backed vulture population and a 97% decline in the long-billed vulture population between 1985 and 1999 (35). Similar observations have been published elsewhere by different authors (8, 36, 37). In 2000, nationwide surveys revealed a similar decline in vulture populations throughout India (38). Later, a severe reduction in populations of *Gyps* species was observed by several ornithologists in neighboring countries, such as Nepal and Pakistan (16, 17, 27, 37, 39).

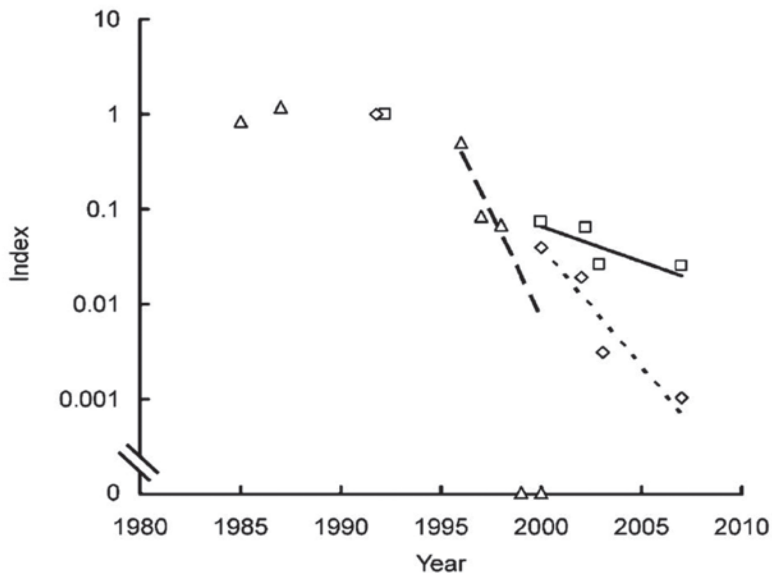

Fig. 3. Declining populations of *Gyps* vultures in India (reproduced from Pain *et al.* (17)). Points show population size index values on a logarithmic scale plotted against calendar year, which depicts the population as a proportion of its initial size. Triangles represent the number of active *Gyps bengalensis* nests in Keoladeo National Park, from Prakash et al. (36), expressed as a proportion of the average number in the 1980s. Indices of population size, relative to 1992, of *G. bengalensis* (diamonds) and *G. indicus* and *G. tenuirostris* combined (squares) in northern India were calculated from road transect count data as described by Prakash et al. (37). Lines depict fitted log-linear regression models (the dashed line represents 5 *G. bengalensis* at Keoladeo; the dotted line represents 5 *G. bengalensis* from road transects, and the solid line represents 5 *G. indicus/tenuirostris* from road transects).

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3. Is diclofenac biomagnification the only cause of vulture mortality? : Multiple reasons have been assigned to the loss of scavengers, including vultures, in specific habitats (14, 40), and a number of studies have highlighted the persistent problems related to the causes and consequences of vulture extinction (17, 19, 35-37, 41-43, Fig. 2). Several reasons are believed to contribute to vulture mortality (Table 1). On the other hand, postmortem analyses of vulture carcasses from different locations have found traces of diclofenac and its derivative compounds in their tissues. While the biomagnification of a drug in an organism may not be the sole cause of mass mortality of its population, many authors have opined that diclofenac contamination, which contributes to renal failure and hepatotoxicity, is the only cause of vulture population decline in India (17-31). However, without properly designed experiments and data analysis, this claim may seem inconclusive. The available reports are inadequate for arguing whether diclofenac is the only main cause of vulture mortality or whether, following diclofenac biomagnification, the increased susceptibility of vultures to microbial pathogens, diseases or physiological disorders, such as OS followed by metabolic depression, are responsible for their mortality in large scale. The argument is that the detection of drugs such as diclofenac would be expected if an animal consumes diclofenac-contaminated food, including carcasses, more often. However, detecting diclofenac or its derivatives in the carcasses of animals cannot be the sole indication that diclofenac is the only cause of death. The discussions in this article

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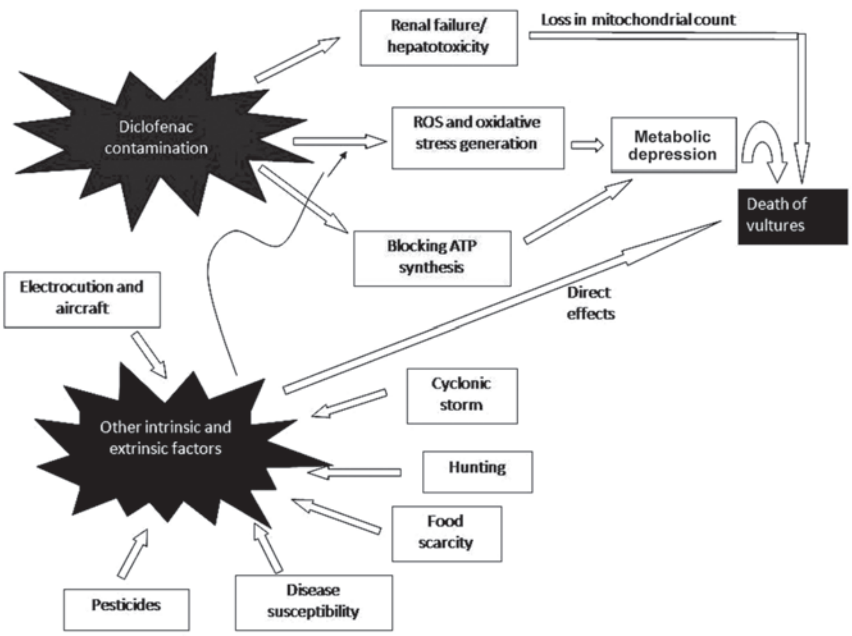

Fig. 4. Ray diagram showing the causes of declining vulture populations.

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regard whether the biomagnification of diclofenac is the only cause of vulture mortality or if there are other factors based on the available literature. An attempt has been made here to note the possible role of elevated OS due to the consumption of diclofenac-contaminated carcasses in vulture mortality. An alternative viewpoint is given in an attempt to identify other possible causes that mediate and remedial measures that can arrest the rapid decline of vulture populations particularly in Indian subcontinent.

3.1. Global status of the research on diclofenac contamination and vulture mortality : A large number of pharmaceutical agents have been found to cause toxicity in several avian species, including vultures (23, 28, 29, 44). For example, the involvement of drugs such as carprofen, flunixin, phenylbutazone and ibuprofen in the deaths of vultures and other avian scavengers has been reported by several authors in India and other countries such as Spain and America (21, 28, 32, 45, 46, 47), and some articles also hint at the role of diclofenac contamination from domestic animal carcasses (17-31).

Diclofenac (2-[(2, 6-dichlorophenyl) amino] benzeneacetic acid, monopotassium salt) contamination from cattle carcasses provides a good example for discussion. It has been mostly studied in the context of vulture mortality by researchers in some Asian countries, such as India, Nepal and Pakistan. Diclofenac was first introduced in the UK in 1979 after being developed by Novartis (formerly named Ciba-Geigy) and was later used extensively in many countries (48). Diclofenac is used as a non-steroidal, anti-inflammatory and analgesic agent (25, 26), and it is not restricted to human beings but is widely used to treat domestic animals, especially cattle and goats (30, 31). According to Prakash et al. (43), if 10–20% of the estimated 503 millions of livestock in India die and become available for consumption by vultures annually (apart from the small portion eaten by people), 5-10% of these carcasses would have detectable

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Table-1. Multiple reasons for the vulture mortality other than diclofenac contamination.

Reasons of Vulture mortality	***Place and year of Research***	***References***
Cyclone	Guntur and Prakasham area of Andhra Pradesh state, India, 1990	(60)
Mycoplasma infection	Changa Manga forest plantation area of Pakistan, 2004	(61)
Malaria	India, 2009	(36, 62)
Neck drooping	India, 2003	
Neck drooping and temperature		(65)
Hunting of vultures for meat by Bandola (Banda) people	Guntur and Prakasham area of Andhra Pradesh state, India, 1990	(60)
Consumption of infected carcasses by pesticides	Moyar Valley and different area in India, 2008	(25, 26, 32, 68, 69)
Metabolic depression related to oxidative stress and blocking in ATP synthesis	Across the world	(24, 51, 113)
Improper nesting and resting place	Turkey, 2008	(13)
Food scarcity and cannibalism	India, 1988, Keoladeo National Park, Bharatpur, Rajasthan, 2003	(41, 82)
Breeding related problems	India, 2003	(42, 87)
Pathological susceptibility	India and abroad, 2011	**(57, 69)**
Electrocution and aircraft	All part of the world	**(69, 90-92)**
Super cyclone	Coastal belt of the state Odisha, India, 1999	Personal observations of the authors

concentrations of diclofenac. However, given the short residence time of diclofenac in tissues, this would only be the case if all of the treated animals died within a week of receiving diclofenac. Taggart et al. (31) observed a 10% prevalence of diclofenac in samples from the carcasses of domesticated ungulates, which suggests that considerably more than 5 million courses of treatment are given annually and/or that the majority of animals being treated are fatally ill (44).

According to the American Society of Health-System Pharmacists, the overall bioaccumulation and retention rate of diclofenac is much higher than other anti-inflammatory and analgesic drugs and it is species specific (49). Therefore, bioaccumulation might be the main reason for the disruption of avian physiology in those animals that feed on the carcasses of domestic animals treated with diclofenac (24). Long-term administration of diclofenac to animals may result in severe pathological conditions, such as peptic ulceration, gastrointestinal bleeding, hepatotoxicity, renal papillary necrosis and renal failure, followed by death (19). If the above symptoms manifest themselves in vultures after they feed on carcasses contaminated with diclofenac, it can be inferred that diclofenac is the only cause of vulture mortality. However, the point remains as to whether this is the only major cause of the depletion of vulture populations from their habitats. It is true that diclofenac contamination has been detected in the carcasses of vultures, and many authors have strongly opined that diclofenac contributes to the catastrophic decline of vulture populations in

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many south Asian countries (21, 50). The major causes of death in vultures contaminated by diclofenac are renal failure and hepatotoxicity (24, 51, 52).

A total of 25 articles on diclofenac-related vulture deaths are currently available on the PubMed website (http://www.ncbi.nlm.nih.gov/pubmed?term=diclofenac%20vulture%20), but none of these articles aimed to discuss whether diclofenac contamination was either a "cause" or an "effect" or "both a cause and an effect" of vulture mortality. Additionally, none of the studies report whether vultures become susceptible to pathogens or other disorders after contamination or if death was mediated by diclofenac. The antimicrobial properties of diclofenac have been studied in microorganisms such as *Entamoeba coli* and *Mycobacterium tuberculosis* (53-55). Based on a preliminary investigation, Cunningham et al. (56, 57) claimed that an infectious disease with a viral etiology may be the cause of declining vulture populations. Although the authors could not find any pathogens in vulture carcasses, this does not necessarily mean that vultures are not susceptible to infectious diseases. Furthermore, the study was limited to particular areas, so it might be possible that diclofenac may impact the physiology of vultures both as an "effect" and also as a "cause" of susceptibility to other pathogens and disorders that can lead to death. On the other hand, Naidoo et al. (58) stated that, depending on its rate of elimination from the body, diclofenac is not likely to be a toxic agent in crow species but can be toxic to vulture species. Ramakrishnani et al. (32) also observed that diclofenac is not the only cause of vulture mortality. Based on such contradictory reports, further investigations are needed to find out whether multiple factors or diclofenac alone threaten vultures with large-scale destruction (10). Recently, Prakash et al. (18) reported that the rate of decline of *Gyps* vultures has slowed in India and Nepal due to the restricted use of diclofenac. In their 2011 survey, however, they observed that the populations of three species of vultures (*G. indicus, G. tenuirostris* and *G. bengalensis*) in both countries not only remained low but *G. bengalensis* showed a reverse trend. The reason for the slowing population decline was attributed to the ban on the use of diclofenac for veterinary purposes, but the authors did not consider other measures, such as captive breeding, the establishment of vulture restaurants and the rapid treatment of morbid birds, adopted by both government and NGOs to arrest the decline of vulture populations in these countries. Therefore, pointing to diclofenac contamination as the sole reason for the death of vultures seems unsupported. Research carried out by organizations and individuals using both laboratory and field methods has generated a list of possible causes other than diclofenac contamination for vulture mortality.

4. Other reasons for vulture mortality

4.1. Environmental factors : Biotic and abiotic environmental factors, such as *extreme* heat or cold, air and water pollution, cyclones, habitat loss, loss of forest canopy, imbalanced food chains, etc., are always detrimental to the survival of a species. For example, variable environmental drivers are found to push turkey vultures (*Cathartes aura*) for frequent movement and to change their habitat in North and South America (59). The magnitude of the adverse effects of such environmental factors is further enhanced by anthropogenic activities that threaten the lives of the species inhabiting an area. Although specific studies that attribute the mass destruction of vultures in India or elsewhere to such forces are lacking, environmental factors are viewed as playing a significant role in the decline of vulture populations. The author (BRP) has personally observed that no vultures were found along the coastal belt of Jagatsinghpur District in Odisha State, India, in the areas of Patrapada, Kakatpur, Astaranga, Kusupur, Nandhara, Olara, Padmapur, Paradeep, Belapur, etc. after the super cyclone with a cyclonic gale of 300 km /h which struck the area on the 29th and 30th October, 1999. At least 11 vulture colonies totaling more than 125 individuals

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disappeared from these areas after the storm. Similarly, in 1990, a severe cyclone reduced a local vulture population of approximately 100 to almost 0 in the Guntur and Prakasham areas of Andhra Pradesh, India (60). This implies that super cyclones might be a factor in the destruction of vultures by destroying the large trees used for nesting. Another cause of reduced vulture populations in the cyclone-affected areas could be the relocation of the birds prior to the arrival of the cyclonic storms, but no such documentation exists.

4.2. Pollution and other factors : Many vultures are colonial in nature (42), so it is possible that mass die-offs could occur after drinking water polluted with pathogenic organisms and organic or inorganic poisons. For example, the presence of a novel *Mycoplasma* species (*Mycoplasma vulturii*) in tissues of an oriental white-backed vulture (*G. bengalensis*) in the Changa Manga forest area of Pakistan (61) indicates that this *Mycoplasma* might be responsible for the decline of the vulture population. Although diclofenac was described as the major cause of death, it can be presumed that the presence of *Mycoplasma* species was responsible for the heterophilic inflammation in the trachea and bronchi of the bird, which could have caused its death (61). Although such studies have not been conducted, it could be helpful to identify both the primary (such as diclofenac) and secondary causes of death in vultures. However, the above study indicates that vultures can be affected by specific pathogens, and malaria pathogens can grow better in areas with polluted water. Although malaria is not a waterborne disease, due to the growth of the malaria vector *Anopheles* mosquito in waterlogged areas, the pathogens that cause malaria could be a cause of death in the wild population of the Indian white-backed vulture (62). Several organochlorine pesticide residues (p,p'-DDE, p,p'-DDT, HCH, dieldrin, etc.) have been detected in the tissues and eggs of *G. bengalensis* from different locations in India, such as Delhi and Mudumalai. Avian scavengers including vultures contaminated with organochlorine compounds also have been observed in Argentinean Patagonia of South America (63). Carneiro et al. (64) have detected heavy metal mainly Pb (24.15 to 25.98 µg/dl) in blood of Griffon vultures (*G. fulvus*) from Portugal and Catalonia, Spain. The authors propose that above concentration of Pb is sufficient to contribute for their impaired physiology especially in alleviating cellular redox regulatory capacity (64). The populations of Indian white-backed and long-billed vultures have been observed to exhibit a drooped neck posture followed by death due to pesticide contamination (36). Gilbert et al. (65) observed that neck-drooping is highly temperature dependent as the behavior serves thermoregulatory purposes, but the same group of authors has also suggested that neck drooping is not a purely temperature dependent behavior in adult vultures (65). Perhaps a combined analysis of neck-drooping as a function of temperature and pesticide contamination will clarify the above contradiction. It is assumed that pesticide contamination correlates with neck-drooping behavior in vultures, so neck drooping could be attributed to the drinking of contaminated water. In such cases, the death of vultures due to the biomagnification of diclofenac would be less probable. Therefore, it might be logical to say that water pollution might be a major reason for declining vulture populations, and future research may substantiate this claim. In the absence of specific experimental results, it may also be presumed that air pollution could drive vulture species to rapidly change habitats (59). As a consequence, adapting to new habitats with different ecosystem dynamics may be difficult; food scarcity would be the most important challenge. Improved techniques for spraying agricultural chemicals and pesticides and the pre-treatment of the gaseous pollutants from industry (especially from chimneys) and other activities (especially from vehicles) may diminish the threat of air pollution to arboreal animals such vultures.

Due to the arboreal and colonial nature of most vultures, environmental extremes, such as high rainfall, high or low temperatures and

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cyclonic storms, may also affect their life span. Irregular and unexpected weather conditions have resulted from the loss of ecosystem balance in general and the loss of green forests in particular. The loss of green canopy always disturbs biogeochemical cycles, and disturbances to biogeochemical cycles, such as the water and carbon cycles, directly influence rainfall and temperature, respectively, while the nitrogen cycle influences canopy growth and development. All of the above processes influence avian life to a great extent (1, 66). As has already been mentioned, neck-drooping followed by mortality in oriental white-backed vultures (*G. bengalensis*) is correlated with a failure to thermoregulate under increased environmental temperature (65). On the other hand, some additional factors have also been found to be responsible for population decline. For example, after the decline in the vulture population due to a cyclone in 1990, the remaining vultures were further reduced by hunting (for meat) by the Bandola (Banda) people in the districts of Guntur and Prakasham of Andhra Pradesh (60). Similarly, one may repeat the personal observation by the author that the vulture population was almost absent from the coastal belt (especially in the coastal areas of Jagatsinghpur District and a few locations in Kendrapara and Puri Districts) of Odisha, India, after the super cyclone of October 1999. Such calamities cannot be avoided, but any further deterioration due to anthropogenic activities can surely be restricted.

4.3. Ingestion of contaminated food : The consumption of contaminated food can create multiple physiological disorders that can ultimately lead to death (24), and this may be the reason why Gilbert et al. (21, 65) suggested the establishment of vulture restaurants and monitoring with satellite transmitters. Such approaches could certainly reduce the risk of the consumption of contaminated food by the birds. Vultures feed in groups (67), which augments the probability of community destruction through feeding on contaminated carcasses. Therefore, dead animals contaminated with pathogens or poisons must be properly disposed of to avoid consumption by vultures. Many farmers spray cattle carcasses with pesticides, such as organochlorine and organophosphorous, to prevent the spread of foul odors (68), and these contaminated carcasses are eaten by vultures. Instances of hundreds of dead vultures due to the consumption of pesticide-contaminated carcasses are not difficult to find (69). It is reported that antimicrobial agents such as marbofloxacin in *Gyps fulvus* (70), penicillins and enrofloxacin in Egyptian vultures (71) can retain in their tissues. As a result this could lead to alter the balance in growth of the indigenous gut microflora, or the immunological status of these birds, making them more vulnerable to suffer infectious diseases and physiological depression. In this connection, geographical variation in cloacal microflora and bacterial antibiotic resistance in threatened vultures in relation to diet and livestock farming practices have been reported (71). It is clear from the above discussion that as with diclofenac contamination, other chemical factors could also be responsible for toxicity in vultures and other scavenger birds (32), and these factors need to be considered as priorities in vulture recovery plans (17, 72).

4.4. Food poisoning : Food poisoning may contribute to habitat-specific vulture mortality due to natural causes, such as toxic fungal or bacterial growth on carcasses or their improper disposal. Unfortunately, reports of vulture deaths due to fungal or bacterial infections caused by food are scarce. Although the occurrence of microorganisms, such as *Mycoplasma* (61), *Clostridium vulturis* (73) and several other microbiome (74) have been reported in oriental white backed vultures, cinereous vulture (*Aegypius monachus*) and new world vultures, respectively, and *Staphylococcus aureus* in Eurasian griffon vulture from Spain (75), investigations into microbial infections and their causative agents, such as food poisoning, should be included in vulture recovery plans.

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4.5. Lack of proper nesting and resting places : Most vultures are arboreal and colonize large trees. Anthropogenic activities, such as encroachment on wild habitats and the cutting of large trees for various purposes, cause habitat destruction that displaces animals. Such phenomena have been reported for vultures (13). According to records available at the Regional Museum of Natural History in Bhubaneswar, India in 1992, slender-billed vultures (*G. tenuirostris)* were known to make their nests in large trees, such as banyan, mango, bullet wood etc., in the coastal belt of the Jagatsinghpur District of Odisha, but when the trees were cleared, the vulture population declined sharply.

4.6. Genotoxic factors : Genetic selection is the most important factor by which the fittest organisms survive to reproduce; this selection is automatic and includes changes at both the phenotypic and genotypic levels. Although molecular studies have been performed to determine the genetic evolution of vultures (76), research into the genotoxic factors responsible for declining vulture populations have received scant attention (77).

4.7. Nutritional problems : According to the report by the BNHS, nutritional factors are not a significant factor in vulture mortality. On the other hand, consumption of contaminated food may disturb digestion and its associated processes and thus result in the loss of nutrition and subsequent retarded growth and development. The problem could be severe in baby vultures as they consume a relatively high amount of food compared to adult birds. An audio-visual report has suggested that baby vultures are more susceptible to death related to food and nutrition (78).

4.8. Food availability : Carcasses are the only important source of food for vultures (1, 79), so the method of disposal of dead animals, such as releasing them into rivers, burying them in the soil and burning them in electric furnaces, can create food shortages. Therefore, if dead animals are disposed of in the open and away from human habitats, vultures might be able to consume the carcasses, which illustrates the concept of vulture restaurants. With a specific strategy and follow-up actions, the proper disposal of dead animals can mitigate negative consequences such as pollution and infection (80). Polis (81) claimed that most cases of cannibalism in nature occur due to food scarcity, and according to BNHS, although food scarcity may not be a cause of vulture mortality, the occurrence of cannibalism in vultures raises questions in this context (41, 82). Therefore, research is needed to ascertain whether food scarcity is a cause of vulture mortality, which can be addressed by studying the behavior of vultures during or prior to instances of cannibalism (83-85). Although vulture restaurants have already been established in a few locations in India, they can still be expanded into other parts of the country (21). They may serve as a beneficial and safe way to provide vultures with food and resting places, which may help them survive and reproduce to allow the rapid recovery of their populations.

4.9. Problems related to breeding : Declining populations can be revived by either slowing the death rate or raising natality (birth rate) along with increasing survivorship. If unnatural death is the reason behind the decline of a species then high natality may not be sufficient for species recovery. In this regard, little information is available on the problems faced by vultures during breeding and in sustaining their life, so identifying such factors may aid the recovery process through the adoption of artificial breeding techniques (72).

4.9.1. Induced breeding : Longevity and the breeding success of captive endangered birds are usually higher than in natural populations (86), so induced vulture breeding under conditions of captivity is therefore suggested if the natural natality is insufficient. Although induced breeding techniques are being applied in many countries, including India, the output is yet to increase remarkably. In India, a few captive care centers have been constructed, such as those at the Birshikargha Wildlife Sanctuary and

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the Nandankanan Zoo. However, more such centers need to be set up in other parts of the country (42).

4.9.2. Incubation : Compared to adults, juvenile birds are usually more susceptible to mortality (78). Apart from induced breeding, the eggs of vultures may be collected from their natural habitat for incubation *in vitro*. Young vultures may then be released into the wild at a stage when their offensive and defensive abilities have developed and they can survive on their own. Instances of inceptive breeding failures in vultures suggest that proper care should be taken while breeding vultures in captive conditions (87).

4.10. Captivity plan for pathological susceptibility : Diseased or morbid vultures need to be identified and supervised to mitigate susceptibility to pathogens. It is believed that morbid birds exhibit signs of illness, including neck-drooping syndrome and inactivity, for nearly 30 days prior to death (36). Therefore, investigations at this stage would help in the assessment of the causes of morbidity and mortality of vultures. The analysis of infected birds may be helpful for drawing a clear picture of their patho-physiology, which has yet to be explored. The infected birds may be released following a complete recovery.

4.11. Epidemic and endemic diseases : Any endemic or epidemic disease must be investigated to take the appropriate remedial measures. Instances of vultures suffering from malaria have been observed (69), and Cunningham et al. (57) investigated 28 vulture carcasses, including adults and juveniles of both *G. bengalensis* and *G. indicus,* for any epidemic epidermal infections. These authors performed postmortem analysis of gut viscera, enteritis, vasculitis and gliosis, and although they did not identify the causative agents, the results of the pathological studies were mostly consistent with an avian viral etiology. It is important to note that Mallophaga species are known to attack bird populations and cause death (88, 89). Therefore, endemic or epidemic diseases could be the reasons for vulture mortality, so the patho-physiologies of vultures in different parts of the world require careful consideration.

4.12. Electrocution and air traffic : Electrocution as a probable cause of vulture death has been discussed by Gupta (69), but the author has maintained that this could not be a strong driver of extinction in avian populations, such as vultures. But a particular study indicated that a vulture without any known disorder died after coming into contact with high voltage electrical wires (69). The increased risk of electrocution in vultures has been attributed to their larger size and wing span, and the destruction of large trees leads them to use high voltage wires as perches. Some other species that are threatened by electrocution are the great Indian bustard, the sarus crane and some species of eagles. Nevertheless, more data are needed to support electrocution as a major cause of vulture mortality (90). However, the concern over collisions between aircraft and vultures, due to their large size, has already been discussed (91). In addition to electrocution, the large feathered forearms of vultures increase their overall risk from accidents, particularly with aircrafts (91, 92).

5. Reports by different organizations on vulture conservation : Out of nine species of vultures recorded in India, two species, namely, the Indian white-backed and long-billed, were the most abundant vultures in the Indian subcontinent approximately four decades ago. However, at present, they are on the verge of extinction and are classified as critically endangered by the IUCN (12). A report by the BNHS suggests that the decline in vulture populations cannot be due to factors such as food shortages, habitat loss, and toxicity, rather it could be due to a multifunctional disorder that needs to be properly studied. Over a decade ago, the scenario was reviewed by Indian and international experts at the “International Meeting on the Vulture Situation in India”, which was held in New Delhi from 18th-20th September, 2000, and organized by the BNHS and supported by the

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Royal Society for the Protection of Birds of the United Kingdom and the Ministry of Environment and Forests of the Government of India. The expert committee recommended the establishment of a nation-wide monitoring program to identify the reasons for the decline of vulture populations and suggest appropriate recovery plans. The establishment of a captive care facility for both sick as well as healthy vultures was another important issue raised by the committee. Accordingly, the Indian Vulture Disease Investigation Centre, the BNHS and the Poultry Diagnostic Research Centre (PDRC) of India, in collaboration with the Institute of Zoology of the Zoological Society of London, have been actively working on vulture diseases in India and abroad. The Darwin Vulture Project is one of the programs that established a dedicated vulture disease investigation center at the PDRC in Pune. On 11th June 2001, India's premier "Vulture Disease Investigative Centre" was opened to investigate the reasons behind declining vulture populations, and following that, a "Workshop on Monitoring Bird Populations" was organized in January, 2002, as part of the Darwin Vulture Project in collaboration with the Forest and Wildlife Department of Haryana. A total of 45 wildlife NGOs, wildlife departments from different states, the coordinators of the Indian Bird Conservation Network and research personnel studying vultures in India and neighboring countries participated in this workshop. They have developed a common protocol for monitoring vulture populations, including the identification and collection of *Gyps* species carcasses, postmortem examinations, etc. The details of the plans have been carefully explained, and they continue to be updated at www.vulturedeclines.org.

A training program is also conducted regularly for interested researchers in collaboration with the National Birds of Prey Centre of Newent, Gloucestershire, U.K. A "Vulture Care Center" in Panchkula District of Haryana was established at Birshikargha Wildlife Sanctuary in Pinjore. Internationally, two reputed organizations are working to conserve vulture populations. According to Pain et al. (17), the work of two research groups is especially important in this regard. The first group comprises the Forest Department of the state government of Haryana, the Zoological Society of London and the National Birds of Prey Trust, which later expanded to include a wide range of national and international organizations. The second group is composed of the Peregrine Fund, Washington State University and the Ornithological Society of Pakistan. While the first group, the BNHS consortium, is focused largely on India, the TPF/OSP group conducts complementary research programs in Pakistan and Nepal.

6. Modern research and vulture mortality : Under the present scenario of declining vulture populations, it may be difficult to obtain a clearance from the Department of Forests and Environment of the Government of India to sacrifice vultures for study. Furthermore, a comparative study of chemicals and other toxic materials, i.e., between diclofenac and agricultural organophosphorous compounds, which are consumed by Indian cattle while grazing, would be difficult to conduct. The majority of carcasses is usually destroyed, which limits further research. In this context, a report by Swan et al. (29) on Asian vultures demonstrates the recovery of their populations. In a comparative study of *G. africanus, G. bengalensis* and *G. indicus*, the authors observed that meloxicam, a veterinary medicine used in India, is of low toxicity to *Gyps* vultures and that its use in place of diclofenac would substantially reduce vulture mortality in the Indian subcontinent (29). Maryam et al. (93) recently discussed the use of different tissue culture techniques as a tool for the conservation of different endangered or threatened species. Tissue culture techniques are valuable because of their broad applications, and one of the most popular techniques is cryopreservation, which is the most effective technique for conserving the germplasm. Many endangered plants have been saved through this

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method of propagation, but there are some limitations as tissue culture methods can cause somaclonal variations. Apart from these limitations, tissue culture remains a major technique for saving commercially and medicinally important endangered species, but such techniques are widely used in the plant sciences but not in animal sciences due to various constraints (93). Therefore, if tissue culture techniques can be used on samples collected from morbid and healthy vultures, the factors responsible for their mortality may come to light.

Grivas et al. (78) described an automated surveillance system used to study siblicide in a bearded vulture (*Gypaetus barbatus*) nest in Crete from 2003–2006. The system is capable of operating autonomously for more than 1 week causing minimal disturbance to the birds. The system has two parts: 1) a nest-monitoring subsystem (camera, microphone, battery with a charge controller and transmitter with an antenna) supported by a solar panel and (2) a recording subsystem (antenna receiver, video signal controller and a PC remotely controlled through a GSM modem) that compresses the audio–video signal and provides real-time monitoring. Two-egg clutches with 7-day laying and hatching intervals were recorded while hatching asynchrony was determined through an analysis of the bioacoustic data. The food delivery rate and number of feedings to the first chick increased after its sibling hatched. Furthermore, it was observed that 98% of the aggressive interactions were initiated by the dominant chick and resulted in an average of 246 pecks per day. Supplementary feeding did not produce a significant correlation between feeding rate and sibling aggression, and the 2nd chick survived for 5 days before dying as a result of starvation accelerated by sibling aggression. On the basis of these results, the authors concluded that measures aimed at increasing the survival of the second chick should be undertaken when it is 1–2 days old. Although the above surveillance technique was used for monitoring the ethology between newborn and mother birds, it enables research on the morbidity of birds in nature, and it will undoubtedly help to reduce the steep decline of vulture populations in nature.

The analysis of metabolic indices is immensely important to the study of several core evolutionary concepts in animal biology, such as population ecology, life history tradeoffs, senescence, longevity and sexual selection in free-ranging organisms (94). Oxidative metabolism is one such metabolic pathway where O_2 performs a major role in directly or indirectly regulating the biochemical processes related to the oxidation of nutrients to produce energy. The physiology of OS comprises the respiration of oxygen (O_2) by mitochondria, the leaking of O_2 to produce reactive oxygen species (ROS), the oxidation of tissues by ROS, the response of both enzymatic and non-enzymatic redox regulatory molecules or antioxidants against the level of ROS produced and the generation of ATP molecules. The status of all of the above biomolecules has a direct or indirect relationship to health and longevity. Therefore, the analysis of OS indices and antioxidant defense parameters is also of immense importance to animal biology, such as in the study of disease susceptibility, organ failure, and longevity in free-ranging organisms (34, 94). Therefore, the role of OS in relation to both the external and internal factors responsible for vulture mortality may be extrapolated.

7. Role of oxidative stress and metabolic depression in vulture mortality : During the exposure of an animal to various insults in their natural habitat, the normal physiology of animals is compromised, and one of the important outcomes is the interruption of oxygen (O_2) consumption and the availability of O_2 to mitochondria under conditions of stress (95-97). Stress can limit or elevate the supply of O_2 to the mitochondria depending on an organism's metabolic status (97). As a result, it may concomitantly generate oxidants ROS under either a limited or elevated O_2 supply because both of these conditions are known to trigger OS

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pathways in animal tissues (34, 98, 99, 100). Reactive oxidants or ROS, such as the superoxide radical ($O_2^{\bullet-}$), H_2O_2 and the hydroxyl radical ($^{\bullet}OH$), are generated as byproducts of normal oxidative metabolism (101). During internal respiration, electrons are leaked from the mitochondrial matrix to the inter-membrane space through the complex I and III enzymes of the electron transport chain (ETC) (34, 95, 96). Therefore, ETC complex I and III enzymes act as the main hub for $O_2^{\bullet-}$ generation (102-105). Under normal physiological conditions, approximately 1-5% of the O_2 consumed by mitochondria is incompletely reduced to $O_2^{\bullet-}$ radicals, and this incomplete reduction of O_2 becomes > 5% under stress. In mitochondria, the 1st, 2nd and 3rd reductions of O_2 produce $O_2^{\bullet-}$, H_2O_2 and the highly reactive $^{\bullet}OH$ molecules, respectively (34, 106). All of the above molecules have high oxidant activity and, if not neutralized, can oxidize biological macromolecules including lipids, proteins and nucleic acids. $O_2^{\bullet-}$ is catalyzed to H_2O_2 by superoxide dismutase (SOD) (107, 108), and H_2O_2 is neutralized to H_2O and O_2 by the catalase enzyme (CAT) (101, 109). H_2O_2 is also broken down by the glutathione peroxidase (GPx) enzyme in the presence of reduced glutathione (GSH), and oxidized glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase (GR) with the help of NADPH. $^{\bullet}OH$ and other ROS are neutralized by small molecular-weight, non-enzymatic antioxidants, such as ascorbic acid (AA), GSH, non-protein sulfhydryl (-SH), vit-E, vit-C, etc (110, 111). Under normal physiological conditions, a balance is maintained between ROS and antioxidants in the cells of aerobic organisms (95); the lower level of ROS, especially H_2O_2, can also be useful for animals due to its role in various signal transduction processes (112), but an insufficient level of antioxidants can result in greater ROS accumulation in cells. Due to an imbalance between O_2 availability and consumption by the animal and ultimately by the mitochondria, the activities of complex mitochondrial enzymes and antioxidants create favorable conditions for more ROS production and tissue oxidation. This results in increased lipid peroxidation (LPx), protein oxidation (carbonylation: PC), nucleic acid adduct formation and, finally, a disorder known as OS (34, 95, 96, 98, 111). It should be noted that the levels of all of the above biomolecules involved in OS physiology are altered as a function of any internal or external insult, such as environmental extremes, possible genotoxic stress, pollution, food scarcity, infection by contaminated food, food poisoning, nutritional problems, susceptibility to pathogens and stress due to epidemic and endemic diseases. Thus, there could be a correlation between the role of OS in the decline of vulture populations under the above conditions of stress.

In relation to the generation of ROS, birds have been found to be susceptible to OS after exposure to diclofenac (102). Considering the increase in the level of LPx and the enhanced activity of the two major redox regulating enzymes, SOD and CAT, with the decrease in the concentration of GSH in the liver, chicks have been observed to experience OS under a minimum diclofenac dose of 0.8 mg/kg body weight (51). The level of LPx as an index of OS has been found to increase in the liver of Japanese quail (*Coturnix coturnix japonica*) with the intramuscular administration of 5 mg/kg of diclofenac. This has been observed in a variety of birds, including vultures (24, 51, 52). Diclofenac (0.42 ìmol) has also been found to induce a substantial increase in the level of ROS in the cultured, isolated kidney cells of both the African white-backed vulture (*Gyps africanus*) and the domestic chicken (*Gallus domesticus*) (24). A recent review found that the acute hepatotoxic effects of diclofenac reduce/impair ATP synthesis (113); ATP synthesis is the final step in oxidative phosphorylation, and this pathway is highly linked to the generation of ROS and OS (98-111). Therefore, the role of cellular biochemical processes that have toxic end products, such as ROS in OS, may have exaggerated effects on nephrotoxicity and hepatotoxicity by diclofenac in vultures. Such

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biochemical processes may amplify the toxic effects of diclofenac in birds in general and in vultures in particular. This prediction is supported by the observation that the susceptibility of vultures to renal tubular damage caused by diclofenac resulted from a combination of increased ROS production, interference with uric acid transport, and the duration of exposure (24). On the other hand, both OS and the blocking of ATP synthesis are responsible for the initiation of metabolic depression in animals which ends in cell death (102). So, in addition to renal failure and hepatic toxicity caused by diclofenac in vultures, ROS toxicity and OS-mediated metabolic depression seem to be coupled with the above physiological disorders. In combination or alone, all of the above factors could contribute to mortality.

The mechanism under which diclofenac can induce ROS followed by cell death in vultures could be explained through the loss of mitochondrial membrane fluidity in the kidney (102). *In vitro* experiments with the cultured kidney cells of vultures showed that diclofenac can induce cell death as evaluated by the inability of the cell culture to reduce the dye 3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyltetrazolium bromide. With the mitochondria being the only organelle capable of reducing the above dye to formazan, the resultant cell death could only be due to the death of the cellular mitochondria. This explanation is supported by the findings of Ng et al. (114, 115), especially with the toxicity of diclofenac being associated with a 200% increase in ROS production in vulture kidneys. Therefore, the hepatotoxic or nephrotoxic effects of diclofenac in vultures seem to be associated with the generation of ROS and the subsequent OS which could lead to mortality in birds.

8. Possible strategies to revive vulture populations : If a species is facing the possibility of extinction, then investigations into the health problems related to its life cycle and pathology must be conducted so that appropriate measures can be taken. India is ahead of other countries in taking steps to develop and implement recovery plans for vultures. As a result, the rate of population decline of the long-billed vulture, though rapid, is slowing substantially being 16% per year as opposed to the catastrophic decline of the oriental white-backed vulture at 44% per year (Fig. 3, 17). The following steps might be taken to recover their population.

Vigorous research into its biology, pathology and metabolism must be performed, and proper funding should be provided for research programs on their nesting ecology, roosting sites, breeding success and feeding ecology. Research laboratories in and around the vulture community must be established, which should also keep records on morbid vultures. General awareness programs should be put into practice to promote the proper disposal of dead animals while assessing their condition, especially as related to disease, while normal carcasses should be made available to vultures for consumption. The proper health and nutritional care of vultures both in captivity and in nature should be ensured through the establishment of vulture restaurants. Captive breeding and rearing should be encouraged and the identification of natural and artificial hazards, and remedial measures must be undertaken. Regular workshops and hands-on training programs (for carcass submission, preliminary analysis, treatment of ailing birds etc.) must be organized for researchers and other interested people laying emphasis on vulture surveillance and monitoring.

9. Concluding remarks : Based on the preceding discussion, it seems that there could be multiple issues behind declining vulture populations besides diclofenac contamination, so restricting the veterinary use of diclofenac may not be sufficient to restrict the decline. On the other hand, irrespective of the cause, morbid birds face a variety of physiological insults related to metabolic depression, such as the generation of ROS and OS, the loss of mitochondrial count, etc.

It is also clear that it is not too late to prepare an action plan to find the causes and

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corresponding remedial measures to address the decline in vulture populations. Specific scientific and social strategies should be put forward to revive vulture populations, and there are instances such as the recovery of the rhinoceros population in India that suggest that the reversal of a declining trend of a particular population is possible. Programs such as the "Coastal Ocean Monitoring and Prediction System", the "Integrated Coastal Mapping and Management", and the "Bay of Bengal Program", which were instituted to protect coastal ecosystems in India, must be executed at the level of both government and organizations to protect declining vulture populations.

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