



Vultures as a model for testing molecular adaptations of dietary specialization in birds



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ABSTRACT

Vultures are the only obligate scavengers among extant vertebrates. They provide valuable ecological services in ecosystems through removing carcasses, thus preventing the growth of other scavenger populations and the spread of pathogens. Moreover, their specific diets expose them to various deadly pathogens, which makes them potential candidates for studying molecular adaptations required to survive this extremely specialized scavenging habit. In this review, we summarize the morphological characteristics and behavioral habits, origin and phylogeny, and molecular adaptations to scavenging in both Old and New World vultures. The two groups of vultures share a similar appearance, indicative of convergent evolution. Vultures have experienced different degrees of specialization in their sensory organs; Old World vultures depend on sight, while New World ones depend on both smell and sight. Combined fossil records and molecular data suggest that vultures evolved independently, with distinct phylogenetic positions. We also explored their adaptation to scavenging in facial and intestinal microbiomes, gastric acid secretion and immunity. Compared with the facial microbiome, the intestinal microbiome had a lower diversity, dominated by Fusobacteria and Clostridia. The phages and single invertebrate species *Adineta vaga*, which feeds on dead bacteria and protozoa, present in the gut suggest a possible alternative defense mechanism. Several genes involved in gastric acidic secretion (including *ATP4B*, *SLC26A7* and *SST*) and immunity (including *BCL6*, *STING*, and *TLRs*) undergoing positive selection likely have essential roles in eliminating invasive pathogens and initiating an innate immune response. Taken together, this review presents the current research status of vultures and highlights the use of vultures as a model for exploring molecular adaptations of dietary specialization in birds. It also provides a theoretical basis for the study of the genetic mechanisms of vultures to scavenging, and contributes to the formulation of vulture conservation strategies.

1. Introduction

Feeding strategies of birds include typical carnivores, granivores, folivores, frugivores, nectarivores, insectivores, and omnivores (Wang and Zhao, 2015). These feeding lineages are interspersed throughout the avian phylogeny, suggesting that dietary transitions among birds are frequent. Although evolutionary driving forces and genetic mechanisms underlying specific diets remain largely unknown, each of these lineages occupies indispensable ecological niches. For instance, within the class of Accipitriformes, only the vultures (22 species) are obligate scavengers that feed on dead or decaying animal matter even among vertebrates,

except for the Palm-nut Vulture (*Gypohierax angolensis*) that mainly feeds on fruits and grains. By contrast, other Accipitriformes or vertebrates have completely different feeding habits or are facultative scavengers (Zou et al., 2021). Notably, previous studies on molecular mechanisms of vultures underlying scavenging were mostly based on comparative genomics and limited to a few species. Scavenging—the consumption of carrion—plays a critical role in decomposition and nutrition recycling (Campbell, 2015). In some ecosystems, vultures can consume up to 90% of carrion, thus effectively preventing the spread of dangerous pathogens, such as chronic wasting, mad cow, rabies, foot and mouth, and bubonic plague (Houston, 1986). In addition, the ability of vultures to

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quickly locate carrion during flight can outcompete and suppress the population of other facultative scavengers, such as rats, feral dogs, insects, and microorganisms (Buechley and Şekercioğlu, 2016b). At the top of the food chain, vultures provide valuable ecosystem services through removing the carrion of animals, such as lions and tigers, and also serve as good indicators of ecosystem health (Buechley and Şekercioğlu, 2016b).

Due to their unsightly appearance and consumption of large, rotten carcasses, vultures in ancient Greece symbolized ugliness, rivalry, greed and ruthlessness (Aristotle, 1902). However, in ancient Egypt and other ancient civilizations, vultures were considered sacred birds because they usually live on mountain cliffs or towering trees (Ma et al., 2017). People worship vultures as gods or totems, displaying vultures on national emblems or flags, such as the two-headed vulture national emblem of the early Tsarist Russian invaders and Costa Rica's national bird the King Vulture (*Sarcorampus papa*) (Ma et al., 2017). People in Xinjiang, Qinghai, Tibet and other parts of China still maintain the traditional custom of sky burials, in which human corpses are broken into fragments and placed on certain sky burial platforms for vultures to eat, as they believe that the soul of the dead could go to heaven in this way (Ma et al., 2017).

According to the red list of IUCN, the number of vulture species assessed as “Critically Endangered”, “Endangered”, “Vulnerable”, and “Near Threatened” are nine, two, two and three, respectively (Fig. 1). Only seven species are Least Concern (IUCN, 2022). Over the past three decades, environmental pollution, veterinary drug abuse, food poisoning, habitat loss, poaching, and collisions with power lines are the main

factors contributing to the mortalities of vultures (Ma et al., 2017). Among them, veterinary drug abuse is a primary extrinsic threat to vultures’ survival. For instance, in South Asia, especially in India, the abuse of diclofenac (an anti-inflammatory and analgesic veterinary drug) caused a 99% and 96% decline in the populations of White-rumped Vultures (*Gyps bengalensis*) and Indian Vultures (*G. indicus*) between 1992 and 2007, respectively (Ma et al., 2017). In Africa, poisoning caused by Strychnine and synthetic organic pesticides also caused the vulture population to decline by an average of 61%, with seven species crashing by 80% (Ogada et al., 2016). In 2013, one poisoned elephant carcass in Namibia caused the death of up to 600 vultures (Smith, 2014). The vulture population decline subsequently led to the increase of facultative scavengers (feral dogs and rats), accelerating the spread of dangerous diseases such as rabies and anthrax, which then caused enormous threats to economic and human health. Thus, immediate actions, such as regulation of lethal dietary toxins, improving relevant laws and regulations, creating vulture-safe zones, and investigating the status of vulture populations regularly, must be implemented to further prevent the extinction of vultures and the associated loss of valuable ecological services they provide.

2. Morphological and behavioral adaptations to obligate scavenging

Considering the scavenging habits that evolved independently in both Old and New World vultures, the term vulture is not actually a systematically meaningful word, but rather an ecological term to represent

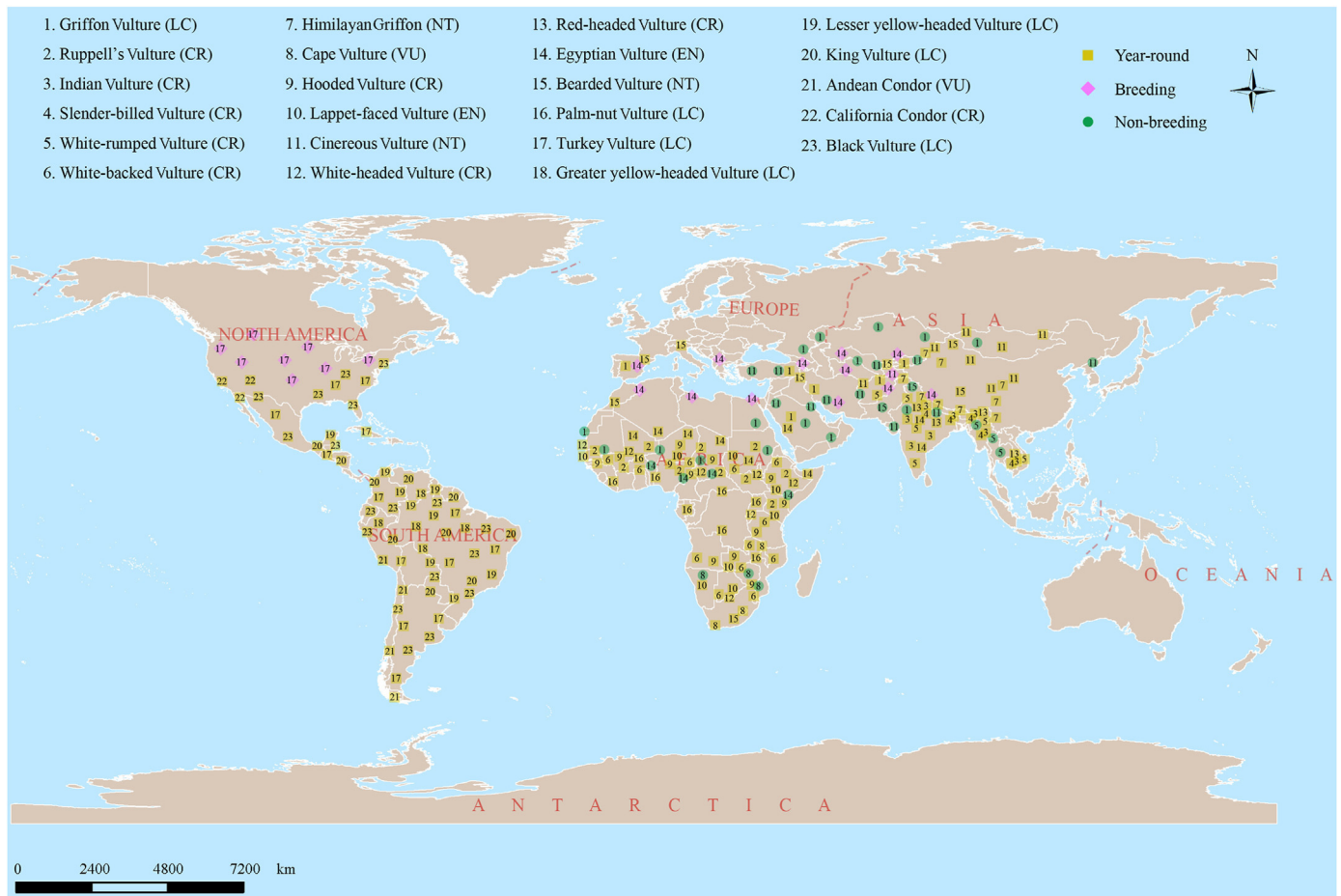


Fig. 1. Distribution ranges of 23 vulture species. Distribution information was taken from the book titled “Birds of the World” (del Hoyo, J.A., Elliott, J.A., Sargatal, J., Christie, D.A., de Juana, E. (Eds.), Cornell Lab of Ornithology, Ithaca, NY). Words in parentheses refer to the conservation status of corresponding species according to the red list of IUCN. The world map was created by ArcGIS (ESRI, 2011. ArcGIS Desktop: Release 10.2. Redlands, CA: Environmental Systems Research Institute).

groups that possess similar lifestyles, morphological characteristics and feeding habits (Campbell, 2015; Buechley and Şekerciöglu, 2016b) (Fig. 2). Old and New World vultures are among the largest flying birds in the world, and locate carcasses by soaring (Ruxton and Houston, 2004). The most notable feature of scavenging vultures is their featherless faces with bald or down-covered heads and long necks, which was thought to be an adaptation to facilitate thrusting into corpses and entrails, and preventing feathers from getting covered in blood. However, several researchers have linked this baldness to thermoregulation (Ward et al., 2008). Their hooked beaks have become more powerful, making it easier to tear through the tough skin and drag out the heavy entrails, and their large wingspans are adapted to soaring without effort while locating carrion, rather than chasing prey (Campbell, 2015). Furthermore, their sturdy feet are better suited for walking on the ground instead of grasping prey, and for New World vultures, the habit of urinating and defecating down their legs (urohydrolysis) helps cool them by evaporation (Campbell, 2015; Buechley and Şekerciöglu, 2016b). Other shared characteristics include strong stomach acids to kill deadly pathogens and even digest bones, a specialized host-microbial alliance, and an excellent immune system allowing carrion consumption without adverse health reactions (Houston and Cooper, 1975). The phenomenon of such morphological and anatomical similarity between the Old and New World vultures is thought to have undergone convergent evolution for scavenging.

To efficiently locate carrion, vultures have experienced different degrees of specialization in their sensory organs. For the Old World vultures, found in mountainous areas, plateaus, plains, desert wadis and highlands in Africa, Asia and Europe, sight rather than olfaction is usually necessary for foraging (Campbell, 2015). Among the New World vultures, cathartid vultures have a highly developed sense of smell, allowing them to detect ethyl mercaptan (a gas emanating from decayed flesh), and find carrion or carcasses below the forest canopy (Houston, 1985; Buechley and Şekerciöglu, 2016a). Other vultures such as King Vultures, Black Vultures (*Coragyps atratus*) and California Condors (*Gymnogyps californianus*) have a very weak, or no sense of smell, but can follow the cathartid vultures to carrion or carcasses (Nielsen, 2006). Therefore, New World vultures use both sight and smell to locate carcasses and occur in mixed terrain that may include neotropical forests or desert patches (Campbell, 2015).

Considering the differences in their primary sensory organs and environmental factors, there are significant differences in dietary composition between the Old and New World vultures (Table 1). The Old World vultures tend to eat larger carcasses, including larger ungulates such as hippopotami, horses, and buffalo, while the New World vultures



Fig. 2. Photo of the Bearded Vulture (Image credit: Donglin Zhou).

Table 1

Dietary sources of 23 species of vultures.

Family/Species name	Dietary sources	References
Accipitridae/Aegyptiinae		
<i>Gyps fulvus</i>	Sheep, goat, cattle, horses, grey wolf, red foxes, jackals, wild ungulates	Karimov and Guliyev, 2017
<i>Gyps rueppellii</i>	Wildebeest, zebra, gazelle, camel, cattle, goat, horses, donkeys	Boone et al., 2006; Wachter et al., 2013
<i>Gyps indicus</i>	Human corpses, goat, deer, gazelle	Clark et al., 2020
<i>Gyps tenuirostris</i>	Deer, pigs, livestock	Sarker and Sarker, 1985; Harris, 2013
<i>Gyps coprotheres</i>	Kudu, cattle, eland, horses	Schultz, 2016
<i>Gyps africanus</i>	Wildebeest, zebra, livestock	Baino et al., 2022
<i>Gyps himalayensis</i>	Yak, human corpses, sheep, goats, dogs, horses, cows, buffalo, red foxes, deer	Lu et al., 2009; Fazili et al., 2021
<i>Gyps bengalensis</i>	Fish, cattle, human corpses, duck	Dharmakumarsinhji, 1955; Brown and Amadon, 1968
<i>Necrosyrtes monachus</i>	Hippopotami, elephant, buffalo, mussels, mollusks, fish, lobsters	Petrides, 1959
<i>Torgos tracheliotos</i>	Reptiles, fish, birds, jackals, mammals, livestock	Mundy, 1982; Mundy et al., 1992; McCulloch, 2006
<i>Aegyptius monachus</i>	Horses, sheep, grey wolf, red foxes, jackals, rabbits, swine, deer	Karimov and Guliyev, 2017
<i>Trigonoceps occipitalis</i>	Lizards, snakes, insects, mongooses, piglets, and birds	Linnaeus, 1758
<i>Sarcogyps calvus</i>	Deer, jackals, livestock, turtles, fish, birds	del Hoyo et al., 1994
Accipitridae/Gypaetinae		
<i>Neophron percnopterus</i>	Red foxes, hares, reptiles, birds, jackals, sheep, cats, dogs, stone martens, badgers	Prakash, 1988; Karimov and Guliyev, 2017
<i>Gypaetus barbatus</i>	Sheep, goat, cow, horses, cats, deer, buffalo, dogs, Tibetan wolf (prefer bone marrow than meat)	Karimov and Guliyev, 2017; Fazili et al., 2021
<i>Gypohierax angolensis</i>	Fruit of oil palm, raffia palm, and fruits and grains of other plants, small mammals, birds, reptiles, amphibians, fish, and invertebrates	del Hoyo et al., 1994; Ferguson-Lees and Christie, 2001; Thomson and Moreau, 2008
Cathartidae		
<i>Cathartes aura</i>	Opossums, coatis, toads, cotton rats, reptiles, amphibians, fish, invertebrates	Platt and Rainwater, 2009; Platt et al., 2021
<i>Cathartes melanbrotus</i>	Primates, sloths and opossums	Campbell, 2015
<i>Cathartes burrovianus</i>	Fishes, reptiles, cats, dogs, anteaters, crab-eating foxes, nutrias, coatis, capybaras, toads	Wetmore, 1965; Pyle and Howell, 1993; Campbell, 2015
<i>Sarcoramphus papa</i>	Raccoons, calves, lizards	Ferguson-Lees and Christie, 2001; Campbell, 2015
<i>Vultur gryphus</i>	Deer, boars, hares, cattle, birds	Duclos et al., 2020
<i>Gymnogyps californianus</i>	Whales, fish, sea lions, sea salmon, calves, deer, feral pigs	Chamberlain et al., 2005
<i>Coragyps atratus</i>	Monkeys, coyotes, fish, raccoons, opossums, skunks, moles, squirrels, cottontails, turkeys, snakes	Farner et al., 1952; Sick, 1993; Kelly et al., 2007

often eat smaller carcasses, predominantly monkeys, rodents, birds and sloths (Table 1) (Buechley and Şekerciöglü, 2016a). In addition, vultures possess broad and highly specialized feeding niches. In the African savannah, the strapping Lappet-faced Vultures (*Torgos tracheliotos*) are the first to eat the carcasses, as they use their powerful beaks to eat tougher hide, sinew and muscles; then the White-backed Vultures (*G. africanus*) and Ruppell's Vultures (*G. rueppellii*) gorge on softer internal organs; the Hooded Vultures (*Necrosyrtes monachus*) and White-headed Vultures (*Trigonoceps occipitalis*) pick up scraps thereafter; and finally, the Bearded Vultures (*Gypaetus barbatus*)—the world's only vertebrate bone-eating specialist, whose diet consists of 85–90% bone marrow—feed on the bones (Buechley and Şekerciöglü, 2016b; Ma et al., 2017). For large bones that are hard to swallow, Bearded Vultures carry them to a height of over 50 m and drop them onto rocks below to break them into smaller pieces (Ferguson-Lees and Christie, 2001). In the Americas, King Vultures are usually dominant over the smaller, weaker billed Black and *Cathartes* Vultures, and also differ from California Condors and Andean Condors (*Vultur gryphus*) (Koford, 1953; Wallace and Temple, 1987; Lemon, 1991; Houston, 1994). Condors prefer to eat viscera and muscle, while Black Vultures remove flesh from bones using their rasp-like tongue and tend to feed on skin and harder tissues (Wallace and Temple, 1987; Nielsen, 2006). The differentiation of dietary ecological niches effectively avoids interspecific competition among vultures, and forms a complicated interspecific relationship formed by the feeding order. Meanwhile, differences in bones and muscles of vultures' heads and necks also indicate various feeding behaviors. The feeding patterns and anatomical adaptations of extant vultures provide important evidence for restoring the ecological details of extinct species.

3. Evolutionary relationships of vultures

Extant vultures contain 16 species of Old World vultures and 7 species of New World vultures, inhabiting diverse biomes from East African savannahs and high Himalayas, to the Amazonian rainforest and the Sahara

Desert (Campbell, 2015) (Fig. 1). Molecular evidence indicates the Old World vultures belonging to Accipitridae are closely related to hawks and eagles, and New World vultures belonging to Cathartidae are the sister group to Old World vultures (Fig. 3) (Johnson et al., 2016; Mindell et al., 2018; Kuhl et al., 2021). Moreover, Old World vultures consist of two clades: Aegypiinae and Gypaetinae. The former is composed of eight species of Griffons of the genus *Gyps* (Cape Griffon *G. coprotheres*, Griffon Vulture *G. fulvus*, Himalayan Vulture *G. himalayensis*, Slender-billed Vulture *G. tenuirostris*, Indian Vulture, Ruppell's Vulture, White-backed Vulture, and White-rumped Vulture), and five species of non-Griffons (Cinereous Vulture *Aegypius monachus*, Red-headed Vulture *Sarcogyps calvus*, Hooded Vulture, Lappet-faced Vulture and White-headed Vulture), closely related to eagles and hawks. Gypaetinae contains three species (Egyptian Vulture *Neophron percnopterus*, Bearded Vulture, and Palm-nut Vulture) closely related to harrier hawks and honey buzzards. In contrast, the New World vultures include seven species of the family Cathartidae: Greater Yellow-headed Vulture (*Cathartes melambrotus*), Lesser Yellow-headed Vulture (*C. burrovianus*), Turkey Vulture (*C. aura*), King Vulture, Andean Condor, California Condor, and Black Vulture.

3.1. Phylogeny of New World vultures

New World vultures and storks present the same unusual behaviors, such as urohidrosis, the flapping of their beaks, beak contact during mating and nurturing between male and female, and enlargement of the air sac in their neck (Konig, 1982; Rea, 1983). They also share many similarities in physiological structures, such as a degenerated or non-functional back toe, bare face and neck, and lack of syrinx resulting in basically no sound (Campbell, 2015). Hence, the cathartid-stork hypothesis has been proposed based on behavioral and morphological characteristics since the 19th century (Garrod, 1874; Ligon, 1967), and was further supported by nuclear DNA-DNA hybridization (Sibley and Ahlquist, 1990) and mitochondrial cytochrome *b* (*cytb*) sequences (Avisé

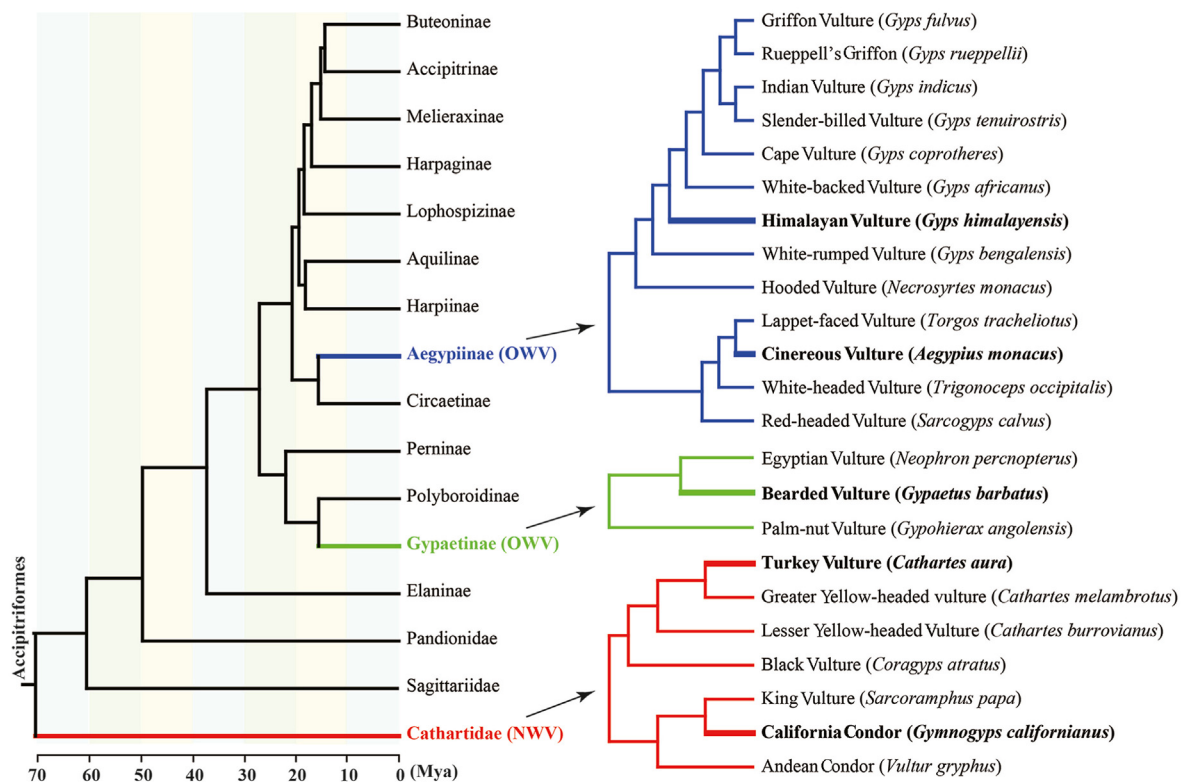


Fig. 3. Phylogenetic relationships among the major groups of Accipitriformes (Johnson et al., 2016; Mindell et al., 2018; Kuhl et al., 2021). Species names in bold refer to vultures with available genomic data. OWV = Old World vultures, NWV = New World vultures.

et al., 1994). For instance, Sibley and Ahlquist (1990) suggested that the New World vultures and storks originate from a common ancestor, and the Old and New World vultures are distant relatives with different origins. Avise et al. (1994) claimed that these two groups have a close relationship and should be considered two Ciconiidae subfamilies. In the early 21st century, a series of studies used broad taxon sampling and informative sites for phylogenetic reconstruction, which no longer supported the cathartid–stork hypothesis, and even proposed that New World vultures should be promoted to a new family—the Cathartidae—or even a higher taxonomic category, the Cathartiformes (Ericson et al., 2006; Livezey and Zusi, 2007; Hackett et al., 2008; Kimball et al., 2013; Mindell et al., 2018). Later, the theory supporting a sister relationship between the New World vultures and Accipitridae (which includes the Old World vultures) and the placement of New World vultures into the Accipitriformes became widely accepted (McCormack et al., 2013; Jarvis et al., 2014; Zhang et al., 2014; Prum et al., 2015).

Similarly, the cathartid species-level relationships remain unclear due to incomplete taxonomic sampling and limited informative sites. Based on the mitochondrial *cytb* gene, previous analyses suggested that the Turkey and Lesser Yellow-headed Vulture form one clade, and the Black and California Condor form a second clade (Wink, 1995; Wink and Sauer-Gürth, 2004). However, a different conclusion was revealed using two mitochondrial and five nuclear genes, which proposed one sister relationship between the Black and *Cathartes* Vultures, and another between the condors and King Vulture (Johnson et al., 2016). Molecular clock analyses, calibrated with fossil records, have estimated the origin of the Cathartidae to be around 69 Ma, and these two primary clades diverging around 14 Ma, with genera ranging in age from about 3 to 12 Ma (Johnson et al., 2016; Mindell et al., 2018). The radiation time of extant Cathartidae vultures is consistent with increased mammalian diversity, particularly predatory mammals and large herbivorous ungulates in open environments, during the middle Miocene (Janis et al., 2000). In the late Pleistocene, the remarkable decrease of terrestrial mammalian megafauna diversity may have caused the reduction of food supply for natural obligate scavengers, contributing to the mass extinction of Cathartidae vultures that fed on terrestrial animal carcasses (Fox-Dobbs et al., 2006; Koch and Barnosky, 2006). In contrast, cathartid vultures having a marine-based diet were likely to survive, possibly because marine megafauna populations did not experience the same magnitude of decline as terrestrial megafauna in the late Pleistocene (Fox-Dobbs et al., 2006).

3.2. Phylogeny of Old World vultures

Early studies proposed that Old World vultures are monophyletic (Brown and Amadon, 1968) or polyphyletic with Neophron, Gypaetus, and Gypohierax, forming one or more clades separate from the other Accipitrids (Mundy et al., 1992; Seibold and Helbig, 1995; Lerner and Mindell, 2005; Mindell et al., 2018; Urantowka et al., 2021). Current taxonomists tend to favor the latter contention, with Old World vultures divided into two subfamilies: Gypaetinae and Aegyptiinae, with the former positioned at the base of the Accipitrid tree (Wink, 1995; Lerner and Mindell, 2005). At the turn of the Paleo-Neogene, the Gypaetinae diverged first from the Perninae, then the Aegyptiinae diverged slightly later from the Circaetinae, followed by the divergence of other Accipitrids (Fig. 3) (Lerner and Mindell, 2005; Mindell et al., 2018). The Aegyptiinae consists of five monotypic genera and eight Griffons of the genus *Gyps*, with the Red-headed Vulture, Lappet-faced Vulture, White-headed Vulture, and Cinereous Vulture forming one group, and the Hooded Vulture and eight Griffons forming another group (Fig. 3). The two groups diverged in the late Miocene, and all monotypic genera emerged from the late Miocene to Pliocene. In contrast, the radiation of the genus *Gyps* occurred within the Pleistocene (Mindell et al., 2018). Among Aegyptiinae, the Lappet-faced Vulture is clustered with the Cinereous Vulture, leaving the Red-headed Vulture as the most primitive of this group (Mindell et al., 2018). Overall, the phylogeny of Old World

vultures, although contradictory with the DNA-DNA hybridization phylogeny proposed by Sibley and Ahlquist (1990), is well supported by morphological, molecular and other phenotypic evidence, indicative of fundamental differences between the two subfamilies (Seibold and Helbig, 1995; Lerner and Mindell, 2005).

4. Evolutionary adaptations to obligate scavenging

Vultures are known as “nature's clean-up crew”, feeding on dead animals that have died mostly from predation, accidents, malnutrition or diseases (Houston, 2009). In order to reach into the body cavities of carcasses, vultures often insert their heads through natural orifices, in particular the anus, thereby exposing themselves to a variety of pathogenic bacteria that could cause tuberculosis, brucellosis, and other diseases (Houston and Cooper, 1975). Thus, how vultures protect themselves against health challenges posed by dietary pathogens has attracted the attention of most vulture biologists. Genetic, physiological and genomic analyses on several species of vultures revealed specific facial and gut microbiomes, gastric acid secretion and immune systems as possible adaptations to their scavenging diets (Mateos-Hernández et al., 2013; Chung et al., 2015; Zou et al., 2021) (Fig. 4).

4.1. Adaptations of facial and gut microbiomes

Gut microbiomes are large and diverse microbial communities, which play crucial roles in maintaining organism health by promoting energy harvest, nutrient acquisition, host defense, and immune regulation (Semova et al., 2012). By investigating face and gut microbiomes from multiple individuals of Black and Turkey Vultures, a previous study has demonstrated that compared to facial skin samples, gut samples showed an extremely low diversity of microbial flora (Fig. 4) (Roggenbuck et al., 2014). Facial skin samples possess an average of 528 operational taxonomic units (OTUs), compared with 76 OTUs of gut samples. Of the two important bacteria groups—Fusobacteria and Clostridia—the former can cause severe food poisoning in vertebrates such as chickens and humans, and periodic die-off of wild birds, particularly waterfowl and shorebirds. The latter have low frequencies in humans, and have been shown to promote colon cancer (Shayegani et al., 1984; Van Immerseel et al., 2004; Rubinstein et al., 2013). Both bacteria groups dominate the vultures' gut microbiota. Interestingly, vultures' facial microbiomes also include these two bacteria groups that are uncommon in other vertebrate microbiomes (Roggenbuck et al., 2014). It was speculated that both bacteria groups probably originate from soil adhering to the carcass or from physical contact with animal carcasses, and are further shaped by the oral-gut passage and the hindgut properties. The most plausible scenario is that these two bacterial groups outcompete other microbes, and form a specialized host-microbial alliance to supply nutrients for vultures from bacterial breakdown of carrion while releasing bacterial toxins (Roggenbuck et al., 2014). Thus, vultures might have evolved to be highly tolerant to bacterial toxins.

Further vulture metagenomic research revealed the presence of a large number of microbial taxa and genes in facial skin associated with diseases such as gas gangrene and pneumonia; in the gut, they are associated with diseases such as gas gangrene and gastroenteritis (Zepeda Mendoza et al., 2018). Potential beneficial bacterial taxa such as anti-listerial bacterium in both facial skin and the gut, as well as genes for the biosynthesis of insecticides, antiparasites and fungicides in facial skin indicated the protective mechanism against possible eukaryotic pathogens in carcasses. Meanwhile, phages present in facial skin and the gut suggest a possible alternative defense mechanism for the elimination and balance of potential pathogens, as in phage therapy (Zepeda Mendoza et al., 2018). One of the most interesting findings is the invertebrate *Adineta vaga*, which feeds on dead bacteria and protozoa, which is more abundant in the gut core than in the facial skin core (Zepeda Mendoza et al., 2018). Finally, the two bacterial groups dominant in vultures' gut microbiota identified by Roggenbuck et al. (2014)—Fusobacteria and

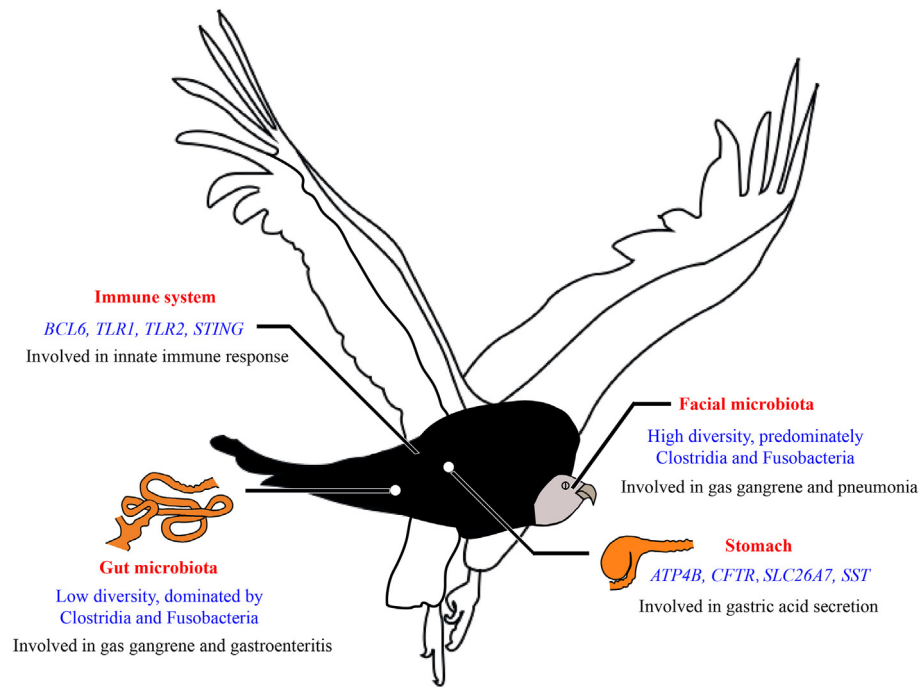


Fig. 4. Evolutionary adaptations of facial and gut microbiomes, gastric acid secretion and the immune system to vultures' scavenging diets.

Clostridia—were further considered to play a role in colonization resistance through biofilm formation (Zepeda Mendoza et al., 2018).

4.2. Adaptation of the gastric acid secretion pathway

Many studies have clarified the peculiarity of the vulture stomach and genes associated with gastric acid secretion (Fig. 5). Vultures have an extremely acidic stomach (lowest PH is 1.0) to inactivate microorganisms encountered from ingested carcasses (Houston and Cooper, 1975). Gastric acid is produced by the proventriculus, and is a major defense mechanism serving as an ecological filter that prevents many infectious viruses and bacteria from gaining access to the intestinal tract. By comparing the genomes of vultures to those of other birds such as chickens, downy woodpeckers, and several eagles, certain genes in the gastric acid secretion pathway were identified to have undergone adaptive evolution in several vulture species. For instance, the Somatostatin (*SST*) gene, encoding a regulatory peptide that suppresses gastric acid secretion through activating the *SSTR2* receptor, was suggested to have been positively or rapidly selected in Bearded and Himalayan Vultures, as well as in the Accipitrimorphae clade (Chung et al., 2015; Zou et al., 2021). *SSTR2* was also found to have undergone rapid evolution in the ancestral branch of Accipitridae (Chung et al., 2015). *SLC26A7* is a basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchanger in gastric parietal cells and plays an important role in gastric acidification (Petrovic et al., 2003). The *SLC26A7* gene was found to be under positive selection in Bearded Vultures, and showed signals of convergence in Bearded, Himalayan and Turkey Vultures (Zou et al., 2021). Analysis of conserved non-exonic elements (CNEs) near or within introns of *SLC26A7* also indicated that several CNEs have undergone convergent evolutionary rate shifts, or have faster evolutionary rates in these three vultures. Given the potential regulatory function of introns in gene expression, researchers suggested that not only the coding region of *SLC26A7*, but also its regulatory region plays an important role in controlling gastric acid secretion (Zou et al., 2021).

Two additional key genes (*ATP4B* and *CFTR*) were also discovered to have undergone molecular adaptation during the secretion of stomach acid in vultures. *ATP4B* forms the β subunit ($\text{H}/\text{K}\beta$) of the gastric proton pump, a heterodimeric gastric H^+/K^+ ATPase that is vital in the transport

of protons (H^+) (Nguyen et al., 2004). *CFTR* is a cystic fibrosis transmembrane conductance regulator, and builds a channel located on the cell surface, responsible for Cl^- transport (Borowitz, 2015). *ATP4B* was found to be under positive selection in Himalayan Vultures, and *CFTR* rapidly evolved in Turkey Vultures. Combined with the molecular adaptation of several other genes in the gastric acid secretion pathway, including *ADCY5*, *CALM2*, *CALM3*, *CAMK2G*, *KCNQ1* and *MYLK*, the genetic mechanism of the evolution of high stomach acid in vultures was further uncovered (Chung et al., 2015; Zou et al., 2021). Genes that were identified to be under rapid or accelerated evolution provide good candidates for further functional studies.

4.3. Adaptation of the immune system

The innate immune system is an important part of an organism's immune defense, providing a natural barrier against microbial invaders and foreign proteins. Given that carcasses are full of large amounts of bacteria and toxins, obligate scavenging vultures were predicted to have evolved a strong immune system to cope with the high risk of contracting deadly parasites. Indeed, some immune-related genes were discovered to have special structures or evolve adaptively in several vultures (Fig. 4). The toll-like receptor 1 (*TLR1*), a member of the TLR family, is a key player in the recognition of microorganisms and initiation of an appropriate immune response during host defense, and is conserved from invertebrates to vertebrates. It has been verified that *TLR1* of the Griffon Vulture and chicken differ in amino acid sequence, with only a 64% similarity; these sequence differences might contribute to immunological functional differences (de la Lastra and de la Fuente, 2007).

By comparing the genomes of vultures and other birds including but not limited to several eagles, researchers have conducted comprehensive analyses on genes associated with the immunity of vultures like the Turkey Vulture in the New World, and Cinereous, Himalayan, and Bearded Vultures in the Old World (Chung et al., 2015; Zhou et al., 2019; Zou et al., 2021). For instance, *AHSG* and *PDCD6* were under positive selection in both Cinereous and Turkey Vultures (Chung et al., 2015). *AHSG* is a serum glycoprotein synthesized by liver cells; it is involved in endocytosis and has opsonic properties by which pathogens are labeled after ingestion and cleared by phagocytes. *PDCD6*, also known as the

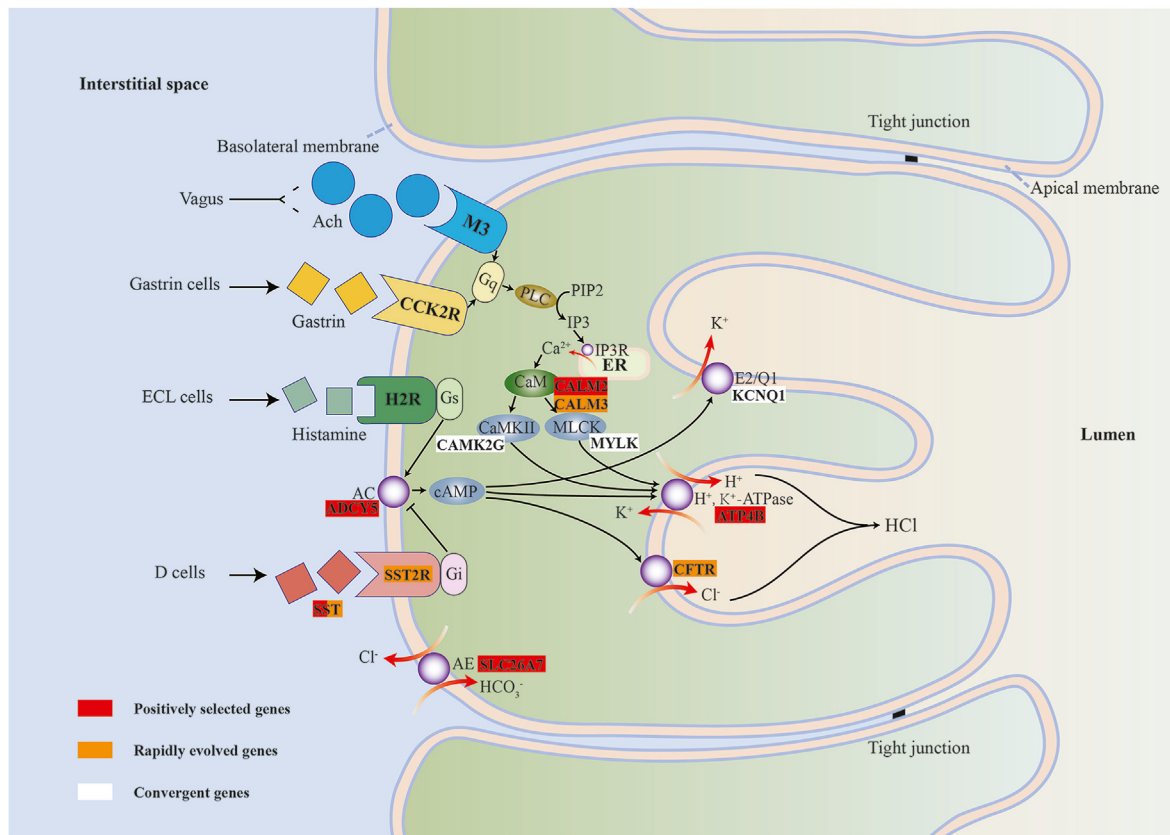


Fig. 5. Diagram of gastric acidic secretion pathway evolution in vultures. Positively selected genes are shown in red box, rapidly evolved genes are shown in orange box, and convergent genes are shown in white box.

programmed cell death protein six, encodes a calcium-binding protein necessary for glucocorticoid-induced cell death, and is essential for virus-induced cell apoptosis. Analyses on the Turkey Vulture suggested another five immune-related positive selected genes (*BCL6*, *CARD9*, *MX1*, *PTPN2* and *SH2D1A*), showing a considerable difference from those of Chung et al. (2015) (Zhou et al., 2019). Researchers also discovered that *PIGR* was positively selected in Turkey, Himalayan and Bearded Vultures, while *PTGES* was positively selected only in the Bearded Vultures (Zou et al., 2021). Proteins encoded by these two genes play vital roles in the transport of polymeric immunoglobulin IgA and IgM and in stimulating mucus secretion, respectively.

In addition to the positively selected immune-related genes, the rapid evolution and convergent evolution among vultures, as well as vulture-specific amino acid changes in immune system pathways also reflect the molecular adaptation of vultures to scavenging. TLR2, another member of the TLR family, was also found to be convergent between Himalayan and Bearded Vultures (Zou et al., 2021). Another three genes, *STING*, *JCHAIN* and *EPCAM*, were shown to have undergone convergent evolution among Turkey, Himalayan and Bearded Vultures (Zou et al., 2021). *STING* (stimulator of interferon genes), independent of TLRs, generates type 1 interferons (IFNs) by activating NF- κ B and IRF3 (Ishikawa and Barber, 2008). *JCHAIN*, as part of polymeric IgA and IgM, is secreted into the mucosa and plays an essential role in antibody production. *EPCAM*, a single channel type I transmembrane glycoprotein expressed in various epithelial tissues, mediates the adhesion of isotype calcium-independent epithelial cells and serves as the first line of defense in the immune barrier against mucosal infection (Nochi et al., 2004). Moreover, a specific amino acid substitution observed in one gene (*BCL6*) of the Turkey Vulture and three genes (*TBK1*, *PIK3AP1* and *TNFAIP3*) shared by Cinereous and Turkey Vultures perhaps alter protein function (Chung et al., 2015; Zhou et al., 2019), considering their key roles in regulating the response to viruses and microbiomes, presenting antigens

or activating NF- κ B (Chung et al., 2015; Zhou et al., 2019). *BCL6* is a nuclear phosphoprotein belonging to the BTB/POZ zinc finger family of transcription factors, and is highly expressed in germinal center B cells. Previous studies have demonstrated that *BCL6* is an endogenous factor that determines Tfh cell development and function, while Tfh cells can interact with B cells in the germinal center and stimulate B cells to proliferate and differentiate into long-acting plasma cells and memory cells (Johnston et al., 2009). *TBK1* (Tank-bound kinase 1) plays a pivotal role in the antiviral innate immune signaling pathway. It can be activated through a variety of pattern recognition receptors (PRR), serving as a kinase that phosphorylates substrates such as transcription factors IRF3/IRF7 to initiate antiviral innate immune responses (tenOever et al., 2004). *PIK3AP1* is a signaling adaptor that contributes to B cell development and can prevent excessive inflammatory cytokine production (Yamazaki et al., 2002). *TNFAIP3*, a zinc finger protein, can be rapidly induced by tumor necrosis factor (TNF), inhibit NF- κ B activation, and is involved in cytokine-mediated immune and inflammatory responses (Song et al., 1996). Taken together, these findings reflect that the evolution of vultures' scavenging is accompanied by the selection of immune-related genes, and these special immune-related genes likely enhance their immune defense against invasive pathogens encountered in their diet.

5. Future directions

Vultures are the only obligate scavengers among birds and other vertebrates, known as "nature's clean-up crew". Through efficiently removing animal carcasses, they provide valuable ecological services in ecosystems, such as suppressing the rapid population growth of other facultative scavengers (hyenas, rats, etc.) and removing deadly pathogens that might cause anthrax, rabies, and plague. One of the most attractive traits of vultures is that they eat carrion without getting sick.

Since the survival of vultures is highly dependent on dead animals (especially vertebrates), we suggest that vultures are strong candidates to become new models for testing the molecular adaptation of dietary specialization in birds.

In this review, we present a comprehensive research overview of vultures, including both the Old and New World vultures. First, we compare the similarities in the morphological characteristics and behavioral habits of two groups of vultures, suggesting that vultures have experienced convergent evolution for scavenging. Meanwhile, vultures have different degrees of specialization in their sensory organs to locate the carrion, and highly specialized feeding niches to eat the carrion. Second, the combined fossil records and molecular data suggested that vultures have evolved independently with a clear phylogenetic relationship. Third, adaptation to scavenging in vultures was shown to be particularly relevant in facial and intestinal microbiomes, gastric acidic secretion and the immune system. Several key genes related to the gastric acidic secretion pathway and immunity, such as *ATP4B*, *SLC26A7*, *SST*, *TLRs* and *STING*, were found to be under positive selection, rapid or convergent evolution, or have vulture-specific amino acid substitutions indicative of potential functional alteration to eliminate invasive pathogens. Since the current understanding of vultures is still rather limited, the mystery of vulture scavenging remains largely uncovered. For instance, as a group of large Accipitriformes birds, what is the reason for vultures to choose a non-predatory scavenging lifestyle? Is there adaptive evolution of scavenging in the ancestors of the Accipitriformes? How exactly do genes related to gastric acid secretion and immunity, as revealed by comparative genomics, enhance immunity in vultures?

To date, genomic data of five vultures (Bearded Vulture, Cinereous Vulture, Himalayan Vulture, California Condor and Turkey Vulture) are available, which provides us the opportunity to further explore the genetic mechanisms of obligate scavenging in vultures (Fig. 4). Based on the available genomic data of Accipitriformes (including five vultures and other Accipitrids with public genomic data) and several other bird species with high-quality genomes (such as chicken, zebra finch, crested ibis and downy woodpecker), we could take a molecular phylogeographical approach which employs phylogenetic evolutionary analyses to reconstruct ancestral traits using molecular markers indicative of trait states. By examining the adaptive evolution of genes related to the gastric acid secretion pathway and immunity, we could infer whether the ancestor of Accipitriformes was a scavenger. Likewise, by comparing the vulture genomes to those of other Accipitriformes birds, we can better understand the evolutionary pattern of genes related to immunity in vultures. Through functional experiments on these genes, we can further reveal the mechanisms of scavenging adaptation in vultures and gain new insights into the evolution of immune-related genes in other facultative animals such as feral dogs and rats.

Given that many vulture species are currently at risk and rely heavily on carrion, we recommend that any livestock that has died accidentally within the range of vultures should not be sold or buried. Instead, these carcasses should be left in the wild for vultures to feed on. To raise awareness about vulture conservation among local residents, it is important to regularly promote and educate them. However, despite these efforts, vultures are still threatened by habitat loss, large-scale mining, high-voltage grids, and heavy metal poisoning. Therefore, relevant departments must formulate protective policies to ensure their survival. In addition, effective, professional, and standardized rescue methods should be employed to establish more vulture rescue centers. These centers will provide food, treatment, and rehabilitation training for local vultures that are hungry, injured or poisoned. Recovered vultures should be released in suitable wild habitats in a timely manner.

Together, we systematically review the current research status of vultures, especially the possible molecular adaptations of obligate scavenging in several vulture species. As the only obligate scavenging vertebrates, vultures are more specialized in their feeding habits than facultative scavenging birds. Hence, we highlight the possibility of vultures serving as a new model for testing molecular adaptations of dietary

specialization in birds, along with providing theoretical bases and new insights for studying the genetic mechanisms of scavenging in vultures and other facultative scavengers.

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Ethics statement

Not applicable.

Authors' contributions

H.Z. conceived and designed the research. Y.C., L.X., P.C. and H.Z. wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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