Drift of oxygen concentrations and gene loss

There have been several mass extinctions during animal evolution [1–4]. Among them, the biggest extinction is believed to have occurred due to the global drop in oxygen concentration in the atmosphere at the PT boundary (Table 1) [1–4]. At this PT boundary, over 90% of species were wiped out due to severe hypoxia (oxygen concentration at around 11–15%) [1–4]. All terrestrial animals must have been exposed to strong selection pressure for adaptation to this low-oxygen condition [1–4]. This mass extinction led to a total change in the basic body plan of vertebrates, allowing them to adapt to low oxygen levels [1–4]. A novel body plan may have included the development of more efficient gas exchange systems, thus affording a greater chance of survival under the low-oxygen conditions and allowing new animals with this novel body plan to become dominant during the Triassic period [5,6]. At the PT boundary, the oxygen concentration sharply went from 30% down to 11–15% [1,2]. The next periods (the late Jurassic, Cretaceous, and Tertiary periods) showed a basic trend of increase in atmospheric oxygen, with a transient drop around the Cretaceous/Tertiary (KT) boundary [1,2].

What was needed for a global change in body plan? A reduced genome size is considered to have been one of the most effective drivers [7–12]. Under hypoxic conditions, theropods totally changed their body plan [13,14] by reducing genome size [7–12]. A recent estimation (Table 2) of vertebrate genome size suggests that genome size has become smaller and smaller during the evolution of the bird lineage (diapsid → little theropod → Neoaves) [7,8]. This estimate shows that gene loss was a main driver of evolution in the bird lineage [9–12].
Research has shown that this gene loss began to occur across the PT boundary when theropod lineages became divided from other diapsids, such as crocodiles, and repeatedly occurred during evolution from reptiles to the Neoaves [7–12]. The basic body plans of theropods and birds [13,14] were changed by intermittent gene loss [7–12]. By contrast, mammals have maintained the same genome size up to the present time [11,12]. The biggest gene loss within a very short time (250–230 million years) occurred just after passage of the PT boundary, when the total change in body plan became dedicated to an adaptation to a lower oxygen level [7–12].

As a result of gene loss, the theropod genome became smaller than mammalian genomes in terms of the number of repetitive and noncoding sequences [7,8]. In addition, the genomes of birds have only about 15 000 protein-coding genes, whereas those of mammals contains more than 20 000 genes [9,10]. Small genome sizes may have been favored by the demands of flight, thus explaining the constricted genome sizes seen among birds, pterosaurs, and bats [11,12].

Table 1. Drift of atmospheric oxygen concentrations

| Geographic periods | Duration (million years ago) | Oxygen concentrations (%) | Trends |
|--------------------|-------------------------------|---------------------------|--------|
| Carboniferous      | 359–299                       | 16 → 28                   | Increase |
| Permian            | 299–252                       | 28 → 30                   | Stabilize |
| PT boundary        |                               |                           |        |
| Triassic           | 252–201                       | 20 → 14                   | Decrease |
| Early Jurassic     | 201–190                       | 14 → 11                   | Decrease |
| Late Jurassic      | 190–145                       | 11 → 15                   | Increase |
| Cretaceous         | 145–66                        | 15 → 19                   | Increase |
| KT boundary        |                               |                           |        |
| Tertiary           | 66–26                         | 17 → 20                   | Stabilize |

*There should have been two phases since the end of the Carboniferous period in terms of a transition of oxygen concentrations [1]. Phase 1 (the Permian, Triassic, and early Jurassic geological periods): the determinant selection pressure on animals was an adaptation to low oxygen [1,3]. Phase 2 (the late Jurassic, Cretaceous, and Tertiary geological periods): the selection pressure served as protection against reactive oxygen species (ROS) leakage induced by increasing oxygen concentrations [1,3].

Abbreviations: KT, Cretaceous–Tertiary; PT, Permian–Triassic.

Table 2. Gene loss of the theropod and bird lineages

| Genus     | Class/order/family | Genome size (billion bp) | Relative size (%) |
|-----------|--------------------|--------------------------|-------------------|
| Human     | Mammal             | 3.5                      | 100               |
| Crocodile | Reptile            | 3.1                      | 89                |
| Triceratops | Ornithischia     | 3.2                      | 91                |
| Apatosaurs | Sauropoda         | 2.15                     | 61                |
| Tyrannosaurs | Theropod        | 1.9                      | 54                |
| Deinonychus | Theropod         | 1.58                     | 45                |
| Emu       | Palaeognathae     | 1.6                      | 46                |
| Crow      | Neoaves            | 1.2                      | 34                |

*Osteocyte size correlates well with genome size in vertebrates. It is possible to approximate osteocyte size from fossilized bones. Thus, the genome size of extinct animals can be estimated by osteocyte sizes in the fossils [7].
Loss of insulin sensitivity to adapt to a low-oxygen atmosphere

In birds, the insulin signaling pathway was apparently shut down in terms of insulin receptor and insulin receptor substrate-1 (IRS-1) phosphorylation in the fat tissue and skeletal muscle, although insulin sensitivity was maintained in the liver [15–17], leading to continuously elevated levels of blood glucose and ketone bodies, both of which are supplied as energy substrates needed to maintain a high metabolic rate and to enable birds to do sustained heavy exercise, such as long flight [18,19]. It has been suggested that insulin sensitivity was lost in the theropods during the Mesozoic era, which was associated with gene loss.

In response to the low oxygen level of the Triassic period, there may have been two options for adaptation, as shown in Figure 1. Mammals took the first option, option A [20,21], whereas theropods took the second option, option B [22–24]. At that time, theropods may have totally changed their body plan by gene loss [7–12], which was dedicated for the purpose of maximizing the efficiency of oxygen usage [22–24], while mammals underwent only a very small model change [10,11]. This response determined the ecological status of mammals and theropods in the Mesozoic era, during which theropods outcompeted mammals and reptiles [1–3].

In response to hypoxia, activation of hypoxia-induced factor-1 (HIF-1) may play a central role in the inhibition of mitochondrial metabolism and activation of glycolysis [25,26]. In addition, insulin is known to activate HIF-1 under normoxic conditions [25,26]. Thus, HIF-1, at least partially, mediates the insulin effects on mitochondrial inhibition [25,26]. Insulin and hypoxia enhance each other and cooperatively inhibit mitochondrial metabolism [25,26]. Although this cooperation may have occurred in the ancestors of mammals, insulin resistance of the bird lineage may have led theropods to lose the insulin-mediated activation of HIF-1 [25,26].

As indicated later, birds and presumably theropods during the Triassic period supposedly made three remarkable physiological innovations, two of which, (i) and (ii), were macroscopic and the third, (iii), was microscopic. This microscopic innovation, insulin resistance, must have been

(A) [Insulin-sensitive]  
(B) [Insulin-resistant]

![Diagram](https://via.placeholder.com/150)

Figure 1. ‘Insulin resistance’ was introduced to adapt to a low-oxygen atmosphere. Basically, in the presence of abundant oxygen and in the absence of insulin, glucose is completely oxidized to H₂O and CO₂ [32–34]. But the presence of insulin inhibits the flow of electrons and protons to mitochondria and activates lactate fermentation, even in the presence of abundant oxygen [29]. In the face of low oxygen, there may be two options. (A) One adaptation is a reduction in oxygen consumption by increasing lactate fermentation while maintaining the insulin system [29]. Mammals must have adopted this system to minimize reactive oxygen species (ROS) leakage, resulting in suppression of the metabolic rate [20,21]. In this model, animals may reduce oxygen consumption so as to adapt themselves to hypoxic conditions [29]. (B) Another adaptation is maximization of oxygen consumption, increasing the metabolic rate by becoming insulin resistant [30]. Birds, and presumably theropods, must have adopted this system and may have faced ROS leakage during the upcoming periods [30]. In this model, animals may have increased oxygen consumption so as to maximize the efficiency of oxygen usage [22–24]. ROS leakage may be a potential adverse effect of this system [22–24].
closely correlated with gene loss at the PT boundary and may have provided cellular platforms to perform the two macroscopic innovations.

(i) The structure of the pelvis, which theropods carry up vertically, enabled them to move quickly without energy loss [13,14].

(ii) Introduction of the air sac to maximize air exchange, which enabled theropods to perform heavy and sustained exercise [5,6]. Birds, and presumably theropods, developed more efficient gas exchange mechanisms to tolerate the hypoxic environment [27,28]. The avian respiratory system is designed for unidirectional airflow to help maximally extract oxygen from the inspired air [27,28]. In addition, birds have a four-chambered heart that prevents the mixing of oxygenated and unoxygenated blood [27,28]. Birds have maintained a high capacity to adapt themselves to hypoxia because they have nucleated and metabolically active erythrocytes [27,28]. They can also increase the amount of hemoglobin inside their erythrocytes under hypoxic conditions [27,28].

(iii) Insulin resistance to maximize the efficiency of oxygen usage in mitochondria [29–31]. Insulin resistance in the fat tissue and skeletal muscle of the chicken may lead to activation of mitochondrial metabolism and increased oxygen consumption [15–17].

Insulin inhibits catabolism (e.g., glucose → H₂O + CO₂) while promoting anabolism (e.g., glucose → glycogen) [32–35]. Therefore, the primary functions of insulin are energy storage (synthesis of glycogen, proteins, and lipids) and prevention of energy consumption (mitochondrial metabolism) [32–35].

Effects of insulin resistance on mitochondrial functions
To increase mitochondrial metabolic rate and oxygen usage, the functions of insulin may have to be constantly suppressed [15–17,30,31]. This notion is supported by two sides of research, the positive side (addition of insulin) and the negative side [insulin receptor knockout (KO) mice], as indicated below.

The addition of insulin action inhibits mitochondrial function [29]
Insulin decreases mitochondrial mass, mitochondrial DNA, intracellular ATP content, and oxygen consumption. Insulin also inhibits transcription of the peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) gene, the activity of which is a key stimulator of mitochondrial biogenesis.

The absence of insulin action [e.g., fat-specific insulin receptor KO (FILKO) mice] activates mitochondrial function [30]
FILKO mice show increased oxygen consumption and extended longevity. FIRKO stimulates PGC-1α expression, activates mitochondrial biogenesis, induces genes that protect cells from oxidative stress by reducing ROS accumulation, and increases the mitochondrial metabolic rate.

What consequences did insulin resistance have for mitochondrial function itself (Figure 2)? Normally, mitochondria are incompletely coupled (10–50% proton leakage, which induces an increase in thermogenesis [22]). According to physiological studies using FILKO mice, insulin resistance leads to the activation of this mechanism [30]. There are distinctive theories as to how mitochondrial metabolism should be associated with evolution and longevity [19–24]. The free radical theory of aging (FRTA) predicts that mitochondria are a main ROS producer, and, therefore, an increase in metabolic rate is not a good thing [20,21]. By contrast, the ‘uncoupling to survive’ hypothesis says that mitochondria are a potent ROS sink, and, thus,
an increase in the metabolic rate is a good thing [22–24]. The FRTA [20,21] favors slow mitochondrial metabolism, while the uncoupling to survive hypothesis [22–24] suggests a high metabolic rate for longevity. Many studies on insulin or insulin-like growth factor-1 (IGF-1) receptor KO mice have concluded that a high metabolic rate and high oxygen consumption may lead to longevity, thus favoring the ‘uncoupling to survive’ hypothesis [29,30]. In fact, the uncoupling rate of mitochondria in birds, an insulin-resistant group, is significantly greater than that of mammals, an insulin-sensitive group [36].

Establishment of insulin resistance

Recent molecular genetic studies have shown that several genes supposedly involved in the endocrine system, including those involved in insulin sensitivity, are missing in the bird genome [37–41]. Four genes that supposedly maintain insulin sensitivity are missing in the bird genome [42–45] and encode the following: (i) omentin [37], (ii) GLUT4 [38,39], (iii) uncoupling protein 1 (UCP1)/UCP2 [40], and (iv) plasminogen receptor [41]. The loss of these four genes may have contributed to insulin resistance in the Triassic period, the development of which may have transformed theropods to become hyperathletic.

Omentin, secreted from adipocytes, is supposed to suppress insulin resistance and can restore insulin sensitivity [42–45]. Dakovic et al. [37] reported that omentin as well as resistin, tumor necrosis factor-α (TNF-α), and plasminogen activator inhibitor are missing in the bird genome. In mammals, insulin sensitivity is notably regulated by the actions of omentin secreted by adipose tissue, which can modulate the activity of the insulin receptor [42–45].

Figure 2. Mitochondrial activation predicted by the ‘uncoupling to survive’ hypothesis [22]. The introduction of insulin resistance increases mitochondrial oxygen consumption, biogenesis, and metabolic rate [29,30]. According to the hypothesis of ‘uncoupling to survive’, the uncoupling rate is increased in accordance with increased mitochondrial activation [22]. To simplify the issue, oxygen consumption and uncoupling rate are supposed to double (a hypothetical example). Because heat production is proportional to uncoupled oxygen consumption, these parameters are increased fourfold. Since ATP synthesis, metabolic rate, and reactive oxygen species (ROS) leakage are proportional to coupled oxygen consumption, they are increased just 1.5-fold, enabling theropods to have endothermy by maximum heat production (fourfold) with minimum ROS leakage. In the case of hypoxia, a diapsid (A) may have insulin resistance to maximize the efficiency of oxygen usage (B) [30]. This shift may increase the several parameters of mitochondrial activities (C) [36].
Xiong et al. [39] reported that GLUT4 is absent from the bird genome; instead, GLUT8 may be broadly expressed, suggesting that constitutive high blood glucose may be due to the loss of GLUT4. The transport of glucose across cell membranes is mediated by members of the GLUT family [46,47], and insulin is an important hormone that regulates glucose uptake by stimulating GLUT4 expression and translocation [46,47]. Unlike mammals, birds are thought to have high blood glucose levels and insulin resistance due to the loss of GLUT4 [38].

Newman et al. reported that UCP1 and UCP2 are also missing from the bird genome; however, UCP3 is expressed, suggesting that UCP3 is closely linked with bird thermogenesis [40,48].

**Insulin resistance and bird paradox**

Insulin resistance [15–17] has several consequences, including high levels of glucose and ketone bodies and high longevity [49–51], all of which contribute to the establishment of the bird paradox associated with mitochondrial activation [52,53]. In addition, this paradox may be a ‘byproduct’ of the adaptation to low oxygen. Insulin/IGF-1 is proposed as an aging hormone in the animal world [54,55], and bird longevity may be a result of avian insulin resistance [32–35].

The paradigm of antiaging research has greatly shifted within the past 20 years, with the central focus of antiaging research having moved from FRTA [20,21] to the insulin hypothesis, suggesting that insulin/IGF-1 is an aging hormone in various animals [54,55]. The insulin hypothesis has been accepted with great impact since Kenyon et al. [54] showed that mutations of the dauer formation 2 (DAF2) gene, which encodes an insulin/IGF-1 receptor, double the lifespan of Caenorhabditis elegans. In addition, a later paper showed that inhibiting insulin/IGF-1 signaling changes the lifespan through activating DAF-16 [55], a forkhead box protein O3 (FOXO3) transcription factor [56,57]. Several models of insulin/IGF-1 receptor KO mice [58–60] have lower fat mass and lower body weight than control mice and show an increase in mean lifespan [58–60]. The results of these studies may justify the statement that the birds are in the ‘DAF2 mutation’ group, whose insulin/IGF-1 receptors are constitutively inactive, doubling the lifespan of the wildtype (mammals).

**Constitutive NRF2 activation in the Neoaves**

The adaptation to low oxygen by introduction of insulin resistance may have been highly effective and not troublesome under hypoxic conditions [1–3]. However, with the increasing concentration of oxygen during the late Jurassic, Cretaceous, and Tertiary periods, as shown in the Table 1, therapsids and birds faced the serious problem of ROS leakage from their mitochondria [1–3]. A series of gene losses may have taken place for adaptation to ROS leakage just after passage of the Cretaceous–Tertiary (KT) boundary (66 million years ago), with deletion of the C-terminal part of the Kelch-like ECH-associated protein (KEAP1), constitutively allowing NRF2 to activate antioxidant enzymes (Figure 3), the activation of which may have taken place during the early Tertiary period (Figure 4) [61,62]. Eventually, birds (Neoaves) have expanded up to the present time [7–12].

At the end of the Mesozoic era (the KT boundary), the earth faced a mass extinction caused by a large comet impact, and 70% of species were lost, including all theropods [1,3]. But just after the KT boundary, the oxygen concentration rose to the present level during the Tertiary period [1,3]. Birds, including Palaeognathae (e.g., chicken and ostrich) and Neoaves (e.g., swallow and crow) [11–13,63], survived to expand to the next stage.

NRF2, a master transcriptional regulator of antioxidant enzymes, is constitutively activated by the deletion of the C-terminal Kelch domain of its repressor protein KEAP1, which tightly binds to the
N-terminal ETGE domain of NRF2 [64–66]. Normally, KEAP1 tightly binds to NRF2, preventing the latter from being translocated into the nucleus, although NRF2 is released following activation by electrophiles to allow the induction of antioxidant enzymes [67–69]. In fact, the NRF2 system has been evolving during these past 1.5 billion years to adapt to the increasing levels of atmospheric oxygen [61]. Since up to the Carboniferous period, the oxygen concentration showed a

Figure 3. A reactive oxygen species (ROS) countermeasure was introduced to address a high-oxygen atmosphere. Constitutive nuclear factor erythroid 2-related factor 2 (NRF2) activation was suggested as a kind of ROS countermeasure to suppress ROS leakage in birds [62]. The mutated Kelch-like ECH-associated protein (KEAP1)/NRF2 system may allow Neognathae (*neovae* represent 95% of all bird species) to overcome ROS leakage induced by their high metabolic rate [62]. Abbreviation: ARE, antioxidant response element.

(The geological periods)

| Paleozoic era | Mesozoic era | Cenozoic era |
|---------------|--------------|--------------|
| Carboniferous (359-299 million) | Triassic (252-201 million) | Cretaceous (145-66 million) |
| Permian (299-252 million) | Jurassic (201-145 million) | Tertiary (66-26 million) |
| Anapsid | Diapsid | Mammal-like reptile |
| Synapsid | Theropod | Mammal |
| Diapsid | Other diapsids | Large theropods |
| Theropod | Little theropods | Palaeognathae |
| Euornithes | Insulin resistance | Body size expansion |
| Neognathae | Constitutive NRF2 activation | [PT boundary] |

Figure 4. Insulin resistance and constitutive nuclear factor erythroid 2-related factor 2 (NRF2) activation essential for the evolution from reptiles (anapsid) to birds (Neognathae). In the Carboniferous period, reptiles (anapsid) became divided into two groups: synapsid (a mammalian lineage) and diapsid (a bird lineage) [7–12]. In the Permian period, mammal-like reptiles were dominant, but all of them became extinct by the end of the Triassic period [1–4]. Theropods (a bird lineage) became dominant, associated with insulin resistance and gene loss [22–24]. In the late Jurassic period, theropods expanded their body size, associated with increasing oxygen concentrations [73–76]. Euornithes (a primitive bird) became separated from the little theropods during the Cretaceous period [73–76]. Associated with a further increase in oxygen concentration [63], Neoaves (swallow and crow) and Palaeognathae (chicken and ostrich) became separated [63]. Neoaves became equipped with constitutive NRF2 activation against reactive oxygen species (ROS) leakage and expanded over the surface of the whole earth [62]. Abbreviations: KT, Cretaceous–Tertiary; PT, Permian–Triassic.
basic trend of increasing [1,3], the NRF2 system, a master regulator of antioxidant enzymes, has become important among the vertebrates [61,62].

One adverse effect of constitutive activation of NRF2 is hyperkeratosis in the digestive tract, identified in KEAP1 KO mice [70]. Neoaves not only overcame this, due to activation of glutathione S-transferase A2 (GSTA2), but also took advantage of hyperkeratosis to produce their highly effective feathers of various colors [62]. The ability of Neoaves to fly may have been enabled by the development of feathers [62].

Another adverse effect of constitutive activation of NRF2 is tumorigenesis [71]. Recent studies showed that constitutive NRF2 activation may be linked with tumorigenicity in humans [71]. Insulin resistance (e.g., in birds) may prevent tumorigenesis induced by constitutive NRF2 activation [52,53,61,62]. In fact, fibroblasts from null IGF-1 receptor KO mice have lost their transformation ability induced by certain activated oncogenes [72].

**Concluding remarks**

One of the most striking outcomes of paleontology over the past 60 years has been the discovery of a very close relationship between birds and little theropods [73–76]. Up to the present time, it was widely accepted that birds evolved from certain little theropods [73–76]. Thus, anatomical and physiological features (air sac, feathers, athletic capacity, and endothermal system) must have been shared between birds and little theropods [73–76]. Since theropods evolved from other diapsids between 230 and 250 million years ago, many researchers suppose that certain critical events for adaptation to low oxygen would have taken place just after passage of the PT boundary [1–3] (Figure 5).

It is noteworthy that the most primitive theropod, herrerasaurus, had hollow bones (possibly with air sacs, which closely correlate with athletic capability) and clearly had a small genome (<1.76 billion bp), suggesting that global gene loss may have taken place before 230 million years ago [7]. The total change in body plan, including insulin resistance, may have occurred just after the PT boundary had been passed within a very short (10–20 million years) time [7]. It is highly possible that herrerasaurus may have been insulin resistant and hyperathletic, similar to the following theropods (e.g., ceratosaurus and allosaurs).

The introduction of molecular genetics to compare avian and mammalian genomes has revealed that some essential genes of mammals are missing in birds and that the genome size of birds is

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**Figure 5. Proposed sequential events for bird evolution.**

**Outstanding questions**

The loss of what genes contributed to insulin resistance in birds? Up to the present, there is only fragmental information available on gene loss in the bird genome. The whole and precise picture of gene loss in the bird lineage [7–12], which is essential for understanding the evolution of the bird lineage (anapsid → diapsid → theropod → little theropod → Euornithes → Neoaves), remains to be determined [7–12]. Here, I describe the possibility of the following physiological axis in bird evolution: selection pressure of hypoxia–global gene loss–insulin resistance–mitochondrial activation–structure of pelvis–installation of air sac–hyperathletic capability–flight and longevity (Figure 5). The most important part for this hypothesis is the causal relationship between ‘Global gene loss’ and ‘Insulin resistance’, and we must establish the whole and precise picture of this event during evolution.

What mechanism was responsible for insulin resistance during avian development (Figure 6)? Insulin and IGF-1 systems are supposedly essential for embryonic development both in mammals and in birds [77,78]. However, mammals retain insulin sensitivity up to death, whereas birds become insulin resistant early in life. This difference may begin to occur just after hatching; in other words, birds lose insulin sensitivity during their development [79]. In the chick sclera, insulin and IGF-1 binding are rapidly downregulated just after hatching, suggesting that there is some mechanism underlying the loss of insulin sensitivity [79]. Determination of this precise mechanism is a key to understanding the bird paradox.

What completely suppresses the phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1) [15–17]? This question suggests that some critical components involved in the events just after insulin and insulin receptor binding [32–35] may be missing in the bird genome. These proteins may be identified by molecular genetics-based comparisons of bird and mammalian genomes [32–35] and may be determinative for the difference between bird and mammalian lineages.
just 30–50% of the genome size of mammals. In addition, the genome size of theropods is just 50–70% of the genome size of mammals, suggesting that the total change in basic body plan associated with global gene loss may have taken place just after passage of the PT boundary [7–12]. Here, I insist that ‘introduction of insulin resistance’ by the loss of omentin, GLUT4, UCP1/UCP2, and plasminogen receptor genes for adaptation of low oxygen caused theropods to become dominant during the Mesozoic era (see Outstanding questions).

Acknowledgements
The author thanks Dr Koujiro Tohyama, DVM (previous Professor of Iwate Medical College, School of Medicine) and Dr Tadashi Yamashita, DVM (Professor of Azabu University, School of Veterinary Medicine) for their critical comments as well as Dr Larry D. Frye for his helpful English editing.

Declaration of interests
No conflict of interest is declared.

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