

# New insight into the evolution of the vertebrate respiratory system and the discovery of unidirectional airflow in iguana lungs

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The generally accepted framework for the evolution of a key feature of the avian respiratory system, unidirectional airflow, is that it is an adaptation for efficiency of gas exchange and expanded aerobic capacities, and therefore it has historically been viewed as important to the ability of birds to fly and to maintain an endothermic metabolism. This pattern of flow has been presumed to arise from specific features of the respiratory system, such as an enclosed intrapulmonary bronchus and parabronchi. Here we show unidirectional airflow in the green iguana, a lizard with a strikingly different natural history from that of birds and lacking these anatomical features. This discovery indicates a paradigm shift is needed. The selective drivers of the trait, its date of origin, and the fundamental aerodynamic mechanisms by which unidirectional flow arises must be reassessed to be congruent with the natural history of this lineage. Unidirectional flow may serve functions other than expanded aerobic capacity; it may have been present in the ancestral diapsid; and it can occur in structurally simple lungs.

diapsid | evolution | lung | lizard | respiratory system

Energetically demanding forms of locomotion, such as powered flight, require a great capacity for gas exchange and selection for aerobic stamina may underlie many unique features of the avian respiratory system (1, 2). The avian respiratory system consists of highly vascularized lungs and avascular air sacs, which are membranous structures that effect ventilation and, in some species, extend between the muscles and even enter the bones (3). The topography of the conducting airways is complex; they form a circular system of tubes, analogous to the loop formed by the blood circulatory system in which arteries connect to veins through numerous small diameter vessels, the capillaries. Likewise, the avian conducting airways connect to each other through numerous tubules, the parabronchi, to form a circular path for respiratory gases (3). Gases flow through most of the parabronchi in the same direction during both inhalation and exhalation (unidirectional flow). This is due to the presence of aerodynamic valves (4–10). In contrast, the mammalian conducting airways arborize with the branch tips ending in blind sacs, there are no valves, and gases travel in the opposite direction along the conducting airways during expiration from the direction followed during inspiration (tidal flow). The presence of aerodynamic valves and unidirectional flow has generally been thought to be a highly derived feature found, among extant animals, only in birds and having evolved either in the crown group with flight or somewhere along the saurischian lineage leading to birds (11), perhaps as a mechanism to meet the high energetic demands of endothermy.

The discovery of unidirectional flow in the lungs of alligators (12, 13) and the savannah monitor lizard (14) indicates that we do not understand the distribution of this phenomenon among different lineages of vertebrates and raises questions about its underlying value. It is possible that unidirectional flow evolved convergently in crocodylians and monitor lizards and serves to expand aerobic capacity. Although monitor lizards are ectotherms,

their lifestyles are largely convergent with small predatory mammals (15) and they have high aerobic capacities compared with other lizards (16). In contrast, extant alligators have limited aerobic stamina (17) but their common ancestor with birds may have had a great aerobic capacity (18) or may have been endothermic (19, 20). Crocodylians and monitor lizards also share a suite of features of their pulmonary and cardiac anatomy that have been purported to give rise to, or coevolve with, birdlike patterns of flow. These features are: (i) a bronchus that has grown deep into the lung as a mesobronchium, (ii) partitioning of the respiratory system into a mechanical part that functions in ventilation and a gas-exchanging region, (iii) intercameral perforations, and (iv) separation of the heart into right and left sides (1, 21). Crocodylians and monitors are also derived in having evolved mechanisms to supplement costal ventilation while exercising (18, 22, 23). Thus, unidirectional flow in these lineages may be one of many derived traits underpinning exceptionally high rates of oxygen consumption during activity.

It is also possible, however, that this pattern of flow evolved before the split of Diapsida into the Lepidosauromorpha (tuatara, lizards, snakes) and Archosauromorpha (crocodylians and birds) in an ectothermic ancestor lacking expanded aerobic capacities and living as long ago as the Permian Period. Unidirectional flow has been purported to serve ectotherms by harnessing the heart as a pump for air during periods of breath-holding (apnea) (12). Light can be shed on this pattern of evolution with observations of more squamates (snakes, lizards), which are the most diverse and largest (~9,000 species) group of living reptiles (24).

## Significance

The avian respiratory system appears strikingly distinct from all other animals. Purported key innovations underpinning avian patterns of airflow are an enclosed intrapulmonary bronchus, intercameral perforations, heterogeneous parenchyma; these traits allegedly coevolved with separation of the cardiac ventricle into right and left sides and are presumed to have been favored by selection because they facilitate high activity metabolisms. In contradistinction to these prevailing theories, here we show that unidirectional flow is present in the lungs of the green iguana, an ectothermic animal with low aerobic capacity, no intrapulmonary bronchus, and no intercameral perforations. This discovery indicates a transformation in our understanding of the evolution of the vertebrate respiratory system is needed.

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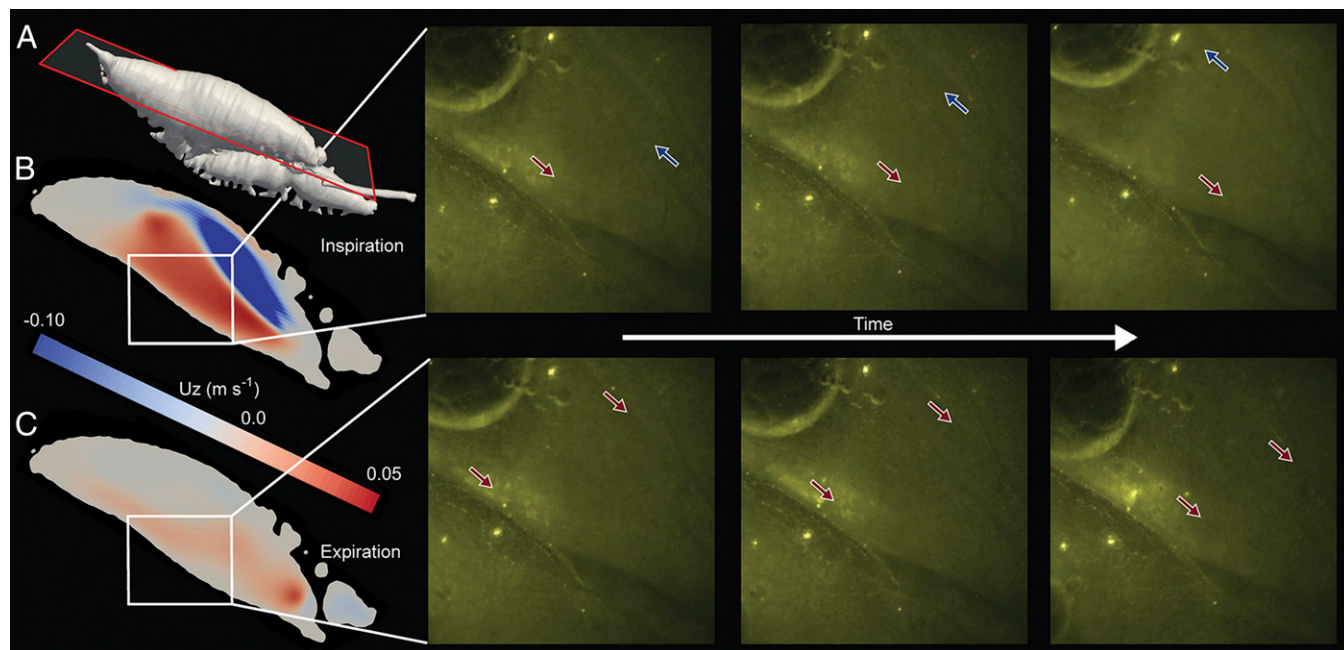
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**Fig. 3.** Flow simulation and ex vivo visualization of flow. (A) Ventromedial view of model with coronal plane of section projected in *B* and *C*. (B) Inspiration: Simulation shows a high-velocity fluid stream emanating caudad (cool colors) and laterad with flow along the medial wall craniad (warm colors). White square marks the approximate location on the model where flow was visualized in excised lungs using fluorescent microspheres in water. Green square panels show three consecutive frames of video (Video S3) of microspheres moving in the lungs while fluid was injected (top three panels) and while it was withdrawn (bottom three panels). (C) Expiration: simulated flow is largely craniad. Flow magnitude (meters per second). Red and blue arrows track microspheres moving craniad and caudad respectively.

a Luxxor Video Camera System (LXX-VBSM), which was interfaced to a computer with a USB 2.0 Image Capture Interface (VC-USB2), and recorded with Debut Video Capture software. The rate of frame capture was 29.97 frames per second. For the purpose of making still images from the movies to display in the printed manuscript, clumps of smoke were tracked visually by advancing through the frames one at a time. The videos of moving smoke did not quantify distances that the particles moved. The videos provide evidence of direction of flow and a qualitative assessment of speed. The velocity of the smoke was not measured. Direction of airflow was determined visually. Both chambers were monitored in all five animals although the exact views differed depending on the location where the probe entered. A minimum of 20 breathing cycles was observed in each location studied. Airflow at the nares was measured with a pneumotachograph (Hans Rudolph) or dual heated thermistor flow meter (HEC 132C, Hector Engineering).

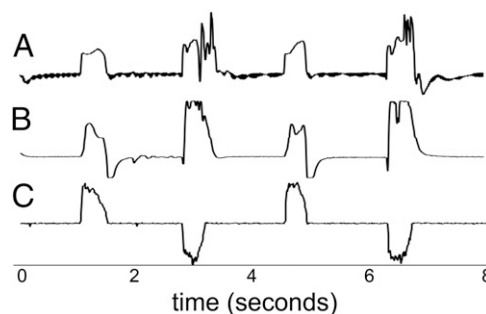
**In Situ and ex Vivo Experiments.** Airflow was measured in separate experiments in situ and in excised lungs ( $n = 10$ ) by implanting a dual thermistor flow meter (HEC 132C, Hector Engineering) into both the dorsal and ventral chambers (Fig. 6). The trachea was intubated and the lung ventilated using a 60-cm<sup>3</sup> syringe. Tracheal flow was measured using a pneumotachograph (Hans Rudolph). The signal was amplified by an A.C./D.C. strain gauge amplifier (P122, Grass Instruments). All analog signals were converted to digital (Biopac Systems) with a sampling rate of 60 Hz and recorded on a computer using AcqKnowledge software (Biopac Systems).

Flow was visualized in excised lungs ( $n = 5$ ) by intubating the trachea and filling the lungs with saline containing microspheres (222  $\mu\text{m}$  in diameter, Thermo Scientific; or C14837, Invitrogen) or pollen collected from sunflowers (*Helianthus annuus*) or citrus pulp. Fluid was withdrawn or pushed into the lung using a 60-cm<sup>3</sup> syringe, and the movement of these particles was visualized using dissection scopes and filmed with a Canon EOS T2i (resolution of 1,080 pixels) digital camera.

**Anatomical Methods.** To assess the presence of intercameral perforations, the lungs ( $n = 5$ ) were excised and the ventral chamber opened. The ostium between the cranial and caudal chambers was sealed with latex. After the latex cured, air was injected into the cranial chamber through the trachea or primary bronchus and the lung placed under water. Gentle pressure was applied manually to the cranial chamber and the intercameral septum was inspected visually for air bubbles filtering through the septum.

Microcomputed tomography ( $\mu\text{CT}$ ) images of excised lungs that had been stained with a KI solution were acquired with an Inveon  $\mu\text{CT}$  scanner (Siemens Preclinical Solutions). Images consisted of 360 degrees rotation with 1,100 steps. The exposure time was 1,700 ms, with detector settings at 80 peak kilovolts and 200  $\mu\text{Amps}$  and a filter of 0.5 mm. Data were reconstructed onto a 2,048  $\times$  2,048  $\times$  1,792 image matrix using the COBRA software package (Exxim Computing). The effective image pixel size was 28.56  $\mu\text{m}$ . Reconstructed images were cropped and visualized using the Siemens Inveon Research Workplace (IRW) and OsiriX software.

Computed tomography data were collected on a Siemens Somatom Definition Flash Scanner at 100 peak kilovolts and 400 milliamp tube current. A series of images were acquired along the long axis of the body. The thickness of each image (slice) was 0.6 mm, and the slices were acquired at intervals of 0.4 mm along the long axis such that 0.2 mm of each slice overlapped with the previous slice. A surface model was then reconstructed from a manual segmentation of the CT data using Avizo 7.1 ([www.vsg3d.com/avizo/standard](http://www.vsg3d.com/avizo/standard)) and a Wacom Intuos4 pen tablet.



**Fig. 4.** Pulmonary and tracheal airflow in an excised lung. Direction of airflow measured along the walls of the lung with heated thermistor flow meters in the cranial chamber (A) and caudal chamber (B). Positive trace is cranial flow. (C) Direction of airflow in the trachea measured with a pneumotachograph. Positive trace is exhalation. Note air moved craniad in both chambers during both phases of ventilation.



discretization schemes. No-slip boundary conditions were applied on the walls of the lung and a custom mesh motion boundary condition was developed to expand and contract the lung at a rate of 15 breaths per minute with a tidal volume of 11 mL, which was based on resting data for green iguanas (25). A physiologically realistic motion of the lung walls was prescribed to match in vivo observations of resting breathing. In particular, the caudal chamber was made to expand and contract more than the cranial chamber and the motion of both chambers was prescribed to be greater laterally than medially. A transient simulation of five breathing cycles was carried out to obtain a time-periodic steady state solution. The computation was performed on 96 processors of a high-performance parallel computer cluster at Penn State University. Airflow patterns in the lung were extracted directly from the numerical solutions using the open-source visualization software ParaView ([www.paraview.org](http://www.paraview.org)).

To ensure that the computational solution is independent of the mesh resolution and time step size, a second simulation was carried out using a significantly finer mesh and a much smaller time step. In particular, the finer mesh contained 3.9 million computational elements, more than twice the number of elements in the coarser mesh, and the simulation was performed on 192 processors using a time step size of 0.0005 s, an order of magnitude smaller than that used in the coarser simulation (0.005 s). In summary, the overall flow patterns in the lung for the coarse and fine mesh simulations are extremely similar (Fig. 7), thereby confirming that the reported results are mesh- and time step-independent.

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