Structural failures of the blood–gas barrier and the epithelial–epithelial cell connections in the different vascular regions of the lung of the domestic fowl, Gallus gallus variant domesticus, at rest and during exercise

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Summary
Structural failure of blood–gas barrier (BGB) and epithelial–epithelial cell connections (EECCs) in different vascular regions of the exchange tissue of the lung was studied in rested and exercised chickens. The number of red blood cells (nRBCs) was counted and protein concentration (PC) measured after lavaging the respiratory system, and blood was sampled to determine the blood lactate levels (BLLs). The numbers of complete BGB breaks (nBGBBs) and those of the EECCs (nEECCBs) were counted in the different vascular territories of the lung. The nRBCs and the PCs increased with increasing exercise intensities but the rate of increase decreased at higher workloads. From rest to the fastest experimental treadmill speed of 2.95 m·sec⁻¹, BLLs increased 4-fold. In all cases, the nEECCBs exceeded those of the BGB, showing that structurally the BGB is relatively weaker than the EECC. The increase in the number of breaks with increasing exercise can be attributed to increase in the pulmonary capillary blood pressure (PCBP) from faster heart rates and higher cardiac outputs, while the leveling out of the measurements made at higher workloads may have arisen from hemodynamic changes that initially ensued from exudation of blood plasma and then flow of blood into the air capillaries on failure of the BGB. The relative differences in the nBGBBs and the nEECCBs in the different vascular regions of the lung were ascribed to diameters of the branches and their points of origin and angles of bifurcation from the pulmonary artery. Presence of RBCs in the air capillaries of the lungs of rested chickens showed that failure of the BGB commonly occurs even in healthy and unstressed birds. Rapid repair and/or defense responses, which were observed, may explain how birds cope with mechanical injuries of the BGB.

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Key words: Birds, Exercise, Rest, Chicken, Lung, Blood–gas barrier, Structural failure

Introduction
“Like most machines, components of the body eventually fail. Disease states, mechanical insult or normal wear may initiate the process of tissue degradation.” (Li et al., 2010)

The air capillaries and the blood capillaries of the exchange tissue of the avian lung have been reported to behave like ‘rigid’ tubes (Macklem et al., 1979; Powell et al., 1985; West et al., 2007; Watson et al., 2008). Like mammals, birds are endothermic homeotherms; they operate at relatively higher body temperatures (40–42°C) (Aschoff and Pohl, 1970). Oxygen is acquired by a structurally complex and functionally efficient respiratory system, the lung air sac system (e.g. Powell and Scheid, 1989; Maina, 2005). To support an energetic lifestyle, large hearts (Hartman, 1955) with large cardiac outputs (Grubb, 1983; Bishop, 1997; Seymour and Blaylock, 2000) supply tissues with oxygen. Compared to mammals, the arterial blood pressures in birds are much higher (Spector, 1956; Speckmann and Ringer, 1963; Erickson, 1994; Seymour and Blaylock, 2000; Lichtenberger, 2005). For example, compared to that of 95 mmHg (12.64 kPa) in a mammal like the cynomologus monkey, Macaca fascicularis, (body mass 4.60 kg), the turkey, Meleagris gallopavo, (4.77 kg) has a higher systolic blood pressure of 150 mmHg (19.95 kPa) (Seymour and Blaylock, 2000). Turkeys have exceptionally high systolic blood pressures of as much as 400 mmHg (53.3kPa) (Beller et al., 2004). In ratites (mainly ostrich, emu, and rhea), sudden deaths have been attributed to aortic rupture (Mitchinson and Keymer, 1977) (2008 factsheet ‘Aortic ruptures’ by Hunter et al.: http://www.agbiosecurity.ca/healthybirds/Factsheets/Disease/AorticRupture.pdf). Termed ‘dissecting aneurism’ or ‘turkey heart attack’, fast
Growing, healthy turkeys (mostly males of between 7 to 24 weeks of age) die suddenly of bleeding after aortic rupture which is caused by high blood pressure (Collins, 1971; Neumann and Unger, 1973; Krista et al., 1986; van Veen, 1999). After autopsy of a visibly (externally) healthy cardinal, *Cardinalis cardinalis*, which died after a short territorial spat, a 7-mm hole (probably caused by acute blood pressure surge during the excitement) was reported in the ventricle of the heart (Erickson, 1994). Birds like the fast growing broiler chickens and turkeys frequently experience hypertensive problems that lead to high myocardial stress, resulting in complications such as aortic aneurysm and ascites (Speckmann and Ringer, 1963; Krista et al., 1970; Julian and Wilson, 1986; Currie, 1999; Issac et al., 2010). In hypertensive turkeys, respectively, Simpson, and Krista et al. (Simpson, 1978; Krista et al., 1967) reported mortality rates from aortic rupture of 20% and 43.7%. It is perplexing that with their relatively high systolic and arterial blood pressures, birds possess a blood–gas barrier (BGB) which is ~3x thinner than those of mammals of equivalent body mass (Gehr et al., 1981; Maina and King, 1982; Maina et al., 1989; Maina and West, 2005; Maina, 2005).

This study investigated the effect of exercise on the structural integrity of the BGB and the epithelial–epithelial cell connections (EECCs) in the different regions of the exchange tissue of the lung of the domestic fowl (chicken), *Gallus gallus* variant *domesticus*, which are supplied by the four main branches of the pulmonary artery (PA). Changes of blood lactate levels (BLLs), numbers of red blood cells (nRBCs) and protein concentrations (PCs) in lavaged fluid were assessed and complete blood–gas barrier breaks (BGBBs) and epithelial–epithelial cell connection breaks (EECCBs) were counted. A list of definition of commonly used abbreviations is given in Table 1 for quick reference.

### Results

At the slowest treadmill speed of 0.66 m.s⁻¹, some of the chickens tried to jump off the treadmill belt but were prevented by a transparent perspex sheet, which was placed well above it. At speeds of 2.53 and 2.95 m.s⁻¹, after 7 minutes of running, the chickens were visibly exhausted; they had to struggle to complete the set exercise time of 10 minutes. The fastest treadmill speed of the experiment (2.95 m.s⁻¹) appeared to be the maximum exercise intensity that the chickens could tolerate for the set exercise program; they had to be physically nudged to keep running and complete the time.

### Blood lactate levels

For the exercised chickens, the pre-exercise BLL values were comparable while the post-exercise ones increased gradually with increasing workload (Fig. 1). In the rested chickens, the difference between the first BLL measurement (1.39±0.01 s.d. mmol.L⁻¹) and the second one (1.27±0.09 s.d. mmol.L⁻¹), measurements taken 10 minutes apart, was not statistically significant (0.5>P>0.1). The small but consistent difference could have ensued from incidental distress of the chickens prior to taking the first measurement. The same can explain the small differences between the resting groups of birds in the different exercise regimens. Between rest and running speed of 0.66 m.s⁻¹, post-exercise BLL rose from 1.29±0.09 s.d. mmol.L⁻¹ to 3.18±0.45 s.d. mmol.L⁻¹, a 2.5-fold increase; between speeds of 1.97 and 2.95 m.s⁻¹, the increase of BLL from 4.03±0.18 s.d. to 5.14±0.61 s.d. mmol.L⁻¹ was not significant (P>0.5) for a 1.5× increase in the running speed (Fig. 1). This shows that under the exercise regimen, BLL approached or reached the highest physiologically tolerable level and that metabolically, the birds were pushed close to their exercise endurance limit.

### Blood–gas barrier breaks and epithelial–epithelial cell connection breaks in different vascular regions of the lung

In rested and exercised chickens, red blood cells (RBCs) were observed in the recovered fluid (Fig. 2); they were indicative of BGB failure. The nRBCs increased up to the treadmill speed of 1.97 m.s⁻¹ after which it leveled out (Fig. 2). While between resting and the treadmill belt speed of 0.98 m.s⁻¹ the nRBCs increased from 3×10⁵±4×10⁴ s.d. per cm² to 5×10⁵±6×10⁴ s.d. per cm², a factorial increase of 1.7, between the speeds of 1.97 (5×10⁵±2×10⁴ s.d. per cm²) and that of 2.95 m.s⁻¹ (6×10⁵±6×10⁴ s.d. per cm²) the increase was only 1.1. At the treadmill speed of 1.97 m.s⁻¹, the chickens started to struggle to keep running, particularly after 7 minutes on the treadmill. In the fluid recovered from the respiratory system of the chickens, PC increased gradually from rest to different exercise intensities (Fig. 3). Between a lower level of exercise and a higher one, the greatest increase occurred between rest (8.85±0.14 s.d. µg.ml⁻¹ and the treadmill speed of 1.97 m.s⁻¹ (34.03±0.23 s.d. µg.ml⁻¹), a factorial increase of ~4. Showing that exercise was affecting them in the same way, the changes in the nRBCs (Fig. 2) matched those of PC (Fig. 3).

### Red blood cells and protein concentrations in lavaged fluid

In both rested and exercised chickens, red blood cells (RBCs) were observed in the recovered fluid (Fig. 2); they were indicative of BGB failure. The nRBCs increased up to the treadmill speed of 1.97 m.s⁻¹ after which it leveled out (Fig. 2). While between resting and the treadmill belt speed of 0.98 m.s⁻¹ the nRBCs increased from 3×10⁵±4×10⁴ s.d. per cm² to 5×10⁵±6×10⁴ s.d. per cm², a factorial increase of 1.7, between the speeds of 1.97 (5×10⁵±2×10⁴ s.d. per cm²) and that of 2.95 m.s⁻¹ (6×10⁵±6×10⁴ s.d. per cm²) the increase was only 1.1. At the treadmill speed of 1.97 m.s⁻¹, the chickens started to struggle to keep running, particularly after 7 minutes on the treadmill. In the fluid recovered from the respiratory system of the chickens, PC increased gradually from rest to different exercise intensities (Fig. 3). Between a lower level of exercise and a higher one, the greatest increase occurred between rest (8.85±0.14 s.d. µg.ml⁻¹ and the treadmill speed of 1.97 m.s⁻¹ (34.03±0.23 s.d. µg.ml⁻¹), a factorial increase of ~4. Showing that exercise was affecting them in the same way, the changes in the nRBCs (Fig. 2) matched those of PC (Fig. 3).

### Structural failure in the chicken lung

Table 1. Definition of abbreviations commonly used in the text.

| Abbreviation | Definition |
|--------------|------------|
| BGB          | Blood–Gas Barrier |
| BGBBs        | Blood–Gas Barrier Breaks |
| BLL          | Blood Lactate Level |
| EECCs        | Epithelial–Epithelial Cell Connection Breaks |
| EECCBs       | Epithelial–Epithelial Cell Connections |
| nBGBBs       | number of Blood–Gas Barrier Breaks |
| nEECCBs      | number of Epithelial–Epithelial Cell Connection Breaks |
| nRBCs        | number of Red Blood Cells |
| PA           | Pulmonary Artery |
| PC           | Protein Concentration |
| PCBPs        | Pulmonary Capillary Blood Pressure |
| RBCs         | Red Blood Cells |
| VO₂          | Oxygen consumption |
| VO₂max       | Maximum Oxygen consumption |
branches was comparable. At the treadmill speed of 0.66 m.sec\(^{-1}\), the region supplied by the cranial branch had the least nBGBBs while that supplied by the caudomedial branch had the greatest number; the number of breaks in areas supplied by the accessory and caudolateral branches was comparable. At the treadmill speeds of 2.53 and 2.95 m.sec\(^{-1}\), the nBGBBs did not show much variation in the four regions. Fig. 6 shows that in rested chickens, the greatest nEECCBs occurred in the region supplied by the accessory branch of the PA while the lowest was in the region supplied by the caudolateral one; the values in the regions supplied by the cranial and caudomedial branches were similar. At treadmill speeds of 0.66 and 0.98 m.sec\(^{-1}\), the nEECCBs in the regions supplied by the accessory and caudomedial branches were comparable and more than in the regions supplied by the cranial and caudolateral branches. At the treadmill speed of 1.97 m.sec\(^{-1}\), the nEECCBs showed modest variation. At treadmill speeds of 2.53 and 2.95 m.sec\(^{-1}\), the accessory and caudomedial branches of the PA had the greatest and the cranial and caudolateral branches the lowest numbers of breaks. Fig. 7 shows the relative differences between the nEECCBs and the nBGBBs in the different vascular regions of the lung. It shows the most common type of break and how the numbers of breaks in the four regions of the exchange tissue compare at rest and during different exercise intensities. In the rested chickens, in the area supplied by the cranial branch of the PA, the EECCBs were the only breaks which were observed while the areas supplied by the other branches showed marked differences of the relative numbers of EECCBs to BGBBs; in the area supplied by the caudomedial branch, the EECCBs contributed 55% of the total number of breaks in the vascular region and for those supplied by the accessory and caudolateral branches the values were 77% and 78% respectively. At 0.66 m.sec\(^{-1}\) treadmill speed, the percentage differences were lower than those in rested chickens, with the area supplied by the

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**Fig. 1. Blood lactate levels (BLLs) of chickens at rest and after exercise.** The post-exercise values increased with increasing exercise intensity but the rate of increase decreased with increasing workload. In rested chickens, the differences between the BLL may be explained by some distress prior to taking the first BLL reading.

**Fig. 2. Changes of the number of red blood cells (nRBCs) in the fluid collected from the respiratory system at rest and after exercise.** The greatest increase of the nRBCs occurred between the treadmill exercise speeds of 0.66 and 1.97 m.sec\(^{-1}\) while the rate of increase of the nRBCs about leveled off at higher workloads.
cranial branch of the PA showing the greatest relative contribution of the EECCBs and the caudomedial one the lowest. At 0.98 m/sec\(^{-1}\), the values were about equal while at the treadmill speed of 1.97 m/sec\(^{-1}\) and higher, the relative proportions between the EECCBs and the BGBBs generally decreased.

Morphologies of the blood–gas barrier breaks and the epithelial–epithelial cell connection breaks

The profuse interdigitation of the air capillaries and the blood capillaries in the exchange tissue of the lung is shown in Fig. 8A,B. The EECCs are for the most part thin cellular extensions that separate the air capillaries while connecting the blood capillaries (Fig. 8A–C). The failure of the EECCs started with small perforations (Fig. 8D) which joined into large slits (Fig. 8E,F), while for the BGB, early injury was heralded by measurable increase of the nRBCs and the PC in the lavage fluid (Figs 2, 3); at lower workloads, linear breaks occurred (Fig. 8G) and at higher ones, the blood capillary wall tore open (Fig. 8H,I).

Discussion

Running on a treadmill is a highly energetic form of exercise that occasions large energy expenditure (Roston et al., 1987; Cabrera et al., 1999). In a horse, Thomas and Fregin observed that at maximum exercise on a treadmill, stroke volume increased by as much 41% above the resting value and cardiac output rose 6-fold (Thomas and Fregin, 1981). Oxygen consumption ($\dot{V}O_2$) of animals running on a treadmill increased linearly as a function of speed (e.g. Gleeson and Baldwin, 1981; Taylor et al., 1981). In the marabou stork, Leptoptilos crumeniferus, Bamford and Maloiy reported a direct relationship between $\dot{V}O_2$ and heart rate for birds running on treadmill (Bamford and Maloiy, 1980), while large increases in the respiratory rate and heart rate were reported by Butler et al., Butler, and Peters et al. in birds flying in a wind tunnel (Butler et al., 1977; Butler, 1991; Peters et al., 2005). Eliassen reported that pulse pressure almost doubled from rest to flight in gulls (Eliassen, 1963), while Hart and Roy recorded 3 to 4 times increase in heart rate over the resting level in exercising pigeon (Hart and Roy, 1966). Increase of BLL in blood during exercise has been shown to occur in three phases (Roston et al., 1987; Cabrera and Chizeck, 1996): in the first phase which corresponds to less than 60% of maximum $\dot{V}O_2$ ($\dot{V}O_{2\text{max}}$) and occurs during mild to moderate exercise, the rate of glycolysis increases several times without significant BLL increase; in the second phase which occurs during heavy exercise
equivalent to 60 to 80% of $\dot{V}O_2\text{max.}$, BLL rises to a higher steady-state level above the resting one, and; during the third phase which occurs when $\dot{V}O_2$ is greater than 80%, anaerobic glycolysis augments the energy derived from aerobic production, causing progressive increase of BLL (Cabrera et al., 1999). Because lactate acid level serves as a diagnostically important oxidizable substrate during exercise, it has been used to access, plan, and predict training and performance endurance programs (Cabrera et al., 1999).

In this study, BLLs rose 4-fold between rested chickens and those run at the fastest treadmill speed of 2.95 m.sec$^{-1}$. The post-exercise BLLs should have been consistent with the exercise intensities since the chickens were not trained to run before the experiments. It cannot, however, be totally ruled out that the chickens may have suffered some degree of stress prior to and during the experiments, even though every care was taken to minimize or eliminate it. The recurrent though insignificant difference between the first and the second BLL measurements in rested birds can be attributed to instinctive stress that may have caused the BLL to remain elevated beyond a 10-minute time interval. The difference between the BLL in the rested chickens and those subjected to the slowest exercise treatment (0.66 m.sec$^{-1}$) can be explained by delay between lactate acid build-up and release of lactate dehydrogenase.

The initial increase of PC in the recovered fluid may have resulted from increased leakiness of the BGB before failure occurred. A similar process was observed in the dog lungs which were subjected to high transient vascular pressure (Maron et al., 2001). Presence of RBCs on the respiratory surface of normal (healthy) birds has been previously reported, e.g. in rock dove, *Columba livia*, (Maina and Cowley, 1998) and the chicken (Nganpiep and Maina, 2002; Kiama et al., 2008). As confirmed here, moderate inconsequential structural failure of the BGB seems to be a common occurrence even in lungs of resting (unstressed) birds. Compared to mammals, birds have higher arterial blood pressures (Seymour and Blaylock, 2000) and much larger hearts (e.g. Hartman, 1955; Seymour and Blaylock, 2000) which generate large cardiac outputs (e.g. Grubb, 1982). Presence of white blood cells and fibrinous strands within minutes of the experiment showed existence of an efficient cellular defense and/or repair mechanism. It may explain how birds contend with BGBBs that appear to occur commonly in their lungs. The production of the fibrillary structures which may plug BGB breaks may be initiated by activation of small GTPases Rho and Cdc42 that are known to stimulate actin filament synthesis (Wood et al., 2002). In the mammalian lung, repair of the alveolar epithelial cells after pressure was increased to injurious levels and then lowered to non-injurious ones was reported by Vlahakis et al. (Vlahakis et al., 2002). In this study, at higher workloads, the chickens may have struggled to complete the 10-minute exercise program due to build-up of lactic acid in the tissues, especially in the leg muscles and occurrence of a
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The greater nEECCBs compared to those BGB, points out to the fact that the former sites are structurally weaker than the latter; the EECCs are the areas of the exchange tissue of the avian lung where thin strands of epithelial cells lie back-to-back, separating the air capillaries and connecting the blood capillaries (Fig. 8A–C). The increase in the relative differences between the nEECCBs and the nBGBBs at higher workloads may have been caused by hemodynamic changes which occurred after failure of the BGB, initially leading to exudation of blood plasma and then streaming of blood into the air capillaries. It is plausible that presence of blood in the air capillaries externally stabilized the BGB while accelerating failure of the EECCs. Interestingly, in chickens infused with 2,4 dinitrophenol (DNP), which caused a 4-fold increase in cardiac output, for unclear reasons, while the pulmonary arterial pressure rose steadily (as cardiac output increased), the increase in the arterial pressure was even less than that in a mammal, where recruitment and distension in the pulmonary capillary system occurs (West et al., 2010).

In concurrence with the physics of fluid flow, the point of origin, the angle of bifurcation, and the diameters of the main branches of the PA should profoundly determine the distribution of blood to different regions of the lung. From the pattern of its branching reported by Abdalla (Abdalla, 1989) and the diameters and angles of bifurcation of the four branches (unpublished observations), more blood should flow into the caudomedial branch because it is the widest and the most direct continuation of the PA while the highest resistance should occur in the accessory branch because it is the first and the narrowest branch that originates from the PA. Measurements made on latex casts of the lung of the domestic fowl (unpublished observations) showed that the angles of bifurcation of the accessory, the cranial, the caudomedial, and the caudolateral branches from the PA were respectively 44°, 56°, 17°, and 90° while the diameters were 0.8, 1.5, 2.2, and 1.4 mm. In the chicken lung, no anastomoses occur between the arterial branches of the PA (Abdalla and King, 1976). Therefore, with certainty, the BGBBs and the EECCBs counted in this study can be associated with the individual branches of the PA. The greatest nBGBBs and nEECCBs occurred in the regions of the lung supplied by the caudomedial and accessory branches in accordance with respectively the expected greater blood flow and resistance (pressure) in the two branches. Pulmonary blood flow redistribution was reported in the lung of the panting ostrich, Struthio camelus, (Jones, 1982).
Conclusion
This study corroborates earlier investigations by Maina and Cowley, Nganpiep and Maina, and Kiama et al. (Maina and Cowley, 1998; Nganpiep and Maina, 2002; Kiama et al., 2008) that structural failure of the BGB is common even in resting (unstressed) and normal (healthy) birds. The high arterial blood pressures in birds (e.g. Seymour and Blaylock, 2000) and systolic pressures that range from 108 to 220 mmHg (14.4–29.3 kPa) (Lumeij and Ritchie, 1994), may lead to high PCBPs, explaining the occurrence BGBBs even in inactive/unstressed birds.

Presence of white blood cells and prompt deposition of fibrin-like material at the failure sites showed existence of an efficient cellular defense and/or repair mechanism of injured BGB. It may explain how birds cope with modest BGBBs. The occurrence of more EECCBs (compared to the BGBBs) showed that as would be expected from their morphologies (Maina and King, 1982; Maina and West, 2005), the EECCs are weaker than the BGBs. The relative numbers of BGBBs and EECCBs in the different parts of the lung, which are supplied with blood by the four main branches of the PA, correlate with the volumes of blood delivered to these regions. Identifying how and under what conditions BGBBs occur in the avian lung should help in formulation of husbandry practices that lower incidences of deaths from such injuries. For birds bred for human consumption, husbandry methods that minimize stress may help reduce losses and therefore improve commercial productivity.
Materials and Methods
Experimental animals
The University of the Witwatersrand’s Animal Ethics Screening Committee granted approval (Clearance St. 007/03/611). Mixed breed free range domestic chickens weighing between 1.4 and 2.8 kg were obtained from a reliable supplier. They were kept in an animal holding unit for 3 weeks to acclimatize to the indoor conditions, become accustomed to handling, and for their health to be properly assessed. The temperature in the well-ventilated room in which they were kept was maintained at 22°C, the birds were exposed to 12-hour light and 12-hour darkness cycles, they were fed a commercial food ration and occasional green vegetables, and water was provided ad libitum. The experimental animals were divided into two groups; the first group comprised of rested chickens while those in the second group were subjected to five exercise regimens. Eight chickens were used for each test; for the 6 experimental set-ups (1 resting and 5 exercise treatments), a total of 48 birds were used, and, in the four experimental formulations (BLL measurement, counting of RBCs, determination of PC, and counting of BGBBs and EECCBs), 192 chickens were used. The microscopic morphologies of the BGBBs and the EECCBs were studied by transmission electron microscopes and scanning electron microscopes.

Pulmonary lavage
The chickens were killed by injection with 5 ml of thiopentone sodium (200 mg cm\(^{-3}\)) (Euthanase\(^{9}\)) into the right brachial vein and the trachea exteriorized and cannulated. PBS (pH 7.4) which was warmed to 40 ˚C was instilled into the respiratory system from a height of 30 cm above the sternum. When the exsanguinated fluid was ligated and the fluid left in the respiratory system for 10 minutes before collecting it by gravity, i.e. by holding the bird’s head down by its legs.

Counting of the red blood cells
A hemocytometer with Neubauer markings and a special coverslip was used to count the RBCs. Counting was done on five squares at a total magnification of 400x, using a 10x eyepiece and a 40x objective. Double counting was avoided by applying an exclusion rule: cells which overlapped the top and the right boundary of the counting area were included while those that touched the bottom and left boundary were excluded. The nRBCs was calculated from the volume of the fluid under the coverslip in the five squares that were counted.

Determination of protein concentration
The Lowry method of PC determination (Lowry et al., 1951) was used. Triplicates of 50 µl each of serially diluted working solution of BSA were put in 1 mm\(^{3}\) Eppendorf tubes. The collected lavage fluid was also prepared in triplicate. Two of 50 µl of reagent A was added to the contents of the Eppendorf tubes which were left on a mechanical shaker for 10 minutes at room temperature (22 ˚C). Fifty µl of reagent B was added to the content of each tube and shaken for another 30 minutes. Absorbance of each of the solutions in a microwell was read at 690 nm wavelength using Labsystems Multiskan Ascent (Amer sham Pharmac ia, Buckinghamshire, UK) spectrophotometer. The absorbance values of the BSA standards were plotted against concentrations to construct a standard curve. The PC in the lavage fluid was calculated using the derived mathematical equation relating the absorbance of the BSA standard to the concentration obtained from the graph.

Experiments on rested chickens
To avoid agitating the birds before the start of the experiment, chickens were kept in isolation (in a cage) for 2 hours. Calming the chickens by sedation was considered but not performed due to possibility of introducing experimental ‘noise’ that would have been difficult to set apart, especially for the exercised birds. After calming down, a chicken was gently removed from the cage and a drop of blood taken by pricking the comb with a lancet. Three measurements of blood lactate level (0.8 mmol/L) were made with Lactate Plus\(^{8}\) (Nova Biomedical, Waltham, MA, USA) hand-held machine. Next, the chicken was injected with 1 ml of saline under the coverslip in the five squares that were counted.

Experiments on exercised chickens
A Collins treadmill machine\(^{8}\) (Upright Merchants, Boulder, CO, USA) powered by a single electric motor with a variable speed of between 0.5 and 3.0 m.sec\(^{-1}\), maximum inclination angle of 5˚ and total running belt length of 150 cm was used. Prior to the experiments, the chickens were allowed to familiarize themselves with the surroundings and become accustomed to the noise made by the machine; they were placed close to it for 1 hour every morning for 2 weeks. Every other care was taken to reduce stress that could increase heart rate or respiratory rate. Measurements of these parameters were avoided in order not to excite the birds. During the experiment, the chickens were quietly approached and gently placed legs first on the treadmill while the machine was running at the slowest speed and zero inclination. When the chicken started walking, the speed of the belt was slowly increased to the desired exercise level. After 10 minutes of running, a sample of blood was immediately taken and the BLL measured. Pulmonary lavage was performed and the nRBCs and the PC determined in the recovered fluid. In the same exercise protocol, the chickens were first injected with heparin through the left brachial vein and after 10 minutes of circulation killed by injecting them with Euthanase\(^{8}\). The coelomic cavity was opened by a ventral approach, the pericardial sac incised, and the adipose tissue around the root of the pulmonary trunk cleared up to where the blood vessel bifurcated into left and right pulmonary arteries. Incisions were made through the right ventricle and also across the tip of the left ventricle to access their cavities. The inflow tube was inserted through the slit in the right ventricle and pushed up to the pulmonary trunk and the outflow tube was inserted through the opening at the apex of the heart and pushed past the atrioventricular valve into the left atrium. The pulmonary circulation was flushed with warm PBS and the lungs fixed as in the rested birds.

Sampling and processing of lung tissues for light microscopy, transmission electron microscopy and scanning electron microscopy
Transverse incisions of the fixed lung were made along the costal sulci and the acquired slices cut in halves. An acetic paper with a quadratic square lattice grid of a total area of 6 mm\(^{3}\) was divided into 1 mm\(^{2}\) numbered squares. The collected lavage fluid was centrifuged at 900 g for 5 minutes and the supernatant reconstituted with 1 ml of reagent A. Two of 1 ml of reagent A was added to the content of each tube and shaken for another 30 minutes. Absorbance of each of the solutions in a microwell was read at 690 nm wavelength using Labsystems Multiskan Ascent (Amer sham Pharmac ia, Buckinghamshire, UK) spectrophotometer. The absorbance values of the BSA standards were plotted against concentrations to construct a standard curve. The PC in the lavage fluid was calculated using the derived mathematical equation relating the absorbance of the BSA standard to the concentration obtained from the graph.

Determination of blood–gas barrier breaks (BGBBs) and epithelial–epithelial cell connection breaks (EECCBs)
The BGBBs and the EECCBs in the vascular regions supplied by the PA were counted on semithin toluidine-blue stained sections at a final magnification of 600x, using a 10x eyepiece and a 40x objective (Fig. 8A). The analysis was made on eight randomly selected microscopic fields per section. The sections were randomly selected from the different vascular regions of the lung. The average nBGBBs was calculated by adding the total number of breaks in the vascular regions of the lung and dividing it by the number of 100 µm\(^{2}\) counting fields; the same was done for the EECCBs. The relative (%) proportions of the BGBBs and the EECCBs in the vascular regions of lung were calculated for comparisons.

Statistical evaluation
A nested analysis of covariance (ANCOVA) was used to test the differences between some of the parameters (Littell et al., 1991), with the statistical level of significance set at P<0.05.

Acknowledgements
This work was funded by the National Research Foundation (NRF) of South Africa. We are grateful to the technical staff of the School of Anatomical Sciences of the University of Witwatersrand for their immense assistance; to Mr C. van der Merwe of University of Pretoria for assistance with scanning electron microscopy; Dr V. Meskieni for training (S.A.J.) in techniques of immuno-electronic microscopy; staff of the Central Animal Service (CAS) Unit of the
University of the Witwatersrand for their help with care of the animals.

Competing Interests

The authors have no competing interests to declare.

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