

## Review

# Pulmonary arterial hypertension (ascites syndrome) in broilers: A review

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**ABSTRACT** Pulmonary arterial hypertension (PAH) syndrome in broilers (also known as ascites syndrome and pulmonary hypertension syndrome) can be attributed to imbalances between cardiac output and the anatomical capacity of the pulmonary vasculature to accommodate ever-increasing rates of blood flow, as well as to an inappropriately elevated tone (degree of constriction) maintained by the pulmonary arterioles. Comparisons of PAH-susceptible and PAH-resistant broilers do not consistently reveal differences in cardiac output, but PAH-susceptible broilers consistently have higher pulmonary arterial pressures and pulmonary vascular resistances compared with PAH-resistant broilers. Efforts to clarify the causes of excessive pulmonary vascular resistance have focused on evaluating the roles of chemical mediators of vasoconstriction and vasodilation, as well as on pathological (structural) changes occurring within the pulmonary arterioles (e.g., vascular remodeling and pathology) during the pathogenesis of PAH. The objectives of this review are to (1) summarize the pathophysiological progression initiated by the onset of pulmonary hypertension and culminating in terminal ascites; (2) review recent information regarding the factors contributing to excessively elevated resistance to blood flow through the lungs; (3) assess the role of the immune system during the pathogenesis of PAH; and (4) present new insights into

the genetic basis of PAH. The cumulative evidence attributes the elevated pulmonary vascular resistance in PAH-susceptible broilers to an anatomically inadequate pulmonary vascular capacity, to excessive vascular tone reflecting the dominance of pulmonary vasoconstrictors over vasodilators, and to vascular pathology elicited by excessive hemodynamic stress. Emerging evidence also demonstrates that the pathogenesis of PAH includes characteristics of an inflammatory/autoimmune disease involving multifactorial genetic, environmental, and immune system components. Pulmonary arterial hypertension susceptibility appears to be multigenic and may be manifested in aberrant stress sensitivity, function, and regulation of pulmonary vascular tissue components, as well as aberrant activities of innate and adaptive immune system components. Major genetic influences and high heritabilities for PAH susceptibility have been demonstrated by numerous investigators. Selection pressures rigorously focused to challenge the pulmonary vascular capacity readily expose the genetic basis for spontaneous PAH in broilers. Chromosomal mapping continues to identify regions associated with ascites susceptibility, and candidate genes have been identified. Ongoing immunological and genomic investigations are likely to continue generating important new knowledge regarding the fundamental biological bases for the PAH/ascites syndrome.

**Key words:** broiler, ascites, pulmonary hypertension, immunology, genetics

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## OVERVIEW

Ascites (serous fluid accumulation in the abdominal cavity) has been observed worldwide in fast growing broilers. The available experimental evidence consistently supports the hypothesis that clinical ascites represents the terminal consequence of a pathophysiological progression initiated by excessively elevated blood pressure within the pulmonary circulation (pulmonary hypertension). Indeed, pulmonary arterial hypertension

(**PAH**), pulmonary hypertension syndrome, and ascites syndrome commonly are used synonymously (Plog, 1973; Sillau and Montalvo, 1982; Huchzermeyer and DeRuyck, 1986; Hernandez, 1987; Julian, 1988; Owen et al., 1994; Wideman et al., 2007). Numerous reviews have summarized the extensive literature related to nutritional, management, environmental, and genetic influences on the pathogenesis of PAH (Wideman, 1984, 1988, 2000, 2001; Huchzermeyer and DeRuyck, 1986; Lopez-Coello et al., 1986; Hernandez, 1987; Julian, 1988, 1993, 2000, 2007; Shlosberg et al., 1992, 1998a; Wideman and Bottje, 1993; Odom, 1994; Bottje and Wideman, 1995; Lister, 1997; Mitchell, 1997; Currie, 1999; DeCuyper et al., 2000; Wideman et al., 2004,

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2007; Olkowski, 2007; Pavlidis et al., 2007; Baghbanzadeh and DeCuyper, 2008). The objectives of this review are to (1) summarize the pathophysiological progression initiated by the onset of pulmonary hypertension and culminating in terminal ascites; (2) review recent information regarding the factors contributing to excessively elevated resistance to blood flow through the lungs; (3) assess the role of the immune system during the pathogenesis of PAH; and (4) present new insights into the genetic basis of PAH.

## PATHOGENESIS

### *Marginal Pulmonary Vascular Capacity*

A broiler chick weighs 40 g at hatch and is capable of growing to 4,000 g in 8 wk. If humans grew at a similar rate, a 3 kg (6.6 lb) newborn baby would weigh 300 kg (660 lb) after 2 mo. Doubling and redoubling of the body mass almost 7 times in 8 wk cannot be sustained without equally dramatic increases in the functional capacities of the heart and lungs. The left ventricle of the heart pumps the oxygenated blood needed to support basal metabolism, activity, and growth. The volume of blood pumped by the left ventricle each minute, known as the cardiac output, averages 200 mL per kilogram of BW per minute (Wideman, 1999). A linear extrapolation of this relative value indicates the absolute cardiac output must increase 100 fold during the 8 wk posthatch, ranging from 8 mL/min for a 40-g chick to approximately 800 mL/min for a 4-kg broiler. The rate at which venous blood returns to the heart must equal the cardiac output; therefore, during the first 2 mo posthatch the blood vessels in a broiler's lungs must develop the pulmonary vascular capacity to receive and oxygenate a 100-fold increase in venous return. We have defined pulmonary vascular capacity to broadly encompass both anatomical and functional characteristics, including the total number and volume of blood vessels in the lungs, the tone (degree of partial constriction) maintained by the precapillary arterioles that offer the primary resistance to pulmonary blood flow, as well as the compliance (ease of distention) and reserve capacity (availability for recruitment) of the capillaries (Wideman and Bottje, 1993; Wideman, 2000, 2001; Wideman et al., 2007). Evaluations of cardio-pulmonary hemodynamics indicate broiler lungs possess a very limited capacity to employ key compensatory mechanisms that enable mammalian lungs to readily accommodate increases in the cardiac output, such as flow-dependent dilation of precapillary arterioles, and vascular distention or recruitment of previously underperfused vascular channels. Instead, in the lungs of rapidly growing broilers all available blood vessels appear to be engorged with blood, indicating the pulmonary vasculature possesses only a modest reserve capacity even under ideal conditions (Wideman and Kirby, 1995b; Wideman et al., 1996a,b, 2007; Martinez-Lemus et al., 1999; Wideman, 2000, 2001; Odom et al.,

2004). The marginal pulmonary vascular capacity of broilers is not surprising in view of the poor correlation between their absolute lung volume and BW, and their low lung volumes relative to BW compared with Single Comb White Leghorn domestic fowl and jungle fowl (Julian, 1989; Vidyadaran et al., 1990; Owen et al., 1995a,c; Silversides et al., 1997; Wideman et al., 1998b; Wideman, 1999; Hassanzadeh et al., 2005; Areiza-Rojas et al., 2011).

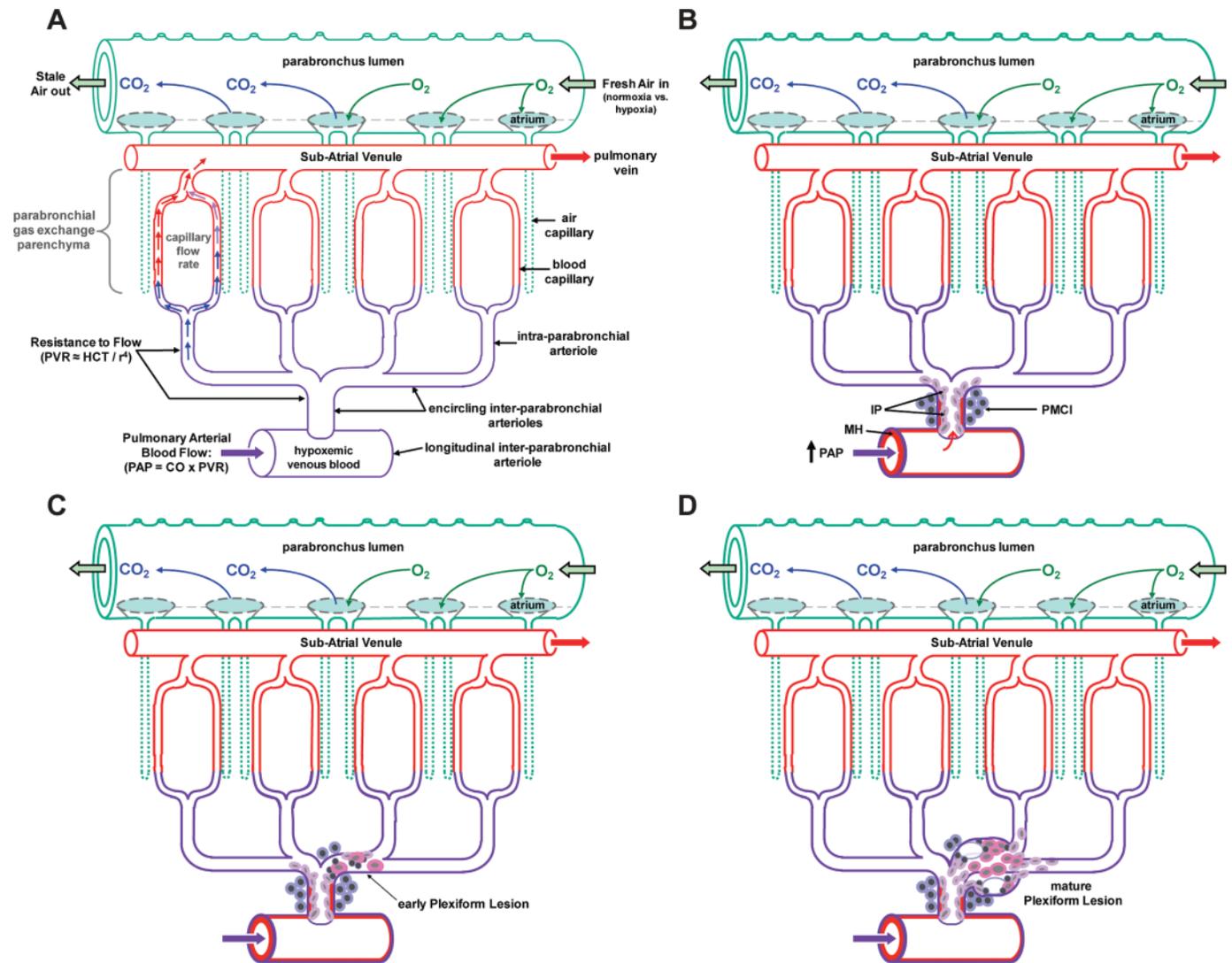
### *Initiation of Pulmonary Hypertension*

In clinically healthy broilers the right ventricle propels the entire cardiac output through the lungs at relatively low ( $\leq 22$  mmHg) pulmonary arterial pressures. The pulmonary circulation normally functions with low hydrostatic pressure gradients to minimize the threat of fluid filtration into the gas exchange spaces (pulmonary edema). Low pressures can be sustained as long as the vasculature maintains a suitably low resistance to blood flow. However, if the pulmonary vascular channels are persistently engorged with blood to the extent that they have become essentially nondistensible and nonrecruitable, or if the pulmonary arterioles (the primary resistance vessels) maintain excessive vascular tone, then the right ventricle is forced to develop increasingly higher pressures to propel growth-related increments in cardiac output through the lungs (Figure 1A). Broilers possessing the most restrictive pulmonary vascular capacity therefore are critically susceptible to the initiation of pulmonary hypertension (Wideman and Bottje, 1993; Wideman, 2000). The sequelae to increases in the pulmonary arterial pressure include cardiac work hypertrophy that is specific for the right ventricle (elevated right-to-total ventricular weight ratios; **RV:TV** ratios), and accelerated rates of blood flow through the lungs. Red blood cells racing too rapidly through the pulmonary vasculature cannot achieve full blood-gas equilibration because a finite amount of residence time at the gas exchange surfaces is required for complete diffusive exchange of O<sub>2</sub> and CO<sub>2</sub> (Henry and Fedde, 1970; Powell et al., 1985; Figure 1A). Inadequate residence time causes blood exiting the lungs to enter the systemic circulation with a lower than normal partial pressure of O<sub>2</sub> (hypoxemia) and a higher than normal partial pressure of CO<sub>2</sub> (hypercapnia). The onset of hypoxemia and hypercapnia serve as reliable predictive indices that apparently healthy broilers will develop PAH (Peacock et al., 1989, 1990; Reeves et al., 1991; Julian and Mirsalimi, 1992; Wideman and Kirby, 1995a,b; Wideman et al., 1996a,b; Roush et al., 1996, 1997; Kirby et al., 1997; Fedde et al., 1998; Wideman et al., 1998c; Forman and Wideman, 1999). Well-established hypoxemia is rapidly and completely reversible if affected birds are provided 100% O<sub>2</sub> to breathe, definitively proving that hyperperfusion of the lungs with blood creates a diffusion limitation attributable to inadequate erythrocyte residence times (Wideman and Tackett, 2000; Wideman et al., 2000; Lorenzoni and

Wideman, 2008a). All major broiler genetics companies routinely use pulse oximetry to assess the adequacy of arterial blood oxygenation. Culling hypoxemic individuals from pedigree lines improves the innate resistance of commercial broilers to PAH, and throughout the past decade the incidence of PAH/ascites has declined dramatically in commercial broiler flocks reared at nominal altitudes (Wideman, 2000, 2001).

Hypoxia refers to a low partial pressure of  $O_2$  in the inspired air. The partial pressure of  $O_2$  decreases with

increasing altitude (e.g., hypobaric hypoxia), and reduced levels of inspired  $O_2$  trigger acute pulmonary vasoconstriction and pulmonary hypertension in broilers (Ruiz-Feria and Wideman, 2001). Thus, hypoxia is a key environmental stressor that contributes significantly to increased incidences of PAH when broilers are reared at higher altitudes (*vide infra*). Hypoxemia refers to blood within the systemic arteries that is under-saturated with  $O_2$  (e.g., has a lower than normal partial pressure of  $O_2$ ). Hypoxemia has no apparent



**Figure 1.** (A) Diagrammatic representation of parabranchial blood flow and air flow patterns. Partially deoxygenated pulmonary arterial blood (broad blue arrow) flows through longitudinal and encircling inter-parabranchial arterioles and then through intra-parabranchial arterioles to perfuse blood capillaries within the gas exchange parenchyma. Oxygen ( $O_2$ ) moves through atrial openings in the parabranchial lumen and diffuses into air capillaries within the gas exchange parenchyma, with  $CO_2$  diffusing in the reverse direction. Gas exchange occurs at the interface between blood- and air-capillaries, and fully oxygenated blood exits the pulmonary vein (broad red arrow). The pressure required to propel blood flow through the pulmonary vasculature (pulmonary arterial pressure: PAP) is directly proportional to the volume of blood pumped by the heart per minute (cardiac output: CO) and the resistance to blood flow through the pulmonary vasculature (pulmonary vascular resistance: PVR). The PVR is directly proportional to the blood's viscosity, which predominately is determined by the hematocrit (HCT), and is inversely proportional to the cumulative vascular radius raised to the fourth power ( $1/r^4$ ). Blood flowing through the gas exchange parenchyma becomes fully saturated with  $O_2$  within the first 20 to 30% of the capillary's length (bright red arrows) unless the flow rate is too rapid for the erythrocytes to achieve full diffusive exchange of  $O_2$  (blue to purple arrows). (B) Increased PAP causes medial hypertrophy (MH) in inter-parabranchial arterioles. Increased turbulence and shear stress at arteriole branch points apparently stimulate the extramural aggregation of arteriolar perivascular mononuclear cell infiltrates (PMCI), accompanied by luminal intimal proliferation (IP; Wideman et al., 2011). (C and D) Plexiform lesions begin developing at sites where intimal proliferation and endothelial damage attract mononuclear cells and heterophils. Mature plexiform lesions have a glomeruloid appearance and are surrounded by the dilated remnants of the arteriolar wall. The semi-obstructive plexiform lesions include proliferating intimal cells, mononuclear cells, and foam-type macrophages (Wideman and Hamal, 2011; Wideman et al., 2011; Hamal et al., 2012).

direct impact on the tone of the pulmonary vasculature. Indeed, the venous blood returning to the lungs normally is hypoxemic, and on a teleological basis it would be functionally counterproductive for deoxygenated venous blood to persistently trigger pulmonary vasoconstriction. Hypoxemia does stimulate erythropoiesis, which increases the hematocrit and enhances the O<sub>2</sub> carrying capacity of blood. Large increases in the hematocrit and the reduced deformability of immature erythrocytes potentially can increase the blood's viscosity and thereby increase the resistance to blood flow (Figure 1A). Elevated hematocrits also increase the risk of thrombotic occlusion of the pulmonary vasculature, which also can increase the pulmonary vascular resistance and contribute to the development of pulmonary hypertension (Burton and Smith, 1967; Maxwell et al., 1990; Maxwell, 1991; Mirsalimi and Julian, 1991; Lubritz and McPherson, 1994; Fedde and Wideman, 1996; Shlosberg et al., 1996b, 1998b; Wideman et al., 1998c).

In the systemic circulation, hypoxemia elicits widespread arteriolar dilation to increase blood flow and restore adequate O<sub>2</sub> delivery to the organs and tissues (Wideman et al., 1996b, 1997, 2000; Wideman and Tackett, 2000; Wideman, 2000; Ruiz-Feria and Wideman, 2001). Systemic arteriolar vasodilation (reduced total peripheral resistance) allows blood to exit the large arteries more rapidly (increased tissue blood flow), leading to reductions in the mean systemic arterial pressure (systemic hypotension) accompanied by increases in the rate at which venous blood returns to the right ventricle. The increase in venous return and the onset of systemic arterial hypotension reflexively stimulate the heart to increase the cardiac output, forcing the right ventricle to develop even higher pulmonary arterial pressures to propel the returning venous blood at ever-increasing flow rates through the pulmonary vasculature (Peacock et al., 1989; Owen et al., 1995b; Wideman et al., 1996a,b, 1998b, 2000; Forman and Wideman, 1999; Wideman and Tackett, 2000). The combination of rapid growth and an inadequate pulmonary vascular capacity triggers a vicious, progressive cascade encompassing hypoxemia, polycythemia, systemic hypotension, increased venous return, and rapidly escalating pulmonary hypertension (Wideman, 2000; Wideman et al., 2007).

### **Terminal Pathogenesis**

Broilers developing early symptoms of PAH, such as visible hypoxemia (cyanosis of the comb and wattles; Peacock et al., 1989, 1990; Reeves et al., 1991; Julian and Mirsalimi, 1992; Wideman and Kirby, 1995a,b; Wideman et al., 1998c) and right ventricular hypertrophy (detected noninvasively by electrocardiography; Odom et al., 1992; Owen et al., 1995c; Wideman and Kirby, 1995a, 1996; Wideman et al., 1998c) often can survive until the flock is harvested. Indeed, death is not inevitable because treatments such as feed restriction in combination with the administration of diuretics (fu-

roseamide, Lasix) that promote sodium and chloride excretion in the urine can restore complete clinical health to broilers that previously had been suffering from full "water-belly" ascites (Wideman et al., 1995a; Wideman and French, 1999; Wideman, 2000; Forman and Wideman, 2001). Nevertheless, the most susceptible individuals do succumb to a progressive suite of pathophysiological crises. For example, sustained hypoxemia and excessive right ventricular afterloads (high pulmonary arterial pressures) cause cardiac decompensation, with the ventricular muscle progressively weakening and dilating as the deteriorating cardiomyocytes accumulate excessive calcium and release protective reserves of taurine and contractile proteins such as troponin T (Maxwell et al., 1993, 1994, 1995; Ruiz-Feria et al., 1999; Ruiz-Feria and Wideman, 2001; Ruiz-Feria et al., 2001; Olkowski, 2007). Cardiac dilation and excessive right ventricular pressures render the right atrio-ventricular valve incompetent, permitting blood to regurgitate into the right atrium during ventricular systole (Chapman and Wideman, 2001). This inability to propel 100% of the returning venous blood through the lungs marks the onset of right-sided congestive heart failure, characterized by the progressive accumulation of unpumped blood in the right ventricle (ventricular dilation accompanies and can supersede work hypertrophy) and in the venous volume reservoir (the large systemic veins), causing an increase in venous pressure (Wideman et al., 1999b; Chapman and Wideman, 2001; Lorenzoni et al., 2008). In response to systemic arterial hypotension, the renin-angiotensin-aldosterone cascade is activated and the kidneys begin retaining excessive quantities of sodium and water (Wideman et al., 1993; Forman and Wideman, 1999). The retained fluid contributes to the increased volume and pressure within the large systemic veins (venous congestion) and ultimately is the source of the ascitic transudate. Venous congestion and hypoxemia detrimentally affect the liver by impeding the inflow of portal blood from the intestinal tract, thereby reducing the supply of O<sub>2</sub> needed to support metabolically active hepatocytes. The ensuing cellular necrosis and scar tissue formation (cirrhosis) reduce the compliance of the hepatic sinusoids which, in combination with elevated sinusoidal pressures attributable to venous congestion, leads to plasma transudation from the surface of the liver into the abdominal cavity (ascites). Growth decelerates, presumably due to severe hypoxemia (O<sub>2</sub> is an important nutrient) accompanied by inanition (Roush and Wideman, 2000; Roush et al., 2001). Death has been attributed to profound hypoxemia, terminal congestive heart failure, respiratory distress caused by pulmonary edema and ascitic compression of the abdominal air sacs, and starvation (Ploog, 1973; Wideman, 1984, 1988, 1999, 2000, 2001; Huchzermeyer and DeRuyck, 1986; Julian et al., 1987; Julian, 1988, 1993; Peacock et al., 1989, 1990; Julian and Mirsalimi, 1992; Wideman and Bottje, 1993; Fedde and Wideman, 1996; Forman and Wideman, 1999; Wideman et al., 1999b, 2000; Wideman and Tackett, 2000).

## RESISTANCE TO PULMONARY BLOOD FLOW

### *Pulmonary Vascular Resistance*

Consistent reports of specific right ventricular work hypertrophy (elevated RV:TV ratios) and direct measurements from catheterized pulmonary arteries have incontrovertibly established the central role of elevated pulmonary arterial pressures in the pathogenesis of PAH (Ploog, 1973; Cueva et al., 1974; Guthrie et al., 1987; Julian et al., 1987; Huchzermeyer and DeRuyck, 1986; Huchzermeyer et al., 1988; Julian, 1988, 1989, 1993; Lubritz et al., 1995; Owen et al., 1995b; Wideman and French, 1999; Wideman, 2000; Chapman and Wideman, 2001, 2006c; Bowen et al., 2006a; Lorenzoni et al., 2008). Increases in the pulmonary arterial pressure theoretically can be attributed to increases in cardiac output as well as to increases in the resistance to blood flow through the pulmonary vasculature (Wideman and Bottje, 1993; Wideman, 2000; Wideman et al., 2007; Figure 1A). Fast growth, full-feeding, acclimation to cool environmental temperatures, heat stress, hypoxemia, hypercapnia, and metabolic acidosis all clearly contribute to the pathogenesis of pulmonary hypertension in broilers by increasing the cardiac output, either directly by increasing the metabolic demand for O<sub>2</sub>, or indirectly by dilating the systemic arterioles and thereby increasing venous return (Peacock et al., 1989; Reeves et al., 1991; Owen et al., 1994; Fedde et al., 1998; Wideman et al., 1998a,c, 1999a,b, 2000, 2003a,b; Wideman, 1999; Wideman and Tackett, 2000; Ruiz-Feria and Wideman, 2001). Comparisons of PAH-susceptible and PAH-resistant broilers do not consistently reveal differences in cardiac output, but PAH-susceptible broilers consistently have higher pulmonary arterial pressures and pulmonary vascular resistances compared with PAH-resistant broilers (Chapman and Wideman, 2001, 2006c; Wideman et al., 2002, 2006, 2007; Bowen et al., 2006a; Lorenzoni et al., 2008). Because the resistance to flow is inversely related to the radius raised to the fourth power ( $1/r^4$ ), relatively small reductions in the pulmonary vascular capacity (e.g., modest vasoconstriction, partial vascular obstruction, pulmonary disease, delayed angiogenesis) can profoundly elevate the cumulative pulmonary vascular resistance (Figure 1A). Indeed, any factor contributing to a reduction in the pulmonary vascular capacity leading to an overall increase in the pulmonary vascular resistance can initiate or accelerate the pathophysiological progression leading to PAH (Wideman and Bottje, 1993; Wideman, 2000).

Broilers developing pulmonary hypertension have a higher pulmonary vascular resistance compared with clinically healthy flock-mates. Pulmonary vascular pressure profiles have consistently demonstrated that the precapillary arteries and arterioles serve as the principal site of increased resistance to blood flow in susceptible broilers, thereby confirming the underlying

etiology as PAH rather than pulmonary venous hypertension (Chapman and Wideman, 2001; Wideman et al., 2007, 2010; Lorenzoni et al., 2008; Kluess et al., 2012). The entire pathogenesis leading to terminal PAH has been replicated by experimentally blocking one pulmonary artery or a portion of the pulmonary arterioles to simultaneously reduce pulmonary vascular capacity and increase pulmonary vascular resistance (Powell et al., 1985; Wideman and Kirby, 1995a,b, 1996; Wideman et al., 1996a,b, 1997, 1998b, 1999b, 2002, 2005b, 2006; Forman and Wideman, 1999, 2001; Ruiz-Feria et al., 1999; Wideman and Erf, 2002). Broiler breeders that thrived in spite of chronic reductions in pulmonary vascular capacity, subsequently produced progeny exhibiting reduced pulmonary arterial pressures, low RV:TV values, and markedly improved resistance to PAH (Wideman and French, 1999, 2000; Chapman and Wideman, 2001; Wideman et al., 2002, 2006). The available evidence therefore overwhelmingly implicates excessive resistance to pulmonary blood flow as one of the principal initiating events that ultimately leads to terminal PAH (Peacock et al., 1989; Wideman, 2000, 2001; Chapman and Wideman, 2001, 2006a,b; Wideman et al., 2004, 2007). Efforts to further clarify the causes of excessive resistance to pulmonary blood flow have focused on evaluating the roles of chemical mediators of vasoconstriction and vasodilation, as well as on pathological (structural) changes occurring within the pulmonary arterioles.

### *Mediators of Pulmonary Vasoconstriction*

Anything that increases the pulmonary vascular resistance can initiate or accelerate the pathophysiological progression leading to PAH. Factors known to increase the resistance to blood flow through broiler lungs include hypoxia, adrenergic neurotransmitters, eicosanoids, methylglyoxal, endothelin-1, serotonin, respiratory damage or disease, and endotoxin, as summarized below.

Exposure to a low atmospheric partial pressure of O<sub>2</sub> (hypoxia) triggers acute pulmonary vasoconstriction and pulmonary hypertension (Ruiz-Feria and Wideman, 2001). Sustained pulmonary vasoconstriction attributable to hypoxia is responsible for increasing the incidence of PAH in commercial broilers reared at high altitudes, particularly when accompanied by subthermoneutral environmental temperatures that increase the cardiac output and induce the release of stress hormones. Exposure to hypobaric hypoxia has been used extensively as a research model for triggering PAH under experimental conditions (Burton and Smith, 1967; Burton et al., 1968; Cueva et al., 1974; Sillau et al., 1980; Sillau and Montalvo, 1982; Owen et al., 1990, 1994; 1995a,b,c; Yersin et al., 1992; Beker et al., 1995; Wideman and Tackett, 2000; Wideman et al., 2000; Ruiz-Feria and Wideman, 2001; Odom et al., 2004; Julian, 2007; Pavlidis et al., 2007; Zoer et al., 2009; Bautista-Ortega and Ruiz-Feria, 2010).

Putative sympathetic (adrenergic) and parasympathetic (cholinergic) nerve terminals have been observed in association with the interparabronchial arterioles of avian lungs (Akester, 1971; Bennett, 1971; King et al., 1978). Epinephrine and norepinephrine constrict avian pulmonary arteries, and intravenously administered epinephrine elicits immediate pulmonary vasoconstriction accompanied by pulmonary hypertension (Somlyo and Woo, 1967; Wideman, 1999; Villamor et al., 2002; Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009; Bautista-Ortega and Ruiz-Feria, 2010). Norepinephrine released by sympathetic nerve terminals and epinephrine released into the circulation by the adrenal glands in response to stress may contribute to the increase in pulmonary vascular resistance during exposure to hypoxia and cool temperatures (Lin and Sturkie, 1968; Wideman, 1999). Adenosine triphosphate (**ATP**) also can be released from sympathetic nerves, and purinergic-2 (**P2X**) receptors for ATP have been linked to lung disease in mammals. Pulmonary arteries isolated from broilers exhibited dose-dependent vasoconstriction in response to ATP, implicating the presence of P2X receptors (Kluess et al., 2012). Vasoconstriction elicited by epinephrine, norepinephrine, ATP, and perhaps other neurotransmitters (e.g., serotonin, *vide infra*) all should be considered factors that potentially may contribute to the excessive vascular resistance in broilers exposed to environmental stressors.

Arachidonic acid (**AA**) cleaved from cell membrane phospholipids is converted by constitutive and inducible cyclooxygenases into common intermediates for the synthesis of eicosanoids (oxygenated metabolites of AA), including thromboxane A<sub>2</sub> (**TxA<sub>2</sub>**). Intravenous AA infusions trigger rapid increases in the pulmonary vascular resistance and pulmonary arterial pressure in broilers, and these responses are blocked by inhibiting cyclooxygenases (Wideman et al., 2005a, 2009). Thromboxane A<sub>2</sub>, whether administered intravenously as the TxA<sub>2</sub> mimetic U44069 or produced by activated thrombocytes, consistently causes pulmonary vasoconstriction and pulmonary hypertension in broilers (Wideman et al., 1996b, 1997, 1998a, 1999a, 2001, 2004, 2005a, 2009; Villamor et al., 2002; Chapman and Wideman, 2006b). The precise contribution of TxA<sub>2</sub> to the progression of pulmonary hypertension in PAH-susceptible broilers remains to be determined. Vasoconstrictive eicosanoids are considered likely to exert a major impact during respiratory inflammation and vascular occlusion (Wideman et al., 2004; Lorenzoni and Wideman, 2008b), but recent evidence does not support their involvement during the pulmonary hypertensive response to bacterial endotoxin (*vide infra*; Wideman et al., 2009).

Methylglyoxal (**MG**) is formed from carbohydrates, fatty acids and proteins and, based on its dicarbonyl structure, is capable of causing widespread oxidative damage. Methylglyoxal has been shown to damage the vascular endothelium and has been implicated in vascular remodeling and systemic arterial vasoconstriction

and hypertension in mammals. Intravenous and intramuscular injections of MG both rapidly elicited pulmonary vasoconstriction and pulmonary hypertension in broilers (Khajali and Wideman, 2011), which potentially may reveal a mechanistic link between full-feeding, oxidative stress, endothelial damage, and the ascites syndrome (Khajali and Fahimi, 2010).

Endothelin-1 (**ET-1**) is intimately involved in the pathogenesis of PAH in mammals and broiler chickens. Endothelin-1 binds to type A receptors (**ET<sub>A</sub>R**) expressed on pulmonary artery smooth muscle cells (**PASMC**), or to type B receptors (**ET<sub>B</sub>R**) expressed predominately on endothelial cells but also to a lesser degree on PASMC. Binding of ET-1 to ET<sub>A</sub>R leads to vasoconstriction and PASMC proliferation, whereas binding to ET<sub>B</sub>R on the endothelium promotes vasodilation via increased production of nitric oxide (*vide infra*). In broilers, ET-1 elicits dose-dependent constriction of pulmonary arteries that can be modulated by nitric oxide (Martinez-Lemus et al., 1999, 2003; Villamor et al., 2002; Odom et al., 2004). Repeated intravenous injections of ET-1 triggered PAH in broilers (Zhou et al., 2008), and pharmacologic ET<sub>A</sub>R blockade reduced the incidence of PAH in broilers exposed to cool temperatures (Yang et al., 2005). Lungs from broilers with pulmonary hypertension expressed higher levels of ET-1 mRNA and lower levels of ET<sub>A</sub>R mRNA compared with lungs from nonhypertensive broilers (Gomez et al., 2007). Cardiac expression of ET-1 and ET<sub>A</sub>R mRNA was higher in the right but not left ventricle of broilers compared with egg-laying chickens, and broilers had higher serum levels of ET-1 than layers (Hassanpour et al., 2010). Broilers developing PAH had higher serum levels of ET-1 than nonhypertensive control broilers, but cardiac ET-1 and ET<sub>A</sub>R mRNA expression were impaired in both ventricles of pulmonary hypertensive broilers compared with controls. The latter observation presumably reflects the deterioration of cardiac myocytes during ventricular decompensation (Hassanpour et al., 2011). Obstructing pulmonary arterioles in numbers sufficient to trigger pulmonary hypertension caused a greater increase in pulmonary ET-1 mRNA expression and equivalent increases in pulmonary ET<sub>A</sub>R mRNA expression in broilers from a PAH-susceptible line compared with broilers from a PAH-resistant line. In contrast, the resistant line exhibited greater expression of ET<sub>B</sub>R than the susceptible line (Hamal et al., 2010a). Cumulatively these observations reflect a consistent association between excessive ET-1 production and pulmonary hypertension, as well as the likelihood that enhanced ET<sub>B</sub>R expression on endothelial cells helps to confer resistance to the vasoconstrictive potency of ET-1 (Hamal et al., 2010a).

Serotonin (5-hydroxytryptamine, **5-HT**) is an extremely potent pulmonary vasoconstrictor that triggers pulmonary hypertension by activating receptors expressed on PASMC (Chapman and Wideman, 2002, 2006b,c; Hamal et al., 2010a; Kluess et al., 2012). Plasma serotonin levels normally are quite low, but se-

rotonin can be released from activated thrombocytes and from pulmonary neuroendocrine cells or serotonergic nerves (Meyer and Sturkie, 1974; Chapman et al., 2008). Broilers fed diets supplemented with high levels of tryptophan, an essential amino acid and precursor for serotonin, developed higher pulmonary arterial pressures than broilers fed diets containing adequate levels of tryptophan (Kluess et al., 2012). Pretreating broilers with the serotonin receptor blocker methiothepin reduced the pulmonary arterial pressure below baseline values, demonstrating serotonin likely exerts tonic control of pulmonary vascular resistance. Methiothepin pretreatment virtually eliminated the increases in pulmonary vascular resistance and pulmonary arterial pressure that normally are elicited by serotonin infusion. Pretreatment with methiothepin also prevented the pulmonary hypertension and mortality that otherwise ensue when microparticles are injected in doses sufficient to obstruct  $\geq 15\%$  of the pulmonary arterioles in ascites-susceptible broilers (Chapman and Wideman, 2006b,c). Obstructing pulmonary arterioles in numbers sufficient to trigger pulmonary hypertension without causing acute mortality evoked a greater increase in the expression of pulmonary serotonin receptor types 1A (5-HT<sub>1A</sub>) and 2B (5-HT<sub>2B</sub>) in broilers from a PAH-susceptible line when compared with broilers from a PAH-resistant line (Hamal et al., 2010a). Serotonin clearly plays a key role in increasing the basal tone (partial state of contracture) of the pulmonary resistance vessels, and potentially can act as a dominant pulmonary vasoconstrictor in broilers (Chapman and Wideman, 2002, 2006b,c). It appears likely that susceptibility to PAH in broilers may, in part, involve excessive serotonin biosynthesis, inhibited uptake or enhanced release of serotonin by thrombocytes, enhanced receptor-mediated vasoconstrictive responsiveness to serotonin, or altered internalization of serotonin by a specific transporter associated with vascular remodeling (*vide infra*; Wideman and Hamal, 2011).

Ascites outbreaks have been attributed in several reports to poor air quality (e.g., dusty conditions), poor ventilation (e.g., elevated ammonia, carbon monoxide, or carbon dioxide levels), and respiratory damage or airway obstruction due to pathogens or pulmonary inflammation (Wideman, 1984, 1988; Julian and Goryo, 1990; Shlosberg et al., 1992, 1996a; Bottje and Wideman, 1995; Tottori et al., 1997; Wideman et al., 1997; Bottje et al., 1998). Exposure to bacterial lipopolysaccharide (**LPS**, endotoxin) has been used to experimentally simulate the responses of broiler lungs to inflammatory responses. Bacterial lipopolysaccharide is an integral component of the cell wall of gram-negative bacteria such as *Escherichia coli*. The respiratory tract is constantly challenged with aerosolized bacteria (e.g., poultry house “dust”) or bacteria translocated into the blood from the gastrointestinal tract or integument. Administering LPS intravenously or via an inhaled aerosol triggers pulmonary hypertension attributable to vasoconstriction (Wideman et al., 2001, 2004, 2009;

Chapman et al., 2005, 2008; Lorenzoni and Wideman, 2008b), and respiratory exposure to *E. coli* amplifies the incidence of PAH (Tottori et al., 1997; Yamaguchi et al., 2000). The pulmonary hypertension elicited by LPS in mammals has been associated with the release or synthesis of several vasoconstrictors, including ET-1, platelet activating factor, TxA<sub>2</sub>, and 5-HT (Wideman et al., 2004). Recent experiments demonstrated that the pulmonary hypertensive response to LPS in broilers cannot predominately be attributed to either 5-HT or vasoconstrictive eicosanoids derived from AA (e.g., TxA<sub>2</sub>), therefore the vasoconstrictive pathways activated by LPS remain to be identified (Chapman and Wideman, 2006c; Chapman et al., 2008; Wideman et al., 2009).

### **Mediators of Pulmonary Vasodilation**

Factors that dilate the pulmonary vasculature and thereby lower the resistance to blood flow can delay or inhibit the onset of pulmonary hypertension. The neurotransmitter acetylcholine (**ACh**) causes relaxation of isolated pulmonary arteries, but experimental evidence linking ACh-mediated vasodilation to reduced susceptibility to PAH is lacking (Martinez-Lemus et al., 1999, 2003; Villamor et al., 2002; Odom et al., 2004). The eicosanoids prostaglandin I<sub>2</sub> (**PGI<sub>2</sub>**, prostacyclin) and prostaglandin E<sub>2</sub> (**PGE<sub>2</sub>**) are pulmonary vasodilators in several mammalian species but do not reduce pulmonary vascular resistance when infused intravenously into clinically healthy broilers, broilers whose pulmonary vasculature had been precontracted with AA, or broilers with preexisting pulmonary hypertension. Accordingly, PGI<sub>2</sub> and PGE<sub>2</sub> do not appear to dilate the pulmonary vasculature in broilers, although PGI<sub>2</sub> may significantly modulate thrombocyte activation and thereby attenuate thrombocytic release of TxA<sub>2</sub> and 5-HT (Wideman et al., 2004, 2005a; Stebel and Wideman, 2008). Adrenomedullin (**AM**) is a vasodilator that reduces pulmonary arterial pressure and triggers increased urinary excretion of sodium chloride and water in mammals. Lungs from broilers developing PAH expressed higher levels of AM compared with lungs from control broilers, suggesting AM potentially may modulate or counteract the onset of hypoxic pulmonary hypertension in broilers (Gomez et al., 2007).

Arginine is an essential amino acid for poultry and serves as the precursor for the potent pulmonary vasodilator nitric oxide (**NO**). The enzyme nitric oxide synthase (**NOS**) utilizes arginine as a substrate for the production of NO. Endothelial cells lining the internal surfaces of blood vessels contain constitutively expressed endothelial NOS (eNOS or NOS-3), which rapidly produces NO to facilitate flow-dependent vasodilation whenever increased blood flow exerts shear stress on the endothelium (Wideman et al., 1996a,b, 1998b; Martinez-Lemus et al., 1999). Activated monocytes/macrophages express inducible NOS (iNOS or NOS-2) which, after an initial delay of hours rather

than minutes, generates copious, sustained quantities of NO during intrapulmonary inflammatory responses (Bowen et al., 2006a,b; Hamal et al., 2008). When eNOS and iNOS are inhibited, the ensuing reduction in NO synthesis leads to pulmonary arterial vasoconstriction, pulmonary hypertension, and PAH (Wideman et al., 1995b, 1996a, 1998b, 2004, 2005a,b, 2006; Grabarevic et al., 1997; Martinez-Lemus et al., 1999, 2003; Ruiz-Feria et al., 2001; Villamor et al., 2002; Wang et al., 2002; Weidong et al., 2002; Moreno de Sandino and Hernandez, 2003, 2006; Odom et al., 2004; Wideman and Chapman, 2004; Bowen et al., 2006a,b; Ruiz-Feria, 2009). Reduced pulmonary arteriolar eNOS expression was reported in broilers developing PAH during chronic exposure to hypobaric hypoxia (Moreno de Sandino and Hernandez, 2003, 2006), but pulmonary eNOS and iNOS expression levels were not correlated with the onset of PAH induced by chronic exposure to sub-thermoneutral temperatures (Teshfam et al., 2006). Cardiac gene expression for iNOS was impaired in broilers with pulmonary hypertension compared with control broilers (Hassanpour et al., 2009). Obstructing a portion of the pulmonary arterioles evoked a greater increase in the expression of both eNOS and iNOS in broilers from a PAH-resistant line compared with broilers from a PAH-susceptible line (Hamal et al., 2010a). Adding supplemental arginine to broiler diets facilitated pulmonary vasodilation in response to large increases in blood flow (Wideman et al., 1996a) and modulated the pulmonary hypertension elicited by epinephrine (Lorenzoni and Ruiz-Feria, 2006; Ruiz-Feria, 2009; Bautista-Ortega and Ruiz-Feria, 2010). Supplemental dietary arginine tended to reduce the incidence of ascites in broilers exposed to cool temperatures in one experiment (Wideman et al., 1995b) but not in another (Ruiz-Feria et al., 1999). Diets supplemented with arginine increased plasma NO levels and attenuated the reduced growth performance and symptoms of pulmonary hypertension in broilers exposed to the combined challenges of cool temperatures and hypobaric hypoxia (Khajali et al., 2011b; Basoo et al., 2012). A recent review of the interrelationships between dietary arginine and NO in poultry concluded that under certain environmental conditions dietary arginine may be limiting for optimal growth and immune competence, and that NO opposes the pathogenesis of PAH by acting as the key pulmonary vasodilator and modulator (inhibitor) of vasoconstriction in broilers (Khajali and Wideman, 2010).

### **Pulmonary Vascular Pathology**

For human patients developing idiopathic PAH (IPAH), increases in pulmonary vascular resistance have been attributed primarily to vasoconstriction and remodeling of the pulmonary vasculature. Vascular remodeling initially involves hypertrophy and hyperplasia of the medial smooth muscle layer in small muscular arteries, distal extension of smooth muscle into non-

muscularized arterioles, and intimal thickening attributable to the accumulation of one or more layers of myofibroblasts and fibrous matrix proteins. In patients with rapidly progressing IPAH, occlusive plexiform lesions form in arterioles immediately downstream from branching points where localized turbulent blood flow and shear stress are thought to damage the endothelium. Dysregulated endothelial cells proliferate until the lumen of the arteriole is functionally obstructed. Slit-like anastomosing endothelial channels supported by connective tissue and myofibroblasts canalize the plexiform obstruction. Inflammatory cells infiltrate both the core and periphery of plexiform lesions, and fibrosis can develop in the intimal and adventitial layers of established lesions. Ongoing plexiform lesion development irreversibly obliterates small pulmonary arteries, pruning the pulmonary vasculature and fueling a positive feedback cycle in which accumulating vascular obstructions progressively increase the resistance to blood flow and amplify the pressure and shear stress imposed upon the endothelium lining the vessels that remain unobstructed (reviewed by Wideman and Hamal, 2011; Wideman et al., 2011).

Pulmonary arterioles of broilers developing PAH consistently exhibit medial hypertrophy that directly elevates the precapillary vascular resistance and also may enhance the responsiveness of the vessels to pulmonary vasoconstrictors (Figure 1; Cueva et al., 1974; Sillau and Montalvo, 1982; Huchzermeyer and DeRuyck, 1986; Hernandez, 1987; Julian, 1988; Peacock et al., 1989; Maxwell, 1991; Enkvetchakul et al., 1995; Xiang et al., 2002, 2004; Moreno de Sandino and Hernandez, 2003, 2006; Pan et al., 2005, 2008; Tan et al., 2005a,b; Gomez et al., 2007; Bautista-Ortega and Ruiz-Feria, 2012). Medial hypertrophy and intimal proliferation are readily detected but appear to be unevenly distributed within the lungs of PAH-susceptible broilers (Wideman and Hamal, 2011), perhaps due to regional differences in the distribution of blood flow (Weidner et al., 2012). Protein kinase C  $\alpha$  promotes the proliferation of vascular smooth muscle cells and has been implicated in arteriole muscularization in broilers developing pulmonary hypertension (Tan et al., 2005a,b; Pan et al., 2008). Lungs from broilers with pulmonary hypertension also expressed higher levels of factors involved in vascular remodeling and angiogenesis (e.g., connective tissue growth factor and adrenomedullin) compared with lungs from nonhypertensive broilers (Gomez et al., 2007).

Until recently, there were no reports of plexiform lesions in avian lungs. We surveyed the lungs of broilers from a PAH-susceptible line to estimate the relative age- and sex-specific incidences of plexiform lesion development. Plexiform lesions were detected as early as the first week posthatch, and between 30 d of age through 52 wk of age the lesions were observed in approximately 40% of the lung sections regardless of sex (Figures 2 and 3). These lesions developed primarily in regions of the lungs where muscularized interpara-

bronchial arterioles exhibited intimal proliferation (Figure 1). Our observations revealed a maturational process through which early compact lesions having a relatively homogeneous endothelial matrix and sparse vascular channels transition into larger mature plexiform lesions exhibiting numerous vascular channels and multiple cell types including connective tissue, inflammatory cells, and proliferating intimal cells (Figure 2) (Wideman et al., 2011; Wideman and Hamal, 2011; Kluess et al., 2012). Broilers fed diets supplemented with excess tryptophan, the precursor for serotonin, developed higher ( $P = 0.11$ ) plexiform lesion incidences by 30 d of age and higher ( $P < 0.01$ ) pulmonary arterial pressures compared with broilers fed diets adequate in tryptophan (Kluess et al., 2012). Serotonin and enhanced expression of the serotonin transporter (SERT, 5-HTT) have been implicated in the etiology of IPAH and plexogenic arteriopathy in humans (Ed-dahibi et al., 1999, 2000, 2001, 2003; Wideman and Hamal, 2011). Immunohistochemical studies confirmed that the plexiform lesions of broilers contain immune/inflammatory cells (e.g., monocytes/macrophages, cytotoxic lymphocytes, B cells, and MHC class II cells) and express the same angioproliferative factors (e.g., von Willebrand factor,  $\alpha$  smooth muscle actin, vascular endothelial growth factor and its type 2 receptor, hypoxia inducible factor-1 $\alpha$ , survivin, and tenascin) that have been identified in human plexiform lesions (Hamal et al., 2012). The lesion densities in broilers are so low that the associated vascular obstruction is considered unlikely to significantly increase the resistance to pulmonary blood flow. Accordingly, plexiform lesion development appears to be a consequence of pulmonary hypertension and the resulting excessive shear stress rather than the proximate cause of the increased pulmonary vascular resistance in PAH-susceptible broilers. Plexogenic arteriopathy may serve to prune arterioles in which turbulent (instead of laminar) blood flow is exacerbated by increasing pulmonary arterial pressures in combination with developmental misalignments of the maturing pulmonary vascular tree (Wideman and Hamal, 2011; Wideman et al., 2011).

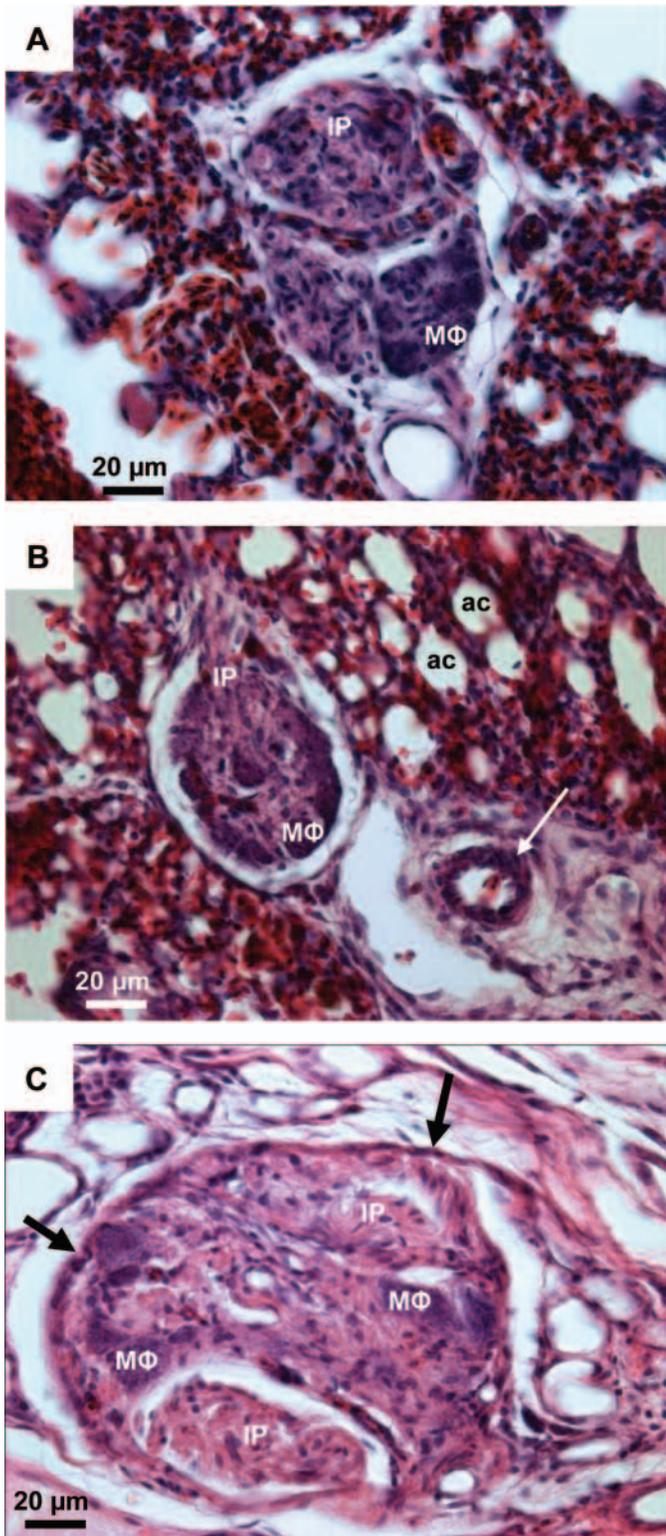
## ROLE OF THE IMMUNE SYSTEM IN THE PATHOGENESIS OF PAH

In both humans and broilers, PAH has been found to involve a significant immune system/inflammatory component and immunopathology. The concept of an autoimmune nature of PAH has been discussed in the literature for some time, and evidence is mounting that the immune system plays a critical role in the etiology and progression of the pulmonary pathology observed in PAH. In humans, an important role of inflammation is suggested by the observed accumulations of perivascular mononuclear cell infiltrates including macrophages, dendritic cells, T and B lymphocytes, and mast cells in pathological specimen from patients. Re-

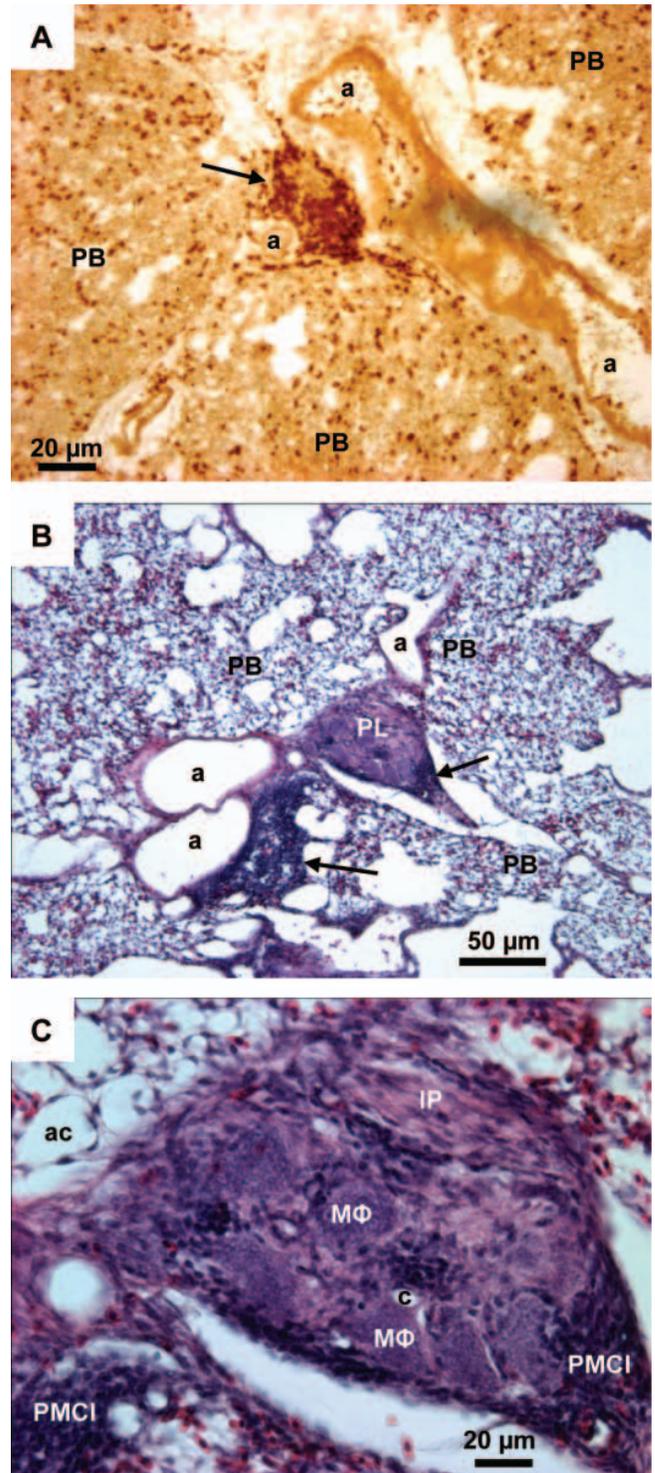
cent pathogenic studies of PAH have highlighted the expression of inflammatory cytokines such as tumor necrosis factor- $\alpha$ , interleukin (IL)-1, IL-6, chemokines (RANTES/CCL5, CXCL10/Fractalkine CCL2), T helper cell-type 1 cytokines (i.e., interferon- $\gamma$ ; IFN- $\gamma$ ), and T helper cell-type 1-associated activities down-stream of IFN- $\gamma$ , such as IL-18 and chemokine CXCL10 (Tuder and Voelkel, 1998; Kherbeck et al., 2011; Price et al., 2012; Ross et al., 2012; Stacher et al., 2012). Specifically, IL-18 and CXCL10 have been implicated in the perpetuation of an inflammatory milieu that eventually contributes to the pulmonary vascular pathology and obstruction characteristic of PAH (Ross et al., 2012). Local pulmonary inflammatory activity is reflected by elevated circulating levels of inflammatory cytokines (IL-1, IL-6) and chemokines (IL-8) as well as C-reactive protein that may correlate with a worse clinical outcome (Dorfmüller et al., 2003; Soon et al., 2010; Kherbeck et al., 2011). An autoimmune etiology of PAH is further supported by PAH development in individuals with scleroderma-like disorders with established autoimmune phenotype and the presence of antinuclear antibodies and autoantibodies directed against EC and fibroblasts (Wick et al., 2010; Kherbeck et al., 2011; Bussone et al., 2012). Moreover, PAH has been associated with a lack of CD4+ T cells, specifically T cells with a regulatory phenotype (Treg) in affected lungs that would normally function to limit vascular endothelial injury and inflammatory/autoimmune activity and hence prevent pulmonary hypertension (Tamosiuniene et al., 2011).

Like most inflammatory/autoimmune diseases, PAH in humans is multifactorial, involving genetic, environmental, and immune system components. Susceptibility to PAH appears to be multigenic and may be manifested in aberrant stress sensitivity, function, and regulation of pulmonary vascular tissue components, as well as aberrant activities of innate and adaptive immune system components. Expression of PAH in susceptible individuals may be triggered by variety of environmental insults that cause pulmonary vascular stress and inflammation [e.g., shear stress, ischemia, infections (viral, bacterial, parasites), toxins (pollutants, endotoxin), and autoimmunity; Austin et al., 2010, Wick et al., 2010; Tamosiuniene et al., 2011)].

Evidence for a role of the immune system in PAH in broilers is in agreement with reports in humans. Lungs from broilers with pulmonary hypertension are infiltrated with mononuclear leukocytes, consisting of T cells, B cells, and macrophages that accumulate in perivascular aggregates (Figure 3). Additionally, MHC class II expression, indicative of IFN- $\gamma$  production and activation of cell-mediated inflammatory activity, is greatly increased throughout the lung tissue, but strikingly so on arterioles that are associated with perivascular mononuclear cell infiltrates and exhibit signs of vascular remodeling (G. F. Erf, unpublished). Perivascular mononuclear cell infiltrates vary in size and complexity, from small aggregates surrounding arterioles



**Figure 2.** (A and B) Photomicrographs of sections from the lungs of 7-d-old broiler chicks showing immature plexiform lesions forming in interparabronchial arterioles, including multiple macrophages (MΦ) and proliferating intimal cells (IP). A small muscular arteriole (arrow) exhibits medial hyperplasia and hypertrophy, and intimal proliferation. Air capillaries (ac) are surrounded by blood capillaries in the gas exchange parenchyma. Original magnification 400×. (C) A mature plexiform lesion in a section from the lung of a 21-d-old broiler exhibits glomeruloid dilation within the remnants of the arteriolar wall (arrows) and contains multiple foam-type MΦ and swaths of proliferating IP.



**Figure 3.** (A) Photomicrograph showing immunostaining of a cryostat section from a broiler lung, showing positive staining for individual CD8+ cells dispersed throughout the parabronchial parenchyma (PB) and spontaneously aggregating around the branch point of an interparabronchial arteriole. Original magnification 400×. (B) Low-power (100×) photomicrograph showing a mature plexiform lesion (PL) at the branching point of several interparabronchial arterioles (a) coursing between 3 adjacent PB. The parabronchial gas exchange parenchyma exhibits little evidence of inflammation, whereas perivascular mononuclear cell infiltrates (PMCI) are aggregating around arterioles in the vicinity of the plexiform lesion (arrows). (C) Higher power (400×) photomicrograph of the same mature plexiform lesion, showing the presence of multiple foam-type macrophages (MΦ), proliferating intimal cells (IP), capillaries (c), and aggregates of PMCI. ac = air capillary.

to large aggregates that extend into the lumen of the arterioles and exhibit plexiform lesion characteristics described by Wideman and Hamal (2011), Wideman et al. (2011), and Hamal et al. (2012; Figures 2 and 3). Immune activity in PAH lungs was also associated with altered proportions among peripheral blood leukocytes, including greatly increased heterophil-to-lymphocyte ratios in broilers with PAH compared with controls (Fritts et al., 1998, abstract). In addition to inflammation and immunopathology, other important parallels between PAH in broilers and humans include genetic susceptibility predisposing individuals to PAH development (see above) and environmental factors that promote expression of PAH in susceptible individuals [high altitude, cold-stress, infection, lipopolysaccharide (LPS), and so on].

A role of the immune system in early etiology of PAH in broilers has been effectively demonstrated using experimental approaches that provide an initiating insult to the pulmonary vasculature. Lipopolysaccharide (endotoxin), derived from the cell walls of gram-negative bacteria, is a potent stimulatory of innate inflammatory activities. Intravenous administration of LPS to broilers from the same genetic line resulted in large increases in pulmonary arterial pressure in some individuals but others failed to exhibit any response to the same supra-maximal dose of LPS. Histological manifestations of the intravenous (i.v.) LPS induced intrapulmonary inflammatory response included vascular congestion, endothelial cell swelling, and notable increases in both large and small mononuclear cells within the pulmonary microvasculature (Wideman et al., 2004). Additionally, LPS activated the vascular endothelium and leukocytes to release a cascade of factors known to constrict the pulmonary vasculature (TxA<sub>2</sub>, 5-HT). The ability to modulate the production and the biological impact of this vasoconstrictive response through production of vasodilatory factors, such as NO, appears to be critical in attenuating the LPS-triggered pulmonary hypertensive response and in the dissemination of the associated focal inflammation of the pulmonary vasculature (Wideman et al., 2004). The extensive variability in the pulmonary hypertensive response to i.v. LPS administration among broilers appears to reflect variability in innate immune activity stimulated by LPS, including an imbalance in the production of vasoactive factors with vasoconstrictive versus vasodilatory (NO) activity (Wideman et al., 2004).

The use of broiler lines selected for PAH susceptibility and resistance (Anthony et al., 2001; Pavlidis et al., 2007) together with induction of pulmonary arteriolar inflammation using i.v. injection of cellulose microparticles (MP; Wideman and Erf, 2002; Wideman et al., 2002) has proven to be an excellent model to examine the role of the immune system in the early etiology of PAH. Intravenously injected MP are carried to the lungs by the venous blood where they occlude the precapillary arterioles resulting in PAH due to increased pulmonary vascular resistance (Wideman

and Erf, 2002). Following i.v. MP injection, broilers with the lowest pulmonary capacity succumb within 48 h to PAH and respiratory insufficiency; those with marginal pulmonary vascular capacity develop terminal ascites within 2 wk, and those with the most robust pulmonary vascular capacity thrive as clinically healthy, PAH-resistant survivors (Wideman and Erf, 2002; Wideman et al., 2002). The MP entrapped in the lungs initiate a vigorous, focal inflammatory response. Within minutes, thrombocytes aggregate around MP lodged in the pulmonary arterioles. This is followed by infiltration and aggregation of mononuclear cells (monocytes/macrophages and lymphocytes) in the perivascular region surrounding MP-occluded arterioles (Wideman et al., 2002, 2007; Wang et al., 2003). In addition to vascular occlusion, vasoactive compounds produced and released by vascular endothelial cells, thrombocytes, and leukocytes were found to contribute to the observed PAH following i.v. MP injection (Wideman, 2001; Wideman et al., 2007). The ability of the broilers to effectively cope with i.v. MP-induced PAH in broilers from the resistant compared with the susceptible line appears to be due in part to qualitative and quantitative differences in the i.v. MP-induced pulmonary inflammatory response during the first 48 h post-MP injection (Hamal et al., 2008, 2010a,b). In both resistant and susceptible broilers, monocytes/macrophage accumulation could be observed within 2 h in the vicinity of entrapped MP, inside the vessels as well as outside. Monocyte/macrophage infiltration continued over the 48 h post-MP injection with large accumulations in the perivascular region of occluded vessels. Histochemical staining for NOS activity coincided with the location, intensity, and amount of macrophage infiltration from 0 to 48 h. These observations suggest activation of iNOS in macrophages, with higher levels of NO production in broilers from the resistant line. Relative iNOS mRNA expression levels in MP-injected lungs were elevated within 2 h in all broilers and continued to increase in resistant lungs throughout the 48-h period; whereas in susceptible broilers, iNOS mRNA expression levels had returned to preinjection levels at 24 h but increased again to levels observed in resistant broilers at 48 h. Considering the need for NO production to overcome inflammation-associated production of vasoconstrictive factors, the higher infiltration levels of macrophages with NOS activity and the sustained expression of iNOS in the lungs of resistant broilers favors their survival when challenged with i.v. MP-induced PAH (Hamal et al., 2008) and pulmonary inflammation. Using the same i.v. MP-injection model, divergent expression patterns of inflammatory chemokines and cytokines in lungs of resistant and susceptible broilers were observed, reflecting qualitative and quantitative differences in the early inflammatory response in PAH-susceptible and resistant individuals. Expression of chemokines IL-8 and K60 which are important in leukocyte recruitment increased in all broilers within 2 h, but their expression reached higher levels in resis-

tant broilers by 6 h and remained higher at 12 and 24 h (K60 only) post i.v. MP-injection. This difference in chemokine expression is in agreement with the observed differences in mononuclear cell infiltration between resistant and susceptible broilers post-i.v. MP injection. Similar differences in inflammatory cytokine expression levels were observed for IL-1 $\beta$  and IL-6 as well as IFN- $\gamma$  and IL-4. The observed differences in the inflammatory response in lungs from i.v. MP-injected resistant compared with susceptible broilers reflect more efficient recruitment, infiltration, and activation of leukocytes associated with resistance. More efficient initiation of an appropriate inflammatory response would result in the initiation of mechanisms designed to overcome effects of vasoconstriction (**NO**), to repair the vasculature, wall off and remove lodged MP, resolve the inflammation, and ultimately restore normal function and capacity of the lung (Hamal et al., 2010a,b).

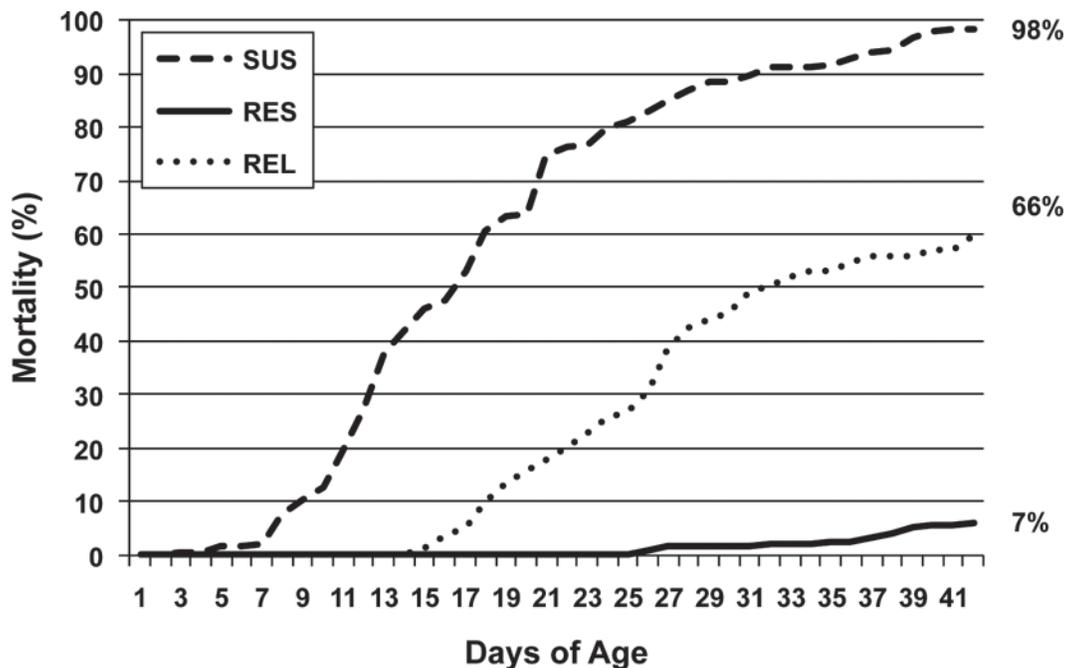
## GENETIC BASIS FOR THE ASCITES SYNDROME

A genetic component of susceptibility to the PAH-ascites syndrome has been suggested by numerous investigators (Huchzermeyer et al., 1988; Peacock et al., 1990; Decuyper et al., 1994; Jones, 1994; Lubritz and McPherson, 1994; Lubritz et al., 1995; Shlosberg et al., 1996b, 1998b; Wideman and French, 1999, 2000; Anthony et al., 2001; de Greef et al., 2001a,b; Pakdel et al., 2002, 2005; Pavlidis et al., 2007; Closter et al., 2012). In addition, several researchers have speculated regarding the number of genes that might be responsible for conferring resistance or susceptibility to the ascites syndrome. Based on heritability estimates of 0.4 to 0.5 and the rapid progress achieved during selection for PAH resistance, Wideman and French (2000) suggested that only a few major genes were likely to be involved. Summarizing 15 generations of blood oxygen saturation data, Navarro and coworkers (2001) suggested an overdominant gene model for ascites. Druyan and Cahaner (2007) proposed epistatic effects of 2 major complementary genes. Contrasting these simplified modes is that of a more complex polygenic trait proposed independently by several research groups (De Greef et al., 2001a,b; Rabie et al., 2005; Hamal et al., 2010a,b).

Ascites has been successfully induced using both invasive (Wideman and Kirby, 1995a; Wideman et al., 1997; Wideman and Erf, 2002; Wideman et al., 2003a) and noninvasive protocols (Wideman et al., 1998c; Julian, 1993, 2000; Deeb et al., 2002; Anthony et al., 2001; Anthony and Balog, 2003). This has resulted in the ability to consistently induce the syndrome and more accurately predict its mode of inheritance. Through this work, moderate to high heritabilities have been reported for ascites (Huchzermeyer et al., 1988; Peacock et al., 1989; Lubritz et al., 1995; Shlosberg et al., 1998b; Wideman and French, 1999, 2000; Moghadam et al., 2001; Navarro et al., 2001; Deeb et al., 2002; Pakdel et

al., 2002; Anthony and Balog, 2003; Ledur et al., 2006; Druyan et al., 2007a,b; Pavlidis et al., 2007). In addition, these selection tools have been applied to research and commercial populations (Shlosberg and Bellaiche, 1996; Wideman and French, 1999, 2000; Anthony et al., 2001; Druyan et al., 2007a,b; Pavlidis et al., 2007).

Long-term divergent selection for ascites susceptibility was initiated at the University of Arkansas using a pedigree elite base population. Research lines were developed using sib selection of birds exposed to sustained hypobaric hypoxia at a simulated altitude of 9,500 ft (2,896 m; Anthony et al., 2001; Balog, 2003; Pavlidis et al., 2007). The commercial elite line, hereafter referred to as the relaxed line (**REL**), exhibited an incidence of ascites of 75% and served as the founder population for the susceptible (**SUS**) and the resistant (**RES**) lines. The response to selection was very rapid with the incidence of ascites by generation 14 reaching 98% for the SUS line and 7% for the RES line (Figure 4). The lines are currently in generation 19 and have to be selected under different simulated altitudes (SUS, 8,000 ft and RES, 12,000 ft; 2,438 and 3,658 m, respectively). Ascites mortality for the SUS line is apparent by 3 d post-hatch, whereas ascites-related mortality in the RES line is substantially delayed. The heritabilities for ascites were estimated to be  $0.30 \pm 0.05$  and  $0.55 \pm 0.05$  for the SUS and RES lines, respectively. These heritability estimates are consistent with what has been previously reported for ascites measured using other conditions (Huchzermeyer et al., 1988; Peacock et al., 1989; Lubritz et al., 1995; Wideman and French, 1999, 2000; Moghadam et al., 2001; Navarro et al., 2001; Deeb et al., 2002; Pakdel et al., 2002). The rapid selection response observed for ascites, coupled with the moderate to high heritabilities, suggest that a few major genes may control ascites. Birds from the SUS line also succumb to ascites during cool temperature exposure or microparticles injections, whereas birds from the RES line are markedly resistant (Wideman et al., 2002; Chapman and Wideman, 2006b). Clinically healthy broilers from the SUS line had higher pulmonary arterial pressures and pulmonary vascular resistances compared with clinically healthy individuals from the RES line (Wideman et al., 2002; Bowen et al., 2006a,b; Chapman and Wideman, 2006b; Lorenzoni et al., 2008). Lung volume as a percentage of BW does not differ between the SUS and RES lines (unpublished observations). The cumulative evidence demonstrates that selection pressures rigorously focused to challenge the pulmonary vascular capacity readily expose the genetic basis for spontaneous PAH in broilers (Wideman, 2001; Wideman et al., 2007, 2011). Selection for ascites in the SUS line has resulted in an increase in total heart weight due to increased weights of both the right and left ventricles. The increase in right ventricle weight was expected due to the positive genetic correlation between the RV:TV ratio (e.g., right-sided work hypertrophy), pulmonary hypertension, and ascites (Lubritz et al., 1995; Wideman et al., 2007); however, the increase in left ventricle



**Figure 4.** Cumulative pulmonary arterial hypertension (PAH)/ascites mortality for progeny from 3 broiler lines that remained unselected (REL, relaxed) or that had been selected for 14 generations under conditions of hypobaric hypoxia for susceptibility (SUS) or resistance (RES) to PAH/ascites (Anthony et al., 2001; Anthony and Balog, 2003; Pavlidis et al., 2007). For the experiment shown, the SUS and REL lines were reared at a simulated altitude of 8,000 feet (2,438 m), whereas the RES line was challenged at a substantially higher simulated altitude of 11,000 feet (3,353 m).

weight was not expected. Presumably left ventricular hypertrophy reflects increased cardiac output in compensation for systemic arterial hypoxemia (Wideman, 1999). Overall, these modifications in heart and lung capacities have potentially created a cardio-pulmonary system that is not sufficiently robust to support rapid growth and muscle deposition, thereby causing the SUS line to very rapidly develop ascites when exposed to stressors such as cold or hypobaric stress.

Although several experiments have shown that genetic selection for ascites resistance can be successfully accomplished, correlated responses for economically important traits have not been promising. The ascites incidence is known to have increased due to selection for rapid growth; therefore, it might be anticipated that selection for resistance could reduce growth potential. In fact, multiple generations of selection for ascites resistance resulted in the RES line being approximately 163 g lighter than the SUS line at 42 d of age (Pavlidis et al., 2007). Pectoralis weights measured at 42 d also showed that SUS line males had significantly heavier breast yields than RES line males (Pavlidis, 2003). The only improvement with respect to traits of economic importance was the fact that the RES line had a better feed conversion ratio (FCR) compared with the SUS line (Pavlidis, 2003). Correlated responses to ascites selection have been reported from other research lines (De Greef et al., 2001a,b; Druyan et al., 2008, 2009).

To map chromosomal regions contributing to ascites susceptibility, the RES and SUS lines were crossed, gen-

erating an F<sub>2</sub> population that was phenotyped for ascites susceptibility or resistance during exposure to hypobaric hypoxia (Pavlidis et al., 2007). The DNA from each bird was genotyped using a genome-wide panel of 3,072 SNP (Muir et al., 2008). The results identified 7 regions on 4 chromosomes that showed significant association with ascites phenotype. Further genotyping in additional populations, including different commercial lines, has demonstrated that at least 3 of these regions show association with ascites phenotype in several different lines (unpublished). The 3 regions are chromosome 9:13.5–14.8 Mbp, chromosome 9:15.5–16.3 Mbp, and chromosome 27: 2.0–2.3 Mbp (map positions based on the May 2006 *Gallus gallus* v2.1 genome assembly). It is noteworthy that, depending on the particular line examined, these regions can show sex-specific differences in statistical associations with ascites. Inspection of the genes from these regions has identified a few candidate genes based on the physiological evidence from birds or mammals. The most likely candidate genes are Gga9:13.5–14.8: AGTR1, angiotensin II type 1 receptor; and UTS2D, urotensin receptor 2 D; Gga9:16–5HT2B, serotonin receptor/transporter type 2B; and Gga27:2- ACE, angiotensinogen cleaving enzyme. Each of these genes has been implicated in some aspect of hypertension or hypoxic response in mouse or humans (Simonneau et al., 2004; Watanabe et al., 2006; Djordjevic and Görlach, 2007; MacLean, 2007; Chung et al., 2009). Genes that we did not find include BMPR2, which has been implicated in human hypertension (De Caestecker

and Meyrick, 2001), in agreement with a focused examination of BMPR2 in our lines (Cisar et al., 2003).

Resequencing of the 5HT2B region from SUS and RES samples identified 3 SNP, one in intron 2, and 2 in exon 3 (Burks and Rhoads, 2011). The exonic SNP are silent third base changes for similarly used codons. Sequence analysis of the promoter region identified 2 SNP that affect 2 potential transcription factor binding sites (unpublished). Resequencing of AGTR1 identified 3 SNP in the single coding exon, exon 3. Two substitutions are silent, whereas the third would substitute glutamate for aspartate 236, the first Asp residue in a motif (RNDDIF) that is conserved in all mammals and frogs (unpublished).

Recent genotype data for the Gga9:14 Mbp region in a commercial line phenotyped for ascites in the hypobaric chamber and different birds from the same line phenotyped for production traits, showed that the VNTR genotype significantly associated with resistance to ascites, had the highest body fat values ( $P = 0.07$ ), and had the highest BW ( $P = 0.06$ ; unpublished). The genotype most associated with susceptibility to ascites had the highest feed conversion. Therefore, in commercial lines this region affects ascites and production traits.

## CONCLUSIONS AND PERSPECTIVES

Broilers are susceptible to PAH/ascites syndrome when their pulmonary vascular capacity is inadequate to accommodate the requisite cardiac output. Consistent reports of specific right ventricular work hypertrophy (elevated RV:TV ratios) and direct measurements via catheterized pulmonary arteries have incontrovertibly established the central role of elevated pulmonary arterial pressures in the pathogenesis of PAH. The onset of pulmonary hypertension consistently has been associated with excessive resistance to blood flow through the pulmonary vasculature. Efforts to deduce the causes of elevated pulmonary vascular resistance in PAH-susceptible broilers have focused on evaluating the roles of environmental and chemical mediators of vasoconstriction and vasodilation, as well as on pathological (structural) changes occurring within the pulmonary arterioles. All factors that increase the pulmonary vascular resistance can initiate or accelerate the pathophysiological progression leading to PAH, including hypoxia, adrenergic neurotransmitters, TxA<sub>2</sub>, MG, ET-1, 5HT, respiratory damage or disease, and LPS. The key pulmonary vasodilator for broilers is NO and evidence is mounting that L-arginine, the substrate for NO production, may be limiting when broilers are exposed to conditions that promote the onset of PAH/ascites. The cumulative evidence attributes the elevated pulmonary vascular resistance in PAH-susceptible broilers to an anatomically inadequate pulmonary vascular capacity, to excessive vascular tone reflecting the dominance of pulmonary vasoconstrictors over vasodilators, and to vascular re-

modeling elicited by excessive hemodynamic stresses affecting the terminal pulmonary arterioles. The primary vasoconstrictor(s) responsible for inappropriately elevating the tone maintained by the pulmonary arterioles and the key promoter(s) of vascular remodeling remain to be conclusively identified. In both humans and broilers, PAH has been found to involve a significant immune system/inflammatory component and immunopathology. Emerging evidence demonstrates that the pathogenesis of PAH includes characteristics of an autoimmune disease, involving multi-factorial genetic, environmental, and immune system components. Recent experiments demonstrated key differences in the intrapulmonary inflammatory responses to vascular occlusion in the lungs of PAH-resistant compared with PAH-susceptible broilers. Resistance to PAH was associated with more efficient recruitment, infiltration, and activation of leukocytes, thereby emphasizing processes designed to generate additional vasodilator (NO), repair the vasculature, wall off and remove obstructions, resolve the inflammation, and ultimately restore normal function and capacity of the lung. Susceptibility to PAH appears to be multigenic and may be manifested in aberrant stress sensitivity, function, and regulation of pulmonary vascular tissue components, as well as aberrant activities of innate and adaptive immune system components. Investigating the role of the immune system in the initiation and pathogenesis of PAH will continue to provide a productive and informative avenue for investigation. Evidence that the immune system influences the pathogenesis of PAH demonstrates the need to assess potential shifts in innate and adaptive immune responsiveness as broilers continue to be aggressively selected for resistance to PAH. Major genetic influences and high heritabilities for PAH susceptibility have been demonstrated by numerous investigators. The cumulative evidence demonstrates that selection pressures rigorously focused to challenge the pulmonary vascular capacity readily expose the genetic basis for spontaneous PAH in broilers. Long-term divergent selection at the University of Arkansas employed hypobaric hypoxia to induce sustained pulmonary vasoconstriction. The PAH phenotype diverged rapidly between SUS and RES lines, with heritabilities estimated to be  $0.30 \pm 0.05$  and  $0.55 \pm 0.05$  for the SUS and RES lines, respectively. Resistance was positively associated with FCR and negatively associated with BW gain. Chromosomal mapping continues to identify regions associated with PAH susceptibility, and several likely candidate genes have been identified. Specific alleles responsible for susceptibility or resistance to PAH remain to be identified and must be aggressively pursued. Desirable production traits that are positively correlated with PAH susceptibility must continue to be monitored in broiler lines undergoing aggressive selection for PAH resistance. Ongoing immunological and genomic investigations are likely to continue generating important new knowledge regarding the fundamental biological bases for the PAH/ascites syndrome.

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