

RESEARCH ARTICLE

Ott's protein osmotic pressure of serum and interstitial fluid in chickens (*Gallus gallus*): effect of age and gender

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SUMMARY

Protein, or colloid, osmotic pressure (COP) is of sufficient magnitude to exert a powerful effect on Starling forces across the capillary wall. Although avian skin lacks active mechanisms for fluid transmission, such as sweating, it is now hypothesized that passive oncotic forces may regulate fluid flow and distribution in the skin and related phenomena. In this study, serum protein profiles, as well as COP in serum (COP_s) and in suction blister fluid (COP_{sbf}), were determined in juvenile, young and adult female and male chickens. For assessing COP, the Ott–Ahlqvist equation was applied. This equation can formulate the effects of multiple serum proteins on COP, according to their molecular mass. The COP values determined in chickens were lower than those previously found in mammals. COP_s increased with age in males, and was higher in adult males than in adult females. In contrast, COP_{sbf} decreased with age in females, although it was better preserved in adult males. The age-dependent decrease in COP was associated with a parallel decrease in the concentration of α_1 -globulin and albumin, and a positive correlation between packed cell volume (PCV) and COP was found. It is concluded that ageing affects Ott's COP around the vessel wall, and that an oncotic mechanism preserves plasma volume. The preservation of COP_{sbf} in males suggests better maintenance of the interstitial ground substance. Females seem to lose more of a tissue's COP_s counteracting force than males, probably as a result of gender-specific changes in the composition of the interstitial matrix. Further studies are required to elucidate the structure and function of avian lymphatics and their role in skin rheology.

Key words: COP, interstitial fluid, ageing, gender, bird, *Gallus gallus*.

INTRODUCTION

The skin has fundamental influence on the control of whole body homeostasis by being the largest organ of the animal body, and by holding a substantial volume of the total extracellular fluid (ECF) (Aukland and Nicolaysen, 1981; Levick, 1991; Aukland and Reed, 1993; Holliday, 1999). Studies in various animal species have revealed that about half of the total ECF volume in adult animals is located in the skin and the connective tissue interstitium; in juvenile animals, because of the higher surface-to-volume ratio, it is about 5% more (Holliday, 1999). In birds, the skin and its unique functional plasticity have shown to be crucial in adaptational responses to various stressors, such as heat, cold and dehydration, which all involve modifications in water transmission between the blood, tissues and the environment (Arad et al., 1983; Marder, 1983; Marder and Ben-Asher, 1983; Menon et al., 1986; Arad et al., 1989; Menon et al., 1989; Peltonen et al., 2000; Muñoz-García and Williams, 2008). Characteristically, thermal fluid transmission between a bird and its environment does not involve exocrine glands, and could be characterized as a passive mechanism, driven mainly by physical factors (Ophir et al., 2003). However, fluid and electrolytes may be stored in the skin, probably as a part of various transmission processes. In mammals, the skin of humans and rats has been shown to store Na⁺ (Heer et al., 2000; Titze et al., 2003; Titze et al., 2004). In avians, studies in chickens using suction blister fluid (SBF) have revealed an accumulation of monovalent cations (Peltonen et al.,

2006). In both cases, the accumulation of electrolytes took place without a significant influence on osmotic pressure. In rats, accumulation has been associated with the storage capacity of the interstitium, which is, in turn, regulated by processes involving the inhibition and disinhibition of glycosaminoglycan (GAG) chain polymerization (Titze et al., 2004). GAGs, together with proteoglycans, glycoproteins and the structural proteins collagen and elastin, form the highly organized extracellular matrix, which endows skin with its water-binding properties (Stern and Maibach, 2008). A similar mechanism for controlling skin storage capacity may be feasible also in chickens, owing to high concentrations of GAGs in their skin (Nakano and Sim, 1989; Nakano and Sim, 1991).

The osmotic pressure of a solution reflects its colligative properties and its behaviour when it is separated from another solution by a semipermeable membrane. About 95% of total osmotic pressure is built by inorganic electrolytes, 4–5% by glucose, urea and large-molecular-weight organic compounds, such as GAGs, and only about 0.5% by proteins (Guyton and Hall, 2006). Even though most of the total osmotic pressure is built by inorganic electrolytes, they do not exert osmotic pressure across the capillary wall in tissues; they are dissolved in water and move freely with it. In contrast, proteins exert a powerful effect on the convective fluid flow. This is because of the finite permeability of capillaries and the exclusion of proteins by the endothelium (Levick, 1991). Holding to the classical Starling hypothesis (Starling, 1896), fluid

shifts across the capillary endothelium respond only to changes in the balance between protein osmotic pressure, i.e. difference in COP, and hydrostatic pressure (Boulpaep, 2009). However, discrepancies between the experimental data and the classical Starling predictions have resulted in a revised model in which the gradient of the Starling forces exists across the glycocalyx, not the endothelium. By this revision, the fluid flow is smaller than that predicted by Starling for bulk driving forces and, for a given driving force, the fluxes are greater for filtration than for absorption (Levick, 2004; Michel, 2004; Curry, 2005). The Donnan forces, tending towards electroneutrality, further emphasize the role of plasma proteins in fluid flow: owing to the net negative charge carried by proteins in normal pH, they affect COP by retaining cations and repelling anions (Hitchcock, 1924). Recently, protein charge has been shown to account for one-third of the total COP in rats (Rügheimer et al., 2008). Once filtrated, water will occupy the interstitial ground substance by binding to collagen and GAGs, especially to hyaluronan (reviewed by Stern and Maibach, 2008). These molecules maintain the space and make the extracellular fluid for a gel, in which water diffuses rather than flows (Guyton and Hall, 2006). Simply for geometrical reasons, collagen and GAGs exclude albumin and other plasma proteins from the densely packed gel, creating an osmotically, as well as rheologically, heterogeneous interstitium (Ogston and Phelps, 1961).

Now, a hypothesis is put forward that the variation of Starling forces across the capillary wall may function as a regulatory factor in the response to various homeostatic imbalances in birds of different age and gender. Homeostatic response often includes the transmission and holding of fluid and electrolytes, which obviously does not involve strictly regulated secretory mechanisms in the practically glandless avian skin. However, there are some previous findings to support the special role of COP as a dynamic regulatory variable. Firstly, plasma albumin and globulin profiles change considerably during development, especially in females. Early electrophoretic studies in Galliform species show that plasma protein compositions differ between females and males of the same species more than those between the males of different species (Sibley and Johnsgard, 1959). Secondly, the relative distribution of total body fluid is influenced by gender and age. In chickens, as the relative body fluid content (percent of body mass) decreases by age, the relative intracellular fluid increases and the ECF decreases, both in blood and interstitium (Sturkie, 1986). In some species, female blood tends to be also more diluted than that of the males, as indicated by lower values of packed cell volume (PCV) (Hebert et al., 1989). PCV seems to increase with age, even though this effect may be dependent on gender (Mitchell and Johns, 2008). Thirdly, the avian lymphatic system differs structurally, and probably functionally, from its mammalian counterpart. In mammals, lymph circulation is the major unidirectional pathway for returning proteins and excess fluid back to plasma, and for restoring the COP of the interstitial fluid (Aukland and Nicolaysen, 1981; Levick, 1991; Aukland and Reed, 1993; Levick, 2004; Michel, 2004). In birds, the lower numbers of lymphatic vessels and the less frequent valves in them (King and McLelland, 1975; Rose, 1985) imply a reduced capacity to drain fluid from tissues.

In order to elucidate the possible influence of the Starling COP difference on fluid transmission and accumulation in avian skin, we assessed, for the first time, the COP of serum and interstitial fluid in female and males chickens, and followed these values during the development of a number of individuals. The COP values were calculated by using the equation of Ahlqvist (Ahlqvist 2004), originally based on the experimental results of Ott (Ott, 1956), which take into account the whole spectrum of plasma protein fractions and, thus, the variability of plasma protein profiles during ageing.

MATERIALS AND METHODS

Animals

Serum protein profiles and Ott's COP in blood and in interstitial fluid were determined in female and male White Leghorn chickens (*Gallus gallus domesticus* Linnaeus 1758) at three successive ages: 10 weeks (juvenile), 20 weeks (transition to sexual maturity) and 30–33 weeks (sexual maturity). Birds were sexed and housed in 20 m² rooms in ambient temperature of 20–23°C. Birds were fed with food manufactured for juvenile, growing and adult chickens. The onset of laying took place at the age of 20 weeks. At the age of approximately 33 weeks, all females had regular laying cycles of one egg per day. During the study, body mass increased almost linearly by 52% in females and by 53% in males. Birds were kept constantly under a steady 12h:12h light:dark photoperiod. All the experiments conformed to the guidelines for proper animal care and use, and were authorized by the local ethical committee.

Sample collection

The SBF and blood samples were collected under injectional anaesthesia by intramuscular ketamine in combination with xylazine in doses of 20 mg kg⁻¹ (Ketalar, Pfizer Animal Health, Helsinki, Finland) and 5 mg kg⁻¹ (Rompun, Bayer, Leverkusen, Germany). Blood samples were taken at the same time of the light:dark cycle and SBF samples within a two-hour time range. SBF was collected *in vivo* by the method first developed for humans by Kiistala and Mustakallio (Kiistala and Mustakallio, 1964). Individual suction cups were attached on the area of thoracic arterial skin devoid of large cutaneous blood vessels. Blood was removed from the brachial vein to 500 µl vials through a 21–22G needle. Serum was separated and collected after 1–2 h of incubation at room temperature and centrifugation. All samples were frozen for storage. PCV was determined immediately from fresh venous blood using 10 µl microhematocrit vials. Separation of proteins was carried out by agarose gel electrophoresis (SPE P/N 655900, Beckman Coulter, Fullerton, USA), according to the manufacturer's instructions. Quantitative results were obtained by software for quantitative gel analysis (ImageJ 1.36b, Wayne Rasband, National Institutes of Health, USA). Total protein (TPR) was determined by BCA Protein Assay (Pierce, Rockford, IL, USA), based on the biuret reaction by proteins, sensitized with bicinchoninic acid.

Equations and calculations

To determine the protein osmotic pressure (Π) of serum, we used an equation by Ahlqvist (Ahlqvist, 2004). Eqn 1 formulates the effects of each protein fraction on the Π of serum. These effects were first presented by Ott (Ott, 1956) and were later modified by Guyton (Guyton, 1981). Eqn 1 states that the effect of globulin concentration on Π is dependent on the molecular mass (M_r). It was revealed that the same concentrations of albumin or of various globulin fractions alone contribute to Π in the order: α_1 -globulin, albumin, α_2 -globulin, β -globulin and γ -globulin (Ahlqvist, 2004).

Ott's colloid osmotic pressure (COP_{Ott, 2°C}) is formulated as follows:

$$\begin{aligned} \text{COP}_{\text{Ott}, 2^\circ\text{C}} = & x_{\alpha-1}(0.518C_{\text{tot}} + 0.0107C_{\text{tot}}^2) \\ & + x_{\text{alb}}(0.338C_{\text{tot}} + 0.00339C_{\text{tot}}^2) \\ & + x_{\alpha-2}(0.203C_{\text{tot}} + 0.00155C_{\text{tot}}^2) \\ & + x_{\beta-1}(0.187C_{\text{tot}} + 0.000577C_{\text{tot}}^2) \\ & + x_{\beta-2}(0.187C_{\text{tot}} + 0.000577C_{\text{tot}}^2) \\ & + x_{\gamma}(0.161C_{\text{tot}} + 0.000223C_{\text{tot}}^2). \end{aligned} \quad (1)$$

In Eqn 1, $x_{\alpha-1}$ is the fractional concentration of α_1 -globulin ($C_{\alpha-1}/C_{\text{tot}}$), x_{alb} is the fractional concentration of albumin, and so

on. The final COP of serum and of SBF (COP_s and COP_{sbf}) at avian body temperature (40°C) were calculated as:

$$COP_{\text{Ott}} = (313\text{ K}/275\text{ K})COP_{\text{Ott}, 2^\circ\text{C}}, \quad (2)$$

where K is degrees Kelvin.

The first-order terms represent the van't Hoff law for osmotic pressure, and the square terms represent the deviations from that law caused by Donnan effects and interactions between proteins.

Statistics

One-way analyses of variance (ANOVA) were used for statistical analyses. If the overall P -value for statistical significance was <0.05 , various *post hoc* tests were used. These were the test for increasing and decreasing trends, based on one-way analyses of variance, and Tukey's or Bonferroni's tests for multiple comparisons. Selected data were analyzed also by Pearson's correlation.

RESULTS

Electrophoretic analysis of serum and suction blister fluid proteins

The electrophoretic pattern of serum and SBF consisted of seven protein fractions: transthyretin, albumin, α_1 -, α_2 -, β_1 -, β_2 - and γ -globulin. Transthyretin was present in both serum and SBF, and

there was gender-specific variation by age in serum in male birds (one-way ANOVA, $P<0.01$): the percentage of total protein in serum peaked in 20-week-old male birds, the value being 1.3% and the mean concentration being $0.60\pm 0.044\text{ g l}^{-1}$. No such variation was observed in females. Densitometrical profiles of serum proteins during development to sexual maturity are shown in Fig. 1. The most relevant proteins for COP, albumin and α_1 -globulin, varied differently with age. Although albumin concentration (g l^{-1}) did not change by age and its concentration was independent of gender (Fig. 2C,D), α_1 -globulin concentration decreased in females, but not in males (Fig. 2A,B). As expected, the total amount of globulin in serum increased rather steeply in females (Fig. 1C,E; one-way ANOVA, $P<0.001$, *post hoc* test for increasing trend, slope 5.89, $R^2=0.545$, $P<0.001$). Globulin concentration increased also in males, but levelled after the transitional phase (Fig. 1B,D; *post hoc* test for increasing trend, slope 1.81, $R^2=0.193$, $P<0.05$). To summarize, the various serum globulins revealed the following differences in their profiles: (1) α_1 -globulin concentration was higher in young adult and adult males than in females of the same age (one-way ANOVA, Tukey-Kramer *post hoc* test, $P<0.001$ in both age groups); (2) β_1 -globulin concentration was higher in laying young adult and adult females than in males of the same age (one-way ANOVA, Tukey-Kramer *post hoc* test, $P<0.05$ for 20- and

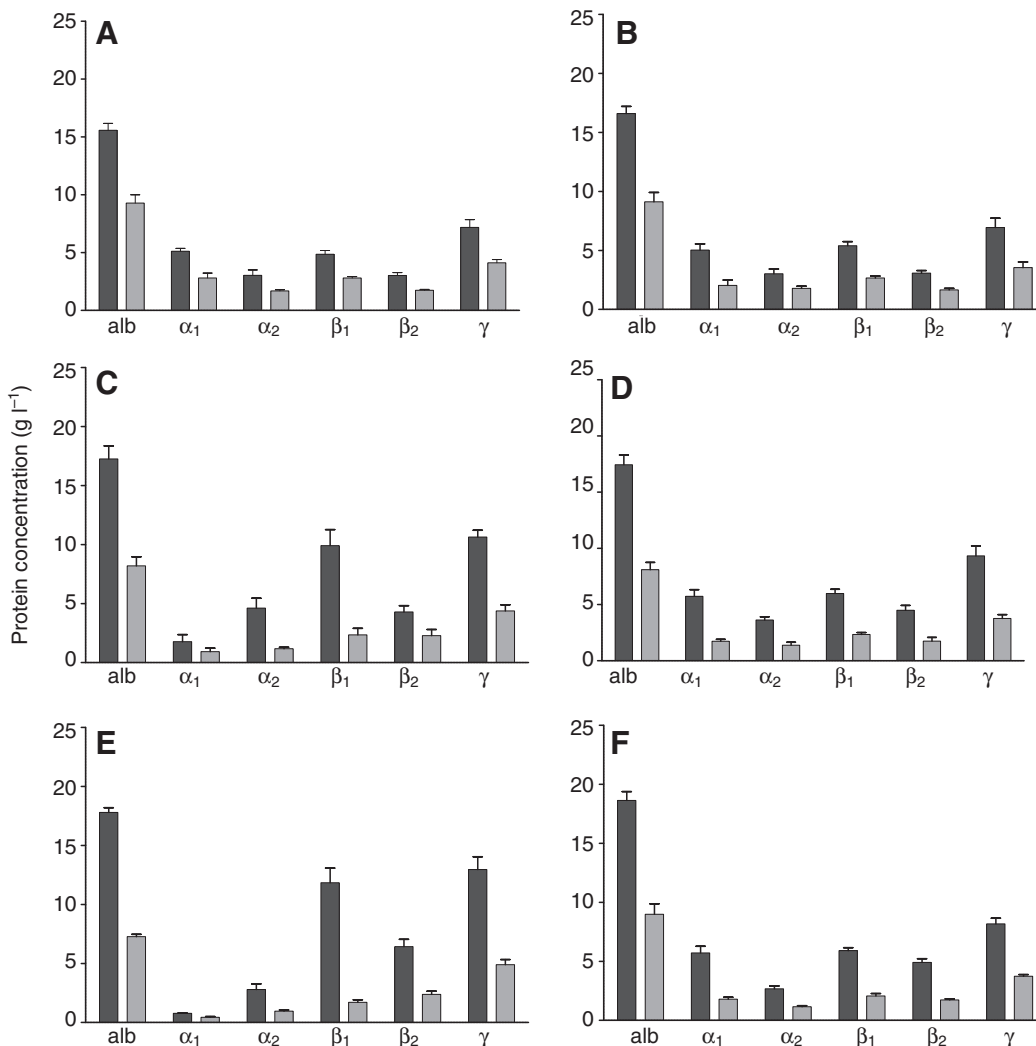


Fig. 1. Protein profiles in serum and suction blister fluid (SBF) in female and male chickens. (A,C,E) Females; (B,D,F) males. (A,B) Juveniles, 10-weeks old. (C,D) Young adults, 20-weeks old. (E,F) Adults, 30- to 33-weeks old. Black bars indicate serum; gray bars, SBF. Alb, albumin; α_1 , α_1 -globulin; α_2 , α_2 -globulin; β_1 , β_1 -globulin; β_2 , β_2 -globulin; γ , γ -globulin. The total protein content of serum and SBF, TPR_s (g l^{-1})/ TPR_{sbf} (g l^{-1}), was: (A) 38.1/22.4, (B) 40.1/21.0, (C) 48.5/19.3, (D) 46.7/19.1, (E) 52.6/17.7, (F) 46.0/19.5.

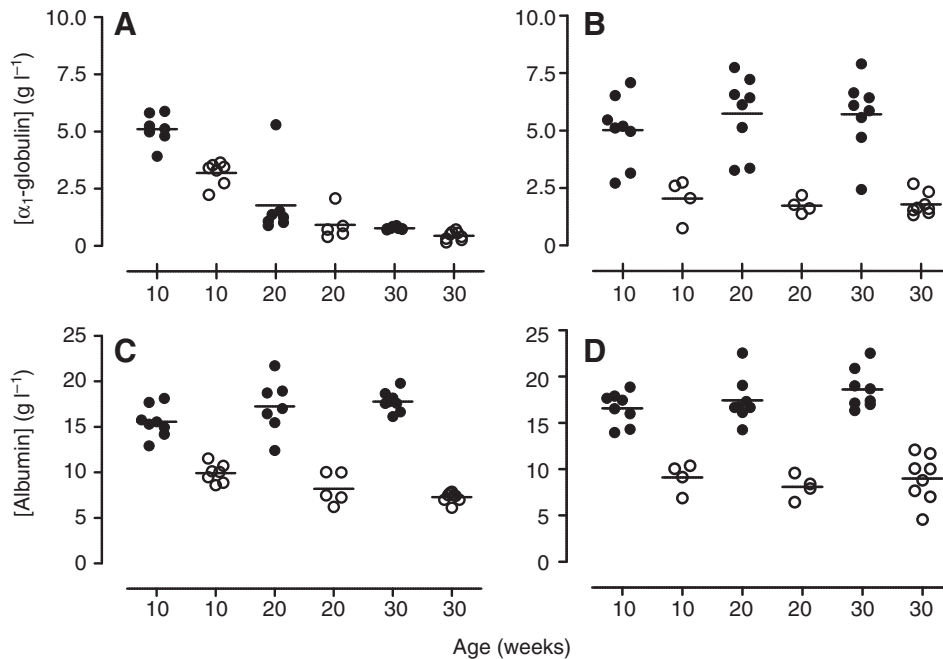


Fig. 2. Age- and gender-dependent variations of α_1 -globulin and albumin concentrations in serum and SBF. (A,C) Females; (B,D) males. Black dots indicate serum; open dots, SBF. (A) A decreasing trend by age can be observed in females for α_1 -globulin concentration in serum (one-way ANOVA, *post hoc* test, $R=-2.17$, $R^2=0.76$, $P<0.001$), and in SBF (one-way ANOVA, *post hoc* test, $R=-1.37$, $R^2=0.82$, $P<0.001$). (C) A decreasing trend by age can be observed in females for albumin concentration in SBF (one-way ANOVA, *post hoc* test, $R=-1.32$, $R^2=0.56$, $P<0.001$).

$P<0.001$ for 30-week-old birds); and (3) γ -globulin concentration was significantly higher in adult females, but not in juvenile and young adult females, than in males of the same age (one-way ANOVA, Tukey–Kramer *post hoc* test, $P<0.01$ for 30-week-old birds). Serum β_1 -, β_2 - and γ -globulin concentrations increased with age in females, whereas α_1 - and α_2 -globulin concentrations decreased or were stable. Only β_2 -globulin concentration increased with age in males; other serum proteins remained unchanged.

Neither albumin nor the total globulin concentrations in the SBF differed between genders with age (Fig. 1). However, in females the SBF versus serum ratios ($[SBF]/[S]$) for both protein fractions declined at the onset of laying and levelled after that: for albumin the decrease was about 20% (one-way ANOVA, *post hoc* test for decreasing trend, slope -9.24 , $R^2=0.26$, $P<0.05$) and for globulin was 28–30% (one-way ANOVA, *post hoc* test for decreasing trend, slope -15.45 , $R^2=0.44$, $P<0.01$). In males, albumin and total globulin concentrations in the SBF remained stable during development. A gender-specific difference was observed in α_1 -globulin and albumin concentrations (Fig. 2), which were higher in adult males than in adult females (one-way ANOVA, Tukey–Kramer *post hoc* test, $P<0.01$). In female SBF, both α_1 -globulin and albumin concentration showed an age-dependent decreasing trend (Fig. 2A,C).

Ott's protein osmotic pressures of serum and suction blister fluid

The calculated mean Ott's COP_s values increased with age in males (one-way ANOVA, $P=0.022$, *post hoc* test for increasing trend, slope 1.3, $R^2=0.24$ and $P<0.05$). In females, no such trend was observed (Fig. 3). By Bonferroni's *post hoc* test for selected pairs, mean COP_s values were shown to be different in transitional and adult, but not in juvenile, birds (P -values <0.05). As expected, COP_{sbf} values were lower than corresponding COP_s values, varying between 35 and 55% of that of the serum. The mean COP_{sbf} values of female and male chickens of varying ages were significantly different (one-way ANOVA, $P=0.004$). However, although a significant declining trend in females was shown (one-way ANOVA, *post hoc* test for linear

trend, slope -1.20 , $R^2=0.49$, $P<0.001$), COP_{sbf} values in males remained stable (Fig. 3).

Derived from the COP data, the Starling oncotic gradient tended to increase with age in females, as shown by the variation of mean ΔCOP values with age (one-way ANOVA, $P<0.001$, *post hoc* test for linear trend, slope 2.1, $R^2=0.51$, $P<0.001$). Generally, the oncotic gradient was better preserved in adult males (Fig. 3, insert).

PCV and COP_{Ott}

The mean PCV value averaged 33.7% ($\pm 0.88\%$) in young adult, and 29.7% ($\pm 0.47\%$) in adult female chickens. The corresponding values in males were significantly higher and showed less variation, being 50.1% ($\pm 0.13\%$) and 47.7% ($\pm 0.13\%$), respectively (one-way ANOVA, $P<0.001$). Derived from the PCV and the corresponding Ott's COP data, intravascular and extravascular oncotic pressures decreased by hemodilution (Fig. 4). However, the decrease was not parallel, resulting in a steeper oncotic gradient in more hemoconcentrated blood.

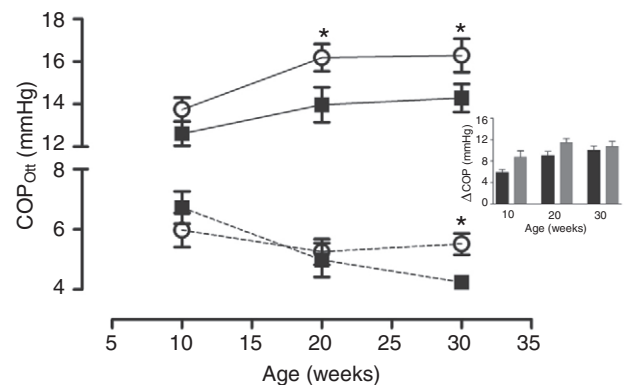


Fig. 3. Ott's protein osmotic pressure in chickens. Black squares indicate females; open circles, males. Solid line indicates serum; dotted line, SBF. Inset shows the difference between COP_{Ott} of serum and SBF (ΔCOP). Black bars indicate females; gray bars, males. * $P<0.05$, *post hoc* tests.

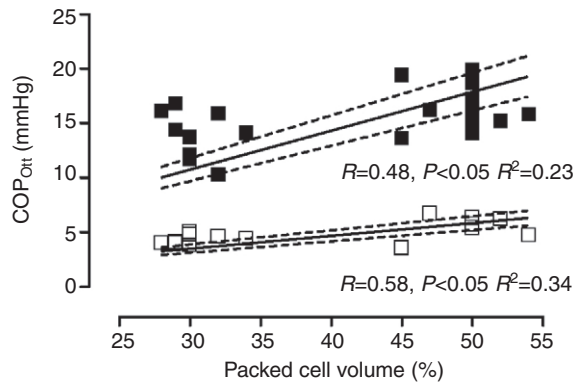


Fig. 4. Correlation (Pearson) between packed cell volume and COP. Black squares indicate COP_s ; open squares, COP_{sbf} . Dotted lines denote the 95% confidence band of the best-fit curve (solid line).

DISCUSSION

Plasma protein composition has been shown to be both an age- and a gender-dependent variable in chickens, and it was hypothesized that this variability would reflect on Starling forces affecting fluid transmissions between the blood and skin tissue. The Starling principle states that transendothelial filtration is driven by capillary hydrostatic pressure and interstitial COP, and that the counteracting absorptive force is exerted by intravascular COP and interstitial hydrostatic pressure. In the present study, the Ott's protein osmotic pressures of serum and SBF, calculated from multiple protein fractions, changed reciprocally with age and showed gender-related differences at sexual maturity. This oncotic component alone implies an increased capacity of the blood to hold fluid and protect plasma volume with age. At the same time, the counteracting interstitial forces appear to weaken. However, males seem to be better able to preserve the oncotic gradient across the vessel wall by preserving COP_{sbf} . Functionally, this would mean that the skin of adult male birds is able to compensate for a higher increase in net transcapillary filtration than the skin of laying females. In the skin, compensation would depend on the fluid-binding capacity of the tissue, on the composition and constancy of the ground substance, and on the capacity of the lymph system to accumulate or drain fluid. There is some evidence to support the better compensation observed in males. Visual inspection of the male skin reveals 'a young or hydrated skin', as it is less transparent and its stereoscopic effect ('relief') is finer and less visible than that of coeval females, similar to humans (Zahouani and Vargiolu, 2004). Male skin is also thicker and more resilient (Peltonen et al., 2006; Peltonen and Mänttari, 2008), implying a greater mass of collagen and elastic fibres in the ground substance. Furthermore, GAGs are present in high concentrations in males, especially in the combs and wattles, hyaluronan being the main species (Nakano and Sim, 1989; Nakano and Sim, 1991).

The present results show a negative correlation between age and albumin and α_1 -globulin concentrations in SBF in females, parallel to the COP decrease, whereas no change was found in males. In rodents, it has been suggested that the decrease in general perimicrovascular protein content with age is associated with the loss of maintenance of the interstitial space (Barber et al., 1995). Conversely, the preservation of the interstitial albumin concentration during ageing seems to be connected with the tissue space becoming increasingly inaccessible to albumin (Parameswaran et al., 1995). Thus, in females the weakening of protein osmotic activity suggests

a susceptibility to age-dependent modulation of the interstitial space available for plasma proteins. In humans, the ground substance is modulated as collagen becomes more sparse, less soluble and thinner, and GAGs become aberrantly localized, interfering with normal water binding irrespective of their amount (Waller and Maibach, 2006). The volume of the interstitial fluid increases, as does the amount of 'free' water that binds to itself, rather than to other components of the interstitium (Wright et al., 1998). So far, the protein exclusion effect *per se* has not been studied in birds.

Estrogen is an important modulator of the interstitial ground substance, as it stimulates collagen production and prevents its loss, and increases skin thickness and elasticity via its effects on water-binding capacity and GAG content (reviewed by Verdier-Sévrain et al., 2006; Farage et al., 2009). Hence, in egg-laying chickens estrogen may be involved in the short-term regulation of skin composition and function owing to its diurnally oscillating secretion pattern (Johnson and van Tienhoven, 1980). The cellular and subcellular mechanisms for the 'collagen (or GAG) effect' of estrogen are complex and, besides estrogen, other sex steroid hormones and their receptors may also be involved. Estrogen concentration in chickens increases markedly just before sexual maturity, peaks during the laying period and drops dramatically only at 70 weeks of age (reviewed by Beck and Hansen, 2004). Therefore, it could be speculated that the age-related decrease in COP_{sbf} found in the present study may not be related to circulating estrogen, but to a decrease in the density of estrogen receptors (Hansen et al., 2003). The well-established downregulating effect of progesterone on estrogen receptors may diminish the estrogen effect in laying females, which display increased progesterone concentrations (Johnson and van Tienhoven, 1980; Herremans et al., 1993; Gahr, 2001). The lower levels of circulating progesterone in younger females and males may enhance the effects of estrogen and result in the higher COP_{sbf} found in these birds.

The present serum COP values in chickens are considerably lower than those reported in mammals. The TPR of birds is generally lower (Carpenter et al., 2001), and thus is the most evident cause for the lower COP values. Depending on age and gender, the values in the present study varied between 33 and 55 g l⁻¹, being maximally 35% below the mammalian level. However, it was found that the causal relationship between TPR and COP differed between genders. In juvenile birds, having uniform fractional protein profiles, males had higher COP values than females in association with their higher TPR, whereas, in the transitional and adult phase, male serum COP peaked above that of females despite having a lower mean TPR. Based on the work of Ott (Ott, 1956), the greater protein osmotic force of serum is probably due to the higher fraction of α_1 -globulin in the male blood. According to the computed model of COP_{Ott} at body temperature in humans, at constant albumin concentration the influence of α_1 -globulin on COP is twofold compared with the influence of γ -globulins (Ahlqvist, 2004). In concert with TPR and α_1 -globulin, the relative amount of plasma may partly explain the observed differences in COP between genders. Even though the total volume of plasma could be estimated to be greater in male chickens in all age groups (Sturkie, 1986), the relative plasma volume in the present study is roughly 20% lower. Because the plasma (the loose intercellular ground substance of blood) provides less available space for proteins in males, it may function more efficiently in maintaining adequate blood volume than does the more diluted plasma in females.

To our knowledge, interstitial COP has not been measured in chickens. However, direct measurements of plasma carried out in 5- to 8-week-old male chicks (McDonagh and Gore, 1982) have

shown approximately 1 mmHg lower values than our present calculations in juvenile males. As the male birds used in this earlier study were two to five weeks younger, the age-dependent increase in the TPR may partly explain the higher values in the present study. Otherwise, the relationship of plasma TPR and measured COP in young male chicks was found to be similar to that found by Ott (Ott, 1956) in humans.

Owing to the insufficient knowledge of COP in birds, the comparison between calculated COP_{Ott} and measured COP cannot be made. In other species, such as in horses, the calculated COP values have been shown to differ from the measured values when the calculations have been based on total protein or albumin and total globulin concentrations, ignoring the effect of low-molecular-weight globulin fractions (Boscan et al., 2007). In humans, the difference between the measured COP and Ott's COP has been estimated to be $\pm 4.5\%$ when rare conditions, such as analbuminemia and nephrotic syndrome, have been excluded (J. Ahlqvist, personal communication). The calculated interstitial Ott's pressure is probably underestimated because of the lack of GAG influence. As yet, the impact of GAGs on COP_{sbf} is not known.

Mobilization of extravascular fluid

The early argument of a greater capacity of birds to mobilize extravascular fluid (Djojogugito et al., 1968; Wyse and Nickerson, 1971) was based on findings of a better tolerance of avian species compared with mammals to severe blood loss (Kováč et al., 1969). Since that, evidence has been accumulated on the ability of birds to tolerate also other severe homeostatic fluid imbalances. Birds seem to stand extremely well osmotic stress during severe thermal dehydration (Arad et al., 1989), as well as dehydration stress at high altitudes during long-distance migration (Landys et al., 2000) or in the desert habitat (Marder, 1983; Muñoz-García and Williams, 2008). Alternatively, in the condition of hydrothorax, the pathological effect of local overhydration on respiration may be avoided by shifting the excess fluid locally from the abdominal air sacs to the microvilli of the adjacent mesothelial cells (Weidner, 2000). A good capacity to mobilize fluid would mean either effective lymph flow in order to return the ultrafiltrated, proteinaceous fluid back to circulation, or effective absorption of fluid back to the venous capillaries. The present results suggest that the effectiveness of the absorption mechanism at the venous end of the capillary network may be related to age. However, little is known about the effects of the various unique characteristics of the avian circulatory and lymph system on the fluid flow between capillaries, tissues and lymphatics. These characteristics include a more efficient heart and a higher systemic blood pressure than mammals, a higher blood pressure of males than of females (Sturkie, 1986; Ruiz-Feria et al., 2004), relative thinness of the capillary endothelial cells compared with mammalian species (Watson et al., 2007), uniformity of the capillary wall 'thinness' and, yet, a large variation of the luminal cross-sectional area along very short distances in some capillary networks (West et al., 2010), and the existence of a thick and curious hyaline coating around the capillaries of specialized organs, such as the intraocular pecten (Goodman and Bercovich, 2008). In theory, these factors challenge the way the Starling hypothesis can be applied in birds, thus leaving the functional 'dimensions' of the protein osmotic gradient still unclear.

The present findings of intravascular and interstitial COP open up new vistas for explaining the phenomenon of thermoregulatory cooling via glandless skin. Cutaneous water evaporation (CWE) is a cooling mechanism, which is primarily driven by the vapour pressure gradient between the bird and its surroundings (Marder

and Arieli, 1988; Wolf and Walsberg, 1996; Ophir et al., 2003). In hot and dry environments, CWE may be enhanced by making the pressure gradient steeper by increasing the hydration of the outermost layers of the skin (Peltonen et al., 1998). Thus, changes in the COP around the cutaneous capillary wall will influence fluid shifts in the skin and, eventually, the process of CWE. Evidence of a specific epidermal fluid build-up has been found in rock pigeons (*Columba livia* Gmelin 1789) acclimated to high ambient temperatures, but not in those acclimated to mesic or cold environments (Peltonen et al., 1998; Peltonen et al., 2000). The associated dermal changes have been found to be an increased number of skin capillaries in the superficial dermis (Peltonen et al., 1998), and increased capillary fenestrations and endothelial gaps, the latter implying an increased extravasation of plasma proteins (Arieli et al., 1999). CWE has also been shown to involve both central and local adrenergic control, the local control involving a β_2 -adrenergic decrease in the arterial resistance to blood flow and an increase in the venous resistance (Marder and Raber, 1989; Ophir et al., 2000). Obviously, these changes tend to increase filtration, creating a driving force for CWE (Ophir et al., 2003). However, the relative importance of CWE to thermoregulatory cooling varies among species. In columbids, CWE becomes the major cooling mechanism in adaptation to a hot and dry environment (Marder et al., 1986; Marder and Arieli, 1988; Withers and Williams, 1990; Marder et al., 2003; Dawson and Whittow, 2000), whereas in other diurnally active species its relative importance decreases with increasing ambient temperature (Wolf and Walsberg, 1996). Together, fluid shifts driven by Starling forces within the body, or out of the body, may function as crucial local and systemic regulatory factors in fighting homeostatic imbalances.

As for the lymph flow, more research is needed to elucidate its physiological role in the homeostatic responses of birds. The lack of flow-driving lymph hearts, the lower number of lymphatic vessels and the less frequent valves compared with mammals (King and McLelland, 1975; Rose, 1985) suggest either a reduced capacity for fluid drainage from the tissues, or a different strategy for recirculating fluid between the circulatory system and the tissues. During the course of evolution, the loss of a permanent external water environment has been compensated for by an internal water reservoir and a lymphatic drainage system. Many amphibian species have a large-volume lymph space, drained externally from the skin and internally from skin capillaries, and pumped by multiple lymph hearts that beat 60–70 times per minute (Priestley, 1878). Many avian species, however, have lost their lymph hearts, but have probably preserved the capacity for fluid storage and a multidirectional flow pattern because of the more primitive structure of the lymph system (von Rautenfeld and Schacht, 2006). The age-dependent decrease in COP_{sbf} in the present study suggests that ageing may increase the volume of the cutaneous interstitial fluid or, at least, the volume that is available for plasma proteins. Besides the possible changes in the composition of the interstitial ground substance, ageing may impair the function of the lymph transport system. It has been shown in rodents that the negative impact of ageing is associated with the loss of the contractile capability of the lymph vessel as a result of atrophy of the smooth muscle cells and elastic matrix (Gashev and Zawieja, 2010). Further studies are warranted in order to elucidate how ageing and natural changes in the physiology of birds modulate the osmotic activity and volume of the various fluid spaces of the body.

To summarize, Ott's protein osmotic pressure, which takes into account the graded osmotic effect of various serum proteins, showed age- and gender-dependent variation in chickens (*Gallus gallus*). Serum COP_{Ott} increased with age in males, in association

with increasing α_1 -globulin concentration. In contrast, COP of the bulk interstitial fluid collected by suction, showed an age-dependent decrease in females, associated with decreasing concentrations of α_1 -globulin and albumin. The affect of other molecules on COP, such as lactose, glucose and GAGs, was not assessed in the present study. However, the specific changes in the serum protein concentrations suggest that ageing modulates the composition of the interstitial ground substance so that the volume of the space occupied by serum proteins changes, affecting water binding and fluid diffusion/flow within the interstitium. The positive correlation between serum COP_{Ott} and PCV suggests that the geometrical space available for proteins may affect the Starling forces also on the luminal side of the vessel wall. We did not measure the volume of the interstitial fluid in the present study. However, the age- and gender-dependent decrease in the COP_{sbf} values is indicative of increased fluid retention/storage in the interstitium. Further studies are required in order to elucidate the role of lymphatics in, and the effects of ageing on, the recycling of serum proteins and fluid back to the circulation in birds.

LIST OF ABBREVIATIONS

COP _{Ott}	Ott's colloid osmotic pressure (Ott's protein osmotic pressure)
COP _s	colloid osmotic pressure of serum
COP _{sbf}	colloid osmotic pressure of suction blister fluid
ECF	extracellular fluid
GAG	glycosaminoglycan
IF	interstitial fluid
PCV	packed cell volume
SBF	suction blister fluid
TPR	total protein

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REFERENCES

- Ahlqvist, J. (2004). Equation for osmotic pressure of serum protein (fractions). *J. Appl. Physiol.* **96**, 762-764.
- Arad, Z., Marder, J. and Eylath, U. (1983). Serum electrolyte and enzyme responses to heat stress and dehydration in the fowl (*Gallus domesticus*). *Comp. Biochem. Physiol.* **74A**, 448-453.
- Arad, Z., Horowitz, M., Eylath, U. and Marder, J. (1989). Osmoregulation and body fluid compartmentalization in dehydrated heat-exposed pigeons. *Am. J. Physiol.* **257**, R377-R382.
- Arieli, Y., Feinstein, N., Raber, P., Horowitz, M. and Marder, J. (1999). Heat stress induces ultrastructural changes in cutaneous capillary wall of heat-acclimated rock pigeon. *Am. J. Physiol.* **277**, R967-R974.
- Aukland, K. and Nicolaysen, G. (1981). Interstitial fluid volume: local regulatory mechanisms. *Physiological Reviews*, **61**, 556-643.
- Aukland, K. and Reed, R. K. (1993). Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiological Reviews*, **73**, 1-78.
- Barber, B. J., Dutta, S., Parameswaran, S. and Babbitt, R. A. (1995). Age-related changes in perimicrovascular protein distribution. *Am. J. Physiol.* **269**, H1213-H1220.
- Beck, M. M. and Hansen, K. K. (2004). Role of estrogen in avian osteoporosis. *Poultry Sci.* **83**, 200-206.
- Boscan, P., Watson, Z. and Steffey, E. P. (2007). Plasma colloid osmotic pressure and total protein trends in horses during anesthesia. *Vet. Anaesth. Analg.* **34**, 275-283.
- Boulpaep, E. L. (2009). The microcirculation. In *Medical Physiology* (ed. W. F. Boron and E. L. Boulpaep), pp. 482-503. Philadelphia, PA: Saunders Elsevier.
- Carpenter, J. W., Mashima, T. Y. and Rupiper, D. J. (2001). *Exotic Animal Formulary*. Philadelphia, PA: Saunders.
- Curry, F.-R. E. (2005). Microvascular solute and water transport. *Microcirculation* **12**, 17-31.
- Dawson, W. R. and Whittow, G. C. (2000). Regulation of body temperature. In *Sturkie's Avian Physiology* (ed. G. C. Whittow), pp. 343-390. New York: Academic Press.
- Djojosugito, A. M., Folkow, B. and Kovách, A. G. (1968). The mechanisms behind the rapid blood volume restoration after hemorrhage in birds. *Acta Physiol Scand.* **74**, 114-122.
- Farage, M. A., Neill, S. and MacLean, A. B. (2009). Physiological changes associated with the menstrual cycle, a review. *Obstet. Gynecol. Surv.* **64**, 58-72.
- Gahr, M. (2001). Distribution of sex hormone receptors in the avian brain: functional implications for neural sex differences and sexual behaviors. *Microsc. Res. Tech.* **55**, 1-11.
- Gashev, A. A. and Zawieja, D. C. (2010). Hydrodynamic regulation of lymphatic transport and the impact of ageing. *Pathophysiology* **17**, 277-287.
- Goodman, G. and Bercovich, D. (2008). Melanin directly converts light for vertebrate metabolic use: heuristic thoughts on birds, Icarus and dark human skin. *Med. Hypotheses* **71**, 190-202.
- Guyton, A. C. (1981). *Textbook of Medical Physiology*, 6th edition. Philadelphia, PA: Saunders.
- Guyton, A. C. and Hall, J. E. (2006). *Textbook of Medical Physiology* (11th edn). Philadelphia, PA: Saunders.
- Hansen, K. K., Kittok, R. J., Sarath, G., Toombs, C. F., Caceres, N. and Beck, M. M. (2003). Estrogen receptor- α populations change with age in commercial laying hens. *Poultry Sci.* **82**, 1624-1629.
- Hebert, R., Nanney, J., Spano, J. S., Pedersoli, W. M. and Krista, L. M. (1989). Erythrocyte distribution in ducks. *Am. J. Vet. Res.* **50**, 958-960.
- Heer, M., Baisch, F., Kropp, J., Gerzer, R. and Drummer, C. (2000). High dietary sodium chloride consumption may not induce body fluid retention in humans. *Am. J. Physiol. Renal Physiol.* **278**, F585-F595.
- Herremans, M., Berghman, L., Decuypere, E. and Vandesande, F. (1993). Immunocytochemical demonstration of progesterone and estrogen receptors in feathers and skin of adult hens. *Acta Biol. Hung.* **44**, 353-366.
- Hitchcock, D. I. (1924). Proteins and Donnan equilibrium. *Phys. Rev.* **4**, 505-531.
- Holliday, M. A. (1999). Extracellular fluid and its proteins: dehydration, shock, and recovery. *Pediatr. Nephrol.* **13**, 989-995.
- Johnson, A. L. and van Tienhoven, A. (1980). Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen, *Gallus domesticus*. *Biol. Reprod.* **23**, 386-393.
- Kiistala, U. and Mustakallio, K. K. (1964). In-vivo separation of epidermis by production of suction blisters. *Lancet* **27**, 1444-1445.
- King, A. S. and McLelland, J. (1975). Lymphatic system. In *Outlines of Avian Anatomy*, pp. 103-105. Baltimore, MD: Williams and Wilkins.
- Kováč, A. G., Szász, E. and Pilmayer, N. (1969). Mortality of various avian and mammalian species following blood loss. *Acta Physiol. Acad. Sci. Hung.* **35**, 109-116.
- Landys, M. M., Piersma, T., Visser, G. H., Jukema, J. and Wijker, A. (2000). Water balance during real and simulated long-distance migratory flight in the bar-tailed godwit. *Condor* **102**, 645-652.
- Levick, J. R. (1991). Capillary filtration – absorption balance reconsidered in light of dynamic extravascular factors. *Exp. Physiol.* **76**, 825-857.
- Levick, J. R. (2004). Revision of the Starling principle: new views of tissue fluid balance. *J. Physiol.* **557**, 704.
- Marder, J. (1983). Cutaneous water evaporation. II. Survival of birds under extreme thermal stress. *Comp. Biochem. Physiol.* **75A**, 433-439.
- Marder, J. and Arieli, Y. (1988). Heat balance of acclimated pigeons exposed to temperature up to 60°C Ta. *Comp. Biochem. Physiol.* **91A**, 165-170.
- Marder, J. and Ben-Asher, J. (1983). Cutaneous water evaporation. Its significance in heat-stressed birds. *Comp. Biochem. Physiol.* **75A**, 425-431.
- Marder, J. and Raber, P. (1989). Beta-adrenergic control of transtaneous evaporative cooling mechanism in birds. *J. Comp. Physiol. B* **159**, 97-103.
- Marder, J., Gavrieli-Levin, I. and Raber, P. (1986). Cutaneous evaporation in heat-stressed spotted sandgrouse. *Condor* **88**, 99-100.
- Marder, J., Withers, P. C. and Philpot, R. G. (2003). Patterns of cutaneous water evaporation in Australian pigeons. *Isr. J. Zool.* **40**, 540-552.
- McDonagh, P. F. and Gore R. W. (1982). A comparison of capillary hydraulic conductivities in postural and locomotor muscle. *Am. J. Physiol.* **243**, H491-H497.
- Menon, G. K., Brown, B. E. and Elias, P. M. (1986). Avian epidermal differentiation: role of lipids in permeability barrier formation. *Tissue Cell* **18**, 71-82.
- Menon, G. K., Baptista, L. F., Brown, B. E. and Elias, P. M. (1989). Avian epidermal differentiation. II. Adaptive response of permeability barrier to water deprivation and replenishment. *Tissue Cell* **21**, 83-92.
- Michel, C. C. (2004). Fluid exchange in the microcirculation. *J. Physiol.* **557**, 701-702.
- Mitchell, E. B. and Johns, J. (2008). Avian hematology and related disorders. *Vet. Clin. Exot. Anim.* **11**, 501-522.
- Muñoz-García, A. and Williams, J. B. (2008). Developmental plasticity of cutaneous water loss and lipid composition in stratum corneum in desert and mesic nestling house sparrows. *Proc. Natl Acad. Sci. USA* **105**, 15611-15616.
- Nakano, T. and Sim, J. S. (1989). Glycosaminoglycans from the rooster comb and wattle. *Poult. Sci.* **68**, 1303-1306.
- Nakano, T. and Sim, J. S. (1991). Chemical composition of glycosaminoglycan fractions from the comb and wattle of single comb white leghorn roosters. *Poult. Sci.* **70**, 2524-2528.
- Ogston, A. G. and Phelps, C. F. (1961). The partition of solutes between buffer solutions and solutions containing hyaluronic acid. *Biochem. J.* **78**, 827-833.
- Ophir, E., Arieli, Y., Raber, P. and Marder, J. (2000). The role of β -adrenergic receptors in the cutaneous water evaporation mechanism in the heat-acclimated pigeon (*Columba livia*). *Comp. Biochem. Physiol.* **125A**, 63-74.
- Ophir, E., Peltonen, L. and Arieli, Y. (2003). Cutaneous water evaporation in the heat-acclimated rock pigeon (*Columba livia*) – physiological and biochemical aspects. *Isr. J. Zool.* **49**, 131-148.
- Ott, H. (1956). Die Errechnung des kolloidosmotischen Serumdruckes aus dem Eiweiss-Spektrum und das mittlere Molekulargewicht der Serum-eiweiss-fraktionen. *Klin. Wochenschr.* **34**, 1079-1083.
- Parameswaran, S., Barber, B. J., Babbitt, R. A. and Dutta, S. (1995). Age-related changes in albumin-excluded volume fraction. *Microvasc. Res.* **50**, 373-380.
- Peltonen, L. M. and Mänttärä, S. (2008). Is there life in the horny layer? Dihydropyridine and ryanodine receptors in the skin of female and male chickens (*Gallus domesticus*). *J. Exp. Biol.* **211**, 1394-1401.

- Peltonen, L., Arieli, Y. and Marder, J. (1998). Adaptive changes in the epidermal structure of the heat-acclimated rock pigeon (*Columba livia*): a comparative electron microscopy study. *J. Morphol.* **235**, 17-29.
- Peltonen L., Arieli, Y., Harjula, R., Pyörnilä, A. and Marder, J. (2000). Local cutaneous water barrier in cold- and heat acclimated pigeons (*Columba livia*) in relation to cutaneous water evaporation. *J. Morphol.* **246**, 118-130.
- Peltonen, L. M., Sankari, S., Kivimäki, A. and Autio, P. (2006). Novel function of the skin in calcium metabolism in female and male chickens (*Gallus domesticus*). *Comp Biochem Physiol* **144B**, 432-441.
- Priestley, J. (1878). An account of the anatomy and physiology of Batrachian lymph-hearts. *J. Physiol.* **1**, 1-18.
- Rose, M. E. (1985). Lymphatic system. In *Form and Function of Birds* (ed. A. S. King and J. McLelland), pp. 341-379. London: Academic Press.
- Rügheimer, L., Hansell, P. and Wolgast, M. (2008). Determination of the charge of the plasma proteins and consequent Donnan equilibrium across the capillary barriers in the rat microvasculature. *Acta Physiol.* **194**, 335-339.
- Ruiz-Feria, C. A., Zhang, D. and Nishimura, H. (2004). Age- and sex-dependent changes in pulse pressure in fowl aorta. *Comp. Biochem. Physiol.* **137A**, 311-320.
- Sibley, C. G. and Johnsgard, P. A. (1959). Variability in the electrophoretic patterns of avian serum proteins. *Condor* **61**, 85-95.
- Starling, E. H. (1896). On the absorption of fluids from the connective tissue spaces. *J. Physiol.* **19**, 312-326.
- Stern, R. and Maibach, H. I. (2008). Hyaluronan in skin: aspects of aging and its pharmacologic modulation. *Clin. Dermatol.* **26**, 106-122.
- Sturkie, P. D. (1986). Heart and circulation: anatomy, hemodynamics, blood pressure, blood flow, and body fluids. In *Avian Physiology* (ed. P. D. Sturkie), pp. 76-101. New York, Heidelberg, Berlin: Springer-Verlag.
- Titze, J., Lang, R., Ilies, C., Schwind, K. H., Kirsch, K. A., Dietsch, P., Luft, F. C. and Hilgers, K. F. (2003). Osmotically inactive skin Na⁺ storage in rats. *Am. J. Physiol. Renal Physiol.* **285**, F1108-F1117.
- Titze, J., Shakibaef, M., Schaffhuber, M., Schulze-Tanzil, G., Porst, M., Schwind, K. H., Dietsch, P. and Hilgers, K. F. (2004). Glycosaminoglycan polymerization may enable osmotically inactive Na⁺ storage in the skin. *Am. J. Physiol. Heart Circ. Physiol.* **287**, H203-H208.
- Verdier-Sévrain, S., Bonté, S. and Gilchrist, B. (2006). Biology of estrogens in skin: implications for skin ageing. *Exp. Dermatol.* **15**, 83-94.
- von Rautenfeld, D. B. and Schacht, V. (2006). Fundamentals of comparative lymphology. In *Földi's Textbook of Lymphology* (ed. M. Földi and E. Földi), pp. 163-178. Munich: Elsevier.
- Waller, J. M. and Maibach, H. I. (2006). Age and skin structure and function, a quantitative approach (II): protein, glycosaminoglycan, water, and lipid content and structure. *Skin Res. Technol.* **12**, 145-154.
- Watson, R. R., Fu, Z. and West, J. B. (2007). Morphometry of the extremely thin pulmonary blood-gas barrier in the chicken lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **292**, L769-L777.
- Weidner, W. J. (2000). Response of air sac mesothelium to expansion of extracellular fluid volume in *Gallus domesticus*. *J. Comp Pathol.* **123**, 182-185.
- West, J. B., Fu, Z., Deerinck, T. J., Mackey, M. R., Obayashi, J. T. and Ellisman, M. H. (2010). Structure-function studies of blood and air capillaries in chicken lung using 3D electron microscopy. *Resp. Physiol. Neurobiol.* **170**, 202-209.
- Withers, P. C. and Williams, J. B. (1990). Metabolic and respiratory physiology of an arid-adapted Australian bird, the spinifex pigeon. *Condor* **92**, 961-969.
- Wolf, B. O. and Walsberg, G. E. (1996). Respiratory and cutaneous evaporatory water loss in high environmental temperatures in a small bird. *J. Exp. Biol.* **199**, 451-457.
- Wright, A. C., Bohning, D. E., Pecheny, A. P. and Spicer, K. M. (1998). Magnetic resonance chemical shift microimaging of aging human skin in initial findings. *Skin Res. Technol.* **4**, 55-62.
- Wyse, D. G. and Nickerson, M. (1971). Studies on hemorrhagic hypotension in domestic fowl. *Can. J. Physiol. Pharmacol.* **49**, 919-926.
- Zahouani, H. and Vargiolu, R. (2004). Skin line morphology: tree and branches. In *Measuring the Skin: Non-invasive Investigations, Physiology, Normal Constants* (ed. P. Agache and P. Hubert), pp. 40-59. Berlin, Heidelberg: Springer-Verlag.