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INFLUENCE OF MOLT ON PLASMA PROTEIN ELECTROPHORETIC PATTERNS IN BAR-HEADED GEESE (*ANSER INDICUS*)

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ABSTRACT: Plasma protein electrophoresis is recognized as a reliable diagnostic tool in avian medicine; however, the influence of circannual phenomena such as molt on protein electrophoregrams is poorly documented. The molt is a period of heavy hormonal and metabolic change in birds. The purpose of this study was to investigate the effects of molt on total protein concentration and electrophoresis patterns in birds. Blood samples were taken from 19 Bar-headed Geese (*Anser indicus*) from mid-May to mid-August, at 15-day intervals. At the same time, molting stage of each bird was recorded. Total protein concentrations were measured and plasma agarose gel electrophoresis was performed on these samples. The Bar-headed Goose was chosen as a model, because they molt over a very short period. The total protein concentration and albumin, alpha-2, beta, and gamma fractions were at their minimum values during molt, whereas the prealbumin and alpha-1 fractions rose to their maximum levels. This study provides baseline information relevant to changes occurring in avian proteinograms throughout the molt. The increase in prealbumin and alpha-1 fractions may be related to an increase in plasma thyroid hormones during molt. The decrease observed in albumin, alpha-2, beta, and gamma fractions may be related to protein and energy shifts toward feather growth, as well as to an expansion of the circulatory system located around the feather follicles with secondary dilutional effects on protein fractions. From a clinical point of view, the observed changes associated with molting were less significant than initially expected, and would not likely results in incorrect diagnoses based on interpretation of the protein electrophoretic patterns.

Key words: *Anser indicus*, Bar-headed Goose, bird, molt, electrophoresis, plasma protein.

INTRODUCTION

Serum protein electrophoresis has been used for several decades in human laboratory medicine. Over the last 15 yr, its use has been extrapolated to avian medicine and it is now commonly recognized as a very reliable diagnostic tool in this field (Cray and Tatum, 1998; Werner and Reavill, 1999). However, some circannual physiologic events, such as molting, may induce changes that could potentially cause practitioners to make incorrect diagnoses based on interpretation of the protein electrophoretic patterns. Feathers are composed of 95% protein and represent nearly 25% of a bird's dry mass (Murphy and King, 1992). The loss and regeneration of feathers during the molt occurs over a period of a few weeks to a few months, and may therefore be associated with heavy protein transport and synthesis. The molt is also a period of intensive energy demand (Klaassen, 1995). Oxygen consumption has been shown to be 9% to 46% greater in molting birds than in nonmolting birds (Walsberg, 1983). This energy expenditure arises from several components: the energy content of feathers, the cost of biosynthesis of feather material, body temperature regulation due to decreased insulation and changes in activity, and the energy intake required to supply the sulphur-containing amino acids necessary for feather synthesis (Klaassen, 1995). In addition to this metabolic aspect, results of studies have shown that a significant increase in total body protein synthesis, osteoporosis, loss of body fat, and suppression of the immune system occur during this event

(Mrosovsky and Sherry, 1980; Murphy and Todd, 1995; Kuenzel, 2003). Also, heavy hormonal changes occur during this time. Thyroid hormones increase concomitantly with a decrease in sexual steroids (Sauveur and De Reviers, 1988). In ducks and geese, the postnuptial molt coincides with high triiodothyronin (T3) and tetraiodothyronine (or thyroxine [T4]) plasma levels, and low levels of circulating luteinizing hormone in females and testosterone levels in males (Assenmasher and Jallageas, 1978; John et al., 1983). In Canada Geese (*Branta canadensis*), growth hormone was observed to be at its highest level during molt (John et al., 1983). Corticosterone levels were shown either to be stable (Rehder et al., 1986; Otsuka et al., 1998; Piersma and Ramenofsky, 1998), or to decrease, depending on which species was studied (Romero and Remage-Healey, 2000; Rich and Romero, 2001).

Within the last 40 yr, a great deal has been written about molting in birds, in particular about the types of molt of various avian species (Delacour, 1964; Oring, 1968; Saint Jalme et al., 1995; Todd, 1996), the physiologic mechanisms of molting (Assenmasher and Jallageas, 1978; John et al., 1983; Walsberg, 1983; Rehder et al., 1986; Klaassen, 1995; Piersma and Ramenofsky, 1998; Johnson, 2000; Romero and Remage-Healey, 2000; Rich and Romero, 2001; Otsuka et al., 2004), and the evaluation of methods used to induce molting in domestic birds (Brake, 1993; Berry, 2003; Park et al., 2004). However, our review of the literature identified only one reference dealing with plasma electrophoretic changes that occur during molt. Results from that study, conducted on Mallards (*Anas platyrhynchos*), showed changes in albumin and alpha-2 globulin fractions determined by use of cellulose acetate electrophoresis (Driver, 1981). This is the only study to have dealt with this topic, and electrophoretic techniques have improved considerably over the last 20 yr.

The purpose of the present study was to more thoroughly investigate changes in the protein electrophoretic patterns in birds during molt, through the example of the Bar-headed Goose (*Anser indicus*). Understanding these changes could help the practitioner with the interpretation of avian electrophoregrams, and improve understanding of avian molt physiology. To this end, geese were chosen as a model because these birds molt only once a year at the end of the reproductive season (Delacour, 1964). This prebasic (or postnuptial) molt leads to growth of both flight and body feathers. All wing feathers are shed and replaced simultaneously, to such an extent that, in general, geese completely loose their flight capacity for 5 to 6 wk (Delacour, 1964; Todd, 1996). In the Barnacle Goose (*Branta leucopsis*) and Canada Goose, wing feather elongation was showed to last 35 to 40 days, with growth speeds averaging 7.5 mm daily (Owen and Ogilvie, 1979; Todd, 1996). Therefore it could be expected that such an intense phenomenon would have significant influence on plasma protein electrophoretic patterns.

In this study we investigated the influence of molting on agarose gel plasma protein electrophoretic patterns using Bar-headed Geese.

MATERIAL AND METHODS

Experimental animals

Nineteen 1-yr-old Bar-headed Geese held at the Clères zoological park (France) were sampled; birds were hand-reared for 1 yr prior to the study in order to limit handling-induced stress. All birds were sexually immature which negated potential changes related to reproduction status. Geese were housed outdoors in a 2-ha meadow, and were dewormed with ivermectin (200 μg/kg) twice a year, in spring and autumn. In mid-June, one male goose fell ill and was excluded from the study.

Samples

Blood samples (2 ml) were collected from the brachial vein into tubes containing lithium heparin (Venosafe vacutainers, Terumo Eur-

ope, Leuven, Belgium) at intervals of 15 days, from mid-May to mid-August 2004. Because hemoglobin is known to interfere with protein concentration determination and plasma electrophoresis, care was taken to avoid hemolysis by using 21-gauge needles and 2-ml syringes (Terumo Europe, Leuven, Belgium). Heparinized blood samples were centrifuged at 3,000 × G for 5 min and the plasma samples were stored in cryotubes (Micronic systems, Lelystad, Holland) at a temperature of −20 C until they were analyzed. Samples were then thawed for 1 hr prior to analysis and rehomogenized by gentle mixing. Analyses were carried out on plasma, because in birds this medium is less prone to hemolysis than serum and it contains fibrinogen, which is an acute-phase protein (Hochleithner, 1994; Cray and Tatum, 1998).

Molt investigation

Members of the Anserinae subfamily are known to molt only once a year, at which time both body and flight feathers are replaced (Delacour, 1964). Samples were sorted into four chronologic stages, which were devised for the purposes of our study. These stages were based on the molt chronology described by Delacour (1964): A) basic plumage I, B) molt of the body feathers, C) primary and secondary remige development (blood feathers measuring one-third to two-thirds of the final feather length), and D) basic plumage II. Heinroth (cited by Romero et al. (2005) described three phases of feather growth: "firstly, a slow initial phase confined to the feather germ at the base of the follicle, secondly a long phase of daily elongation during the major part of which growth is more or less linear, thirdly a progressive slowing down of growth, a withdrawal of pulp from the calamus and the cornification of the feather base". Stages B and C, respectively, corresponded to body and wing feather elongation.

Molt was assessed every 15 days at the times when the birds were captured for blood sampling. Molt score was not investigated more precisely in order to limit handling of the birds and potential stress-induced artifacts on the protein electrophoretic pattern (Grieninger et al., 1978; Chamanza et al., 1999). All geese were synchronous in molting. During the sample collection in mid-June and at the beginning of July all geese were at the growth phase of body feathers (B) and flight feathers (C). Samples from mid-May and early June, and from mid-July to mid-August corresponded to times when birds had basic plumage (A or D).

Total protein concentration

Total protein concentration was determined by the Biuret reaction, using a Roche Integra 400 wet chemistry analyzer (Roche diagnostics GmbH, Mannheim, Germany). Readings were made at a wavelength of 552 nm.

Plasma protein agarose gel electrophoresis

Agarose gel electrophoresis of plasma proteins was carried out using a Hydrasys© semiautomated system (Sebia, Evry, France), with Hydragel protein 15/30© set (Sebia, Evry, France). It was operated according to the manufacturer's instructions, using version 7.00 F0.1 of the system software. Samples of plasma (10 μl) were manually distributed onto the sample template, and were allowed to diffuse for a period of 5 min in a wet chamber. Application of the samples to the gel, electrophoresis, and drying of the gel were all performed automatically in the migration compartment of the instrument. The temperature was maintained at 20 C using a Peltier device during the complete migration process, and gels were dried by heating to 65 C. Electrophoretic separation was obtained on 8 g/l agarose gels in a Tris-barbital buffer (pH 9.2), at a constant power level of 20 W, until 33 Vh had been accumulated. Once dried, the gels were manually transferred to the staining compartment of the instrument where amido-black staining, destaining, and drying were performed automatically. Once these operations had been completed, the gels were scanned with a high-resolution Epson perfect V700 photo scanner (Epson France, Nanterre, France). Electrophoretic curves and dosages of the different fractions were acquired using Phoresis© software, version 5.50 (Sebia, Evry, France). Albumin was identified as having the strongest anodal peak. The beta peak was defined as fibrinogen (Roman et al., 2007). Globulins were divided into five fractions. The alpha fractions were located between the albumin and beta peaks, and the gamma fraction was located beyond the beta peak. As previously described in other studies of avian protein electrophoresis, the albumin/globulin (A/G) ratio was calculated by dividing the sum of the prealbumin and albumin fractions by the sum of the globulin fractions (Lumeij, 1987; Cray and Tatum, 1998).

Statistical analysis

Systat 7.0 software (SPSS, Inc., London, England) was used for all analyses. Because of the small size of the studied population, we

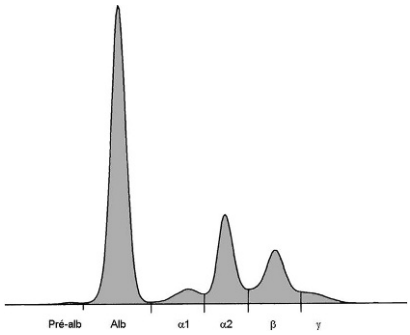

FIGURE 1. Example of plasma protein electrophoretic pattern of a Bar-headed Goose (*Anser indicus*) including albumin, alpha-1, alpha-2, beta, and gamma fractions.

used Friedman's two-way variance analysis and Wilcoxon's signed rank test.

RESULTS

Bar-headed Geese protein electrophoretic patterns were divided into six fractions (Fig. 1): one prealbumin fraction, one albumin fraction, two alpha fractions, one beta fraction, and one gamma fraction. In both sexes, total protein concentration appeared to vary significantly as a function of time (Friedman's test: $P=0$). In both males and females, total protein concentration significantly decreased from mid-June to early July (respectively: $Z=-2.366$, $P<0.02$; $Z=-2.667$, $P<0.01$; Fig. 2). Thereafter, total protein concentration remained at a low value until the end of the study.

Several fractions, such as the prealbumin and alpha-1 fractions, were observed to increase during molt in both males and females. The prealbumin fraction increased from mid-June to early July (Fig. 3a). This increase was significant in females (Table 1) and marginally significant in males ($Z=1.859$, $p=0.063$). Thereafter, it significantly decreased in both sexes, from early July to mid-July (Tables 1 and 2). The alpha-1 fraction tended to be higher around the molt period (Fig. 3c). However, it reached its maximum value earlier in females than in males. The alpha-1 fraction increased significantly from early June to mid-June in females (Table 1), reaching its maximum value during molt of body feathers. Thereafter, it decreased significantly from early July to mid-August. In males, the alpha-1 fraction tended to increase from mid-May to early July, reaching a maximum during molt of flight feathers. This increase was only marginally significant ($Z=1.859$, $P=0.063$). The alpha-1 fraction then remained stable from early June to early August, and significantly decreased until the end of the study (Table 2).

Other protein fractions, such as the albumin, alpha-2, beta, and gamma fractions, decreased at the time of molting. The albumin concentration decreased from mid-June to early July in both males and females (Tables 1 and 2, Fig. 3b). The albumin fraction then increased significantly for males from early July to mid-July. The alpha-2 fraction decreased significantly in males and females from early July to mid-July (Tables 1 and 2, Fig. 3d) and reached its minimum value during molt, and then increased significantly from early July to mid-July. The beta fraction significantly decreased in both males and females, from mid-June to early July (Tables 1 and 2, Fig. 3e) and then remained stable until the end of the study. The gamma fraction decreased significantly in both males and females from early June to early July (Tables 1 and 2, Fig. 3f), and again increased significantly from early July to early August.

In both sexes, the A/G ratio did not appear to fluctuate in relation to the molt period.

Except for the alpha-1 and gamma fractions, no significant difference was found between males and females for any of the measured parameters. In general, the alpha-1 fraction was significantly higher in females than in males ($P<0.02$), except from early June to early August when no significant difference was found. The gamma fraction was significantly higher in females than in males ($P<0.05$).

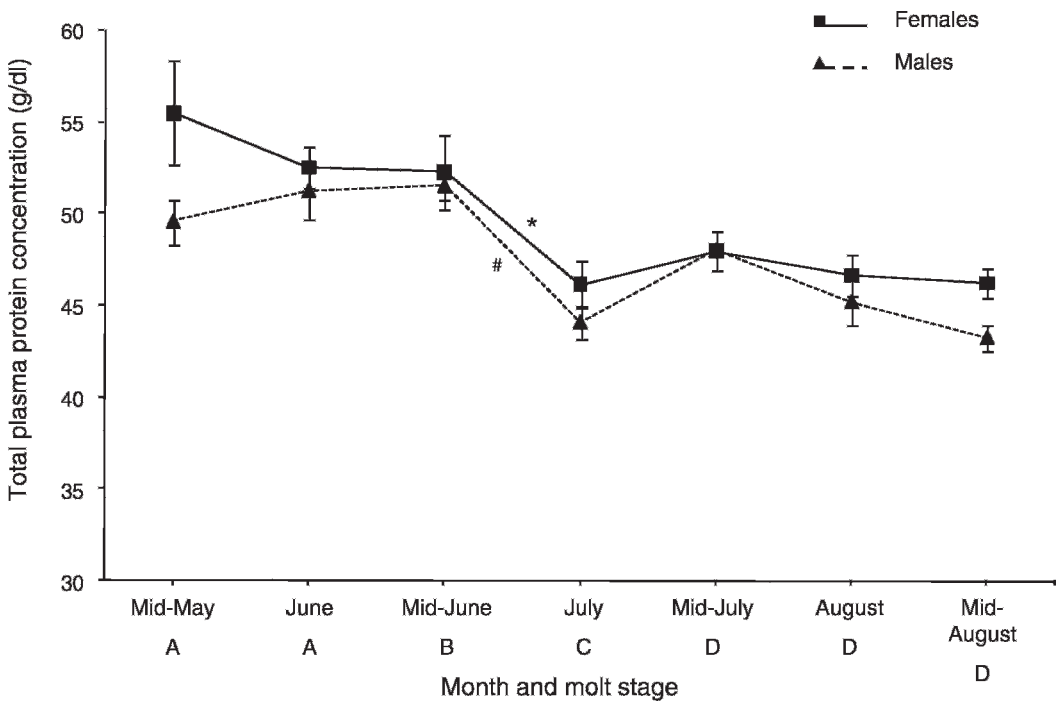

FIGURE 2. Variation of total plasma protein concentrations during molt in Bar-headed Geese (*Anser indicus*) in bimonthly blood samples taken between mid-May and mid-August. Molt stages are indicated as A) basic plumage I, B) molt of the body feathers, C) primary and secondary flight feather development, and D) basic plumage II. The data points are expressed as mean±SEM. Significant differences between values from two successive samples (15-day interval) are represented by "*" in females and "#" in males. When significant differences apply to time intervals greater than 15 days, the latter are enclosed in brackets.

DISCUSSION

Results of the present study provide baseline information on changes that occur in avian proteinograms throughout the molt. It is clear that molting coincides with a decrease in total protein concentration and albumin, alpha-2, beta, and gamma fractions, followed by an increase in total protein and albumin, alpha-2, and gamma fractions once molting is finished. Also, prealbumin was increased during the molt. The alpha-1 fraction was higher around the molt than after or before. This study is the first to have investigated changes in plasma proteins of both male and female birds during molting.

Our results are similar to those of previous publications, which showed a decrease in albumin and total protein concentrations during the molt of chickens (Gildersleeve et al., 1983), passerine birds (De Graw and Kern, 1985), and tropical seabirds (Work, 1996). For Mallard, Driver (1981) demonstrated that the total protein concentration decreased significantly, reaching a minimum during molt, and then increased. The albumin concentration was 10% to 20% lower during molt than after molt and the alpha-2 fraction was lower during molt than beforehand. No changes were found in the other protein fractions. This may have been the result of smaller sample size and use of less accurate electrophoresis technique (cellulose acetate electrophoresis), than the work presented here. Our results provide data concerning changes observed in prealbumin, alpha-1, beta, and gamma fractions.

Results of the study reported here showed total protein concentration de-

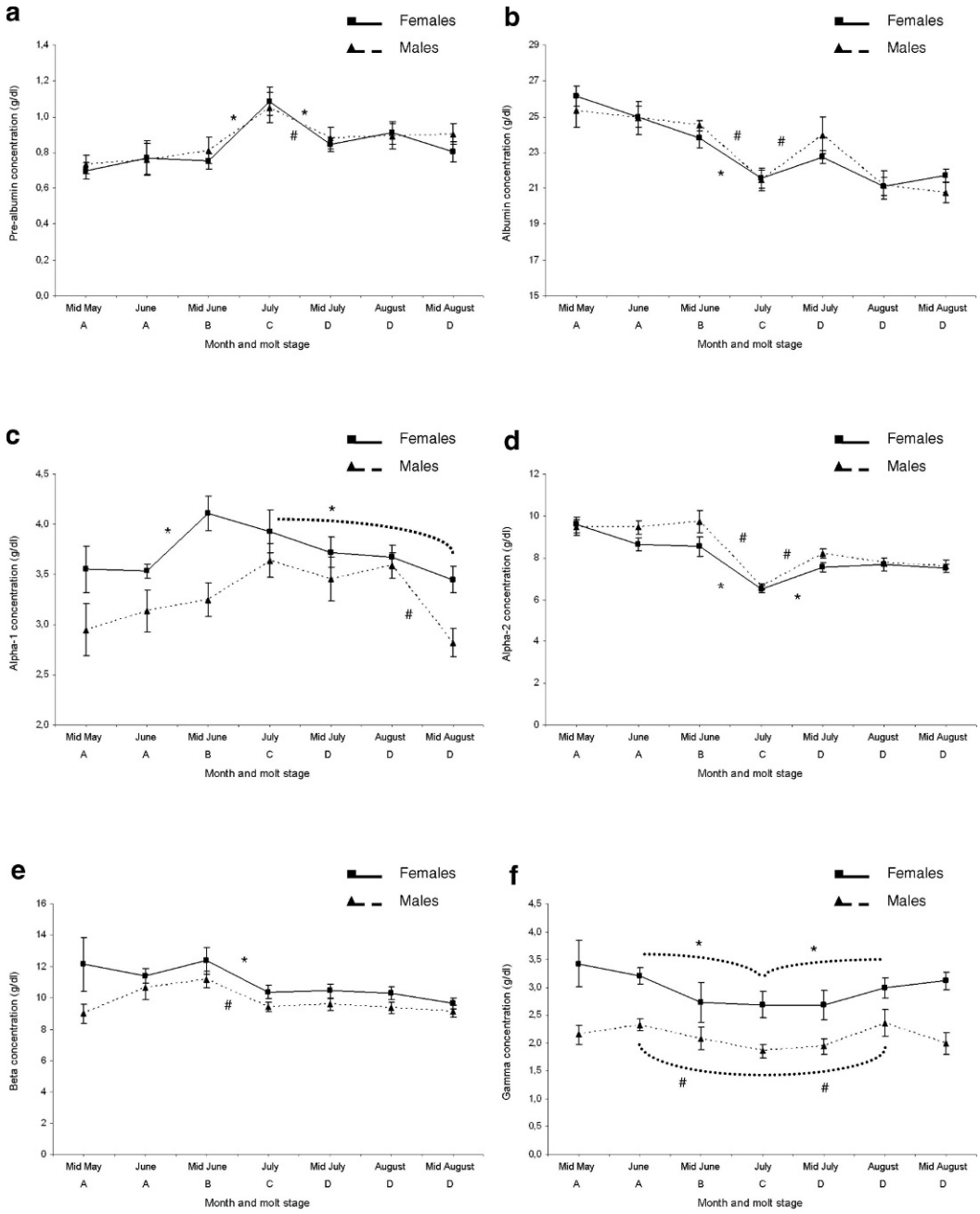

FIGURE 3. Variation of concentrations in (a) prealbumin, (b) albumin, (c) alpha-1, (d) alpha-2, (d) beta, and (e) gamma fractions during the molt in Bar-headed Geese (*Anser indicus*) in bi-monthly blood samples taken between mid-May and mid-August. Molt stages are indicated as A) basic plumage I, B) molt of the body feathers, C) primary and secondary flight feather development, and D) basic plumage II. The data points are expressed as mean±SEM. Significant differences between values from two successive samples (15-day interval) are represented by "*" in females and "#" in males. When significant differences apply to time intervals greater than 15 days, the latter are enclosed in brackets.

TABLE 1. Wilcoxon's Z and *P* values for significant differences between two measurements in female Bar-headed Geese (*Anser indicus*).

	Total protein	Pre-albumin	Albumin	Alpha-1	Alpha-2	Beta	Gamma
Mid-May							
Early-June				Z = 2.824 P < 0.05			
Mid-June	Z = -2.667 P < 0.01	Z = 2.981 P < 0.05	Z = -3.059 P < 0.01		Z = -2.903 P < 0.01	Z = -2.884 P < 0.05	Z = -2.118 P < 0.05
Early-July		Z = -2.824 P < 0.01			Z = 3.059 P < 0.01		
Mid-July				Z = -1.961 P < 0.05			Z = 2.040 P < 0.05
Early-August							
Mid-August							

TABLE 2. Wilcoxon's Z and *P* values for significant differences between two measurements in male Bar-headed Geese (*Anser indicus*).

	Total protein	Pre-albumin	Albumin	Alpha-1	Alpha-2	Beta	Gamma
Mid-May							
Early-June							
Mid-June	Z = -2.366 P < 0.02		Z = -2.366 P < 0.02		Z = -2.366 P < 0.02	Z = -2.366 P < 0.02	Z = -2.366 P < 0.02
Early-July		Z = -2.201 P < 0.05	Z = -2.201 P < 0.05		Z = 2.201 P < 0.05		
Mid-July							Z = 1.992 P < 0.05
Early-August							
Mid-August				Z = -2.201 P < 0.05			

creased mainly as the result of reduced albumin, alpha 2, and gamma fractions during the molt. Albumin is the most abundant plasma protein and has important roles as a carrier protein, in regulation of colloidal osmotic pressure of blood, and as a mobile source of amino acids in nutritional emergencies (Butler, 1971; Kaneko, 1989). The observed decline in albumin concentration during molting suggests that this amino acid reserve was used for feather production. In molting passerine birds, there was an acceleration of whole body protein turnover, which could enable an increase in the animal's metabolic plasticity and allow such adaptations (Murphy and Todd, 1995). The fact that in our study, the albumin values increased after the molt in male birds before decreasing again, could be explained by a "rebound" effect related to competition between the homeostatic phenomenon aimed at restoring albumin to its original concentration and the consumption of albumin as an amino acid reserve used for feather production.

Our results highlight the fact that the gamma fraction, which contains immunoglobulins (Kaneko, 1989; Cray and Tatum, 1998; Werner and Reavill, 1999), decreased during the molt, which is a period of intense energy expenditure (Klaassen, 1995). Energetically expensive conditions that are not directly related to molting, such as immune responses (Ots et al., 2001; Martin et al., 2003), may be temporarily reduced (Raberg et al., 1998). Depletion of the immune system during molt was first demonstrated in the Smyth-line chicken, which expresses the gene of an autoimmune disease similar to vitiligo, with resulting loss of melanocytes from feathers. During molt, some of the new emerging feathers are pigmented, which suggests that the autoimmune disease is not completely expressed during this period because the immune system is temporarily depleted (Boyle and Smyth, 1984). This is in agreement with results from a recent publication which showed that T3 has an immunosuppressive effect on humoral immunity in Common Eiders (*Somateria mollissima*; Bourgeon and Raclot, 2007). A dramatic increase in the concentration of this hormone, which likely occurs throughout the molt (Assenmacher and Jallageas, 1978; John et al., 1983), is likely responsible for a transfer of energy toward feather replacement and may explain the decrease in the gamma fraction observed in the present study. Once the molt was over, this fraction increased.

A decrease in some protein fractions may be related to hemodilution effects from expansion of the circulatory system located around feather follicles. Results of some studies have showed that, in passerine birds, molting causes an increase in plasma volume (Chilgren and De Graw, 1977; De Graw and Kern, 1985). The changes observed in the alpha-2 or beta fractions in our birds may be related to one or more of the phenomena described above.

Our findings show that prealbumin and alpha-1 fractions tend to increase during molt. Increased plasma levels of thyroid hormones, which promote feather replacement and regulate impaired homeothermy of defeathered birds (Johnson, 2000), may have contributed to increases in these fractions. It has been demonstrated that in chickens, both T3 and T4 selectively stimulate the synthesis of four major plasma proteins in hepatocyte cultures, including lipoproteins, prealbumin B, fibrinogen, and alpha-1-globulin M (hemopexin) which are acute-phase proteins (Hertzberg et al., 1981; Grieninger et al., 1986). A short period of exposure, of approximately 30 min, is sufficient to trigger a nearly full thyroid hormone effect on plasma protein synthesis (Hertzberg et al., 1981). Results of another study demonstrated the existence of an association between increase in concentration of plasma transthyretin and molting in birds (Cookson et al., 1988). This carrier protein, also called thyroxine-binding preal-

bumin, may be responsible for increase in prealbumin fraction during remige molt observed in our study. Our finding of decreased beta fraction (which contains fibrinogen) during the molt remains unexplained.

Except for the alpha-1 fraction, the main changes observed in plasma protein fractions closely coincided with molt of flight feathers and may correspond to maximum changes in the protein metabolism in geese. The alpha-1 fraction reached its maximum value 15 days earlier in females than in males. This could be related to slightly earlier molt in females than in males, which our relatively imprecise molt investigation method was not able to detect. Thorough investigation of feather molt was not feasible in this study, because the required frequent handlings of the birds would have resulted in stress-induced changes in the proteinograms (Grieninger et al., 1978; Chamanza et al., 1999).

The main protein differences between males and females occurred in alpha-1, beta, and gamma fractions. These fractions were shown to be greater in females than in males. Such differences between males and females have seldom been investigated in birds outside the breeding season. In Black Storks (*Ciconia nigra*), higher gamma values were observed in females than in males, a finding that remains unexplained (Lanzarot et al., 2005). In the results of our study, differences might have been caused by the presence of underlying inflammatory conditions in some female birds. Further studies are needed to investigate differences between male and female electrophoregrams outside the breeding season.

From a clinical point of view, changes related to molting were less significant than initially expected, and may not be sufficient to result in an incorrect diagnosis based on interpretation of the protein electrophoretic patterns.

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