

PLASMA PROTEIN CONCENTRATIONS OF THE YOUNG AND ADULT *AMAZONA BRASILIENSIS* PARROTS

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Abstract

Introduction. The Red-tailed Amazon parrot (*Amazona brasiliensis*) is an endangered species of the Psittacine family, and for which various data are important for a comprehensive preservation plan. Data about plasma protein gel electrophoresis of Amazon parrot blood are scarce. The purpose of this study was to determine plasma protein concentrations and concentrations of major protein bands in blood of young and adult Red-tailed Amazon parrot (*Amazona brasiliensis*).

Materials and Methods. Blood samples from eight young and eight adult healthy free-living parrots were obtained. Plasma protein concentration and fractions were determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Mann–Whitney U test was used to compare variables.

Results and Conclusions. Six major protein bands with the following molecular weights were identified by SDS-PAGE: 170 kDa, 117 kDa, 85 kDa (putative ovotransferrin), 60 kDa, 45 kDa and 23 kDa. Adult parrots had significantly higher concentrations of total proteins, albumin and other proteins with similar mobility (around 60 kDa). Young birds had significantly higher levels of 23kDa proteins. The concentration of putative ovotransferrin (85 kDa) was not different between young and adult parrots. Plasma protein gel electrophoresis patterns in Red-tailed Amazon parrots are similar between young and adult animals, but specific protein bands differ in their absolute concentrations. This finding should be taken into consideration when clinical pathology data are analysed.

Key Words: *Amazona brasiliensis*, plasma, SDS-PAGE, proteins, Red-tailed Amazon parrots

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INTRODUCTION

The Red-tailed Amazon parrot (*Amazona brasiliensis*) (Linnaeus, 1758) is listed as a vulnerable species and is endemic to a narrow coastal region in southern Brazil. Habitat loss and trapping for illegal trade are major threats to this bird species (IUCN, 2016).

Adult Red-tailed Amazon parrots weight around 420g with approximately 36 cm of body length. The plumage is mostly green with a broad red band across the tail. Juveniles have a duller plumage. This parrot reaches sexual maturity after four years of age. In nature, the diet of this parrot comprises fruits, flowers, and leaves (Sick, 2001).

Serum and plasma proteins in avian production have been investigated extensively by gel electrophoresis (Kuryl and Gasparska, 1985; Brandt et al., 1951). Despite this, there are only few studies regarding gel electrophoresis and health status of wild free-living birds, mainly because of difficulties in collecting blood samples (Tothova et al., 2016; Lumeij, 2008). Electrophoresis is a useful technique to evaluate species-specific protein patterns and dysproteinaemias. However, there are considerable differences in protein fractions in different avian species, especially in psittacine birds (Cray et al., 2007). This is why the interpretation of electrophoresis data should be recognized as a continuous challenge to clinicians and researchers (Tothova et al., 2016; Cray et al., 2007; Archer and Battison, 1997; Brandt et al., 1951).

Age of the birds could influence plasma or serum protein electrophoretic pattern. However, little has been reported regarding the influence of age on protein electrophoresis of Psittaciformes. Clubb et al. (1991), reported that young macaws under eight months of age have low albumin and γ -globulin concentrations, while Vaz et al. (2015) reported that total plasma protein concentration was increased in free-living red-tailed Amazon parrot weighing over 400g. Detailed studies on clinical haematology and biochemistry parameters of Red-tailed Amazon parrot have been published recently (Vaz et al., 2015; Vaz et al., 2016), but no data on protein fractions obtained after plasma electrophoresis are available for this species. Indeed, only 3-4 μ L of plasma can provide important clinical information after proteins are separated using electrophoresis. Separation of proteins with electrophoresis could be useful for health monitoring and taxonomic investigations, relevant to conservation and management of this bird species.

The objective of this study was to determine plasma protein concentrations of free-living Red-tailed Amazon parrots and to investigate whether age influences total plasma protein and concentration of protein bands separated on SDS-PAGE.

MATERIALS AND METHODS

The birds investigated in this study belonged to an area of continuous Atlantic forest at the Environmental Protection Area of Guaraqueçaba, southern Brazil (25°15' - 25°30'S; 48°20' - 48°30'W). All approaches were approved by the Ethics Committee of the School of Veterinary Medicine and Animal Sciences (CEUA 145/2010), São Paulo

State University, (FMVZ-UNESP), Brazil, and were carried out in accordance with current legislation on animal protection.

All of the 16 parrots analysed in this study were evaluated for the purpose of identification under the biodiversity conservation program (SPVS/Brazil). Parrots, without signs of illness, were removed from the nest and then manually restrained for physical examination and blood collection. The samples were obtained from the jugular vein of eight young and eight adult Red-tailed Amazon parrots. Parrots were considered young if less than 8 months-old and less than 420g. Aliquots of each blood sample were collected in 1mL syringes treated with 1,000 UI lithium heparin and transferred immediately to 2 mL glass tubes for centrifugation ($1,500 \times g$ for 5 min). The harvested plasmas were stored in Eppendorf microtubes (Eppendorf, Hamburg, Germany), at -20°C , with protein analyses performed within eight months.

Total plasma protein was determined with a commercial kit (Intertek Katal, São Paulo, Brazil) in an automatized spectrophotometer (Cobas Mira Plus; Roche Diagnostic Systems, Indianapolis, IN, USA) by the biuret method. Plasma protein fractions were determined by unidimensional electrophoresis by means of sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) as described by Laemmli (1970), in 10% acrylamide gels. The gels were stained with 0.2% Coomassie Brilliant Blue. Plasma samples were prepared with 5% mercaptoethanol and 0.001% bromophenol blue in a reducing condition (5 min at 95°C), and 5% glycerol was added afterwards.

The estimated concentrations of plasma protein band or peaks were determined via computer-assisted densitometry (CS 9301® Shimadzu Scientific Instrument, Kyoto, Japan). Protein peaks were identified using reference markers (SDS6H2, Sigma-Aldrich®, St. Louis, USA) with molecular weights (MW) of 20, 24, 29, 45, 55, 66, 97, 116, and 200 kDa. Protein concentration estimation in each single electrophoretic band (mg/dL) was obtained when surface under specific peak (%) on an electrophoretogram was multiplied with total protein concentration (g/L) and divided by 100.

No haemolysis or lipaemia were detected in any of the samples analysed. All variables were first assessed for normality using the Shapiro-Wilk test. Mann-Whitney U test was used as the data were not normally distributed. Data are reported as median and range. Statistical significance was set at $P < 0.05$ for all analyses. All statistics were performed using statistical software (GraphPad Version 6 for Windows, GraphPad Software Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

This is the first report of plasma protein gel electrophoresis with a comparative approach on total plasma protein and major protein band concentration between free-living young and adult Red-tailed Amazon parrots.

In our study, total median plasma protein levels were 3.1 g/dL [2.2-3.7] in young and 4.9 g/dL [4.2-6.6] in adult parrots. It was already shown that total protein concentration in birds increases with age (Brindt et al., 1951), but also with oestrogen

levels in females (Lumeij, 2008). In our study, the range of total protein concentration for young parrots was comparable to that reported in a previous investigation of Red-tailed Amazon nestlings (Vaz et al., 2016). Adult parrots in the current study had higher total proteins than nestling parrots weighing over 400g (Vaz et al., 2015), but values were comparable to those reported for *Pionopsitta pileata* and *Amazona vinacea* (Schmidt et al., 2009). These differences could be due to both mentioned variables: age and oestrogen level. A limitation of this study is that there it was not possible to determine the sex of the birds investigated.

The SDS-PAGE technique allowed the fractionation of six major protein bands in both age groups (Figure 1). Their molecular weights were: 170 kDa, 116 kDa, 85 kDa (putative ovotransferrin), 60 kDa (albumin and different proteins with similar mobility in SDS PAGE), 45 kDa and 23 kDa (Figure 1). Concentrations of major protein bands with their calculated molecular weights in six young Red-tailed Amazon parrots are presented in Table 1. Our results demonstrated that adult parrots had higher concentrations of 60 kDa proteins and lower concentrations of 23 kDa protein band. Proteins with 170 kDa, 116 kDa and 45 kDa were not found to correspond to specific bird proteins, so they are not discussed further in this text.

The most abundant protein band in our study was broad, with MW range from 55 to 66 kDa and a peak at 60 kDa (Figure 1 – band 4). In a previous report dealing with chickens and cockatiels, it has been shown that MW of serum albumin is 66 kDa

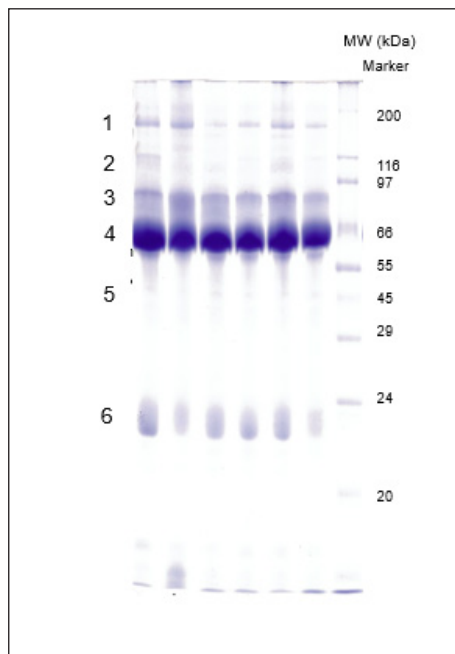


Figure 1. SDS-PAGE of Red-tailed Amazon parrot plasma on a pore gradient gel (10%) under reducing conditions. All six lanes refer to young parrots. Molecular weight markers (SDS6H2, Sigma-Aldrich®, St. Louis, USA) are shown on the right side of the gel. Major protein bands identified were: 1 (170 kDa), 2 (116 kDa), 3 (85 kDa) 4 (60 kDa), 5 (45 kDa), 6 (23 kDa).

(Archer and Battison, 2007). Additionally, the heavy IgG chain in young and adult pigeons had a molecular weight of 58 kDa (Engberg et al., 1992), so we consider that in our study, a protein band containing plasma albumin overlapped a band containing heavy IgG chain. In our study, the concentration of the putative albumin and IgG heavy chain band was two times higher in adult than in young parrots. Previously, it was estimated that concentration of IgG in pigeon is around 6.5mg/mL (Engberg et al., 1992), and also, is very similar in young macaw parrots (Clubb et al., 1991). As IgG probably was minor protein fraction in band number 4, it could be inferred that the major protein fraction in this band are albumins. The concentration of proteins in band 4 was higher in adult than in young parrots and the band could be ascribed to both albumins and heavy IgG chains. It should be noted that for better separation of albumins and other proteins in birds, non-reducing SDS PAGE or gradient gels (4-20%) should be used.

Table 1. Comparison of plasma total protein and protein bands of young and adult Red-tailed Amazon parrot (*Amazona brasiliensis*).

Band number	Molecular weight (kDa)	Putative protein	Median [range] mg/dL (young) (n=8)	Median [range] mg/dL (adult) (n=8)	P Value
1	170	NI	79 [26-222]	123 [63-186]	0.08
2	116	NI	35.4 [16.7-86]	45 [29-96]	0.39
3	85	Ovotransferrin	269.4 [186-694]	432.5 [330-648]	0.06
4	60	Albumin*	2.25 [1.5-2.7]	4.1 [3.1-5.3]	0.0002
5	45	NI	23.8 [6.5-108]	37.5 [18.8-104]	0.56
6	23	NI	288.9 [176.7-433]	171 [0-266]	0.02

*Albumin and different proteins with similar mobility in SDS PAGE; NI: Not identified.

The second most abundant protein band had MW of 85 kDa (Figure 1 – band 3). It had the same electrophoretic pattern as chicken ovotransferrin (Tohjo et al., 1995). Thus, we consider that herein we identified ovotransferrin by SDS-PAGE electrophoresis in Red-tailed amazon parrots. The concentration of this protein fraction was lower in young parrots, but not significantly. Identification of ovotransferrin is important because this protein serves as a biomarker of inflammation in birds (Tohjo et al., 1995).

In this study, a broad band having a peak at 23kDa (Figure 1 – band 6) was found in both adult and young Red-tailed Amazon parrots, with significantly higher values for young birds. This band could represent light IgG chains that are not separated from

other low molecular weight proteins. One of the low molecular weight proteins could be a monomer of the alpha chain of Hp in ruminants, which has been found in horses, dogs, and cattle. However, more detailed analysis should be performed using other types of electrophoresis, as stated before.

These findings in Red-tailed Amazon parrots provide an electrophoretic pattern to support clinical approaches and conservation planning for this bird species. Ideally, individual measurements of each protein for healthy or diseased birds with validated assays would be useful in order to properly characterize euproteinaemia and dysproteinaemia in Red-tailed Amazon parrots.

CONCLUSION

In this study, the plasma protein gel electrophoresis for Red-tailed Amazon parrots has demonstrated differences between adult and young birds. Adult birds had higher concentrations of total proteins, albumin and different proteins with similar mobility in SDS PAGE and lower concentrations of 23 kDa protein. An 85kDa protein was probably ovotransferrin, and was not significantly different between adult and young birds. Overall, despite variations in protein band concentrations, there is a similar plasma protein electrophoresis pattern for both adult and young Red-tailed Amazon parrots that could be a useful tool to monitor health status of Red-tailed Amazon parrots.

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Authors' contribution

EMSS and ACP participated in the design of the study, method development, laboratory analyses, statistics, and preparation of the manuscript. PPS and EAS participated in sample collection. All authors read and approved the final manuscript.

Declaration of conflicting interests

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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KONCENTRACIJE PLAZMA PROTEINA KOD MLADIH I ODRASLIH *AMAZONA BRASILIENSIS* PAPAGAJA

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Kratak sadržaj

Uvod: Crvenorepi Amazona papagaj (*Amazona brasiliensis*) je ugrožena vrsta iz porodice *Psittacine* i za obuhvatni plan zaštite potrebni su brojni podaci. Podaci o gel elektroforezi proteina plazme kod papagaja roda *Amazona* su retki. Cilj ove studije je bio da utvrdi koncentracije plazma proteina kao i glavnih proteinskih traka kod mladih i odraslih Crvenorepih Amazona papagaja (*A. brasiliensis*).

Materijal i metode: Dobijeni su uzorci krvi od osam mladih i osam odraslih zdravih papagaja koji žive slobodno. Koncentracije proteina plazme i njegove frakcije utvrđene su korišćenjem natrijum dodecil sulfat poliakrilamid gel elektroforeze (SDS-PAGE). Za upoređivanje varijabli korišćen je Mann–Whitney U test.

Rezultati i zaključak: Šest proteinskih traka sa pratećom molekularnom težinom identifikovane su kao glavne pomoću SDS-PAGE: 170 kDa, 117 kDa, 85 kDa (putativni ovotransferin), 60 kDa, 45 kDa and 23 kDa. Odrasli papagaji su imali značajno veće koncentracije ukupnih proteina, albumina i drugih proteina slične mobilnosti (oko 60 kDa). Mlade ptice su imale značajno više nivoe 23kDa proteina. Koncentracija putativnog ovotransferina (85 kDa) nije se razlikovala između mladih i odraslih papagaja. Plazma proteinski gel elektroforeza obrazac kod Crvenorepog Amazona papagaja je sličan kod mladih i odraslih životinja, ali se specifične proteinske trake razlikuju u pogledu svoje apsolutne koncentracije. Ovi nalazi bi trebalo da se uzmu u obzir prilikom analize podataka kliničke patologije.

Ključne reči: *Amazona brasiliensis*, plazma, SDS-PAGE, proteini, Crvenorepi Amazona papagaj