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Research paper

Urofecal steroid profiles of captive Blue-fronted parrots (*Amazona aestiva*) with different reproductive outcomes

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ABSTRACT

Despite Psitaciformes (parrots) being the third largest nonpasserine order (398 species), it currently ranks second in number of threatened species (28%) according to the Internatinal Union for Conservation of Nature (IUCN) criteria. Since most of the literature concerning reproductive endocrinology in avian species derives from domestic and song birds, it is puzzling that advances in reproductive science for the Psitaciformes order lags far behind, in spite of the growing threats against them. In order to expand our knowledge of Neotropical parrots (Psittacidae), we examined annual changes in urofecal sex steroid metabolites of Blue-fronted amazon pairs (*Amazona aestiva*) exhibiting successful (nestlings) and unsuccessful breeding (infertile or no eggs). Urofecal samples were collected over a year from eight breeding pairs housed under the same environmental and management conditions. Fecal androgen and progestagen concentrations were determined in males and females, respectively, by enzyme immunoassays previously validated for this species. All eggs were registered between late winter and mid-spring, and egg-laying intervals varied between females (range: 1–8 days; average 3.60 ± 0.51 days). Similar profiles of urofecal progestagens were observed in reproductively successful females and females producing infertile eggs, with progestagen peaks preceding egg laying events (1.77 ± 0.50 days). In contrast, non-laying females had no rises in progestagens during the year. Successful and unsuccessful males did not displayed distinct annual patterns of androgen production, and apart from the peaks during the breeding season, more than half of the individuals intriguingly presented significant increases from late summer to early autumn, a period without reproductive activity. Finally, we noticed that samples with progestagen levels exceeding 40 ng/g had very high probability (>97.5%) to be from females in pre-laying or laying phases, suggesting a feasible application of this characteristic to noninvasively discriminate the reproductive status in amazon females with an accuracy and sensitivity of 94.55% and 58.13%, respectively. Our findings confirmed that urofecal progestagens and androgens are good indicators of the gonadal condition in Blue-fronted amazons, but there is still much to be done for their extensive use in artificial insemination or selection of the most suitable breeding birds for the season.

1. Introduction

Psittaciformes is one of the most popular orders among pet owners not just for its colorful appearance and charismatic behavior, but also because of its high levels of intelligence and aptitude to imitate a wide range of sounds including the human voice (Olah et al., 2016). Unfortunately, such attributes led to a huge demand for these birds in the pet market. Today psittaciformes represents 20% of bird species traded globally, second only to Passeriformes (song birds) which represents 70% of wild birds commercialized worldwide (FAO, 2011). One example of this popularity is depicted by Weston and Memon (2009), who estimated that 75% of the 11.2 million pet birds in US households were psittacines. As a consequence, international trade contributed to the decline of several parrot populations to the point of having 111 of the 398 known species listed as threatened. Remarkably, recent assessments indicate that 56% of all psittacine species are still

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experiencing declining numbers worldwide (IUCN, 2014; Olah et al., 2016). This adverse scenario is not different in Brazil, where in spite of exhibiting the greatest diversity of the Psittacidae family (86 species), currently 23 are threatened, one species is presumed extinct, and one species is extinct in the wild (Piacentini et al., 2015; BirdLife International, 2017).

These numbers emphasize the importance of captive breeding to promote either sustainability of global trade of parrots or their conservation. However, large-scale production of psittacines to satisfy pet market demand is restricted to a few species (mostly budgerigars, cockatiels, lovebirds and small conures; Engebretson, 2006), and even for them very little information exists on reproductive endocrinology (Myers et al., 1989; Lee et al., 1999; Costantini et al., 2009; Lovas et al., 2010; Hahn et al., 2011). This lack of endocrinological data for Neotropical parrots hinders future research involving manipulation of reproductive cycles (through artificial lighting or hormone treatments) or adjustments to existing biotechnologies (e.g., artificial insemination). To aid in filling this gap, we sought to use the Blue-fronted amazon (*Amazona aestiva*) as a model for the genus *Amazona* (comprising 34 species). This species is assigned to the IUCN Least Concern category (Birdlife International, 2016), has a sizable population in Brazilian zoos and private breeders, and breeds fairly well under appropriate conditions. These parrots are monogamous, seasonal breeders (July–November) and usually produce 2–4 eggs clutch per year that they incubated for about 24–29 days (Sick, 2001; Seixas et al., 2002; Seixas, 2007). Hence, the aims of this study were (1) to examine annual fluctuations of urofecal androgen and progestagen metabolites in male and female Blue-fronted amazons, respectively, and (2) to define whether or not measurements of sex steroid metabolites can be used to determine reproductive status in potential breeders. In male birds, testosterone regulates the development of sexual male characteristics, activation of sexual behavior (e.g., courtship and copulation) and sperm production (Deviche et al., 2011; Riters and Alger, 2011). Progesterone is assumed to be the main steroid in female birds involved with ovulation and egg laying, while data shows this hormone to be related to the transition from courtship to incubation behavior (Johnson, 2011; Riters and Alger, 2011).

2. Material and methods

2.1. Animals

This research was conducted at Brisa commercial breeder located in São Paulo State, South Brazil (21°16′46″S, 48°13′43″W; Licenses IBAMA CTF: 263703, AM: 00024/2008-SP and SMA AM: 118748/2015). Eight pairs of Blue-fronted amazon (*Amazona aestiva*) were kept in suspended breeding aviaries (1 m high × 1 m wide × 2 m deep), half-roofed, equipped with external vertical wooden nest boxes (45 cm high × 20 cm wide × 20 cm deep). Adult males and females (>5 years old) were obtained from rescue centers (i.e. seized from illegal parrot traders) and, after a full health check; pairs were formed two months prior to the onset of the study. Once formed, pairs were visually isolated from other pairs. Water and food (extruded pellets manufactured by the breeder) were provided *ad libitum* through automatic feeders and waterers. Additionally, seasonal fruits (e.g., banana, papaya, mango, guava, grapes, etc.) were provided as behavioral enrichment. Parrots were disturbed as little as possible during the whole monitoring period. All laid eggs were incubated naturally, and unhatched eggs were only withdrawn after females abandoned them. Chicks were removed from the parents for hand feeding at 20 days of age. Experimental procedures for this study comply with the current regulations establish by the Institutional Animal Care and Use Committee at the Faculdade de Ciências Agrárias e Veterinárias – Universidade Estadual Paulista (Protocol No. 009350/11-CEUA/ FCAVJ).

2.2. Sample collection, reproductive monitoring and environmental data

Parrot droppings consist of feces and urine, which are excreted together through the cloaca, and therefore are referred to as urofecal samples. Excreted urofecal samples were collected weekly (on the same day of the week) from each individual from June 2011 to May 2012. Collection of all samples proceeded as follows: beginning at 02:00 PM wire partitions were inserted to separate members of a pair. Next, the aviary floors were lined with clean polyethylene tarpaulins (one per individual bird, to prevent cross-contamination of male and female samples). All fresh urofecal samples voided until 05:00 PM were collected and placed within individually labeled plastic microtubes, and then stored at −20 °C until steroid extraction. Sampling frequency was increased to daily whenever egg laying, incubation or parental care was perceived. Parrot pairs were monitored daily through inspections of nests to assess nesting activity (e.g. use of wood pieces deliberately left by investigators inside nests for the parrots to use for making their own bedding material), egg laying and incubation, and chick development. Fertility was confirmed through egg candling following 10 days of incubation. Molt in all individuals was concentrated between January and mid-February. Meteorological data were provided by a weather station at the Faculdade de Ciências Agrárias e Veterinárias – Universidade Estadual Paulista located exactly 7.53 km away from Brisa commercial breeder (21°14′19″S, 48°16′55″W).

2.3. Steroid extraction and analysis

Urofecal samples were dried (57 °C, 72 hs), pulverized, homogenized and extracted as described by Pereira et al. (2010). In general, 0.1 g of the resulting powder was mixed to 2 mL of 80% methanol, while smaller samples (<0.1 g) were placed in tubes containing proportional amounts of solvent (e.g., 0.03 g in 0.6 mL of 80% methanol). These suspensions were vortexed for 30 s, shaken for 12 h (250 rpm), and then vortexed again for 30 s before centrifugation (400*g* for 20 min). Subsequently, supernatants were transferred into a clean tube and stored at −20 °C.

Enzyme immunoassay protocols (EIA) were performed following Brown et al. (2004), and antisera for the detection of androgen and progestagen metabolites were supplied by C. Munro (University of California, CA, USA). Cross-reactivities for testosterone antiserum (R156/7) are 100% with testosterone; 57.3% with 5a-dihydrotestosterone; 0.2% with androstenedione; 0.4% with androsterone; and <0.04% with other tested metabolites (as reported by the manufacturer). Cross-reactivities for progesterone antiserum (CL425) are 100% with progesterone; 188% with 4-pregnen-3α-ol-3.20-dione; 172% with 4-pregnen-3β-ol-20-one; 94% with 5α-pregnan-3β-ol-20-one; 64% with 5α-pregnan-3α-ol-20-one; 55% with 5α-pregnan-3,20-dione; 12.5% with 5β-pregnan-3β-ol-20-one and ≤10% with for all other metabolites tested (Graham et al., 2001). Standard assay validation procedures included assessment of parallelism between serial dilution of Blue-fronted amazon urofecal extracts and the respective standard curves, recovery of exogenous analyte, and biological relevance of hormonal data. Recovery tests were performed by combining equal volumes of diluted urofecal extract and known amounts of exogenous hormone and calculating the difference between the expected and observed concentrations of exogenous hormone (androgens: $y = 1.06x + 1.59$, $R^2 = 0.99$; and progestagens: $y = 0.80 x - 4.24$, $R^2 = 0.98$;). To assess the physiological relevance of