Use of computer-assisted semen analysis for evaluation of Rosy-faced lovebird (Agapornis roseicollis) semen collected in different periods of the year.

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Use of computer-assisted semen analysis for evaluation of Rosy-faced lovebird (*Agapornis roseicollis*) semen collected in different periods of the year

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Abstract

The seminal characteristics of the Rosy-faced lovebird (*Agapornis roseicollis*) were analyzed, both in and out of season, using a computer-aided sperm analyzer (CASA). Avian semen collection and artificial insemination techniques have great potential for captive breeding programs, and CASA allows an objective and quantitative assessment of sperm motility and kinetics. Although *Agapornis roseicollis* is a largely diffuse species, its seminal parameters have never been fully investigated. Using the massage technique, 38 ejaculates were collected in the breeding season, and 6 ejaculates were collected outside of the breeding season. Semen color, volume, degree of contamination, spermatozoa concentration, total and progressive motility and kinetics parameters were recorded. Seasonal significant differences were found in the ejaculate volume (1.6±0.6 and 1.1±0.2µl in and out season, respectively $P<0.01$) and spermatozoa concentration (7194.0±6735.1x10^6 and 327.5±314.0 x10^6 spermatozoa/ml $P<0.01$); among the motility parameters, only BCF, indicating the frequency of flagellar beats, was significantly higher out of the reproductive season (29.8±2.6 vs 24.5±3.8 Hz $P<0.01$). There was very large individual variation in semen characteristics that could

qualify a male as a potentially good or bad semen donor for future assisted reproduction in captivity.

Keywords: Rosy-faced lovebird; *Agapornis roseicollis*; Semen analysis; Season

1. Introduction

Many psittacine species are endangered due to human activity and poaching, loss of natural habitat and climatic changes. Captive breeding programs have therefore become strategic to preserve these species' genetic material and to allow the return of the captive bred parrots back to the wild [1]. Avian artificial insemination is claimed to be an effective technique for conservation programs, having the potential to increase the number of offspring and to maintain the genetic diversity of a population [2,3]. For the benefit of rare and endangered Psittaciformes, common pet species can be used as a model, and the budgerigar (*Melopsittacus undulatus*) represents the most extensively studied model species [4,5].

The massage method is the usual technique for semen collection in birds. Adopted for the first time in poultry in 1935 [6], it has been slightly modified according to species and size of the birds [1, 7]. Semen collection from Psittaciformes has occasionally been reported, both for analysis purposes and for artificial insemination attempts [1,3,4,8,9,10,11], with variable success rates [9,11]. Either because of the limited volume of the ejaculates or due to the need for maximal sample volume for artificial insemination [11], complete semen evaluation has been carried out in only few species, such as the budgerigar (*Melopsittacus undulatus*) [4,5,12], Hispaniolan parrot (*Amazona ventralis*) [8], Blue-fronted Amazon (*Amazona aestiva*) [10], Quaker parakeet (*Myiopsitta monachus*) [3] and a few others [9,11]. The use of a computer-aided sperm analyzer has recently been reported only for the budgerigar [12]. For this species and a few other companion psittacine birds, spermatozoa

morphological characteristics have been described [7]. Seasonal variations in psittacine semen quality have not been the object of previous investigations.

The aim of this work was to characterize the semen quality of a potential model species, the Rosy-faced lovebird (*Agapornis roseicollis*), a very popular companion psittacine bird, in different periods of the year by utilizing a computer-aided sperm analysis system.

2. Materials and methods

2.1. Birds

The birds of the species *Agapornis roseicollis* for this study came from three amateur collections located in the Department of Veterinary Sciences at the University of Turin. All of the collections had been tested and were found free from the main avian viral diseases (avian polyomavirus and psittacine beak and feather disease) and from psittacosis. The 60 males included in this study, aged 1-2 years, were evaluated through celioscopy both to confirm the gender and to exclude any gonadal pathology [13]. All the birds were clinically healthy, and their nutritional condition was good. They were housed at the 'Centro Animali Non Convenzionali' (C.A.N.C.) of the Department for the period of semen collection, i.e., about one week. The parrots were kept in groups and fed with commercial seed mixtures provided by the breeders.

2.2. Semen collection and dilution

Semen was collected in two different periods of the year: outside the breeding season (September) and during the breeding season (December-January). After 3-4 days of acclimatization at the C.A.N.C., semen collection was attempted using the sacro-abdominal-cloacal massage technique [6,14]. Attempts at semen collection were made with 10 birds in September and on 50 birds in

December-January. Each bird was physically restrained by an assistant using a knotted towel in the form of a doughnut. In this way, with the head and beak contained in the towel, the assistant only had to hold the wings and legs wide open. Ejaculation was produced by massaging, with the thumb and index or middle finger, the dorsal aspect of the abdomen towards the cloaca, followed by gentle rhythmic squeezing at the base of the cloaca with the same finger of the other hand. The ejaculate was collected in graduated microcapillary tubes (Microcaps® - Drummond Science Company). The parameters that were immediately recorded were color and volume.

2.3. Semen analysis

Immediately after collection, semen was empirically diluted depending on the expected spermatozoa concentration. Outside of the breeding season, the dilution was 1:50, while during the breeding season, it was 1:100 or 1:200. Medium M199-no Hepes (M5017 Sigma-Aldrich, St. Louis, MO, USA) was supplemented with 19.9 μM sodium pyruvate, 1% bovine serum albumin, 26.2 Mm sodium bicarbonate and water. The medium, calibrated to pH 8.2 and maintained at 37.5°C, was used as a semen extender after preliminary trials (data not shown). The time from semen collection to analysis was within 5 minutes. The degree of contamination of the diluted ejaculates was visually classified from 1 to 5, and the type of contaminant was recorded (urates, erythrocytes and feces). Sperm concentration was determined using a Makler chamber after 1:200 dilution of 10 μl of extended sample with a solution of distilled water and 4% formaldehyde. Semen motility and motility characteristics were evaluated using a Computer Aided Sperm Analyzer (CASA; CEROS, Hamilton Thorne Research Inc., Version 14 Build 008, IMV Technologies France) on 10 μl of extended semen placed in a pre-heated Makler chamber (37.5°C). The parameters evaluated were total motility (TM %), progressive motility (PM %), average path velocity (VAP μm/s), straight line velocity (VSL μm/s), curvilinear line velocity (VCL μm/s), amplitude of later head displacement (ALH μm), beat cross frequency (BCF Hz), straightness of track (STR %) and

linearity of track (LIN %). The settings of the instrument were as follows: 60 frames per second (Hz), 30 frames per field, minimum contrast=20, minimum cell size=8. Static cells were defined as VAP< 5.0 μ/s and VSL<13.0 μ/s. These parameters were chosen after different trials with *Agapornis roseicollis* semen (data not shown).

2.4. Statistical analysis

The data are presented as the mean±standard deviation (SD). Kruskal–Wallis one-way analysis of variance testing was used to determine differences in sperm volume, concentration, motility and motility parameters in and outside of the breeding season (SPSS 21.0, SPSS Inc. Chicago, Ill, USA).

3. Results

3.1. Semen collection

Six out of ten birds gave an examinable ejaculate outside of the breeding season, resulting in a success rate of 60%. During the breeding season the success rate was higher (76%), and 38 ejaculates were obtained from 50 birds.

3.2. Semen analysis

Outside of the breeding season, the semen color ranged from transparent to yellow. The mean volume was 1.1±0.2 μl. Contamination (from urates) occurred in 33.3% of samples and was considered grade 1. The spermatozoa concentration was 327.5±314.0 $x10^6$ sperm/ml. The total and

progressive motility were 31.0±29.2% and 22.7±27.2%, respectively. The number of motile spermatozoa/ml was 155.5±292.2 $x10^6$ and 0.2±0.3 $x10^6$/ejaculate (Table 1).

In the breeding season, the semen color was creamy white, indicating high spermatozoa concentration. The mean volume of the ejaculates was 1.6±0.6 μl. Contamination was present in 89.5% of the ejaculates. Contamination was mainly due to urates, but erythrocytes were also present in approximately 18% of cases. Contamination was considered grade 1 in 26.3% of the samples, grade 2 in 28.9%, grade 3 in 21.0% and 4 in 10.5%. The 4 ejaculates with grade 4 contamination were not analyzed. The sperm concentration was 7194.0±6735.1 $x10^6$ sperm/ml. The total and progressive motility were 48.4±28.1% and 44.4±26.3%, respectively. The number of motile spermatozoa/ml was 3476.9±3147.9 $x10^6$ and 5.1±5.9 $x10^6$/ejaculate (Table 1).

The sperm volume was significantly smaller outside of the breeding season ($P<0.01$), and the spermatozoa concentration was significantly lower ($P<0.01$), including both the number of motile spermatozoa per milliliter and per ejaculate ($P<0.01$) (Table 1).

The mean values of the motility parameters both during and outside of the breeding season are shown in Table 1. The only parameter that significantly differed during or outside of the breeding season was BCF ($P < 0.01$).

4. Discussion

Although *Agapornis roseicollis* is one of the more widely bred parrot species is the most popular lovebird among the nine existing species, its seminal characteristics have never been reported in the literature [11,15]. Deeper knowledge of male reproductive biology can increase the success of breeding programs and enable the use of *Agapornis roseicollis* as a model species.

In our work, in addition to evaluating *Agapornis roseicollis* semen using a CASA system, we characterized an aspect of the species' reproductive biology, i.e., semen quality during and outside of the breeding season. Season significantly affected sperm volume and concentration, while the

155 percentage of total and progressive motility of spermatozoa, although numerically lower outside of
156 the breeding season, did not differ significantly. This seasonal variation is consistent with the
157 morphometric and functional gonadal modifications observed during the two periods. A greater
158 volume of mature testicular tissue in the breeding season is likely to lead to an increase in sperm
159 production. Additionally, the attempts at semen collection had a higher likelihood of success during
160 the breeding season, but the number of birds studied should be increased to strengthen the statistical
161 analysis.
162 Among the kinetic parameters, only BCF was significantly higher outside of the breeding season;
163 however, because VCL and ALH were numerically higher, a trend toward hyperactivated
164 spermatozoal motility is suggested, a pattern that, in mammalian spermatozoa, occurs in
165 capacitating conditions and favors penetration of the oocyte [16]. High BCF, ALH and VCL are
166 indicative of spermatic vigor, but spermatozoa may exhaust their energy reserves and show reduced
167 longevity under these conditions [16]. Some of the extender components, like bovine serum
168 albumin, may have induced this effect in conditions of reduced spermatozoa concentration [17].
169 We found very high individual variation in semen quality, especially outside of the breeding season,
170 and a higher number of ejaculates could be very useful to better evaluate seasonal differences. A
171 very large individual variation in semen characteristics is commonly reported in different psittacine
172 birds [3,9,10,11,12] so that a male can be qualified as a potentially good or bad semen donor. In the
173 domestic fowl, phenotypic differences in the straight-line velocity (VSL) of spermatozoa can be
174 related to differences in individual fertility because they account for at least a part of the variation in
175 sperm mobility observed among males, i.e., the net movement of a sperm cell population against
176 resistance at body temperature [18]. This observation could also be tested in psittacine species.
177 The data in the literature are not highly comparable with our study data because the species,
178 sampling technique, semen extender and dilution, and analysis protocols are very heterogeneous.
179 The success rates of the attempts at semen collection, when reported, appear to be very variable
180 among species, including 74.2% in *Nymphicus hollandicus* [1], 34.9% to 83.3% in *Amazona*

181 *ventralis* [8], and 54.5% to 96.6% in various other species [11]. The success rates are also
182 influenced by the method of collection. The collection technique used in our work is similar to that
183 reported by Samour [5], except for our use of a softer device and the aid of a second operator. For
184 ethical reasons and to respect of animal welfare, we did not consider electroejaculation as a
185 collection method, although it has been used previously in psittacine birds [11].
186 In our work, the degree of ejaculate contamination was rarely high enough to hamper semen
187 analysis. Data in the literature regarding percentages of contaminated ejaculates are limited (9.9%
188 in *Nymphicus hollandicus*) [1]; more often, previous works reported only that contaminated samples
189 were excluded from examination.
190 Due to the small volume of semen, similar to that reported for other psittacine birds [1,3,9,10,12],
191 we could not measure pH or osmolarity. The extender was chosen after preliminary trials. Semen
192 was handled and analyzed at 37.5°C because, in a preliminary trial, we measured semen
193 temperature at the moment of ejaculation in 10 Rosy-faced lovebirds and registered values from
194 35.7 to 38.2 °C, which rapidly decreasing towards the environmental temperature due to the small
195 volume. We also registered a difference in temperature between the cloaca and the body (data not
196 shown), as reported in some Passerine birds [19]. Values in the range of 37-38°C are considered the
197 standard temperature for bird semen analysis [12,20,21,22].
198 The use of a computer-aided sperm analyzer (CASA) for objective evaluation of the motility and
199 kinetic parameters of small psittacine bird semen is limited to a recent study involving
200 *Melopsittacus undulates* [12]. In *Melopsittacus undulatus,* Gloria et al. [12] showed different semen
201 volumes and motility parameters depending on the husbandry method, with higher values when
202 parrots were kept with a single female instead of in promiscuous aviaries. Our data are similar to
203 those from birds kept in the latter setting, as the parrots used in our study were indeed kept in group
204 housing. Other literature data regarding psittacine sperm motility are limited to a few species, and
205 the values, measured by direct microscopic observation, are quite variable: *Amazona ventralis* (33-
206 82%) [8], *Aratinga auricapilla* (39-54%) and *Tanygnathus lucionensis* (83-88%) [9]. The sperm

concentration that we measured for *Agapornis roseicollis* in the breeding season is higher than that previously reported in *Myiopsitta monachus* [3], *Amazona ventralis* [8], *Aratinga auricapilla* [9] and *Melopsittacus undulatus* [12]. However, another work involving *Melopsittacus undulatus* [15] found much higher semen concentrations, even higher than our measurements for *Agapornis roseicollis*.

The seminal characteristics of *Agapornis roseicollis* are worth further investigation, with the aim of relating semen quality to fertility and defining a minimum inseminating dose both for breeding purposes and for research regarding the challenges of assisted reproduction in captivity.

Acknowledgements

The authors wish to thank Dr. Luca Ferreri for his help in data analysis and all of the breeders who provided lovebirds for this research.

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Table 1 Mean values (± SD) of ejaculate volume, sperm concentration, motility and motility parameters measured by the CEROS analyzer during and outside of the breeding season. Mean values that differ significantly within a row ($P < 0.01$) have different small letters. (TM = total motility, PM = progressive motility, VAP = velocity average pathway, VSL = velocity straight line, VCL = curvilinear velocity, ALH = amplitude lateral head, BCF= beat cross frequency, STR = straightness, LIN = linearity.)

	Out of season (N=6)	**In season (N=34)**
Volume of the ejaculate (µl)	1.1±0.2 [a]	1.6±0.6 [b]
Concentration (N spermatozoa/ml x10^6)	327.5±314.0 [a]	7194.0±6735.1 [b]
TM (%)	31.0±29.2	48.4±28.1
PM (%)	22.7±27.2	44.4±26.3
Motile spermatozoa/ml (x10^6)	155.5±292.2 [a]	3476.9±3147.9 [b]
Motile spermatozoa/ejaculate x10^6)	0.2±0.3 [a]	5.1±5.9 [b]
VAP (µm/s)	45.1±15.8	42.9±10.1
VSL (µm/s)	34.1±12.7	34.1±8.1
VCL (µm/s)	73.8±22.3	67.6±17.8
ALH (µm)	4.5±1.7	3.9±1.2
BCF (Hz)	29.8±2.6[a]	24.5±3.8[b]
STR (%)	78.8±6.6	80.0±6.1
LIN (%)	47.8±6.4	54.0±7.7