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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 ( $1.6 \pm 0.6$  and  $1.1 \pm 0.2 \mu\text{l}$  in and out season, respectively  $P < 0.01$ ) and spermatozoa concentration ( $7194.0 \pm 6735.1 \times 10^6$  and  $327.5 \pm 314.0 \times 10^6$  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 ( $29.8 \pm 2.6$  vs  $24.5 \pm 3.8$  Hz  $P < 0.01$ ). There was very large individual variation in semen characteristics that could

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

29

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

31

## 32 **1. Introduction**

33

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

42 The massage method is the usual technique for semen collection in birds. Adopted for the first time  
43 in poultry in 1935 [6], it has been slightly modified according to species and size of the birds [1, 7].  
44 Semen collection from Psittaciformes has occasionally been reported, both for analysis purposes  
45 and for artificial insemination attempts [1,3,4,8,9,10,11], with variable success rates [9,11]. Either  
46 because of the limited volume of the ejaculates or due to the need for maximal sample volume for  
47 artificial insemination [11], complete semen evaluation has been carried out in only few species,  
48 such as the budgerigar (*Melopsittacus undulatus*) [4,5,12], Hispaniolan parrot (*Amazona ventralis*)  
49 [8], Blue-fronted Amazon (*Amazona aestiva*) [10], Quaker parakeet (*Myiopsitta monachus*) [3] and  
50 a few others [9,11]. The use of a computer-aided sperm analyzer has recently been reported only for  
51 the budgerigar [12]. For this species and a few other companion psittacine birds, spermatozoa

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

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

57

## 58 **2. Materials and methods**

59

### 60 *2.1. Birds*

61

62 The birds of the species *Agapornis roseicollis* for this study came from three amateur collections  
63 located in the Department of Veterinary Sciences at the University of Turin. All of the collections  
64 had been tested and were found free from the main avian viral diseases (avian polyomavirus and  
65 psittacine beak and feather disease) and from psittacosis. The 60 males included in this study, aged  
66 1-2 years, were evaluated through celioscopy both to confirm the gender and to exclude any  
67 gonadal pathology [13]. All the birds were clinically healthy, and their nutritional condition was  
68 good. They were housed at the ‘Centro Animali Non Convenzionali’ (C.A.N.C.) of the Department  
69 for the period of semen collection, i.e., about one week. The parrots were kept in groups and fed  
70 with commercial seed mixtures provided by the breeders.

71

### 72 *2.2. Semen collection and dilution*

73

74 Semen was collected in two different periods of the year: outside the breeding season (September)  
75 and during the breeding season (December-January). After 3-4 days of acclimatization at the  
76 C.A.N.C., semen collection was attempted using the sacro-abdominal-cloacal massage technique  
77 [6,14]. Attempts at semen collection were made with 10 birds in September and on 50 birds in

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

85

### 86 *2.3. Semen analysis*

87

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

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

108

#### 109 *2.4. Statistical analysis*

110

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

115

### 116 **3. Results**

117

#### 118 *3.1. Semen collection*

119

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

123

#### 124 *3.2. Semen analysis*

125

126 Outside of the breeding season, the semen color ranged from transparent to yellow. The mean  
127 volume was 1.1 $\pm$ 0.2  $\mu$ l. Contamination (from urates) occurred in 33.3% of samples and was  
128 considered grade 1. The spermatozoa concentration was 327.5 $\pm$ 314.0  $\times 10^6$  sperm/ml. The total and

129 progressive motility were  $31.0\pm 29.2\%$  and  $22.7\pm 27.2\%$ , respectively. The number of motile  
130 spermatozoa/ml was  $155.5\pm 292.2 \times 10^6$  and  $0.2\pm 0.3 \times 10^6$ /ejaculate (Table 1).

131 In the breeding season, the semen color was creamy white, indicating high spermatozoa  
132 concentration. The mean volume of the ejaculates was  $1.6\pm 0.6 \mu\text{l}$ . Contamination was present in  
133 89.5% of the ejaculates. Contamination was mainly due to urates, but erythrocytes were also present  
134 in approximately 18% of cases. Contamination was considered grade 1 in 26.3% of the samples,  
135 grade 2 in 28.9%, grade 3 in 21.0% and 4 in 10.5%. The 4 ejaculates with grade 4 contamination  
136 were not analyzed. The sperm concentration was  $7194.0\pm 6735.1 \times 10^6$  sperm/ml. The total and  
137 progressive motility were  $48.4\pm 28.1\%$  and  $44.4\pm 26.3\%$ , respectively. The number of motile  
138 spermatozoa/ml was  $3476.9\pm 3147.9 \times 10^6$  and  $5.1\pm 5.9 \times 10^6$ /ejaculate (Table 1).

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

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

145

#### 146 **4. Discussion**

147

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

152 In our work, in addition to evaluating *Agapornis roseicollis* semen using a CASA system, we  
153 characterized an aspect of the species' reproductive biology, i.e., semen quality during and outside  
154 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

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

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

215

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217

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219 provided lovebirds for this research.

220

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278

279

280

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

	<b>Out of season (N=6)</b>	<b>In season (N=34)</b>
<b>Volume of the ejaculate (<math>\mu</math>l)</b>	1.1 $\pm$ 0.2 <sup>a</sup>	1.6 $\pm$ 0.6 <sup>b</sup>
<b>Concentration (N spermatozoa/ml <math>\times 10^6</math>)</b>	327.5 $\pm$ 314.0 <sup>a</sup>	7194.0 $\pm$ 6735.1 <sup>b</sup>
<b>TM (%)</b>	31.0 $\pm$ 29.2	48.4 $\pm$ 28.1
<b>PM (%)</b>	22.7 $\pm$ 27.2	44.4 $\pm$ 26.3
<b>Motile spermatozoa/ml (<math>\times 10^6</math>)</b>	155.5 $\pm$ 292.2 <sup>a</sup>	3476.9 $\pm$ 3147.9 <sup>b</sup>
<b>Motile spermatozoa/ejaculate <math>\times 10^6</math>)</b>	0.2 $\pm$ 0.3 <sup>a</sup>	5.1 $\pm$ 5.9 <sup>b</sup>
<b>VAP (<math>\mu</math>m/s)</b>	45.1 $\pm$ 15.8	42.9 $\pm$ 10.1
<b>VSL (<math>\mu</math>m/s)</b>	34.1 $\pm$ 12.7	34.1 $\pm$ 8.1
<b>VCL (<math>\mu</math>m/s)</b>	73.8 $\pm$ 22.3	67.6 $\pm$ 17.8
<b>ALH (<math>\mu</math>m)</b>	4.5 $\pm$ 1.7	3.9 $\pm$ 1.2
<b>BCF (Hz)</b>	29.8 $\pm$ 2.6 <sup>a</sup>	24.5 $\pm$ 3.8 <sup>b</sup>
<b>STR (%)</b>	78.8 $\pm$ 6.6	80.0 $\pm$ 6.1
<b>LIN (%)</b>	47.8 $\pm$ 6.4	54.0 $\pm$ 7.7

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288