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**Semen evaluation in four autochthonous wild raptor species using CASA**

A. Dogliero\*, A. Rota, R. Lofiego, M. Mauthe von Degerfeld, G. Quaranta

Dipartimento Scienze Veterinarie – Università di Torino, Grugliasco– Italy

*\*Corresponding Author: Tel +39 0116709057 Fax +39 011 2369049*

*E-mail address: andrea.dogliero@gmail.com*

12 **Abstract**

13

14 At least ten percent of the approximately 300 species of the order *Falconiformes* are listed as being  
15 globally threatened. The present work describes the seminal characteristics of three diurnal and one  
16 nocturnal raptor species. Semen was collected from clinically healthy *Accipiter nisus* (N=1), *Falco*  
17 *subbuteo* (N=6) and *F.tinnunculus* (N=5) adult males that were housed at the ‘Centro Animali Non  
18 Convenzionali’ of the Department of Veterinary Sciences of the University of Turin. The semen was  
19 collected after a period of recovery and before their release as well as from 7 *Bubo bubo* males bred  
20 in captivity as part of a raptor conservation project. All of the potential semen donors were trained in  
21 semen collection during the breeding season via a ritualized procedure. Ejaculation was achieved  
22 using a massaging technique. Each sample was evaluated for volume, degree of contamination and  
23 spermatozoa concentration. The semen motility and kinetic parameters were assessed in diluted semen  
24 (modified TALP, pH 7.5, temperature 37.5°C) using a Computer Aided Sperm Analyzer. Semen  
25 collection was successful in all of the diurnal species and in five *Bubo bubo* individuals. The sperm  
26 motility and sperm kinetic parameters were very variable both among and within species. In contrast  
27 with previous studies that involved raptors bred in captivity and imprinted on humans, we worked  
28 with wild birds and attempted to overcome the problem of poor semen quality, which is strongly  
29 influenced by stress, by adopting a ritualized procedure that has never been reported for semen  
30 collection purposes.

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32 *Keywords:* avian semen, birds of prey, computer-assisted semen analysis

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## 39 **1. Introduction**

40

41 Knowledge of the seminal characteristics of a species is crucial for understanding their reproductive  
42 biology and particularly for planning captive breeding programs that adopt artificial  
43 insemination(AI). AI can be used in avian conservation programs to assist in creating viable, self-  
44 sustaining populations [1]. At least 10% of the approximately 300 species of the Order *Falconiformes*  
45 are listed as being globally threatened [2]. Apart from the excellent results obtained with selected  
46 Peregrine falcons [3, 4] and California condors [5], captive breeding, especially that of endangered  
47 eagles and hawks, is far from successful [6]. This is partly due to the inadequate knowledge of the  
48 normal reproductive parameters of various species but it is also due to the likely consequence of  
49 captivity stress [7]. Additional problems with wild raptors include the unavailability of founders,  
50 inbreeding depression, female-male incompatibility, asynchrony, inability to naturally copulate, poor  
51 semen quality and urine contamination when birds are brought into an *ex situ* environment, sperm  
52 transport inefficiency and diseases [6]. Common species can be used as a model for endangered ones,  
53 both for semen collection, processing and preservation as well as for captive breeding programs [8].  
54 Although many wild raptor species are not endangered, very little is known about their seminal  
55 characteristics [9,10]. The common kestrel (*Falco tinnunculus*) and Eurasian sparrowhawk (*Accipiter*  
56 *nisus*) are two raptor species that are considered to be residents in Italy. Only Northern Europe  
57 populations migrate south for the winter, while their southern counterparts, at most and in rare cases,  
58 show limited dispersive movements [2,11]. These two species represent the most common birds of  
59 prey in Europe. Conversely, the Eurasian hobby (*Falco subbuteo*) is a long-distance migrant that  
60 winters in Africa and Asia. This species is largely present in Italy, but it is a more vulnerable species  
61 that mainly eats insects, swifts and house martins. The Eurasian eagle-owl (*Bubo bubo*) is a species  
62 of eagle-owl that resides in much of Eurasia; besides being one of the largest living species of owl, it  
63 is also one of the more widely distributed. With a total range in Europe and Asia of approximately

64 32 million square kilometres and a total population estimated to be between 250 thousand and 2.5  
65 million individuals, the 'International Union for Conservation of Nature' lists its conservation status  
66 as being of "least concern" [12]. Various studies have investigated the biology or breeding behaviour  
67 of this species and report reproductive programs in captivity [13,14], but none has addressed semen  
68 collection and evaluation.

69 The present study aims to improve the knowledge of the seminal characteristics of four common  
70 raptor species using CASA analysis, which is a standardized and objective evaluation method.

71

## 72 **2. Materials and Methods**

73

74 The 'Centro Animali Non Convenzionali' (C.A.N.C.) of the Department of Veterinary Sciences of  
75 the University of Turin treats injured wild animals with the goal of releasing the ones that recover.  
76 C.A.N.C. has a project to build a wild avian species semen bank for conservation purposes, and the  
77 present study is part of that project. In the breeding season (April-June) of three consecutive years  
78 (2013-2015), attempts at semen collection were made in all of the clinically healthy adult males of  
79 *Accipiter nisus* (N=1), *F. subbuteo* (N=6) and *F. tinnunculus* (N=5) that arrived at the 'Centro'. At  
80 the same time and in the full breeding season (February to May) of 2014, seven adult Eurasian eagle-  
81 owl males (*B. bubo*) between 7 and 15 years old were included in the study. All of the owls were  
82 housed in outdoor pens, coupled with a female, and bred in captivity, in agreement with C.A.N.C.  
83 raptor recovery and conservation projects. Every raptor species, both diurnal and nocturnal, was fed  
84 a diet consisting of rabbits, quails, rats, day-old chicks, mealworms and locusts (only *F. subbuteo*) in  
85 varying percentages, depending on the species.

86 All of the males were both macroscopically and endoscopically evaluated for the confirmation of  
87 good clinical conditions and for the assessment of gonadal status and functionality. The potential  
88 semen donors were trained in semen collection twice weekly, beginning with a ritualized procedure  
89 consisting of a fixed hour of performance, the affixation of a falconry hood immediately after capture,

90 precise positioning, bandaging of the talons with cohesive bandaging tape (Vetrap®) and a series of  
91 simulated semen collection manipulations. The procedure was always consolidated with positive  
92 reinforcement consisting of the daily meal at the conclusion of the process. In the diurnal raptor  
93 species, the semen was collected in the early morning (between 8:00 and 10:00 a.m.) and in the early  
94 afternoon (between 02:00 and 04:00 p.m.) in the Eurasian eagle-owl. For semen collection, each bird  
95 was physically restrained by an operator using a soft towel to contain the front half of the bird's body  
96 to avoid struggling and stress and to ensure safety. Ejaculation was achieved using a modified  
97 massaging technique [15] with the thumb and index or middle finger on the dorsal aspect of the  
98 abdomen towards the cloaca, followed by gentle rhythmic squeezing at the base of the cloaca with  
99 the same finger of the other hand. The ejaculate was collected in graduated microcapillary tubes  
100 (Microcaps, Drummond Science Company Broomall, PA, USA) and directly evaluated for colour and  
101 volume. Immediately after collection, the semen was empirically diluted, from a minimum of 1:2 (*B.*  
102 *bubo*) to a maximum of 1:50 (*Falco sp.*), in modified TALP (100 mM sodium chloride; 3.1 mM  
103 potassium chloride; 25 mM sodium carbonate; 0.3 mM sodium dihydrogen phosphate; 10 mM  
104 HEPES; 2 mM calcium chloride; 0.4 mM magnesium chloride and 1 mg/ml sodium pyruvate). All of  
105 the components were from Sigma-Aldrich (St. Louis, MO, USA), calibrated at pH 7.5 and maintained  
106 at 37.5°C. The time from semen collection to analysis was within 5 minutes. The degree of  
107 contamination of the diluted ejaculates was visually classified from 1 to 5, and the type of the  
108 contaminants was recorded (urates, erythrocytes and faeces). When the contamination degree was  
109 >4, the samples were discarded. The sperm concentration was determined using a Makler chamber  
110 after a standard 1:100 dilution of 10 µl of the extended sample with a solution of distilled water and  
111 4% formaldehyde. Semen motility and the motility parameters of 10 µl of the extended semen placed  
112 in a pre-heated Makler chamber (37.5°C) were evaluated using a Computer Aided Sperm Analyzer  
113 (CASA; CEROS, Hamilton Thorne Research Inc., Version 14 Build 008, IMV Technologies, France).  
114 The evaluated parameters were total motility (TM %), progressive motility (PM %), average path  
115 velocity (VAP µm/s), straight line velocity (VSL µm/s), curvilinear line velocity (VCL µm/s),

116 amplitude of later head displacement (ALH  $\mu\text{m}$ ), beat cross frequency (BCF Hz), straightness of the  
117 track (STR %), and the linearity of the track (LIN %). The settings of the instrument were as follows:  
118 60 frames per second (Hz), 30 frames per field, minimum contrast=15, minimum cell size=10; and  
119 static cells were considered when VAP < 10.0  $\mu\text{/s}$  and VSL < 13.0  $\mu\text{/s}$ . These parameters were chosen  
120 after the different trials with raptor species semen (data not shown).

121

### 122 **3. Results**

123

124 The semen collection was successful in 1/1 individual birds of *A. nisus*, 6/6 *F. subbuteo*, 5/5 *F.*  
125 *tinnunculus* and 5/7 *B. bubo*. The number of attempts at semen collection in the different species is  
126 reported in Table 1. *A. nisus* and *F. tinnunculus* required an average of three weeks of training,  
127 whereas *F. subbuteo* had to be trained for a longer period of 4-5 weeks. Although an analysable  
128 sample could be collected from some *B. bubo* birds immediately and without training, other  
129 individuals were difficult to handle and stimulate to produce an ejaculate. Many samples of all of the  
130 species showed a very high degree of contamination, especially at the beginning of the training period,  
131 and had to be discarded (Table 1). The colour of analysable ejaculates was whitish in *Falco sp.*,  
132 variable from whitish to turbid yellowish in *B. bubo* or whitish to a pale yellow in *A. nisus*. The colour  
133 was a good index of spermatozoa concentration and/or contamination. When contaminated with  
134 urates or blood, the yellow colour was more whitish or reddish. The semen characteristics are shown  
135 in Table 2, and the kinetic parameters appear in Table 3; large intra- and interspecific variability is  
136 evident. The single bird of the species *A. nisus* showed very poor seminal quality, a likely  
137 consequence of the stress from captivity and manipulation. The semen parameters of *F. subbuteo*  
138 were generally lower than those of the other species of *Falco*, similar morphometric characteristics  
139 notwithstanding. *B. bubo* produced a higher mean ejaculate volume ( $8.97 \pm 4.03 \mu\text{l}$ ) but lower mean  
140 sperm concentration ( $37.7 \pm 53.0 \text{ spermatozoa} \times 10^6/\text{ml}$ ); the individual variability of these parameters  
141 was very high: semen volume ranged from 2 to 15.4  $\mu\text{l}$  and spermatozoa concentration from 9 to

142 144spermatozoa  $\times 10^6$ /ml. The seminal kinetic parameters in this species were generally rather poor  
143 (Table 3).

144

#### 145 **4. Discussion**

146

147 Several methods of collecting semen in raptors are described in the literature, from cloacal massage  
148 [1,6,15-17] to cooperative copulation [9,10]. Contrary to other wild bird species, such as ducks and  
149 geese [18], pigeons [19] and many psittacine [20], experimental electroejaculation has never been  
150 performed in raptors. Despite the fact that cooperative copulation may provide better semen quality  
151 results [10], we adopted the cloacal massage technique because it has several advantages when  
152 working with wild birds. First, it does not require a long training period. Second, it can be adapted to  
153 many different species, variable for both dimensions and phylogeny and is not an invasive procedure  
154 [1,6].The large majority of the previous research on raptors was conducted on birds bred in captivity  
155 and imprinted on humans [8,9,21-23]. However, we worked with wild birds, with the goal of releasing  
156 them into the wild without affecting their natural habits and survival ability. Wild animals and birds  
157 should be kept in captivity for as short a time as possible after recovery to give them the greatest  
158 opportunity to survive in their natural environment. Cooperative copulation requires a very long  
159 training period and can be adopted in birds that will not be released because of the strong imprinting  
160 on humans that this method implies [9,10]. Wild raptors often show poor semen quality when in  
161 captivity, mainly because of stress [6], and we tried to overcome this problem by adopting a ritualized  
162 procedure that originated from falconry. The adoption of procedures from falconry could be useful  
163 in obtaining samples from valuable individuals, although the protocol that we described, which has  
164 never been previously reported for such purposes, should undergo further experimentation and  
165 validation.

166 Semen contamination may represent a problem in wild raptors kept in captivity [6], and many  
167 ejaculates showed a very high degree of contamination and had to be discarded; we observed higher

168 contamination in the first attempts at semen collection in all of the species, particularly in the Eurasian  
169 hobby and the Eurasian eagle-owl, which suggests that the training of the birds can improve semen  
170 quality. Contamination is extensively reported in the literature [6,23,24]. In our case, it consisted of  
171 urine and sometimes erythrocytes, as a result of minor damage to the delicate cloacal mucosa, and  
172 more rarely faecal material. Low faecal contamination of the ejaculates appeared to be correlated to  
173 fasting before collection. We also found that the operator's experience can be significant in obtaining  
174 only mildly urine-contaminated samples, due to the correct stimulation of ejaculation and not  
175 urination, despite the vicinity of the anatomic structures.

176 In general, the semen characteristics of the autochthonous diurnal raptors that we analysed were  
177 comparable to those reported in the literature, at least for similar species regarding both size and  
178 phylogenetic aspects. The semen volume appears to be related to bird size, and spermatozoa  
179 concentration tends to decrease when the ejaculate volume increases [25,26]. The semen volume and  
180 quality may also vary throughout the reproductive season [27].

181 The values of the American kestrel (*Falco sparverius*) are similar to those of the common kestrel (*F.*  
182 *tinnunculus*), with a semen volume range of 10-15 $\mu$ l, a mean spermatozoa number of  
183  $614.0 \pm 352.3$ /ejaculate and a success rate in semen collection ranging from 7 to 55% [21]. With  
184 respect to the Peregrine falcon (*Falco peregrinus*) [22],  
185 both the common kestrel (*F. tinnunculus*) and Eurasian hobby (*F. subbuteo*) showed a higher semen  
186 collection success rate and sperm concentration ( $295 \pm 190 \times 10^6$ /ml and  $130.0 \pm 99.0 \times 10^6$ /ml,  
187 respectively) but a much smaller semen volume (Peregrine falcon: 27-208  $\mu$ l [22]). Neither the  
188 seminal characteristics of *B. bubo* (Eurasian eagle-owl) nor those of the other owls have been  
189 previously investigated or reported. When comparing our findings with data from other diurnal raptor  
190 species of similar size, such as the Indian white-backed vulture (*Gyps bengalensis*), a similar CASA  
191 system (HTM IVOS 10, Hamilton Throne Research, Inc., Danvers, MA) was used [24], whereas for  
192 the golden eagle (*Aquila chrysaetos*), a SCA® system (Microptic SL, Barcelona 08029, Spain) was  
193 used [10]: using these two systems, the semen volume was  $370 \pm 260 \mu$ l and  $42.2 \pm 31.8 \mu$ l, respectively,

194 and the sperm concentration was  $58.4 \pm 33.2 \times 10^6/\text{ml}$  and  $467.7 \pm 392 \times 10^6$  sperm/ml, respectively. The  
195 kinetic parameters of *B. bubo* are more similar to the values reported for *Aquila chrysaetos* (despite  
196 the different computer-aided image analysis systems used) than to the much higher values reported  
197 for *Gyps bengalensis*.

198 Most of the cited research on raptors' semen did not assess the kinetic parameters, and this fact  
199 hinders further comparisons and a better understanding of both intra and interspecific semen  
200 differences.

201 In our work, the results of the sperm parameters were very variable both among and within species,  
202 as shown in many cases by high standard deviations (which reached a maximum in some parameters  
203 of *B. bubo*). The variability in the semen parameters is a peculiarity of wild avian species [28-30] and  
204 is also typical of wild raptors [1,6,10]. Very different ejaculates were also collected from a single  
205 bird, as shown from the 112 highly heterogeneous semen samples of a single *Aquila chrysaetos*,  
206 resulting in a large variability both in volume and in spermatozoa concentration [10]. A lower  
207 variability can be obtained by increasing the number of birds and samples, which was previously  
208 shown by Umaphathy et al. in four Indian white-backed vultures [24].

209 The high intraspecific variability that we observed suggests that the birds showing better semen  
210 quality could be selected as potential semen donors in captive breeding programs; if we suppose that  
211 in birds, similar to mammals, semen quality can be related to fertility [31,32], then good semen donors  
212 could have a higher possibility to produce progeny in captivity. Intraspecific variability could also be  
213 partly due to natural factors, such as the age of the bird or different periods in the breeding season  
214 [10], or to different stress responses to temporary captivity conditions and handling. All of the species  
215 involved in our study are monogamous (which was also previously observed by Villaverde-Morcillo  
216 et al. [10]); therefore, variability in semen quality cannot be due to sperm competition and the degree  
217 of polygamy.

218 Many problems need to be overcome when collecting semen from wild birds, and this preliminary  
219 work shows a possible technique that could be further investigated to obtain semen samples while

220 minimizing the training period and human contact. Common raptor species should also be studied  
221 because they can represent useful models for similar but threatened species.

222

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<b>Species (N of birds)</b>	<b>Attempts at semen collection (N)</b>	<b>Collection success (N)</b>	<b>Discarded ejaculates (N)</b>	<b>Analysable ejaculates (N)</b>
<i>Accipiter nisus</i> (1)	5	3	2	1
<i>Falco subbuteo</i> (6)	48	28	22	6
<i>Falco tinnunculus</i> (5)	20	8	3	5
<i>Bubo bubo</i> (7)	140	61	56	5

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**Table 1** The total number (N) of attempts at semen collection, the N of the samples collected, discarded samples (because of a contamination degree>4) and analysable ejaculates in four wild raptor species.

	<i>Accipiter nisus</i> (N=1)	<i>Falco subbuteo</i> (N=6)	<i>Falco tinnunculus</i> (N=5)	<i>Bubo bubo</i> (N=5)
<b>Volume of the ejaculate (µl)</b>	2	3.60±2.41	2.78±1.27	8.97±4.03
<b>Degree of contamination (1-4)</b>	1	2.33±1.21	3.20±0.84	2.75±0.46
<b>N spermatozoa × 10<sup>6</sup>/ml</b>	5	130.0±98.9	295±190	37.7±53.0
<b>N spermatozoa × 10<sup>3</sup>/ejaculate</b>	10	160.0±56.6	614.0±352.3	453.8±862.3

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**Table 2** The mean values ± standard deviation of ejaculate volume, degree of contamination, spermatozoa concentration and spermatozoa number in the analysable ejaculates of four wild raptor species.

	<i>Accipiter nisus</i> (N=1)	<i>Falco subbuteo</i> (N=6)	<i>Falco tinnunculus</i> (N=5)	<i>Bubo bubo</i> (N=5)
<b>TM (%)</b>	18	49.17±37.07	59.20±27.14	25.25±17.99
<b>PM (%)</b>	3	17.00±12.23	36.80±17.25	13.00±9.58
<b>VAP (µm/s)</b>	23.20	45.28±26.60	33.90±3.60	31.64±6.31
<b>VSL (µm/s)</b>	20.60	28.80±18.72	28.08±1.99	27.83±5.45
<b>VCL (µm/s)</b>	37.60	74.80±40.91	53.70±8.19	46.96±9.25
<b>ALH (µm)</b>	8.90	4.05±2.15	2.90±0.59	2.35±0.81
<b>BCF (Hz)</b>	89.00	21.43±6.59	24.66±3.62	26.76±7.81
<b>STR (%)</b>	55	69.00±4.29	84.40±6.23	88.00±6.14
<b>LIN (%)</b>	55	40.33±4.50	60.00±6.63	63.25±9.59

326

327 **Table 3** Spermatozoa motility (TM = total motility and PM = progressive motility) and motility  
328 parameters measured by the CEROS analyser in N ejaculates of four raptor species (the mean values  
329  $\pm$  standard deviation). VAP = velocity average pathway, VSL = velocity straight line, VCL =  
330 curvilinear velocity, ALH = amplitude lateral head, BCF= beat cross frequency, STR = straightness,  
331 LIN = linearity