Semen evaluation in four autochthonous wild raptor species using CASA.

REVISED

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

Abstract

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

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

1. Introduction

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

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

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

2. Materials and Methods

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

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

precise positioning, bandaging of the talons with cohesive bandaging tape (Vetrap®) and a series of simulated semen collection manipulations. The procedure was always consolidated with positive reinforcement consisting of the daily meal at the conclusion of the process. In the diurnal raptor species, the semen was collected in the early morning (between 8:00 and 10:00 a.m.) and in the early afternoon (between 02:00 and 04:00 p.m.) in the Eurasian eagle-owl. For semen collection, each bird was physically restrained by an operator using a soft towel to contain the front half of the bird's body to avoid struggling and stress and to ensure safety. Ejaculation was achieved using a modified massaging technique [15] with the thumb and index or middle finger on 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 Broomall, PA, USA) and directly evaluated for colour and volume. Immediately after collection, the semen was empirically diluted, from a minimum of 1:2 (*B. bubo*) to a maximum of 1:50 (*Falco sp.*), in modified TALP (100 mM sodium chloride; 3.1 mM potassium chloride; 25 mM sodium carbonate; 0.3 mM sodium dihydrogen phosphate; 10 mM HEPES; 2 mM calcium chloride; 0.4 mM magnesium chloride and 1 mg/ml sodium pyruvate). All of the components were from Sigma-Aldrich (St. Louis, MO, USA), calibrated at pH 7.5 and maintained at 37.5°C. 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 the contaminants was recorded (urates, erythrocytes and faeces). When the contamination degree was >4, the samples were discarded. The sperm concentration was determined using a Makler chamber after a standard 1:100 dilution of 10 μl of the extended sample with a solution of distilled water and 4% formaldehyde. Semen motility and the motility parameters of 10 μl of the extended semen placed in a pre-heated Makler chamber (37.5°C) were evaluated using a Computer Aided Sperm Analyzer (CASA; CEROS, Hamilton Thorne Research Inc., Version 14 Build 008, IMV Technologies, France). The evaluated parameters 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 the track (STR %), and the linearity of the track (LIN %). The settings of the instrument were as follows: 60 frames per second (Hz), 30 frames per field, minimum contrast=15, minimum cell size=10; and static cells were considered when VAP< 10.0 μ/s and VSL<13.0 μ/s. These parameters were chosen after the different trials with raptor species semen (data not shown).

3. Results

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

142 144spermatozoa × 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

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

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

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

and the sperm concentration was 58.4±33.2 x10^6/ml and 467.7±392 x10^6 sperm/ml, respectively. The kinetic parameters of *B. bubo* are more similar to the values reported for *Aquila chrysaetos* (despite the different computer-aided image analysis systems used) than to the much higher values reported for *Gyps bengalensis*.

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

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

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

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

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

References

[1] Blanco JM, Wildt DE, Höfle U, Voelker W, Donoghue AM. Implementing artificial insemination as an effective tool for ex situ conservation of endangered avian species. Theriogenology 2009;71:200–13.

[2] del Hoyo J, Elliott A, Sargatal J. Handbook of the Birds of the World, vol. 2: New World Vultures to Guineafowl. Barcelona, Lynx Ed; 1994.

[3] Weaver JD, Cade TJ. Falcon propagation: a manual of captive breeding. Boise ID: The Peregrine Fund Inc; 1983.

[4] Hoffman C. Peregrine to soar off endangered species list. Endangered Species Bull 1998;23:20-1.

[5] Saint Jalme M. Endangered avian species propagation: an overview of functions and techniques. Avian PoultBiol Rev 2002;13:187–202.

[6] Blanco JM, Gee GF, Wildt DE, Donoghue AM. Producing progeny from endangered birds of prey: treatment of urine-contaminated semen and a novel intramagnal insemination approach. J Zoo Wildl Med 2002;33:1-7.

[7] Gee GF, Bertschinger H, Donoghue AM, Blanco JM, Soley J. Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. Avian PoultBiol Rev 2004;15:47-101.

[8] Brock MK, Bird DM. Prefreeze and postthaw effect of glycerol and dimethylacetamide on motility and fertilizing ability of American Kestrel (*Falco sparvierus*) spermatozoa. J. Zoo Wildl Med 1991;22:453-59.

[9] Temple SA. Artificial insemination with imprinted birds of prey. Nature 1972;237:287-8.

[10] Villaverde-Morcillo S, García Sánchez R, Castaño C, Rodríguez E, González F, Esteso MC, Santiago-Moreno J. Characterization of natural ejaculates and sperm cryopreservation in a golden eagle (*Aquila chrysaetus*). J Zoo Wildl Med 2015;46:335-8.

[11] Ferguson-Lees L, Christie DA. Raptors of the world. London, Christopher Helm; 2001.

[12] http://www.birdlife.org/datazone/speciesfactsheet.php?id=2215

[13] McKeever K. Care and rehabilitation of injured owls: a user's guide to the medical treatment of raptorial birds and the housing, release training and captive breeding of native owls. Ontario, Canada, The Owl Rehabilitation Research Foundation; 1979.

[14] Parry-Jones J. Understanding owls: biology, management, breeding, training. Devon, United Kingdom, David & Charles; 1998.

[15] Burrows WH, Quinn JP. A method of obtaining spermatozoa from the domestic fowl. Poult Sci 1935;15:251–4.

[16] Burrows WH, Quinn JP. The collection of spermatozoa from the domestic fowl and turkey. Poult Sci 1937;16:19-24.

[17] Samour JH. Semen collection, spermatozoa cryopreservation and artificial insemination in nondomestic birds. J Avian Med Surg 2004;18:219-23.

[18] Samour HJ, Sprat DMJ, Hutton RE, Jones DM. Studies on semen collection in waterfowl by electrical stimulation. Br Vet J 1985;141:265.

[19] Betzen KM. Techniques for electrical semen collection of birds. MSc Thesis. Stillwater, Oklahoma State University; 1985.

[20] Harrison GJ, Wasmund D. Preliminary studies of electroejaculation to facilitate manual semen collection in psittacines. Proc An Meet Ass Avian Vet, Krakow, Poland, 1983;207-213.

[21] Brock MK. Cryopreservation of semen of the American Kestrel (*Falco sparverius*). Thesis. Master of Science Degree, Macdonald College of McGill University, Montreal, Quebec, Canada; 1986: 85.

[22] Hoolihan J, Burnham W. Peregrine falcon semen: a quantitative and qualitative examination. J Raptor Res 1985;19:125-7.

[23] Bird DM, Lague PC, Buckland RB. Artificial insemination vs. natural mating in captive American kestrels. Can J Zool 1983;54:1183–91.

[24] Umapathy G, Sontakke S, Reddy A, Ahmed S, Shivaji S. Semen characteristics of the captive Indian white-backed vulture (*Gyps bengalensis*). Biol Reprod 2005;73:1039-45.

[25] Chen G, Gee GF, Nicolich JM, Taylor JA. Semen collection and fertility in naturally-fertile sandhill cranes. Proc N Am Crane Workshop 1997;7:258.

[26] Gee GF, Bertschinger H, Donoghue AM, Blanco JP, Soley J. Reproduction in nondomestic birds: physiology, semen collection, artificial insemination and cryopreservation. Avian Poult Biol Rev 2004;15:47-101.

[27] Birkhead TR, Fletcher F. Depletion determines sperm numbers in male zebra finches. Anim Behav 1995;49:4516.

[28] Sontakke SD, Umapathy G, Sivaram V, Kholkute SD, Shivaji S. Semen characteristic, cryopreservation, and successfull artificial insemination in the Blue rock pigeon. Theriogenology 2004;62:139-52.

[29] Gloria A, Contri A, Carluccio A, Parrillo S, Cicconi M, Robbe D. The breeding management affects fresh and cryopreserved semen characteristics in *Melopsittacus undulatus*. Anim Reprod Sci 2014;144:48–53.

[30] Dogliero A, Rota A, Mauthe von Degerfeld M, Quaranta G. Use of computer-assisted semen analysis for evaluation of Rosy-faced lovebird (*Agapornis roseicollis*) semen collected in different periods of the year. Theriogenology 2015;83:103-6.

[31] Farrell PB, Presicce GA, Brockett CC, Foote RH. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 1998;49:871–9.

[32] Colenbrander B, Gadella BM, Stout TA. The predictive value of semen analysis in the evaluation of stallion fertility. Reprod Domest Anim 2003;38:305-11.

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

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.

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

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.

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

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