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Semen evaluation in four autochthonous wild raptor species using CASA

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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 7Bubo 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 in 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 \( \mu m \)), 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 \( \mu/s \) and VSL<13.0 \( \mu/s \). 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 \( \mu l \)) but lower mean sperm concentration (37.7±53.0 spermatozoa \times 10^6/ml); the individual variability of these parameters was very high: semen volume ranged from 2 to 15.4 \( \mu l \) and spermatozoa concentration from 9 to
144 spermatozoa × 10⁶/ml. The seminal kinetic parameters in this species were generally rather poor (Table 3).

4. Discussion

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

Semen contamination may represent a problem in wild raptors kept in captivity [6], and many 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 ×10⁶/ml and 130.0±99.0 ×10⁶/ml,
respectively) but a much smaller semen volume (Peregrine falcon:27-208 μl [22]). Neither the
semenal 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 x10⁶/ml and 467.7±392 x10⁶ 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


### 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.

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

### 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.

<table>
<thead>
<tr>
<th>Species (N of birds)</th>
<th>Accipiter nisus (N=1)</th>
<th>Falco subbuteo (N=6)</th>
<th>Falco tinnunculus (N=5)</th>
<th>Bubo bubo (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of the ejaculate (µl)</td>
<td>2</td>
<td>3.60±2.41</td>
<td>2.78±1.27</td>
<td>8.97±4.03</td>
</tr>
<tr>
<td>Degree of contamination (1-4)</td>
<td>1</td>
<td>2.33±1.21</td>
<td>3.20±0.84</td>
<td>2.75±0.46</td>
</tr>
<tr>
<td>N spermatozoa × 10⁶/ml</td>
<td>5</td>
<td>130.0±98.9</td>
<td>295±190</td>
<td>37.7±53.0</td>
</tr>
<tr>
<td>N spermatozoa × 10³/ejaculate</td>
<td>10</td>
<td>160.0±56.6</td>
<td>614.0±352.3</td>
<td>453.8±862.3</td>
</tr>
</tbody>
</table>

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