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Original paper

Sex determination of eurasian woodcock (*Scolopax rusticola* L.) by genetic and imaging diagnostic methods

ATTILA BENDE[1*], NÓRA PÁLINKÁS-BODZSÁR[2], LÁSZLÓ BOA[3] & RICHÁRD LÁSZLÓ[1,]

[1] Department of Wildlife Biology and Management, University of Sopron, 9400 Sopron, Bajcsy-Zsilinszky út 4. Sopron, Hungary, [2] National Centre for Biodiversity and Gene Conservation, Gödöllő, Isaszegi út 200., 2100, Hungary, [3] BOA-VET Veterinary Medicine, Nagykanizsa, Teleki utca 56., 8800, Hungary.

Abstract
In the study of the behavioural ecology, migration, and habitat use of the Eurasian woodcock (*Scolopax rusticola* L.), the knowledge of sexes would be of great importance. In our work, we tested the sex determination possibilities - in terms of practicality, reliability, and cost-effectiveness - and developed a protocol for professionals dealing with woodcock that is easy to implement in the field and offers reliable sex determination without damaging the captured birds. While evaluating the applicability of each method, we compared the genetic sex determination procedure using blood and feather samples with the X-ray, ultrasound, and endoscopic sex determination possibilities of birds. For our study, blood and feather samples were collected from birds with known sex (dissected birds) in the vicinity of Sopron (Hungary) during autumn ringings and spring hunts. In terms of sampling and taking into consideration the stress affecting the birds and the cost-effectiveness as well, the analysis of DNA samples from feathers and blood proved to be the most favourable. Sex determination was 100% successful for both type of samples. The field sampling methodology required for the genetic analysis and proposed by us is simple, fast and gentle, easy to master, and at the same time, based on itsresults, DNA of suitable quality and quantity can be obtained for geneticsex determination. DNA can be isolated from freshly taken feather samples with high reliability, so we recommend that this easy-to-collect, well-stored sample type should be used in large sample population genetic analysis instead of difficult-to-store muscle tissue samples.

Keywords: molecular sexing sex determination, sampling procedure, blood sampling, feathers DNA, blood DNA, wading bird

Introduction

In the case of woodcock, there are just slight differences between sexes, so it is hard to separate them based on their appearance traits, plumage colouration and markings as well as leg colour are useless as sexual characteristics (Clausager 1973; Cramp & Simmons 1983; Ferrand & Gossman 2009). However, knowledge of sex would be of great importance in migration research and behavioural ecology studies because the behavioural patterns of males and females are different (Clausager 1973; Cramp & Simmons 1983). Clausager (1973) was the first to point out the usability of the quotient of central feathes of tail and beak length. Subsequently, a number of studies (McCabe & Brackbill 1973; Glutz von Blotzheim 1977; Artmann & Schroeder 1976; Rochford & Wilson 1982; Hoodless 1994; Sorace et al. 1999; Ferrand & Gossmann 2009; Aradis et al. 2015) have been carried out to determine sex based on individual body sizes (e.g., bill, tail, wing measurement, body weight), but based on these, genders cannot be distinguished with sufficient reliability. According to Glutz von Blotzheim (1977) a woodcock with a beak longer than 77 mm and a tarsus longer than 38 mm is most likely to be a female, but no information is given on the reliability of this method.

Stronach et al. (1974) used a binary regression formula to separate the sexes according to the length of the beak and tail. Their results show that the model can be used with 72% reliability for males and 75% for females. The probability of error was 28% if young birds were included in the analysis. Birds less than 12 months of age may have been excluded by examining the tips and proximal edges of outer primaries (ragged outline on first years; smooth on older birds, at least until April) and the terminal lighter bar on primary coverts (broader and browner on young birds). When all birds that had not yet undergone full molting were excluded, an accuracy of around 95–98% was achieved (Shorten 1975). In a similar study, Ferrand & Gossmann (2009) obtained worse results. Their results showed that males on average had shorter bills and longer tails than females. The authors pointed to the fact that there was so much overlap between the data that it was impossible to reliably determine the sex of the majority. They propose several criterions for sexing using bill, tail length and their ratio for both adult and juveniles. However, they also pointed out that method does not allow sexing to be more accurate than 45% for adult birds and 25% for juveniles.

Detailed statistical studies based on differences in morphometric data — linear models, discriminant and principal component analysis — did not provide definite results for other Charadriiformes species either (Remisiewicz & Wennerberg 2006; Schroeder et al. 2008).

According to Hoodless (1994), the difference in body weight between the sexes in the laying phase of the nesting period may be suitable for sexing some individual woodcocks, but Aradis et al. (2015) reported that the method is not sufficiently reliable even in this narrow time interval.

Furthermore, Aradis et al. (2015) reported that discriminant function analysis applied to a set of selected morphometric traits did not achieve 80% confidence in

* Corresponding author: A. Bende. Email: bende.attila@uni-sopron.hu

the case of juvenile and even adult woodcocks (79.6% for females and 77.1% for males).
In the course of our study, we looked for an easy-to-apply, low-cost, yet highly reliable method of sex determination in the field, comparing it with other alternative sex determination options, the applicability of which could also be considered in the case of woodcock. The procedures tested were ultrasound, X-ray, and invasive endoscopic analyses, as well as genetic sex determination from feather and blood samples. In the case of birds equipped with a high-value radiotelemetry transmitter, knowledge of the sexes during migration or in the evaluation of the data concerning habitat use would be particularly important in order to understand how males and females differ in their behaviour patterns.
Unfortunately, either in the case of birds equipped with transmitter or in the case of ringed birds, the sex is generally unknown, despite the fact that turning ups often involve the death of birds, and destructive procedures could be used. Due to the problems detailed above, it would be useful to determine the sex of the birds at the time of marking, so that we can get to know this species better by having sex-differentiated data sets.

Material and method

Sampling

The cover net method used to catch living woodcock has long been known in the ornithological literature (Bub 1996). But it was first used for woodcock only in 1976 (Mansoori 1977), despite the fact that this method proved successful as early as 1939 in the case of American woodcock (*Scolopax minor* L.) (Merovka 1939). The method became widely general in France first (Gossmann et al. 1988; Ferrand & Gossmann 1989, 1990; Gossmann & Ibanez 1991), and has been used in Hungary since the 2000s. At night, birds feeding in the open, typically on short-grass land, are searched for and captured with the help of a reflector and cover net/drop net (Glasgow 1958) (Figure 1).

Figure 1. Woodcock catch with cover net method in the field of Sopron Mountain (Photo: A. Bende).

During the live capture, we searched for feeding woodcocks around Sopron (Hungary, Coordinates: 47.644599, 16.579262) with the help of a 1300 lumen reflector and a thermal imaging camera (Pulsar Axion Xm38), and then approached the continuously illuminated bird, covering it with a 1-meter diameter net attached loosely to its frame at the end of an 8-meter-long telescopic rod.
For the success, it was crucial that the catchers worked well together, standing behind the light source from beginning to end, and that the cover net was above the light beam until the last minute, otherwise, the bird would easily notice the device and fly away.
After capture, blood was drawn from the wing vein *(vena cutanea ulnaris)* according to the blood sampling protocol for small birds (Owen 2011), for which the feathers on the upper arm were not removed, only smoothed with wet cotton wool to make the vein clearly visible. Blood samples were taken with a 2 ml syringe and a 25G needle. Due to the high venous pressure and thin skin, a smaller hematoma may develop around the blood collection area, so after the blood collection, the bleeding was tamponed with dry sterile cotton wool while keeping the bird in a stable position, so the bleeding stopped quickly and the bird was able to be released after a few minutes. The amount of blood drawn was approximately 0.5–1 ml, which contains enough DNA for a successful genetic analysis (Harcourt & Brown 2000) (Figure 2).

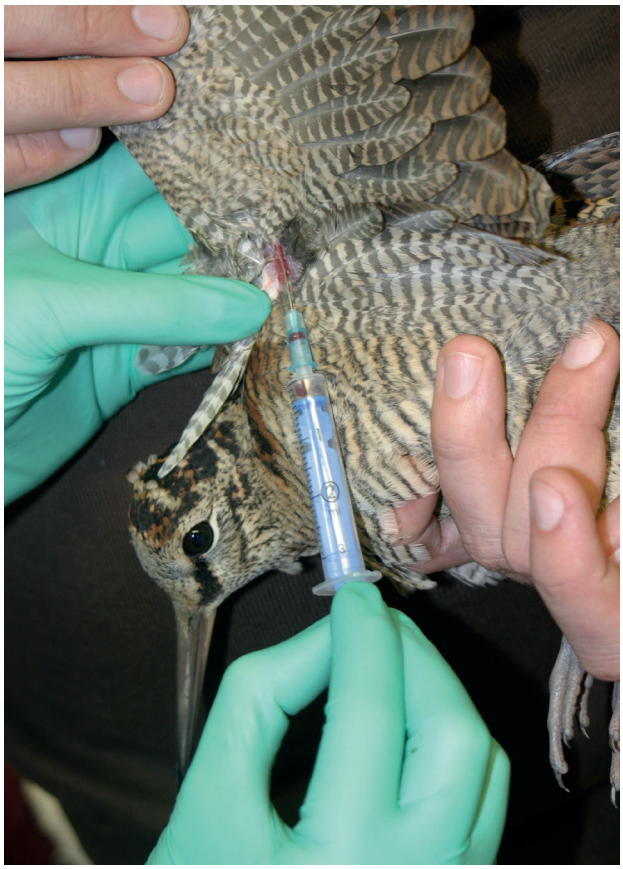

Figure 2. Blood sampling from the wing vein of the Woodcock (vena cutanea ulnaris) (Photo: A. Bende).

The blood sample was collected in a syringe and in blood collection tubes filled with anticoagulant (Na-EDTA)

solution used in haematology test. Autumn samples were stored refrigerated (5 °C) for 5 days, while spring samples were stored frozen (-20 °C) for several months as an effect of delayed processing due to the pandemic situation. In our experience, the efficiency of the method was not affected by any of the applied sample storage methods, as the sex determination could be performed with 100% success in the case of blood samples stored for a short time without anticoagulant solution and even in the case of Na-EDTA blood samples frozen for months. To prevent DNA degradation, if analyses cannot be performed within a few days, it is recommended that the samples should be frozen. The feather samples required for genetic analysis were also collected from the primaries of birds bagged in March during the spring sampling in the Sopron area, which contained enough blood for the study.

Vili et al. (2009) found that fresh feathers were best suited for sex determination because in the case of shed feathers the quality of extractable DNA deteriorates sharply in a few months. Feather samples (3 scapulars from each bird) were stored in the same manner and for the same time as blood samples until genetic analysis. For genetic analysis, the superior umbilicus was removed with a scalpel to make the blood on which DNA extraction is based available (Horváth et al. 2005). The wing feathers and blood samples were not collected from the same birds, but the sex was known in all cases because the samples were collected from birds taken during the hunt.

For the dissection, we used woodcocks captured during the hunt for monitoring purposes. The woodcock can be hunted for research purposes in Hungary during the spring migration of the species in accordance with the law 79/2004. (V. 4.) FVM, appendix 1. Blood and feather samples collected from these birds provided an opportunity to verify the genetic identification method. They have also been used as a reference in imaging laboratory diagnostic tests to verify the reliability of each method tested.

By virtue of the fact that the research is conducted on wild species, we acted in accordance with Section 1§ 4 (f) of Government Decree 40/2013 (14.II.) on the regulation of animal experiments, which states that the research does not qualify as animal experimentation, and therefore does not require a permit.

Sex determination by genetic methods

The performed genetic analysis is based on different sex chromosomes, because in the case of birds, the females have heterogametic (WZ) and the males have homogametic (ZZ) sex chromosomes. With the help of the applied method, individual sexes can be separated by detecting sequences specific for the W chromosome, because in the female sex - in the case of most species - the so-called CHD-W gene binding to W encodes a chromo-helicase DNA binding protein (Griffiths & Tiwari 1995). In males Z-chromosome-binding version (CHD-Z) is also known. These sex-linked genes are located outside the recombinant pseudoautosomal region of the sex chromosomes, so they are least variable and, due to this property, suitable for defining sexes (Fridolfsson & Ellegren 1999). In our analysis, DNA samples from a total of 20 birds were extracted from blood samples (n=5 captured alive bird [November 2019.] n=15 hunted bird [March 2020.]) by the conventional salting out method modified for birds (Bodzsar et al. 2009; Miller et al. 1988). The DNA isolation protocol of the collected feather samples (20 individuals, 3 feathers from each bird) differed in that the bloody feather ends had been added directly to the seed lysis-SDS mixture (Bodzsar et al. 2009) by adding proteinase-K enzyme, followed by a so-called digestion process (56 °C, overnight). Concentrations of DNA samples were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Then, they were equalized to 20 ng/µl density and stored frozen at -20 °C until further use. DNA-based sex determination was performed using the P2/P8 primer pair designed by Griffiths et al. (1998), which amplifies DNA fragments of different sizes on the aforementioned CHD-Z and CHD-W (Chromobox-Helicase-DNA-binding) genes; this results in a fragment of one size in the male sex and two in the female. The final volume of 15 uL master mix contained 10x Dream Taq Buffer with 20mM $MgCl_2$ (Thermo Fisher Scientific), 5 µM primer, 25mM dNTP mix (Thermo Fisher Scientific), 20 mg/ml BSA (Bovine Serum Albumin, Thermo Fisher Scientific), 5U/µL Taq DNA polymerase (DreamTaq DNA polymerase, Thermo Fisher Scientific) and 100 ng genomic DNA. PCR profile was determined according to the protocol reported by Griffiths et al. (1998) with some modifications: 95°C for 4 min denaturation followed by 30 cycles of amplification: 94°C for 30 sec, 48°C for 45 sec and 72°C for 45 sec and final extension at 72°C for 5 min (Kyratec Trinity Supercycler). The PCR products were detected on 1.5 % agarose gel (Bio-Rad) using 10000x GelGreen® nucleic acid stain (Biotum) with gel electrophoresis.

Imaging diagnostic procedures

The imaging laboratory diagnostic procedures were tested on individuals from which blood and feather samples were obtained (n=15 hunted bird, n=5 captured bird). Endoscopy was performed on non-living birds (n=4) that were also hunted during spring sampling.

The possibility of physical analysis of the reproductive organs of birds is greatly limited due to their physiological and anatomical features. As an alternative, the applicability of widespread imaging diagnostic procedures (ultrasound, X-ray, and invasive endoscopy) to determine the sex of the woodcock is brought up. The analysis necessary for the evaluation and comparison of the methods were performed by veterinarians dr. László Boa and dr. Fanni Molnár.

For the **X-ray analysis** at the Sopron Veterinary Center, we used a Gierth RHF 200 ML portable X-ray machine with Jungwon Precision Ind. Co. LTD X-ray cartridges and green-sensitive intensifying screens (speed: 400) with Retina XOE green-sensitive films. In the ventrodorsal

position, the bird was placed with its back on the cassette, with wings fixed to the side, legs pulled slightly back and to the side, and head turned to the side and fixed at the jaw joint. In the latero-lateral position, pictures were taken, with the wings of the birds placed consistently to the right, folded out, and fixed above the body in the direction of the back. Taking latero-lateral radial images in the case of live wild birds may even lead to spontaneous respiratory arrest due to the increased stress situation, so the study should be performed with caution (Molnár et al. 2007).

For **ultrasound diagnostic analysis**, a Mindray Digiprince DP-6900 Vet mobile ultrasound device with a micro-convex transducer at 8.5 MHz was used at the Sopron Veterinary Centre. The analysis method was tested on freshly captured woodcocks during spring sampling. There are only two areas on the body that provide a suitable echo-window for the analysis. These are the ventromedial part of the abdominal wall between the processus xiphoideus of the sternum and the pelvic bone, and in the parasternal direction on the dorsolateral side of the abdomen between the femoral joint and the last rib arch (Beregi 2007). In our own analysis, we examined the body cavity of the woodcock using a probe behind the caudal end of the sternum in the dorsally laid birds, slightly to the right of the midline, thus bypassing the gizzard, which degrades the quality of imaging due to the food residues it contains.

Invasive endoscopy is an expensive procedure that can also be used in wild bird research, one of the indications of which is typically the separation of the sexes in bird species without sexual dimorphism. In most bird species, only the left ovary, including fallopian tube, develops, so the standard endoscopic entry site is situated on the right side. The body cavity of the bird placed in the lateral position was penetrated behind the last rib, below the musculus flexor cruris medialis muscle and the os pubis, in front of the cranial edge of the vastus muscle (Taylor 1994). For the analysis of live birds weighing around 300 g, isoflurane anaesthesia without premedication is recommended; it is used successfully in the vast majority of bird species (Sós et al. 2007). The woodcock endoscopic analyses used in the comparison were performed on four specimens by dr. László Boa at the Veterinary Clinic of the Budapest Zoo & Botanical Garden with a Karl Storz type rigid endoscope, with a viewing angle of 0–30 degrees and a diameter of 2.7 mm.

Results and Discussion

Sex determination from feather and blood samples

Genetic analyses are gaining ground in ornithological research, but they are still not considered commonplace. One common type of genetic analysis targets sex determination (Fridolfsson & Ellegren 1999; Hipkiss et al. 2002), as many bird species have no visible sex dimorphisms. In the case of woodcock, the possibilities of the separation of sexes by genetic methods have also been studied (Väli & Elts 2002; Vučićević et al. 2012). However, these studies do not approach the issue from a field applicability perspective. Non-invasive sampling can be carried out without significant disturbance of the analysed specimens, but at the same time in the case of the woodcock - since it is a rare nesting species in Hungary, appearing in larger numbers only during its autumn-spring migration (Hadarics & Zalai 2008) - finding moulted feathers is uncertain, and the DNA obtained from them is of poorer quality than the sample obtained by blood sampling after capturing the animals during the semi-invasive procedure (Taberlet & Luikart 1999). In all cases, usable amounts and qualities of DNA could be extracted from the blood samples we collected, stored, and processed, so sex determination was successful in all cases. Of the 20 samples, 8 proved to be females and 12 specimens to be males (Figure 3).

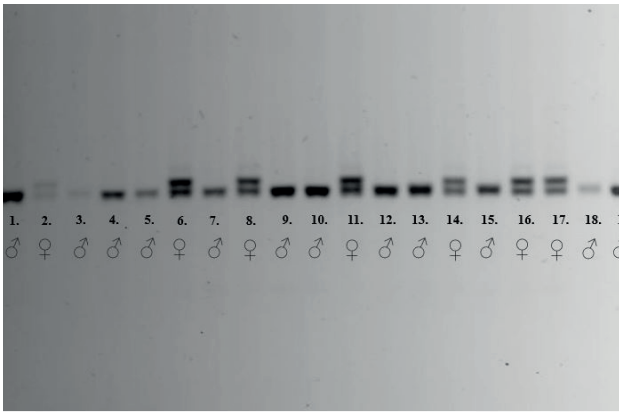

Figure 3. Sexes detected as a result of gel electrophoresis from blood samples during the PCR reaction used (Photo: N. Pálinkás-Bodzsár).

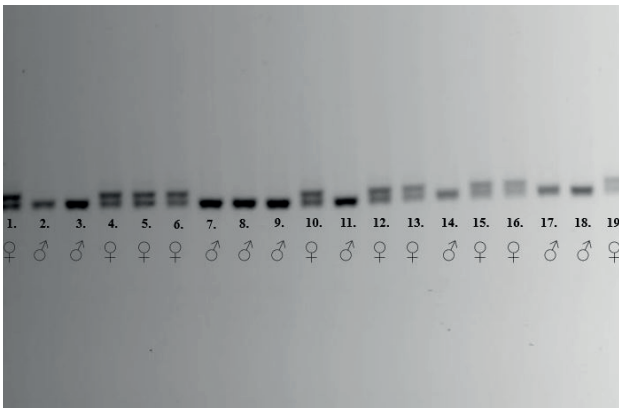

Figure 4. Sexes detected as a result of gel electrophoresis from feather samples during the PCR reaction used (Photo: N. Pálinkás-Bodzsár).

The method is recommended for ringing, and for determining the sex of birds marked with a telemetry transmitter. The sampling method can be mastered with a little practice, and the collection of samples does not require significant equipment, so the procedure can be carried out easily and safely in the field during the marking. In the case of feather samples, all PCR reactions were

evaluable during sex determination. Of the 20 samples analysed, 11 were females and 9 were males (Figure 4).
Efficiency is greatly enhanced by DNA extraction from fresh feather samples (Väli & Elts 2002), but at the same time removal of primaries from live birds is not recommended due to the significant stress effect.
Based on our results, fresh feather samples after hunting utilization may be optimal for genetics studies, since sufficient quality and amount of DNA can be extracted from the samples for analysis and sampling does not require special training and sampling equipment, so a wide range of data providers can be included in the sampling. The easily collected, deep-frozen, easy-to-store feather samples could be used to replace muscle tissue samples collected in a 2 ml "Micro test tube" with 70% concentration of ethyl alcohol, in Hungary in 2015 during the National Eurasian Woodcock Monitoring Program.

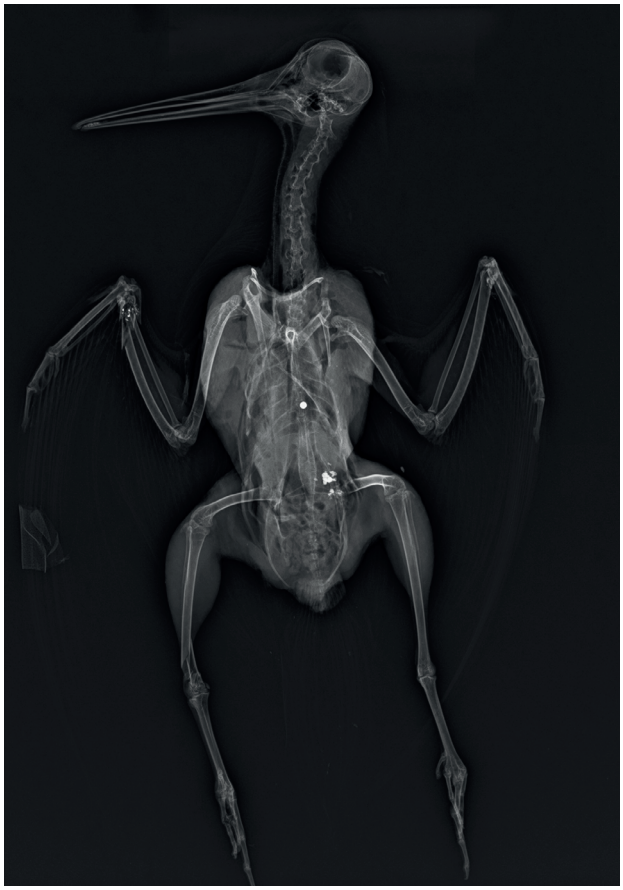

Figure 5. Ventrodorsal radiological recording of the Eurasian woodcock (Scolopax rusticola L.) (Photo: A. Bende).

Imaging diagnostic procedures
The applicability of these methods is significantly influenced by the age of the birds to be analysed and the time of the analysis, since the possibility to detect juvenile or inactive adult gonads by imaging diagnostic procedures is severely limited. The juvenile ovary is flat, elongated, finely granular in structure, and during maturing the similarity to a bunch of grapes due to the differently developed follicles will become more and more dominant. Fully or almost fully developed eggs also increase the chances of sex detection. Juvenile testicles are better visualized as they stand out more, so even both gonads can be identified on the images. Based on the above, these abdominal organs can be analysed with radiological methods with greater success in the sexually active phase and in adults due to their hyperplasia.
During the **X-ray analysis**, the dense plumage covering the body and the internal organs having prominently indistinguishable contrasts made the evaluation of radiological recordings more difficult (Figure 5). In the recordings, even the gizzard is not always a reliable orientation point, since in the case of woodcock it rarely contains solid mineral parts of lime structure. Molnár et al. (2007) found that gonads between the lungs and kidneys had been observable only occasionally. In our X-ray analysis, we were able to successfully determine the sex of the specimen in only a few cases. While analysing a live bird, proper fixation is a basic criterion for successful recording, which must be done very carefully, taking into consideration the stress sensitivity generally characteristic of wild birds. Based on the above, due to its low reliability, this method is not recommended for determining the sex of the woodcock.

Ultrasound diagnosis is of less importance in birds than in mammals for anatomical reasons. This is explained in part by the dense plumage and in part by the abdominal and anterior/posterior thoracic air sacs systems with high air content in the bodies of birds, and the intestinal tract with the gizzard, which also significantly reduces the quality of ultrasound imaging. According to Faragó et al. (2000) results, in the case of woodcocks weighing about 350–400 g, the gonads (paired testicles of several centimetres and ovaries with a few millimetres of well-developed follicles and oocyte) are quite small and difficult to image even in the state of sexually active hyperplasia. It is likely that eggs formed in the fallopian tube, especially in the advanced state of egg development, are clearly visible during ultrasound imaging. The performed analyses have shown that the applicability of the non-invasive ultrasound procedure is limited. In the case of the analysed woodcocks (n=20), in no case was the genital organ recognized with complete certainty.

Invasive endoscopy analyses were previously performed by dr. László Boa, during which the genitals could be well visualized in all cases (n=4), which proves that endoscopy would be an applicable procedure for the clear determination of the sexes in the research of the woodcock. It is an invasive procedure that can be used with relatively little risk; it is safe, and provides immediate results, with the disadvantages of anaesthesia and high infrastructure requirements, which cannot be provided in all cases and not necessarily immediately and is a significant cost. Bleeding may be mentioned as a risk factor, which in part impairs visibility and in part may endanger the analysed individual. In case

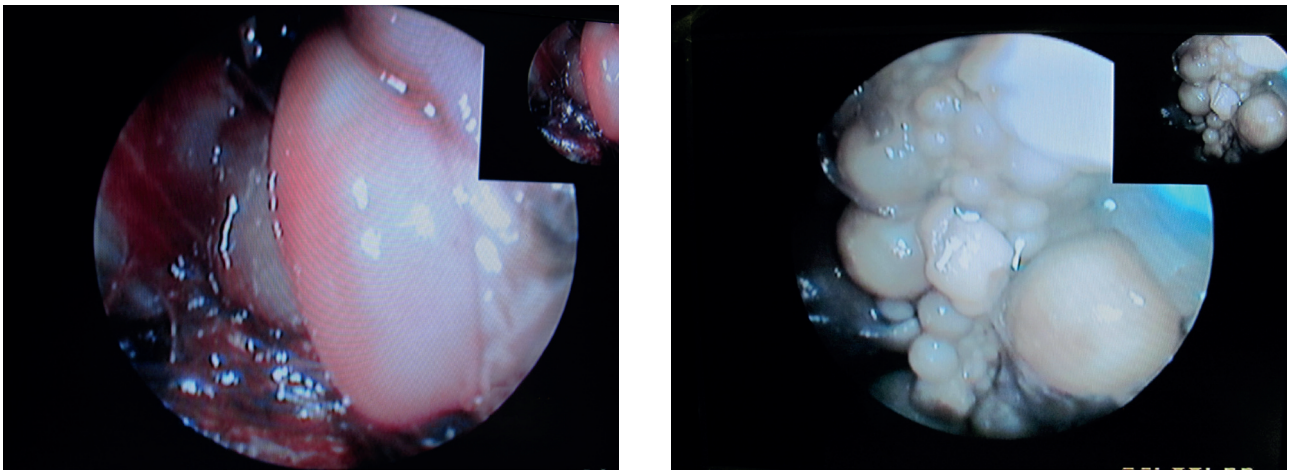

Figure 6. Invasive endoscopic analysis of the Eurasian woodcock (*Scolopax rusticola* L.). a) shows the female´s reproductive organs, b) the male's reproductive organs (Photo: L. Boa).

of bleeding, the analysis should be interrupted to clean the optics and to detect the location of the bleeding. The advantage of the method is mentioned by Sós et al. (2007) that the coelomic cavity of birds is relatively resistant to bacterial infections, so the likelihood of septic complications is minimal. However, in our view, the practical applicability of the method is limited given its high infrastructural and anaesthetic needs, the stress affecting the captured birds, and also the costs (Figure 6a, b).

Proposed sampling and sex determination procedure

We recommend an easy-to-perform, easy-to-learn protocol for sex determination in the following terrain during ringing and telemetry transmitter marking of the woodcock:

Take blood after capturing the woodcock with a cover net in addition to the data recorded during the usual ringing; this requires two people. One keeps the bird stable, while the other draws a few drops of blood (0.5 to 1 ml) from its wing vein *(vena cutanea ulnaris)* with a 2 ml syringe and a 25G needle, according to the sampling protocol for small birds.

Do not remove feathers from the blood collection area; smoothing aside with 70% alcohol cotton wool will make the vein clearly visible.

Keep the bird stable, as the bird trying to escape can be easily injured, and high venous pressure around the blood collection area can cause hematomas. After blood collection, the bleeding is tamponed with 70% alcohol cotton wool so that the bleeding stops quickly and the bird can be released after a few minutes.

The blood sample can be stored in blood collection tubes filled with anticoagulant (Na-EDTA) solution or even in a sampling syringe. Chilled (5 °C) samples should not be stored for more than a week. Frozen (-20 °C) samples can be stored for up to months without DNA damage. The genetic laboratory will perform sex determination from blood samples within a few days to make the sex of the marked live birds known, allowing gender-differentiated processing of research results.

Removal of feathers from live birds is not recommended. Fresh samples of feathers, which are suitable for population genetics studies with a large number of elements, should be collected after the hunt. These feather samples could be used to replace muscle tissue samples that are difficult to collect and store.

Acknowledgments

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