Video Article

Avian Semen Collection by Cloacal Massage and Isolation of DNA from Sperm

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Abstract

Collection of semen may be useful for a wide range of applications including studies involving sperm quality, sperm telomere dynamics, and epigenetics. Birds are widely used subjects in biological research and are ideal for studies involving repeated sperm samples. However, few resources are currently available for those wishing to learn how to collect and extract DNA from avian sperm. Here we describe cloacal massage, a gentle, non-invasive manual technique for collecting avian sperm. Although this technique is established in the literature, it can be difficult to learn from the available descriptions. We also provide information for extracting DNA from avian semen using a commercial extraction kit with modifications. Cloacal massage can be easily used on any small- to medium-sized male bird in reproductive condition. Following collection, the semen can be used immediately for motility assays, or frozen for DNA extraction following the protocol described herein. This extraction protocol was refined for avian sperm and has been successfully used on samples collected from several passerine species (*Passer domesticus*, *Spizella passerina*, *Haemorhous mexicanus*, and *Turdus migratorius*) and one columbid (*Columba livia*).

Video Link

The video component of this article can be found at https://www.jove.com/video/55324/

Introduction

Birds are ideal subjects for studies involving sperm quality and competition[1], sperm telomere dynamics[2], epigenetics[3], and similar topics, as they are widely used in biological research and sperm can be easily sampled using cloacal massage. Cloacal massage is a gentle, non-invasive manual technique for collecting semen from birds[4,5,6]. Repeated samples can be obtained easily and no special tools are required, making it simple to perform in the field or lab. Although cloacal massage has been in use for decades, it is difficult to learn from the available written descriptions. This publication is intended to reduce the time and uncertainty involved in learning cloacal massage.

Semen collected from birds using cloacal massage can be used immediately for motility assessment[4,7] or artificial insemination[8], or frozen for other uses such as advanced imaging and DNA extraction. Semen samples from passerine birds are small but contain densely packed sperm. DNA is extracted using a commercial extraction kit for simplicity, with modifications to overcome the specialized protective features of sperm[9]. After extraction, sperm telomeres can be measured using qPCR[10].

Protocol

This protocol involves vertebrate animal subjects and has been approved by the Institutional Animal Care and Use Committee (IACUC) at North Dakota State University.

1. Semen collection from a passerine bird using cloacal massage

NOTE: Cloacal massage is an effective semen collection technique on reproductively active birds only, but can be performed successfully outside of the breeding season in appropriate captive situations. Reproductive activity should be determined for target species prior to using cloacal massage. This technique can be used on wild birds upon capture or on birds held in captivity.

1. Starting with a male bird, (in breeding condition) bander's grip in the non-dominant hand, transfer the bird to the dominant hand with its ventral side touching the palm (**Figure 1**).
    1. Ensure that the bird's head is nearest to the lateral edge of the palm. Lightly secure the head and body using the pinky, ring, and middle fingers. This leaves the bird's vent and tail exposed.
    2. Leave the bird's legs loose or lightly restrained using the palm.

Figure 1: Grip for cloacal massage. The bird is secured lightly in the dominant hand with its ventral side touching the palm and head secured by the pinky finger. Please click here to view a larger version of this figure.

2. Place the dominant index finger and thumb on either side of the superior end of the cloaca and lightly pinch the fingers together while applying very slight pressure toward the tail (**Figure 2**). The cloaca will evert, exposing the cloacal mucosa (pink interior).
   NOTE: If the bird is not currently producing semen, the cloacal protuberance can be small and the cloaca is difficult to evert. With experience, it is easy to differentiate between the cloacas of reproductively active and inactive males[5,11].

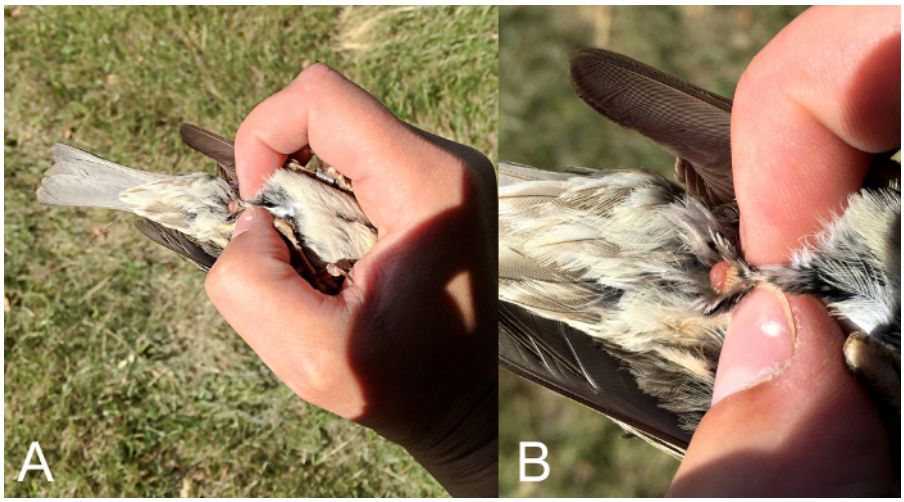

Figure 2: Finger position and cloacal protuberance. Evert the cloaca by applying slight pressure to the superior end (A). In a reproductively active male passerine, the cloacal protuberance is obvious and firm (B). Please click here to view a larger version of this figure.

3. With the non-dominant hand, place the pad of the index finger on the dorsal side of the base of the tail to stabilize the hand, and then place the tip of the thumb just below the everted cloaca.
   NOTE: A short and smooth thumbnail is advantageous for performing cloacal massage and protects the bird from accidental injury.
4. Move the non-dominant thumb deep and cranially in a repetitive motion with medium pressure, staying below the cloaca. Continue until the bird ejaculates. Depending on the individual, this typically takes between 1 and several dozen strokes.
5. As soon as the bird ejaculates, collect the semen in a microhematocrit tube. Repeat cloacal massage to obtain multiple ejaculates.
   NOTE: Passerine semen is light brown and typically has a somewhat thick consistency. White or dark substances ejected from the cloaca are not semen, and may contaminate the semen sample. The appearance of a small amount of blood on the cloaca or mixed in with the semen is usually caused by too much pressure during massage.
6. For DNA extraction, place the semen into a microcentrifuge tube with 20 µL of 1x PBS (pH 7.4). NOTE: Semen stored in 1x PBS is not suitable for artificial insemination, but can be used for DNA extraction at a later date when stored at -80 °C. The PBS can be room temperature or chilled, but should be in a liquid state when the semen sample is deposited.
   1. Confirm the presence of sperm in the sample by viewing a small amount of the diluted sample under a compound microscope (**Figure 3**).
7. Store the diluted semen at -80 °C until extraction.

2. Extraction of DNA from bird semen

NOTE: This protocol has been tested with multiple commercial DNA extraction kits, but it has only been successful when used with one DNA kit[12]. Steps that are modified from the kit protocol are indicated by *.

1. Prepare a 1 M solution of dithiothreitol (DTT) in water and mix using a vortex.*

1. Aliquot the DTT solution and store it at -20 °C until immediately before use. Each sample to be extracted requires 10 µL of 1 M DTT solution.*

2. Prepare a buffer containing 20 mM Tris-Cl, pH 8.0; 20 mM EDTA; 200 mM NaCL; and 4% SDS. Each sample to be extracted requires 90 µL of this buffer.*
    1. Store the buffer at room temperature. If it has precipitated, warm it to 56 °C to dissolve.*
3. Preheat an incubator containing a rocker or shaker to 65 °C.*
4. Immediately before beginning the extraction, mix 10 µL DTT solution with 90 µL of the buffer prepared in step 2.2 for each sample to be extracted, *e.g.* 900 µL buffer plus 100 µL DTT solution if extracting 10 samples. This mixture will be called DTT-buffer.*
5. Add 70 µL 1x PBS to the entire diluted semen sample and mix well using a vortex.*
6. Add 100 µL DTT-buffer and 10 µL proteinase K solution to the sample. Vortex for 20 seconds, then incubate in the preheated incubator for 1 hour on a rocker or shaker.*
7. Add 200 µL buffer AL and 200 µL fresh EtOH (100%) and vortex for 20 seconds.*
8. Transfer the entire sample to a spin column and centrifuge 1 minute at 6,000 x g. Discard the collection tube.
9. Place the column on a new tube and add 500 µL prepared buffer AW1. Centrifuge 1 minute at 6,000 x g. Discard the collection tube.
10. Place the column on a new tube and add 500 µL prepared buffer AW2. Centrifuge for 1 minute at 6,000 x g. Discard the flow-through and place the column back on the tube.
11. Centrifuge 3 minutes at 20,000 x g. Discard the collection tube and place the column on a 1.5 mL microcentrifuge tube.
12. Pipet 35 µL of AE buffer directly onto the column membrane and incubate at room temperature for 5 minutes.*
13. Centrifuge 1 minute at 14,000 x g. Discard the spin column and store extracted samples at -80 °C.

Representative Results

Cloacal massage and DNA extraction using the described protocol has been performed on several passerine species and one columbid (**Table 1**). The presence of sperm in semen samples collected by cloacal massage was confirmed by viewing a small amount of the diluted samples under a compound microscope at 400X magnification (**Figure 3**). After extracting DNA from semen samples collected from house sparrows using the protocol described here, eight samples were run on gel electrophoresis with a DNA ladder (three samples can be seen in **Figure 4**). Distinct bands without smearing were seen in all eight of the sample lanes, indicating successful isolation of high quality DNA. All other samples extracted using this protocol were assessed for DNA concentration using a spectrophotometer (See **Table 1**).

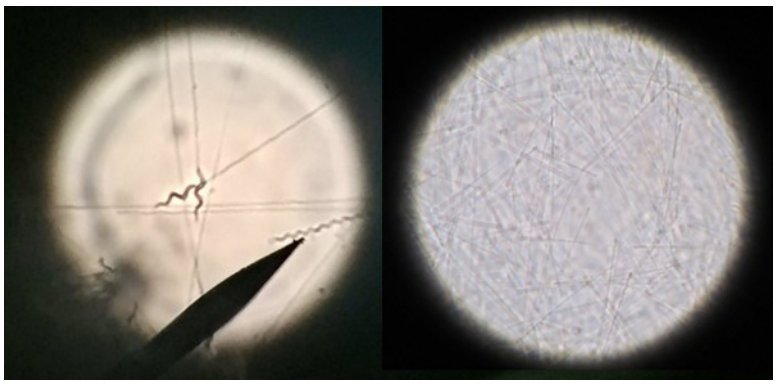

Figure 3: **Sperm from two small passerines.** These sperm samples were obtained by cloacal massage from a house finch (*Haemorhous mexicanus*), left, and a house sparrow (*Passer domesticus*), right, trapped in Fargo, ND in 2016. The samples were each diluted in 20 µL PBS and viewed under a compound microscope at 1,000X and 400X magnification, respectively. Please click here to view a larger version of this figure.

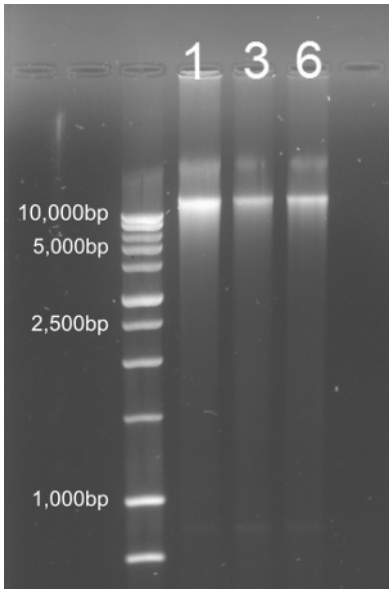

Figure 4: DNA gel electrophoresis of sperm DNA. Three samples of DNA extracted from house sparrow semen run on a gel with a DNA ladder. Note the presence of distinct bands, indicating successful extraction of high quality DNA. Please click here to view a larger version of this figure.

	House sparrow (*Passer domesticus*)	Chipping sparrow (*Spizella passerina*)	House finch (*Haemorhous mexicanus*)	American robin (*Turdus migratorius*)	Rock pigeon (*Columba livia*)
Number of samples collected	156	3	2	2	2
Number of samples extracted	90	3	2	2	2
Semen collection success rate	98%	75%	100%	67%	67%

Table 1: Species and samples collected and extracted to date. At the time of publication, samples have been processed using the described protocol from 4 passerine species and 1 columbid. Semen collection success rate refers to the percentage of each for which a sample was successfully obtained out of the total number attempted.

Discussion

We describe a simple and reliable method for collecting semen from small and medium birds in reproductively active condition, and extracting DNA from avian sperm.

The described semen DNA extraction protocol is modified from a kit for simplicity, but was refined for use on avian semen. Sperm are resistant to lysis by standard extraction chemicals[9,13], and semen samples collected from passerines are fairly small (typically a few microliters). These factors present challenges that are successfully overcome by using an appropriate concentration of DTT and incubating at 65 °C. Using this method, we have consistently isolated usable amounts of DNA for qPCR analysis of sperm telomere length (typically between 40 - 250 ng/µL, depending on ejaculate volume).

DTT has previously been used to extract DNA from avian sperm in combination with a phenol/chloroform reaction[13]. Phenol/chloroform reactions are effective for isolating DNA, but they are more time consuming, are more hazardous and require a fume hood. A notable limitation of our extraction protocol is that it has only been successful when used with one brand and type of extraction kit, possibly due to the small volume of starting sample. Very small semen samples (less than 1 µL) or samples with low sperm density may prevent successful extraction of DNA due to insufficient DNA present in the sample, but we have not encountered this problem when cloacal massage is performed on birds in breeding condition. To avoid this issue, cloacal massage can be repeated immediately to collect multiple ejaculates that can be pooled for extraction.

Cloacal massage has been in use for decades and in many species[1,4,5,6,7,15,16,17], and is a quick and effective means of collecting semen from birds in many cases. For studies involving wild or untrained birds that can be captured, it is more effective than collection methods that rely on convincing a male bird to copulate with an item and deposit his semen willingly[4]. Another semen collection method is by dissection of the testes or seminal glomera, but this is not an option for conservation purposes or repeated sampling[4,14]. The primary limitation of cloacal massage is the difficulty in learning the technique without demonstration. Written descriptions are often insufficient to understand and learn to implement the process. By illustrating cloacal massage in detail, we hope to reduce learning time and difficulty for those wishing to learn the technique.

Disclosures

The authors declare that they have no competing financial interests.

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