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DIFFICULTIES STORING AND PRESERVING TYRANT FLYCATCHER BLOOD SAMPLES USED FOR GENETIC ANALYSES[1]

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Abstract. We stored blood samples of Eastern Phoebes (*Sayornis phoebe*) in a lysis buffer ("QLB") that has been used successfully to preserve blood samples of many other species. We found that although samples from adults were not affected greatly, samples of nestling blood stored for more than a few days did not reliably produce the quantity and quality of DNA useful for multi-locus DNA fingerprinting. We also were unable to extract usable DNA from blood samples collected from Eastern Kingbird (*Tyrannus tyrannus*) nestlings, but obtained usable DNA from blood of Least Flycatcher (*Empidonax minimus*) nestlings stored for more than a year. We recommend that anyone planning DNA research with tyrant flycatchers should conduct their DNA extractions as soon as possible after collection. A pilot study to test methods of storage, preservation, and extraction may be necessary before beginning a large-scale project.

Key words: blood preservation, DNA fingerprinting, paternity analysis, tyrant flycatchers.

Molecular genetic analysis of DNA samples has become a common complement to avian field studies. Although the processing of DNA samples is often the responsibility of molecular biologists, the collection, preservation, transportation, and storage of DNA samples often falls to the field researcher. The technique used to store DNA samples in the field needs to be reliable, stable, easy to use, and, ideally, broadly applicable. Seutin et al. (1991) developed "Queen's Lysis Buffer" (QLB) and found it suitable for avian blood storage without refrigeration. Furthermore, avian blood stored in QLB yielded the high volumes of quality DNA needed for multi-locus fingerprinting, making it ideal for field workers to use when collecting samples for molecular genetic analysis. In this paper, we document difficulties with preserving blood sampled for DNA studies from Eastern Phoebe (*Sayornis phoebe*) nestlings. We provide evidence that similar problems may occur with nestling blood of other tyrant flycatchers and suggest that to minimize loss of data, DNA should be extracted from tyrant flycatcher blood samples as soon as possible after they have been collected.

METHODS

We conducted this study near the Queen's University Biological Station, Chaffeys Locks, Ontario, Canada (44°34′N, 76°9′W) from May to August 1993 and April to August 1994. In 1993, we collected 50–225 μl of blood in 50 μl nonheparinized capillary tubes by brachial venipuncture from 21 adult phoebes. From 64 nestlings, we collected 10–220 μl blood samples from the tibiotarsal vein or the brachial vein in 25 μl nonheparinized capillary tubes. We estimated the volumes collected to the nearest 5 μl from the portion of the capillary tubes filled. The blood samples were suspended in 1 ml of QLB in 1.5 ml microcentrifuge tubes and stored at 4°C for 1–16 weeks ($\bar{x}$ = 61.2 days) until we performed the DNA extractions on them.

In 1994, we collected 26 adult samples and 86 nestling samples, but we collected no more than 150 μl per microcentrifuge tube to keep the buffer : blood ratio high. With nearly all samples, the 3-day extraction process was begun as quickly as possible and all extractions were completed within 3 to 8 days ($\bar{x}$ = 3.2 days) of collection. In both years, the tubes of QLB were obtained from a common pool shared with other researchers conducting concurrent studies on Eastern Bluebirds (*Sialia sialis*) and Tree Swallows (*Tachycineta bicolor*).

To test for the effects of longer storage in 1994, we collected two 50 μl samples in QLB from each nestling in two broods of five nestlings, when the nestlings were 10 days old. One-half of the duplicate samples were extracted immediately and the other half were stored at 4°C for two weeks to examine how storage time affected the DNA extracted. We also collected blood samples in QLB from eight Least Flycatcher (*Empidonax minimus*) nestlings (two broods) and five Eastern Kingbird (*Tyrannus tyrannus*) nestlings (two broods), to determine whether the blood storage techniques worked in related flycatchers.

We extracted the DNA from our blood samples following the general methods of Sambrook et al. (1989). Specific details of the procedure may be found in Meek et al. (1994) and Põldmaa et al. (1995). The DNA was extracted and purified from our blood samples using two phenol/chloroform (70:30) extractions and one chloroform extraction (Põldmaa et al. 1995). We precipitated the DNA using 3 M sodium acetate

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(pH = 5.3, ~1/10 volume) and 2-propanol (~1 volume). The visible, fibrous strands of DNA that appeared were spooled out on a sterile glass rod and air dried. The DNA was then resuspended in 1.0 ml of TNE2. We called DNA that was recovered this way "spoolable."

If an extraction failed to produce spoolable DNA, we added more 3 M sodium acetate and 2-propanol (~1/2 original volume of each added), placed the samples at −20°C overnight, and then centrifuged them at 3,000 rpm for 30 min. The pellet of DNA obtained was air dried and resuspended in TNE2 as above. DNA recovered by these additional procedures we called "unspoolable." Note that even unspoolable samples produced some DNA.

DNA quality and quantity were assessed by electrophoresing 4 μl aliquots of the stock suspension on 0.8% Agarose gels, staining the gels with ethidium bromide, and visualizing the DNA under short wavelength UV light (Seutin et al. 1991). To assess quantity, we cleaved a 4 μl aliquot with *Eco*RI (Promega Corp., Madison, Wisconsin), following the manufacturer's instructions, and visually compared the resulting profile with a standard of known concentration in an adjacent lane. We estimated the concentration of DNA in each sample and calculated the yield of DNA in μg. Means are presented ± SE.

RESULTS

The method of DNA storage (QLB and refrigeration) used in this study and the extraction technique followed are usually very reliable. In fact, all 47 of the adult blood samples collected provided spoolable DNA and samples of Eastern Bluebird (MacDougall-Shackleton et al. 1996) and Tree Swallow (Barber et al. 1996) nestlings collected in the same batch of QLB on the same days using the same procedure all produced spoolable DNA. The average yield per adult was 245 ± 9 μg (n = 47). In general, spoolable samples contained reasonable quantities of high molecular weight DNA.

However, 43/64 (67%) of the nestling samples in 1993 did not produce spoolable DNA. The DNA yield was significantly less for unspoolable (73.6 ± 9 μg, n = 43) than spoolable samples (149.6 ± 9 μg, n = 21; $t_{62} = 5.2$, $P < 0.001$). DNA from unspoolable samples was usually sheared, of low molecular weight, and proved not to be useful for multi-locus fingerprinting.

We gave two of the 1993 families (nine nestlings, four parents) of Eastern Phoebe samples to another researcher (C. Barber, Queen's University). She processed Tree Swallow and Eastern Phoebe samples simultaneously using her own reagents. All of the Tree Swallow samples and all of the adult Eastern Phoebe samples produced spoolable DNA, whereas only two of the Eastern Phoebe nestling samples did. Furthermore, we successfully performed extractions of Tree Swallow nestling samples (unpubl. data) and Bell Miners (*Manorina melanophrys*; Conrad et al. 1998), using the same reagents and protocol. Technique of the researcher and differences in reagents cannot account for the poor yields from nestling samples.

There was a significant negative correlation between the DNA yield and the number of days between collection and extraction of the samples for nestlings in 1993 (controlling for blood volume collected, partial correlation, $r_{61} = -0.53$, $P < 0.001$) but not for adults (partial correlation, $r_{18} = -0.13$, $P > 0.50$). We scored the quality of the DNA using a scale of 1 to 5, where 1 indicated a sample of such low quality it was unlikely to produce a multi-locus fingerprint, and 5 indicated a sample of high quality. The quality score of nestling samples decreased significantly with the time to extraction ($r_s = -0.36$, n = 86, $P < 0.01$). Moreover, the time to extraction was significantly shorter for nestling samples that produced spoolable DNA (45.5 ± 5.0 days, n = 21) than those that did not (67.3 ± 4.4 days, n = 43; $t_{62} = -3.02$, $P < 0.01$).

In 1994, when extractions were performed shortly after the blood samples were collected, DNA yield was still negatively correlated with time to extraction (partial correlation $r_{83} = -0.39$, $P < 0.001$) for nestlings, but not for adults (partial correlation $r_{22} = -0.31$, P = 0.16). Despite the relatively short time to extraction for all samples, the quality score of nestling samples still decreased significantly with the time to extraction ($r_s = -0.40$, n = 86, $P < 0.001$). However, only 5/86 (6%) failed to produce spoolable DNA. Again, the DNA yield was significantly greater for samples that produced spoolable DNA (252 ± 4 μg, n = 81) than those that did not (220 ± 12 μg, n = 5; $t_{84} = 2.1$, P < 0.05) and the time to extraction was significantly shorter for nestling samples that produced spoolable DNA (3.3 ± 0.2 days, n = 81) than those that did not (6.4 ± 1.2 days, n = 5, $t_{84} = 3.05$, $P < 0.01$). We attribute the overall greater success at extracting nestling samples in 1994 to the fact that the samples were extracted as soon as possible after collection, but even a delay of 3 days appears to decrease the probability of obtaining spoolable DNA.

All 10 pairs of nestling samples collected in QLB to test the effect of storage time produced spoolable DNA. However, the samples stored for two weeks yielded an average of 15% less DNA than samples extracted right away (215 ± 8 vs. 252 ± 17 μg, paired $t_9 = 2.6$, $P < 0.05$). Spoolable DNA could not be extracted from any of the Eastern Kingbird nestling samples collected, even those that were extracted right away. Surprisingly, all blood samples of nestling Least Flycatchers provided "spoolable" DNA, including some samples that were not extracted until a year after they were collected.

DISCUSSION

The difficulties preserving the DNA in blood of Eastern Phoebe nestlings did not result from problems with the techniques, given that the same process worked on the adults and samples from other species collected by the same people at the same time. Moreover, the technique has been highly successful with many species (P. T. Boag, unpubl. data). Starting the extraction of DNA as quickly as possible after the samples were collected was necessary to be reliably successful. Even then, the extraction process was not as likely to be successful as with other species. Extraction from QLB-preserved Tree Swallow blood samples by the same procedure, for example, is nearly 100% successful (C. Barber, pers. comm.).

Interestingly, we experienced similar problems recovering DNA from preserved nestling blood of Eastern Kingbirds, but not of Least Flycatchers. We also are aware of similar difficulties with Acadian Flycatchers (*Empidonax virescens*) and other populations of kingbirds (R. Fleischer, pers. comm.). As yet, we do not know why nestling blood samples of these species should be unusually unstable. We recommend that anyone planning DNA research with tyrant flycatchers conduct their DNA extractions as soon as possible after collection, or test alternate methods of preservation or tissue collection and extraction beforehand.

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WINTER ANDROGEN LEVELS AND WATTLE SIZE IN MALE COMMON PHEASANTS

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Abstract. We report the results of morphological and hormonal measurements of 101 male Common Pheasants (*Phasianus colchicus*) captured during winter and at the beginning of the breeding season in order to identify correlates of ornament size. Androgen levels in January were bimodally distributed with one group with low hormone levels and a second group with high levels. In February, log transformed androgen levels were normally distributed, with all males showing values similar to the high-level group in January. Wattle size was positively correlated with androgens in January but not in February, suggesting that this male trait can indicate the ability of quality males to start earlier androgen production.

Key words: androgens, Common Pheasant, ornaments, Phasianus colchicus, *sexual selection, wattles.*

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Recent experiments have shown that in the Common Pheasant (*Phasianus colchicus*) female choice is influenced by features of some male ornaments, such as tail length and the presence of black points in the wattle (Mateos and Carranza 1995, Mateos 1998), and that male dominance rank depends on wattle size (Mateos and Carranza 1997). Moreover, von Schantz et al. (1989) observed a relationship between male spur length and harem size in natural conditions, even though the role of spur length on female choice in the Common Pheasant has been challenged by the results of recent experiments (Hillgarth 1990, Mateos and Carranza 1996).

It is still not clear what a female gains from ornament-based mate choice in a species like the Common Pheasant nor why male dominance is based on the size of the wattle, a "soft part" that is not used in fighting. Indeed, there is a lack of data on the correlates of male ornaments in this species, especially under natural conditions. Recent studies have failed to clarify the relationships between male ornaments and territory qual-