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EFFECTS OF POSTMORTEM INTERVAL AND PRESERVATION METHOD ON RNA ISOLATED FROM FIELD-PRESERVED AVIAN TISSUES Efectos del Intervalo Post-Mortem y del Método de Preservación en el ARN Aislado de Tejidos de Aves Preservados en el Campo

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EFFECTS OF POSTMORTEM INTERVAL AND PRESERVATION METHOD ON RNA ISOLATED FROM FIELD-PRESERVED AVIAN TISSUES

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Abstract. Studies of gene expression and transcriptome profiling often require the isolation of high-quality mRNA. Here, we examined how preservation method and the lag between collection and processing (postmortem interval; PMI) affect RNA quality and yield in field-collected samples. We subjected tissue samples of 11 House Sparrows (*Passer domesticus*) to two preservation treatments (flash freezing in liquid nitrogen versus treatment with Ambion's RNAlater prior to freezing) and eight PMI treatments (intervals ranging from 0 to 360 min). Mean RNA yields were significantly higher for flash-frozen tissues. Total RNA yield, as measured by a UV-vis spectrophotometer (Nanodrop), was not negatively associated with PMI in either preservation treatment, and we were able to extract RNA of quality (28S:18S fluorescence ratio >0.9) sufficient for most gene-expression applications from specimens that were held at ambient temperature for up to 6 hr postmortem. These results demonstrate that sufficient quantities of high-quality RNA can be extracted from avian tissues collected under typical field conditions. Although RNA remains stable for several hours postmortem, other studies have demonstrated that the transcriptional profile changes as a function of PMI. To increase the value of tissue samples for RNA studies we recommend that collectors include with a specimen detailed notes on the duration of the PMI as well as other extrinsic (e.g., time of day, temperature) and intrinsic (e.g., age of bird, sex) factors that influence levels of gene expression.

Key words: gene expression, genetic resources, mRNA, natural history collections, postmortem interval, transcriptomics.

Efectos del Intervalo Post-Mortem y del Método de Preservación en el ARN Aislado de Tejidos de Aves Preservados en el Campo

Resumen. Con frecuencia, los estudios de expresión génica y transcriptómica requieren el aislamiento de ARNm de alta calidad. Examinamos cómo la calidad y cantidad del ARN obtenido a partir de muestras recolectadas en el campo se ven afectadas por el método de preservación y el intervalo entre la muerte y el procesamiento. Preservamos muestras de tejido de 11 individuos de la especie *Passer domesticus* mediante dos tratamientos (congelamiento rápido en nitrógeno líquido versus tratamiento con RNAlater de Ambion antes de congelar) y ocho intervalos post-mortem en un rango de 0 a 360 minutos. La cantidad promedio de ARN obtenida fue significativamente mayor a partir de los tejidos congelados rápidamente. El ARN total, medido empleando un espectrofotómetro UV–visual (Nanodrop), no se relacionó negativamente con el intervalo post-mortem en ninguno de los tratamientos de preservación y fuimos capaces de extraer ARN de calidad suficiente (cociente de fluorescencia 28S:18S > 0.9) para la mayoría de las aplicaciones de expresión génica a partir de especímenes que fueron mantenidos a temperatura ambiente por hasta 6 horas después de su muerte. Estos resultados demuestran que es posible extraer cantidades suficientes de ARN de alta calidad a partir de tejidos de aves obtenidos bajo condiciones de campo típicas. Aunque el ARN permanece estable por varias horas post-mortem, otros estudios han demostrado que el perfil transcripcional cambia como función del intervalo post-mortem. Para incrementar el valor de las muestras de tejido para los estudios de ARN, recomendamos que los colectores incluyan con el espécimen notas detalladas sobre la duración del intervalo transcurrido entre la muerte y la preservación de los tejidos, así como otros factores extrínsecos (e.g., hora del día, temperatura) e intrínsecos (e.g., edad y sexo del ave) que afectan los niveles de expresión génica.

INTRODUCTION

The study of differential gene expression in natural populations represents one of the modern frontiers of avian ecological genomics (Lerner and Fleischer 2010). Geographic, ontogenetic, or temporal changes in gene expression are likely to be relevant to many research topics in avian biology, including the timing of migration (Jones et al. 2008), ecological physiology (Cheviron et al. 2008), territoriality (Mukai et al. 2009), learning and development (Wada et al. 2006, Replogle et al. 2008, Künstner et al. 2010), adaptive evolution (Carleton and Kocher 2001, Shapiro et al. 2004, Lai et al. 2006,

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Whitehead and Crawford 2006), and speciation (Adams 2007, Landry et al. 2007, Wolf et al. 2010).

Molecular techniques available for studying variation in gene expression, such as microarray analysis and quantitative PCR, often require relatively large amounts of high-quality transcripts of mRNA. After an organism's death, these transcripts degrade because of the activity of cellular and environmental RNA-specific endonucleases (RNAses) so that steps must be taken to stabilize the mRNA (Florell et al. 2001, Sambrook and Russel 2001, Lorkowski and Cullen 2006). These are easily implemented under laboratory conditions but may be challenging for ornithologists collecting samples in remote areas.

Because of its medical and forensic relevance, the effect of the lag between death and preservation of a sample (postmortem interval, PMI) on the stability and quality of mRNA isolated from human tissues has been evaluated in numerous studies (Johnson et al. 1986, Finger et al. 1987, Barton et al. 1993, Yasojima et al. 2001, Malik et al. 2003, Baechler et al. 2004, Miller et al. 2004, Ohashi et al. 2004, Kuliwaba et al. 2005, Micke et al. 2006, Walter et al. 2006, Ervin et al. 2007, Gopee and Howard 2007, Heinrich et al. 2007, Chevyreva et al. 2008, Fajardy et al. 2009, Durrenberger et al. 2010, Rudloff et al. 2010). The main results of these studies are that for some applications, such as RT-PCR, degraded mRNA retains its utility even with a PMI on the scale of several years. For example, PCR has been successful with mRNA isolated from dried blood 15 years old (Bauer et al. 2003). The high-quality mRNA required for other molecular techniques (e.g., microarray analysis) can retain its basic structural integrity, as measured by microchip assays and the 28S/18S ratio, up to 48 hr postmortem at room temperature (Ervin et al. 2007), but some studies have reported degradation of RNA with increasing PMI. For example, Broniscer et al. (2010) examined RNA isolated from 33 human brains with a median PMI of 7.7 hr and found that 70% of the isolates from samples with PMI ≤5 hr were of high quality, in contrast to only 21% of the samples from tissues with PMI >5 hr.

Studies of the relationship between PMI and RNA quality in other animals, including the mouse (Auer et al. 2003), dog (von Euler et al. 2005), pig (Malik et al. 2003, Fontanesi et al. 2008), cow (Bahar et al. 2007), rabbit (Marchuk et al. 1998), and Atlantic salmon (Seear and Sweeney 2008) have largely corroborated the human studies. The relative postmortem stability of RNA documented in the mammal studies was hypothesized to be due in part to the average body temperature in mammals, which tends to be high (~36–40 ºC) relative to the temperatures (4–24 ºC) of the experimental preservative examined in the studies (Seear and Sweeney 2008). Thus cellular endonucleases in these postmortem tissues were functioning at temperatures well below their optimum. To our knowledge, similar studies have not been published for birds, which also have relatively high body temperatures (~38–43 ºC) (Prinzinger et al. 1991).

Here, we assessed the effect of PMI on RNA quality and yields from vouchered bird samples collected under field conditions. Part of our goal was to elucidate the potential of existing collections of frozen tissue for RNA studies. One of the most effective methods of RNA preservation, flash-freezing of tissues in liquid nitrogen (Mutter et al. 2004, Wang et al. 2006), is the standard operating protocol at many museums for preservation of tissue in the field. Thus frozen tissues archived at museums may represent a substantial resource of high-quality mRNA for studies of avian gene expression and transcriptomics (Edwards et al. 2005).

METHODS

SAMPLING

We collected 11 adult male House Sparrows (*Passer domesticus*) in mist nets in March and April 2006 (Appendix). A House Sparrow's body temperature ranges from ~38 to 43 ºC (Hudson and Kimzey 1966). From each individual, we collected approximately 200 mg of pectoral muscle and liver tissue. We assigned tissue samples from each individual randomly to one of eight PMI treatments (0, 5, 10, 30, 60, 120, 180, 360 min at the outdoor ambient temperature, ~20º C, before preservation) and one of two tissue-preservation treatments (Appendix). For example, if an individual was assigned to the 180-min PMI treatment, it was sacrificed, left at ambient temperature for 180 min, and then tissues were removed and preserved according to the assigned preservation treatment. For the 0-min PMI treatment, tissues were removed and preserved immediately after sacrifice. Tissues were either flash frozen in liquid nitrogen (–180 ºC) (flash-freezing treatment) or removed and placed in approximately 2 mL of a commercial RNA stabilization buffer (RNAlater buffer from Ambion), left at ambient temperature for 2 hr, and then flash frozen in liquid nitrogen (RNAlater treatment). The RNAlater treatment was designed to simulate a field condition in which tissue samples can not be frozen immediately, such as the collection of a specimen away from camp.

RNA EXTRACTION AND QUANTIFICATION

We extracted total RNA from each tissue sample by using TRIzol (Invitrogen) in conjunction with an RNeasy mini kit (Qiagen). We homogenized tissue samples in TRIzol (1 mL TRIzol per 40mg tissue) and then added 500 µL of chloroform to the homogenate. Samples were transferred to 1.5-mL microcentrifuge tubes, incubated at room temperature for 5 min, and centrifuged at 10 000g at 4 ºC for 15 min to allow for phase separation. The RNA-containing aqueous phase was transferred to a 1.5-mL microcentrifuge tube and mixed with an equal volume of diethyl pyrocarbonate treated 70% ethanol. The sample was then added to an RNeasy spin column (Qiagen) and centrifuged at 12 000g for 15 sec at room temperature. Following centrifugation, we added 700 µL

of the Qiagen-supplied buffer RW1 to the spin column and centrifuged the sample at 12 000g at room temperature for 15 sec. We then added 500 µL of the Qiagen-supplied buffer RPE to the spin column and centrifuged the sample at 12 000g at room temperature for 15 sec. We performed a second wash by adding 500 µL of RPE buffer to the spin column and centrifuging the sample at 12 000g at room temperature for 3 min. We eluted total RNA in 1.5-mL microcentrifuge tubes by adding 40 µL of RNase-free water (Qiagen) to the spin columns and centrifuging the sample at 12 000g at room temperature for 1 min. RNA extracts were stored at –80 ºC.

We quantified total RNA with a Nanodrop ND-1000 spectrophotometer and calculated RNA yield as the concentration of total RNA (ng μL^{-1}) per unit of tissue used in the extraction (mg). Approximately 85% of the total RNA in eukaryotic cells is ribosomal RNA (rRNA), whereas mRNA makes up only 1 to 5% of total cellular RNA (Sambrook and Russel 2001). The remaining fraction consists of other RNAs of low molecular weight (tRNAs, siRNA, miRNA, etc.). The integrity of rRNA can be used as a proxy to assess the integrity of mRNA in a sample indirectly (Sambrook and Russel 2001). We assessed rRNA quality both qualitatively and quantitatively by means of gel electrophoresis on an Experion Automated Electrophoresis Station (BioRad) and inspected the gel images visually to confirm the presence of bright, clean bands corresponding to the 28S and 18S ribosomal subunits, providing a qualitative assessment of rRNA integrity. We also calculated the ratio of fluorescence intensities between the 28S and 18S bands (28S:18S ratio) to provide a quantitative assessment of rRNA integrity(Sambrook and Russel 2001). Extractions with concentrations of at least 3.25ng μL^{-1} per mg of tissue with clean 28S and 18S bands and a 28S:18S fluorescence ratio of 0.9 or higher typically yield quantities of mRNA sufficient for microarray analysis (Cheviron et al. 2008) and next-generation transcriptome sequencing (Wolf et al. 2010). We considered extractions meeting these criteria to be "high-quality" extractions, acknowledging that this term is relative to other methods of measuring rRNA quality and to the mRNA analysis to be performed (Copois et al. 2007).

STATISTICAL ANALYSIS

We performed all statistical tests with the computer program JMP (SAS Institute 2000). We used paired, two-tailed *t*-tests to detect significant differences in RNA yields by experimental treatment, and linear regressions to assess the association between PMI and RNA yield and quality.

RESULTS

We isolated relatively large quantities of RNA (flash-frozen mean yield = 11.4 ng μL^{-1} per mg of tissue; RNAlater mean yield = 6.7 ng μL^{-1} per mg of tissue) from tissue samples in all experimental treatments (Table 1, Fig. 1). Mean yields of RNA were significantly higher for flash-frozen tissues ($t = 2.696$, $P = 0.01$). All extractions were of sufficient quality for gene-expression applications (28S:18S > 0.9). We did not find a significant negative association between RNA quantity and PMI in either preservation treatment (flash frozen $r^2 = 0.008$, $P = 0.74$; RNAlater $r^2 = 0.405$, $P = 0.01$). Although there was a significant positive relationship between RNA yield and PMI for the RNAlater treatment, this relationship is likely due to our small sample sizes. The PMI is known to affect the transcript profile of skeletal muscle (see Discussion), and in some cases it is associated with upregulation of genes involved in protein biosynthesis (Sanoudou et al. 2004). It is possible that such processes contribute to this counterintuitive pattern, but because we did not observe similar results in the flash-freezing treatment or RNAlater-preserved liver samples (see below), it seems more likely that this result is an artifact of our small samples sizes. Neither did we observe a reduction in rRNA integrity as a function of PMI (Fig. 1).

To determine whether these results held for a different tissue type, we extracted RNA from RNAlater-preserved liver tissue from a subset of individuals representing the range of PMI treatments (Table 2). Again, we did not observe a significant negative association between RNA yield and PMI ($r^2 = 0.005$, $P = 0.87$). We extracted RNA of quality and quantity sufficient for most current gene-expression and transcriptomic applications from all PMI treatments (Table 2).

TABLE 1. Mean yield of RNA from muscle of House Sparrows preserved by two methods and at various intervals postmortem (time in minutes). Two replicates of each combination of treatments; ranges are given in parentheses. See Appendix for voucher numbers for each tissue sample.

Treatment	RNA yield (ng μL^{-1} per mg)
Flash freezing	
0	7.5 (3.3–11.7)
5	19.1 (11.2–26.9)
10	10.6 (7.5–13.7)
30	15.0 (12.0–18.1)
60	4.0 (2.1–5.9)
120	4.6 (3.7–5.5)
180	15.5 (14.1–16.9)
360	14.0 (12.0–16.0)
RNAlater	
0	3.3 (2.8–3.7)
5	4.0 (3.7–4.3)
10	9.0 (8.0–9.9)
30	6.4 (3.5–9.2)
60	5.5 (3.8–7.2)
120	6.7 (5.9–7.3)
180	8.2 (8.0–8.4)
360	10.5 (9.8–11.1)

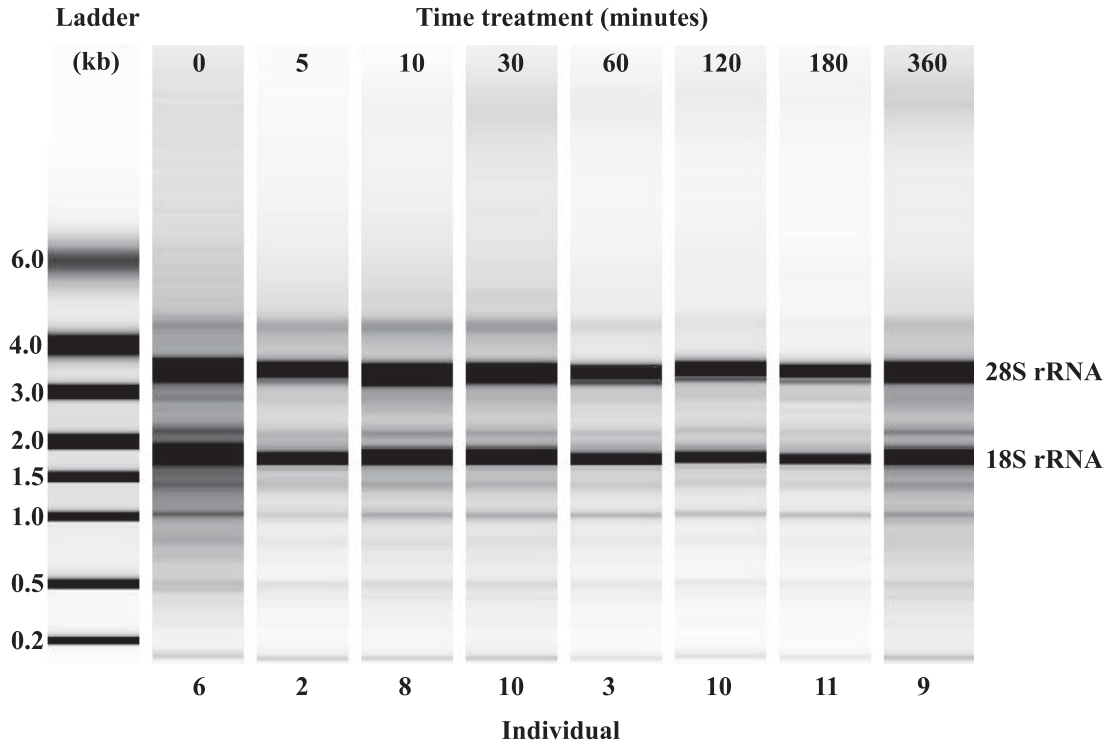

FIGURE 1. Representative electrophoretic gel image produced by an Experion Automated Gel Electrophoresis Station. Each well contains 2 µL of total RNA extracted from one individual. For individual numbers and time treatments, see Appendix. Bands at 3.5 and 1.8 kb represent the 28S and 18S ribosomal subunits, respectively. The dark 28S and 18S bands indicate little rRNA degradation, and the similarity among samples taken at various intervals postmortem indicates there is no substantial reduction in RNA integrity over the time investigated, 6 hr.

TABLE 2. Yield of RNA from liver tissue of House Sparrows preserved with RNAlater at various intervals postmortem (time in minutes). See appendix for voucher numbers for each tissue sample.

Postmortem interval (min)	RNA yield (ng μL^{-1} per mg)
0	10.17
5	16.18
10	28.19
30	47.42
60	7.57
120	24.55
180	25.57
360	23.48

DISCUSSION

Our results corroborate previous studies of mammals and fishes (see references in Introduction) in finding that RNA degradation is minimal for PMIs of several hours. As genomic tools for nonmodel organisms become more widely available, studies of gene expression and transcriptomics in natural populations of birds will become increasingly common (e.g. Cheviron et al. 2008, Bulgarella et al. 2009, Künstner et al. 2010, Wolf et al. 2010). Such studies will require the isolation of high-quality mRNA. Although, at least anecdotally, many ornithologists considered preservation of mRNA to be logistically challenging under field conditions, our results demonstrate that RNA of quality and quantity sufficient for many gene-expression assays can be isolated from avian tissues that were preserved in the field.

The maximum PMI we evaluated was only 6 hr, but nondegraded RNA has been isolated from tissue samples up to 48 hr postmortem (Ervin et al. 2007). Although RNA has been reported to degrade faster in tissues such as liver, kidney, spleen, and pancreas that are rich in RNA endonuclease (Fontanesi et al. 2008), we observed no difference in quality between liver and pectoral muscle samples at the PMIs we evaluated.

Although high-quality mRNA can be isolated from tissues after a long PMI, the transcript profile is known to change with the PMI (Copois et al. 2007), a process we did not examine. Using a transcript array, Birdsill et al. (2010) found decreasing levels of expression of genes with increasing PMI, and Sanoudou et al. (2004) discovered postmortem upregulation of genes involved with protein biosynthesis. Postmortem changes in the transcript profile introduce an additional source of variation for qPCR, microarray, or transcriptomic analyses attempting to identify differences in expression

among experimental treatments (Auer et al. 2003, Pérez-Novo et al. 2005). Therefore, one needs to consider PMI along with the myriad of other variables that influence gene-expression levels in individuals.

Our results suggest that collections of frozen genetic resources represent important storehouses of material for many RNA-based studies of gene expression. RNA-Seq-based transcriptome profiling and gene-expression studies (Wang et al. 2009) rely on next-generation sequencing technologies that currently yield sequences approximately 30–500 base pairs in length. These methods in particular are less sensitive to mRNA degradation (Wang et al. 2006). Cycles of freezing and thawing have been shown to affect the recovery of high-quality RNA adversely (Botling et al. 2009), but treatment of tissue samples with RNAlater prior to freezing greatly reduces the negative effects (Forster et al. 2008). In a microarray analysis of cancer-gene transcripts in humans, Jochumsen et al. (2007) noted that tissues preserved in RNAlater could experience up to three cycles of freezing and thawing before any loss of RNA quality or quantity. If ornithologists anticipate that samples will be thawed and frozen repeatedly in the future, then addition of an RNA-stabilizing buffer prior to freezing would be worthwhile.

Many, or perhaps most, tissues housed in collections of ornithological genetic resources at museums are the product of general collecting. These tissue samples capture native RNA-expression profiles of wild birds at the time of euthanasia (plus any changes that occur during the PMI). Because of the inherent plasticity of gene-expression patterns, differences among individuals in variables such as PMI, age, condition, sex, fat deposition, reproductive status, molt, time of year, time of day, behavior, mating status, and locality all introduce variation in expression profiles that could confound differences in gene-expression patterns between samples of interest. Because of the multifarious factors that influence gene-expression levels in individuals—levels that can change dramatically in minutes—studies seeking to elucidate genetically encoded differences in gene-expression levels often rely on controlled common garden experiments (Cheviron et al. 2008). Although the bulk of tissues housed in museums will not have been collected under controlled experimental conditions, the utility of the tissues for RNA studies increases dramatically if data on the variables mentioned above, plus other relevant variables, are carefully documented for each specimen. These samples provide a snapshot of gene-expression patterns in an individual's native habitat, and they are likely to increase in value as studies of gene expression in natural populations of nonmodel organisms become more commonplace.

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APPENDIX. Voucher specimens of the House Sparrow in the Louisiana State University Museum of Zoology tested for effect of post-mortem interval and method of preservation on quality of RNA.

Individual	Voucher[a]	Treatments[b]
1	B-55164	FF, 5 min; R, 5 min
2	B-55165	FF, 0 min; FF, 30 min; R 5 min
3	B-55166	FF, 0 min; FF, 360 min; R, 60 min
4	B-55167	FF, 5 min; FF, 60 min; R 0 min
5	B-55168	FF, 10 min; FF, 180 min; R 10 min
6	B-55169	R, 0 min; R, 60 min
7	B-55171	FF, 120 min; R, 30 min; R 120 min
8	B-55172	FF, 60 min; R, 10 min
9	B-55173	FF, 30 min; FF, 120 min; R, 360 min
10	B-55174	R, 30 min; R, 120 min; R, 360 min
11	B-55175	FF, 10 min; FF, 360 min; R 180 min

[a]All specimens from Louisiana, East Baton Rouge Parish, Baton Rouge, 2150 Terrace Ave., 30° 26.1′ N, 91° 9.3′ W.
[b]FF, flash frozen; R, RNAlater.