SHORT COMMUNICATIONS

OBTAINING OFFSPRING GENETIC MATERIAL: A NEW METHOD FOR SPECIES WITH HIGH NEST PREDATION RATES

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Abstract. Over the past decade, the field of molecular genetics has revolutionized our understanding of avian mating systems, by demonstrating that social bonds might not accurately reflect parentage because of unknown levels of cryptic mating (e.g., extra-pair copulations). Use of molecular genetics tools for paternity analysis requires genetic material from putative parents and nestlings. Unfortunately, high nest predation rates often preclude detailed genetic studies of tropical taxa. Here, we describe a nondestructive method that increases the efficiency of obtaining genetic material from offspring for a group of tropical passerines (Pipridae). The method entails replacing eggs with plaster replicas, incubating eggs artificially, and returning hatchlings to their original nests for further development. This method greatly improved our ability to sample offspring, as we collected genetic material from 100% of manipulated nests, compared to 52% of unmanipulated nests.

Key words: ex situ incubation, manakin, nest predation, Pipridae, plaster eggs.

Obtención de Material Genético de la Progenie: un Nuevo Método para Especies con Alta Taza de Depredación de Nidos

Resumen. Durante la última década, el campo de la genética molecular ha revolucionado el entendimiento de los sistemas de apareamiento de las aves, demostrando que los lazos sociales observados pueden no reflejar paternidad con precisión, debido a niveles desconocidos de apareamiento críptico (e.g., copulaciones extra-pareja). El uso de herramientas de genética molecular para el análisis de paternidad requiere material genético de padres putativos y polluelos. Desafortunadamente, la alta tasa de depredación de nidos en especies tropicales usualmente impide la realización de estudios genéticos detallados. Aquí se describe un método no destructivo que incrementa la eficacia para obtener material genético de polluelos de un grupo de aves paseriformes tropicales (Pipridae). El método consiste en reemplazar huevos con replicas de yeso, incubar los huevos artificialmente y devolver los polluelos a los nidos originales para su posterior desarrollo. Este método incrementó significativamente nuestra habilidad de muestrear polluelos, pudiendo colectar material genético del 100% de los nidos manipulados, comparado con una colecta de material genético de sólo el 52% de los nidos no manipulados.

The study of avian mating systems is key to understanding avian life-history strategies. Tropical systems are critical to this understanding, given the incredible diversity of species in this region. Unfortunately, the nesting and breeding habits of many species of tropical birds remain poorly known because nests are difficult to find and nest predation rates are high (Skutch 1985, Roper 2005). As a result, the development of alternative nest-sampling techniques for tropical birds is essential to further our understanding of both avian mating systems and life history.

Recently, the study of avian mating systems has been greatly advanced by the use of molecular genetics tools (Avise 1996, Hughes 1998, Snow and Parker 1998), which have unveiled previously overlooked behaviors (e.g., extra-pair copulations, intraspecific brood parasitism). The use of molecular tools is essential for estimating variance in male and

female reproductive success (Snow and Parker 1998); however, sampling reproductive success through parental analysis requires genetic material from putative parents and offspring. Methods to obtain genetic material from birds encompass extracting DNA from blood, feathers (Segelbacher 2002), tissues (e.g., skin; Mundy et al. 1997), and eggshells (maternal DNA; Strausberger and Ashley 2001). In the case of offspring, the most common protocol to obtain genetic samples is to wait until eggs hatch and to take samples from hatchlings (in situ incubation method). However, if nests are depredated during the incubation stage, researchers lose the opportunity of obtaining genetic data. Alternatively, researchers may be able to obtain blood samples by puncturing eggs and drawing blood from an embryo vein. This method, however, has drawbacks. First, eggs need to be in the right stage of development to be punctured (i.e., when veins are visible [Langenberg et al. 1997] or when eggs show the first signs of hatching [Lecomte et al. 2006]). If eggs are not at a suitable stage, this method is not applicable and researchers may again lose the opportunity to obtain a genetic sample due to nest predation. Second, this method has been designed for and tested in birds that have large and robust eggs (i.e., birds generally >100 g). Small and fragile eggs present considerable difficulties, as punctures are more likely to result in fatal damage to eggs (WPT, pers. obs.). Lastly, genetic material can be obtained from offspring by collecting the entire clutch (i.e., artificial predation). This technique, however, has many ethical considerations and may not be compatible with some study objectives (e.g., determining reproductive success) or study systems (e.g., rare or endangered species).

Here, we describe an innovative and nondestructive method that increases the efficiency of obtaining genetic material from offspring of four small tropical passerines (Blue-crowned Manakin [*Lepidothrix coronata*], Blue-backed Manakin [*Chiroxiphia pareola*], White-crowned Manakin [*Pipra pipra*], and Wire-tailed Manakin [*P. filicauda*]) that suffer high nest predation rates. The ex situ incubation method entails replacing eggs from active nests with plaster replicas, incubating eggs to hatching in captivity, taking a blood sample from hatchlings, and then returning hatchlings to their original nests for further development.

METHODS

This research was conducted at Tiputini Biodiversity Station (0°38′S, 76°08′W), located along the Tiputini River, Orellana Province, eastern Ecuador (see Karubian et al. [2005] for a detailed site description). Tiputini Biodiversity Station has more than 20 km of trails and two gridded 100 ha plots (ca. 1 km × 1 km each).

We worked with four species of manakin (Pipridae)—Blue-crowned Manakin, Blue-backed Manakin, White-crowned Manakin, and Wire-tailed Manakin—that range in weight from 8.5 to 17.5 g. Manakins are small birds that inhabit the understory and subcanopy of forests in warm, humid regions of Central and South America (Hilty and Brown 1986,

Ridgely and Tudor 1994). Manakin nests are typically shallow, pendent, open cups built in a forked branch (Hilty and Brown 1986), and are subject to high predation (Skutch 1985).

Nests were located by systematically searching within the two 100 ha study plots and around leks located off the study plots from November 2003 to March 2004 and November 2004 to March 2005, corresponding to the main breeding season in the region. Systematic searches were supplemented by radio-tracking breeding female manakins that were captured on or off the study plots. Radio-transmitters (Holohil Systems Ltd., Ontario, Canada) weighing 0.54–0.75 g (i.e., no more than 5% of the female's body mass) were attached using a Rappole harness (Rappole and Tipton 1991). Females were then tracked to find the location of their nests and later recaptured to remove transmitters.

Over the course of our research we employed two different strategies to obtain genetic samples from offspring. During the 2003–2004 field season, we left eggs in monitored nests and waited until they hatched to take a blood sample from nestlings (in situ incubation method). High nest predation rates (81% of nests depredated) led us to adopt a different approach. During the 2004–2005 field season, we replaced eggs with plaster replicas and incubated eggs in the laboratory (ex situ incubation method). Hatchlings were returned to their original nests after blood samples were taken. Data from the first field season are used here to evaluate the efficacy of the ex situ incubation method.

We prepared plaster eggs using DO-IT® brand sinker molds (model EG-9-M2, Do-it Corporation, Denver, Iowa). Plaster mix (plaster of paris) was prepared following brand instructions (in a ratio of three parts powder to two parts water). The same size mold (3/4 oz) was used for all four species despite slight variation in egg size; size did not appear to influence acceptance of eggs by females. Plaster eggs were sanded and painted (water-based, nontoxic acrylic paint) to mimic the natural color and speckling of real manakin eggs (Fig. 1). A coat of varnish was added to protect the fake eggs from humidity. When we found an active manakin nest, we waited until the female left the nest and then replaced the eggs with the same number of plaster replicas. Eggs were immediately brought back to the field station in small cushioned containers, candled to examine developmental stage (Lokemoen and Koford 1996), and placed in a Brinsea® Octagon 20 incubator (Brinsea Products Inc., Titusville, Florida) at 37.7°C. We maintained the relative humidity inside the incubator between 45% and 55% to mimic natural conditions.

Nests were monitored approximately every three days (range of interval between checks: 1–9 days) to check their status (i.e., active, depredated, or abandoned). Nests were considered: (1) active, if the female was seen incubating; (2) depredated, if the female was absent and the fake eggs had disappeared or showed any indication of predator activity (e.g., teeth marks); or (3) abandoned, if the fake eggs were intact in the nest, the female was not present, and there were signs of abandonment (e.g., wet eggs, dead
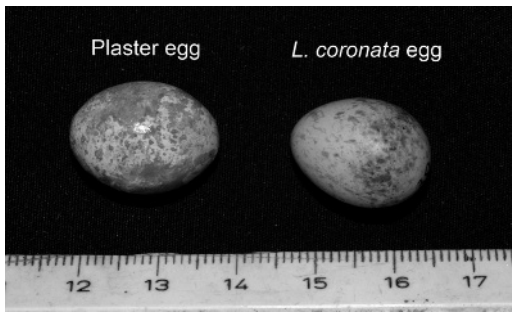
FIGURE 1. A comparison of a real Blue-crowned Manakin (*Lepidothrix coronata*) egg and a plaster replica, used in the ex situ incubation method conducted at Tiputini Biodiversity Station, Ecuador. Plaster eggs were used to stimulate females to continue incubating, while real eggs were incubated artificially in the laboratory.

leaves covering the nest). When eggs were intact, there were no signs of abandonment, and the female was not present we revisited the nest several times or visited the nest before dawn prior to assigning abandoned status. The status of six nests could not be determined and they were placed in an "unknown fate" category.

Eggs in the incubator were candled every three days to ensure that they were developing properly. As soon as the chicks hatched, we punctured their jugular or brachial vein and extracted ~12.5 μl of blood. Chicks were then returned to their original nest to be raised by the attendant female. We returned to the nest and took a second blood sample from nestlings when they were 3–5 days old. During resampling, chicks had grown large enough to extract ~25 μl of blood. In cases where nests were depredated before the real eggs hatched in the incubator, we incubated the eggs until the embryos were well developed (12 to 16 days) and then collected and preserved the eggs in 90% ethanol. Experience in the laboratory shows that offspring DNA can be extracted from chick blood samples and eggs in all developmental stages, and that a small amount of avian blood, such as 12.5 μl, contains enough DNA to allow molecular analyses (WPT, pers. obs.).

RESULTS

We found 61 manakin nests, 31 of which were monitored in situ and 30 using the ex situ incubation method (Table 1). Twenty-two of 31 nests monitored in situ were depredated (29 eggs and 14 chicks), three were successful (four fledglings), two were abandoned (two eggs), and four could not be assigned status (six chicks; Table 2). We obtained genetic material from 16 in situ nests, including 22 blood samples from chicks, and three eggs and two chicks from abandoned nests. The fates of nests with plaster eggs closely matched those of in situ nests; 19 of 30 nest were depredated (31 eggs and two chicks), two were successful (four fledglings), two were artificially depredated following loss of eggs in the incubator (three eggs, see below), five were abandoned (10 eggs) and two nests (four eggs) could not be assigned status (Table 2). In this case, however, we obtained genetic material from 100% of the nests (18 blood samples and 36 eggs of depredated or abandoned nests).

Over the course of the 2004–2005 field season, we replaced a total of 54 eggs (in 30 nests) with plaster replicas and incubated them in the laboratory. All but three females accepted and incubated the plaster eggs. The "rejection" of fake eggs by two of these females, however, is questionable. In one case the replacement of eggs coincided with a tree falling 1.5 m from the nest and in the second case the female was never seen at the nest, so the nest may have been abandoned before we found it.

Forty-three of these 54 eggs were successfully incubated to hatching. Initially, we lost several eggs due to hairline fractures on the egg's surface, which were attributed to eggs colliding with divider walls and other eggs in the incubator. Hairline fractures were sufficient to arrest development of the embryo. After augmenting cushioning material on divider walls and between eggs, this problem was eliminated. We found that the incubation period in the laboratory was similar to that observed in undisturbed active nests in situ (~16–18 days). Moreover, "incubator hatchlings" were readily accepted by females after being returned to their original nests.

DISCUSSION

Our results corroborate previous findings that manakins, like most other tropical open-cup nesters, suffer high rates of nest predation (75% of nests with known fates lost to predation in this study). The use

TABLE 1. Number of Blue-crowned Manakin (*Lepidothrix coronata*), Blue-backed Manakin (*Chiroxiphia pareola*), White-crowned Manakin (*Pipra pipra*), and Wire-tailed Manakin (*P. filicauda*) nests and eggs found for the in situ and ex situ incubation methods conducted at Tiputini Biodiversity Station, Ecuador.

Species	In situ incubation		Ex situ incubation		Total nests	Total eggs
	Nests	Eggs	Nests	Eggs		
Blue-crowned Manakin	9	16	12	21	21	37
Blue-backed Manakin	3	4	2	4	5	8
White-crowned Manakin	2	4	2	4	4	8
Wire-tailed Manakin	17	31	14	25	31	56
Total	31	55	30	54	61	109

TABLE 2. Percentages of depredated, successful, and abandoned nests for the in situ and ex situ incubation methods (artificially depredated nests [i.e., eggs lost during ex situ incubation] and nests of unknown fate were excluded from these calculations), and percentages of nests and offspring from which we could get a genetic sample (all nests were included in these calculations). Sample sizes are given in parentheses.

	Depredated nests	Successful nests	Abandoned nests	Genetic material[a]	
				Nests sampled	Offspring sampled
In situ	81% (22)	11% (3)	7% (2)	52% (16)	49% (27)
Ex situ	73% (19)	8% (2)	19% (5)	100% (30)	100% (54)
Total	75% (41)	9% (5)	13% (7)		

[a] Blood or tissue sample.

of ex situ incubation doubled our success of obtaining genetic material from nests, in terms of both the number of nests and offspring sampled. This significant increase was largely a result of our ability to collect genetic samples from nests that would have been depredated in the incubation stage; in the past, nests depredated during the incubation stage eliminated the opportunity to take blood samples from the offspring.

The ex situ method, however, also imposes certain challenges. First, one needs to ensure that females accept plaster eggs, so that hatchlings can be returned to the nest at a later date. In this study, we made fake eggs that resembled real eggs as closely as possible and females readily accepted and incubated these plaster eggs. Second, eggs need to be made of nontoxic materials to prevent harm to predators that may consume the eggs. Third, eggs are extremely fragile, so care must be taken in transporting eggs and storing them in the incubator. Fourth, the incubator must operate 24 hours a day. At our study site, and most study sites in remote tropical field stations, electricity is not always reliable. To ensure a continuous supply of electricity to the incubator at our site, we complemented the energy supply with two 12-volt car batteries.

In addition to the challenges of the ex situ method, all studies that require nest manipulation have an undesirable risk of nest abandonment. Manakins exhibited fairly high rates of nest abandonment (13%). Besides researcher disturbance, possible causes of nest abandonment include failed predation attempts, weather conditions (George et al. 1992, Robinson et al. 2005), female body condition (Fort and Otter 2004, Heath and Frederick 2005), non-predator nest disturbance (e.g., brood parasitism; Ortega and Ortega 2001), infertile eggs, and resource availability (Fort and Otter 2004). Further, nest abandonment may vary as a function of female experience and age (K. McFarland, Vermont Institute of Natural Science, pers. comm.). We did find a slightly elevated risk of nest abandonment (five vs. two nests) for nests with plaster eggs, but most females readily accepted plaster eggs and hatchlings transferred from the incubator. Moreover, daily survival rates of nests did not differ statistically between our manipulated and unmanipulated nests (TBR et al., unpubl. data). Given the high nest predation rates observed in this study system, it is not surprising that some females abandon nests. Significant predation pressure may result in a relatively low threshold of tolerance before abandoning the nest. Most nests were found in early stages of development (i.e., nest construction, egg-laying, or early incubation) and females may be more likely to abandon nests in earlier stages. In both strategies (in situ and ex situ), we tried to minimize nest disturbance by approaching nests when females were not present and, when possible, checking the status of nests from a distance. We believe that the greatest source of disturbance came during attempts to capture the nesting female to obtain a blood sample. Capturing the female at the nest is essential for parental analysis and researchers need to make decisions about the capture time frame. Here, the obvious balance is between minimizing disturbance to the nest and female while maximizing number of females captured (i.e., prior to loss of the nest from predation). In this study, our protocol was to capture and bleed the female one or two days after the nest was found to increase the chances of obtaining maternal DNA. In systems with lower nest predation, the proposed time frame could be prolonged to potentially reduce the percentage of nests abandoned.

We tested an ex situ incubation method that proved to be highly successful for obtaining genetic material from offspring of four species of manakin. We believe this method may be applicable to many other passerines, especially in the tropics, for which detailed knowledge of genetic mating systems is lacking. Furthermore, by increasing the number of genetic samples from offspring, we can answer new questions that were previously precluded because of small sample sizes and low statistical power. Thus, this method not only allows us to study the reproductive behavior of a new set of species (e.g., rare species), but also provides the opportunity to increase our power to answer new questions that will enhance our current knowledge about mating systems and avian life history.

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