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Marking of Insemination Encounters with Cloacal Microspheres

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Introduction

This report describes a method for marking insemination encounters between free-living birds, particularly passerines. Most birds of this group have continuous, spontaneous release of sperm, and include "sperm balls" in their method of insemination (Quay 1986a, 1987a). Individual birds that have participated in such behavior within a brief test period can be identified by means of this method.

This marking technique depends upon banding of wild birds and upon cloacal lavages (Quay 1984) to assay the transfer of microspheres from males to females, and to simultaneously obtain information on the reproductive status of donors and recipients of the microspheres.

Materials and Methods

Instructions for cloacal lavaging are given in Quay (1984).

For installation of microspheres. Microspheres (MSs): Many types, colors and compositions are available commercially. I used 5 kinds of MSs (code #, mean + standard deviation of diameter, as stated on the labels): ① 50 + 10, ② 25 + 5, ③ 15 + 5, ④ 15 + 1.9, ⑤ 5.7 + 1.5 µm. Their sources are: ① to ③ - Nuclear Products, 3M Center, St. Paul MN; ④ - Pharmacia, Uppsala, Sweden; ⑤ - Dow Chemical Co., Indianapolis IN. Spheres smaller than 1.0 µm diameter were too small and nondescript to be reliably distinguished by light microscopy from extraneous particles in the lavages. Characteristics of ①-⑤ are given in Figure 1. The frequency distributions of the diameters represent patterns of variation beyond what was expected on the basis of the standard deviations noted above. MSs used here had innate, non-fluorescent colors.

Cloacal lavages (CLs). All birds, males and females, were first weighed, measured and banded if not a recapture, within 5 to 30 minutes of capture by mist-netting. They then received a standardized series of 4 CLs. The first three were without avoidable stimulation, and the fourth was taken immediately following 20 quick and gentle anterior to posterior strokes on each side of the vent region, either with thumb and forefinger (for medium to large birds) or the rubberized or tubing-covered tips of a small hemostat or forceps set with a gape of about 4 mm (for small birds). When some of the lavage aliquot was lost due to retention or expulsion by the bird, additional aliquots were used in order to bring the total lavage volume on the slide to a near standard volume, as judged by the area covered. Each lavage slide (slides 1 to 4) received a small (approximately 0.01 ml) drop of concentrated formaldehyde solution in the center of the marked ellipse before the addition of lavage aliquots. Lavage slides were numbered (slide #s 1 to 4 in sequence, and the CL specimen number). Each pipet was discarded after use with a single bird. The bulbs were rinsed and internally blotted immediately after use. CL slides were air dried and immediately stored in dust-proof boxes. Slides were protected from spatter and other sources of con-

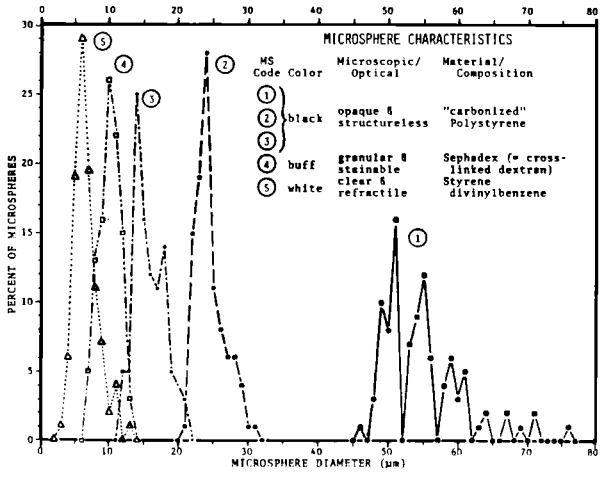

Figure 1. Tabulated characteristics and plotted frequency distributions of diameters of microspheres used in this study. Frequency distributions were based upon microscopic diameter measurements of 100 microspheres in "standard" slides.

Table 1. Male birds that received cloacal installations (CIs) in 1986

Species	Dates	Total No. Individuals	Number of Reinstillations 1	2	3	4	5	6	7	8	Total CIs
Indigo Bunting (*Passerina cyanea*)	May 6-30	*41	2	2							47
House Sparrow (*Passer domesticus*)	Apr 9-May 31	*24	*3	3	2	2				1	55
N. (Baltimore) Oriole (*Icterus galbula*)	Apr 29-May 31	*18		*2		*1					26
Red-winged Blackbird (*Agelius phoenicius*)	May 4-30	*14		*1							16
Brown-headed Cowbird (*Molothrus ater*)	May 5-8	5	5	2							14
American Robin (*Turdus migratorius*)	May 9-10	1	1								2
Whip-poor-will (*Caprimulgus vociferus*)	May 2	1									1
											Total=154

* One or more birds were relavaged in May-June 1987 and seen to be reproductively active and normal.

tamination. Additional procedural notes are available (Quay 1984, 1985ab, 1986ac).

Cloacal installations (CIs). After the final CL from a male, a CI was administered, consisting of 1 type of microsphere (Table 1). If recaptured a male would again receive the same microsphere. The volume of the CI varied from 0.5 to 1.0 ml, depending on bird size (as for the CLs above) and was placed by pipet tip 3 to 5 mm within the vent without pressure. The vent area was then gently pressed with the finger tip for about 30 seconds before release of the bird. A major amount of the CI was usually expelled by the bird soon after release. This and other variables (clumping after mixing, etc.) prevented estimation of amount or number of microspheres for effective CI. However the stock CI suspension mixtures in water were roughly 5% microspheres by weight. Avoidance of cross-contamination is very important.

Sites and subjects. This study was made in mixed habitats 5km NNW of Foley, Lincoln County, Missouri (39° 08'N, 90° 46'W). Quantitative characteristics of the netting and banding activities have been described (Quay 1987b). Subject species are listed in Table 1.

Microscopy. The cloacal lavage slides (without staining or covering) were studied by light microscopy for identification of sperm (using phase contrast optics) and of microspheres (using brightfield, phase contrast and fluorescence light microscopy). A magnification of 100x was adequate for the survey scanning for sperm of medium to large size and for microspheres; 400x was used for closer examinations and survey for smaller objects. Measurements were made using a calibrated (with a stage micrometer rule) ocular micrometer at a magnification of 1000x. Frequency distributions (Figures 1 and 2) were based upon samples of 100 microspheres per standard or CL slide, in microscope fields along grid lines at arbitrary intervals facilitated by use of a mechanical stage. Small objects on the slide were relocated by using a "Levins Microslide Field Finder No 7100."

Recognition of CI microspheres was aided by comparison with standards; these were blank slides dipped in 2% gelatin in water, dried and given a drop of diluted aqueous suspension from one of the stock CI suspension mixtures. In the preparation of these suspensions it is essential to mix them thoroughly in order to breakup aggregations just prior to pipetting and transfer to a "standard" slide or to the cloaca of a male to prevent a bias in particulate size or composition.

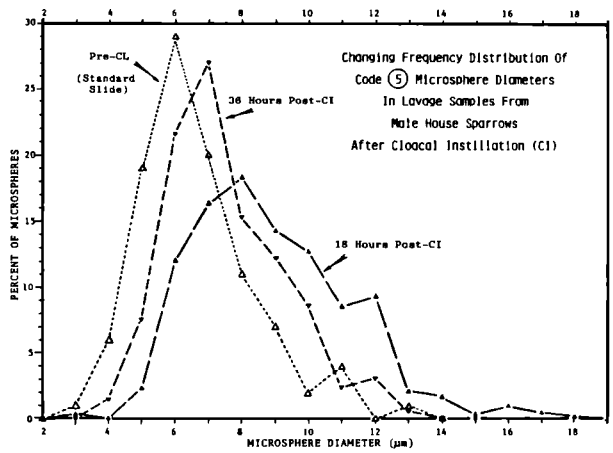

Figure 2. Effect of time after cloacal instillation upon the frequency distribution of diameters of code ⑤ microspheres lavaged from male House Sparrows.

Gelatin on the "standard" slides cemented the mixtures when dried, as did the natural mucoid contents of the cloaca on the CL slides.

Results and Discussion

Marker recognition and comparative merits. The five kinds of microspheres (codes ① to ⑤) were readily recognized in CL slides, although caution was necessary to avoid mistaking other spheroidal objects such as some kinds of pollen and spores for microspheres. Unique characteristics of the synthetic microspheres include: near absolute spherical form, distinctive optical characteristics, uniformity of coloration, and lack of internal structural features. Optical and chromatic characteristics of the smaller microspheres (codes ④ to ⑤)often required close study to differentiate them from such things as lipoidal droplets or residue. These had optically variable imperfections in their refractile concentric patterns and lost their spherical shape when dried.

Optimal microsphere diameter appeared to be 10 to 50 μm. The manufacturers' stated means and standard deviations for the diameters were not exact (see above and Figure 1).

Recovery of microspheres from males. Practicality of the CI technique depends on male retention of sufficient MSs to enable transfer of some to one or more females. Some males that received CIs in this study were relavaged within one to several days post-CI (Tables 1 and 2). Counts and diameters of MSs in the CL slides were analyzed. Table 2 presents the results for numbers of MSs found at different times post-CI. The relatively few males that received MS ④ installations were not recaptured until many days later, and no ④ MSs were found. For ①, ②, ③, and ⑤ recoveries in CLs occurred for from 1 to 5 days (Table 2). The data suggest that cloacal retention of microspheres in males was inversely related to MS size. This is most clearly illustrated by the three kinds of MSs (①, ② and ③) that had the same composition and differed only in diameter. The number of MSs per installation was also inversely related to MS size. Other evidence supports the importance of both size and composition of the MSs for their relative cloacal retention. This is borne out by the results from MS ⑤ showing retention of very large numbers (Table 2). Examination of the frequency distributions of MS ⑤ sizes in males lavaged at different times post-CI suggest that larger MSs tend to be more easily extracted early and that smaller MSs tend to progressively predominate when lavaging is done later (Figure 2).

A probably related trend is seen when one compares MS ⑤ sizes in the four consecutive CL slides. The only statistically significant difference occurred between slide 1 and all the others. Mean MS diameter in such cases was always greater in slide 1. Therefore ⑤ MSs were not only retained longer (Table 2) but the smaller ones were retained longer than the larger ones (Figure 2). These results indicate the presence of an artifactual difference in MS behavior in relation to size, and this could bias comparisons of different males in MS retention or transfer. An analogous kind of non-homogeneous distribution of microspheres in large blood vessels has been observed in studies of blood flow (Rudolph and Heymann 1971, Sabto et al. 1978, Rosenberg et al. 1983). The explanations sometimes offered, laminar flow and axial streaming, appear unlikely for cloacal contents under normal conditions in passerines. A more probable explanation for MS behavior within the cloaca may be differences in binding of MSs to the cloacal lining in relation to size and chemical composition. Intrinsic differences in their

Table 2. Numbers of microspheres (MSs) recovered from males by cloacal lavage after cloacal instillation (CI).

	MS Code #:①			②			③			⑤		
Post CI day	Hours	# MSs	Species	Hours	# MSs	Species	Hours	# MSs	Species	Hours	# MSs	Species
Day 1	24	2	AMRO	24	3	NOOR	24	23	HOSP	18	52,4000	HOSP
Day 2	25	0	HOSP	41	6	NOOR	42	51	INBU	26	395	HOSP
	47	0	RWBL	42	0	HOSP				29	26,900	INBU
				43	111	INBU				30	27,300	INBU
				43	5	HOSP				36	8,940	HOSP
										39	35,800	INBU
Day 3	49	0	RWBL	67	2	HOSP	52	1	INBU			
Day 4							94	8	HOSP			
Day 5							106	11	HOSP			
Day 6				123	0	NOOR						
Day 10							243	0	NOOR			

chemistry and surface charge may be important in this phenomenon. Some preliminary support for this idea can be found in the technical literature. Ion capturing and exchanging behaviors can be induced in the specific copolymer constituting ⑤ microspheres (Schwartz and Goodman 1982 p. 190).

No contamination with microspheres other than those instilled in that particular bird was found in any male CL slide.

Recovery of microspheres from females. Results pertaining to cloacal MSs in females are summarized in Tables 3 and 4. Also Figure 3 illustrates the simultaneous occurrence of microspheres from three different male Indigo Buntings in the cloaca of one female. These results demonstrate the efficacy of the CI technique for providing direct evidence of insemination encounters between specific individuals. A variety of biological factors contribute to the relative success or failure of the method as applied to a particular species. Beyond the factor of probability itself (having a large number of birds and CIs) several biological factors can be noted:

(1) Known timing or patterns of sperm release and insemination characterizing the species or population. The data of Table 4 reflect the fact that inseminations in Indigo Buntings were centered in early to mid May, although males were given CIs throughout the month. In contrast House Sparrows were involved in inseminations throughout April and May.

(2) Age-class differences in behavior. The population of Indigo Buntings had a large contingent of transient SY males; these were equal to older males in sperm fecundity (Quay unpublished), and comparably active in insemination encounters (Table 4).

(3) Pair-bonding, territoriality and social system. Lack of success in detecting transfer of MSs in Red-winged Blackbirds resulted from failure to obtain many recaptures, but behavioral observations of multi-year banded individuals indicated the importance of social factors as well. Older males, banded

Table 4. Detected male-to-female transfers of microspheres.*

Species / Dates	Males: Age Class	Ind.	CI: MS Code	CI: Time (CST)	Approx. Time Interval (Hours)	Females: Approx. Capture Time (CST)	Females: No. MSs in CLs	Ind.
Indigo Bunting								
May 6	SY	A♂	①	08:14	10.	18:30	1	A♀
May 12	SY	B♂	②	10:23	0.5	10:30	2	B♀
May 12-13	ASY	C♂	③	16:40	13.	05:30	5	C♀
	SY	D♂	①	20:23	9.		2	
	SY	E♂	②	21:20	8.5		3	
May 13	SY	F♂	③	10:48	4.	15:00	2	D♀
House Sparrow								
Apr. 9-10	AHY	A♂	⑤	14:05	16.	06:00	10	A♀
	ASY	B♂	②	08:35	21.5		2	
					28	12:45	1	B♀
Apr. 13-15	AHY	C♂	③	19:05	41.5	12:30	1	C♀
May 18	AHY	A♂	⑤	10:18	5.	15:10	1	D♀
May 19-20	ASY	D♂	②	20:30	9.	05:30	1	E♀
May 21-22	ASY	B♂	②	15:22	24.	15:30	1	D♀
May 22-23	ASY	D♂	②	15:58	16.	08:00	1	F♀
May 23-24	ASY	B♂	②	19:20	20.5	06:00	1	C♀
	TY	E♂	③	13:50	16.		2	
					21.5	11:30	1	H♀
Brown-headed Cowbird								
May 5-6	AHY	A♂	②	12:11	22.5	10:30	1	A♀

*Key: Age classes (based upon bandings in previous years and plumage characteristics; Quay 1987b, Pyle et al. 1987) - AHY = in at least its second calendar year; ASY = in at least its third calendar year; SY = in its second calendar year (the calendar year after that of hatching); ATY = in at least its fourth calendar year. Capture Time = estimated time of capture by mist net. CI = cloacal instillation of microspheres. CLs = cloacal lavages (cloacal lavage slide contents). CST = Central Standard Time. Ind. = individual bird, each designated by a particular letter (A-H) in relation to species and sex. MSs = microspheres; code numbers and characteristics as in Fig. 1.

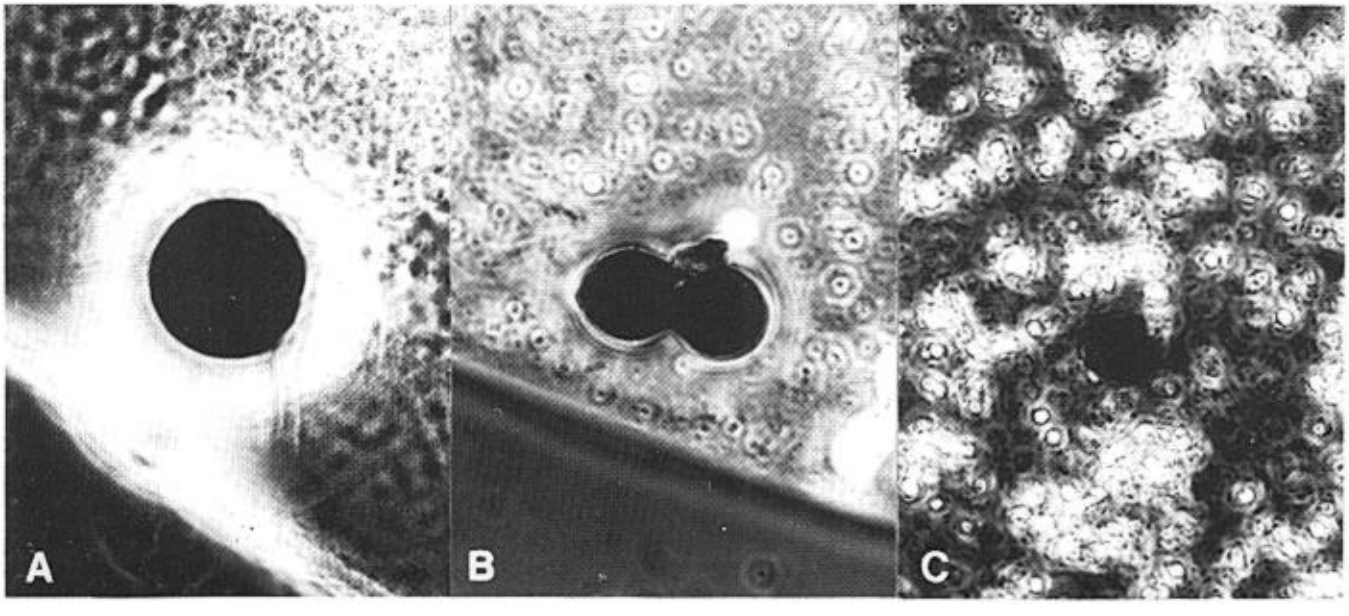

Figure 3. Photomicrographs at the same magnification of examples of the three kinds of microspheres lavaged from Indigo Bunting female C (Table 4.). A - a code ①. B - two code ②)s. C - a code ③.

Table 3. Adult females lavaged following cloacal instillation of males of the same species

Species	Dates	Cloacal lavages: Total Individuals	Recurrences 1	2	3	4	5	6	7	Total lavages	With microspheres: Total Individuals	Recurrences 1	2	Total Occurrences
Indigo Bunting	May 6-31	*80	*10	2						94	4			4
House Sparrow	Apr 9-June 1	*27	5	3	1	1	1	*1	1	63	*8		1	10
N. (Baltimore) Oriole	Apr 30-May 31	*11	*1		1		1			20	0			0
Red-winged Blackbird	May 4-30	*22								22	0			0
Brown-headed Cowbird	May 5-11	*4		1						6	1			1
Total										205	13			15

*One or more birds were relavaged in May-June 1987 and seen to be reproductively active and normal.

in former years and occupying all of the prime territories, were net-wary. Most males that were captured and that received CIs were first nuptial transients that generally were quickly driven from the resident territories, to which the local females remained closely associated.

Impacts, applications and limitations of the CI technique. None of the birds involved in either the CI or CL techniques showed any adverse effects, within the season, or upon recapture the following year (1987, Tables 1 and 3). Cloacal installation along with cloacal lavage has applications in studies of reproductive and associated behaviors of passerine species under natural conditions. This methodology opens a previously untapped resource in avian reproductive studies, whereby the quantity, quality and timing of an individual male's sperm output and insemination attempts can be evaluated. These reproductive parameters can be examined in comparison with the behavioral, hormonal, and other characteristics of the same birds and on a long term basis under natural conditions.

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