PITFALLS AND IMPROVED TECHNIQUES IN AVIAN PARENTAGE STUDIES

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ABSTRACT.—Recent studies on avian parentage have used both biochemical techniques and field observations to detect intraspecific brood parasitism and extra-pair fertilizations (EPFs). In long-term parentage studies of the European Starling (*Sturnus vulgaris*) using electrophoresis and field observations, we have encountered several methodological problems. Based on our findings and the existing literature, we describe these pitfalls and suggest improvements in techniques.

The pitfalls include difficulty in obtaining large sample sizes, manipulation of natural populations, inaccurate allelic frequencies based on electrophoretic screening of too few individuals, catching the wrong putative parents, improper storage and handling of tissue samples, confounding effects of developmental changes in allelic expression, and misinterpretation of electrophoretic results.

To improve parentage studies, we suggest censusing nest boxes around the peak times of laying to detect more than one egg per day, matching nestlings with eggs, establishing strict criteria for identifying putative parents at a nest, collecting pectoral muscle using an incision that goes in the direction of the superficial pectoral muscle fibers, and assessing the relative efficiency of laboratory and field methods to decide which would yield maximum results.
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THE use of electrophoresis to study avian parentage has increased rapidly (Gowaty and Karlin 1984, Fleischer et al. 1985, Gavin and Bollinger 1985, Joste et al. 1985, Mumme et al. 1985, Evarts and Williams 1987, Westneat 1987, Wrege and Emlen 1987, Kendra et al. 1988). More recently, DNA technology has been applied to birds for the same purpose (Quinn et al. 1987, Wetton et al. 1987). The goal is to detect intraspecific brood parasitism and extra-pair fertilizations (EPFs). Intraspecific brood parasitism involves egg laying in the nest of a conspecific with no subsequent parental investment by the parasite (Yom-Tov 1980). EPFs can, but do not always have to, produce cuckoldry (Power et al. 1981). The detection of intraspecific brood parasitism or EPFs is an indication that several reproductive strategies exist in a population of birds. Comparing protein phenotypes and DNA fragment patterns between putative parent and offspring for the purpose of parental exclusion may be the only way to detect these phenomena in some cases. This is especially true of EPFs where observation (such as witnessed copulation) cannot confirm parentage. In the case of intraspecific brood parasitism, both biochemical techniques (e.g. electrophoresis and DNA fingerprinting) *and* field observations may be used to detect parasite eggs or offspring.

The general methods of parentage studies are to collect eggs for studies of maternity only (Fleischer et al. 1985, Kendra et al. 1988) or to collect tissue (such as blood, pectoral muscle, feather pulp, or liver) from adults and nestlings for studies of maternity or paternity (Gowaty and Karlin 1984, Gavin and Bollinger 1985, Mumme et al. 1985, Evarts and Williams 1987, Quinn et al. 1987, Westneat 1987, Wetton et al. 1987, Wrege and Emlen 1987). The collected eggs or tissues are then examined for evidence of nonparentage. Field observations may be used as additional data or to supplement existing biochemical data (Quinn et al. 1987).

Our purpose was to review the pitfalls of techniques used to study avian parentage and to suggest improved techniques based on our own experiences with European Starlings (*Sturnus vulgaris*). This review primarily pertains to field methods and electrophoresis. It is apparent from the recent literature that researchers may not be aware of all of these difficulties or the need for improved techniques. We will refer to these studies (including our own) in the spirit of constructive criticism. We feel strongly that

understanding the drawbacks of certain practices before they become common in parentage studies is important.

FIELD METHODS

Sample size.—Sample size should be a major consideration when choosing a study species (see any basic statistical text for a discussion of this topic). For example, based on 22 early clutches (those laid in April), we estimated the rate of intraspecific brood parasitism in a population of starlings to be 13.6%; but after 3 yr and 111 early clutches, we estimated the rate of parasitism to be 26.1%.

Effects of manipulation.—Consideration must be given to the consequences of manipulating birds. When nest boxes are provided, care should be taken to position them to accommodate the territorial requirements of the study species. If this is not taken into account, it is possible to inflate rates of brood parasitism and EPFs (Semel and Sherman 1986). If boxes are placed closer than that found in nature, territories might overlap or birds might change their territorial boundaries to avoid overlap. In the first case, birds will encounter each other more often than under natural conditions and may spend more time defending their territories and resources against unnaturally close competitors. In the second case, birds will have to forage at greater average distances from their nests because of the high rate of prey harvest near their nests caused by population packing. In both cases, the amount of time spent on nest defense and mate guarding decreases, and rates of brood parasitism and EPFs may be artificially increased. Gowaty's bluebird nest boxes were only a fraction of a territory diameter apart (Gowaty 1980: 28, table XVII), calling into question the meaning of the high rate (25% for complete families) of multiple parentage Gowaty and Karlin (1984) reported.

Censusing nests to detect brood parasite eggs.—One criterion for detecting a brood-parasite egg is the presence of >1 egg/day (Yom-Tov 1980). Normally, birds do not lay >1 egg/day (Woodard and Mather 1964, Fraps 1965, Gilbert 1971, Lofts and Murton 1973). Daily censusing during the laying period will verify the laying rate and allow detection of parasite eggs (Brown 1984, Emlen and Wrege 1986). Censusing times are crucial for accurate estimates of brood parasitism and should be scheduled around the population's peak time of laying. For example, if most eggs are laid between 0800 and 1000, then censusing just before and just after this time interval (at the same time each day) should maximize immediate detection and marking of eggs (see below). In turn, this will help establish host laying patterns and facilitate identification of brood-parasite eggs. However, some birds cannot tolerate the disturbances of censusing. For example, Cliff Swallow (*Hirundo pyrrhonata*) laying will be disrupted if nests are checked before eggs are laid (C. R. Brown pers. comm.)

Parasite eggs can easily be missed if nests are censused only once daily or around nonpeak laying times. We feel that this was the case in 1983 when we censused twice daily *after* the peak laying interval. Parasitism during host laying was detected in only 9.1% of early clutches. By contrast, in 1985, we censused our boxes 3 times daily during laying: at 0700–0800 EST, 1100–1200 EST (at least 76% of all first-brood eggs were laid between 0700 and 1200), and 1500–1700 EST. Parasitism was detected in 28.9% of early clutches.

Despite ambitious censusing techniques, brood-parasite eggs can be missed if laid just before or just after the host begins to lay. These eggs are not distinguishable from host eggs because there is no apparent deviation in the laying cycle of the host (Frederick and Shields 1986). Brood-parasite eggs can also be missed if they are removed by hosts before censusing (Stouffer et al. 1987).

Marking eggs and nestlings.—The ability to determine the temporal components of their respective strategies is crucial to understanding the strategies of brood parasites and their hosts, and participants in EPFs. This information may explain mate guarding and aggressiveness. To this end, the ability to identify which nestling came from which egg is required. Consequently, nestling/adult electrophoretic mismatches can show behavioral patterns otherwise obscure. By marking eggs and nestlings, we determined that brood parasites laid their eggs primarily on the second and third days of the host laying cycle when males still guarded mates and incubation had not yet begun. In addition, we determined the reproductive success of host vs. parasite with respect to hatching and fledging (Romagnano 1987).

For identification, eggs were marked sequentially with indelible ink at the time of laying. Identification at time of hatching may be dif-

ficult because the researcher may not be at the nest when all eggs hatch. Food dye can be used to circumvent this problem (Rotterman and Monnett 1984). We injected 30 μl of one color (blue, red, green, and yellow of *McCormick* and *Durkee* brands) into the air space of each pipped egg with a 50-μl syringe (Hoffenberg et al. 1988). The hatched nestlings retained some of the food dye on their down feathers, skin or beak for 1–2 days. *McCormick's* yellow dye caused 7 cases of prehatching mortality while no mortality was attributed to the other colors. This was corrected by using only 15 μl of yellow dye. Yellow color was still visible and no further deaths occurred.

Colored nestling claws were clipped in a unique pattern upon hatching. This allowed identity of a nestling's position in the egg-laying sequence until it could be permanently identified with a numbered USFWS band at the time of biopsy (biopsy occurred 20 days after hatching). Clipped claws usually remained blunt, but some nestlings had to have their claws reclipped 12–15 days after hatching.

Electrophoretic and Biopsy Techniques

Preliminary electrophoretic screening.—Preliminary screening of tissues to assess both the number of variable loci and the number of alleles at those loci is mandatory before a large project is undertaken (Mumme et al. 1985). A minimum of 50 adults should be screened to obtain accurate allelic frequencies upon which to judge the utility of a protein system (Lewontin 1974). Screening <50 adults may give false preliminary allelic frequencies and some variable loci will be missed. For example, based on 30 adults, we estimated the ratio of the frequencies of two alleles at one locus to be 2:1. However, on subsequent sampling of 175 adults, we found the ratio to be 3:2. Similarly, preliminary estimates based on a small sample of starlings suggested that plasma cholinesterase was a variable locus. After sampling >100 starlings, we discovered that a third rare allele was found for both plasma cholinesterase and pectoral muscle esterase I. These were, in fact, the same enzyme.

Ideally, one would like to have a system with many variable loci, multiple alleles at those loci, and all alleles in equal frequency so that the chances of parental exclusion are high (Westneat et al. 1987, Wrege and Emlen 1987). If the allelic frequencies at a single locus are 0.95 and 0.05, respectively (assuming the loci are unlinked and in Hardy-Weinberg equilibrium), then the probability of excluding parentage would be quite small (0.045) when both parents are known. However, the probability of excluding parentage is four times greater with one locus that has two alleles at equal frequency (Wiener et al. 1930) or eight times greater if the locus has three alleles at equal frequency (Wiener 1968). The probability of parental exclusion based on a combination of loci will vary according to the probability of exclusion for each locus.

The low degree of genetic variability in birds (Barrowclough et al. 1985) reduces the utility of electrophoresis as a tool for parentage studies in many avian species. Using only one or two types of tissue for analysis restricts the number of proteins present for detection (Mumme et al. 1985). Although we screened 33 loci, only 3 were used in our study. One of these loci (plasma amylase) had approximately equal allelic frequencies while the other two loci (pectoral muscle esterases 1 and 2) had allelic frequencies approaching monomorphism.

Choice of electrophoretic medium.—Most studies on avian parentage have used either starch or polyacrylamide gel electrophoresis. Both of these methods have advantages and disadvantages. Starch gel electrophoresis is more commonly used and produces results more quickly than polyacrylamide (i.e. on a per gel basis, polyacrylamide gels can usually be stained to detect only one enzyme, while starch gels can be stained to detect several enzymes). However, the degree of enzyme resolvability may vary from lot to lot with starch (G. F. Barrowclough pers. comm.).

Polyacrylamide gel electrophoresis has a high degree of sensitivity in detecting protein variants (Coyne et al. 1979, Ramshaw et al. 1979) and costs approximately the same as starch on a per gram basis (Sigma). However, in its liquid form, polyacrylamide is a neurotoxin and must be handled appropriately. We favor the use of polyacrylamide when only a few systems are being examined. We have used both starch and polyacrylamide for preliminary screening and found that polyacrylamide afforded greater resolution of enzymes.

Identifying the right adults.—Strict criteria to determine which birds are putative parents at a given nest must be established to prevent tis-

sue collection from the wrong birds. Such mistakes are especially likely in species with visitors or helpers at nests. Criteria for parentage might include behaviors such as incubation, brooding young, cleaning nests, feeding nestlings, or nest defense. The criteria we used to identify the parents at starling nests were brooding young at night (females only) (Litovich 1982), feeding, and nest cleaning.

Careful observations of individually recognizable birds at nests (e.g. bands or distinct markers) can eliminate questionable birds. Birds that do not meet the criteria should be excluded from estimates of rates of genetic mismatches as this may artificially inflate these rates. For example, Gowaty and Karlin (1984) caught a female that did not match any of the nestlings in "her" nest; this bird should not have been included in their estimate of the frequency of multiple parentage for all broods because the "observations on this family were incomplete and ambiguous" (see Gowaty and Karlin 1984: footnote to table 1). Overestimates of rates of brood parasitism or EPFs might lead to wrong interpretations of the importance of reproductive strategies.

All birds that meet the criteria for parents should be sampled. The probability of detecting brood parasitism decreases markedly when only the female is sampled (Wiener 1952) and EPFs cannot be detected at all. In addition, the wrong conclusions can be reached if *all* attending adults are not sampled. At one starling nest we observed two females and one male in attendance. Electrophoretic analysis showed that the nestling phenotypes resulted from the male mating with both females and was thus a case of communal nesting. Had we sampled only one of the females, we would have reached the erroneous conclusion that some of the nestlings were the result of intraspecific brood parasitism (Stouffer et al. 1988).

Obtaining tissue.—Biopsy techniques should not cause trauma which could lead to nest desertion. We sampled seven females on the night of the third day after the first nestling hatched. Five of these seven nests failed the next day, most likely due to female desertion after early biopsy or exposure of young nestlings while their mothers tended their own wounds. Thereafter, all females were sampled on the night of the sixth day after hatching of the first nestling; no further nest failures were attributable to this procedure.

We collected both blood and pectoral muscle tissue. Blood was collected from the brachial vein (Hoffenberg et al. 1988). Occasionally the punctured brachial vein of one wing formed a hematoma with little subsequent blood flow. In these cases, the brachial vein from the other wing was used with no apparent increase in trauma to the bird. Lack of trauma after venipuncture has also been observed in other birds (Stangel 1986).

We initially collected pectoral muscle tissue by making a cut longitudinal to the keel through the skin and superficial pectoral muscle and removing a small piece with a fine scissors (Seidensticker 1970, Baker 1981). This technique has been used with satisfactory results and no apparent detrimental effects (Westneat et al. 1986, this study, except see Frederick 1986). However, we modified this technique by making the incision along the grain of the muscle. Scar tissue formation was minimized with a diagonal incision; birds showed no visible evidence of scar tissue after 28 days. We feel it important to determine the orientation of the superficial pectoral muscle before doing large-scale biopsies (orientation may vary from species to species) so that cuts can be made in the direction of the muscle fibers rather than across fibers.

Handling and storage of tissue.—Spurious electrophoretic results of plasma proteins are possible if tissue is not handled properly once collected. In some studies, blood was frozen immediately in the field (Gowaty and Karlin 1984, Gavin and Bollinger 1985). In others, plasma and cells were separated before freezing (Joste et al. 1985, Mumme et al. 1985, this study). Although it may be impossible to bring blood back to a lab for processing before freezing, we strongly recommend that every attempt be made to do so. We immediately froze some samples of whole blood at −80°C before plasma was separated from cells by centrifugation. This blood was later centrifuged to obtain a plasma fraction, but red blood cell (RBC) lysis had occurred. Electrophoretic analysis of plasma with RBC lysis products and plasma separated from RBCs before freezing revealed differences in electrophoretic patterns of the same animal. One system in which this was notable was albumin (Fig. 1). "Clean" plasma was single banded for this system while "contaminated" plasma was double banded. To prove that we were seeing the effects of RBC lysis on plasma patterns, we ran seven individuals for which we had "con-

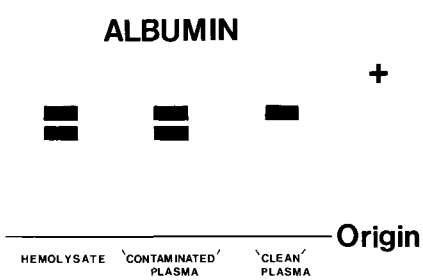

Fig. 1. Diagram of Coomassie-stained electrophoretic gels of hemolysate, "contaminated" plasma and "clean" plasma for albumin of the same individual starling. Patterns differ between treatments.

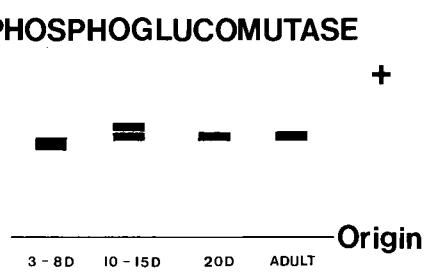

Fig. 2. Diagram of an electrophoretic gel of phosphoglucomutase showing effects of age on allelic expression in starlings. Pre-fledgling age nestlings (3–8 days and 10–15 days) show patterns not seen in fledglings (20 days) or adults.

taminated" plasma, "clean" plasma, and hemolysate (Bush 1967). In all cases, "contaminated" plasma and hemolysate patterns matched. The pattern seen for "contaminated" plasma is a result of components of RBCs (Fig. 1). Another system which showed a RBC "allele" was pectoral muscle esterase 2.

Clearly, electrophoretic patterns can be influenced by tissue preparation and handling. Thus, the results from use of whole blood must be interpreted with caution.

Developmental effects.—Nestlings present a special problem when dealing with tissue collection due to developmental effects on protein expression (Bush 1967, Ohno et al. 1969). Hatchling House Sparrow (*Passer domesticus*) plasma had only half the number of electrophoretic bands as adults (Bush 1967). But, as they approached fledging, nestling banding patterns became more adultlike. We noted this for nestlings collected at various stages of development. We collected pectoral muscle tissue from starling nestlings at days 3–8 ($n = 23$), 10–15 ($n = 12$), and 20 ($n = 331$). (Starlings normally fledge on day 20; day 1 is the day of hatching of the first nestling.) Nestlings aged 3–8 days showed a diffuse banding pattern for phosphoglucomutase (Pgm). Nestlings aged 10–15 days showed a double-banded Pgm pattern. Day 20 nestlings and adults ($n = 205$) expressed only a single band for this locus (Fig. 2). Major growth of pectoral muscle mass occurs in starlings from days 10–15 (Ricklefs 1979), thus changes in gene expression may be expected during this time.

While offering useful ontogenetic information for developmental biologists, the apparent age-developmental changes can confound the conclusions in avian parentage studies. Failure to consider these differences in phenotypic patterns can lead to false mismatches if nestlings are compared to adults that do not display these patterns. This could be the single most important source of error in parentage studies. We strongly recommend that nestling tissue collection be as close to fledging as possible.

The importance of developmental effects should encourage the researcher to develop methods whereby nestlings can be sampled close to fledging but be restricted from fledging prematurely until the traumatic effects of biopsy and handling have passed. We prevented young birds from fledging right after biopsy by attaching boards to the nest-box opening to restrict its size so that nestlings could not fledge but parents could still feed. We restricted nestlings on day 18 and performed biopsies on day 20. The boards were removed on day 21 allowing nestlings to fledge normally (Hoffenberg et al. 1988).

Interpretation and Usefulness of Data

Nature of mismatches.—Once a phenotypic mismatch has been detected, it is important to decide the source of the mismatch. All nonbiological reasons should be ruled out (i.e. catching the wrong adult, developmental effects, and tissue artifacts). Where mismatches are ambiguous, field observations might distinguish among the possibilities. For example, at one starling nest, both the female and male had an FF genotype for plasma amylase. Nestling 5 at this box had an FS genotype. The other four nestlings at this box were FF. This nestling could have resulted from a brood-parasite egg or cuckoldry. Due to the lack of any field evidence (e.g. egg color differences within the clutch) that would have allowed us to categorize this nestling, we did not include it in our results. How-

ever, if the intent of the experiment is to determine overall rates of multiple parentage, then all cases should be reported.

Westneat et al. (1987) proposed a method—based on the models developed by Wiener et al. (1930) to determine human paternity—that allows a prediction of the source of genetic mismatches based on a comparison of the distributions of expected and observed types of parental exclusions. However, this method works best for species that have primarily one source of genetic mismatches (Wrege and Emlen 1987).

Relative efficiency of laboratory and field methods.—The relative efficiency of laboratory and field methods will vary according to individual circumstances. We found that field techniques were far more sensitive than laboratory techniques in the detection of brood parasitism. This was primarily due to the allelic frequencies of the three loci used for electrophoretic analysis. Electrophoresis of 72 early brood families detected six unambiguous cases of parasitism at five nests (6.9%). By contrast, 29 of 111 early clutches (26.1%) that were censused contained parasite eggs.

Field observations could not be used to detect EPFs in starlings. We had to rely solely on electrophoresis. For families where three loci were examined (95), we detected cuckoldry in two broods (2.1%) (Hoffenberg et al. 1988). Statistical methods have been developed to provide less biased estimates of cuckoldry based on the genotypic data (Evarts and Williams 1987). Although the recent emphasis on mathematical models to estimate various parameters of brood parasitism or EPFs is applauded (Evarts and Williams 1987, Frederick and Shields 1986, Westneat et al. 1987, Wrege and Emlen 1987), it should be stressed that the numbers generated by these models are only as good as the quality of the data used in their calculation.

OTHER METHODS TO STUDY AVIAN PARENTAGE

Other means of detecting intraspecific brood parasitism and EPFs are available. Ovarian examination can show if the number of eggs in a nest matches the number of ruptured follicles in the ovary (Davis 1958, Kennedy 1989). Genetic markers have been used successfully with poultry (Compton et al. 1978, Payne and Kahrs 1961) and might be useful in wild birds if they can be found. Restriction fragment length polymorphisms (RFLPs) have been compared within families (Quinn et al. 1987). This technique uses restriction enzymes to generate DNA fragments which are then detected with DNA probes. Offspring should show the same DNA fragment patterns as their parents. DNA "fingerprinting" has also been used successfully in birds (Wetton et al. 1987). After DNA fragments are generated by restriction enzymes, the fragments are probed with DNA that is a clone of the core regions of areas of the genome known as minisatellites. These are highly variable, tandem-repetitive regions of DNA located in the heterochromatin. DNA "fingerprinting" is a more sensitive technique than RFLPs (Lewin 1986). Although RFLPs and DNA "fingerprinting" seem attractive alternatives to electrophoresis, the cost of such a project would be prohibitive to anyone not already set up to do molecular genetics studies. More importantly, many of the same pitfalls that apply to electrophoresis and parentage studies in general would apply here as well.

RECOMMENDATIONS

We recommend that avian studies of parentage include the following improvements:

1. Carefully consider sample sizes needed to yield meaningful results.
2. Assess the effects of manipulation, such as nest-box placement, on your population.
3. Census nests at least twice daily to detect brood parasite eggs. Censuses should take place at the same times each day before and after the peak laying times. Do not census if birds are greatly disturbed.
4. Mark eggs sequentially during laying and maintain the egg/nestling identification until biopsy.
5. Screen at least 50 adults for variable protein systems before starting a full scale project. The loci used should yield enough information to phenotypically discriminate among a large proportion of individuals. If not, then don't use electrophoresis.
6. Use strict criteria for deciding which adult birds are putative parents at a nest, e.g. incubation, feeding, nest defense, etc.
7. Minimize the trauma of pectoral muscle biopsy by making an incision that goes with the grain of the superficial pectoral muscle.
8. Separate plasma from RBCs when possible and look for artifacts when not possible.

9. Avoid loci with developmental effects or use them only after their adult phenotypes emerge, e.g. on or as close as possible to the day of fledging.
10. Use field and laboratory techniques to complement each other.
11. Assess the relative efficiency of laboratory and field techniques early on in the project.

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