The Condor 86:79–80

AN EMBRYO-DYEING TECHNIQUE FOR IDENTIFICATION THROUGH HATCHING

LISA MIGNON ROTTERMAN

AND

CHARLES MONNETT

The clutches of many birds tend to hatch synchronously (Lack 1968) and may also hatch at night (pers. observ.). Thus, it is difficult to determine which young hatched from which egg, even with frequent nest visits (e.g., Fiala 1981). The ability to easily assign nestlings to specific eggs would aid in accumulating the large samples that are needed to elucidate the effects of different patterns of parental investment. Precise relationships of egg-laying order, egg size, hatching order and clutch size to hatchling quality (e.g., probability of fledging) could be determined.

In this paper we present a method of dyeing embryos while still in the egg, thus permitting such questions to be examined directly.

STUDY AREA AND METHODS

We studied a population of Red-winged Blackbirds (*Agelaius phoeniceus*) breeding in marshes on or adjacent to the Columbia National Wildlife Refuge in central Washington. The study area is near, and similar to, that described by Horn (1968) and Orians (1980). Nests were located before egg-laying began and were visited daily. We sequentially numbered each egg on the day it was laid by marking directly on the shell with a black fine-point felt-tipped marker for non-porous surfaces. After the tenth day of incubation we visited each nest twice daily to examine eggs for openings in the shell caused by embryo "pipping." We always waited until pipping had occurred before piercing eggs in order to minimize the possibility of harming the embryo.

Within a nest, eggs were selected as experimentals or controls by laying order according to a predetermined rotating schedule. In no case did we dye all nestlings within a nest. Before piercing, experimental eggs were first lightly scratched on the side of the egg opposite the pipped opening. Scratching roughened the surface, a step which kept the piercing instrument from sliding on the shell, thereby making it easier to control the size and the location of the opening. For piercing we usually used a stainless steel wire, approximately 0.4 mm in diameter, but occasionally we employed a small pin or needle. The wire used to string the Fish and Wildlife Service bands works well for this purpose. We punctured the shell with the wire directed nearly parallel to it, rather than perpendicular to the surface, so as to lessen the likelihood of stabbing the embryo.

Green, red, blue, or yellow McCormick-Schilling food coloring (containing dyes FD & C yellow #5, reds #3 and #4, and blue #1) was used as the dyeing agent. Green chicks were sometimes difficult to tell from blue chicks and hence we rarely used both colors in the same nest. The other colors dyed unambiguously if used properly. One very small drop of coloring was applied to the outside of the shell, adjacent to the puncture, and usually the dye inspired immediately into the egg. If inspiration did not occur (usually due to blockage by a piece of shell or membrane, or because piercing was insufficient) reinsertion of the wire tip laterally against the shell inner surface served as a conduit and caused the dye to flow rapidly into the egg. Excess dye was wiped from the outside of the egg. The egg was then returned to the nest, taking care that neither the puncture site nor the pipping hole was placed downwards. The embryo could suffocate from the latter condition and other embryos could be accidentally dyed from the former.

Although a small drop of blood occasionally collected at the puncture site, the embryos hatched and appeared normal. Two embryos died due to our handling. In both cases we used pins to puncture the egg, and the eggs lost a relatively large amount of blood immediately after piercing. The embryos were shriveled within their shells the following day.

When a small amount of dye was used as described, nestlings were usually dyed on an isolated part of their plumage. Usually, the only areas colored were the down on the top of the head or on the back. On the first day after hatching, nestlings were individually marked on their tarsi, again with the felt-tipped marker, since the dye was often undetectable after 24 h. On the eighth day after hatching, nestlings were weighed in a small plastic bag on a 100-gram Pesola scale, and their right tarsus measured. Fledging outcome was based, for each nestling, on its condition and the age when last seen in the nest.

Dyeing could affect two major external factors that influence the immediate welfare of the nestlings: the willingness of the parent(s) to care for the young, and the ability of predators to detect the young. Differences in growth rates and fledging success for experimental and control nestlings within each nest would reflect the former. Differences in the probability of nest predation of nests containing some dyed young vs. those without would indicate the latter. Partial predation (not all eggs or nestlings within a nest are predated) does occur, but it was rare during this study.

We compared growth of dyed vs. control nestlings based on weight at eight days of age. Because the number of nestlings in the developing brood and the relative ages of the nestlings probably influences individual growth rates, we used only the first three hatchlings from broods fledging at least three young in our weight comparisons. The fourth and fifth hatchlings are usually one and two days younger than the first three, respectively. Sample sizes were too small to permit comparisons within these two ranks. We also compared the probability of fledging using clutches hatching at least three young. Since Red-winged Blackbirds are sexually dimorphic by eight days of age, we tested the effect of dye on growth within each sex, using Student's *t*-test and one-way ANOVA. Nestlings were sexed by interpretation of weight and tarsal measurement data. By the eighth day post-hatching, there is virtually no overlap in tarsus length (unpubl. data) or weight (Williams 1940, Haigh 1968, Holcomb and Twiest 1970, Fiala 1981, unpubl. data) of the two sexes for healthy blackbird nestlings.

We compared the probabilities of fledging for dyed and control nestlings by chi-square analysis with the sexes

TABLE 1. Weights (in grams) of eight-day-old Red-winged Blackbird nestlings subjected to pre-hatching dye treatment.

		No dye	Red	Yellow	Blue or green*
Female nestlings	Mean	31.7	32.3	32.8	31.7
	SD	2.7	3.0	1.9	2.6
	n	13	5	9	11
Male nestlings	Mean	42.9	42.5	42.0	42.1
	SD	2.5	3.4	4.9	4.5
	n	12	10	7	7

* Blue and green were combined due to small sample sizes and similar appearance of treated nestlings.

combined using two groupings: the oldest three nestlings and the fourth.

RESULTS AND DISCUSSION

Weights did not differ significantly between dyed vs. control eight-day-old male and female nestlings. Males averaged 42.9 ± 2.5 g (SD) and 42.2 ± 4.0 g ($t = 0.48$, 34 df, $P > .50$) while females averaged 31.7 ± 2.7 g and 32.2 ± 2.4 g ($t = 0.61$, 36 df, $P > .50$) for control and dyed nestlings, respectively. Analysis of variance for treatments of control, red, yellow, and blue or green nestlings also indicated that there was no specific color effect for either sex (females: $F = 1.26$, 3, 34 df, $P > .25$; males: $F = 0.10$, 3, 32 df, $P > .25$; see Table 1).

Nests containing dyed young showed no significant increase in probability of predation during the nestling stage compared with nests containing only control young. The probability of predation for nests containing dyed young was 0.28 ($n = 87$); the probability of predation for nests containing only control nestlings was 0.30 ($n = 43$) ($\chi^2 = 0.11$, 3 df, $P > .95$). Within-nest comparison of fledging success for the oldest three nestlings showed no difference between dyed and control nestlings ($\chi^2 = 0.00$, 3 df, $P > .99$), as 97% of the nestlings fledged (72 of 74 and 33 of 34) in each category, respectively. A within-nest dye effect might be most detectable for the fourth- or fifth-ranked nestlings because they are already at a disadvantage due to their relative immaturity; these nestlings starve to death more frequently than their siblings (unpubl. data). We had insufficient data to test growth, but probability of fledging was not significantly different for dyed vs. control nestlings when tested on the fourth-ranked nestlings ($\chi^2 = 2.1$, 3 df, $P > .50$).

Our results indicate that dyeing embryos shortly before hatching does not appear to have short-term adverse effects on either nestling condition or survival. However, condition at fledging is not necessarily a good indicator of an individual nestling's reproductive prospects. Possible long-term effects from the dye (e.g., carcinogenic or mutagenic effects) could not be assessed from our study. Nevertheless, we feel that such effects are unlikely because, as mentioned previously, the dye normally colored the down only on the back or the head and was usually undetectable within one to several days.

The preferred amount, color and type of dye used to mark embryos would depend on the species and the nature of the study. In our initial experiments with this method we used as much as two small drops of dye, which brilliantly and extensively colored the nestlings, including the beak. These nestlings were still obviously colored on their down feathers at fledging. However, we were quickly able to minimize the dyeing as described above. This technique was preferable for our study, as we needed to distinguish nestlings for less than 24 h for the questions we were chiefly addressing. If the interval between dyeing and subsequent hatching or visitation were greater, or if nestlings could not be marked satisfactorily in any other way post-hatching, heavier dyeing might be desirable.

The dye could probably be injected before pipping, but this might increase the risk of dehydration, internal uptake of the dye by the embryo, or other dangers to the embryo. We have no systematic data regarding such risk. Carol Vleck (pers. comm.) suggested that the following procedure could be tested on chicken eggs: pierce the egg over the air cell (to avoid the chorio-allantoic circulation) using either a sterilized piercing object or a non-sterilized one and antibiotics. Use sterilized dye and, lastly, seal the hole with either wax or with plastic (or, our suggestion, possibly "liquid band-aid"). We encourage such a pilot study, since injection before pipping would further lessen the required frequency of nest checks.

Several potential problems related to both the color and amount of dye also warrant consideration. Adults of some species might reject oddly colored nestlings although we found no evidence of parental rejection within our population. Our findings concur with Haigh's (1968), who reported similar insensitivity to nestling color in adult Red-winged Blackbirds after partially marking nestlings with a soft red crayon.

Certain colors might increase conspicuousness and, hence, vulnerability of nestlings to predators. Our findings do not support such an effect for Red-winged Blackbirds. We believe our data to be a good test of a nestling color effect, because a predominant predator in our study areas was the Black-billed Magpie (*Pica pica*), which hunts primarily by vision. For birds that are more dependent on nesting concealment, however, the color of the nestling relative to its background might be more important.

In summary, we have presented a quick and simple technique that permits continuous identification of individual birds through hatching, and which does not appear to significantly affect their short-term survival or condition.

We thank James Wittenberger, Dee Boersma, Gordon Orians and Nat Wheelwright for reading and commenting on the manuscript, and Eve Hiatt for useful advice. We especially thank Robert Paine and James Wittenberger for encouragement throughout our research. We thank Carol Vleck for suggesting the pre-pipping dyeing procedure. David Goeke granted permission for work on the Columbia National Wildlife Refuge, and Para Brothers' Ranch permitted us to work on adjacent private lands. Field work was partially supported by NSF grant BNS8004873.

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Department of Zoology, University of Washington, Seattle, Washington, 98195. Current address: Department of Ecology and Behavioral Biology and Bell Museum of Natural History, 318 Church Street, S. E., University of Minnesota, Minneapolis, Minnesota 55455. Received 23 December 1982. Final acceptance 15 June 1983.