

## Morphology versus molecules: sexing Red-winged Blackbird nestlings

Patrick J. Weatherhead,<sup>1,4</sup> Katherine E. Muma,<sup>2</sup> J. Dylan Maddox,<sup>1</sup>  
Jenny M. Knox,<sup>1</sup> and Kevin W. Dufour<sup>3</sup>

<sup>1</sup>Program in Ecology and Evolutionary Biology, University of Illinois, Champaign, Illinois 61820, USA

<sup>2</sup>Department of Biology, Ithaca College, Ithaca, New York 14850, USA

<sup>3</sup>Canadian Wildlife Service, 115 Perimeter Road, Saskatoon, Saskatchewan S7N 0X4, Canada

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**ABSTRACT.** Past studies of offspring sex ratios in birds have often relied on sexually size dimorphic species where nestling sex could be determined based on weight at a given age. DNA-based sexing techniques allow us to assess the accuracy of those techniques and to refine them for use when costs or convenience make DNA methods impractical. Using nestling Red-winged Blackbirds (*Agelaius phoeniceus*) whose sex was determined using DNA, we compared sex ratios obtained using different morphological criteria. Conservative criteria from previous studies were completely accurate, but allowed sexing of few nestlings younger than 8 d old, and were more successful for sexing males than females. A new method was developed that allowed accurate sexing of nestlings beginning at day 6 posthatching and was less biased relative to known sex ratios. Using 11 years of data, the conservative method left an average of 55% of nestlings and 36% of fledglings unsexed, compared to 31% and 9% using the new method. Furthermore, the male bias in sex ratio estimates using the conservative method was greater, both absolutely and relative to estimates based on the new method, when the proportion of unsexed nestlings (because they were not weighed when older) was higher. Thus, estimates of population sex ratios will be more accurate as the number of nestlings measured on day 8 or older increases. However, if some nestlings that were not weighed past day 7 fledge, the new method allows more of those individuals to be sexed than the conservative method, and the population sex ratio estimate should be more reliable. Although our approach should apply to other sexually dimorphic species, the criteria used must be developed based on such species-specific attributes as growth patterns and degree of hatching asynchrony.

### SIÑOPSIS. Sexado morfológico vs. molecular en pichones de *Agelaius phoeniceus*

Los estudios hechos en el pasado, sobre la relación de sexos en aves, dependieron (en gran medida) del dimorfismo en los pichones en donde el sexo podía ser determinado por el peso de estos a una edad particular. El sexado utilizando ADN permite determinar la exactitud de estas técnicas y a refinarlas cuando el costo o la conveniencia hace al ADN un método impráctico. Utilizando como ejemplo a pichones de *Agelaius phoeniceus* cuyo sexo había sido determinado utilizando ADN, comparamos la tasa de sexos utilizando diferentes criterios morfológicos. Criterios, utilizados en estudios previos, resultaron ser completamente confiables aunque no permitieron el sexado adecuado de pichones menores a ocho días y resultaron ser más exitosos sexando machos que hembras. Desarrollamos un nuevo método que nos permite sexar con exactitud pichones mayores a seis días, y que tiene menos sesgo para determinar la tasa de sexos. Utilizando los datos coleccionados de la misma población, durante 11 años previos, el método conservador dejó sin sexar un promedio del 55% de los pichones y el 36% de los volantones, comparado con el 31% y el 9%, respectivamente, utilizando el método novel. Más aún, el sesgo en la tasa anual de sexos de machos en la población, fue mayor al utilizarse el método conservador, en términos absolutos y relativos, que los estimados basados en el nuevo método y en la mayor proporción de pichones sin sexar. La razón por la cual los pichones no fueron sexados, fue que estos no fueron pesados cuando ya estaban crecidos (mas viejos). La exactitud de los estimados de la tasa de sexos poblacionales irá en aumento según se vayan pesando los pichones más viejos.

**Key words:** DNA sexing, Red-winged Blackbirds, sex identification, sex ratio

Ornithologists have long been interested in sex ratios. Ideas such as those of Fisher (1930) and Trivers and Willard (1973) identified circumstances where optimal sex ratios should deviate from 1:1. Testing these and other hypotheses have provided evidence that birds do adjust sex

ratios facultatively in response to a variety of factors (West and Sheldon 2002, Cassey et al. 2006). Sexually dimorphic birds have been of particular interest in sex ratio research for two reasons. First, size differences between male and female nestlings mean that costs of producing sons and daughters differ (Fiala and Congdon 1983, Slagsvold et al. 1986, Teather and Weatherhead 1988). Such differences lead to

<sup>4</sup>Corresponding author. Email: pweather@uiuc.edu

expectations of nonrandom sex ratios arising either through excess production or excess mortality of nestlings of one sex (Fisher 1930, Weatherhead and Teather 1991). Second, size differences allow investigators to determine nestling sex in the field, until recently the easiest way to test theory. DNA-based sexing techniques (Griffiths et al. 1998, Kahn et al. 1998, Fridolfsson and Ellegren 1999) now permit sexing without reliance on morphology. These techniques also permit evaluation of the accuracy of morphological methods used previously, and the refining of morphological methods for future use where sexing nestlings in the field is required. Our goal was to use DNA methods to evaluate and refine morphological sexing methods for Red-winged Blackbirds (*Agelaius phoeniceus*).

Red-winged Blackbirds are sexually size dimorphic and have been the subject of numerous studies of nestling sex ratios (Weatherhead and Teather 1991, Beletsky 1996). McIlhenny (1940) claimed that nestling sex could be determined with certainty after the fifth day, apparently based on general appearance (Williams 1940), and determined that males outnumbered females by more than three to one. Subsequent studies using more objective approaches have generated estimated sex ratios much closer to equality. Although most investigators have used bimodality of weight distributions of older nestlings to assign sex (Williams 1940, Nero 1961, Holcomb and Twiest 1970, Fiala 1981, Westneat et al. 1995, Weatherhead and Dufour 2000), laparotomy (Fiala 1981), and necropsy (Williams 1940, Weatherhead 1983, 1985) have also been used.

Using size differences to sex Red-winged Blackbird nestlings is simple and noninvasive, and the accuracy of this approach has been confirmed using both gonadal inspection (Williams 1940, Fiala 1981) and sightings of adults sexed as nestlings (Weatherhead and Dufour 2000). The shortcoming of using weight differences to sex nestlings is that the distributions of male and female weights overlap until late in the nestling period (Williams 1940, Holcomb and Twiest 1970). Thus, if late measurements are missing (e.g., predation, premature fledging, or infrequent nest checks), male and female weights overlap and the sex of some individuals is unknown. Furthermore, it is not known whether both sexes are equally represented among those unsexed nestlings, and thus whether estimated sex ratios are accurate. Our objectives were to use

weight measurements from Red-winged Blackbird nestlings sexed using DNA to (1) assess the accuracy of existing criteria for sexing based on weight, (2) determine whether the sex ratio of unsexed nestlings (i.e., with masses in the area of overlap) differs from that of sexed nestlings, (3) determine whether sexing criteria can be modified to improve accuracy and reduce the number of unsexed nestlings, and (4) apply these modified criteria to previously published data to determine whether sex ratio estimates are altered.

## METHODS

Field work was conducted at two beaver ponds previously used for long-term studies of Red-winged Blackbirds (Weatherhead and Dufour 2000) at the Queen's University Biological Station at Chaffey's Locks, Ontario (44°34'N, 76°20'W). From early May to early July 2005, we searched both marshes every 2–3 days for new nests and visited nests found previously. Almost all nests were found prior to or during egg laying, so we were able to accurately determine first egg dates and hatch dates. We marked nestlings individually after hatching and weighed them during most nest visits. When nestlings were 4–6 days old, we collected approximately 35  $\mu$ l of blood by brachial venipuncture. Blood was stored in 1 ml of lysis buffer (Seutin et al. 1991) and refrigerated at 4°C until used for sex determination.

**Genetic sexing methods.** Because commonly available primers (Griffiths et al. 1998, Kahn et al. 1998, Fridolfsson and Ellegren 1999) used to determine sex in birds do not produce easily discernible size differences between the Z- and W-specific products in Red-winged Blackbirds, we substituted 1296rev\_Agpho (M. K. Babcock and F. K. Barker, pers. comm.), a primer more specific to Passerines and *Agelaius*, for 1272H of the primer pair 1237L/1272H (Kahn et al. 1998).

We extracted DNA using DNeasy Tissue Kits (Qiagen, Valencia, California). All polymerase chain reactions (PCRs) were performed in 10- $\mu$ l volumes in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) following the thermal profile described by Fridolfsson and Ellegren (1999). The final concentration of all reactions was 11 mM Tris-HCl, 70 mM KCl, 2.5 mM of MgCl<sub>2</sub>, 10  $\mu$ g BSA (New England Biolabs, Ipswich, Massachusetts), 400  $\mu$ M dNTPs,

0.5  $\mu$ M each of 1237L (5'-GAGAACTGTG CAAAACAG-3') and 1296rev\_Agpho (5'-CTTTCTGAGACKGAGTCACTAT-3'), 0.05 U of AmpliTaq (Applied Biosystems, Foster City, California), and 2  $\mu$ l of the DNA extraction solution. PCR products were visualized via electrophoresis using a 4% agarose gel containing ethidium bromide (0.6  $\mu$ g/ml) in 1X TAE buffer. Sex was assigned as female when two bands were present (from the Z and W chromosomes) and male when a single band was present (from the two Z chromosomes).

**Sexing based on mass.** Assigning sex based on mass requires knowing nestling age. We knew hatch dates for many nestlings that were seen shortly after hatching. Using the mass of these nestlings and assuming that the last-hatched egg hatched at most 24 h after the first-hatched egg (incubation often begins when the penultimate egg in the clutch is laid; Weatherhead 1985), nestlings weighing less than 5 g were assumed to have hatched that day (day 0). We assigned sex to nestlings using two existing methods. The first of these (the "conservative method") is based on the most conservative criteria from several previous studies (Holcomb and Twiest 1970, Fiala 1981, Westneat et al. 1995) that were themselves more conservative than earlier criteria (Williams 1940, Nero 1961). We assigned sex using the last measurements available for each nestling. Prior to day 7, there is too much overlap in mass to be able to assign sex. On day 7, nestlings weighing <26 g were considered females and >33 g males. On day 8, nestlings weighing <31 g were considered female and >33 g male. On day 9 and later, nestlings weighing <33 g were considered female and >35 g male. Nestlings with weights between these values were considered to be of unknown sex. The second method was modified from the conservative method by Weatherhead and Dufour (2000). This "liberal method" was developed to allow sexing of nestlings measured prior to day 7 and to sex some nestlings previously classified as unknown using additional information such as relative growth patterns within nests. For example, nestlings of unknown sex were assigned a sex if they maintained a consistent growth pattern relative to nest mates for which sex was assigned using the conservative criteria (i.e., nestlings consistently several grams lighter than nest mates assigned as males were considered females, and vice versa). Hatching order was also considered because last-hatched

nestlings tend to grow more slowly than earlier-hatched nestlings of the same sex.

The liberal method allows more nestlings to be sexed, but involves an element of subjectivity. Our goal in developing the third approach (the "new method") was to develop more refined, but objective, criteria for assigning sex. After assigning sex using the conservative and liberal methods, we assessed the accuracy of our assignments using DNA-based assignments and determined whether the known (i.e., using DNA data) sex ratio of unassigned nestlings differed from the sex ratio of those that were assigned. We used the known sex of unassigned nestlings to develop the new method for sexing based on weight. Two steps were involved. First, as with the liberal method, we considered last-hatched nestlings separately. Second, we reassessed our criterion for assigning age (i.e., the weight threshold between day 0 and day 1) because nestlings that could not be sexed by the conservative and liberal methods were females heavy for their age and males light for their age. Some of that overlap likely resulted from the heaviest females actually being a day older than we had considered, and the lightest males being a day younger. This was an iterative process whereby we lowered the weight threshold between day 0 and day 1 until we minimized the number of unassigned nestlings at later ages.

Finally, we applied our revised criteria (the new method) to an 11-year sex ratio data set to determine how these criteria affected sex ratio estimates. For 10 years, all nestlings had been sexed using both the conservative and liberal methods (Weatherhead and Dufour 2005) and we applied the same methods for the additional year. All data were collected at the same study site used in 2005. Details of field methods are provided by Weatherhead and Dufour (2005).

Sex ratios were compared to expected values of 50% males using binomial tests. Values are given as means  $\pm$  1 SE, and alpha levels for statistical significance were set at  $P < 0.05$ . Throughout, we use percent males as our measure of "sex ratio."

## RESULTS

We located 170 Red-winged Blackbird nests, and obtained blood samples from 171 nestlings from 52 nests. Of the nestlings sampled, 148 fledged (hereafter referred to as fledglings). DNA analysis revealed that 82 (48.0%) nestlings were

Table 1. Percentage of Red-winged Blackbird nestlings whose sex could be assigned using the conservative, liberal, and new methods relative to nestling age (day 0 is the estimated hatch day).

Day	Sample size	% of sample sexed (% errors)		
		Conservative	Liberal	New
5	78	0 (0)	53.8 (4.8)	70.5 (5.5)
6	50	20.0 (0)	82.0 (2.0)	70.0 (0)
7	29	31.0 (0)	89.7 (0)	75.9 (0)
8	8	100 (0)	100 (0)	100 (0)

males and 89 (52%) were females. Fledglings included 66 males (46.5%) and 76 females. Neither of these sex ratios differed from 1:1 (binomial tests,  $P = 0.27$  and  $0.16$ , respectively). For many nests, we stopped measuring nestlings well before fledging, making this a good test of assigning sex based on nestling mass because less pronounced sexual dimorphism makes younger nestlings more difficult to sex.

Because of the limited number of measurements of older nestlings, only 26 of 172 (15.1%) nestlings could be sexed by the conservative method, with most being older nestlings (Table 1). No nestlings were sexed incorrectly using the conservative method. The liberal method increased sexing success to 63.7%, with success increasing substantially for older nestlings (Table 1). Again, the low success was due to few nestlings being measured when older. Two sexing errors occurred using the liberal method, with a large female incorrectly classified as a male on day 6 and a small male incorrectly classified as a female on day 5.

To develop the new sexing criteria, we examine cases where nestling mass at a given age was inconsistent with the mass of same-sex nestlings of the same age, where sex was known from DNA analysis. These mismatches were largely rectified by redefining the criterion for mass on hatch day (day 0) as  $\leq 4.0$  g, thereby altering the estimated hatch day for some lighter nestlings. For all but last-hatched nestlings, our new sexing criteria based on mass for females and males, respectively, were  $< 25$  g and  $> 27$  g on day 5,  $\leq 27$  g and  $> 30$  g on day 6,  $\leq 31$  g and  $> 32$  g on day 7, and  $\leq 31$  g and  $> 33$  g on day 8. Note that by day 8, these criteria were almost identical to those for the conservative method. For last-hatched nestlings that tended to grow more slowly independent of sex, we modified the respective criteria for females and males as

$< 24$  g and  $> 25$  g on day 5, and  $\leq 26$  g and  $> 29$  g on day 6. Thereafter, the criteria were the same as for other nestlings. Because these new criteria represented an attempt to formalize the liberal method, overall sexing success using these two approaches was similar (liberal: 63.7%; new: 61.4%). The new method allowed more nestlings to be sexed on day 5 (Table 1), but resulted in three sexing errors (all small males). Thus, for subsequent analyses, we use only mass for day 6 or later, where no errors occurred.

The conservative method left many nestlings unsexed and was less successful for sexing female nestlings. Of 26 nestlings sexed, 21 (80.8%) were male, a ratio significantly different from 1:1 ( $P < 0.001$ ) and from the known nestling sex ratio of 48.0% males determined using DNA. Relative to the known sex ratio, both the liberal method and our new method left a slightly higher proportion of male nestlings unsexed, producing estimates of 45.0% and 40.0% males, respectively. For fledglings, the percentage of males determined by the liberal and new methods was 42.1% and 37.4%, respectively, again underestimating the proportion of males.

For 11 years prior to 2005, we had data for 2750 nestlings and 1644 fledglings (Table 2). Given that our new method was an attempt to formalize the liberal method, it was not surprising that when we applied the new method to these data, estimated sex ratios were correlated with those estimated previously using the liberal method ( $r = 0.95$ ,  $P < 0.001$  in both cases). Similarly, mean annual sex ratios estimated by the two methods were not significantly different (paired  $t$ -tests: nestlings:  $t = 1.47$ ,  $P = 0.17$ ; fledglings:  $t = 0.47$ ,  $P = 0.65$ ). Thus, we restrict subsequent comparisons to the conservative and new methods.

Using the conservative method, across years, an average of  $54.9 \pm 3.6\%$  of nestlings and  $31.4 \pm 5.5\%$  of fledglings could not be sexed. Sexing success improved substantially using the new method, with only  $35.9 \pm 2.5\%$  of nestlings and 9.3% of fledglings unsexed. These were both significant improvements over the conservative method (Table 2, both  $P < 0.001$ ). Mean sex ratios over all years using the conservative method were  $58.1 \pm 3.5\%$  males for nestlings and  $58.1 \pm 3.8\%$  males for fledglings. Using the new method, overall mean sex ratios were

Table 2. Percentage of nestling and fledgling Red-winged Blackbirds either identified as males or not sexed using the conservative, liberal, and new sexing methods, 1985–1995.

Sexing method	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995
	Number of nestlings										
	179	224	305	379	334	399	232	225	209	174	90
Percent males											
Conservative	44.0	51.8	51.5	69.6	56.0	51.8	75.0	59.2	46.8	54.4	78.9
Liberal	39.1	47.3	45.7	55.6	50.7	45.6	45.5	51.6	45.1	48.6	50.0
New	34.2	44.7	45.9	57.3	48.3	45.1	47.7	50.9	42.3	48.2	50.0
Percent unknown sex											
Conservative	58.1	50.0	56.7	54.9	49.7	38.6	81.0	68.4	40.7	48.3	57.8
Liberal	48.6	42.4	46.9	41.2	36.2	25.3	62.1	57.8	31.1	36.2	33.3
New	34.6	33.0	44.3	32.7	29.9	23.8	44.0	52.9	32.1	36.8	31.1
	Number of fledglings										
	102	119	137	221	207	279	151	110	135	115	68
Percent males											
Conservative	44.9	50.5	50.5	69.8	54.8	49.1	80.0	58.0	46.9	55.2	78.9
Liberal	42.5	49.6	45.0	56.5	49.7	44.2	47.3	51.7	46.2	49.1	58.3
New	37.6	46.2	45.2	60.1	48.5	44.6	50.0	51.6	43.7	48.6	60.0
Percent unknown sex											
Conservative	41.2	11.8	21.9	26.7	24.2	20.4	76.8	37.3	16.3	24.3	44.1
Liberal	21.6	1.7	5.8	5.4	5.8	3.6	51.0	19.1	2.2	6.1	11.8
New	3.9	1.7	9.5	5.9	5.3	6.8	29.8	15.5	5.2	7.0	11.8

significantly less male biased for both nestlings ( $46.8 \pm 1.7\%$ ) and fledglings ( $48.7 \pm 2.0\%$ ) than those estimated using the conservative method (nestlings: paired  $t = 4.4$ ,  $P = 0.001$ ; fledglings: paired  $t = 3.8$ ,  $P = 0.003$ ). The apparent over-estimation of the proportion of males by the conservative method appears to be a function of the proportion of young that were sexed. The estimated annual values of the percent male young increased as the proportion of unsexed nestlings increased (nestlings:  $r = 0.57$ ,  $P = 0.067$ ; fledglings:  $r = 0.71$ ,  $P = 0.01$ ). This pattern did not occur using the new method (nestlings:  $r = 0.07$ ,  $P = 0.83$ ; fledglings:  $r = 0.27$ ,  $P = 0.41$ ). As a consequence, the discrepancy between the annual sex ratios estimated using the conservative and new methods increased as the proportion of unsexed nestlings increased (nestlings:  $r = 0.64$ ,  $P = 0.03$ ; fledglings:  $r = 0.92$ ,  $P < 0.001$ ). The more nestlings or fledglings that were unsexed using the conservative method, the greater the apparent over-estimate of the percent males by the conservative method relative to the new method (Fig. 1).

## DISCUSSION

Male and female Red-winged Blackbirds hatch from the same-sized eggs and then diverge in size over 9–10 days until there is almost no overlap. Thus, sexing nestlings based on morphology is highly accurate for nestlings weighed just prior to fledging. This was reflected in our study by the convergence of the three different sexing methods on the same criteria for older nestlings. However, for a variety of reasons (e.g., predation, avoiding causing premature fledging, and nest visitation schedules), nestlings are not always measured just before fledging. Using nestlings sexed using a DNA-based method, we found that different methods of sexing using nestling mass produced different outcomes. Here we restrict our discussion to comparing the conservative and new sexing methods. The liberal method involved subjective criteria and our new method was essentially the liberal method with all criteria made objective. Comparison of the performance of the new and liberal methods confirmed their similarity.

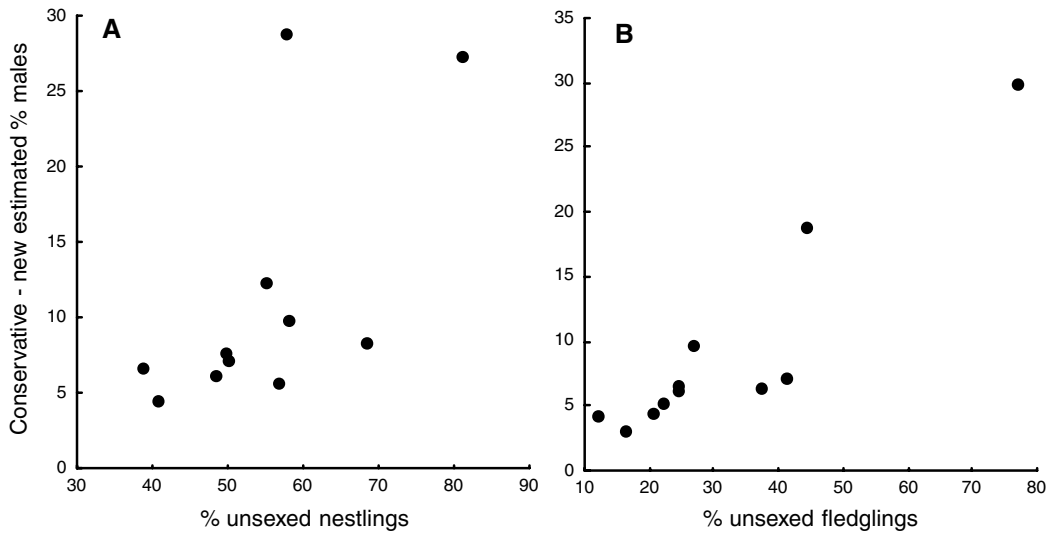


Fig. 1. The difference between the conservative and new sexing methods in the estimated annual percentage of male nestlings (A) and fledglings (B) relative to the proportion of young that could not be sexed using the conservative method.

The value of the conservative method is that it uses criteria known to apply across populations (Holcomb and Twiest 1970, Fiala 1981, Westneat et al. 1995) and appears to be completely accurate. However, it also requires measurements late in the nestling stage that are not always available. Furthermore, the conservative criteria were more successful at sexing males than females, presumably reflecting greater variance among females than males in the age that they reach their asymptotic weights. The new method allowed us to sex nestlings accurately beginning at day 6 (where the hatch date was day 0), and thus to sex a greater proportion of nestlings than the conservative method. Applied retrospectively to 11 years of data (for which true sex ratios were unknown), the two methods produced sex ratio estimates that were correlated across years, but the conservative method produced estimates that were significantly male biased relative to the new method and the extent of that bias increased as the proportion of unsexed nestlings increased (because they were not weighed when they were older).

The practical implications of our results are straightforward. If the goal is to estimate population sex ratios, accuracy of estimates will increase as the number of nestlings measured on day 8 or older increases because the estimate will be more

accurate if a greater proportion of nestlings are sexed. Because criteria for sexing older nestlings are the same for the various sexing methods, all methods produce the same results. Thus, estimates of fledging sex ratios when all fledglings are measured late in the nestling period will be very accurate. If some nestlings that were not weighed past day 7 fledged, the new method allows more of those individuals to be sexed than does the conservative method, and the population sex ratio estimate should be more reliable. The conservative method appears to become increasingly unreliable for generating population sex ratio estimates as the proportion of unsexed nestlings increases. If the goal is to sex individual nestlings, the conservative and new methods are both accurate, but the latter allows more individuals to be sexed when some nestlings are not weighed after day 7.

Several points should be considered regarding the applicability of our sexing methods to other Red-winged Blackbird populations. On the positive side, our sexing criteria converged on those of the conservative method for older nestlings. Because the conservative criteria come from several studies conducted in different parts of the species' range, it appears that nestling growth patterns are similar across the range. Thus, our criteria should apply to other populations. On

the negative side, for younger nestlings, sexing criteria are sensitive to how age is assigned. Even if nestling growth trajectories are the same in two populations, differences in methods could have an important influence on aging nestlings. For example, if nestlings are weighed exclusively early in the morning in one study and late in the day in another, weight thresholds for assigning hatch day will differ. Therefore, it is important that this threshold be assessed empirically as we did here. More broadly, our approach should apply to other sexually dimorphic species, although obviously the criteria must be developed based on such species-specific attributes as growth patterns and degree of hatching asynchrony.

An additional caveat regarding our sexing criteria (and any criteria based on morphology) is that they allow sex determination only late in the nestling period. Nestlings that die prior to that will not be sexed. Given that size differences between the sexes could lead to sex-biased mortality (Weatherhead and Teather 1991), one cannot assume that sex ratios near the end of the nestling period are the same as those at hatching. Molecular methods will be necessary if the goal is to determine sex at hatching, assuming blood samples can be obtained from very young nestlings.

DNA-based sexing techniques (Griffiths et al. 1998, Kahn et al. 1998, Fridolfsson and Ellegren 1999) now allow determination of the sex of birds without relying on differences in morphology. Thus, it could be argued that this whole exercise is moot because there is no longer a need to rely on morphological approaches. However, the costs and inconvenience of DNA-based sexing could still make sexing based on morphology an attractive option for studies of sexually dimorphic species, as long as acceptable levels of accuracy can be achieved. By combining molecular and morphological methods as we did, investigators can assess and refine morphological methods that will be useful for determining how to apply those methods in future research, and to assess how much confidence to place in results obtained using those approaches in the past.

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