Primary Sex-ratio Variation in Two Brood Parasitic Birds: Brown-headed Cowbird (*Molothrus ater*) and Common Cuckoo (*Cuculus canorus*)

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ABSTRACT.—We examined primary sex-ratios of two brood parasitic species, Brown-headed Cowbird (*Molothrus ater*) and the Common Cuckoo (*Cuculus canorus*), to determine whether there was any evidence of primary sex-ratio manipulation as has been demonstrated in other species of birds. Despite good reasons for why female brood parasites should manipulate the primary sex-ratio of their young, we found a lack of evidence for a bias in the sex-ratio of eggs produced at the population level, with respect to the host species parasitized or time of breeding season, or in terms of the sex ratio of eggs produced by individual females. Thus, this study provides another example in birds of little evidence for sex-ratio variation in relation to environmental factors.

RESUMEN.—Examinamos el cociente primario de sexos de dos especies que parasitan nidos, *Molothrus ater* y *Cuculus canorus*, para determinar si existe alguna evidencia de la manipulación de dicho cociente, como ha sido demostrado para otras especies de aves. A pesar de que existen buenas razones por las cuales las hembras podrían manipular el cociente primario de sexos de sus pichones, no encontramos evidencia sobre un sesgo en el cociente de sexos de huevos producidos a nivel poblacional, con respecto a la especie hospedera parasitada o al momento de la estación reproductiva, ni en relación a la proporción de sexos de huevos producidos por cada hembra. Así, este estudio brinda otro ejemplo acerca de la escasa evidencia que existe sobre la variación en el cociente de sexos en relación a factores ambientales en las aves.

In birds, females of some species might manipulate the sex ratio of their young in response to external factors such as territory or mate quality (Burley 1981, 1986; Ellegren et al. 1996; Komdeur et al. 1997; Kilner 1998; Kölliker et al. 1999). That manipulation can occur through primary (Oddie 1998) or secondary sex-ratio manipulation (Blank and Nolan 1983). There are now a number of studies in which pattern and mechanism of secondary sex-manipulation are well documented (Røskaft and Slagsvold 1985, Kilner 1998, Lessels 1998, Westerdahl et al. 2000), whereas there are fewer examples of the patterns and causes of primary sex-ratio manipulation in birds (but see Anderson et al. 1997, Heinsohn et al. 1997, Komdeur et al. 1997).

Obligate brood-parasitic birds offer an excellent system to study possible patterns and causes of primary sex ratio manipulation because they do not provide any parental care for their offspring. Hence, the observed sex-ratio is a direct function of what the female produces and is not confounded by any parental care at the secondary stage (Payne and Payne 1998). Because host nestlings remain in the nest, the parasite must compete with host nestlings for food (Dearborn 1997). Therefore, there are two possible patterns of sex-ratio bias. First, Weatherhead (1989) suggested that in sexually size-dimorphic parasite species, larger male nestlings should be laid at a greater frequency in the nests of large hosts because they should compete better than females with large host nest-mates. On the other hand, because smaller host nestlings receive less food (Dearborn 1997), they are out competed by the larger cowbird nestlings due to size differences (Dearborn 1997) and begging height reached (Lichtenstein and Sealy 1998); and because adults at parasitized nests exhibited higher provisioning rates than adults at unparasitized nests (Dearborn et al. 1998), cowbird nestlings should be provisioned at a higher rate in nests of smaller hosts. Hence, the more costly sex (e.g. larger males) should be placed in the nests of smaller hosts (Weatherhead 1989).

Despite the fact that Weatherhead (1989) found no evidence for either pattern in Brown-headed Cowbirds (*Molothrus ater*), where males are larger than females, his results need to be confirmed in that species with larger samples of nestlings over longer time periods. In addition, to determine its generality, this hypothesis should be examined in other size-dimor-

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phic species such as the Common Cuckoo (*Cuculus canorus*). Given that nestling cuckoos eject host eggs, competition with host nestlings is not important, though cuckoo hosts vary substantially in size (Wylie 1981) and reducing provisioning costs for host parents may act as a selective pressure that could bias the sex of cuckoos laid in nests of different hosts.

Here, we use recently developed molecular sexing techniques (Griffths et al. 1998) to analyze the primary sex-ratios in nestlings collected in the course of multiyear studies of populations of two species of brood parasite: the Brown-headed Cowbird and Common Cuckoo. Specifically, we analyzed the nestling sex ratios of both species to (1) determine whether biases existed at the level of the population and individual female, and (2) whether variation occurs within factors such as year, time of season, and in relation to host species.

Methods.—Egg samples from both species came from long-term studies of individually marked populations where the goal of both studies was to use genetic analyses of parentage to determine reproductive success of adult males and females. In each case, the general approach was to mark and sample a large portion (60–95%) of adults in a given area and then to use genetic techniques to match parents with eggs that had been collected from host nests in the study area.

For cowbirds, we used DNA from 332 samples of incubated eggs (mean number per year: 47.43 ± 17.04; range: 25–65) that had been collected mainly from three hosts (Red-winged Blackbird [*Agelaius phoencieus*], Yellow Warbler [*Dendroica petechia*], and Song Sparrow [*Melospiza melodia*]) over a seven-year period (1993–1999) at the University of Manitoba Field Station (Delta Marsh) in Manitoba. Detailed descriptions of the study site, collection methods, and genetic methods are provided in Alderson et al. (1999) and Woolfenden (2000). For cuckoos, DNA was analyzed from 129 samples (mean number per year: 32.25 ± 16.26; range: 16–54) obtained from eggs laid in the nests of two hosts (Great Reed Warbler [*Acrocephalus arundiaceus*] and Azure-winged Magpie [*Cyanopica cyana*]) over a four-year period (1991–1994) from a study site on the Chikuma River, Nagano, in central Japan. Details of that study site, sample collection, and methods of genetic analysis are described in Nakamura and Miyazawa (1997) and Marchetti et al. (1998). In both studies, due to high predation rates, eggs from parasites were collected from host nests as soon as possible (1–3 days) after laying and were artificially incubated until a large enough tissue sample could be collected from developed embryos. Thus, samples used represent a reasonable measure of the primary sex-ratio for each species.

We determined sex of nestling samples using primers (P2 and P8) developed by Griffiths et al.

(1998) that amplify conserved regions on the Z and W chromosomes that differ in size in a wide range of birds. Since females are the heterogametic sex (ZW), female profiles should contain two bands (Z and W products), whereas males should produce a profile consisting of a single band (two copies of the Z product).

To sex nestlings, we amplified W or Z fragments, or both, in 10 µL PCR reactions consisting of 1 M Tris-HCL pH 8.3, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 200 ng of each primer, 0.05 units of *Taq* polymerase and 250 ng of nestling genomic DNA. Cycling conditions consisted of an initial denaturing step at 94°C for 3 min and then 32 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 55 s. Ten microliters of each sample was then run on a 3% agarose gel stained with ethidium bromide at 90 V for 60–70 min.

To test whether those primers could be used to reliably determine sex in these species, we used a panel of 10 adult birds (5 males and 5 females) whose sex had been determined on the basis of plumage, behavior, or both. When genotyped using the above procedure, all males from both species produced a single band profile (~300 bp in cowbirds and cuckoos), whereas all females produced two band profiles (~300 and ~350 bp in cowbirds and cuckoos). On that basis, we subsequently scored all singleband profiles in each species as males and all doubleband profiles as females.

We used a replicated goodness-of-fit test (*G*-statistic) to determine if sex ratio of eggs produced deviated from an expectation of 50:50 in relation to different factors. To obtain overall patterns we (1) pooled data across years into a single ratio, and (2) treated each year as a replicate and combined probabilities for tests done for each year into a single test of significance as described by Sokal and Rohlf (1981). A crude power analysis for overall sex-ratio for cowbirds and cuckoos was also done by determining what deviation would be required to achieve significance at the 0.05 level given the number of offspring tested.

Results.—Proportion of males produced in each year varied from 0.38 to 0.64 and the sex-ratio was significantly male-biased in 1994 and female-biased in 1998 (Table 1). When data were pooled across years, the overall ratio of males:females was 163:169 (1:0.96) and was not significantly different from 0.5 (Table 1) although for significance to be obtained, the proportion of one sex would have to be >0.55 or <0.45. The combined probability based on *G*-values for tests done for each year was also not significant ($G = 11.739$, df = 7, $P > 0.05$). Therefore, in most years and for the overall data set, the primary sex-ratio in cowbirds did not deviate significantly from equality at the population level.

Of the 332 samples collected, 279 were from known-host nests. Within years, proportion of males

TABLE 1. Proportion of male cowbirds laid each year. *P*-value refers to whether the proportion of males produced was significantly different from 0.5.

Year	*n*	Proportion of males	df	*G*	*P*
1993	25	0.44	1	0.360	0.55
1994	61	0.64	1	4.801	0.02
1995	26	0.46	1	0.154	0.69
1996	47	0.45	1	0.533	0.47
1997	44	0.59	1	1.462	0.23
1998	64	0.38	1	4.043	0.04
1999	65	0.46	1	0.385	0.53
Pooled	332	0.49	1	0.108	0.74
Total	—	—	7	11.739	0.11

found in each host nest varied substantially although sample sizes also differed (Red-winged Blackbirds: 0.20–0.81; Yellow Warblers: 0.20–0.70; Song Sparrows: 0.0–0.58; Table 2). Sex ratio did not differ in the number of males produced in any of the years. When pooled across years, 57, 44, and 48% of the samples found in blackbird, warbler, and sparrow nests, respectively, were males. Those proportions did not deviate significantly from each other (Table 2). Because no cowbird eggs were found in Song Sparrow nests in 1993, a 2×2 analysis was performed and therefore the *G*-value was not used in the calculation of the combined probability test. For the remaining years, the combined probability for tests is also not significant ($G = 25.612$, df = 30, $P > 0.05$). Thus, there is no strong, consistent association between the sex of a cowbird chick and the identity of the host nest in which it is laid.

Finally, to examine whether the sex of nestlings varied with the time of the season, each breeding season was divided into five one-week intervals, with the beginning of the first week determined by the date on which the first cowbird egg was found in a host nest. Due to low sample sizes data were pooled across years and the proportion of males laid in nests within each one-week block was compared. Proportion of males produced ranged from 36.8 to 52.7% (n = 24 to 82 chicks per week) but did not differ significantly from 50% in any of the five weeks ($G < 2.6$; all $P > 0.11$). The combined probability across multiple tests is also not significant ($G = 7.1$, df = 5, $P > 0.05$).

Using parentage results from Woolfenden (2000), the sex of offspring assigned to individual females was examined to see whether there was any evidence for bias in the production of young of a particular sex. Although 41 adult females were assigned to eggs laid on the study site over the seven years, we arbitrarily focused on 14 females that laid five or more eggs in one or more years (Table 3). None of the females produced offspring with significantly biased sex ratios (all $P > 0.05$). Combined-probabilities test was also not significant ($G = 15.21$, df = 14, $P > 0.05$). Thus, we found no evidence that individual female cowbirds were biasing the sex of the young they produced.

Although sample sizes are smaller, we also found no deviations from unity in nestling sex ratios of cuckoos. In only one of four years were there significantly more males produced (Table 4). The overall sex ratio (proportion of males, 0.54) did not differ significantly from unity (Table 4) although our power to detect significant deviations close to 50:50 is limited because to achieve significance given the size of the pooled sample, the proportion of males would have to be ≥ 0.58 or ≤ 0.41. The combined probability is also not significant ($G = 5.88$, df = 4, $P > 0.05$).

Almost all cuckoo nestlings ($n = 129$, 97.6%) were found among nests of two host species, the Great Reed Warbler and the Azure-winged Magpie. However, no difference was observed in proportion of males found in either type of host within or across years (Table 5); the combined *G*-value is also not significant ($G = 3.27$, df = 12, $P > 0.05$). Overall, as with cowbirds, nestling sex ratios did not deviate from unity in any analysis.

TABLE 2. Proportion of male cowbird eggs laid in Red-winged Blackbird (RWBL), Yellow Warbler (YWAR) and Song Sparrow (SOSP) nests. The values in parentheses represent the number of host nests found containing cowbird eggs. *P*-value refers to whether the proportion of males produced in each host nest was significantly different from 0.5.

Year	*n*	RWBL	YWAR	SOSP	df	*G*	*P*
1993	23	0.33 (12)	0.64 (11)	0.00 (0)	5	2.144	0.83
1994	58	0.82 (28)	0.70 (10)	0.40 (20)	5	9.311	0.10
1995	23	0.36 (11)	0.56 (9)	0.67 (3)	5	1.236	0.94
1996	23	1.00 (1)	0.67 (6)	0.25 (16)	5	5.156	0.40
1997	34	0.67 (6)	0.50 (4)	0.58 (24)	5	0.285	0.99
1998	57	0.20 (6)	0.32 (31)	0.45 (20)	5	1.953	0.86
1999	61	0.33 (3)	0.20 (15)	0.53 (43)	5	5.527	0.35
Pooled	279	0.57 (67)	0.44 (86)	0.48 (126)	5	2.488	0.78
Total	—	—	—	—	35	25.613	0.88

TABLE 3. Proportion of male offspring produced by a single female pooled over the total years of the study. Only females that produced five or more offspring (*n*) in total were used for this analysis. *P*-value refers to whether proportion of males produced by a single female was significantly different from 0.5.

Individual[a]	*n*	Proportion of males	df	*G*	*P*
Cowbirds					
15912	18	0.667	1	2.039	0.15
15915	8	0.750	1	2.093	0.15
15917	7	0.286	1	1.328	0.25
15936	8	0.250	1	2.093	0.15
15946	6	0.500	1	0	1.00
15967	5	0.200	1	1.927	0.17
15995	9	0.556	1	0.111	0.74
90101	6	0.667	1	0.680	0.41
90115	6	0.500	1	0	1.00
90147	5	0.800	1	1.927	0.17
90816	20	0.550	1	0.200	0.65
90827	5	0.600	1	0.201	0.65
90832	5	0.800	1	1.927	0.17
98mom10[b]	6	0.333	1	0.680	0.41
Cuckoos					
518153	8	0.500	1	0	1.00
6095125	8	0.750	1	2.093	0.15

[a] Numbers represent partial band numbers of each individual.

[b] Nonsampled female whose hypothetical genotype explained all six offspring (see Woolfenden 2000).

Because of the smaller sample sizes, there were only two female cuckoos that were assigned more than five young (Table 3). Neither overproduced nestlings of a particular sex (*P* > 0.11) and the combined probability test is also not significant (*G* = 2.09, df = 2, *P* > 0.05).

Discussion.—We found no strong evidence for primary sex-ratio bias at the population or individual level, or in relation to factors such as host species or time of season in either cowbirds or cuckoos. Thus, our study represents another example in birds (e.g. Koenig and Dickinson 1996, Bradbury et al. 1997, Westerdahl et al. 1997) in which, despite good theoretical reasons to expect biases to exist, there is no evidence for sex-ratio variation in relation to parental attributes or environmental factors. It is also consistent with previous work by Weatherhead (1989) on cowbirds, which showed no strong deviation in secondary sex-ratios at the population level or in relation to host.

However, as discussed by Koenig and Dickinson (1996), there are several related reasons why sex-ratio biases may not be present despite good conceptual reasons to expect them. First, the frequency-dependent selection to maintain population sex ratios at 50:50 (Fischer 1958, Frank 1990) may be strong relative to any selection to bias sex ratios away from

TABLE 4. Proportion of male cuckoos laid each year. *P*-value refers to whether the proportion of males produced was significantly different from 0.5.

Year	*n*	Proportion of males	df	*G*	*P*
1991	25	0.400	1	1.001	0.32
1992	54	0.537	1	0.297	0.59
1993	16	0.438	1	0.251	0.62
1994	34	0.676	1	4.328	0.04
Pooled	129	0.535	1	0.628	0.43
Total	—	—	4	5.882	0.21

unity. Second, costs of altering the primary sex ratio may be high in birds (Oddie 1998, Krackow 1999) and, therefore, because brood-parasitic species have no opportunity to manipulate secondary sex-ratios by withholding parental care, the net selection to alter sex ratios may be even weaker than in birds that provide care to their young. Third, selective pressures to alter sex ratios may exist, but act in opposite directions leading to a lack of net selection for ratios away from unity in pooled samples. Fourth, sample sizes larger than those obtained in most field studies of birds may be required to detect subtle changes in sex ratio that result from weak selection to bias the sex of nestlings that are produced. At present, we do not have the information to determine which of those possibilities apply to these birds, but any one or combination of them explain the lack of biases shown in these parasite species.

Finally, the lack of deviation from unity in the primary sex ratio of cowbirds at Delta Marsh confirms that the significant male bias in many adult populations (Woolfenden et al. 2001) is not due to the increased production of male nestlings but can be explained as a result of the increased mortality of adult females relative to adult males. In support of this, Woolfenden et al. (2001) showed that annual adult survivorship of males was substantially higher (90%) than that of adult females (70%).

TABLE 5. Proportion of male cuckoos laid in Azure-winged Magpie (AWMA) or Great Reed Warbler (GRWA) nests. The values in parentheses represent the number of host nests found containing cuckoo eggs. *P*-value refers to whether the proportion of males produced in each host nest was significantly different from 0.5.

Year	*n*	AWMA	GRWA	df	*G*	*P*
1991	22	0.25 (12)	0.50 (10)	3	1.482	0.69
1992	52	0.54 (13)	0.56 (39)	3	0.026	0.99
1993	15	0.50 (4)	0.36 (11)	3	0.225	0.97
1994	33	0.33 (3)	0.70 (30)	3	1.539	0.67
Pooled	122	0.41 (32)	0.58 (90)	3	2.793	0.42
Total	—	—	—	12	3.27	0.99

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