

Pre-ovulation control of hatchling sex ratio in the Seychelles warbler

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Females of some bird species have a high degree of control over the sex ratio of their offspring at laying. Although several mechanisms have been put forward to explain how females might control the sex of their eggs, virtually nothing is known. As females are the heterogametic sex in birds, adjustment of the clutch sex ratio could arise either by pre- or post-ovulation control mechanisms. The Seychelles warbler (*Acrocephalus sechellensis*) exhibits extreme adaptive egg sex ratio bias. Typically, warblers produce only single-egg clutches, but by translocating pairs to vacant habitat of very high quality, most females were induced to produce two-egg clutches. Overall, females skewed clutch sex ratios strongly towards daughters (86.6%). This bias was evident in the first egg, but critically, also in the second eggs laid a day apart, even when all absent, unhatched, or unsexed second eggs were assumed to be male. Although a bias in the first egg may arise through either pre- or post-ovulation mechanisms, the skew observed in second eggs could only arise through pre-ovulation control. Post-ovulation adjustment may also contribute to skewed hatchling sex ratios, but as sex-biased release of gametes is likely to be a more efficient process of control, pre-ovulation mechanisms may be the sole means of adjustment in this species. High fitness differentials between sons and daughters, as apparent in the Seychelles warblers, may be necessary for primary sex ratio adjustment to evolve.

Keywords: egg sex ratio; pre-ovulation control; post-ovulation control; Seychelles warbler

1. INTRODUCTION

Sex allocation theory predicts that parents should benefit from the ability to control their relative investment in male and female offspring if their profitability sometimes varies (Charnov 1982). This idea has received strong support among taxa, especially some haplodiploid insects, that have precise and well understood mechanisms for the adjustment of offspring sex ratios (Hamilton 1967; Werren 1980; Godfray & Werren 1996). However, an increasing number of studies show apparent control of sex ratios at birth in mammals (Clutton-Brock & Iason 1986; Cockburn 1990; Davison & Ward 1998; Kruuk *et al.* 1999) and hatching in birds (Ankney 1982; Dijkstra *et al.* 1990; Ligon & Ligon 1990; Ellegren *et al.* 1996; Lessells & Mateman 1996; Anderson *et al.* 1997; Heinsohn *et al.* 1997; Komdeur *et al.* 1997; Kilner 1998; Nishiumi 1998; Nager *et al.* 1999; Sheldon *et al.* 1999; Legge *et al.* 2001), although mechanisms for adjustment in these taxa remain largely unknown (Krackow 1995, 1999; Emlen 1997; Hardy 1997; James 1997; Sheldon 1998). While fascinating in their own right, understanding the mechanisms and costs of sex ratio modification is critical if the adaptive benefits to sex ratio control are to be quantified and predictions made about optimal patterns of sex allocation (Krackow 1995; Oddie 1998). Indeed, in principle, it is inappropriate to argue that any observed sex ratio biases are adaptive unless both the benefits and costs of adjustment are known (James 1993; Krackow 1995, 1999).

Birds have chromosomal sex determination (Williams 1999) and possess no known physiological or genetic

mechanisms for skewing the sex ratio at laying (Krackow 1995; Emlen 1997; Hardy 1997; Oddie 1998; Sheldon 1998; Komdeur & Pen 2002). The female reproductive tract comprises a single functional ovary that releases follicles (ova) into the oviduct during each breeding attempt. A hierarchy of follicles develops in the ovary, with the largest becoming the next ovum to be released in the process of ovulation. Shortly after ovulation, the ovum is fertilized by sperm that are present in the upper region of the oviduct, known as the infundibulum. The fertilized ovum then passes down the oviduct over a period of about 24 h, where albumen and then shell is secreted around it in the shell gland. In species that lay eggs daily, follicles are ovulated at 24 h intervals (Sturkie 1986).

Importantly, female birds are the heterogametic sex (producing W- and Z-bearing ova), so mechanisms of sex ratio adjustment could potentially function either pre- or post-ovulation (Hardy 1997; Oddie 1998). In the chicken (*Gallus domesticus*), sex is determined shortly before ovulation, during the first meiotic division, when segregation of the sex chromosomes leaves either the W or Z chromosome included in the developing ovum and the other sex chromosome consigned to part of the polar body (Sturkie 1986). It has been proposed that pre-ovulation (or primary) control could occur through segregation distortion at the first meiotic division or through differential provisioning of ova of different sex to influence the order in which they are released from the ovary (Ankney 1982; Krackow 1995; Oddie 1998, but see Krackow 1999). Post-ovulation (or secondary) control could operate through sex-selective re-absorption of the ova in the oviduct (pre- or post-fertilization) or dump laying of eggs of the 'unwanted' sex (Emlen 1997). A key difference

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between pre- and post-ovulation mechanisms of adjustment is that post-ovulation control presumably requires skipping a day when an egg could have been laid and, in the case of dump laying, wasting the resources that were provisioned to that egg. Skipping day(s) at the start or during the ovulation sequence (Emlen 1997) would result in either delayed clutch completion or a smaller clutch. Females could also skip laying the last egg(s) of a clutch if they were of the 'unwanted' sex, resulting in a smaller clutch and delayed start of incubation. Pre-ovulation control by segregation distortion would not suffer from these costs, and control by pre-ovulation sex-differential follicle retardation would infer only minor costs when yolk could be re-absorbed. Therefore, in principle, pre-ovulation control of any kind would be the more efficient mechanism of control (Myers 1978; Oddie 1998; Komdeur & Pen 2002). In turn, more efficient mechanisms of control should broaden the scope for adaptive sex ratio adjustment, because the fitness differential between sons and daughters need not be as great to balance the costs of adjustment (Pen & Weissing 2002).

Previous studies that report hatchling sex ratio biases (see above) have not explicitly considered whether sex ratio adjustment occurred pre- or post-ovulation. In this study, we aim to distinguish between primary and secondary egg sex ratio adjustment in the Seychelles warbler, (*Acrocephalus secellensis*), a species known to exhibit extreme control of hatching sex ratio (Komdeur *et al.* 1997). The Seychelles warbler is a rare island endemic, until 1988 known only to come from Cousin Island (29 ha) in the Seychelles. On this island, the warbler population has reached carrying capacity, and many breeding pairs are aided by helpers that are usually daughters from previous broods. Having helpers is beneficial to pairs on high-quality territories, because reproductive success is enhanced. However, on low-quality territories, helpers are costly because they deplete insect prey (Komdeur 1994). Breeding pairs maximize their inclusive fitness by modifying the sex ratio of the single-egg clutch towards sons (77%, $n = 57$), the dispersing sex, when breeding on poor territories, and towards daughters (87%, $n = 32$) when breeding on rich territories (Komdeur *et al.* 1997). However, because only single-egg clutches were produced by these pairs, it was not possible to distinguish between pre- and post-ovulation mechanism of egg sex adjustment.

In efforts to conserve the species, an additional population was established on the nearby Aride Island (68 ha) in 1988. Pairs transferred to this island started breeding immediately in very high-quality habitat and produced mainly two-egg clutches (Komdeur *et al.* 1995). The high quality of habitat and absence of helpers led females to skew brood sex ratios strongly towards daughters. This preference for daughters, coupled with the fact that most females produced two-egg clutches, allowed us to investigate mechanisms of egg sex ratio adjustment. A bias in the sex ratio of first-laid eggs could result from either pre- or post-ovulation processes, whereas a skewed sex ratio of the second egg laid within 24 h of the first could only arise through pre-ovulation adjustment. In this paper, we examine egg sex ratios of warbler pairs on Aride Island over 3 years to establish if there is evidence for pre-ovulation sex ratio adjustment in this species. In addition, we examine if there is any dependence or difference between the sex

of first and second eggs, which could provide further insights into mechanisms of adjustment.

2. MATERIAL AND METHODS

(a) *Study population and data collection*

Following relocation, the entire warbler population on Aride Island was studied between September 1988 and October 1991. All warbler territories were situated in habitat where food abundance was on average 1.6 times higher than on high-quality habitat on Cousin Island (Komdeur 1996; Komdeur & Edelaar 2001). Furthermore, territories on Aride were initially 4.4 times larger than on Cousin, providing the warblers with access to an average of about seven times more insect prey than the warblers occupying high-quality territories on Cousin Island (Komdeur 1996). A total of 19 breeding pairs were studied. Initially, cooperative breeding was absent, but was observed in some pairs from November 1990 to October 1991. Because the presence of helpers has been shown to affect hatchling sex ratios (Komdeur *et al.* 1997), pairs were excluded from analysis once they had helpers on their territory. This only reduced the sample slightly because only three clutches produced by two of the study pairs were excluded. All nests of each pair were found and inspected daily, and eggs numbered with a permanent marker pen on the day that they were laid to determine the sequence of laying. Clutches were monitored daily for hatching. Conspecific egg dumping was unlikely because a subsequent study of Seychelles warblers on Cousin Island, using molecular genotyping, revealed that all eggs in multiple-egg clutches produced by breeding pairs were laid by the social mother (Richardson *et al.* 2001).

Upon hatching, each nestling was given a unique marking on its legs and feet with a permanent marker and the egg number from which it hatched recorded. Hatching was asynchronous (usually 24 h apart in two-egg clutches) and it was always possible to determine which chick hatched from which egg in the clutch. The markings were renewed when necessary, until nestlings were fitted with coloured leg bands and a numbered metal band when between 5 and 9 days old. All nestlings survived to be re-trapped in their second year of life for sex determination. At the time, molecular sexing techniques were not available so we sexed all birds using the variables wing length and body mass in a multiple logistic regression model ($\chi^2 = 148.23$, d.f. = 2, $p < 0.001$). The sexes of the warblers on Aride were separated using the function $y = 1/(1 + e^{-z})$, where $z = 79.88 - 2.72 \times \text{body mass} - 0.544 \times \text{wing}$. A bird was classified as female if y was close to zero or male if y was close to unity. A substantial proportion of these nestlings on Aride Island were subsequently bled as adults between 1993 and 1996 (58 males and 109 females) and sexed using random amplified polymorphic DNA markers (Lessells & Mateman 1996, 1998; Komdeur *et al.* 1997). Based on these individuals, all sexes assigned using the multiple logistic regression model were in agreement with the sex determined using this molecular technique.

(b) *Statistical analyses*

We first provide the binomial probability for the overall sex ratio of hatchlings to determine if the anticipated excess production of daughters was evident. Most of the 19 females contributed multiple clutches to the dataset (range 1–9). To avoid pseudoreplication and biased test statistics in small samples of broods of variable size (Krackow & Tkadlec 2001; Krackow *et al.* 2002), one sex ratio for first, second and all eggs combined

was derived for each female in our sample by summing these eggs in all of her clutches. We then used Wilcoxon's signed rank test to evaluate deviations of overall progeny sex ratios and those of first and second eggs from parity. Heterogeneity between females was not analysed any further because the small number of clutches produced by most females afforded little statistical power and, furthermore, explaining variation between females was not the aim of this study.

To evaluate whether post-ovulation mechanisms could have been exclusively responsible for the sex ratio bias, we used the actual sex of second eggs found only if the second egg was laid on the day consecutive to the first egg laid. For those clutches that comprised only one egg, or where the sex of the second egg could not be determined, the second egg was assumed to be the 'less adaptive' sex—male, in this case. For those clutches where the second egg was laid more than one day apart, we assumed that this second-laid egg was really not the second egg to be ovulated and that the actual second unlaidd egg was the 'less adaptive' sex—male in this case. The binomial probability of finding as few or fewer males in second eggs of this 'worst case' dataset estimates the likelihood that the bias in our observed sample was due only to post-ovulation mechanisms. This is a conservative estimate because any genuine single-egg clutch will erroneously inflate this probability. Clearly, this assertion rests on our assumption that the data represent a complete sample of clutches in the population (i.e. not over-representing clutches with non-delayed second eggs).

Finally, we tested if the sex ratio significantly changed between first- and second-laid eggs. In multiple-egg clutches, this was achieved with a McNemar test for change (Siegel & Castellan 1988), by calculating the two-sided binomial probability of the skew in discongruent pairs of clutches (i.e. those containing either males first and females second, or vice versa) that is appropriate for small numbers of such pairs (Siegel & Castellan 1988). In addition, Fisher's exact probabilities were calculated in 2×2 contingency tables (i.e. first and second eggs were treated as independent samples) in comparisons not allowing for paired tests.

3. RESULTS

A total of 86 clutches was produced by 19 females over a three-year period between October 1988 and November 1991. These females produced an average of 4.53 ± 2.57 (s.d.) clutches with a range of 1 to 9. The majority of clutches contained two eggs (72%), but clutch size ranged from 1 to 4 (20×1 egg; 62×2 eggs; 3×3 eggs; 1×4 eggs). Over 90% of laid eggs were sexed (142 out of 157); of the remainder, 13 failed to hatch and were not sexed and two hatched but the young died before sexing. In two clutches, there was a period of two days in the laying sequence between the appearance of the first and second eggs.

Overall, only 19 of the 142 sexed hatchlings were male (binomial test; $p < 0.0001$). The mean sex ratio of hatchlings produced per female was also strongly female-biased (Wilcoxon signed rank test: $Z = 3.87$, $n = 19$, $p < 0.0001$) and this skew was evident for both first ($Z = 3.49$, $n = 19$, $p < 0.0001$) and second, successively laid eggs ($Z = 3.88$, $n = 17$, $p < 0.0001$) (figure 1). The four third-laid eggs and one fourth-laid egg were all female. Critically, we found that the sex ratio of second eggs in the 'worst-case'

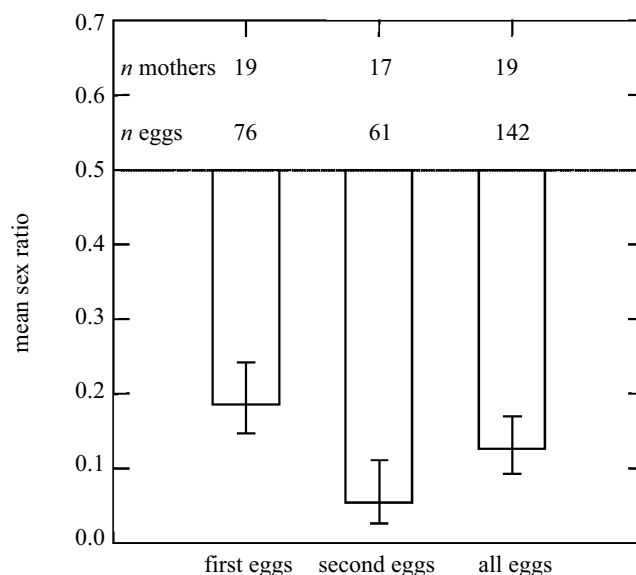


Figure 1. Mean sex ratio of first, second and all eggs produced by female Seychelles warblers on Aride Island. Means were derived from the average values for each of the 19 females and vertical lines are estimates of binomial errors.

dataset was also female-biased (binomial test; 31 males versus 55 females, $p = 0.0063$).

Comparing the sex ratio of first- and second-laid eggs, we found that second eggs were more female-biased than first eggs (table 1). The sex composition did significantly differ between first and second eggs when including all clutches, i.e. treating single eggs as first eggs (Fisher's exact probability for all clutches: $p = 0.044$). The change of sex ratio within multiple-egg clutches did not reach significance (McNemar test: $p = 0.065$). This trend in multiple-egg clutches stems from the fact that mothers with female first eggs produced significantly fewer male eggs at second laying (2 in 47) than expected from the overall sex ratio of first eggs (13 in 61, table 1; Fisher's exact probability: $p = 0.012$), while those with male first eggs did not (2 in 11, table 1; Fisher's exact probability: $p = 1.00$).

4. DISCUSSION

Previous work has demonstrated that Seychelles warbler females adjust their hatchling sex ratio according to the quality of habitat within their territory (Komdeur *et al.* 1997). Consequently, when breeding pairs were relocated to the vacant, extremely high-quality habitat of Aride Island in 1988, it came as little surprise that female warblers were observed to skew clutch sex ratios strongly towards daughters (mean sex ratio = 0.12 ± 0.03 (s.e.), $n = 19$ females). Because of the low population density on Aride, most daughters dispersed from the natal territory, but remained unpaired for some time because breeding pairs were producing so few sons. The extreme high quality of the habitat on Aride also induced females to produce a predominance of two-egg clutches, so it was possible to show that the bias was evident in both the first, and more importantly, the second of eggs laid 24 h apart.

Even assuming that all missing eggs the day after the laying of the first egg were male, a highly significant over-representation of females was still evident in second eggs

Table 1. Sex composition of first- and second-laid eggs in multiple- and single-egg clutches by Seychelles warblers on Aride Island between 1988 and 1991. Virtual number of male and female second eggs under the 'worst-case' hypothesis are given in parentheses (unknown, sex not determined).

multiple-egg clutches		second egg			first-egg totals	single-egg clutches
		male	female	unknown		
first egg	male	2 ^a	9	2	13	2
	female	2	45 ^b	1	48	13
	unknown	0	3	2	5	5
	second-egg totals	4 (31)	57 (55)	5	total number of clutches: 86	

^a One clutch.

^b Two clutches with a period of two days between the first and second eggs.

('worst case' scenario; see § 3). Mechanisms operating after ovulation could not have caused such a bias (see § 1), so we can conclude that pre-ovulation mechanisms must contribute to the sex ratio bias observed in Seychelles warblers.

Current textbook knowledge, mainly derived from the reproductive biology of domestic chickens and turkeys, maintains that follicles in birds form a growth hierarchy in which the second follicle can only ovulate with a delay of about 1 day due to suppression by the primary follicle (Sturkie 1986). As first meiosis (i.e. sex determination) in these domestic species takes place only shortly (0.5 to 3 h) before ovulation, a pre-ovulation mechanism involving differential resorption of 'unwanted' sex follicles would seem unlikely because a delay in laying would be observed. Most plausibly, pre-ovulation sex ratio adjustment in Seychelles warblers results from distorted segregation of the sex chromosomes at the first meiotic division (Ankney 1982; Krackow 1995). Although it is unclear how this process could operate (Krackow 1999; Williams 1999), recent evidence in peafowl (*Pavo cristatus*) suggests that maternal steroid levels might cause segregation distortion (Petrie *et al.* 2001).

Nevertheless, we suggest that mechanisms of sex ratio adjustment without laying delay involving differential mortality or provisioning of resources to ova before ovulation (Ankney 1982; Krackow 1995; Oddie 1998) cannot be completely ruled out. As pointed out by others (Ankney 1982; Oddie 1998), our current understanding of these events is derived only from domestic species where biased sex ratios are rarely observed and that are phylogenetically very distinct from those species shown to exhibit appreciable sex ratio variation. Clearly, there is a need for studies of the processes of gamete formation and ovulation in species where sex ratio adjustment is known to occur.

In this study, we found tentative evidence that the sex ratio of the second, successively laid egg in multiple-egg clutches was even more female-biased than the first. This implies that the mechanism of control operates more effectively for second eggs, or that several mechanisms are involved and that their relative influence differs between eggs. An adaptive advantage to a greater skew of the second egg is unclear. However, if genuine, this pattern could reflect a relationship between offspring sex ratio and variation in female quality or condition, independent of habitat quality.

Mechanisms of sex ratio adjustment should only evolve where the benefits of control outweigh the costs. To what

extent do Seychelles warblers benefit from the ability to control the hatchling sex ratio? Preliminary data indicate that inclusive fitness benefits to females raising daughters on high-quality habitat were 9.8 times higher than for females raising sons (J. Komdeur, D. S. Richardson and T. Burke, unpublished data), suggesting strong selection for the evolution of sex ratio control in this population.

In principle, pre-ovulation control of sex ratio should be more efficient than post-ovulation adjustment (see § 1). Nevertheless, all mechanisms of sex ratio control are likely to entail some cost. The threshold costs (percentage of total reproductive resources wasted) of discarding the undesired sex have recently been compared with the benefit ratio of producing the desired sex versus the undesired sex (Pen & Weissing 2002). In the Seychelles warbler, after the 10-day period in the nest, parents continue to feed the young for about a further three months after hatching (mean: 88.5 ± 19.7 (s.d.); range 70–240 days; $n = 189$) (Komdeur 1996). If we assume, for argument's sake, that investment in each egg represents 10% of total maternal investment in parental care (almost certainly larger than the real figure), the model of Pen & Weissing (2002) predicts that a fitness ratio of only 1.5 is required to sustain this level of wasted investment. This fitness differential is six times less than the 10-fold advantage estimated for this population (see above). Furthermore, as female warblers appear to exert pre-ovulation control, which is potentially far cheaper than dumping eggs, the cost of sex ratio control may be trivial relative to the fitness benefits.

In theory, a more serious obstacle to the evolution of pre-ovulation adjustment may be the potential conflict of interest between mother and gamete (Reiss 1987; Krackow 1999). It has been argued that the sex chromosomes of gametes have no genetic interest in sex ratio adjustment, while gametic automosomes should only agree on sex ratio distortion if the fitness differential between the sexes exceeded three (Reiss 1987). While this represents a substantially higher threshold than the costs of wasted resources, it is still well inside the estimated fitness differential for the warbler. Parent–gamete conflict is of particular relevance to pre-ovulation sex ratio control because, after fertilization, the conflict is between parent and offspring, which is much reduced (Trivers 1974).

While surmountable in the Seychelles warbler, the obstacles to pre-ovulation sex ratio control of parent–gamete conflict coupled with resource waste may preclude

such control mechanisms from evolving in all but a few bird species. In most studies that report sex ratio skew at hatching, it is unclear when the bias may have been produced. However, a range of other studies show skews in sex ratio related to position of the egg in the laying sequence. In some of these cases the bias appears to be confined to the first egg (e.g. Harris' hawk (*Parabuteo unicinctus*); Bednartz & Hayden 1991). As discussed earlier, first-egg biases may arise through post-ovulation adjustment (Emlen 1997; Krackow 1999), while sex of the remaining eggs is left to chance. Nevertheless, mechanisms that result in non-random ovulation in first eggs only cannot be excluded in these cases. Other studies either show (lesser snow goose (*Chen caerulescens*), Ankney 1982; ring-billed gull (*Larus delawarensis*), Ryder 1983; bald eagle (*Haliaeetus leucocephalus*), Bortolotti 1986; zebra finch (*Taeniopygia guttata*), Kilner 1998; lesser black-backed gull (*Larus fuscus*), Nager *et al.* 1999) or imply (zebra finch, Clotfelter 1996; great tit (*Parus major*), Lessells *et al.* 1996; blue tit (*Parus caeruleus*), Sheldon *et al.* 1999; laughing kookaburra (*Dacelo novaeguineae*), Legge *et al.* 2001) a relationship between laying order and sex ratio, or a deviation from parity that continues beyond the first-laid egg. These studies certainly suggest the operation of pre-ovulation adjustment mechanisms, although because of the difficulty of obtaining these data, insufficient details on laying order, laying gaps and/or infertility of eggs were presented to rule out the operation of post-ovulation mechanisms. Further, some of these studies did not provide explicit tests for deviation from parity of eggs beyond the first. Still other studies show that male and female eggs within a clutch are provisioned differently, independent of the effects of laying order (white-crowned sparrow (*Zonotrichia leucophrys*), Mead *et al.* 1987; American kestrel (*Falco sparverius*), Anderson *et al.* 1997; house sparrow (*Passes domesticus*), Cordero *et al.* 2000). This implies either non-random ovulation or sex-specific recognition of fertilized ova coupled with control over egg provisioning.

Clearly, further studies are needed at the physiological, genetic and ecological levels to develop an understanding of how some birds are able to adjust the sex ratio of their clutch in an apparently adaptive manner. In this study, we show that pre-ovulation control is evident in the Seychelles warbler, quite possibly through directed haplotype segregation at first meiotic division, though determination of the actual mechanism needs further investigation. The extent to which similar mechanisms may operate in other avian species remains to be explained.

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