

Offspring sex ratio variation in relation to brood size and mortality in a promiscuous species: the Aquatic Warbler *Acrocephalus paludicola*

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We analyse nestling sex ratio variation in the Aquatic Warbler *Acrocephalus paludicola* to test for predictions from sex allocation theory that the brood sex ratio is close to parity. We also tested Fiala's (1980) prediction that there is no difference in sex ratio between broods affected and not affected by mortality, and whether a shift in primary sex ratios or simple differential mortality by sex underlies that difference. Furthermore, we explore additional analytical possibilities for inferring proximate mechanisms through simulation modelling. In the Aquatic Warbler, which is promiscuous, the overall sex ratio determined by molecular sexing of nestlings at 8–11 days of age did not deviate significantly from parity (proportion of females 0.509), nor did we find any predictive effect of brood size, maternal body mass, fat condition, wing and bill length, laying date, mean daily temperature, and multiple-male mating. However, extensive simulation suggested that the whole pattern of sex ratio variation is unlikely to arise purely by chance: (1) there is a diverging sex ratio between complete and partial broods, (2) large broods tended to be female-biased and small broods male-biased, and (3) low ambient temperature prior to the laying period seemed to increase the proportion of female offspring in complete broods. We conclude that most variation in nestling sex ratio is non-adaptive in nature, and results from variation in female nestlings mortality dependent on brood size and sex ratio.

Fisher's theory and potential constraints arising from the sex determination mechanism in heterogametic animals predict sex ratio equity and binomial sex variance (Fisher 1930, Bull & Charnov 1988). In most birds, sex ratios at the nestling stage are close to parity and variation does not exceed that expected due to binomial sampling variation (Harmsen & Cooke 1983, Clutton-Brock 1986, Breitwisch 1989, Hardy 2002, Krackow 2002). However, the view that heterogametic sex determination severely constrains the production of biased sex ratios has recently been challenged by demonstrating flexible sex ratio patterns in several higher vertebrate species, such as birds, in response to environmental uncertainty (Komdeur *et al.* 1997, Oddie 1998, Sheldon *et al.*

1999, Hardy 2002, West & Sheldon 2002). Such findings continue to stimulate the ongoing debate as to whether the observed pattern is adaptive or is merely a consequence of multiple constraints setting limits on an individual. The former conclusion implies parental interference and the assumption that benefits and costs of sex ratio adjustment are known. Unlike for fitness benefit, quantification of the cost cannot be undertaken without a detailed understanding of the proximate mechanisms underlying modification of the sex ratio. As different physiological mechanisms can incur strikingly different costs, their identification should thus be a prerequisite before looking at adaptive explanations for any statistically significant pattern observed (Krackow 2002).

In birds, females are the heterogametic sex, which gives them the potential to employ the most efficient way of adjusting sex ratio, i.e. at ovulation. This ability

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has recently been demonstrated in the Seychelles Warbler *Acrocephalus sechellensis* (Komdeur *et al.* 2002). However, as there are no obvious physiological or genetic mechanisms for skewing the sex ratio at laying (Komdeur *et al.* 2002), parents skewing the sex ratio that are unable to take advantage of the primary control must therefore selectively kill offspring of a particular sex, thereby losing some of the resources already invested in those offspring (Williams 1979). Consequently, this secondary control of sex ratio, by manipulating the survival of male and female offspring differentially, is considered more costly than the primary one. Distinguishing between these two types of sex ratio adjustment is critically important for the study of the adaptiveness of sex allocation, as the vast majority of cases with sex-specific differential mortality resulting from food shortage have not arisen through the adaptive behaviour of parents; rather they appear to have a non-functional explanation (Clutton-Brock 1991).

Here, we analyse nestling sex ratio variation in a population of the Aquatic Warbler *Acrocephalus paludicola*, a small insectivorous passerine bird (about 12 g), in which males are slightly, but significantly, larger and heavier than females (Dyrzcz 1993). It inhabits wet *Carex*-marshland in fertile lowland, which is particularly rich in arthropods, mainly in Belarus, Poland and Ukraine. It has retreated from western Europe because of a loss of suitable habitats. Nests of the Aquatic Warbler are often clustered at places where food resources are rich and, within these clusters, females are aggressive towards one another and defend exclusive foraging grounds (Dyrzcz & Zdunek 1993). Although males have a well-defined song-post, no distinct agonistic behaviour has been observed among males in the field. Telemetric data indicate that males regularly visit large areas of the population range; as a consequence, their home ranges often overlap (Schmidt *et al.* 1999, Schaefer *et al.* 2000). The Aquatic Warbler has a quasi-promiscuous breeding system (Heise 1970, Schulze-Hagen *et al.* 1993, 1995, Dyrzcz *et al.* 2002) in which males are emancipated from any parental duties. The high density of potential prey may explain and facilitate the uniparental rearing of offspring by the female. The percentage of broods with nestlings sired by more than one male is high across years, ranging from 54 to 92% ($n = 64$); a maximum of five fathers was detected in four broods (Dyrzcz *et al.* 2002).

We focus primarily on the question of what proximate mechanisms are involved in shaping the sex

ratio pattern in the Aquatic Warbler. In birds, it is often difficult to assess the variation in primary sex ratio from the nestlings because in some nests not all the offspring can be sexed owing to embryo and/or nestling mortality. Moreover, the primary sex ratio cannot be determined simply by disregarding nests affected by differential mortality prior to sexing. This would introduce a bias in favour of broods that had an excess of the better-surviving sex (Fiala 1980). Similarly, including such nests in the analysis induces the same type of bias by overestimating the proportion of the sex that has greater survivorship. Dividing broods into those with complete and partial numbers of offspring may thus appear to be of little help in inferring the causality behind the production of sex ratio pattern in birds. Here we argue that, contrary to this view, the distinction between complete and partial broods is useful in the analyses of bird sex ratios and should be routinely performed whenever the data on brood losses are available. We demonstrate that, combined with simulation modelling, the analysis of sex ratio variation between complete and partial broods may be an important diagnostic tool providing additional information on the nature of mortality as one of the possible mechanisms altering avian sex ratios.

MATERIALS AND METHODS

Field study

Field studies were carried out in 1993, 1994 and 1997 on a 44-ha plot of fen mires in the southern basin of the Biebrza River in north-east Poland (53°20'N, 22°40'E). A great part of the study area was a bed of *Carex* tussocks with *C. appropinquata* as the dominant species. A characteristic feature of the area was an extensive moss layer on the ground and scattered bushes of *Salix* spp., on average 1 m in height (for further details see Dyrzcz & Zdunek 1993). Water depth among tussocks varied between 1 and 5 cm during the study. A grid of 100 × 100-m squares, marked by coloured poles, was established to allow for orientation in the field and the mapping of nests. Females were mist-netted near their nest and males near their singing post. They were weighed using a Pesola spring balance (range 50 g, sensitivity 0.5 g). Wing length was measured to the nearest 1 mm by the flattened wing method (Svensson 1992), and the length of the bill from the skull was measured to the nearest 0.1 mm. Fat condition was determined visually by one person, according to the six-degree scale

(0–5) used by Operation Baltic in Poland (Busse 1970).

Blood samples were collected from nestlings on days 8–11 of life (counting hatch day as day 1), from breeding females and as many males as possible. A total of 63 broods was available for DNA analysis. Only the first brood per female and year was examined.

Molecular sexing

Molecular sexing was modified according to the methods outlined in Kahn *et al.* (1998), and based on the detection of the *CHD* gene on avian sex chromosomes. In most species, males produce one band and females two, presumably reflecting differing intron sizes of the W vs. Z chromosomes (Kahn *et al.* 1998). PCR used was 1237L: GAGAACTGTGC-AAAACAG and 1272H: TCCAGAATATCTTCT-GCTCC. PCR conditions were as follows. The PCR mix consisted of: 60 ng (2 μ L) total DNA in 25 μ L total volume, 0.12 μ L 1272H Primer (97.45 pmol/ μ L), 0.103 μ L 1237 L Primer (83.1 pmol/ μ L), 1 μ L Nucleotide-mix (100 μ M of GTP, GTP, TP and ATP), 2.5 μ L 10 \times buffer with 15 mM MgCl₂, 0.15 μ L *Taq*-Polymerase (0.6 Units; Pharmacia Biotech, Freiburg, Germany), 0.1 μ L 33P α -dATP (10 μ Ci). The PCR programme comprised: 2 min at 94 °C; 30 cycles of 30 s at 94 °C, 1 min at 56 °C, 2 min at 72 °C and finally 10 min at 72 °C.

PCR products were separated electrophoretically on a denaturing Sequagel matrix at 65 W for 3.5 h (length 40 cm). After drying, the gel was exposed to an X-ray film (Hyperfilm-MP, Amersham, UK) for 2–6 days and developed (X-ray developer and fixer, Kodak).

Statistical analysis

Sixty-three broods born to 63 different mothers were available across the 3 years of the study. As there were no co-variates at the level of an individual nestling to model the probability of becoming a particular sex, we modelled the proportion of females, a descriptive property of broods, as the binomial response variable. We fitted generalized linear mixed models (GLMM), implemented as a macro Glimmix in SAS (Littell *et al.* 1996), to examine whether nestling sex ratios varied predictably with any factor. This method assumes a binomial error distribution and allows both fixed and random effects at different levels of data to determine the response variable transformed using a logit link function. In this case,

a random component arose because broods were sampled across years. Hence, year was fitted as a random effect, leading to a two-level statistical model. The proper covariance structure was decided according to the lowest Akaike Information Criterion (AIC) values. As continuous fixed effects, measured at the level of the brood, we used mean daily temperature (measured for each brood during the 10 days prior to the laying of the first egg), brood size, date of clutch initiation (Julian date with 1 for May 1 and 32 for June 1), and maternal traits including body mass, wing length, bill length and fat condition. We were able to determine whether broods had been affected by offspring mortality at the time of sampling, and also to discriminate between broods fathered by one male or more. These factors, namely brood completeness and paternity, were entered as categorical variables. The model parameters were estimated by restricted pseudo-likelihood. The significance of the fixed effects was assessed by *F*-tests, with the denominator degrees of freedom being specified by Satterthwaite option (see Krackow & Tkadlec 2001 for a methodological review). Similarly, variation in brood size was analysed using GLMM with the year included as a random effect but now setting a Poisson error distribution and log link function. To assess whether there was an excess of female offspring in the sample causing the ratio to deviate from parity, we used a binomial two-sided test (procedure FREQ in SAS). Variation in mortality rate, defined as the proportion of young from the initial clutch size being alive at the time of sampling, i.e. including hatching failure, was analysed by fitting GLMM assuming a binomial error variance as described above.

If sex is allocated to each offspring randomly, i.e. if the probability of becoming a particular sex is constant within and between broods, then the distribution of (fe)males should be binomial owing to Mendelian segregation in heterogametic organisms. To explore this binomial expectation, we applied a statistical method proposed by James (1975) and recommended further by Krackow *et al.* (2002) to be used in cases with small broods of unequal size (see also Krackow 1992). This technique leads to the calculation of a test statistic *z* following a standard normal distribution. Significant positive values of *z* indicate a sex-ratio variance greater than binomial (over-dispersion) and negative values indicate variance smaller than binomial (under-dispersion). Owing to the complexity of this technique, we refer the reader to the review by Krackow *et al.* (2002) for a detailed description.

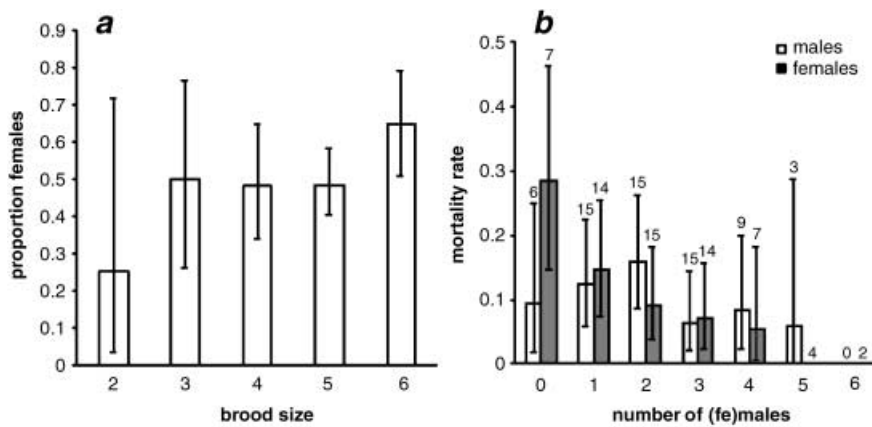


Figure 1. (a) Nestling sex ratios (with the 95% binomial confidence limits) in the Aquatic Warbler in relation to brood size. (b) The relationship between the finite mortality rate (measured as the proportion of offspring lost from the initial clutch size until the time of sampling) relative to the number of (fe)males surviving in the nest. Numbers above the 95% binomial confidence limits bars indicate sample sizes for broods with the given number of (fe)males.

Table 1. Statistical analysis of fixed effects on the proportion of females in broods estimated by single-factor GLMMs.

Variable	No. of broods	Estimate	95% CI	F	P
Maternal traits					
Initial clutch size	63	0.127	-0.347 to 0.602	0.29	0.59
Brood size ^a	63	0.265	-0.062 to 0.592	2.62	0.11
Mass	55	0.220	-0.170 to 0.611	1.28	0.26
Fat condition	55	0.022	-0.215 to 0.259	0.03	0.85
Wing length	57	0.057	-0.217 to 0.332	0.17	0.68
Bill length	57	-0.057	-0.613 to 0.498	0.04	0.84
Date	63	0.018	-0.036 to 0.071	0.43	0.51
Mean temperature	63	-0.068	-0.191 to 0.055	1.21	0.28
Paternity ^b	58	0.168	-0.490 to 0.827	0.26	0.61
Brood completeness ^c	63	0.592	-0.130 to 1.314	2.69	0.11

^aBrood size at the time of sampling. ^bOffspring sired by one (the estimate given here) vs. sired by more than one male. ^cComplete (the estimate given here) vs. partial broods. Note that a traditional GLM yielded $P = 0.04$.

RESULTS

Brood size ranged from two to six, with a mean of 4.56 ($se = \pm 0.133$), and decreased with date ($F_{1,10} = 5.37$, $P = 0.043$). The most frequent size class was five (31 broods, 49%). Across all broods, there were 287 offspring, with 146 females and 141 males, an overall proportion of females of 0.509 (95% confidence interval (CI) 0.449–0.567). No deviation from parity is therefore detected. There was little between-year variation in sex ratios, as indicated by a zero random effect of year as estimated by unconditional GLMM (applying a traditional contingency table analysis leads to $\chi^2 = 1.26$, $df = 2$, $P = 0.54$). The distribution of sex in broods was significantly higher than binomial (over-dispersion), suggesting that the sex ratios do vary among broods (K/I (see Krackow *et al.* 2002) = 0.033, $z = 3.11$, $P = 0.008$).

The proportion of females (pf) tended to be greater in broods of six nestlings ($pf = 0.648$, 95% c.i.

0.521–0.776, $n = 54$ nestlings) and less in broods of two nestlings ($pf = 0.250$, 95% c.i. 0–0.550, $n = 8$; Fig. 1a). This might indicate that variation in nestling sex ratio is clutch-size dependent. However, we found no evidence of this, or for the variation being related to any of the remaining explanatory variables (Table 1). Another mechanism, with the potential to produce this pattern, might be variation in differential mortality by sex depending on some specific brood property, such as clutch size or primary brood sex ratio. Sex-specific mortality might then be concentrated in certain brood classes leaving the remaining broods largely unaffected. Consequently, broods can be divided roughly into two large categories depending on whether they are affected by offspring mortality, and these groups should differ from each other in sex ratio.

It is important to emphasize that simple differential, sex-specific mortality (when mortalities differ between the sexes but stay constant) predicts equal

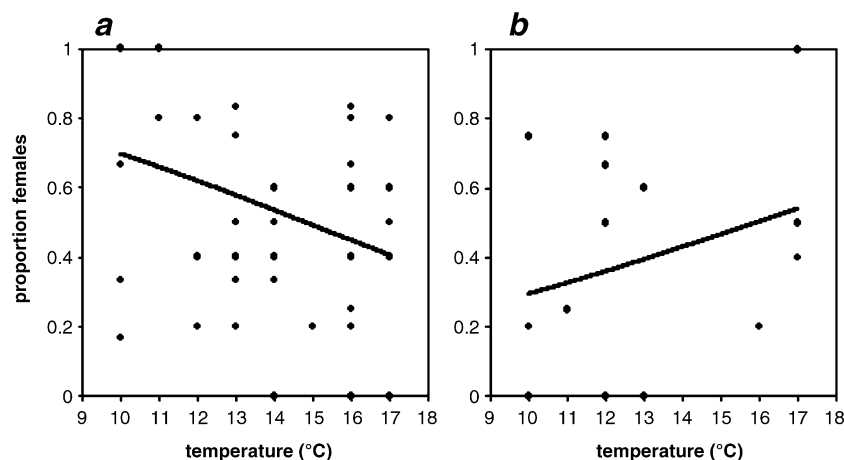


Figure 2. Sex ratios in complete (a) and partial broods (b) as observed (dots) and predicted by GLMM (curves) relative to ambient temperature. The fixed part of the model incorporates brood completeness, temperature and their interaction. Year of the study is included as a random effect.

sex ratios in complete and partial broods (Fiala 1980). We knew from the initial clutch size that, at the time of sampling, the size was reduced in 16 broods. Mean sizes for complete and partial broods were 4.81 ($se = \pm 0.142$) and 3.81 ($se = \pm 0.243$), respectively. This reduction, corresponding on average to one chick, is significant (GLMM, random intercept: $F_{1,61} = 12.14$, $P < 0.001$). The overall finite mortality rate (including unhatched eggs) was 0.106. The highest mortality rate of 0.28 was recorded for an initial clutch size of four. The pattern of variation in mortality did not depend on initial clutch size ($F_{1,60.7} = 2.59$, $P = 0.11$) or temperature ($F_{1,61} = 0.56$, $P = 0.46$) but it did vary with date; broods produced later in the season suffered greater losses ($F_{1,43.5} = 5.30$, $P = 0.026$). However, mortality rates were associated negatively with the number of surviving female offspring ($F_{1,61} = 13.69$, $P = 0.0005$; Fig. 1b) rather than male offspring in the brood ($F_{1,35} = 0.95$, $P = 0.35$), a result that is expected for variation in female mortality. Because the number of female offspring in a brood is correlated with sex ratio and clutch size, mortality affected broods more if they were small and male-biased at the time of sampling. This is confirmed by regressing mortality rates on nestling sex ratios, resulting in a decrease in mortality with an increasing proportion of females ($F_{1,60.4} = 4.14$, $P = 0.046$). Although the difference in sex ratios between complete and partial broods was not statistically significant (Table 1), the tendency for the proportion of females to be higher in complete than in partial broods is impressive (complete: $pf = 0.540$, 95% CI 0.475–0.605, $n = 226$ nestlings; partial: $pf = 0.393$, 95% CI 0.271–0.516, $n = 61$ nestlings). Sex ratios are significantly over-dispersed in complete broods ($K/I = 0.028$, $z = 2.505$, $P = 0.006$),

but no such deviation was found in partial broods ($K/I = 0.008$, $z = 0.372$, $P = 0.36$).

If complete and partial broods had different sex ratios, these could respond differently to other variables. Consequently, we fitted a set of models including the interaction terms for brood completeness and for the effect tested. The only variable whose effect depended on a brood category was the mean daily temperature (brood completeness \times temperature: $b = -0.321$, 95% CI -0.604 to -0.037 , $F_{1,59} = 5.10$, $P = 0.028$). This decreased the proportion of females in complete broods but seems to have been indifferent or slightly to increase the proportion of females in partial broods (Fig. 2). We obtained similar results when analysing these two brood groups separately. With increasing ambient temperature the female proportions decreased in complete broods ($F_{1,45} = 5.58$, $P = 0.023$) and did not change in partial broods ($F_{1,14} = 1.43$, $P = 0.25$), suggesting that ambient temperature may be involved in the mechanisms controlling sex ratio in the Aquatic Warbler.

To summarize the main results, we could not demonstrate any significant variation in nestling sex ratios by applying rigorous statistical tests separately to several predictor variables. However, when considered as a whole, the sex ratio variation in the Aquatic Warbler did appear to exhibit a specific pattern: (1) large broods tend to be female-biased and small broods male-biased; (2) Fiala's prediction holds statistically but the observed difference in sex ratios between the complete and partial broods is fairly large, with the complete broods having more females and the partial broods having fewer females than males; (3) mortality rates were better predicted by the number of female rather than male offspring surviving in the nest and were higher in small

clutches laid late in the season than in large clutches laid early in the season; (4) low ambient temperatures prior to the laying period seem to increase the proportion of female offspring in complete broods but have no effect in partial broods.

Simulation models

To obtain further insight into the proximate causal relationships underlying sex-ratio control in the Aquatic Warbler, we designed computer models to simulate four basic mechanisms thought to be capable of producing the observed pattern of sex ratio variation. These incorporated the basic features of our data: (1) clutch-size-dependent variation in primary sex ratios (cs), with small clutches being male-biased and large clutches being female-biased; (2) temperature-dependent variation in primary sex ratios (t), with clutches at low temperatures being female-biased and those at high temperatures being male-biased; (3) sex-ratio-dependent variation in

female mortality rates ($mort-p$), which increases in male-biased clutches and decreases in female-biased while keeping male mortality low and constant; (4) sex-ratio- and clutch-size-dependent female mortality ($mort-f$) increasing in clutches with a decreasing number of female offspring while keeping male mortality low and constant. When combined, these four functional relationships give another seven complex mechanisms subject to simulation (see Appendix). In fact, we simulated far more model variants and their combinations but these gave no additional insight into causes of the observed pattern, and are not presented here.

First, models incorporating no mechanisms are unlikely to reproduce the important features of the field data, namely the difference in nestling sex ratios between complete and partial broods (Table 2). Thus, as expected, Fiala's prediction holds in 99% of the simulation runs. This also applies to the mechanism based on the effect of temperature on primary sex ratios. Secondly, clutch-size-dependent variation

Table 2. Comparison of parameter values estimated from the field and the simulated data produced by several models for nestling sex ratio variation in the Aquatic Warbler. The parameter values for simulated data are means or grand means based on estimates from 1000 runs, the 95% CI is estimated by a percentile method taking the values cutting off the lower 2.5% and the upper 2.5%.

Data	Brood size (n)		Nestling sex ratio p			Difference in p' (95% CI)	No. of runs with difference > 0.147
	Complete	Partial	All broods	Complete	Partial		
Field	4.81 (47)	3.81 (16)	0.509	0.540	0.393	0.147	–
Simulated							
null model ^a	5.04 (63)	–	0.500	0.501	0.500	–	–
null + mortality ^b	4.56 (38)	4.55 (25)	0.499	0.500	0.499	0.001 (–0.128 to 0.119)	9
null + diff. mort ^c	4.41 (33)	4.41 (29)	0.456	0.456	0.456	0.000 (–0.120 to 0.120)	11
cs ^d	4.55 (38)	4.56 (24)	0.550	0.523	0.588	–0.065 (–0.195 to 0.067)	2
t ^e	4.55 (38)	4.55 (25)	0.500	0.500	0.501	0.000 (–0.130 to 0.129)	11
$mort-p$ ^f	4.45 (36)	4.52 (26)	0.491	0.554	0.404	0.150 (0.020 to 0.280)	521
$mort-f$ ^g	4.98 (35)	3.93 (27)	0.491	0.540	0.409	0.132 (–0.002 to 0.267)	426
$cs + t$ ^h	4.55 (38)	4.55 (25)	0.586	0.564	0.617	–0.053 (–0.194 to 0.081)	5
$cs + mort-p$ ⁱ	4.68 (38)	4.33 (24)	0.537	0.576	0.467	0.109 (–0.032 to 0.248)	312
$cs + mort-f$ ^j	5.19 (35)	3.66 (27)	0.532	0.586	0.430	0.156 (–0.001 to 0.320)	562
$t + mort-p$ ⁱ	4.48 (37)	4.52 (25)	0.492	0.557	0.396	0.161 (0.001 to 0.299)	588
$t + mort-f$ ^k	4.99 (36)	3.97 (26)	0.492	0.549	0.394	0.155 (0.008 to 0.304)	544
$cs + t + mort-p$ ⁱ	4.65 (39)	4.32 (24)	0.536	0.585	0.447	0.138 (–0.017 to 0.297)	457
$cs + t + mort-f$ ^k	5.19 (35)	3.67 (26)	0.532	0.595	0.411	0.184 (0.036 to 0.345)	673

^aThe primary sex ratio is fixed at parity and there is no mortality. ^bThe null model but with both sexes suffering the same mortality rates (i.e. binomial probabilities of death) of 0.1. ^cMortality rates are 0.05 and 0.2 for males and females, respectively. ^dClutch-size-dependent variation in primary sex ratio, with p' being varied between 0.45 and 0.65. ^eTemperature-dependent variation in primary sex ratio, with p' being varied between 0.45 and 0.65. Mortality rate is fixed at 0.1 for either sex. ^fFemale mortality rate is dependent on a primary sex ratio and is varied between 0 and 0.35. ^gClutch-size-dependent and sex-ratio-dependent variation in female mortality rate introduced as dependence on the number of female offspring in the clutch. Female mortality rates are allowed to vary between 0 and 0.4, those for males are fixed at 0.1. ^hThe effects of clutch size and temperature on sex ratios are equal and additive, each having capacity to change p' (= 0.5) by 0.15 up or down (consequently, p' varies between 0.2 and 0.8). ⁱFemale mortality rates are allowed to vary between 0 and 0.35, those for males are fixed at 0.1. ^jFemale mortality rates are allowed to vary between 0 and 0.5, male rates are 0.1. ^kFemale mortality rates are allowed to vary between 0 and 0.4, those of males are 0.1.

Table 3. Comparison of parameter values estimated from the field and the simulated data produced by several models for nestling sex ratio variation in the Aquatic Warbler. The parameter values for simulated data are obtained as mean values from 1000 runs.

Data	Brood size effect on p'	Temperature effect on p'		Overall mortality rate	Estimated effects on mortality rate ^l	
		Complete	Partial		No. of males	No. of females
Field	0.265	-0.173	0.148	0.106	-0.149	-0.573
Simulated						
null model ^a	0.002	-0.000	-0.021	0	-0.145	-0.145
null + mortality ^b	0.004	0.000	0.004	0.100	-0.216	-0.221
null + diff. mort ^c	0.003	-0.003	0.002	0.126	-0.222	-0.224
cs ^d	0.225	0.003	-0.004	0.100	-0.322	-0.133
t ^e	0.001	-0.235	-0.239	0.100	-0.193	-0.192
mort-p ^f	0.048	0.000	0.002	0.114	0.023	-0.397
mort-f ^g	0.081	0.004	-0.006	0.112	-0.202	-0.529
cs + t ^h	0.169	-0.240	-0.251	0.101	-0.253	-0.138
cs + mort-p ⁱ	0.216	0.000	-0.006	0.102	-0.013	-0.341
cs + mort-f ^j	0.245	-0.002	-0.003	0.111	-0.215	-0.516
t + mort-p ⁱ	0.051	-0.265	-0.219	0.109	0.034	-0.378
t + mort-f ^k	0.081	-0.251	-0.225	0.108	-0.139	-0.502
cs + t + mort-p ⁱ	0.226	-0.271	-0.193	0.099	0.046	-0.352
cs + t + mort-f ^k	0.262	-0.266	-0.196	0.110	-0.114	-0.498

^{a-k}For detailed information see the footnote to Table 2. ^lEstimated effects (slopes) for the numbers of surviving male and female offspring on the proportion of offspring lost from the initial clutch size until the time of sexing.

in the primary sex ratio alone can generate this difference but it does so in the opposite way to that observed in the data. Combining the effect of clutch size with that of temperature does little to remove this problem. Thirdly, all other models and their combinations have the capacity to produce nestling sex ratios that diverged between the broods affected and unaffected by mortality. Yet, the model combining all mechanisms seems to be particularly efficient in this respect, reproducing all major data features. Moreover, the best model (cs + t + mort-f) also produces the significant interaction term between the temperature and brood completeness in 358 simulation runs; the slope for complete broods was steeper than that for partial broods and only one significant interaction was produced with the slopes reversed. This best model was closely followed by a model incorporating the effect of temperature and the female mortality varying in response to both clutch size and primary sex ratio. It is notable that unlike the models that include the sex-ratio-based variation in female mortality, these models are even capable of generating the size difference between the complete and partial broods. However, this model fails to induce the dependence of nestling sex ratios on brood size (Table 3). The models comprising a clutch-size effect can do this but they fail to model the temperature-derived features.

Hence, these simulation results confirm that the mechanisms described above do have the potential to produce diverging sex ratios between the complete and partial broods and that Type I error is not necessarily the only viable candidate to explain the pattern of sex ratio variation in the Aquatic Warbler. More importantly, however, the theoretical evidence we have obtained through simulations strengthens our belief in the relative importance of the mechanisms based on the variation in female mortality. Conversely, the view that the primary sex ratio depends on an initial clutch size is weakened as a primary mechanism leading to the pattern of sex ratio variation in the Aquatic Warbler. Moreover, and more importantly, we demonstrate that the pattern observed in field data is unlikely to arise as a random event.

DISCUSSION

Several authors have recently emphasized the need for a shift from adaptive to proximate explanations in studies of the sex ratio variation in higher vertebrates (Oddie 1998, Krackow 2002). Here we examined sex ratio variation in the Aquatic Warbler with an eye to the analytical possibilities of inferring the underlying proximate mechanisms directly from standard field data. As for many other birds (e.g.

Koenig & Dickinson 1996, Westerdahl *et al.* 1997, Hartley *et al.* 1999, Pagliani *et al.* 1999, Saino *et al.* 1999, Heg *et al.* 2000, Buchanan 2001, Leech *et al.* 2001, Hardy 2002, Krackow 2002, South & Wright 2002, Whittingham *et al.* 2002), the overall nestling sex ratio did not deviate from parity. Moreover, no explanatory variable, including brood size, maternal body mass, fat condition, wing and bill length, laying date, mean daily temperature, and multiple-male mating, was found to predict the number of nestlings of either sex. This is consistent with the fact that in passerines biased sex ratios are confined mainly to co-operatively breeding species, such as the Seychelles Warbler (Komdeur *et al.* 1997, Komdeur 1998), Black-eared Miner *Manorina melanotis* (Ewen *et al.* 2001) and Bell Miner *M. melanophrys* (Clarke *et al.* 2002), in which the sex ratio skew toward sons or daughters depends on which sex contributes more to female fitness by helping. However, extensive simulations have suggested that the observed pattern of variation is far from being a purely random event resulting mechanically from cytological machinery: (i) large and complete broods tend to be female-biased and small and partial broods male-biased, and (ii) the proportion of female offspring in complete broods appears to be higher at low temperatures than at high temperatures. Although several putative proximate mechanisms can create such a pattern in data, the evidence obtained through the simulation gives more confidence that the variation in female mortality depends on the brood size and sex ratio. Because mortality mechanisms are generally less likely to be adaptive than modifications of the primary sex ratio, owing to higher manipulation costs and lack of evidence for parental discrimination in birds, these findings therefore suggest that most of the variation observed in nestling sex ratio is non-adaptive in nature, being a consequence of sex differences in offspring responses to environmental constraints, such as food shortage.

From a detailed analysis of data, sex-ratio-dependent variation in female mortality was identified as the most parsimonious proximate mechanism capable of generating the difference in nestling sex ratios observed between broods in relation to offspring mortality. The dependence of mortality on sex ratio has been proposed as a likely mechanism for sex ratio variation in Rooks *Corvus frugilegus* (Røskaft & Slagsvold 1985) and raptors (Dijkstra *et al.* 1998). In both cases, the dependence was attributed to sex-related differences in food demands as a result of sexual size dimorphism (Fiala & Congdon 1983). Broods of

equal size containing more nestlings of the larger or 'expensive' sex suffer higher mortality than broods containing more nestlings of the smaller or 'cheaper' sex. In support of their conclusion, Dijkstra *et al.* (1998) have developed a brood sex-ratio-dependent mortality model to demonstrate the capacity of the postulated mechanism to generate a U-shaped relationship between brood size and sex ratio at fledging. Nestling mortality in their data, although independent of sex, increases with the number of the larger sex (females) in the brood and with brood size. This is not the case with our data, in which mortality tends to decrease rather than increase with both the number of the smaller sex (females) and brood size as a consequence of the higher mortality produced in smaller clutches late in the season. Moreover, sex differences in size and mass were only demonstrated among adult birds (Dyrzcz 1993). An alternative explanation for the sex-ratio-driven variation in mortality is based on the greater competitive ability of the larger-sized sex under adverse feeding conditions. This has been suggested to explain the greater mortality of female Great Tit *Parus major* nestlings (Oddie 2000). Unlike the previous explanation, this is now proposed to account for an excess of the larger and more aggressive sex. Again, this is not the case with our data, as we have no evidence that the overall nestling sex ratio deviates from unity. However, these two mechanisms are both plausible and conceivable as operating in the Aquatic Warbler and neither of them can be excluded on the basis of the evidence presented here.

Our evidence that it is primarily the variation in the mortality of female offspring that matters is relatively weak. Nevertheless, when compared with the mortality in males, female mortality appears to be more plausible as a general mechanism for several reasons: (1) it is the number of female offspring that varies with brood mortality (note that there is a zero mortality rate in broods containing five or six female nestlings); (2) simulation models incorporating varying male mortality were substantially less effective in producing a sex-ratio difference between complete and partial broods; (3) they even reversed the female bias in sex ratios with increasing brood size by inducing more males in larger broods. Therefore, the sex ratio dependence in female mortality appears to be a more parsimonious mechanism than that found in male mortality. Importantly, this conclusion does not imply that males and females are always disproportionately affected by mortality. In fact, the overall mortality rates in best approximating

simulation models are about 0.1 for both male and female nestlings. Furthermore, the sexes did not differ over 1000 simulation runs in the amount of mortality variation (about 24% for both sexes as measured by the coefficient of variation). Hence the mechanisms based on sex-ratio-dependent variation of mortality linked to one sex only do not necessarily lead to differential mortality in the sample mean or variance. This interesting feature further supports the view that analyses of sex ratios need to be performed carefully and in great detail before any conclusion can be drawn.

The proportion of female offspring in complete broods was higher at low temperatures than at high temperatures. During cold and wet weather feeding conditions deteriorate for insectivorous birds. Variation in temperature can therefore be regarded as an operational measure of food availability. There is enough evidence that under adverse feeding conditions both egg and fledgling sex ratios can be biased in favour of the energetically cheaper (smaller) sex, particularly in dimorphic species, such as Blue-footed Booby *Sula nebouxi* (Torres & Drummond 1999), European Shag *Phalacrocorax aristotelis* (Velando *et al.* 2002), Montagu's Harrier *Circus pygargus* (Arroyo 2002), American Kestrel *Falco sparverius* (Wiebe & Bortolotti 1992), Eurasian Kestrel *Falco tinnunculus* (Korpimäki *et al.* 2000), Lesser Black-backed Gull *Larus fuscus* (Nager *et al.* 2000), Tawny Owl *Strix aluco* (Sasvari & Hegyi 2002) and Zebra Finch *Poephila guttata* (Kilner 1998). Experimental manipulation of food supply was correlated with changes in offspring sex ratios in Zebra Finches (Bradbury & Blakey 1998, Kilner 1998) and Kakapos *Strigops habroptilus* (Clout *et al.* 2002). Clearly, passerines are under-represented in the above list. However, the evidence from the Aquatic Warbler is at least consistent with the general pattern in birds suggesting that the temperature-dependent variation in nestling sex ratio cannot be considered merely a Type I error but may reflect a real variation in primary sex ratios (note that mean temperature was not correlated with mortality, which might imply another explanation in terms of variation in differential mortality). This opens the possibility for the direct involvement of a mother in the proximate mechanisms as a necessary, although not sufficient, condition for considering any sex-ratio pattern to be adaptive.

Male attractiveness has recently been demonstrated to have a potential to modify offspring sex ratio in Collared Flycatchers *Ficedula albicollis* (Ellegren *et al.* 1996) and Blue Tits *Parus caeruleus* (Sheldon

et al. 1999). Our results do not support the hypothesis that a male who manages to sire the whole brood is more attractive to females than one siring only part of the brood. We found no difference in sex ratios between multipaternal and monopaternal broods. Similarly, no difference in sex ratios between broods of polygynous and monogamous males was found in the closely related Great Reed Warbler *Acrocephalus arundinaceus* (Westerdahl *et al.* 1997), nor does the sex ratio differ from 1 : 1 in the polygynously breeding Corn Bunting *Miliaria calandra* (Hartley *et al.* 1999). By contrast, the fledgling sex ratios in the nests of second-mated females in the polygynous House Wren *Troglodytes aedon* were more female-biased than fledgling sex ratios in the nests of first-mated females (Albrecht & Johnson 2002). The second-mated females typically receive little or no male parental assistance and fledge fewer and poorer-quality young than do first-mated females. However, parental care in the Aquatic Warbler differs in that the males are emancipated from any parental duties, so invalidating this argument for this species.

We strengthened a traditional statistical analysis of data by simulation modelling to explore the capacity of supposed mechanisms to generate the observed pattern in data. In 1980, Fiala rightly dismissed Mayr's (1939) assertion that the primary sex ratio of birds can be estimated from the nestling sex ratio of complete broods in which all the young survive until they can be sexed. This may challenge the potential value of analyses aimed at comparing sex ratios between complete and partial broods. Here we argue that it is still worth investigating how much nestling sex ratios vary between broods affected or unaffected by mortality and that this analytical step should be routinely undertaken whenever data on nestling mortality are available.

Examining the size of effect and the direction of change can provide an additional source of information, which is particularly important for making inferences about the proximate mechanisms underlying the sex ratio variation. For instance, if an overall nestling sex ratio in the sample is skewed towards one sex, there are two equally plausible hypotheses at hand. Either there is a skew in the primary sex ratio or the sexes differ in the probability of dying unsexed. Under these simplified conditions, no sex-ratio difference is predicted between complete and partial broods (Fiala 1980). If the sex ratio apparently varies with brood size, we again have two possible mechanisms. Either there is a clutch-size-dependent adjustment of the primary sex ratio or there is

clutch-size-dependent variation in the sex difference in the probability of dying unsexed. Now, Fiala's prediction would not hold and the difference in sex ratio between complete and partial broods should be expected to appear. What is important to point out here is that each of the two mechanisms predicts a shift in its own direction, giving us a chance to distinguish between them by simulation modelling. Thus, in the latter case, simulation modelling may serve as a good analytical tool for distinguishing among the processes adjusting sex ratio at fledging. Moreover, even in situations where researchers are left with no statistically significant effects by applying statistical tests sequentially on an array of predictors, the simulation is still of great value, as it could help in assessing the hypothesis that the observed pattern arises by chance.

In this study, we present no conclusive evidence on sex-ratio variation in the Aquatic Warbler. Rather, we add yet another unclear picture in an interesting warbler species and further studies are clearly desirable. Modern empirical sex-ratio research, taking full advantage of the advent of molecular sexing techniques, clearly emphasizes experimental approaches in studying adaptive sex allocation (Komdeur & Pen 2002). The underlying proximate causes and mechanisms shaping the primary and secondary sex ratios in birds can therefore be tackled directly through carefully designed experimental manipulations. However, field ornithology has a long history during which large data sets containing many missing data on the sex of dead nestlings have been accumulated by thousands of field workers. All these data, subjected to rigorous statistical testing supplemented by simulation techniques, can thus become a rich source of information for the modern theory of sex allocation in birds.

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APPENDIX 1

In each of the 1000 simulation runs, 63 clutches (data sample size) were drawn from a Poisson distribution (mean of five eggs). Sex ratios were assigned to each clutch with the binomial probability p subject to the effect of clutch size or temperature. Probability p responding to one effect was allowed to vary between 0.45 and 0.65, in the case of two effects between 0.2 and 0.8. Temperature values were taken from a uniform distribution bounded between 0 and 7 to reflect the variation in field data ranging from 10 to 17 °C. The numbers of deaths per sex and clutch were generated as random deviates with the binomial probability equalling the observed finite mortality rate of 0.1 as a baseline level. Female mortality rates are allowed to vary from 0 to a limiting upper value (usually 0.35–0.5) such that the overall mortality rate approaches 0.1 as observed in field data (see footnote to Table 2). The effects of all variables are introduced by means of logistic regression models ensuring a sigmoid form for all functional relationships. The rationale is that (i) these forms secure

that sex ratios are bounded between 0 and 1 as desired for analysis of proportions, and (ii) we do not know how the various relationships are in nature and sigmoid relationships are the simplest non-linear ones. For example, to scale the effect of clutch size on the binomial probability p' with which sex ratios are generated, we used an equation of the form:

$$p' = p + P \left(\frac{e^{\beta_0 + \beta_1 \text{clutch-size}}}{1 + e^{\beta_0 + \beta_1 \text{clutch-size}}} - 0.5 \right),$$

where p represents the middle value for p' (here 0.5), P is a range for p' (here 0.3, i.e. p' is allowed to vary from 0.45 to 0.65), and the values of regression coefficients are given as $\beta_0 = \log[f/(1-f)]$ and $\beta_1 = -(1/\theta) \log[(2-f)/(1-f)]$. Here, f is the fraction of P that would be attained at the smallest brood size and θ is the brood size at which P attains half its value (i.e. $Pf/2$). The value of f and θ both affect the functional relationship between p and brood size and when f is close to unity, as assumed here, the relationship is sigmoid-shaped. We used 0.99 for f and mean values of predictor variables for θ (e.g. 5 for brood size).