



Short communication

Hatching order and sex ratio in Southern Grey Shrike *Lanius meridionalis* in relation to clutch size

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Despite major advances in sex ratio theory, how offspring sex should vary with hatching order remains unclear. We examine nestling sex ratio in the Southern Grey Shrike *Lanius meridionalis* according to hatching order and clutch size. Southern Grey Shrike nestlings present a different sex ratio with body-mass rank order depending on clutch size. When the clutch size was five eggs (with a very low risk of brood reduction; 13%) the less costly sex (male) was found at the end of the body mass hierarchy. However, when clutch size was six eggs (with a high risk of brood reduction; 42%) the larger sex (female) was found at intermediate positions in the hatching order, possibly to decrease competitive asymmetries.

Keywords: brood reduction, clutch size, nestling hierarchy, sex ratio.

Sex ratio theory predicts that under certain circumstances organisms should vary the sex of their offspring in order to maximize fitness return, leading to a biased sex ratio (West 2009). In fact, several hypotheses, not mutually exclusive, predict adaptive variation in the offspring sex ratio (Cockburn *et al.* 2002, West 2009, Komdeur 2012). In birds, these hypotheses have been particularly successful in predicting sex ratio biases in species with only one offspring per breeding attempt

(e.g. Seychelles Warbler *Acrocephalus sechellensis*; Komdeur *et al.* 1997) but have proved less predictive in species with larger brood sizes (Rosivall 2008, Carranza & Polo 2012, but see Badyaev *et al.* 2002 for a remarkable exception). This situation probably reflects the fact that, in species with large broods, the optimal sex ratio depends on hatching order, making mechanisms more difficult to resolve than for species with only one offspring per brood (Hasselquist & Kempenaers 2002). Predicting sex ratio according to hatching order has proved to be difficult (Komdeur & Pen 2002), to the extent that the existing models have generated opposing predictions (Uller 2006).

Birds usually show brood reduction (Magrath 1990), which typically, but not always, results from the death of later-hatching nestlings and the enhanced survival prospects of earlier-hatched nestlings (Magrath 1989). When environmental conditions are adverse (food shortage), nestlings of the larger sex, whose rearing is costlier in terms of parental effort, suffer higher mortality (Benito & González-Solís 2007), especially when they hatch from the last egg in the laying order (Torres & Drummond 1997) or when brood size is experimentally enlarged (Røskoft & Slagsvold 1985). Therefore, as the amount of food parents provide individual nestlings usually decreases with hatching order (Carranza 2004), the probability of death of the last-hatched nestling is higher if it belongs to the costlier sex than if it belongs to the less costly sex, especially when the probability of brood reduction is high (Benito & González-Solís 2007). Consequently, to reduce the probability of maladaptive brood reduction, i.e. brood reduction with a negative impact on parental fitness (Hillström & Olsson 1994), a higher frequency of the less costly sex would be expected later in the hatching order (Hasselquist & Kempenaers 2002). This 'intra-brood sharing-out hypothesis' (Carranza 2004) thus predicts (for a typical passerine) that the adaptive strategy for parents is that chicks hatching at the end of hatching sequence, the position that receives less food, are of the less costly sex, in order to decrease the probability of loss of the lowest-rank nestling.

On the other hand, an offspring of the smaller sex can be at a disadvantage when competing with siblings of the larger sex (Anderson *et al.* 1993), especially when hatching later in the sequence, which may lead to maladaptive brood reduction (Uller 2006). In such a case, one may expect that the brood would be biased towards the larger sex as the hatching sequence advances in order to reduce undesirable sibling asymmetries (Hasselquist & Kempenaers 2002, Uller 2006). In other words, the 'intra-brood competitive equilibrium' hypothesis (Uller 2006) predicts that the adaptive strategy for parents is that the larger sex (the better competitor, but also the costlier to rear) hatches at the end of the hatching sequence to decrease the probability of loss

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of the lowest-rank nestling. The two hypotheses thus generate opposing and mutually exclusive predictions. Although the two hypotheses were proposed almost 15 years ago (Hasselquist & Kempenaers 2002), it remains unclear how nestling sex ratio should vary with hatching order. Badyaev *et al.* (2002) predicted optimal sex ratio according to hatching order in different populations of House Finches *Haemorhous mexicanus* based on data on the fitness of each sex according to their position in the hatching sequence. However, there is no general rule to predict the pattern of sex ratio with hatching order in birds, as empirical work has reported mixed results for different populations or species including: (i) the costlier sex being less probable with the advance of hatching order (Tryjanowski *et al.* 2011), (ii) the more competitive sex being more probable with the advance of hatching order (Kilner 1998) and (iii) no evidence of sex ratio variation with hatching order (Bowers *et al.* 2013).

In this study, we contribute to the understanding of sex ratio variation with hatching order by examining how nestling sex ratio varies with hatching order and clutch size in the Southern Grey Shrike *Lanius meridionalis*. This species is a predatory passerine in which females are the costlier sex, as they are heavier than males and suffer more mortality when environmental conditions are adverse (Moreno-Rueda *et al.* 2014).

METHODS

The study took place during 2000, 2001 and 2002 in an area of agricultural fields around the town of Olite, Spain (42°29'N, 1°39'W). In the study area, most clutches (92%) have five or six eggs (Campos *et al.* 2011), and a small number of nests with fewer than five eggs were not included in the study. Overall, we considered 48 nests in which no egg or nestling was lost, and thus we could determine the primary sex ratio (*sensu* West 2009) of these broods, i.e. the sex ratio of embryos. We were sure that no eggs or nestlings were lost in these nests because we visited the nests around the end of egg-laying, the hatching day and when nestlings were 13 days old, recording clutch size, number of hatchlings and number of fledglings. Given that we included only nests in which all eggs survived to fledging, our findings were not confounded by sex-dependent mortality of embryos or nestlings, which has been studied specifically elsewhere (Moreno-Rueda *et al.* 2014). In another 19 nests with a known clutch size (of five or six eggs), at least one nestling or egg perished. Overall, brood reduction or loss of eggs occurred in 28.4% of nests (Campos *et al.* 2011).

Hatching of shrikes' eggs is asynchronous because incubation starts with the laying of the last or the penultimate egg (Hernández 1993), so we assessed hatching order based on body mass of chicks, assuming that

body-mass rank reflects hatching order. To test this assumption, we used data from a study population in southern Spain (Moreno-Rueda *et al.* 2016). During 2009–2011, we weighed 5-day-old nestlings ($n = 70$ chicks from 13 nests), which were individually marked with nail polish on their claws. Nestlings were weighed again when 13 days old. Nestling body-mass rank at 5 days old significantly predicted nestling rank at 13 days old (Linear Mixed Model, $\chi^2 = 151.97$, $P < 0.001$; brood identity as random factor; Fig. S1), suggesting that body-mass rank estimated when 13 days old may be used as a proxy of hatching order. This approach, however, results in some errors, mainly among the first few positions in the body mass hierarchy (Fig. S1). For this reason, we grouped nestlings into three categories of rank order: 'first nestlings' for ranks 1 and 2, 'mid nestlings' for ranks 3 and 4, and 'last nestlings' for ranks 5 and 6.

Blood samples (*c.* 0.1 mL) were collected from nestling shrikes by brachial venepuncture using a 30 G needle with blood collected using micropipettes and spotted onto FTA[®] Classic cards (Whatman International Ltd, Maidstone, UK) (Gutiérrez-Corcheró *et al.* 2002). DNA was extracted from FTA[®] Classic cards with ammonium acetate: a piece of dried blood sample (*c.* 2 mm²) was cut in sterilized conditions and transferred to a microcentrifuge tube for incubation in 250 μ L SET buffer (0.15 M NaCl, 0.05 M Tris pH 7.5, 0.001 M EDTA) at 4 °C for 5 h. The sample was treated with 7 μ L of sodium dodecyl sulphate (20%) and 5 μ L of proteinase K (10 mg/mL) at 55 °C overnight. About 250 μ L of ammonium acetate (4 M) was added to the mixture and left at room temperature for 30 min after which the suspension was centrifuged for 10 min at 13 244 $\times g$. The supernatant was transferred to a new vial and the DNA was precipitated by adding 1 mL of absolute ethanol and 10% (v/v) sodium acetate (3 M). After centrifugation, the air-dried DNA pellet was re-suspended in 40 μ L of ddH₂O.

Non-coding regions of DNA of the CDH protein (present in the Z and W sex chromosomes) were amplified using polymerase chain reaction (PCR). Primers were designed by Griffiths *et al.* (1998): P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCA TCGCTAAATCCTTT-3'). The mix included 5 μ L buffer (Tris-HCl 200 mM, pH 8.4, KCl 500 mM), 1.5 μ L MgCl₂ 50 mM, 4 μ L dNTPs 2.5 mM, 0.15 μ L Taq polymerase (Bioline), 8.3 μ L primer D and R, and distilled water to 50 μ L. The reaction began with a 4-min denaturation cycle at 94 °C, 35 cycles at 48 °C for 45 s, 72 °C for 45 s and 94 °C for 30 s, and a final cycle at 48 °C for 1 min and 72 °C for 5 min (Mycycler Thermal Cycler, BioRad). PCR fragments were separated by gel electrophoresis through a 2.5% agarose gel with ethidium bromide at 40 V for 5 min and 100 V for 45 min. According to Griffiths *et al.* (1998), blood

samples with one DNA band (corresponding to the CHD-Z gene) indicate males, and samples with two bands (corresponding to CHD-Z and CHD-W genes) are females.

Given that sex ratio is expressed as a frequency, we analysed how sex ratio varied with hatching order by contrasting observed frequencies with expected frequencies of 0.5 (null hypothesis) with Fisher exact tests (Wilson & Hardy 2002). The relationship between sex ratio and body-mass rank was tested with Spearman correlations. In addition, to test the effects of both brood size (five or six) and hatching order as determined from body-mass rank (first nestlings, mid-nestlings and last nestlings) on nestling sex, we used a generalized linear mixed model (GLMM) with penalized quasi-likelihood (Schall 1991, Zuur *et al.* 2009) using the library nlme (Pinheiro *et al.* 2012). In this statistical model, sex (male or female) of the nestling was the dependent variable with a binomial error distribution, with body-mass rank, brood size and their interaction as fixed categorical effects, and brood identity as a random factor. The significance of the interaction (analysis of deviance type III) indicates different patterns of sex ratio with brood size according to body-mass rank. All analyses were carried out with R (R Core Team 2015).

RESULTS

Brood sex ratio (fraction of male nestlings in the nest) of a complete clutch (those without brood reduction or hatching failure) averaged 0.52 and did not significantly differ from 0.50 (95% CI 0.45–0.58, $n = 48$ nests). In broods of five nestlings (the modal clutch size for broods

without brood reduction, $n = 27$), chicks tended to be males as the body-mass rank order advanced ($r_s = 0.89$, $P = 0.04$; Fig. 1). For the second most common brood size (six nestlings, $n = 21$; the modal clutch size including nests with brood reduction), sex ratio was significantly female-biased for mid-nestlings (31 females vs. 11 males; Fisher exact test, $P = 0.04$), but was unbiased for both first nestlings (19 females vs. 23 males; $P = 0.83$) and last nestlings (24 females vs. 18 males; $P = 0.66$). Comparing the sex ratio in broods of five and six nestlings for each rank position, we found no significant differences for rank orders 1–4 (χ^2 always $P > 0.10$). However, for rank 5, the sex ratio tended to differ between nests according to brood size (0.78 for brood size five vs. 0.48 for brood size six; $\chi^2 = 3.43$, $P = 0.06$). Similarly, the GLMM showed an almost significant effect of the interaction brood size*rank order on nestling sex ratio ($\chi^2_2 = 5.55$, $P = 0.06$), the additive effects of rank ($\chi^2_1 = 0.07$, $P = 0.80$) and brood size ($\chi^2_2 = 3.36$, $P = 0.19$) not being significant. In addition, brood reduction was significantly more frequent in broods with clutches of six eggs (15 of 36 nests) than in broods with clutches of five (only four of 31; $\chi^2 = 6.78$, $P < 0.01$).

DISCUSSION

The findings of this study suggest that in a population of Southern Grey Shrike the primary sex ratio differed from 1 : 1 in some positions in the hatching order. In clutches of five eggs, chicks were most likely to be the cheaper sex to produce when they occupied the last-hatched position in the laying sequence, although the

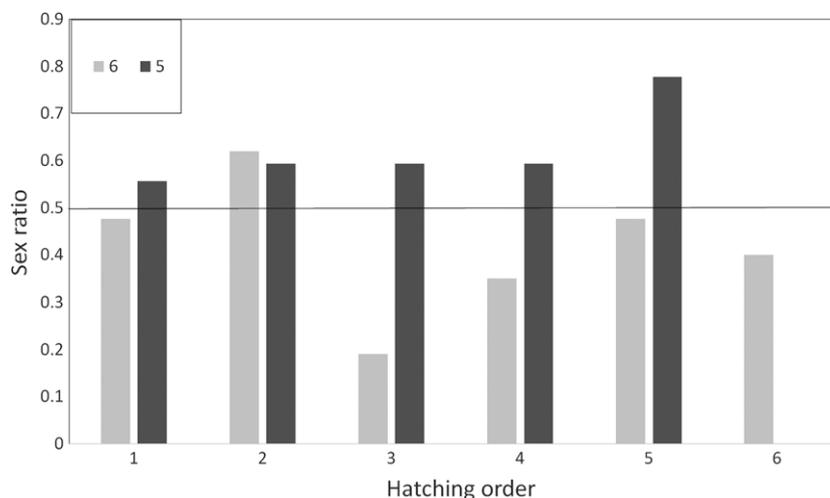


Figure 1. Sex ratio (proportion of male nestlings) according to hatching order in broods of five nestlings (dark grey, $n = 27$ broods) and six nestlings (light grey, $n = 21$ broods) in Southern Grey Shrikes nesting in Olite (Spain) during 2000–2002. The solid horizontal line indicates the 1 : 1 sex ratio (0.5). Only broods in which all eggs produced a fledgling were included.

statistical significance of this effect was marginal. In clutches of six eggs, chicks were most likely to be the costlier sex (females) when they occupied intermediate positions in the laying sequence. It should be noted that sex ratio may be altered by extra-pair paternity, as extra-pair nestlings tend to be males in shrikes (Schwarzová *et al.* 2008), although the frequency of extra-pair nestlings in our population is unknown. In addition, the two clutch sizes also differed in the probability of brood reduction, which was significantly higher for clutch size of six (42%) than for clutch size of five (13%). This last finding suggests that last-hatched nestlings have different risks of mortality (brood reduction) depending on clutch size.

In clutches of five eggs, the probability of having a male offspring increased as the hatching order advanced. This finding supports the intra-brood sharing-out hypothesis (Carranza 2004) because females are the sex that needs more resources (Moreno-Rueda *et al.* 2014), and because chicks in the last position usually receive less food (Moreno-Rueda *et al.* 2009); thus males probably benefit more than females when hatching from last-laid eggs. In other words, given that the last-hatched nestling receives less food, and females need more food than males, females would have less possibility than males of acquiring sufficient food to fledge when hatched in the last position. Similar findings have been reported frequently in other studies of birds (Clutton-Brock 1986).

In contrast, in clutches of six eggs, shrikes showed a different pattern of sex ratio with hatching order. In this case, middle-laid eggs were more likely to be females. Last-hatched nestlings, which die in almost half of broods of six hatchlings and may be considered 'marginal' nestlings (Forbes *et al.* 1997), seem under little selection for sex ratio, which is 1 : 1. This finding supports models that suggest that sex ratio adjustment may be reduced in last-hatched nestlings when they have a low probability of survival (Carranza 2004, Carranza & Polo 2012). The fact that mid-order eggs are more likely to be females may reflect avoidance of non-adaptive brood reduction, by placing the more competitive sex in lower positions in the ranking, and thus reducing sibling competitive asymmetries (lending support to the intra-brood competitive equilibrium hypothesis; Hasselquist & Kempenaers 2002, Uller 2006). The intra-brood competitive equilibrium hypothesis predicted that sex ratio should be biased to the larger sex in last-hatched nestlings. However, in our study, given that last-hatched nestlings in broods of six have a low probability of fledging, the most likely conclusion is that selection should favour a sex ratio biased to the larger sex in the last positions in hatching sequence that have non-negligible probabilities of producing recruits. Such a position corresponds with mid-order nestlings in our study.

A question that rises from these results is why the pattern of sex ratio with hatching order differs

according to clutch size. Based upon the data reported here we propose a new hypothesis, the 'conditional strategy' hypothesis. We hypothesize that the optimal variation in sex ratio with hatching order probably differs according to the risk of mortality of last-hatched nestlings. When mortality of last-hatched nestlings is high (high rate of brood reduction), maladaptive brood reduction would be reduced by decreasing sibling asymmetries, and thus the larger sex should be more frequent in the last-hatched nestlings with realistic survival prospects. However, if brood reduction is rare, then to maximize the fitness return for each hatching position (Carranza 2004), the less costly sex should be found mainly in the position that receives less food, i.e. the last. Our hypothesis thus suggests that the intra-brood sharing-out hypothesis is applicable to situations of low rates of brood reduction, whereas the intra-brood competitive equilibrium hypothesis is applicable to situations of high rates of brood reduction. This hypothesis offers testable predictions and might explain the high diversity of patterns in sex ratio variation with hatching order reported in the literature to date.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Relationship between body-mass rank order when 5 days old and body-mass rank order when 13 days old in 70 nestlings from 13 broods.