It has been hypothesized that parents increase their fitness by biasing the sex ratio of extra-pair offspring (EPO) towards males. Here, we report a male bias among EPO in a wild population of blue tits (*Cyanistes caeruleus*). This resulted from a decline in both the proportion of males and EPO over the laying order of eggs in the clutch. However, previous studies suggest that, unlike the decline in EPO with laying order, the relationship between offspring sex ratio and laying order is not consistent between years and populations in this species. Hence, we caution against treating the decline in proportion of males with laying order, and the resulting male bias among EPO, as support for the above hypothesis. Variable patterns of offspring sex and paternity over the laying order may explain inconsistent associations between offspring sex and paternity, between and within species.

1. Introduction

Because the relative fitness of sons and daughters will often vary depending on environmental conditions and parental traits, adjustment of offspring sex ratio can improve parental fitness [1]. Variance in male reproductive success is typically higher than that of females, which in socially monogamous species results from gains (and losses) of paternity through extra-pair mating [2]. Hence, if male success at acquiring extra-pair paternity is heritable and increases reproductive success (an assumption of ‘good genes’ explanations for extra-pair paternity [3]), females may increase their fitness by biasing the sex ratio of extra-pair offspring (EPO) towards sons [4].

Empirical evidence to support this prediction is equivocal. A study in house wrens (*Troglodytes aedon*), with a large sample size, found a male bias among EPO [5], but the majority of other studies have not (reviewed by Johnson *et al.* [5]). A possible explanation for the absence of a male bias among EPO is the inability of females to directly influence the paternity of specific ova [4]. This can potentially be solved by biasing whole litters or broods towards sons when females have engaged in extra-pair copulation, which may lead to a male bias among EPO at the population level. However, if females are more prone to engage in extra-pair copulation when mated to low-quality males, it may be maladaptive to bias the sex ratio of within-pair offspring towards sons as well. An alternative solution that does enable females to bias the sex ratio of EPO within litters or broods is to vary both offspring sex and paternity systematically with ovulation order [4].
Recent studies show that overrepresentation of EPO early in the laying order may be common among a wide range of bird species [6–9], so biasing early eggs towards sons, irrespective of paternity, would lead to a male bias among EPO within broods in these species. Here, we investigate this possibility in a wild population of blue tits (Cyanistes caeruleus), a species where males increase reproductive success with extra-pair paternity [10]. EPO are more likely in eggs laid earlier in the laying order [8,11,12], and in which a male bias among EPO has been reported previously [13] (but see [8,14,15]).

2. Material and methods

The study was conducted in the breeding season of 2009 in a nest-box population at ‘De Vosbergen’ estate, in The Netherlands (53°08’ N, 06°35’ E). All active nests were checked daily after the start of egg laying and new eggs were marked in order of laying. Complete clutches were collected 1 or 2 days prior to hatching, replaced with model eggs to prevent desertion, and all eggs hatched in separate compartments in incubators (for more details see [8]). This enabled us to link hatchlings to the specific egg they hatched from. Throughout the day, new hatchlings were uniquely marked and returned to their nest of origin within 2 h of hatching. Within 3 days of hatching, a small blood sample (ca 10 μl) was collected from the nestlings and putative parents were caught and blood-sampled 8–12 days after hatching of the first offspring. Sex of hatchlings was determined following Griffiths et al. [16]. See Vedder et al. [17] for details on molecular parentage analyses. Broods from single females or secondary females of polygynous males were excluded from analyses (following [17]).

We tested for effects on offspring sex with generalized linear mixed models with a binomial error distribution and a logit link function, and brood identity as a random effect. We tested two specific questions with two separate initial models. (i) Is the sex ratio among EPO biased towards males (at the individual or the brood level)? (ii) Can this be explained by a decline in sex ratio (proportion of males) with laying order, in parallel with the earlier published decline in proportion of EPO with laying order [11]? To account for the possibility that an effect of laying order is caused by overrepresentation of one sex in small clutches (that do not contain high laying orders), we included clutch size in the second initial model (see [18] for more details on this approach). Final models were obtained by stepwise backwards elimination of least significant terms. Only significant terms (p < 0.05) were retained in the final models. An experiment involving temporary removal of eggs to delay incubation onset and increase clutch size had no effect on offspring paternity and sex in interaction with laying order ([11], O. Vedder 2009, unpublished results). Hence, we pooled all data with respect to experimental treatment. This resulted in a sample size of 743 hatchlings (with known laying order, sex and paternity) from 68 broods, of which 22 contained EPO.

3. Results

Offspring paternity (extra-pair or within-pair) was significantly associated with offspring sex; EPO were more likely to be male (figure 1a and table 1a). The proportion of EPO per brood was not significantly related to offspring sex (table 1a), indicating that offspring of broods that contained a higher proportion of EPO were not more likely to be male, irrespective of individual paternity.

Adding laying order and clutch size to a model that contained offspring paternity to explain variation in offspring sex, revealed that a significant decline in the proportion of males with laying order accounted for the male bias among EPO (table 1b and figure 1b). This can be explained by a parallel decrease in the proportion of EPO with laying order (figure 1b, for statistics see [11]). The absence of a significant association between clutch size and offspring sex (table 1b) indicates that the effect of laying order on offspring sex is not caused by smaller clutches containing more males.

4. Discussion

Although it has been 17 years since Sheldon & Ellegren [4] suggested that female birds could bias the sex ratio of EPO
Table 1. Model summaries of the association between offspring sex and (a) individual offspring paternity and the proportion of EPO in a brood, and (b) laying order, clutch size and individual offspring paternity (*n* = 743 hatchlings from 68 broods). Statistics for non-significant terms were derived by entry back into the final model.

<table>
<thead>
<tr>
<th>Model</th>
<th>dependent variable: offspring sex (0, female; 1, male)</th>
<th>coefficient (s.e.)</th>
<th>( \chi^2 )</th>
<th>( \Delta \text{d.f.} )</th>
<th><em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td></td>
<td>-0.16 (0.08)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>offspring paternity (0, WPO; 1, EPO)</td>
<td></td>
<td>0.67 (0.34)</td>
<td>4.03</td>
<td>1</td>
<td>0.045</td>
</tr>
<tr>
<td>prop. of EPO in brood</td>
<td>non-significant</td>
<td>0.45 (0.66)</td>
<td>0.27</td>
<td>1</td>
<td>0.603</td>
</tr>
<tr>
<td>(b) dependent variable: offspring sex (0, female; 1, male)</td>
<td>offspring paternity (0, WPO; 1, EPO)</td>
<td>0.53 (0.34)</td>
<td>2.41</td>
<td>1</td>
<td>0.121</td>
</tr>
<tr>
<td>laying order</td>
<td></td>
<td>-0.04 (0.02)</td>
<td>5.37</td>
<td>1</td>
<td>0.020</td>
</tr>
<tr>
<td>offspring paternity (0, WPO; 1, EPO)</td>
<td>non-significant</td>
<td>-0.03 (0.04)</td>
<td>0.46</td>
<td>1</td>
<td>0.498</td>
</tr>
<tr>
<td>clutch size</td>
<td></td>
<td>-0.03 (0.04)</td>
<td>0.46</td>
<td>1</td>
<td>0.498</td>
</tr>
</tbody>
</table>

Towards males by varying both in concert over the laying order of eggs within a clutch, this is the first study to specifically test, and find support for, this proposed mechanism. Covariance of paternity and sex with laying order may be responsible for all reported male biases among EPO, as both species in which a male bias among EPO has been reported to date (blue tits; [13], this study, house wrens; [5]) also show a decline in EPO with laying order (blue tits; [8,11,12], house wrens; [7]).

While our findings in this study are consistent with the hypothesis that parents increase their fitness if they bias the sex ratio of EPO towards males [4], we caution against treating male bias among EPO as general support for this hypothesis. In blue tits, the decline in EPO with laying order has been consistently reported in different years and populations [8,11,12]. However, a decrease in proportion of males with laying order was not found in another blue tit population [19], or in different years in our study population (2006–2007; coefficient \( \pm \text{s.e.} = 0.01 \pm 0.02 \), \( \chi^2 = 0.14 \), \( \Delta \text{d.f.} = 1 \), \( p = 0.708 \), 2009 versus 2006–2007; coefficient \( \pm \text{s.e.} = -0.05 \pm 0.02 \), \( \chi^2 = 4.15 \), \( \Delta \text{d.f.} = 1 \), \( p = 0.042 \), see [8] for methodological details). Consequently, more often than not, there is not an association between offspring sex and paternity has not been found [8,14,15]. Hence, the decline in proportion of males with laying order observed in 2009, resulting in a male bias among EPO, may be more likely to be the result of adaptation to year-specific environmental conditions, rather than a general mechanism to achieve a male bias among EPO. Although we can only speculate on what conditions caused the decline in proportion of males with laying order in 2009, environment-specific associations between laying order and offspring sex have been reported in other species [20,21]. In species with a declining proportion of EPO with laying order, this may occasionally result in a male bias among EPO, which would explain why reported associations between offspring sex and paternity have been inconsistent, both between and within species (reviewed by Johnson *et al.* [5]).

The absence of a consistent male bias among EPO, despite an existing mechanism to achieve this (as apparent in this study), suggests that either selection against a decline in the proportion of males with laying order frequently outweighs potential benefits of biasing the sex ratio of EPO towards males, or that a male bias among EPO conveys no fitness benefit. In this respect, it is noteworthy that a key assumption of the hypothesis that parents increase their fitness if they bias the sex ratio of EPO towards males—heritability of male success in acquiring EPO—has been tested only once and was revealed, in that case, to have almost zero heritability [22]. Indeed, studies that test assumptions of the hypothesis are critical to assessing whether male biases in EPO are adaptive.

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**Data accessibility.** The data is deposited in the dryad repository: http://dx.doi.org/10.5061/dryad.b4628.

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