

## Nestling sex ratios in a population of Bluethroats *Luscinia svecica* inferred from AFLP™ analysis

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We studied the sex ratio of Bluethroat *Luscinia svecica* broods using AFLPs. Our aim was to test whether there is a bias towards males that could be explained by sexual selection theories, or conversely, a bias towards females that could help explain the female-biased sex ratio among juveniles observed at a wintering site. The AFLP technique was reliable in sexing the nestlings from even small initial DNA quantities. Given the large number of polymorphic markers that can be obtained for each primer combination, the probability of detecting a W-chromosome-linked fragment is reasonably high. As a consequence, this method could be used in other species for sex-ratio studies and for other genetic purposes. Among 246 nestlings, we found an overall proportion of males of 50.8% at hatching and the sex-ratio variation using broods as independent units was not significantly different from expectation under a binomial distribution. None of the parental and environmental variables tested changed significantly the deviance to the model. Thus, sex determination in the Bluethroat seems to match the classical Mendelian model of a 1:1 sex ratio and cannot explain the biased sex ratio towards juvenile females found at the wintering site.

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The study of offspring sex ratio (proportion of males) variations in a variety of animals provides a wide range of opportunities for studying natural selection and adaptations. Starting with a null hypothesis of an equal number of females and males in a brood (Fisher 1930), finding such an equilibrium can be due to either a random process of Mendelian crosses between male and female gametes (Williams 1979), or to a strong selection favouring a sex ratio of unity at the population or the brood level (Kolman 1960, Verner 1965, Williams 1979, Taylor and Sauer 1980). Departures from the predicted equilibrium have been considered as adaptive in a number of evolutionary explanations (Hamilton 1967, Trivers and Willard 1973, Clark 1978, for a review see Frank 1990). In birds, there was until recently little

evidence for sex-ratio manipulation and adaptive sex-ratio variation (Clutton-Brock 1986). However, some recent studies have shown that it can be adaptive to produce preferentially one sex, even though the mechanisms underlying such variations are poorly known (Krackow 1995).

Adaptiveness is essentially related to the availability of a number of different resources (Blank and Nolan 1983, Dijkstra et al. 1990, Daan et al. 1996, Decoux 1997, Komdeur et al. 1997, Kilner 1998, Nishiumi 1998), including an easier access to potential mates (Burley 1981, Ellegren et al. 1996, Svensson and Nilsson 1996). According to sexual selection theories, if the relative fitness of males and females differs and if the characters that make males attractive are heritable,

it is advantageous for females to produce more sons which will also be attractive and will gain higher breeding success than less attractive males. Such sex-ratio variation has been demonstrated experimentally for the Zebra Finch *Taenopygia guttata* (Burley 1981), Collared Flycatcher *Ficedula albicollis* (Ellegren et al. 1996) and Blue Tit *Parus caeruleus* (Svensson and Nilsson 1996).

The Bluethroat *Luscinia svecica* is a dimorphic territorial passerine. Males have a colourful throat with blue and chestnut surrounding a conspicuous patch (white or red depending on the subspecies), that is exhibited during courtship display. As these male ornaments may play a role in sexual selection (Johnsen and Lifjeld 1995), we investigated the possibility of a male-biased sex ratio in broods of a Bluethroat population (subspecies *L. s. namnetum* with a white throat patch).

If the opposite result was found (female-biased sex ratio), this could help explain the increasingly biased sex ratio towards juvenile females reported over years at a wintering site (Tagus estuary in Portugal), studied for 10 years (Constant and Eybert 1995). We wanted to know whether this bias is due to post-fledging ecological factors or to a bias at the level of the brood at either the hatchling or fledging stage.

However, testing for variations in sex ratio, possibly due to adaptive adjustment, requires the use of a reliable method for sexing the young before any sex-biased mortality has occurred. Molecular techniques have circumvented the drawbacks of classical sexing, such as cytogenetics or laparotomy which are not appropriate if big sample sizes are needed (Ellegren and Sheldon 1997). Since the female in birds is the heterogametic sex (Z/W; males are Z/Z), it is appropriate to use methods based on their ability to detect a DNA fragment specific to the W-chromosome (Ellegren 1996) such as minisatellite or microsatellite multilocus fingerprinting (Millard et al. 1992, Longmire et al. 1993, Austin and Parkin 1995) or RAPD (Random Amplified Polymorphic DNA) patterns (Williams et al. 1990, Sabo et al. 1994, Komdeur et al. 1997, Lessells and Mateman 1998).

In order to investigate the hatchling sex ratio for departure from unity in the Bluethroat we used the AFLP technique (Amplified Fragment Length Polymorphism), a recent fingerprinting method based on double amplification via PCR of selected restriction fragments of a total digest of genomic DNA (Vos et al. 1995). This technique is essentially used in plant mapping and studies of diversity in crops or wild plants (Majer et al. 1996, Schondelmaier et al. 1996, Travis et al. 1996, Greef et al. 1997, Escaravage et al. 1998). With a few exceptions AFLP data on animals are still scarce (Otsen et al. 1996, Vos and Kuiper 1996, Ajmone-Marsan et al. 1997). We also tested if sex-ratio variation was associated with a number of parental and environmental variables.

## Materials and methods

### Field methods and samples

Samples were collected during three breeding seasons (1995, 1996 and 1997) in a population of Bluethroats breeding in the saltmarsh of Guérande (47°20'N, 2°25'W), France. Territories were defined by scoring all the singing posts attributable to a particular male during the breeding season. The outermost singing posts defined the territory limits. The territory size and the area of salt pans included in the territories were calculated using G.I.S. vector and Arc View. As a measure of each territory's ecological resources, the spatial structure was computed using a heterogeneity index calculated in CHLOE (Rodriguez et al. 1997).

Bluethroat pairs were captured with mist-nets or traps. Blood samples were taken from the brachial vein and stored in Queen's lysis buffer (Seutin et al. 1991) until DNA extraction. Each individual was banded with colour rings in addition to a numbered metal ring. Adults were aged as 1-yr-old or older (Svensson 1992). Wing length was measured to the nearest mm with a stopped ruler. As an index of male attractiveness, the white throat patch (width and height) was measured to the nearest 0.1 mm after ruffling and smoothing out the feathers with a finger until the spot no longer varied in size and its area was estimated as:  $\pi \times (\text{width}) \times (\text{height})$ . For females we scored only the presence/absence of a blue bib and/or blue moustaches (scores were as follows: 1 = no ornamentation, 2 = blue moustaches, 3 = blue bib and 4 = blue bib and blue moustaches).

Daily visits permitted us to record the breeding attempts and to measure breeding success. In all nests that did not fail before the nestlings reached 7–8 days old, we colour banded the young and plucked growing feathers which were stored in 80% ethanol. In this study we focus on 55 families in which we sampled all the members (16 in 1995, 21 in 1996 and 18 in 1997). Altogether 246 nestlings were sampled. In four cases we had samples for both first and second broods that we considered as independent samples. A total of 38 adult males and 41 adult females were involved in one or several families within or between breeding seasons. The influence of parental variables on brood sex ratio was assessed by examining the sex ratio of the first brood. This was also done for both broods of a polygynous male in 1996. We found a total of 22 unhatched eggs in the three breeding seasons. Ten of them were checked and three contained an embryo (two of the embryos were sister and brother from the first and second brood, respectively). The three embryos were not included in the calculation of sex ratios.

The occurrence of extra-pair paternity was also investigated from the samples collected in 1995 and 1996 by using AFLPs (Questiau et al. in press). At least one extra-pair young was detected in 63.8% of the nests

totalling 41.9% of all young. As only parentage exclusions were studied, we focus on the total number of extra-pair young ( $n = 68$ ) when examining possible overall sex-ratio variation among the extra-pair offspring. The hypothesis is that males investing time in extra-pair copulations should be of better quality and should have sired more male offspring. Thus, considering all the extra-pair young together it should be possible to detect a male-biased sex ratio.

### DNA isolation

A 3 mm piece of the base of a growing feather for the nestlings or 400  $\mu$ l of the mix of blood and lysis buffer for the parents was digested in 400  $\mu$ l of a proteinase K solution (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, 10 mM NaCl, 1% SDS, 10 mg/ml DTT, 0.5 mg/ml proteinase K). DNA isolation was carried out using two standard phenol-chloroform extractions and one chloroform extraction as described in Taberlet and Bouvet (1991). DNA was precipitated in ethanol and diluted in TE buffer (10 mM Tris-HCl, pH 8, 0.1 mM EDTA) following a classical procedure (Sambrook et al. 1989).

### AFLP procedure

The first step of the AFLP™ technique is an enzymatic digestion of DNA by a rare (*EcoRI*) and a frequent cutter (*MseI*) according to the length of their restriction site. Each resulting fragment is ligated to adapters that serve as a binding site for primers with one to three additional selective nucleotides included at the 3' end that limit the number of scorable fragments on the gel. The result is a multilocus fingerprinting-like pattern which can be analysed on an automated sequencer by the use of fluorescent primers. Amplified fragment length polymorphisms were resolved according to the AFLP™ Plant Mapping Kit protocol (Perkin Elmer). No more than 55 ng of DNA from blood samples were used to eliminate artefactual bands possibly due to the presence in blood of enzymatic reaction inhibitors in the restriction-ligation reaction. Sixty to 200 ng DNA from the growing feathers were used. We performed the restriction-ligation step at 37°C for 2 hours in a final volume of 11  $\mu$ l. The reaction was then diluted to 200  $\mu$ l in  $1 \times TE_{0.1}$  (20 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Four microlitres of the diluted restriction-ligation DNA were added to 1  $\mu$ l of preselective primers with one nucleotide extension at the 3' end and 15  $\mu$ l AFLP Core Mix implemented in the kit. The preselective PCR was run as follows: 2 min at 72°C followed by 35 cycles with the following cycle profile: 30 s at 94°C, 30 s at 60°C and 2 min at 72°C. The amplifica-

tion reaction was diluted 20 times with  $1 \times TE_{0.1}$ . Three microlitres of the preamplified diluted DNA were mixed with 15  $\mu$ l AFLP Core Mix, 5 pmol selective *MseI*-CTA primer and 1 pmol fluorescent (FAM) *EcoRI*-ACT selective primer. The second amplification began with one cycle with a 2 min denaturation step at 94°C, 30 s annealing at 65°C and 2 min elongation at 72°C. Eight cycles followed with 10 s denaturation at 94°C, 30 s annealing with a temperature reduced each cycle by 1°C from 64°C to 57°C, and elongation for 2 min at 72°C. The PCR was continued for 30 cycles (10 s at 94°C, 30 s at 56°C, 2 min at 72°C).

Two microlitres of the selective amplification were added to 1  $\mu$ l of loading buffer containing deionized formamide, blue dye and genescan-500 ROX-labelled size standard, and run on a ABI PRISM™ 377 DNA sequencer (Perkin Elmer) in a 5% Long Ranger™ gel (FMC) during 5 hours.

A 334 bp fragment specific to the females (called the 334 fragment below; Fig. 1) was revealed in the profiles of all 41 adult females analysed. A smaller number of adult females and males would have been sufficient to test the sex-specificity of the 334 fragment. However, as all pairs were analysed in the study of parentage (Questiau et al. in press), we used all the data we had. The occurrence of the fragment was reproducible (data not shown). Consequently, the AFLP technique was considered reliable for sexing Bluethroats. As the female is the heterogametic sex, this fragment is assumed to be situated on the W-chromosome. The probability of finding the fragment in none of the 38 males and all the 41 females if this band was associated with an autosome and was occurring in 41 out of 79 individuals in the population is  $(38/79)^{38} \cdot (41/79)^{41} = 1.7 \times 10^{-24}$ . The presence or absence of the 334 peak was determined for all nestlings using the GeneScan™ 2.0.2 and Genotyper™ 2.0 analysis software.

### Statistical analyses

Preliminary investigations of the overall population and extra-pair young sex ratio, were carried out using a G-test but we also studied sex ratio at the level of the brood to avoid pseudoreplication caused by treating nestlings as independent units. This was achieved by taking binomial errors into account and using a logit link, with number of sons in a brood as the dependent variable and the total number of offspring in a brood as the binomial denominator using GLIM (Crawley 1993). The discrepancy between the model and the data is reported by the deviance which is distributed asymptotically as  $\chi^2$ . To assess the statistical significance of an independent explanatory variable, we looked at the deviance variation when it was added to the model.

## Results

Altogether, 246 nestlings and three embryos (two females and one male) were analysed. The overall proportion of males among the hatchlings for the three breeding seasons combined was 0.508 and did not differ from unity ( $G_1 = 0.065$ , NS). Table 1 gives the proportion of males for each of the three breeding seasons. The highest proportion of males was observed in 1995 and the lowest in 1996 but the variation between years was not significant ( $\chi^2 = 0.8$ , d.f. = 2, NS). The distribution of brood sex ratios is given in Fig. 2. This variation in the proportion of males among broods taken independently ( $0.504 \pm 0.189$ , mean  $\pm$  SD, Table 1) did not differ from that expected from a binomial distribution (deviance in null model = 40.65; d.f. = 54, NS). None of the parental and environmental variables added to the model made the deviance differ significantly from that expected under the binomial assumption (Table 2). Males with a large throat patch did not

have broods whose sex ratio was biased towards males as could be predicted under a model of sex allocation related to male attractiveness. Neither were the resources as measured by several components of the territory related to any sex-ratio variation, nor was time of season or female size as measured by wing length. Considering all the available data on the fledglings, their overall sex ratio (0.524) did not differ significantly from unity ( $G_1 = 0.333$ , NS). Among the extra-pair young analysed, no bias towards either sex was found: 34 were females and 34 were males ( $G_1 = 0$ , NS).

## Discussion

We studied sex-ratio variation in a bird species, the Bluethroat, using AFLP markers. Our aim was to test whether there was a biased sex ratio towards males under sexual selection hypotheses on factors influencing

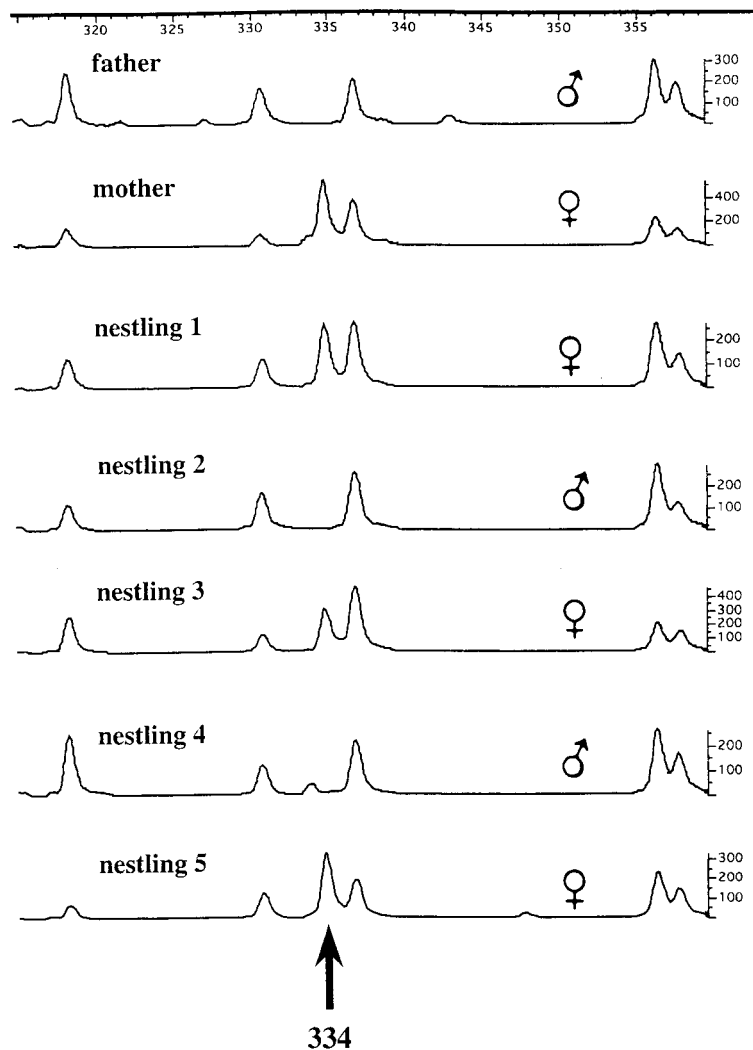


Fig. 1. Part of an AFLP electrophoregram of a Bluethroat brood showing a 334 bp fragment specific to females.

Table 1. Overall proportion of males in Bluethroat broods for the three breeding seasons and mean sex ratio for each brood taken independently. In 1996, three second broods are included in the table, one in 1997.

|           | Number of broods | Number of eggs | Number of sexed chicks | Overall proportion of males | Test for a 0.5 sex ratio, G value | Mean sex ratio $\pm$ SD for each brood taken independently |
|-----------|------------------|----------------|------------------------|-----------------------------|-----------------------------------|--|
| 1995      | 16               | 79             | 70                     | 0.543                       | 0.514, NS                         | 0.535 $\pm$ 0.200  |
| 1996      | 21               | 105            | 97                     | 0.474                       | 0.257, NS                         | 0.468 $\pm$ 0.179  |
| 1997      | 18               | 84             | 79                     | 0.519                       | 0.114, NS                         | 0.516 $\pm$ 0.195  |
| All years | 55               | 268            | 246                    | 0.508                       | 0.065, NS                         | 0.504 $\pm$ 0.189  |

sex allocation. According to these hypotheses, attractive males should gain more breeding success than less attractive ones; therefore, females mated to attractive males should benefit from having more sons, themselves attractive if they inherit their father's attractive traits (Burley 1981). Such results have been found in a few experimental studies (Burley 1981, Ellegren et al. 1996, Svensson and Nilsson 1996). As Bluethroat males have variable conspicuous secondary traits, they potentially provide a good model for testing this hypothesis. In the studied population, we found a mean sex ratio of unity for all broods. In birds, this is the general rule (Clutton-Brock 1986) and cases of adaptive departure from the expected balanced sex ratio are still scarce (Dijkstra et al. 1990, Lessells et al. 1996, Bradbury et al. 1997, Komdeur et al. 1997, Westerdaal et al. 1997). None of the proposed explanatory variables including those related to male attractiveness (size of the throat patch or territory size and quality) changed the deviance in the model significantly. With our sample, we could not reject the null hypothesis of the Mendelian model in which the sex (genetically determined) of each offspring is determined by the random processes of meiosis and fertilisation, giving a variance consistent with a binomial distribution (Williams 1979, Frank 1990). Our test of the influence of male attractiveness on brood sex ratio might have been skewed in this species by a high rate of extra-pair paternity (Krokene et al. 1996, Questiau et al. in press). Since a male is not necessarily the putative father of all nestlings in a given brood, the estimated sex ratio can be attributable to a mixed progeny and can give rise to misleading results. However, if the males that sired the extra-pair young are a priori expected to be preferred by females, we think that we would have found a male-biased sex ratio in all extra-pair young, as in the Blue Tit (Kempenaers et al. 1997). But this was not the case, a result similar to that of Sheldon and Ellegren (1996) for the Collared Flycatcher. Thus, there was neither evidence for selection for a biased sex ratio according to sexual selection theories, nor for selection for a sex ratio of unity (sex ratio variation less than binomial) as could be predicted under Verner's (1965) theory. This suggests that in the Bluethroat sex determination is simply a matter of random association between female and male gametes without any further adaptive significance, and that probably no mechanism controls the brood sex ratio

either before or after hatching (i.e. differential nestling mortality between the sexes).

By contrast, if an initial sex ratio skew towards females had been found at the brood level, this might have explained the bias towards wintering juvenile females reported from the Tagus estuary (Constant and Eybert 1995). As no departure from unity in hatchling sex ratio was found, this result cannot explain their finding. However, a secondary sex ratio biased towards females could have been found if sex-biased mortality had occurred in the nest between hatching and fledging. But no such bias in fledging sex ratio was detected. Therefore, the bias towards juvenile females in the wintering site may be related to ecological factors such as sex-differential mortality during migration, differential settlement in wintering territories or different patterns of dispersal between the sexes (Constant and Eybert 1995). Actually we cannot refute the possibility of a difference in the distribution of the two sexes in the wintering range, with males settling preferentially in areas other than those chosen by young females. The wintering areas of the subspecies *namnetum* range from northern Portugal to Morocco. Other wintering sites north and south of the studied site should be investigated to check for this possibility.

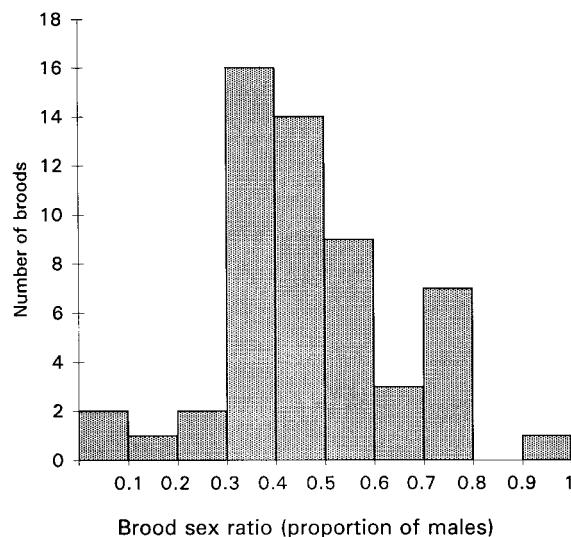


Fig. 2. Distribution of the proportion of males within Bluethroat broods for the three breeding seasons.

Table 2. Values of the change in deviance for each variable taken as single explanatory variable.

| Explanatory variable    | n  | Deviance in the null model | Change in deviance | d.f. | Associated P value |
|-------------------------|----|----------------------------|--------------------|------|--------------------|
| Year                    | 55 | 40.654                     | 0.822, NS          | 2    | 0.663              |
| Female age              | 43 | 35.434                     | 0.042, NS          | 1    | 0.837              |
| Male age                | 39 | 34.551                     | 0.380, NS          | 1    | 0.537              |
| Hatching success        | 55 | 40.654                     | 2.501, NS          | 1    | 0.114              |
| Female wing length      | 35 | 26.940                     | 0.030, NS          | 1    | 0.862              |
| Male wing length        | 28 | 21.789                     | 0.010, NS          | 1    | 0.920              |
| First egg hatching date | 55 | 40.654                     | 0.260, NS          | 1    | 0.610              |
| Female ornaments        | 39 | 27.751                     | 0.249, NS          | 3    | 0.969              |
| Male throat patch       | 27 | 14.728                     | 1.176, NS          | 1    | 0.278              |
| Diversity index         | 49 | 37.668                     | 1.677, NS          | 1    | 0.195              |
| Territory size          | 45 | 27.583                     | 0.900, NS          | 1    | 0.343              |
| Saltpan area            | 45 | 27.583                     | 0.321, NS          | 1    | 0.571              |

This study also demonstrated the use of the AFLP method for sexing nestlings of the Bluethroat. A previous study using a RAPD primer pair failed to detect a sex-linked fragment (Lessells and Mateman 1998). Few studies use AFLP markers in animals (Otsen et al. 1996, Vos and Kuiper 1996). Our study is the first one, to our knowledge, that reports a sex-identification marker using this DNA method. This technique was reliable according to its good reproducibility. Seven nestlings sexed as males returned the following year and their sex was confirmed by field observations. The 334 fragment seems to be specific to Bluethroat females. In four other investigated passerine birds (*Luscinia caliope*, *Saxicola torquata* (Muscicapidae), *Parus major* (Paridae) and *Loxia curvirostra* (Fringillidae)), this fragment, when present, was not sex-specific indicating a different location of the band in the genome (data not shown). However, a multitude of selective primer combinations and different nucleotide elongations at the 3' end provide a high number of amplified fragments and thus, a non negligible probability of revealing a W-linked fragment. With the primer combination used in this study we revealed 23 polymorphic bands. We thus consider that the AFLP technique could potentially be used for such gender identification in other species. By the use of PCR, this technique also allows the use of small DNA quantities (minimum 50 ng) for sexing non-hatched birds or embryos. However, as this method is time consuming and as the finding of a sex-specific fragment is a matter of chance, we recommend to use it for gender identification when other questions about the genetics are to be solved at the same time (population genetic structure, parentage) (Questiau et al. in press). Indeed, AFLP profiles provide a DNA fingerprint useful for a number of genetic purposes (Ajmone-Marsan et al. 1997).

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Great Tit and Siberian Rubythroat samples respectively to test whether the 334 band was specific to the Bluethroat. We are also grateful to two anonymous referees for their constructive suggestions on the first version of the manuscript. This study was supported by the Centre National de la Recherche Scientifique and the Universities Joseph Fourier, Grenoble (France), and Rennes 1 (France).

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