

# Interspecific variation in egg testosterone levels: implications for the evolution of bird song

L. Z. GARAMSZEGI,\* C. BIARD,† M. EENS,\* A. P. MØLLER† & N. SAINO‡

\*Department of Biology, University of Antwerp, Campus Drie Eiken, Wilrijk, Belgium

†Laboratoire de Parasitologie Evolutive, Université Pierre et Marie Curie, Paris Cedex 05, France

‡Dipartimento di Biologia, Università degli Studi di Milano, Milano, Italy

## Keywords:

androgens;  
birds;  
maternal hormones;  
repertoire size;  
sexual selection;  
song;  
yolk testosterone.

## Abstract

Although interspecific variation in maternal effects via testosterone levels can be mediated by natural selection, little is known about the evolutionary consequences of egg testosterone for sexual selection. However, two nonexclusive evolutionary hypotheses predict an interspecific relationship between egg testosterone levels and the elaboration of sexual traits. First, maternal investment may be particularly enhanced in sexually selected species, which should generate a positive relationship. Secondly, high prenatal testosterone levels may constrain the development of sexual characters, which should result in a negative relationship. Here we investigated these hypotheses by exploring the relationship between yolk testosterone levels and features of song in a phylogenetic study of 36 passerine species. We found that song duration and syllable repertoire size were significantly negatively related to testosterone levels in the egg, even if potentially confounding factors were held constant. These relationships imply that high testosterone levels during early development of songs may be detrimental, thus supporting the developmental constraints hypothesis. By contrast, we found significant evidence that song-post exposure relative to the height of the vegetation is positively related to egg testosterone levels. These results support the hypothesis that high levels of maternal testosterone have evolved in species with intense sexual selection acting on the location of song-posts. We found nonsignificant effects for intersong interval and song type repertoire size, which may suggest that none of the above hypothesis apply to these traits, or they act simultaneously and have opposing effects.

## Introduction

The phenomenon of phenotypic plasticity provides females with an opportunity to modify the phenotype of their offspring via maternal effects (Falconer & Mackay, 1996; Mousseau & Fox, 1998). The deposition of biologically active molecules, such as androgen hormones (most importantly testosterone) into egg yolk,

is a chief component of such maternal effects that can have short-term or long-term organizational effects on offspring (Gil, 2003; Groothuis *et al.*, 2005). Increased levels of egg testosterone can enhance begging vigour of chicks and thereby affect their competitive ability and growth rate (Schwabl, 1993, 1996; Eising *et al.*, 2001). Such maternal effects can have long-term consequences for survival (Sockman & Schwabl, 2000; Navara *et al.*, 2005), development of secondary sexual characters (Rubolini *et al.*, 2006) and potentially future reproductive success (Groothuis *et al.*, 2005). In addition, androgens can have long-term organizational effects on the development of the brain, but also other tissues (Dufty *et al.*, 2002; Groothuis *et al.*, 2005). Such effects are likely

*Correspondence:* L.Z. Garamszegi, Department of Biology, University of Antwerp, Campus Drie Eiken, Universiteitsplein 1, B-2610, Wilrijk, Belgium.

Tel.: (+32) 38 20 22 86; fax: (+32) 38 20 22 71;  
e-mail: laszlo.garamszegi@ua.ac.be

to affect the development of the phenotype often years into the future.

Although considerable intraspecific variation in yolk androgen concentration exists (Schwabl, 1999), this is exceeded by the considerable variation that exists among species (Gorman & Williams, 2005; Gil *et al.*, in press). Such variation may be expected to result from the evolutionary constraints that arise from the phylogenetic consequences of maternal effects. Supporting the hypothesis that yolk testosterone levels may evolve, recent comparative studies reported significant repeatabilities within species, and revealed biologically relevant associations at the interspecific level. For example, Gorman & Williams (2005) showed that yolk testosterone levels were negatively related to early developmental traits across 11 passerine species. In contrast, a more detailed analysis using more than 100 species of birds indicated no significant contribution of yolk hormones to either developmental period or growth rate when taking into account the similarity among species because of common ancestry (Gil *et al.*, in press). However, yolk testosterone levels were positively correlated with another yolk androgen, androstenedione, implying that the steroid content of the egg evolves along a single axis. In addition, females of colonial species were found to invest more testosterone in eggs than solitary breeder species (Gil *et al.*, in press). These results generally suggest that selection pressures because of natural selection may mediate patterns of yolk androgens. Although sexual selection may also be hypothesized to play an important role in the evolution of reproductive physiology (see Wingfield *et al.*, 1990; Hirschenhauser *et al.*, 2003; Garamszegi *et al.*, 2005b), including egg androgens, the above comparative studies were unable to explain interspecific variance in yolk hormone levels with differences in mating behaviour.

However, the evolution of sexual characters is likely to be accompanied by the evolution of maternal hormones in the egg, which should mediate an interspecific relationship between the degree of trait elaboration and yolk testosterone levels. First, females seem to carefully adjust the amount of androgen they put into eggs, and this adjustment may partially depend on the quality of their mates as signalled by secondary sexual characters. For example, the differential allocation hypothesis posits that females deposit more testosterone into their eggs when mated to attractive males because such allocation will increase the number of their grand-offspring due to the greater mating success of their sons (Gil *et al.*, 1999, 2004; Strasser & Schwabl, 2004; Uller *et al.*, 2005). However, such differential allocation may on its own have detrimental effects on fitness if daughters suffer from elevated levels of testosterone, as indicated by two experimental studies of birds (Rubolini *et al.*, 2006; Saino *et al.*, 2006). On the other hand, maternal effects may also be compensatory, whereby females allocate more to reproduction if their mate is unattractive and/or low

quality, for example, when it is young and inexperienced (Michl *et al.*, 2005).

Secondly, females may affect the development of sexual traits of their offspring by varying embryonic androgen levels. Early exposure to high levels of testosterone is likely to produce more male-like and less female-like characteristics in adulthood, whereas low levels will have the reverse effect (Mazur & Booth, 1998). Accordingly, a study of house sparrows *Passer domesticus* showed a positive relationship between testosterone levels in eggs and subsequent size of the badge in males (Strasser & Schwabl, 2004). A quantitative genetic study of male sexual behavior of captive zebra finches *Taeniopygia guttata* showed strong maternal effects on aggressiveness and song rate of sons and on daughters' mating preferences for these male traits (Forstmeier *et al.*, 2004). Therefore, sexual selection may be expected to enhance the allocation of maternal hormones, because it could allow females to distribute resources differentially among offspring according to the quality of their father, and/or produce attractive sons. If the exaggeration of sexual characters reflects the strength of sexual selection, and if females invest more in their offspring in terms of maternal hormones in sexually selected species, we predict a positive phylogenetic association between signals of male quality and yolk testosterone.

Contrary to favouring sexual selection, the evolution of maternal testosterone may have negative consequences for such traits by constraining trait development. In general, testosterone is thought to enhance male reproductive displays in adulthood such as the production of song, mainly through an increase in song rate, but also by affecting other male traits (reviews in Folstad & Karter, 1992; Owens & Short, 1995; Ball *et al.*, 2002; Roberts *et al.*, 2004). However, evidence suggests that high testosterone levels during development of song have a suppressive effect. For example, juvenile song sparrows *Melospiza melodia* that were treated with testosterone at 100 days of age produced abnormal songs in adulthood (Whaling *et al.*, 1995). The inhibitory effect of testosterone on vocal production has also been shown in the dark-eyed junco *Junco hyemalis*, in which control males sang at higher rates and had more song types than testosterone-implanted males (Titus *et al.*, 1997). Experimentally raising circulating testosterone concentrations in developing zebra finches resulted in premature song crystallization (Korsia & Bottjer, 1991), while testosterone also reduced adult song plasticity in zebra finches (Williams *et al.*, 2003). Moreover, developmental constraints because of high levels of testosterone may not only apply to vocal traits. Injection of testosterone in eggs of the ring-necked pheasant *Phasianus colchicus* resulted in a reduced length of male tarsal spurs, a trait which positively predicts male success in intra- and intersexual selection and viability (Rubolini *et al.*, 2006). In the striped plateau

lizard *Sceloporus virgatus*, juveniles receiving exogenous testosterone developed blue throat patches more slowly than control animals (Abell, 1998). Currently, the negative effect of yolk testosterone levels is far from being proved for visual signals (e.g. see Strasser & Schwabl, 2004 for positive effects), although available indirect evidence for song traits support the existence of developmental constraints. Hence, if similar detrimental effects on song development can be attributed to maternal testosterone levels, a negative relationship between yolk testosterone levels and expression of song could be predicted in a comparative study.

Here, we tested the predictions of the two evolutionary hypotheses by characterizing the extent to which egg testosterone levels are related interspecifically to an important marker of sexual selection, namely bird song. We have chosen to use bird song, because it is likely to be sensitive to early testosterone exposure (see above), and also because it reflects the intensity of sexual selection. Several aspects of this sexual behavior in terms of song output and song complexity have been quantified in different species, for which information is available from the literature. Large intra- and interspecific variation in bird song of males is generally thought to be attributed to factors associated with male–male competition and female choice (Catchpole & Slater, 1995; Searcy & Yasukawa, 1996; Searcy & Nowicki, 2000). In line with this assumption, comparative evidence shows that song complexity is associated with polygyny, in which sexual selection is presumed to be more intense than under social monogamy (Read & Weary, 1992). Importantly, this does not seem to apply to extra-pair paternity, which reflects sperm competition, suggesting that interspecific variation in song mainly reflects the intensity of sexual selection in terms of mate acquisition (Garamszegi & Møller, 2004). Due to the physiological link between testosterone and development and seasonal control of bird song, an evolutionary relationship between yolk androgens and features of song is more likely to exist than for other sexual traits.

We tested the following two specific predictions. First, given that maternal effects determine reproductive success and fitness, and it is likely that sexual selection, as reflected by song elaboration, plays an important role in the evolution of such mechanisms, we predicted a positive relationship between features of song and yolk testosterone. Secondly, if early development of song is blocked by high levels of embryonic testosterone through their organizational effects on brain tissue and other traits, song may, as a consequence, be less elaborate in males of species in which females allocate high levels of yolk hormones than in species with lower levels of yolk testosterone. Such a by-product of maternal hormones should mediate a negative interspecific relationship between features of song and yolk testosterone.

## Materials and methods

### Egg testosterone levels

We used published data for yolk testosterone levels from Gil *et al.* (in press), who present data for more than 100 species from several parts of the world. Extraction recoveries, cross-reactivity of antibodies, intra- and interassay coefficients of variation, but also within-species and between-laboratory repeatabilities are presented in that paper. Because repeatability between sources was found to be significant and high, we also used information on egg testosterone levels from Gorman & Williams (2005). Although in previous comparative analyses of maternal hormones another androgen, namely androstenedione was considered (Gil *et al.*, in press), here we focused on the effect of yolk testosterone only. We decided to exclude androstenedione from the current analyses, because the hormonal regulation of song, such as androgen-suppressed song development, is generally thought to be achieved via the effect of testosterone (see Introduction), while the effect of androstenedione is unknown. Hence, we primarily expected any interspecific pattern to occur for testosterone. Here we used information on 36 species only, because we were unable to locate information on song variables for the remaining 25 passerine species with data on yolk testosterone.

### Song data

We used a number of variables to represent song output and song complexity following the definitions of Read & Weary (1992). First, song output was measured as song duration(s) and intersong interval(s). We did not estimate song continuity and song rate as defined by Read & Weary (1992), because these variables are directly calculated from song duration and intersong interval. Hence, biological interpretations for song continuity and song rate can be made from statistical and phylogenetic models that simultaneously involve song duration and intersong interval. Secondly, song complexity was measured as song repertoire size (the mean number of different song types possessed by an individual which represents a measure of between-song complexity), and syllable repertoire size (the mean number of different syllable types within a song which represents a measure of within-song complexity). For species having tremendous song complexity, summaries of repertoire size provide near-infinite estimates, because it is difficult to estimate their potential repertoires (Read & Weary, 1992; MacDougall-Shackleton, 1997). In such cases, song elements, such as syllables are combined randomly and thus a particular combination occurs rarely, which leads to the detection of numerous song types. Following a common practice, in species with infinitely large song type repertoire size we assigned an arbitrary value of

1000 (see Read & Weary, 1992; Garamszegi & Møller, 2004). However, we also present comparative results when an infinite song repertoire size instead was set to 300.

We estimated the degree of exposure of song-post locations, because our previous analyses showed that this trait may be an overlooked song feature relevant in the context of sexual selection, as it can signal the ability of a male to bear the cost of being exposed to predators (Møller *et al.*, 2006). We determined the height of the song-post of common passerine birds during 1986–1989 relative to the surrounding vegetation or structures such as buildings. One of us (A.P.M.) systematically noted the height of all song-posts recorded during March–August whenever in the field. See Møller *et al.* (2006) for further information. Although mate choice may mainly occur during spring, choice of extra-pair copulation partners may continue throughout the summer when second and third clutches are fertilized. We only considered the vegetation within a distance of 2 m from the singing bird. In brief, song-post exposure is estimated as the percentage of all song-posts of a given species that are at a height above the surrounding vegetation, ranging from 0 when all song-posts are lower than the surrounding vegetation to 100 when all song-posts are above the vegetation. The number of observations on which each of these estimates for a different species was based ranged from 28 to 500 song-posts. This method for describing song-posts was first developed by Scherrer (1972), who reported quantitative information for 34 common species with little overlap with the species analysed here.

Use of song parameters in comparative analyses raises issues about comparability (Krebs & Kroodsma, 1980). We have previously investigated the extent to which different measures of song complexity and temporal organization provide reliable and comparable information (Garamszegi *et al.*, 2003; Garamszegi & Møller, 2004). Using a number of different tests we found a high degree of consistency among comparable measures of complexity, justifying our approach (see Garamszegi *et al.*, 2003; Garamszegi & Møller, 2004 for details). Finally, the fact that previous comparative studies of song features in birds have shown biologically meaningful results (e.g. Read & Weary, 1992; Székely *et al.*, 1996; Badyaev *et al.*, 2002) suggests that song complexity and temporal organization indeed provide reliable information. There was a high degree of consistency between our estimate of song-post exposure and that of Scherrer (1972) (relying on a larger dataset:  $F = 479.69$ , d.f. = 21, 22,  $P < 0.001$ ; see Møller *et al.*, 2006). This analysis indicates that there is little geographical variation in song-post exposure, and that song-post exposure was estimated in a repeatable and hence reliable way. The significant repeatability of the trait also shows that seasonal variation in song-post exposure seems to be negligible when compared with the variation among species.

We primarily used song data from Read & Weary (1992), supplemented by information from other, more recent sources (see details in Appendix). Although in our literature search, we attempted to find song data for each species with known egg testosterone levels, this effort resulted in 36 species with quantitative information on both song and egg testosterone. Song variables were  $\log_{10}$ -transformed before analyses, and song-post exposure was arcsine-square-root transformed.

### Confounding variables

We investigated several variables that may potentially confound the relationship between features of song and yolk testosterone levels. Data for these variables were derived from handbooks, which provide detailed information on ecological traits by following a standard format and relying on the primary literature, which facilitates cross-species comparisons (Cramp & Perrins, 1985–1994; Glutz von Blotzheim & Bauer, 1985–1997; Marchant & Higgins, 1991–2001; Dunning, 1993; Poole *et al.*, 1993–2002).

Estimates on developmental periods, such as the duration of incubation and nestling period may not only be related to egg testosterone levels (Gorman & Williams, 2005), but also to the duration of exposure of embryos to maternal egg androgens. Hence, we obtained information on duration of developmental periods in days. We used median values when a range of values was reported.

Testosterone levels in adult males have been reported to depend on latitudinal distribution (Garamszegi *et al.*, 2005b). In order to avoid similar effects potentially acting on yolk testosterone levels, we included data on the mean breeding distribution latitudes of species. Distribution maps were derived from our sources, and coordinates for the northernmost and southernmost breeding distribution borders were estimated with a map, and these two estimates were subsequently averaged.

Similarly, a number of confounding factors may influence the evolution of bird song (Read & Weary, 1992). Males of polygynous species have been reported to have low song rates and large syllable repertoire sizes (Read & Weary, 1992). Species were classified as having either (i) no polygyny (a score of 0), (ii) irregular polygyny (species with < 5% of males attracting more than one female were given a score of 1), or (iii) regular polygyny (species with > 5% of males attracting more than one female were given a score of 2).

Species inhabiting open grassland habitats have been reported to have lower song rates and song versatility than species of forested habitats (Read & Weary, 1992), because of different sound transmission properties in these two kinds of habitats (Slabbekoorn & Smith, 2002). Species were classified as inhabiting open (score of 0) or forested habitats (score of 2). Forested habitats were those with forest and woodland, or scrub, if habitat descriptions suggested that the preferred scrub was in

forests or woodlands, and the remainder was classified as open habitats. Species inhabiting both kinds of habitats were categorized as living in mixed habitats (score of 1).

Migratory species have been reported to have large song repertoires and large syllable repertoires (Read & Weary, 1992). Migratory behaviour was scored on a three-point scale as (i) resident (a score of 0), (ii) partial migrant (species having resident and migratory populations; a score of 1), or (iii) migrant (a score of 2).

Gil *et al.* (in press), reported a significant positive relationship between degree of breeding coloniality and concentration of androstenedione, but not of testosterone. As testosterone correlates with androstenedione (Gil *et al.*, in press), and there is no information about the interspecific relationship between song and breeding sociality, we also considered coloniality as a potentially confounding factor. We scored species as solitary species (score of 0) if they breed in large, all-purpose territories, or colonial species (score of 1) that have aggregated territories only containing nest sites. To remove potential allometric effects we used data on body mass as a covariate in the comparative analyses (see Gil *et al.*, in press).

Variables reflecting polygyny, migration, habitat and coloniality were treated as continuous variables in the comparative analyses. The statistical reason behind this choice is that although these variables were scored as discrete, intermediate states are biologically meaningful, and different states are thus arbitrary points along a continuum (Sokal & Rohlf, 1995). In addition, in an evolutionary context, a transition between two states of these variables follows nondiscrete evolutionary changes. Therefore, the continuous treatment is generally applied in comparative studies that are constrained to use qualitative data for a larger set of species (Harvey & Pagel, 1991; Bennett & Owens, 2002). Thus as a result, one can show qualitatively that a given trait plays a role for the evolution of another, but cannot assess its quantitative importance. We adopted the continuous treatment of these variables in order to be able to include them in our robust comparative framework that uses continuous variables (see below). However, we also tested for their confounding effect by using an alternative phylogenetic approach (Grafen, 1989), in which we treat them as categorical variables (see Appendix). The entire data set including references is reported in Appendices 1 and 2.

### Comparative analyses

We applied the general method of comparative analysis for continuous variables based on generalized least squares (GLS) models using the statistical software CONTINUOUS (Pagel, 1997, 1999a). The GLS model characterizes evolutionary changes along each branch of a phylogenetic tree through the variance components of traits (Pagel, 1997). Hypotheses are tested with likelihood

ratio statistics. This compares the log-likelihood of the model corresponding to a null hypothesis ( $H_0$ ) over the model for an alternative hypothesis ( $H_1$ ), where the likelihood ratio =  $-2 \log_e(H_0/H_1)$ . The likelihood ratio statistic is asymptotically distributed as a chi-squared variate with degrees of freedom equal to the difference in the number of parameters between the two models.

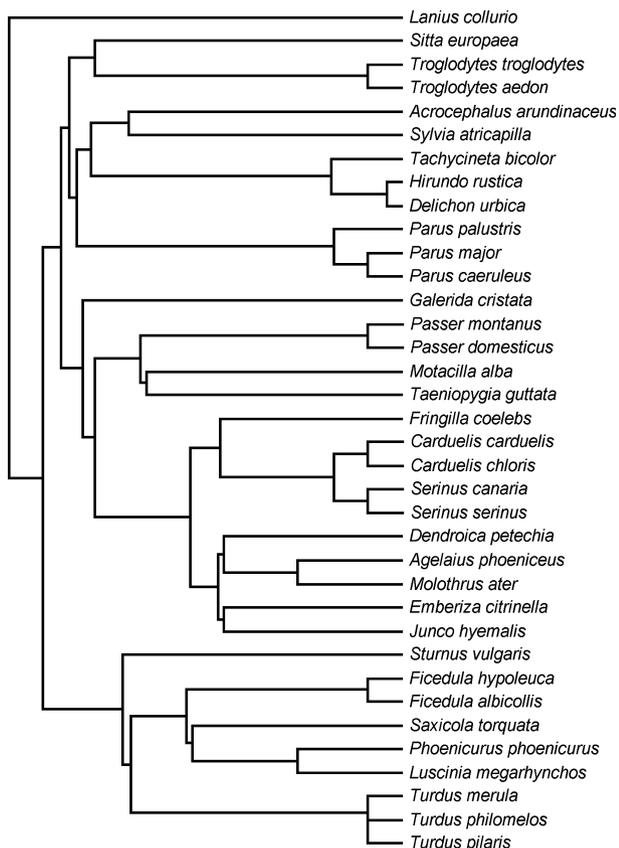
First, we assessed the contribution of scaling parameters sequentially by estimating the maximum likelihood values of the branch length scaling parameter  $\kappa$ , and the phylogeny scaling factor  $\lambda$  [recent simulations showed that the estimation of  $\delta$ , the overall path length scaling factor is biased (Freckleton *et al.*, 2002), and thus we avoided estimating this parameter]. The  $\kappa$  parameter by differentially stretching long and short branches would yield a punctuational mode of trait evolution at  $\kappa = 0$ , whereas  $\kappa \geq 1$  indicates the importance of long branches in trait evolution (gradualism). Values of  $\lambda < 1$  would correspond to traits being less similar amongst species than expected from their phylogenetic relationship, whereas  $\lambda = 1$  suggests the reverse. Any of these potential effects present in the data can be detected by comparing the log-likelihood of a  $H_0$  model containing default (= 1) values for the scaling parameters with the log-likelihood of an alternative  $H_1$  model in which one parameter is permitted to take its maximum likelihood value. If a significant effect was found ( $P < 0.05$ ), the estimated values were used in the final model, otherwise default settings were used.

Secondly, using the appropriate scaling parameters the correlation between pairs of traits was tested by log-likelihood ratio statistics comparing model  $H_0$  that fits the data forcing the correlation to be zero with the alternative  $H_1$  model, permitting correlated evolution of the two characters. Third, using the best model fitting the data we estimated the phylogenetic correlation between traits. We assumed that the evolution of traits followed a standard constant-variance random walk evolutionary model, and thus we used the corresponding settings in CONTINUOUS (Model A). The appropriate scaling parameters and the log-likelihood ratio statistics testing for correlated trait evolution are presented. When we controlled for potentially confounding factors we entered these variables together with the variables of interest in the same model, and assessed the significance of the correlated evolution of the traits considered. If we found significant evidence for correlated evolution, we calculated the partial phylogenetic correlation for the relationship in question. The phylogenetic method implemented in the program CONTINUOUS does not allow insight on the phylogenetically transformed data (Pagel, 1999b). For illustrative purposes we present figures based on the raw species data on which we superimpose the phylogenetically corrected regression lines.

We tested our predictions on five song traits, and thus we applied Bonferroni adjustment on the level of significance for multiple tests by using the Sidak's

method to assess the experiment-wise type I error rate (Ury, 1976). Strict application of this method severely reduces the power of tests (Wright, 1992), but such sacrificial loss of power can be avoided by choosing an experiment-wise error rate higher than the usually accepted 5%. We used 10% as suggested by Wright (1992) and Chandler (1995).

Phylogenetic information for our comparative analyses originated mainly from Sibley & Ahlquist (1990) that relied on extensive studies of DNA–DNA hybridization. This phylogeny was supplemented with information from Suhonen *et al.* (1994) for Paridae, Møller *et al.* (2001) for Hirundinidae and Badyaev (1997) for cardueline finches (Fig. 1). We applied branch lengths from the tapestry tree of Sibley & Ahlquist (1990). Information on branch length for very few species was unavailable from Sibley & Ahlquist (1990). To calculate branch length for these species, we used the convention that within families the distance between different genera is 3.4  $\Delta T_{50H}$  units, and between species within genera is



1  
**Fig. 1** Phylogenetic hypothesis of passerine birds used for comparative analyses of yolk testosterone levels and song variables. The scale is given in the bottom left corner. For sources, see Materials and methods.

1.1  $\Delta T_{50H}$  units (Sibley & Ahlquist, 1990; Bennett & Owens, 2002). The phylogeny is shown in Fig. 1. Recent studies indicate that the phylogeny of Sibley & Ahlquist (1990) may be incorrect for some taxa (Sheldon & Gill, 1996; Barker *et al.*, 2002). Therefore, we also constructed our composite phylogeny based on Barker *et al.* (2002) using equal branch length. The corresponding results, which do not affect the conclusions of this paper, are reported in Appendix 3.

## Results

We found a significant negative phylogenetic correlation between song duration and yolk testosterone levels (Table 1; Fig. 2a). The starling *Sturnus vulgaris* had an extremely long duration of songs for its level of yolk testosterone (Fig. 2a; topmost data point), but exclusion of that species did not affect the conclusion (Fig. 2a). Likewise, there was a significant negative phylogenetic correlation between syllable repertoire size and yolk testosterone levels (Table 1; Fig. 2b). The canary *Serinus canaria* had an extremely large syllable repertoire size for its yolk testosterone level (Fig. 2b), and it is worth noting that this data point was based on domesticated canaries that intentionally or unintentionally have been selected to either increase their syllable repertoire size or their level of yolk testosterone (here we used song data for wild canaries). However, the conclusions did not change after exclusion of that data point (Fig. 2b). Analyses of song type repertoire size and intersong interval revealed no significant associations (Table 1).

Song-post exposure was significantly positively related to testosterone levels in eggs (Table 1; Fig. 2c). One species (great reed warbler *Acrocephalus arundinaceus*) had a very low song-post exposure for its level of yolk testosterone (Fig. 2c). However, exclusion of that species did not change the conclusions (Fig. 2c).

To uncover the role of song rate and song continuity, we included intersong interval and song duration in the same phylogenetic model. However, the only significant predictor of yolk testosterone was song duration, whereas the effect for intersong interval was not significant (partial phylogenetic correlations at  $\kappa = 0$ ,  $\lambda = 0.434$ , when LR = 6.13,  $P = 0.006$  for the correlated evolution: song duration,  $r = -0.691$ ,  $P = 0.006$ ; intersong interval,  $r = 0.308$ ,  $P = 0.283$ ;  $N = 15$ ). Hence, yolk testosterone levels varied independently of song continuity and song rate among species.

Previous comparative analyses demonstrated several significant relationships among song traits (Garamszegi & Møller, 2004; Møller *et al.*, 2006). To reveal the independent effect of song variables on yolk testosterone levels, we introduced song duration, syllable repertoire size and song-post exposure in the same phylogenetic model, and assessed their partial correlation with yolk testosterone levels. Although there are only 14 species for which information is available for all variables, the

**Table 1** Results of generalized least squares (GLS) analyses of the relationship between song variables and yolk testosterone levels in birds.

| Testosterone level       | $\kappa$     | $\lambda$    | Correlation   | <i>P</i>     | <i>N</i> |
|--------------------------|--------------|--------------|---------------|--------------|----------|
| Song duration            | 0.491        | <u>0.582</u> | <b>-0.482</b> | <b>0.018</b> | 21       |
| Intersong interval       | 0.736        | <u>0.963</u> | -0.078        | 0.761        | 15       |
| Song repertoire size     | 0.504        | 0.918        | 0.373         | 0.057        | 24       |
| Syllable repertoire size | <u>0.008</u> | <u>0.465</u> | <b>-0.425</b> | <b>0.020</b> | 27       |
| Song exposure            | 1.158        | 0.981        | <b>0.469</b>  | <b>0.013</b> | 25       |

Scaling parameters ( $\kappa$  and  $\lambda$ ) and phylogenetic correlations were derived as defined in Materials and methods. Significant scaling parameters that were used in the phylogenetic models are underlined. When the maximum likelihood value of these parameters was not significantly different from 1, they were set to one. Significant relationships after Bonferroni adjustment are highlighted (adjusted *P*-level = 0.0208). When infinite song repertoire sizes were set to 300 (instead of 1000), the observed phylogenetic relationship for this trait was, at  $\kappa = 1$  and  $\lambda = 1$ ,  $r = 0.400$ ,  $P = 0.041$  (nonsignificant after Bonferroni adjustment).

phylogenetic model tends to show that the above-detected relationships for different song traits are independent of each other (partial phylogenetic correlations at  $\kappa = 1$ ,  $\lambda = 0$ , when LR = 11.50,  $P < 0.001$  for the correlated evolution: song duration,  $r = -0.462$ ,  $P = 0.130$ ; syllable repertoire size,  $r = -0.678$ ,  $P = 0.015$ ; song-post exposure,  $r = 0.617$ ,  $P = 0.033$ ;  $N = 14$ ).

We investigated the effects of several potentially confounding variables in different evolutionary models, in which we tested for the correlated evolution of yolk testosterone, a song trait and a confounding variable. If we found significant evidence for these three traits to evolve in a correlated fashion, we estimated the partial phylogenetic correlation between the song trait and egg testosterone while holding the effect of the confounding variable constant (Table 2). As we only considered song traits for which we had already found significant evidence for correlated evolution with egg testosterone in a pairwise approach (Table 1), each model adjusted for three traits showed a tendency for correlated evolution, as it inherently involved two correlated traits (Table 2). If the models with three variables were significant, this indicated that the additional confounding variable may also correlate with any of the traits in question. In such cases, the confounding variable may potentially raise the observed correlation between egg testosterone and the song trait. However, the calculated partial correlations revealed little evidence of confounding effects, suggesting that our pairwise findings are not due to the indirect effects of third variables. Only the control for the effect of nestling period on song duration affected the significance level of the relationship in focus. We infer that this apparent sensitivity was caused by the low sample size, as the unsigned effect size was still  $r > 0.35$ . The results were similar when we used migration, polygyny, habitat type and coloniality as categorical variables in an alternative phylogenetic approach (Appendix 4).

## Discussion

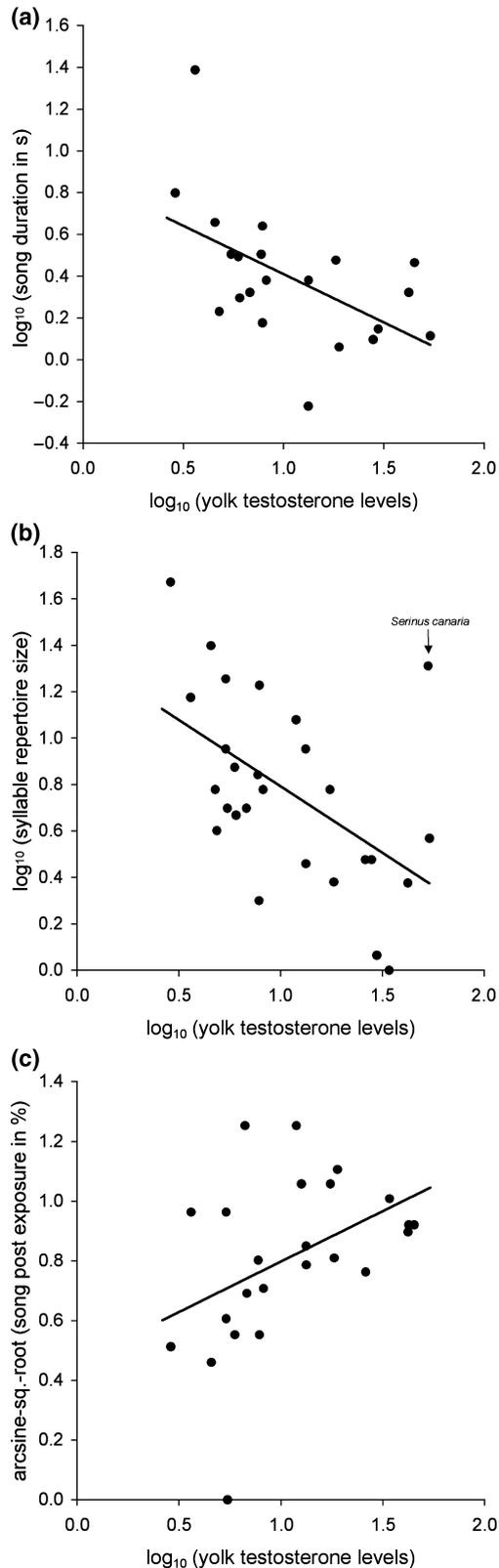
The main findings of this comparative analysis of egg yolk testosterone and song features of passerine birds

were that (i) song duration and syllable repertoire size were negatively related to testosterone; and (ii) song-post exposure increased with yolk testosterone. We will briefly discuss these findings.

We presented two alternative hypotheses for the expected relationships between maternal testosterone and subsequent song quality of their sons. First, females may strategically increase investment of maternal testosterone into their eggs when mated to the most attractive males in order to produce the most attractive or viable sons (Gil *et al.*, 1999, 2004; Strasser & Schwabl, 2004; Uller *et al.*, 2005) or to enhance male-like characteristics in both sexes (Mazur & Booth, 1998). Such a female allocation strategy is likely to be enhanced in sexually selected species, and thus we predicted a positive relationship between features of song and yolk testosterone levels across species. Our findings in association with song-post use tended to support this prediction.

Secondly, the physiological effect of testosterone on song development generally appears to be suppressive (Korsia & Bottjer, 1991; Whaling *et al.*, 1995; Titus *et al.*, 1997; Williams *et al.*, 2003). These developmental constraints should produce a negative interspecific association between song expression and yolk testosterone, as shown for song duration and syllable repertoire size. This suggests that there is an optimal level of testosterone in egg yolk in order to produce normal song development.

Previous comparative analyses of bird song have revealed several significant relationships between different song traits (Garamszegi & Møller, 2004; Møller *et al.*, 2006). Therefore, there may be a high degree of concordance between song features associated with yolk testosterone and phenotypic correlations across species. Apparent covariation between a song trait and yolk testosterone may be due to consistent covariation with another song trait, while an evolutionary constraint may act along a single evolutionary axis. For example, high yolk testosterone levels may select for a singing style, which can be characterized by long and simple songs and high song-post exposure. However, when controlling for associations between song traits, partial correlation



**Fig. 2** The interspecific relationship between yolk testosterone levels (pg/mg, log-transformed) and (a) song duration (sec, log-transformed); (b) syllable repertoire size (log-transformed); and (c) song-post exposure (%), when using species-specific data (for phylogenetically corrected relationships, see Table 1). Lines are phylogenetic regression lines with the following equations: (a)  $y = 0.94 - 51x$ ; (b)  $y = 1.35 - 55x$ ; and (c)  $y = 0.44 + 0.37x$ . Arrow indicates a species for which yolk testosterone data only were available for captive animals [exclusion of this data point from (b) results in a phylogenetic relationship at  $\kappa = 0$  and  $\lambda = 0.382$ :  $r = -0.579$ ,  $P = 0.001$ ,  $N = 26$ ]. Exclusion of the extreme data points from (a) (*Sturnus vulgaris*) and (c) (*Acrocephalus arundinaceus*) did not affect the phylogenetic results (phylogenetic correlations without outliers: song duration:  $r = -0.453$ ,  $P = 0.032$ ,  $N = 20$ , when  $\kappa = 0$  and  $\lambda = 0.352$ ; song-post exposure:  $r = 0.507$ ,  $P = 0.008$ ,  $N = 24$ , when  $\kappa = 1$  and  $\lambda = 1$ ).

coefficients implied that song features were independently related to yolk testosterone levels.

Choice of song-posts may be important for males to increase their mating success, because the perception of song by receivers may be improved by choice of particular locations for the production of song (Dabelsteen *et al.*, 1993; Holland *et al.*, 1998), or exposed song-posts may be attractive for females because they can reflect male risk taking due to elevated susceptibility to predation when using exposed song-posts (Møller *et al.*, 2006). Here we found that species in which males sang from the most exposed sites had mothers that produced eggs with high levels of yolk testosterone. This finding can be interpreted in several different ways depending on the causal relationship between variables.

First, high levels of yolk testosterone may be prevalent in species with intense sexual selection, causing males to take higher risks in sexual signalling than in species with less intense sexual selection. In fact, in species with high egg testosterone levels, sexual signalling via song-post exposure should be enhanced, because such high levels suppress the expression and function of other song traits, such as song duration and syllable repertoire size. Secondly, high levels of maternal testosterone may not only increase the activity level of offspring (e.g. Eising & Groothuis, 2003), but also the activity level of adults, because testosterone implants in adult males cause an increase in locomotory activity (Wada, 1986; Ketterson & Nolan, 1992). Thus, males that hatched from eggs with high levels of testosterone may already be running high risks of predation because of their elevated level of testosterone, thereby reducing the relative importance of song-post exposure for overall predation risk. It would be interesting to investigate to which extent testosterone implants directly affect song-post choice.

Although we did not explicitly study the mechanisms accounting for the findings reported here, we believe that it is worthwhile considering the possibilities. The results indicated that high levels of testosterone had a suppressive effect on song features. This effect could be mediated

| Confounding variables | GLS model for the correlated evolution of three traits                                | Partial correlation between the song trait and yolk testosterone |
|-----------------------|---|--|
|                       | Models for song duration  |  |
| Incubation period     | $\kappa = 0.519$ , $\lambda = \underline{0.350}$ , LR = 3.93, $P = 0.048$             | $r = -0.543$ , $P = 0.014$                                       |
| Nestling period       | $\kappa = 0.646$ , $\lambda = \underline{0.778}$ , LR = 4.93, $P = 0.020$             | $r = -0.371$ , $P = 0.107$                                       |
| Body mass             | $\kappa = 0.684$ , $\lambda = \underline{0.795}$ , LR = 2.84, $P = 0.128$             | Not estimated  |
| Latitude              | $\kappa = 0.761$ , $\lambda = \underline{0.717}$ , LR = 3.52, $P = 0.071$             | Not estimated  |
| Migration             | $\kappa = 0.730$ , $\lambda = \underline{0.753}$ , LR = 3.43, $P = 0.077$             | Not estimated  |
| Polygyny              | $\kappa = 0.737$ , $\lambda = \underline{0.764}$ , LR = 2.61, $P = 0.160$             | Not estimated  |
| Habitat               | $\kappa = 0.728$ , $\lambda = \underline{0.809}$ , LR = 3.53, $P = 0.069$             | Not estimated  |
| Coloniality           | $\kappa = 0.469$ , $\lambda = \underline{0.346}$ , LR = 8.61, $P < 0.001$             | $r = -0.608$ , $P = 0.004$                                       |
|                       | Models for syllable repertoire size   |  |
| Incubation period     | $\kappa = 0.278$ , $\lambda = \underline{0.459}$ , LR = 3.00, $P = 0.111$             | Not estimated  |
| Nestling period       | $\kappa = 0.159$ , $\lambda = \underline{0.656}$ , LR = 3.31, $P = 0.085$             | Not estimated  |
| Body mass             | $\kappa = \underline{0.295}$ , $\lambda = \underline{0.700}$ , LR = 2.16, $P = 0.229$ | Not estimated  |
| Latitude              | $\kappa = \underline{0.000}$ , $\lambda = \underline{0.201}$ , LR = 4.44, $P = 0.031$ | $r = -0.502$ , $P = 0.009$                                       |
| Migration             | $\kappa = \underline{0.068}$ , $\lambda = \underline{0.508}$ , LR = 3.56, $P = 0.068$ | Not estimated  |
| Polygyny              | $\kappa = 0.117$ , $\lambda = \underline{0.485}$ , LR = 3.43, $P = 0.077$             | Not estimated  |
| Habitat               | $\kappa = \underline{0.253}$ , $\lambda = \underline{0.715}$ , LR = 2.88, $P = 0.124$ | Not estimated  |
| Coloniality           | $\kappa = \underline{0.392}$ , $\lambda = \underline{0.770}$ , LR = 2.00, $P = 0.261$ | Not estimated  |
|                       | Models for song-post exposure   |  |
| Incubation period     | $\kappa = 1.366$ , $\lambda = 1.000$ , LR = 3.62, $P = 0.065$                         | Not estimated  |
| Nestling period       | $\kappa = 1.273$ , $\lambda = 0.991$ , LR = 7.07, $P = 0.003$                         | $r = 0.574$ , $P = 0.003$  |
| Body mass             | $\kappa = 0.963$ , $\lambda = 0.936$ , LR = 3.15, $P = 0.097$                         | Not estimated  |
| Latitude              | $\kappa = 1.240$ , $\lambda = 0.987$ , LR = 4.49, $P = 0.029$                         | $r = 0.424$ , $P = 0.039$  |
| Migration             | $\kappa = 0.908$ , $\lambda = 0.955$ , LR = 3.23, $P = 0.091$                         | Not estimated  |
| Polygyny              | $\kappa = 0.868$ , $\lambda = 0.845$ , LR = 5.66, $P = 0.010$                         | $r = 0.542$ , $P = 0.006$  |
| Habitat               | $\kappa = 1.201$ , $\lambda = 0.985$ , LR = 4.12, $P = 0.041$                         | $r = 0.448$ , $P = 0.028$  |
| Coloniality           | $\kappa = 1.391$ , $\lambda = 1.000$ , LR = 4.22, $P = 0.038$                         | $r = 0.434$ , $P = 0.034$  |

Significant scaling parameters that were used in the models are underlined. When the maximum likelihood value of the parameters was not significantly different from 1, they were set to one. Only song traits that were significantly related to yolk testosterone levels were considered (see Table 1). Sample sizes are equal to those in Table 1, d.f. = 3 for each GLS model.

through learning, morphology (neural substrate, song-producing tissue) or physiology (priming effects on testosterone-producing tissue and immunity). First, the results may arise from effects of testosterone on learning ability. Song learning accounts for acquisition of song in passerine birds, and learning ability may be influenced by sex hormones via their negative effects on the bursting of specific neurones in the brain, which are critical for vocal plasticity (e.g. Livingston & Mooney, 2001). Secondly, maternal testosterone may affect the development of the neural substrate involved in processing or producing song. Several studies indicated that androgens could produce early organizational effects on different brain regions that subsequently influenced adult song production (Gurney & Konishi, 1980). For example, the song nucleus (nucleus HVC of the nidopallium) may play an important role in this process, because its size is independently related to syllable repertoire size and song duration (Garamszegi & Eens, 2004), which could explain the patterns we found here. In a similar vein, we cannot exclude the possibility that maternal testosterone has a direct effect on song-

producing tissue in the syrinx (Gahr, 2004). Finally, maternal testosterone may, through its effects on the early endocrine environment, subsequently affect the production of testosterone or the sensitivity of neural tissue to testosterone. Alternatively, maternal testosterone may, through its priming effects, affect development of the immune system (Martin, 2000). Only individuals that are resistant to parasitism would in this case be able to produce testosterone-dependent songs, while simultaneously coping with the immuno-suppressive effects of testosterone (Folstad & Karter, 1992).

Despite the obvious role of song in sexual selection and its potential sensitivity to the suppressive effect of testosterone during development, we found nonsignificant relationships for song type repertoire size and intersong interval. In addition, Gil *et al.* (in press) failed to find an interspecific relationship between yolk testosterone levels and sexual dichromatism. There may be four different explanations as to why the relationship between maternal androgens and sexual signals is not always robust. First, the investigated sexual traits may be

**Table 2** Phylogenetic models testing for the correlated evolution of three traits, in which we assessed the effect of a potentially confounding variable. If the most suitable model assumed the correlated evolution of the three traits, we calculated the partial correlation between the song trait of interest and yolk testosterone levels while holding the potentially confounding effect constant. In each phylogenetic model, the scaling parameter settings were fitted to the data at hand.

poor indicators of male quality, and are thus unable to reflect the intensity of sexual selection. We find this possibility unlikely, because comparative studies have shown that song repertoire size and song intersong interval can evolve under the pressure of sexual selection as they reflect male provisioning rate and annual fecundity, respectively (Read & Weary, 1992). Similarly, sexual dichromatism is a good predictor of extra-pair paternity (Møller & Birkhead, 1994; Bennett & Owens, 2002).

Secondly, these particular traits may develop independently of maternal testosterone. Unfortunately, experimental data for this explanation are unavailable for vocal traits. However, evidence for visual traits exists that support or contradict this explanation (Strasser & Schwabl, 2004; Rubolini *et al.*, 2006). Thirdly, different mechanisms shaping the relationship between egg testosterone and sexual traits may act in concert, as the enhanced effect of sexual selection and developmental constraints caused by testosterone may simultaneously favour an intermediate optimum. Hence, if both processes act, this may mask any simple direct relationship in a correlative interspecific study. Although we cannot address this possibility, relying on the available data, theoretically this seems a likely explanation. For example, females may be able to enhance the development of sexual traits of their sons via increased yolk testosterone levels, but only until a certain threshold, beyond which high egg testosterone levels are suppressive. Finally, the relatively small sample size may have contributed to the lack of significant relationships. Although we could only find information on song and yolk testosterone for 36 species, these species were well distributed on the phylogenetic tree (Fig. 1). In fact, our sample size was still larger than that for the study of Gorman & Williams (2005) that was able to identify evolutionarily meaningful patterns. However, the limited sample size necessitates careful interpretation.

Our comparative study raises a number of different prospects for future studies. First, our findings suggest that it is possible to manipulate male song by inoculation of eggs with testosterone or testosterone-suppressors. Secondly, these effects of testosterone should be specific for song duration and syllable repertoire size. Thirdly, song-post use should be under the influence of circulating testosterone, as mediated by maternal yolk testosterone during early ontogeny. Finally, these effects should be produced by effects of maternal testosterone on learning, morphology and/or physiology of offspring. All these predictions are open to experimental tests.

In conclusion, we have shown clear relationships between maternal testosterone deposited in egg yolk and subsequent features of song and song-post exposure in their sons. These findings suggest that mothers through their maternal effects are able to enhance or suppress not only vocalizations produced by their

offspring, but also the actual singing behaviour once these offspring reach adulthood.

## Acknowledgments

We are highly indebted to D. Gil for kindly providing data on yolk testosterone levels. L.Z.G. received a post-doc grant from the FWO Flanders (Belgium). M.E. was founded by an FWO project (G.0130.07).

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Received 10 November 2006; accepted 18 December 2006

## Appendix

**Appendix 1** Yolk testosterone levels (T) and features of songs in birds. Data used in the phylogenetic analyses, sorted alphabetically by species.

| Species                          | T (pg/mg)             | Song duration (s)     | Intersong interval (s) | Syllable repertoire size | Song type repertoire size | Song-post exposure (%) |
|----------------------------------|-----------------------|-----------------------|------------------------|--------------------------|---------------------------|------------------------|
| <i>Acrocephalus arundinaceus</i> | 5.478 <sup>1</sup>    | 3.2 <sup>2</sup>      | 2 <sup>2</sup>         | 5 <sup>2</sup>           | 1000 <sup>2</sup>         | 0 <sup>3</sup>         |
| <i>Agelaius phoeniceus</i>       | 27.993 <sup>1,4</sup> | 1.25 <sup>2</sup>     | 14 <sup>2</sup>        | 3 <sup>2</sup>           | 5 <sup>2,5</sup>          |                        |
| <i>Carduelis carduelis</i>       | 42.534 <sup>1</sup>   |                       |                        |                          |                           | 75 <sup>3</sup>        |
| <i>Carduelis chloris</i>         | 26.076 <sup>1</sup>   |                       |                        | 3 <sup>2</sup>           | 22 <sup>5</sup>           | 55 <sup>3</sup>        |
| <i>Delichon urbica</i>           | 4.86 <sup>1</sup>     |                       |                        | 4 <sup>5</sup>           |                           |                        |
| <i>Dendroica petechia</i>        | 54 <sup>4</sup>       | 1.3 <sup>2</sup>      | 7.1 <sup>2</sup>       | 3.7 <sup>2</sup>         | 8 <sup>2,5</sup>          |                        |
| <i>Emberiza citrinella</i>       | 42.188 <sup>1</sup>   | 2.1 <sup>2</sup>      |                        | 2.38 <sup>6,7</sup>      | 2.025 <sup>2,5</sup>      | 72 <sup>3</sup>        |
| <i>Ficedula albicollis</i>       | 7.732 <sup>1</sup>    | 3.2 <sup>8,9</sup>    | 10.2 <sup>8,9</sup>    | 6.95 <sup>8,9</sup>      | 1000 <sup>8,9</sup>       | 60 <sup>3</sup>        |
| <i>Ficedula hypoleuca</i>        | 6.048 <sup>1</sup>    | 1.98 <sup>10</sup>    | 8.68 <sup>10</sup>     | 4.66 <sup>10,11</sup>    | 1000 <sup>10,11</sup>     |                        |
| <i>Fringilla coelebs</i>         | 6.805 <sup>1</sup>    | 2.1 <sup>2</sup>      | 8.1 <sup>2</sup>       | 5 <sup>2</sup>           | 2.75 <sup>2,5</sup>       | 46 <sup>3</sup>        |
| <i>Galerida cristata</i>         | 6.67 <sup>1</sup>     |                       |                        |                          |                           | 100 <sup>3</sup>       |
| <i>Hirundo rustica</i>           | 7.869 <sup>1</sup>    | 4.37 <sup>12–14</sup> | 7.95 <sup>12–14</sup>  | 16.9 <sup>12–14</sup>    |                           | 95 <sup>15</sup>       |
| <i>Junco hyemalis</i>            | 29.67 <sup>4</sup>    | 1.4 <sup>2</sup>      | 4.5 <sup>2</sup>       | 1.16 <sup>16</sup>       | 5 <sup>2</sup>            |                        |
| <i>Lanius collurio</i>           | 11.93 <sup>1</sup>    |                       |                        | 12 <sup>2</sup>          |                           | 100 <sup>3</sup>       |
| <i>Luscinia megarhynchos</i>     | 5.95 <sup>1</sup>     | 3.12 <sup>17</sup>    | 3.78 <sup>17</sup>     | 7.5 <sup>17</sup>        | 220 <sup>5</sup>          | 30 <sup>3</sup>        |
| <i>Molothrus ater</i>            | 14.81 <sup>4</sup>    |                       |                        |                          | 4 <sup>5</sup>            |                        |
| <i>Motacilla alba</i>            | 5.38 <sup>1</sup>     |                       |                        | 9 <sup>2</sup>           |                           | 80 <sup>3</sup>        |
| <i>Parus caeruleus</i>           | 13.321 <sup>1</sup>   | 2.4 <sup>2</sup>      | 4.6 <sup>2</sup>       | 2.88 <sup>6,18</sup>     | 4 <sup>2,5</sup>          | 58 <sup>3</sup>        |
| <i>Parus major</i>               | 18.272 <sup>1</sup>   | 3 <sup>2</sup>        |                        | 2.4 <sup>6,19</sup>      | 3.75 <sup>2,5</sup>       | 61 <sup>3</sup>        |
| <i>Parus palustris</i>           | 7.85 <sup>1</sup>     | 1.5 <sup>2</sup>      | 3.5 <sup>2</sup>       | 2 <sup>2</sup>           | 4 <sup>2</sup>            | 30 <sup>3</sup>        |
| <i>Passer domesticus</i>         | 34.155 <sup>1,4</sup> |                       |                        | 1 <sup>20</sup>          |                           | 85 <sup>3</sup>        |
| <i>Passer montanus</i>           | 17.48 <sup>1</sup>    |                       |                        | 6 <sup>2</sup>           |                           | 90 <sup>3</sup>        |
| <i>Phoenicurus phoenicurus</i>   | 5.379 <sup>1</sup>    |                       |                        | 18 <sup>2</sup>          | 200 <sup>5</sup>          | 36 <sup>3</sup>        |
| <i>Saxicola torquata</i>         | 18.95 <sup>1</sup>    | 1.15 <sup>21,22</sup> |                        |                          |                           | 94 <sup>3</sup>        |
| <i>Serinus canaria</i>           | 53.07 <sup>1,4</sup>  |                       |                        | 20.5 <sup>6,23</sup>     | 300 <sup>5</sup>          |                        |
| <i>Serinus serinus</i>           | 44.997 <sup>1</sup>   | 2.92 <sup>24,25</sup> |                        |                          |                           | 75 <sup>3</sup>        |
| <i>Sitta europaea</i>            | 12.64 <sup>1</sup>    |                       |                        |                          |                           | 90 <sup>3</sup>        |
| <i>Sturnus vulgaris</i>          | 3.62 <sup>1,4</sup>   | 24.5 <sup>26</sup>    |                        | 15 <sup>2</sup>          | 40.13 <sup>26</sup>       | 80 <sup>3</sup>        |
| <i>Sylvia atricapilla</i>        | 4.55 <sup>1</sup>     | 5 <sup>2</sup>        | 4.2 <sup>2</sup>       | 25 <sup>2</sup>          |                           | 21 <sup>3</sup>        |
| <i>Tachycineta bicolor</i>       | 2.616 <sup>1,4</sup>  |                       |                        |                          | 2.6 <sup>5</sup>          |                        |
| <i>Taeniopygia guttata</i>       | 4.18 <sup>4</sup>     |                       |                        |                          | 1 <sup>5</sup>            |                        |
| <i>Troglodytes aedon</i>         | 4.78 <sup>4</sup>     | 1.7 <sup>2</sup>      |                        | 6 <sup>2</sup>           | 1000 <sup>2</sup>         |                        |
| <i>Troglodytes troglodytes</i>   | 2.885 <sup>1</sup>    | 6.3 <sup>2</sup>      | 5.6 <sup>2</sup>       | 47 <sup>2</sup>          | 13.75 <sup>2,5</sup>      | 26 <sup>3</sup>        |
| <i>Turdus merula</i>             | 8.218 <sup>1</sup>    | 2.4 <sup>2</sup>      | 1.6 <sup>2</sup>       | 6 <sup>2</sup>           | 33 <sup>2,5</sup>         | 48 <sup>3</sup>        |
| <i>Turdus philomelos</i>         | 13.276 <sup>1</sup>   | 0.6 <sup>2</sup>      | 0.9 <sup>2</sup>       | 9 <sup>2</sup>           | 159.5 <sup>2,5</sup>      | 66 <sup>3</sup>        |
| <i>Turdus pilaris</i>            | 10.852 <sup>1</sup>   |                       |                        |                          | 27 <sup>5</sup>           |                        |

References: (1) Gil *et al.* (in press); (2) Read & Weary (1992); (3) Møller *et al.* (2006); (4) Gorman & Williams (2005); (5) MacDougall-Shackleton (1997); (6) Garamszegi *et al.* (2005a); (7) Hansen (1984); (8) Garamszegi *et al.* (2004); (9) Garamszegi *et al.* (2006); (10) Lampe & Espmark (1994); (11) Lampe & Sætre (1995); (12) Galeotti *et al.* (1997); (13) Galeotti *et al.* (2001); (14) Garamszegi *et al.* (2005c); (15) L.Z. Garamszegi unpublished data; (16) Titus (2002); (17) Kunc *et al.* (2005); (18) Gorissen *et al.* (2002); (19) Van Duyse *et al.* (2002); (20) Cramp & Perrins (1985–1994); (21) Greig-Smith (1982a); (22) Greig-Smith (1982b); (23) Leitner *et al.* (2001); (24) Mota (1999); (25) Mota & Cardoso (2001); (26) Eens (1997).

**Appendix 2** Phenotypic traits of birds that are used as confounding variables in the phylogenetic analyses. Data for Incubation period, nestling period, migration, polygyny, habitat and coloniality are from Cramp & Perrins (1985–1994), and Glutz von Blotzheim & Bauer (1985–1997) except for *Agelaius phoeniceus*, *Dendroica petechia*, *Junco hyemalis*, *Molothrus ater*, *Tachycineta bicolor* and *Troglodytes aedon* for which we used Poole *et al.* (1993–2002); and for *Taeniopygia guttata* for which we relied on Marchant & Higgins (1991–2001). Information on body mass originated from Dunning (1993).

| Species                          | Incubation period (days) | Nestling period (days) | Body mass (g) | Mean breeding latitude | Migration | Polygyny | Habitat | Coloniality |
|----------------------------------|--------------------------|------------------------|---------------|------------------------|-----------|----------|---------|-------------|
| <i>Acrocephalus arundinaceus</i> | 14                       | 13                     | 30.35         | 46.8                   | 2         | 2        | 0       | 0           |
| <i>Agelaius phoeniceus</i>       | 12                       | 10                     | 52.55         | 40.0                   | 1         | 2        | 0       | 0           |
| <i>Carduelis carduelis</i>       | 12.1                     | 14.7                   | 15.6          | 43.6                   | 2         | 0        | 2       | 1           |
| <i>Carduelis chloris</i>         | 12.9                     | 14.4                   | 27.65         | 49.0                   | 2         | 0        | 2       | 1           |
| <i>Delichon urbica</i>           | 14.2                     | 23.9                   | 19.55         | 42.9                   | 2         | 0        | 0       | 1           |
| <i>Dendroica petechia</i>        | 11.3                     | 8.3                    | 9.5           | 43.5                   | 2         | 1        | 0       | 0           |
| <i>Emberiza citrinella</i>       | 13                       | 12.4                   | 26.75         | 54.7                   | 0         | 1        | 1       | 0           |
| <i>Ficedula albicollis</i>       | 12.8                     | 15.9                   | 13.4          | 48.6                   | 2         | 2        | 2       | 0           |
| <i>Ficedula hypoleuca</i>        | 13.3                     | 14.6                   | 14.35         | 50.8                   | 2         | 2        | 2       | 0           |
| <i>Fringilla coelebs</i>         | 12.6                     | 13.9                   | 24.2          | 49.5                   | 1         | 0        | 2       | 0           |
| <i>Galerida cristata</i>         | 12                       | 16                     | 44.65         | 29.9                   | 0         | 0        | 0       | 0           |
| <i>Hirundo rustica</i>           | 14.3                     | 19.5                   | 19.1          | 47.1                   | 2         | 1        | 0       | 1           |
| <i>Junco hyemalis</i>            | 12.5                     | 10.5                   | 19.6          | 49.5                   | 1         | 1        | 1       | 0           |
| <i>Lanius collurio</i>           | 14                       | 14.5                   | 30.7          | 49.5                   | 2         | 0        | 1       | 0           |
| <i>Luscinia megarhynchos</i>     | 13                       | 11                     | 20.15         | 40.9                   | 2         | 0        | 1       | 0           |
| <i>Molothrus ater</i>            | 11                       | 10.5                   | 43.9          | 41.5                   | 1         | 1        | 0       | 0           |
| <i>Motacilla alba</i>            | 12.6                     | 13.7                   | 20.75         | 50.3                   | 1         | 0        | 0       | 0           |
| <i>Parus caeruleus</i>           | 14.2                     | 18                     | 11.75         | 48.9                   | 0         | 1        | 2       | 0           |
| <i>Parus major</i>               | 13.9                     | 18.9                   | 18.5          | 49.6                   | 0         | 1        | 2       | 0           |
| <i>Parus palustris</i>           | 14                       | 18.5                   | 11.9          | 51.4                   | 0         | 0        | 2       | 0           |
| <i>Passer domesticus</i>         | 12                       | 14.1                   | 30.35         | 47.4                   | 0         | 2        | 0       | 1           |
| <i>Passer montanus</i>           | 12.5                     | 17.5                   | 21.7          | 52.9                   | 0         | 0        | 1       | 1           |
| <i>Phoenicurus phoenicurus</i>   | 13                       | 14.5                   | 15.9          | 48.9                   | 2         | 0        | 2       | 0           |
| <i>Saxicola torquata</i>         | 13.5                     | 13.5                   | 14.9          | 15.2                   | 2         | 0        | 0       | 0           |
| <i>Serinus canaria</i>           | 13.5                     | 16                     | 15.25         | 33.4                   | 0         | 0        | 2       | 1           |
| <i>Serinus serinus</i>           | 12.6                     | 15.2                   | 11.95         | 43.8                   | 1         | 0        | 2       | 1           |
| <i>Sitta europaea</i>            | 14.8                     | 23.5                   | 23.9          | 49.2                   | 0         | 0        | 2       | 0           |
| <i>Sturnus vulgaris</i>          | 12.2                     | 21                     | 80.5          | 49.5                   | 1         | 1        | 2       | 1           |
| <i>Sylvia atricapilla</i>        | 11                       | 11                     | 18.85         | 42.6                   | 2         | 0        | 2       | 0           |
| <i>Tachycineta bicolor</i>       | 14.5                     | 21                     | 20.1          | 49.5                   | 2         | 1        | 0       | 0           |
| <i>Taeniopygia guttata</i>       | 13                       | 21                     | 12            | 19.5                   | 0         | 0        | 0       | 1           |
| <i>Troglodytes aedon</i>         | 12.5                     | 17                     | 10.9          | 45.5                   | 2         | 2        | 2       | 0           |
| <i>Troglodytes troglodytes</i>   | 16.02                    | 17.3                   | 8.9           | 50.1                   | 1         | 2        | 2       | 0           |
| <i>Turdus merula</i>             | 13.7                     | 13.6                   | 95.85         | 49.5                   | 0         | 0        | 2       | 0           |
| <i>Turdus philomelos</i>         | 13.4                     | 13.2                   | 70.5          | 53.2                   | 1         | 0        | 2       | 0           |
| <i>Turdus pilaris</i>            | 12                       | 12.9                   | 92.1          | 57.9                   | 1         | 0        | 2       | 1           |

**Appendix 3** Results of generalized least squares (GLS) analyses of the relationship between song variables and yolk testosterone levels, when relying on an alternative phylogeny of birds (Barker *et al.*, 2002). As branch length estimations are unavailable for this phylogeny, we did not adjust the corresponding scaling parameter, and in each model  $\kappa = 1$  was used. The phylogeny scaling parameter ( $\lambda$ ) was estimated and used as described in the paper (underlined when significant).

|                           | $\kappa$ | $\lambda$    | Correlation | $P$   | $N$ |
|---------------------------|----------|--------------|-------------|-------|-----|
| Testosterone level        |          |              |             |       |     |
| Song duration             | 1.000    | 0.000        | -0.477      | 0.020 | 21  |
| Intersong interval        | 1.000    | 1.000        | -0.203      | 0.426 | 15  |
| Song repertoire size      | 1.000    | 1.000        | 0.347       | 0.080 | 24  |
| Syllable repertoire size* | 1.000    | <u>0.674</u> | -0.355      | 0.056 | 27  |
| Song exposure             | 1.000    | 0.621        | 0.390       | 0.042 | 25  |

\*Excluding the domestic *Serinus canaria* (see text for details):  $\lambda = 0.653$ ,  $r = -0.522$ ,  $P = 0.004$ ,  $N = 26$ .

**Appendix 4** Phylogenetic relationships between song variables of interest and yolk testosterone levels when migration, polygyny and habitat type were controlled as categorical variables in phylogenetic regressions used as an alternative comparative approach (Grafen, 1989).

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Controlled confounding variables

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|             |  |
|-------------|--|
|             | Models for song duration                             |
| Migration   | $F_{1,17} = 6.58, P = 0.020, \text{slope} = -0.492$  |
| Polygyny    | $F_{1,17} = 12.81, P = 0.002, \text{slope} = -0.644$ |
| Habitat     | $F_{1,17} = 4.88, P = 0.041, \text{slope} = -0.461$  |
| Coloniality | $F_{1,18} = 10.91, P = 0.004, \text{slope} = -0.460$ |
|             | Models for syllable repertoire size*                 |
| Migration   | $F_{1,23} = 3.23, P = 0.085, \text{slope} = -0.393$  |
| Polygyny    | $F_{1,23} = 4.14, P = 0.053, \text{slope} = -0.428$  |
| Habitat     | $F_{1,23} = 4.31, P = 0.049, \text{slope} = -0.438$  |
| Coloniality | $F_{1,24} = 5.86, P = 0.023, \text{slope} = -0.476$  |
|             | Models for song-post exposure                        |
| Migration   | $F_{1,21} = 5.27, P = 0.032, \text{slope} = 0.333$   |
| Polygyny    | $F_{1,21} = 3.81, P = 0.065, \text{slope} = 0.287$   |
| Habitat     | $F_{1,21} = 4.05, P = 0.057, \text{slope} = 0.342$   |
| Coloniality | $F_{1,22} = 2.61, P = 0.121, \text{slope} = 0.276$   |

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\*Excluding the domestic *Serinus canaria* (see text for details):  $F_{1,22} > 8.88, P < 0.007, \text{slope} < -0.573$