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Simultaneous pituitary–gonadal recrudescence in two Corsican populations of male blue tits with asynchronous breeding dates

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Abstract

Animal populations living in geographically variable environments respond to different selection pressures. The adaptive character of the responses to environmental information determines the degree of synchrony of the breeding period with local optimal conditions. An example is provided by two populations of Mediterranean blue tits (*Parus caeruleus*) in Corsica, breeding in different habitats, with a 1-month difference in the onset of egg laying. This difference in the onset of lay is supposed to be adaptive because, although chicks from both populations are raised mostly on caterpillars, the timing of the appearance of caterpillars is earlier for populations of tits associated with deciduous oak trees than those associated with evergreen oak trees. Here, we show that, despite the difference in the timing of egg laying, males from these two populations start seasonal hypothalamo–hypophysial–testicular development at approximately the same time, in late winter. Specifically, the vernal recrudescence of brain GnRH-I perikarya and fibers, testes volume and song activity began around the same dates and proceeded at the same pace in late winter in both populations. Plasma testosterone and LH levels displayed seasonal variations that were shifted by less than 2 weeks compared to the 1-month difference in egg laying periods. We hypothesize that the strong selection pressures on these two populations to adapt the timing of their breeding seasons to their local environment may have acted mostly on the female egg laying dates, and not so much on the initiation and rate of seasonal recrudescence of the hypothalamo–hypophysial–testicular activity in males.

Keywords: Testosterone; Bird; Gonadotropin; LH; Testes; Song control nuclei; Season; *Parus caeruleus*; Reproduction; Population; Adaptation

Introduction

Free-living organisms are challenged annually to match their reproductive effort with the optimal period for breeding success (Lack, 1968; Murton and Westwood, 1977; Wingfield and Moore, 1987). Each year, a specific combination of environmental factors defines a seasonal pattern of food abundance which in turn influences both parental condition and offspring survival (Lambrechts and Visser, 1999; Thomas et al., 2001). Because the development of the reproductive organs is a relatively long process that must start much earlier than the actual time of maximal food availability, birds must rely on other environmental factors to predict the optimal time for breeding. Among these cues, the best documented are photoperiod, temperature, tree phenology, social interactions and experience from previous years (Dawson et al., 2001; Grieco et al., 2002; Hau, 2001; Schwabl et al., 2005; Silverin, 1991a, 1995; Visser and Lambrechts, 1999; Wingfield et al., 1997; Wingfield and Moore, 1987; Wingfield and Silverin, 2002). Some of these factors are thought to act early in the pre-breeding season, while others probably act in later phases of the reproductive cycle to allow the fine adjustment of egg laying date. As the optimal time for breeding varies both in space and time, the adaptive responses of local populations can be expected to differ. Accordingly, many studies have

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demonstrated that the seasonal patterns of reproduction differ between populations of the same species living in different environments (Moore et al., 2005; Nager and van Noordwijk, 1995; Perfito et al., 2004; Silverin et al., 1993; Tramontin et al., 2001). However, the proximate cues used by the organisms and their differential adaptation to these cues are generally poorly understood largely because analysis of this adaptation requires long-term studies quantifying the sources of selection and the predictive value of the environment (Lambrechts and Visser, 1999; Nager and van Noordwijk, 1995; van Noordwijk et al., 1995).

Blue tit (*Parus caeruleus*) populations living in the Mediterranean region have been studied for several decades (Blondel et al., 1999; Lambrechts et al., 2004). These birds live in heterogeneous habitats, differing in the abundance of broad-leaved deciduous (*Quercus humilis*) and evergreen (*Quercus ilex*) oak trees. Broad-leaved deciduous oak trees present an early phenology with maximal abundance of caterpillars, the main food used to rear chicks, occurring 1 month earlier than in the evergreen habitat. In the Mediterranean region, long-term ecological studies suggest that blue tits living in a broad-leaved deciduous habitat (exemplified by the study site at Muro) have higher fitness when matching the chick rearing period with the caterpillar peak in May. However, blue tits living in an evergreen habitat (exemplified by the study site of Pirio, located at a similar latitude and altitude) have higher fitness when rearing chicks 1 month later, in June when caterpillars are most abundant (Blondel et al., 1993, 1999, 2001; Lambrechts et al., 1997a,b, 2004; Lambrechts and Dias, 1993). Although the two study sites of Muro and Pirio are only 25 km apart, exchanges of individuals between the two populations appear to be rare based on the observation that subjects banded at one site have almost never been recovered at the other site (see Blondel et al., 1999; Caro et al., 2005b; and Lambrechts, M.M. unpublished data based on 12 consecutive years of ringing in these two populations and over 3000 captures). This isolation favors genetic differentiation of these populations for evolutionary important features such as the timing of breeding (Blondel et al., 1999, 2001). Moreover, previous studies in captivity showed that blue tits originating from the deciduous and evergreen habitats differ in their response to photoperiodic cues. Birds from Corsican evergreen habitat that breed later than birds from mainland deciduous oak forests exhibited a higher photoperiodic threshold for the time of laying the first clutch when placed in outdoor aviaries where all other environmental factors were the same (Lambrechts et al., 1996, 1997b). These populations provide an experimental model in which both a major selection factor (seasonal caterpillars peak) and an environmental cue (photoperiodic threshold) acting on the physiology and behavior are well documented to analyze physiological mechanisms (proximate factors) underlying seasonal differences in the temporal changes in components of the hypothalamo–pituitary–gonadal axis.

A preliminary study has described the vernal recrudescence of singing, the development of the testes and changes in plasma testosterone in a limited sample of subjects during one breeding season (Caro et al., 2005a). This study suggested that the rate of testicular development was faster in Muro than in Pirio tits but that the onset of gonadal development could be similar at the two study sites. We describe here more extensive studies during two consecutive breeding seasons involving more than 250 individuals. We assessed the seasonal changes in several aspects of the development of the reproductive system including singing activity, testes size and plasma concentrations of testosterone and of luteinizing hormone (LH) and of gonadotropin-releasing hormone-I (GnRH-I) cells and fibers in the hypothalamus. These results confirm that recrudescence of the hypothalamo–pituitary–testicular activity begins at the same time in both populations of blue tits. A lag in development between males in Muro and Pirio only takes place when females are nearing ovulation, but this lag never exceeds 10 to 15 days, i.e., much less than the 1 month asynchrony between egg laying dates. These data therefore demonstrate that free-living populations are able to respond to the environmental constraints and adjust their egg laying time to the period of optimal abundance of food for the chicks but that, in the tits populations under investigation, the adjustment of testicular recrudescence in males does not fully match the difference in the female's egg laying phenology.

Materials and methods

This study was carried out on Corsican populations of blue tit (*P. caeruleus ogliastrae*) from broad-leaved deciduous (Muro) and evergreen (Pirio) study sites located at similar latitudes (Muro: 42°32′ north; Pirio: 42°22′ north) and altitudes (Muro: 280 m; Pirio: 200 m). The two sites are approximately 25 km apart and separated by low mountain crests (between 400 and 1900 m) (see map in Lambrechts et al., 1997a). The Muro and Pirio populations have been studied since 1993 and 1976 respectively (see Blondel et al., 1999). All data presented here were collected in 2002 and 2003. As previously described, extensive studies on a substantial proportion of the population confirmed that egg laying periods differed markedly between the two populations. All clutches were characterized by the date when the female laid the median egg in the sequence (e.g., 5th out of 9 eggs). During the 2 years under study, there was no overlap in the timing of laying of first clutches at these two sites: egg laying at the Muro site finished before the earliest egg laying started in Pirio (2002: $U = 0$, $n_{\text{Muro}} = 15$, $n_{\text{Pirio}} = 51$, $P < 0.0001$; 2003: $U = 0$, $n_{\text{Muro}} = 11$, $n_{\text{Pirio}} = 42$, $P < 0.0001$; Lambrechs, M.M., unpublished data). All experiments complied with CNRS animal care guidelines (certificate no. 34–96 issued by the French Ministère de l'Agriculture et de la Forêt).

Field procedures

Singing activity

Song activity was monitored at both sites from early February until late May, using the point count method. Sampling consisted of four 10-min point counts performed in the morning of a given day, each divided in 10 1-min periods during which we counted the number of singing males that were heard (see Caro et al., 2005a for more details). These 10 scores were then added to provide a total score for each point count, and the total singing activity in a sampling day was estimated by summing these four point counts. A total of 284 point counts were carried out in this way on 71 different sampling days (28 days in 2002 and 43 days in 2003) that were more or less equally distributed between the two study sites. For statistical analyses, however, one to four sampling dates were pooled to obtain comparable mean sampling dates at each study site. In these analyses, the data from the four point counts were, however, kept separate and used as the measure of variability within a given day or pool of days. In some figures, sampling days were kept separate to allow a better view of the different seasonal patterns.

Plasma and tissue sampling

Blue tits were captured in mist nets using live decoys and tape-recorded male vocalizations. Directly after capture (2 to 10 min), a blood sample was collected from the jugular vein in heparinized syringes (Omnican 30, B. Braun; Melsungen, Germany). Plasma was immediately separated by centrifugation, frozen on dry ice and stored in freezers (−40°C or −80°C) until assayed for hormones. Phenotype measurements were collected for each bird (see Blondel et al., 1999; Lambrechts et al., 1997a for details), which was then ringed using aluminum rings provided by the French CRBPO and released. A total of 258 blood samples were collected (77 in 2002 and 181 in 2003) that were spread more or less evenly over the breeding season. All blood samples that were used in the analyses presented here had been collected on different subjects (all birds sampled were ringed), and no repeated measures were included in these analyses.

After blood sampling, some of the males at both sites were killed by decapitation to provide samples for additional histological investigations. Brains and testes were collected at Muro and Pirio at the end of February, March (2002 and 2003) and December (2003). Another group of males was killed in the evergreen site (Pirio) at the end of April in both years, just before egg laying. No males were killed at this time at Muro since parents are then feeding chicks, and if the male disappears, the female is unable to raise the entire brood alone. After dissection, brains were immersed in a fixative solution of buffered phosphate saline (PBS) containing 5% Acrolein (Acros Organics; Geel, Belgium) for 1 h without shaking and then 3 h with shaking. Brains were cryoprotected in a 30% sucrose solution overnight and frozen on dry ice (−80°C) until analyzed. Testes were immediately measured (length and width) to the nearest 0.1 mm and their volumes calculated using the equation: $V = 4 / 3\pi\alpha^2\beta$ where α is half the testis width and β is half the testis length.

Plasma hormone assays

Circulating levels of luteinizing hormone (LH; samples collected in 2003) and testosterone (samples collected in 2002 and 2003) were measured by radioimmunoassays (RIAs). Both hormones were assayed in duplicates. All LH samples were measured in a single assay, testosterone levels were measured in two different assays (one for each breeding season).

Luteinizing hormone

Plasma LH concentrations were measured using a micro-modification of a chicken LH double-antibody precipitation radioimmunoassay (Sharp et al., 1987). The assay reaction volume was 60 μl comprising 20 μl plasma sample or standard, 20 μl primary rabbit LH antibody and 20 μl I^{125}-labeled LH. The primary antibody was precipitated to separate free and bound I^{125} label using 20 μl donkey anti-rabbit precipitating serum and 20 μl non-immune rabbit serum. A dilution curve generated by serially diluting a tit plasma pool was parallel to the standard curve. The assay sensitivity was 7.3 pg/tube, and the intra-assay coefficient of variation was 7.0%.

Testosterone

Plasma testosterone levels were assayed at the CEBC (Centre d'Etudes Biologiques de Chizé) as detailed in Chastel et al. (2003) and Mazuc et al. (2003) and validated for Corsican blue tits (Caro et al., 2005a). Briefly, testosterone was extracted from a 50 μl plasma sample with 3 ml diethyl-ether. Extracts were re-dissolved in 0.3 ml of 0.01 M phosphate-buffered saline (pH 7.4) containing 0.1% bovine albumin (PBS–BSA). Two aliquots of 100 μl were incubated overnight at 4°C with 100 μl (ca. 9000 cpm) of tritiated testosterone (TRK 921, 250 μCi, Amersham-France) and 100 μl of specific antiserum (Dr. G. Picaper, CHR Orleans, France). The free and bound steroids were separated by addition of 0.5 ml of dextran-coated charcoal. After centrifugation, which precipitated free steroids, radioactivity in the upper phase was measured in a liquid scintillation counter (1600 Packard). The lowest concentration detectable was 0.1 ng/ml, and the intra-assay coefficients in 2002 and 2003 were smaller than 5%. Two assays were conducted, and inter-assay coefficient was 5.2% (n = 6 duplicates).

Brain gonadotropin-releasing hormone

Brains were cut in 30 μm transverse frozen sections on a cryostat. Sections were stored in an antifreeze solution at −20°C until analyzed. Every fifth section was washed three times for 5 min in 0.01 M phosphate-buffered saline (PBS) then 15 min in a PBS solution containing 0.1% of sodium borohydride to block the remaining aldehydes from the Acrolein solution and finally 20 min in a solution of hydrogen peroxide (0.6% H_2O_2 in PBS) to block endogenous peroxidases. Between each of these steps, sections were washed three times 5 min in 0.01 M PBS.

After a 30-min preincubation in 5% normal goat serum (NGS) prepared in 0.01 M PBST (PBS containing 0.2% Triton X-100) to reduce background staining, sections were transferred to the primary antisera directed against chicken gonadotropin-releasing hormone-I (GnRH-I) (see Van Gils et al., 1993 for details concerning antibody preparation and validation) at a dilution of 1/4000 in 0.01 M PBST. Sections were incubated for 1 h with shaking at room temperature and then overnight without shaking at 4°C. On the next day, sections were washed three times in PBST and free avidin and biotin were blocked using the avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA). After three washes in 0.01 M PBST, sections were incubated for 90 min at room temperature with the secondary antibody (biotinylated goat-anti rabbit) at a dilution of 1/400 in 0.01 M PBST. Sections were rinsed three more times in 0.01 M PBST and incubated for 90 min at room temperature in ABC Vectastain elite Kit PK-6100 (Vector Laboratories). They were rinsed three times in 0.01 M PBST. The peroxidase activity was then visualized after 5-min incubation with DAB as a chromogen (20 mg of 3,3′ diaminobenzidine tetrahydrochloride in 50 ml PBST containing 20 μl of 30% hydrogen peroxide). They were washed three times with distilled H_2O, mounted on gelatin-coated slides, dried overnight, covered with an aqueous gelatin medium and coverslipped.

GnRH-I immunolabeling was quantified by counting the number of immunoreactive cell bodies and calculating the density of immunoreactive fibers. Sections were analyzed microscopically using a ×20 objective. Immunoreactive cell bodies were manually counted in every section from the level of the tractus septopallio-mesencephalicus (TSM) to the caudal end of the septum at a level just caudal to the commissura anterior (CoA) where no additional cells could be observed. The total number of cells detected in all immunolabeled sections was summed for the entire brain. Since every fifth section was immunolabeled, these numbers roughly represent one fifth of the total number of GnRH-I neurons in the brain. These total numbers of counted cells were thus multiplied by five. The resulting final numbers are however an overestimation due to double counting. The diameter of 25 neurons selected randomly in five brains was found to be 11.3 ± 1.36 μm. Assuming that the diameter of these neurons is similar in all orientations (which should be the case) and considering a section thickness of 30 μm, the Abercrombie (1946) correction would suggest that actual numbers of GnRH-I cells should be about 72% of the totals presented here [30 / 11.3 + 30)]. We did not correct the numbers presented in the result section since the correction factor is at this point somewhat imprecise but it should be stressed that this systematic error does not affect in any way our conclusions concerning the relative development of the GnRH-I system in the two populations.

For quantifying GnRH-I fibers, all sections between the level of the TSM and the level of largest extent of the CoA were digitized by a video camera connected to a MacIntosh microcomputer. In each section, two fields each measuring 0.18 mm^2 were digitized, captured and analyzed using the NIH Image J computer software, version 1.34S (Wayne Rasband, NIH, Bethesda, MD, USA). These fields (one on the right and one the left side of the brain) were positioned at the edges of the third ventricle at a medial dorso-ventral location where a maximum of GnRH-I cell bodies are observed.

All sections were made binary, and manual thresholding was used to separate labeled from unlabeled pixels. Pixels containing cell bodies were manually erased from the stained area. The computer then calculated the percentage of the field (fractional area) covered by the immunolabeled fibers. The average of all these values was then calculated for each brain. Technical problems (damage to or loss of sections) prevented the collection of complete measures in a number of brains, and these subjects were thus excluded from the analysis.

Statistical analyses

Differences between study populations (Pirio vs. Muro) and effects of the sampling time on different components of the reproductive system (song, LH

and testosterone levels, GnRH-I cell bodies and fibers, testis volumes) were tested using two-way analyses of variance (ANOVA) using the populations and sampling dates as independent factors. It was considered that data points within each population represent independent samples since birds were caught randomly at each site. Individuals within a population are not correlated as far as we could assess (they were randomly selected), and if they were in some way (we could for example have sampled two brothers), there was no way to know what is their degree of relationship and thus no way to take that information into account in the analysis. This problem is however common to most field studies. Sampling dates were also considered as an independent factor since no repeated sampling on the same subjects was performed, except possibly for the estimates of singing activity. However, in this case, the identity of the singing birds was unknown, and it was therefore impossible to use these data as repeated measures.

Data from the 2 years of study (2002 and 2003) were pooled unless otherwise stated. In general, sample sizes were too small or missing at some time points to allow inclusion of year as a factor in the ANOVAs, but partial analyses considering as a block the pooled data from before the breeding season and those during the part of the year when hormone levels, testes size and brain GnRH-I are elevated failed to identify differences between the 2 years (data not shown in detail). Post hoc comparisons were carried out with Fisher Protected Least Significant Difference (PLSD) tests in order to compare the experimental groups or the sampling dates two by two. Egg laying dates were compared using a Mann–Whitney U test. Effects were considered significant at $P \leq 0.05$.

Results

Male singing behavior

The seasonal patterns of song activity at Muro and Pirio were studied for two consecutive years (2002 and 2003). In 2003, the sampling started earlier than in 2002 and was more intense around the egg laying periods. For this reason, 2003 results are more detailed than those observed during the first year. All results are summarized in Fig. 1. Results from 2002 were published earlier (Caro et al., 2005a) but are summarized again in this figure to allow comparisons. During both years, seasonal maximal song activity occurred around the same dates in each population and corresponded almost exactly to

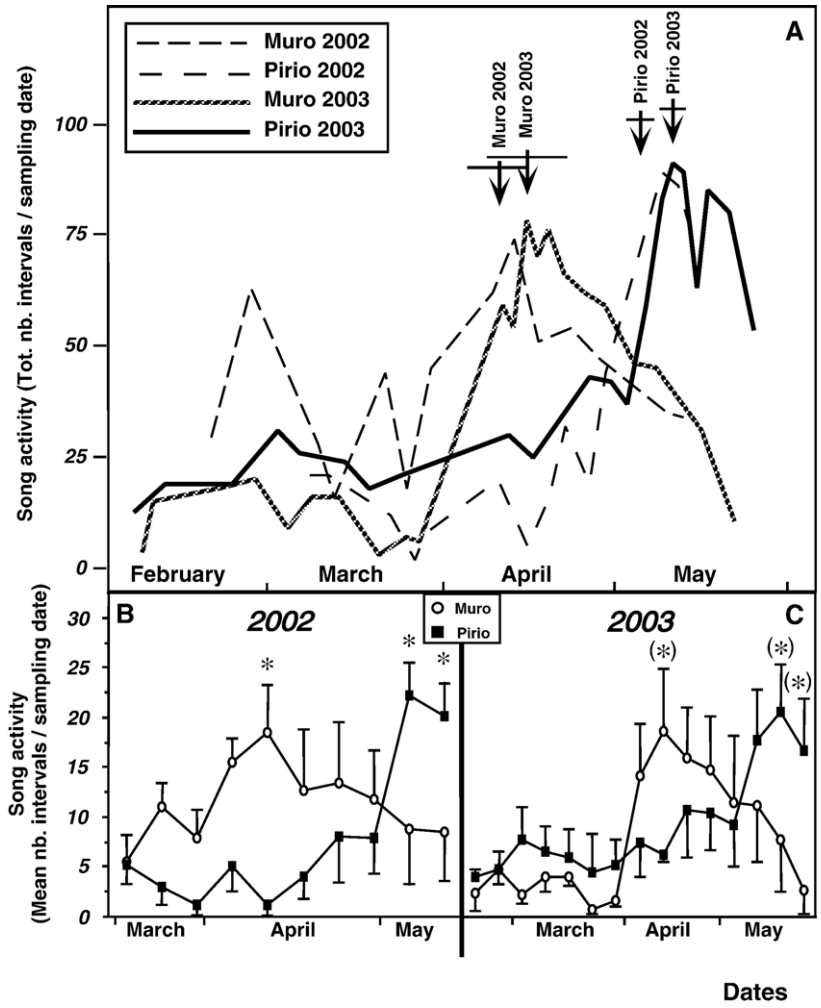

Fig. 1. Seasonal song intervals heard during the four point counts in each population. The arrows on top of each curve represent the median and the interquartile range (horizontal bars) of the egg laying period. There is a close relationship between the maximal levels of male singing activity and the female egg laying periods in each site under study. (B, C) Mean (±SE) number of 1-min song intervals obtained after pooling one to four sampling dates (see text for detail of methods). Results of 2002 (B) and 2003 (C) are shown separately as they were analyzed separately. Results of the post hoc tests comparing the two populations are presented at the top of the bars (*$P < 0.05$ compared to the other study population at the same sampling period). Parentheses around the sign of signification indicate that the interaction between site and month was not significant in 2003 in the ANOVA (see text).

the middle of the respective egg laying periods (see arrows in Fig. 1A).

Statistical analysis by two-way ANOVA of the 2002 results identified a significant effect of the study site and a significant interaction between study sites and time. The overall effect of time was however not significant (see Caro et al., 2005a for details). This interaction resulted essentially from a higher early activity in Muro and a later peak in Pirio (see Fig. 1B for post hoc tests comparing sites at the same date; other results not shown to facilitate reading of the figure).

In 2003, the seasonal song activity pattern was not significantly influenced by the study site ($F_{1,90}$ = 1.057, P = 0.3066). The interaction between study sites and time was nearly significant ($F_{14,90}$ = 1.692, P = 0.0710), and the overall effect of time was highly significant ($F_{14,90}$ = 2.669, P = 0.0027). To allow a direct comparison with the 2002 data, post hoc tests were nevertheless calculated to tentatively identify the dates when singing activity was different at Muro and Pirio. Results of these tests are shown in parentheses in the figure to indicate that they are derived from a data set with a non-significant value after ANOVA (see Fig. 1C). These tests indicate that the maximal levels of singing activity were reached at different times in the two populations corresponding to the respective egg laying periods (see Fig. 1A).

Interestingly, a two-way ANOVA of the data collected in the pre-breeding period in 2003 (from February 5 to the end of March) showed that the singing activity was significantly higher in Pirio than in Muro ($F_{1,42}$ = 5.915, P = 0.0194), but the activity did not vary significantly during this period (Time: $F_{6,42}$ = 0.518, P = 0.7916; Interaction: $F_{6,42}$ = 0.359, P = 0.9006).

Testes size

Analysis by two-way ANOVA (sites and months as factors) of testes volumes (Fig. 2) identified a significant overall effect

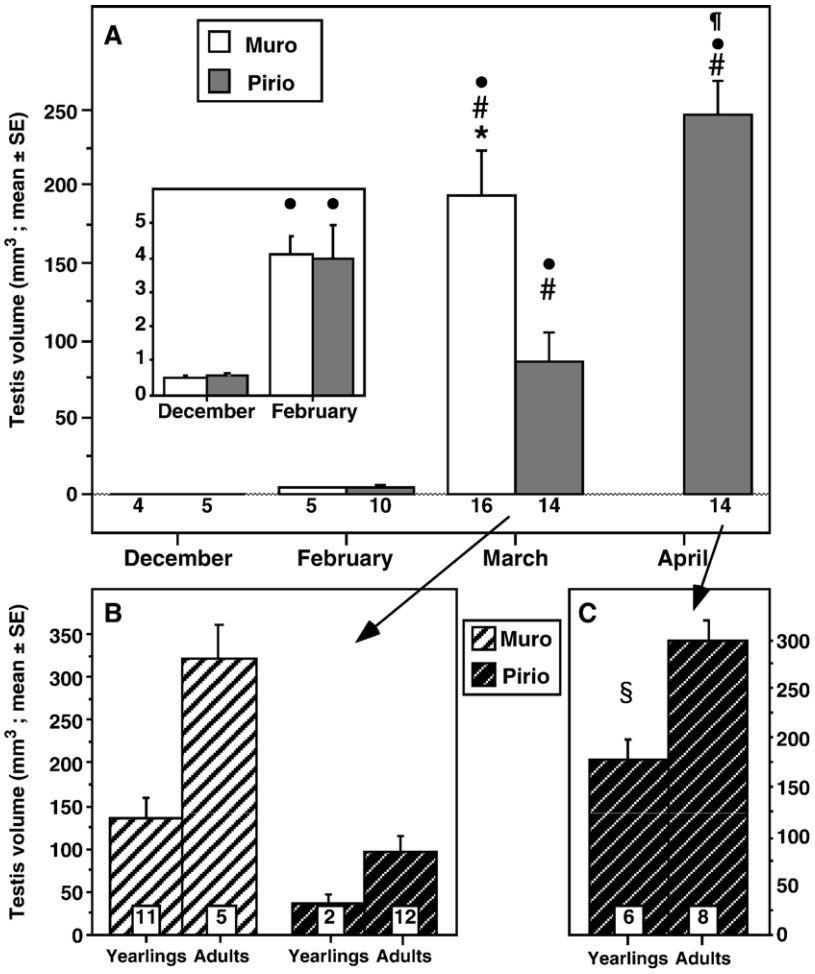

Fig. 2. Seasonal growth of the testis volume of male blue tits at the two study sites of Muro (n = 25) and Pirio (n = 43). Results are presented as means ± SE, and sample sizes are shown in or under the corresponding bars. (A) Seasonal change of testis volumes in the entire populations (pooled data from yearling and adult males); the insert represents the data from the early sampling periods of December and February to illustrate the early synchronized recrudescence. (B, C) Age-related differences in testis volume in Muro and Pirio in samples collected in March and April when sufficient numbers of subjects are available to permit a separate analysis of yearlings and adults. Results of post hoc comparisons following significant ANOVAs are presented at the top of the bars as follows: #P < 0.05 compared to the same site in February; ¶P < 0.05 compared to the same site in March; •P < 0.05 compared to the same site in December; *P < 0.05 compared to the other site at the same sampling period; §P < 0.05 compared to the other age class at the same site and date.

of sampling date ($F_{3,61}$ = 33.796, $P < 0.0001$) and a significant interaction between sites and months ($F_{2,61}$ = 3.150, P = 0.0499). There was, however, no overall difference between sites ($F_{1,61}$ = 2.245, P = 0.1392). Post hoc tests identified the origin of the interaction and of the seasonal changes. In general, testes sizes were larger in March and April than at earlier periods (see detail of post hoc in Fig. 2A). In addition, testes volumes were larger at Muro than at Pirio in March.

This analysis was slightly unbalanced in that no sample had been collected in Muro in April to avoid endangering brood survival (see Materials and methods). A two-way ANOVA excluding data from April confirmed that there was an overall effect of month ($F_{2,48}$ = 21.672, $P < 0.0001$) and a significant interaction between sites and months ($F_{2,48}$ = 3.303, P = 0.0453) but no overall difference between sites ($F_{1,48}$ = 2.354, P = 0.1315).

Post hoc tests identified no difference between testes sizes measured in December and February, but this failure might have been due to the inclusion of very large values (in March and April) in the data set, therefore making small changes (between December and February) negligible by comparison with the larger values and variability observed at the reproductive peak. Qualitative inspection of the raw data indicated, indeed, that all testes sizes measured in February were larger than in December. In a separate two-way ANOVA, we therefore analyzed the testis volume variations during the early sampling periods of December and February (see insert in Fig. 2A). In this analysis, testis size was significantly affected by the sampling month ($F_{1,20}$ = 13.946, P = 0.0013), but not by the study sites ($F_{1,20}$ = 0.004, P = 0.953) and the interaction between these factors was not significant ($F_{1,20}$ = 0.009, P = 0.927), indicating that this early limited development was similar in both populations.

Qualitative analysis also indicated that yearling birds had smaller testes than second year and older birds in March and April (Figs. 2B, C). Samples sizes were not large enough to introduce the age of the subjects as a third factor in the ANOVA, and targeted comparisons were thus performed specifically on data collected in March (both study sites, Fig. 2B) and April (Pirio site only, Fig. 2C). A two-way ANOVA of testes sizes measured in March in yearlings and adults indicated significant overall effects of both factors (Age: $F_{1,26}$ = 12.052, P = 0.0018; Site: $F_{1,26}$ = 21.774, $P < 0.0001$) and a nearly significant effect of the interaction ($F_{1,26}$ = 3.263, P = 0.0824). In April, yearlings also had smaller testes than adults in the subjects sampled in Pirio ($F_{1,12}$ = 14.362, P = 0.0026; see Fig. 2C). In February, the similar testis volumes observed between the two populations were not a consequence of unbalanced numbers of individuals in the two age classes (Muro: 3 yearlings, 2 adults; Pirio: 6 yearlings, 4 adults).

LH

In samples collected in 2003, plasma LH levels varied significantly during the reproductive season ($F_{8,143}$ = 5.094, $P < 0.0001$; see Fig. 3). The influence of the study site was nearly significant ($F_{1,143}$ = 3.596, P = 0.0599), and there was a significant interaction between the study sites and sampling periods ($F_{8,143}$ = 2.199, P = 0.0308). Post hoc tests comparing seasonal changes indicated an overall increase in LH concentrations during the breeding season and a decrease at its end (data not shown to avoid cluttering the figure). Comparisons between the study sites revealed only two significant differences at the beginning of the season (on February 26 and March 11), but the decline at the end of the breeding season was relatively synchronous in both sites, so no difference between sites could be detected at that time.

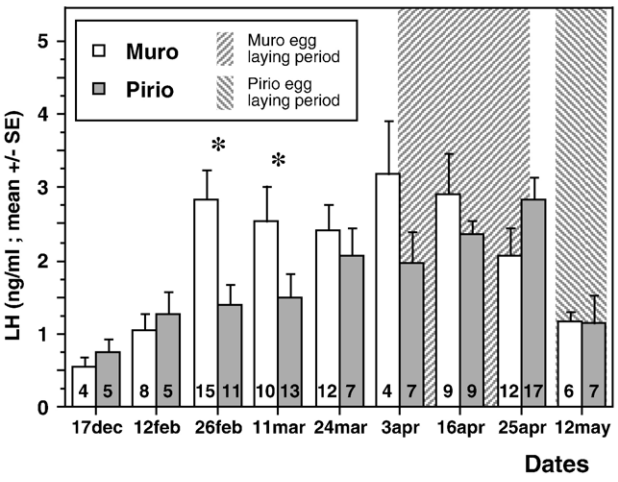

Fig. 3. Seasonal changes of LH plasma concentrations in male blue tits at the two study sites of Muro (n = 78) and Pirio (n = 83). Results are presented as means ± SE with sample sizes indicated in the corresponding bars. Results of the post hoc tests comparing the two populations at the same date are presented at the top of the bars as follows: *$P < 0.05$ compared to the other study population at the same sampling period.

We also examined the possible existence of an age effect (yearlings vs. adults) on LH levels. A three-way ANOVA (study sites, sampling periods, age classes as factors) identified no age effect and no interaction of age with other factors. Because some experimental groups were not represented in this ANOVA (5 out of the 36 groups: 2 sites × 2 ages × 9 sampling periods) thus making it statistically invalid, we also reanalyzed separately all values collected during the period of elevated LH concentrations (between February 26 and April 25) after pooling all dates, keeping only the sites and age as factors. This again revealed no effect of age and no interaction of age with site on plasma LH concentrations (data not shown).

Testosterone

Testosterone concentrations (Fig. 4) varied significantly during the breeding season ($F_{8,193}$ = 4.035, P = 0.0002) but were not affected by the study site ($F_{1,193}$ = 0.956, P = 0.3296). There was a significant interaction between study sites and sampling periods, but the magnitude of this effect was much smaller than the effect of time ($F_{8,193}$ = 2.154, P = 0.0327).

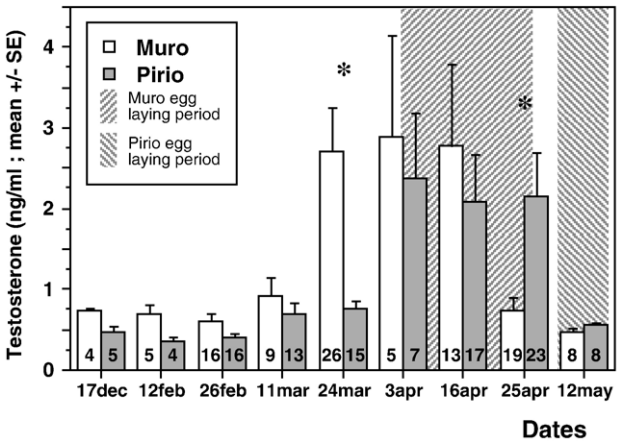

Fig. 4. Seasonal changes of testosterone plasma concentrations in male blue tits at the two study sites of Muro (n = 105) and Pirio (n = 106). Results are presented as means ± SE with sample sizes indicated in the corresponding bars. Results of the post hoc tests comparing the two populations at the same date are presented at the top of the bars as follows: *$P < 0.05$ compared to the other study population at the same sampling period.

Post hoc comparisons between study sites revealed only two significant differences at the beginning or end of the season (on March 24 and April 25) indicating that the seasonal variation in plasma T concentrations was relatively similar in both locations and was only shifted by a maximum of approximately 10–15 days (i.e., one sampling date).

We also examined whether age (yearlings vs. adults) affected testosterone concentrations with a three-way ANOVA (study site, date, age), and this analysis revealed no difference between yearlings and adults ($F_{1,168}$ = 0.900, P = 0.3441) although age may interact with date ($F_{8,168}$ = 2.083, P = 0.0401). Since data at some sampling points were absent or limited, we also analyzed separately all pooled values collected during the pre-breeding (from February 12 to March 11) and the breeding (March 24 to April 25) periods by two-way ANOVAs with age and sites as factors. These two analyses revealed no significant effect of age and no interaction (data not shown).

Gonadotropin-releasing hormone-I (GnRH-I)

Neuroanatomical distribution

GnRH-I-immunolabeled cell bodies and beaded fibers were mainly found in the preoptic, septal and hypothalamic area with cell bodies bilaterally aggregated in the vicinity of the third ventricle (Fig. 5). Rostrally, immunolabeled cells were first observed in the preoptic area at the level of the TSM but were mostly densely aggregated at the level of the medial preoptic nucleus (POM) where they overlapped extensively with this nucleus. The density of these cells decreased in a rostral to caudal direction, and immunolabeled cells were rarely found at the level of the anterior commissure although one or two positive cells could occasionally be seen just caudal to the commissure. Along the rostral to caudal axis, the position of these GnRH-I cell bodies shifted progressively from a ventral to a more dorsal position.

Immunolabeled GnRH fibers were first seen just rostral (approximately 300 μm) to the first cell bodies to be noted, in the most rostral sections containing the TSM. They were found throughout the periventricular hypothalamus occupying an area that increased progressively in the dorsal direction between the level of the TSM and the level of the anterior commissure. In more caudal sections, immunolabeled fibers progressively converged to the ventral part of the hypothalamus and at the most caudal levels, exclusively concentrated in the median eminence.

Quantification of immunolabeled cell bodies and fibers

The estimated total numbers of GnRH-I cells bodies (see Materials and methods) were analyzed by a two-way ANOVA with study sites and sampling periods as factors. This analysis detected a significant effect of sampling period ($F_{3,37}$ = 5.637, P = 0.0028) but no difference between sites ($F_{1,37}$ = 0.277, P = 0.6018) and no interaction ($F_{2,37}$ = 0.635, P = 0.5355). Post hoc PLSD tests comparing the different sampling periods indicated a larger number of immunoreactive cells in March compared to December and in April compared to the other three periods (see Fig. 6). The same conclusions were reached after exclusion of the April data that were available at Pirio only (Months: $F_{2,29}$ = 4.802, P = 0.0158; Sites: $F_{1,29}$ = 0.327, P = 0.5717; Interaction: $F_{2,29}$ = 0.750, P = 0.4812; data in March different from December).

A general three-way ANOVA including age as a factor (in addition to site and date) confirmed the overall effect of the sampling period ($F_{3,31}$ = 6.004, P = 0.0024) but revealed no other significant effect nor interaction and, in particular, no significant effect of age. Since study site had no effect and the effect of date resulted essentially from an increase between December and subsequent periods, data from both sites in February, March and April were reanalyzed by a two-way ANOVA to better assess the potential effect of age. This analysis indicated that adults have overall a larger number of GnRH-I cells than yearlings ($F_{1,31}$ = 5.873, P = 0.0214) and that the number of these cells still varies during the reproductive season ($F_{2,31}$ = 4.710, P = 0.0164), but there was no interaction between these factors ($P > 0.40$).

Analysis of the fractional area covered by GnRH-I-immunolabeled fibers by two-way ANOVA (sites and date as factors) identified a significant effect of month ($F_{3,37}$ = 8.340, P = 0.0002), but no effect of sites and no interaction (respectively $F_{1,37}$ = 0.362, P = 0.5513 and $F_{2,37}$ = 2.113, P = 0.1352). These fractional areas were smaller in December compared to the three other periods (Fisher PLSD $P < 0.05$; see Fig. 6). The same conclusions were obtained if the April data collected at Pirio only were excluded (data not shown).

When data were analyzed by a three-way ANOVA including the age as an additional factor, the effect of months was confirmed ($F_{3,31}$ = 5.826, P = 0.0028) and no other significant effect or interaction was detected although the overall effect of age came close to statistical significance ($F_{1,31}$ = 3.300, P = 0.0790). If data from both sites were pooled (no site effect and no interaction including sites are present) and analysis was focused on the breeding season by excluding the December

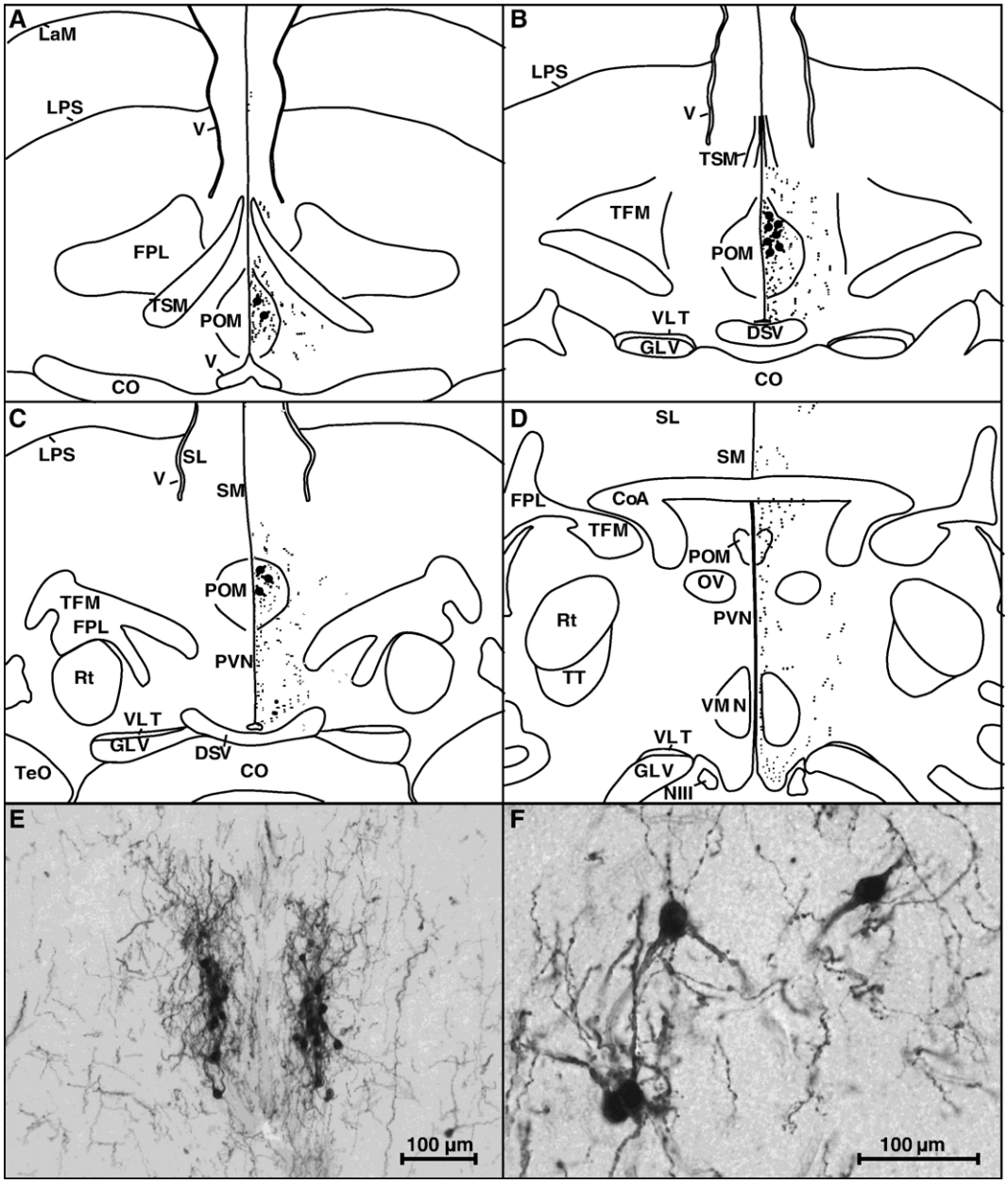

Fig. 5. Schematic illustration of the distribution of GnRH-ir perikarya (black circles) and fibers (little dots) in Corsican male blue tits (A to D, from rostral to caudal sections). CO: chiasma opticum, DSV: decussatio supraoptica ventralis, FPL: fasciculus prosencephali medialis, GLV: nucleus geniculatus lateralis, LaM: lamina mesopallialis, LPS: lamina pallio-subpallialis, NIII: nervus oculomotorius, OV: nucleus ovoidalis, POM: nucleus preopticus medialis, PVN: nucleus periventriculus magnocellularis, Rt: nucleus rotundus, SL: nucleus septalis lateralis, SM: nucleus septalis medialis, TeO: tectum opticum, TFM: tracus thalamo-frontalis, TSM: tractus septopallio-mesencephalicus, TT: tractus tectothalamicus, V: ventriculus, VLT: nucleus ventrolateralis thalami, VMN: nucleus ventromedialis hypothalami. Nomenclature used in this paper is based on the recent revision as described in Reiner et al. (2004). Photomicrographs (E and F) of the hypothalamic regions containing high density of immunolabeled perikarya and fibers.

data, a significant effect of age was then detected with yearlings showing a lower density of GnRH-I fibers than adults (age effect: $F_{1,31} = 6.151$, $P = 0.0188$).

Discussion

In this study, we demonstrate that the timing of the later stages of seasonal testicular growth and associated singing activity differs markedly between the broad-leaved (Muro) and evergreen (Pirio) male blue tit populations in Corsica. The rate of testicular growth between February and March was greater for Muro than for Pirio tits (Fig. 2A) so that the final stages of testicular development and associated singing are synchronized with the egg laying period, which is earlier in the Muro than in the Pirio population (Fig. 1). This difference in the timing of egg laying reflects differences in the local conditions for optimal reproduction (see Introduction). However, the timing of the initiation of reproductive activity did not differ between Muro and Pirio males as demonstrated by the observation that initial testis recrudescence (presumably

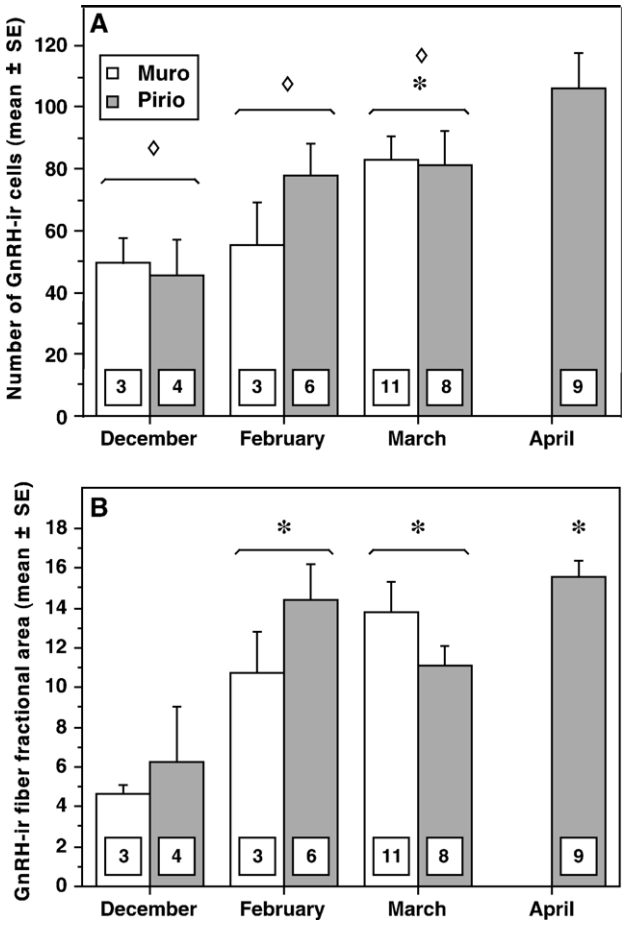

Fig. 6. Seasonal change in the number of GnRH-ir perikarya (A) and in the fractional area covered by GnRH-ir fibers (B) in male blue tits at the two study sites of Muro (n = 17) and Pirio (n = 27). Results are presented as means ± SE with sample sizes indicated in the corresponding bars. Numbers of cells presented are the uncorrected totals obtained by summing the counts in every fifth section. The absolute numbers are thus overestimated since no correction was made for double counting, but this does not affect differences between populations (see Materials and methods for more detail). Results of post hoc comparisons following significant ANOVAs are presented as follows: *$P < 0.05$ compared to December, ◇$P < 0.05$ compared to April.

independent of photostimulation and reflecting endogenous termination of photorefractoriness; see below) occurred simultaneously in both populations between December and February. In February, testis volumes were slightly (but significantly) higher than in December and were the same in Muro and Pirio tits (Fig. 2A insert).

Testicular development then took place rapidly in Muro so that apparently maximal sizes were observed 1 month later (in late March) when females were nearing ovulation. Similarly, the largest testicular size (presumably reflecting the annual peak based on the seasonal changes in other variables) was observed in late April in Pirio, in association with egg laying. Interestingly, however, a significant testicular development was already observed in late March at Pirio, i.e., more than 1 month before the egg laying period of this population (Fig. 2A). Although our sampling interval does not preclude that the Pirio peak was reached earlier, sometimes between late March and late April, these data strongly suggest that the phase of rapid gonadal growth was more rapid in males living in Muro than in Pirio.

It appears that the phenology of testicular recrudescence in these two male populations is not fully adapted to the difference in egg laying periods: males in Pirio initiate their rapid testicular development (in March) and/or reach their apparent seasonal peak of testicular size (sometimes between late March and late April) too early. To our knowledge, this is the first study showing such a clear asynchrony between male reproductive development and female egg laying periods. In similar work that had demonstrated reproductive asynchrony between populations breeding at similar latitudes and altitudes, the seasonal sexual maturation of both sexes appeared to be synchronized (European blackbirds, *Turdus merula*: Partecke et al., 2005; rufous-collared sparrows, *Zonotrichia capensis*: Moore et al., 2005).

Measures of plasma hormone concentrations, which did not require sacrifice of the subjects and could therefore be performed more frequently, allowed us to refine these conclusions. The detailed pattern of seasonal changes in plasma LH and testosterone concentrations provides additional evidence for the absence of a large difference in the timing of reproductive development in males from broad-leaved and evergreen habitats. The testosterone peak at Pirio (3rd April) was reached only 10 days later than at Muro (24th March) (Fig. 4). Although the seasonal pattern of changes in LH concentrations was not as well defined as for testosterone, no major difference between sites could be detected for this hormone (most differences in average levels are not significant), except at the very beginning of the breeding season. LH levels then remained high both in Muro and Pirio for most of the spring, and they decreased at approximately the same time (Fig. 3). Interestingly, at Muro, the peak of testosterone and LH covers both nest building and early egg laying periods while in Pirio testosterone and LH concentrations had already decreased and were close to their basal levels when females were in the first half of the laying period. Thus, although there is some degree of asynchrony in the seasonal patterns of plasma LH and testosterone between Muro and Pirio, this time lag between populations is far smaller (10–15 days) than the difference between egg laying dates (1 month).

Song activity in males is generally associated with both an intra-sexual (territory defense) and inter-sexual (female attraction) function (Catchpole and Slater, 1995). In temperate zone birds, high singing rates have also often been correlated with high levels of testosterone (Balthazart, 1983). However, an earlier study on Mediterranean blue tits showed that plasma testosterone and singing activity were not correlated, suggesting that high plasma levels of testosterone were not essential for males to sing (Caro et al., 2005a). The same conclusion can be drawn from the present study in the Pirio population: males reached their maximal singing activity during the female egg laying period, but at this time, testosterone concentrations had already decreased (Fig. 4). A similar decline in plasma testosterone before the egg laying period has also been reported in a few other species, including great tits (Van Duyse et al., 2003), and a similar temporal uncoupling between

testosterone and singing activity has been demonstrated in several species (Ball et al., 2002; Pinxten et al., 2002; van Duyse et al., 2003). Furthermore, in 2003, song activity peaked only once, late in the breeding season, just before or during the egg laying periods. This suggests that song behavior in these populations was mainly associated with the period of sexual maturity in females, and not with the early establishment of the territories.

It could be shown that even if the seasonal patterns of singing activity present a 1-month difference in the maximal activity (Fig. 1), males had similar levels of activity during the early stages of the pre-breeding period and in 2003, the singing activity was even significantly higher at Pirio than at Muro during February and March. This schematic seasonal pattern of singing can be viewed as a mirror of the vernal recrudescence of the hypothalamo–pituitary–gonadal axis and associated behaviors in Muro and Pirio males: reproductive development very probably begins simultaneously in both populations but then is progressively delayed in Pirio or advanced in Muro so that a limited shift in peak activities is finally observed around the time of egg laying.

Seasonal changes in brain GnRH

The distribution of GnRH-ir cells and fibers observed here in blue tits was very similar to the distribution previously reported for the closely related great tits (Silver et al., 1992) and for other songbird species (Cho et al., 1998; Hahn and Ball, 1995; Stevenson and MacDougall-Shackleton, 2005). We also found no difference between the Muro and Pirio populations in the seasonal recrudescence of brain GnRH-I cells and fibers between December and March (Fig. 6; no significant interaction between sites and periods in the ANOVAs). Most of the increase in GnRH-I cell numbers and fiber density occurred early during the pre-breeding period, between December and February (apparently between December and March for cells in the Muro population).

These data therefore indicate that most of the seasonal recrudescence of the GnRH system occurs during the late winter and precedes by more than 1 month the testicular development and the rapid increase in plasma testosterone concentrations. This recrudescence occurs while birds are still under winter short photoperiods and likely reflects the termination of photorefractoriness that is induced by short days (Sharp et al., 1986; Williams et al., 1987; see also below).

In a previous study on these male tit populations, the same early seasonal recrudescence was observed for two brain song control nuclei, HVC (used as a proper name; see Reiner et al., 2004) and RA, the robust nucleus of the arcopallium (see Caro et al., 2005b). Furthermore, this early increase in HVC and RA volumes was synchronized in Muro and Pirio birds. Together, these results suggest that the seasonal changes occurring in the brain of these males are simultaneous despite the dramatic differences observed in the timing of egg laying periods. Since GnRH is the major central neuropeptide driving seasonal breeding (Ball, 1993; Ball and Hahn, 1997) by stimulating the release of LH and FSH (Sharp et al., 1990), the apparent absence of differences in the rate of development of the GnRH system between Muro and Pirio males lends further support to the view that the initiation of the reproductive function in these tit populations occurs at about the same time despite differences in the optimal breeding date between the two sites.

Age and breeding development

Reliable quantitative differences in reproductive development were also observed between yearlings and adults. In this study, adult male blue tits had larger testes volumes than first year birds in March and April. Adult males also had more GnRH-I perikarya in the hypothalamus–preoptic area than yearlings during the breeding seasons, and they also displayed a higher density of GnRH-I-immunoreactive fibers.

Differences between age classes affecting multiple aspects of the reproductive physiology have previously been reported. These differences concern the testis size, volume of the cloacal protuberance (an androgen-dependent structure), brain neurogenesis, testosterone, FSH and PRL concentrations, clutch size, egg laying date and overall reproductive success (Absil et al., 2003; Corbitt and Deviche, 2005; Dawson, 2003; Deviche et al., 2000; Forslund and Part, 1995; Ketterson and Nolan, 1992; Schwabl et al., 2005; Silverin et al., 1997). Interestingly, in the present study, we failed to detect an effect of age on the plasma concentration of LH and testosterone, despite the fact that testes sizes were different. Unfortunately, plasma FSH could not be assayed given the small volume of the blood samples that could be collected. Changes in FSH should in theory provide a better index of testes volume than LH or testosterone concentrations, which correlate mostly with the steroidogenic testicular compartment (Sertoli cells) that has a small volume, while FSH levels should in theory be related to the volume of the seminiferous tubules, which occupy most of the testis volume in mature males. Other studies have similarly failed to detect age-related differences in plasma levels of LH or testosterone (De Laet et al., 1985; Deviche et al., 2000; Foerster et al., 2002).

Most explanations of these age-related differences in reproductive physiology refer to differential ontogenetic development or to effects of the experience gained during previous breeding seasons (e.g., Deviche et al., 2000), but few experiments have attempted to formally disassociate these two types of causal factors. Recently, however, Sockman et al. (2004) were able to separate these factors by experimentally manipulating photoperiodic cycles so that they could compare groups of starlings (*Sturnus vulgaris*) that were exposed to an increasing photoperiod simulating spring for the first or second time while they were of the same age (Sockman et al., 2004). In juvenile female starlings that received prior experience with photostimulation, the changes in body mass, GnRH-I fibers, LH levels, vitellogenin and ovarian follicle diameter were much more pronounced than in age-matched birds that had never experienced photostimulation. This clearly demonstrates that previous experience has an impact on the functioning of the hypothalamo–pituitary–gonadal axis.

Environmental factors influencing male reproductive development in blue tit

Because preparation of the reproductive system involves a suite of morphological, physiological and behavioral changes, birds have to start this seasonal development several weeks before the optimal time for breeding. Due to the important energetic resources that are needed both for developing and maintaining a functional reproductive system (Murton and Westwood, 1977), birds have evolved to use environmental predictive cues to minimize energy expenditure. It is thus theoretically predicted that populations breeding at different periods but at the same latitude should use different aspects of the environmental information or respond differentially to the same cues in order to start their seasonal development in an asynchronous manner. This prediction has been partly validated in several studies (Moore et al., 2005; Partecke et al., 2004; Perfito et al., 2004; Silverin et al., 1993; Tramontin et al., 2001), and it has also been suggested that this mechanism operates in Mediterranean blue tit populations. Under natural photoperiods, captive pairs of blue tits from broad-leaved and evergreen landscapes maintain their difference in laying dates and this even in pairs of birds hatched in captivity, thus suggesting that this difference has, at least in part, a genetic basis (Lambrechts and Dias, 1993). Supplemental experiments on captive pairs of Mediterranean blue tits have shown that this laying difference was induced by differential threshold responses to photoperiod (Lambrechts et al., 1996, 1997b; Lambrechts and Perret, 2000).

Slow initial testicular growth

Photoperiod is generally considered as the main "initial predictive cue" used by seasonal temperate zone birds to start their reproductive development in late winter–early spring (Wingfield et al., 1997; Wingfield and Moore, 1987). However, the view that increasing daylength above the critical daylength is the only source of photoperiodic information that temperate zone birds use to initiate seasonal breeding is over-simplistic; photoperiodic information provided by the whole seasonal cycle of changes in photoperiod is used to provide predictive information for the timing of the breeding season (Sharp, 1996, 2005). The key seasonal event which initiates a breeding season is the dissipation of either juvenile or adult photorefractoriness by short days (Nicholls et al., 1988; Williams et al., 1987), which is associated with a slow recovery of photoperiodic responsiveness (e.g., Dawson and Goldsmith, 1997; Sharp et al., 1986). The full recovery of photosensitivity, in some birds, is associated with slow gonadal development as observed in tits (Silverin, 1991b), starling (Gwinner and Ganshirt, 1982), and the grey partridge (Sharp et al., 1986) in late winter–early spring. This slow gonadal development can be seen as initiating the onset of the breeding season and has been shown not to depend on increasing short photoperiod in the starling (Gwinner and Ganshirt, 1982), grey partridge (Sharp et al., 1986) or great tit (Silverin et al., 1993). It is therefore likely that the increase in testicular volume observed in the Muro and Pirio tits in February was similarly not dependent on increasing short daylengths.

The mechanism responsible for increased slow gonadal growth on winter-like short days before the breeding season is uncertain but has been most fully investigated in the starling where it has been proposed to be part of the mechanism which generates circannual reproductive rhythms (Schwab and Rutledge, 1975). Irrespective of the mechanism involved, the timing of the initiation of slow gonadal development while still exposed to winter-like daylengths is a potential target for evolutionary pressure to adjust the timing of breeding to optimal environmental conditions. An example is provided by the red grouse (*Lagopus lagopus scoticus*) and the closely related willow ptarmigan (*Lagopus lagopus lagopus*) (Sharp and Moss, 1981). The first eggs are laid by red grouse at the end of April and by willow ptarmigan at the end of May both in captive and natural conditions. There is no difference in critical daylength in the two subspecies, but testicular activity is much greater when exposed to winter-like daylengths in red grouse than in the willow ptarmigan as reflected in the sizes of the testosterone-dependent supra-orbital combs. The earlier breeding season in the red grouse therefore can be accounted for, in part, by greater anticipatory testicular activity in the red grouse than in the willow ptarmigan while exposed to short days. This difference in short day testicular activity is genetically determined as demonstrated by the observation that testicular activity in red grouse–willow ptarmigan hybrids are intermediate between that of red grouse and willow ptarmigan. A similar difference in testicular development in Pirio and Muro blue tits in February could also contribute to the difference in the timing of the breeding season in the two populations. However, as demonstrated in Fig. 2, there is no evidence that testicular growth occurring between December and February differs between the two populations. This growth is likely to be a function of the rate of recovery of full photosensitivity, which is apparently synchronized in the two blue tit populations.

Rapid testicular growth

From late February–early March on, testis growth patterns between Muro and Pirio started to diverge as a function of a different response to photoperiod (differential thresholds or rates of reaction) and/or to non-photoperiodic supplemental factors. Although a difference in response to photoperiod may exist between these two populations of male tits, it apparently only induces an asynchrony in the seasonal patterns of plasma LH and testosterone that is relatively small (10–15 days) compared to the difference between egg laying dates (1 month). Despite the lower sampling frequency, which somehow limits the accuracy of the conclusions that can be drawn, it seems that the asynchrony in testicular development is also much smaller than 1 month. According to Silverin (1994), great tits are indeed able to reach their full testis size after only 2 weeks of photostimulation. If the same rate of growth applies to blue tits, this means that Muro birds started their rapid testicular growth at the latest by mid-March and that Pirio birds had to start at least 1 week before the end of March to reach the half maximal values by the end of March. The initiation of photoperiodic

testicular growth would thus be separated by only 1 or 2 weeks. The differential response to photoperiod can thus be held responsible for only 1 or 2 weeks of asynchrony in the reproductive development of these two male populations.

The differences in the final stages of male maturation (e.g., peak testes size, singing activity and behaviors directly linked to copulation) associated with the 1 month difference in egg laying dates should thus not depend only on a difference in photoperiodic response but should additionally be controlled by supplementary cues.

Among these supplementary cues, temperature, food availability, social factors and phenology of the vegetation are most often cited and are best documented. Temperature, food availability and phenology of the vegetation can probably be excluded based on experiments on captive birds kept in controlled photoperiods. Indeed, birds originating from the broad-leaved and evergreen forests retain their natural adaptive difference in egg laying onset when kept in aviaries containing the same type of vegetation, fed ad libitum and exposed to the same temperature regime (Lambrechts and Perret, 2000). It is therefore most likely that social factors are responsible for and control the differential reproductive growth rate in males. Among those factors, signals provided by the females are likely to play the most important role. In aviary experiments, males were indeed always kept in pairs with a female originating from their own populations. It can thus be hypothesized that the differential breeding dates observed both during the experiments made in captivity and in the field studies reported here are largely driven by the females. The adaptive differentiation of the breeding dates in these two blue tit populations might thus be sex-specific, with females being under stronger local resource-based selection pressures than males. Further studies of female development will probably help in solving this interesting question.

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References

Abercrombie, M., 1946. Estimation of nuclear population from microtome sections. Anat. Rec. 94, 239–247.

Absil, P., Pinxten, R., Balthazart, J., Eens, M., 2003. Effect of age and testosterone on autumnal neurogenesis in male European starlings (*Sturnus vulgaris*). Behav. Brain Res. 143, 15–30.

Ball, G.F., 1993. The neural integration of environmental information by seasonally breeding birds. Am. Zool. 33, 185–199.

Ball, G.F., Hahn, T.P., 1997. GnRH neuronal systems in birds and their relation to the control of seasonal reproduction. In: Parhar, I.S., Sakuma, Y. (Eds.), Brain Shuppan, Tokyo, pp. 325–342.

Ball, G.F., Riters, L.V., Balthazart, J., 2002. Neuroendocrinology of song behavior and avian brain plasticity: multiple sites of action of sex steroid hormones. Front. Neuroendocrinol. 23, 137–178.

Balthazart, J., 1983. Hormonal correlates of behavior. In: Farner, D.S., King, J.R., Parkes, K.C. (Eds.), Avian Biol., VII. Academic Press, New York, pp. 221–365.

Blondel, J., Dias, P.C., Maistre, M., Perret, P., 1993. Habitat heterogeneity and life-history variation of Mediterranean blue tits (*Parus caeruleus*). Auk 110, 511–520.

Blondel, J., Dias, P.C., Perret, P., Maistre, M., Lambrechts, M.M., 1999. Selection-based biodiversity at a small spatial scale in a low-dispersing insular bird. Science 285, 1399–1402.

Blondel, J., Perret, P., Dias, P., Lambrechts, M.M., 2001. Is phenotypic variation of blue tits (*Parus caeruleus*) in Mediterranean mainland and insular landscapes adaptive? Genet. Sel. Evol. 33, S121–S139.

Caro, S.P., Balthazart, J., Thomas, D.W., Lacroix, A., Chastel, O., Lambrechts, M.M., 2005a. Endocrine correlates of the breeding asynchrony between two Corsican populations of blue tits (*Parus caeruleus*). Gen. Comp. Endocrinol. 140, 52–60.

Caro, S.P., Lambrechts, M.M., Balthazart, J., 2005b. Early seasonal development of brain song control nuclei in male blue tits. Neurosci. Lett. 386, 139–144.

Catchpole, C.K., Slater, P.J.B., 1995. Bird Song. Cambridge University Press.

Chastel, O., Lacroix, A., Kersten, M., 2003. Pre-breeding energy requirements: thyroid hormone, metabolism and the timing of reproduction in house sparrows *Passer domesticus*. J. Avian Biol. 34 (3), 298–306.

Cho, R.N., Hahn, T.P., MacDougall-Shackleton, S., Ball, G.F., 1998. Seasonal variation in brain GnRH in free-living breeding and photorefractory house finches (*Carpodacus mexicanus*). Gen. Comp. Endocrinol. 109, 244–250.

Corbitt, C., Deviche, P., 2005. Age-related difference in size of brain regions for song learning in adult male dark-eyed juncos (*Junco hyemalis*). Brain Behav. Evol. 65, 268–277.

Dawson, A., 2003. A comparison of the annual cycles in testicular size and moult in captive European starlings *Sturnus vulgaris* during their first and second years. J. Avian Biol. 34, 119–123.

Dawson, A., Goldsmith, A.R., 1997. Changes in gonadotrophin-releasing hormone (GnRH-I) in the pre-optic area and median eminence of starlings (*Sturnus vulgaris*) during the recovery of photosensitivity and during photostimulation. J. Reprod. Fertil. 111, 1–6.

Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in birds. J. Biol. Rhythms 16, 365–380.

De Laet, J.F., Dhondt, A.A., De Boever, J.G., 1985. Circannual plasma androgen levels in free-living male great tits (*Parus major major* L.). Gen. Comp. Endocrinol. 59, 277–286.

Deviche, P., Wingfield, J.C., Sharp, P.J., 2000. Year-class differences in the reproductive system, plasma prolactin and corticosterone concentrations, and onset of prebasic molt in male dark-eyed juncos (*Junco hyemalis*) during the breeding period. Gen. Comp. Endocrinol. 118, 425–435.

Foerster, K., Poesel, A., Kunc, H., Kempenaers, B., 2002. The natural plasma testosterone profile of male blue tit during the breeding season and its relation to song output. J. Avian Biol. 33, 269–275.

Forslund, P., Part, T., 1995. Age and reproduction in birds—Hypotheses and tests. Trends Ecol. Evol. 10, 374–378.

Grieco, F., van Noordwijk, A.J., Visser, M.E., 2002. Evidence for the effect of learning on timing of reproduction in blue tits. Science 296, 136–138.

Gwinner, E., Ganshirt, G., 1982. Testicular development in European starlings in early spring. Naturwissenschaften 69, 555–557.

Hahn, T.P., Ball, G.F., 1995. Changes in brain GnRH associated with photorefractoriness in house sparrows (*Passer-domesticus*). Gen. Comp. Endocrinol. 99, 349–363.

Hau, M., 2001. Timing of breeding in variable environments: tropical birds as model systems. Horm. Behav. 40, 281–290.
Ketterson, E.D., Nolan, V., 1992. Hormones and life histories—An integrative approach. Am. Nat. 140, S33–S62.
Lack, D., 1968. Ecological Adaptations for Breeding in Birds. Methuen, London.
Lambrechts, M.M., Dias, P.C., 1993. Differences in the onset of laying between island and mainland Mediterranean blue tits *Parus caeruleus*: phenotypic plasticity or genetic differences? Ibis 135, 451–455.
Lambrechts, M.M., Perret, P., 2000. A long photoperiod overrides non-photoperiodic factors in blue tits' timing of reproduction. Proc. R. Soc. London B 267, 585–588.
Lambrechts, M.M., Visser, M.E., 1999. Proximate aspects of the timing of reproduction. In: Adams, N.J., Slotow, R.H. (Eds.), Proc. 22 Int. Ornithol. Congr., Durban. BirdLife, South Africa, Johannesburg, pp. 231–233.
Lambrechts, M.M., Perret, P., Blondel, J., 1996. Adaptive differences in the timing of egg laying between different populations of birds result from variation in photoresponsiveness. Proc. R. Soc. London B 263, 19–22.
Lambrechts, M.M., Blondel, J., Hurtrez-Boussès, S., Maistre, M., Perret, P., 1997a. Adaptive inter-population differences in blue tit life-history traits on Corsica. Evol. Ecol. 11, 599–612.
Lambrechts, M.M., Blondel, J., Maistre, M., Perret, P., 1997b. A single response mechanism is responsible for evolutionary adaptive variation in a bird's laying date. Proc. Natl. Acad. Sci. U. S. A. 94, 5153–5155.
Lambrechts, M.M., Caro, S.P., Charmantier, A., Gross, N., Galan, M.J., Perret, P., Cartan-Son, M., Dias, P.C., Blondel, J., Thomas, D.W., 2004. Habitat quality as a predictor of spatial variation in blue tit reproductive performance: a multi-plot analysis in a heterogeneous landscape. Oecologia 141, 555–561.
Mazuc, J., Bonneaud, C., Chastel, O., Sorci, G., 2003. Social environment affects female and egg testosterone levels in the house sparrow (*Passer domesticus*). Ecol. Lett. 6, 1084–1090.
Moore, I.T., Bonier, F., Wingfield, J.C., 2005. Reproductive asynchrony and population divergence between two tropical bird populations. Behav. Ecol. 16, 755–762.
Murton, R.K., Westwood, N.J., 1977. Avian Breeding Cycles. Oxford University Press, London.
Nager, R.G., van Noordwijk, A.J., 1995. Proximate and ultimate aspects of phenotypic plasticity in timing of great tit breeding in a heterogeneous environment. Am. Nat. 146, 454–474.
Nicholls, T.J., Goldsmith, A.R., Dawson, A., 1988. Photorefractoriness in birds and comparison with mammals. Physiol. Rev. 68, 133–176.
Partecke, J., Van't Hof, T., Gwinner, E., 2004. Differences in the timing of reproduction between urban and forest European blackbirds (*Turdus merula*): result of phenotypic flexibility or genetic differences? Proc. R. Soc. London B 271, 1995–2001.
Partecke, J., Van't Hof, T., Gwinner, E., 2005. Underlying physiological control of reproduction in urban and forest-dwelling European blackbirds *Turdus merula*. J. Avian Biol. 36, 295–305.
Perfito, N., Tramontin, A.D., Meddle, S., Sharp, P., Afik, D., Gee, J., Ishii, S., Kikuchi, M., Wingfield, J.C., 2004. Reproductive development according to elevation in a seasonally breeding male songbird. Oecologia 140, 201–210.
Pinxten, R., De Ridder, E., Balthazart, J., Eens, M., 2002. Context-dependent effects of castration and testosterone treatment on song in male European starlings. Horm. Behav. 42, 307–318.
Reiner, A., Perkel, D.J., Bruce, L.L., Butler, A.B., Csillag, A., Kuenzel, W., Medina, L., Paxinos, G., Shimizu, T., Striedter, G., Wild, M., Ball, G.F., Durand, S., Gunturkun, O., Lee, D.W., Mello, C.V., Powers, A., White, S.A., Hough, G., Kubikova, L., Smulders, T.V., Wada, K., Dugas-Ford, J., Husband, S., Yamamoto, K., Yu, J., Siang, C., Jarvis, E.D., 2004. Revised nomenclature for avian telencephalon and some related brainstem nuclei. J. Comp. Neurol. 473, 377–414.
Schwab, R.G., Rutledge, J.T., 1975. Stable daily light regimes as inductive factors of endogenous testicular cycles in the European starling *Sturnus vulgaris*. Int. J. Biometeorol. 19, 219–231.
Schwabl, H., Flinks, H., Gwinner, E., 2005. Testosterone, reproductive stage, and territorial behavior of male and female European stonechats *Saxicola torquata*. Horm. Behav. 47, 503–512.
Sharp, P.J., 1996. Strategies in avian breeding cycles. Anim. Reprod. Sci. 42, 505–513.
Sharp, P.J., 2005. Photoperiodic regulation of seasonal breeding in birds. Ann. N.Y. Acad. Sci. 1040, 189–199.
Sharp, P.J., Moss, R., 1981. A comparison of the responses of captive Willow Ptarmigan (*Lagopus lagopus lagopus*), Red Grouse (*Lagopus lagopus scoticus*) and hybrids to increasing daylengths with observations on the modifying effects of nutrition and crowding on Red Grouse. Gen. Comp. Endocrinol. 45, 181–188.
Sharp, P.J., Massa, R., Bottoni, L., Lucini, V., Lea, R.W., Dunn, I.C., Trocchi, V., 1986. Photoperiodic and endocrine control of seasonal breeding in grey partridge (*Perdrix perdrix*). J. Zool. Lond. (A) 209, 187–200.
Sharp, P.J., Dunn, I.C., Talbot, R.T., 1987. Sex-differences in the LH responses to chicken LHRH-I and LHRH-Ii in the domestic-fowl. J. Endocrinol. 115, 323–331.
Sharp, P.J., Dunn, I.C., Main, G.M., Sterling, R.J., Talbot, R.T., 1990. Gonadotropin releasing hormones: distribution and function. In: Wada, M., Ishii, S., Scanes, C.G. (Eds.), Endocrinology of Birds: Molecular to Behavioral. Springer Verlag, Berlin, pp. 31–42.
Silver, R., Ramos, C., Machuca, H., Silverin, B., 1992. Immunocytochemical distribution of GnRH in the brain of adult and posthatching great tit *Parus major* and ring dove *Streptopelia roseogrisea*. Orn. Scand. 23, 222–232.
Silverin, B., 1991a. Behavioral, hormonal, and morphological responses of free-living male pied flycatchers to estradiol treatment of their mates. Horm. Behav. 25, 38–56.
Silverin, B., 1991b. Some aspects of seasonal changes in circulating levels of LH and testosterone in willow tits (*Parus montanus*) and great tits (*Parus major*). Proc. Int. Ornithol. Congr. Christchurch, New Zealand, pp. 2081–2091.
Silverin, B., 1994. Photoperiodism in male great tits (*Parus major*). Ethol. Ecol. Evol. 6, 131–157.
Silverin, B., 1995. Reproductive adaptations to breeding in the north. Am. Zool. 35 (3), 191–202.
Silverin, B., Massa, R., Stokkan, K.A., 1993. Photoperiodic adaptation to breeding at different latitudes in great tits. Gen. Comp. Endocrinol. 90, 14–22.
Silverin, B., Kikuchi, M., Ishii, S., 1997. Seasonal changes in follicle-stimulating hormone in free-living great tits. Gen. Comp. Endocrinol. 108, 366–373.
Sockman, K.W., Williams, T.D., Dawson, A., Ball, G.F., 2004. Prior experience with photostimulation enhances photo-induced reproductive development in female European starlings: a possible basis for the age-related increase in avian reproductive performance. Biol. Reprod. 71, 979–986.
Stevenson, T.J., MacDougall-Shackleton, S.A., 2005. Season- and age-related variation in neural cGnRH-I and cGnRH-II immunoreactivity in house sparrows (*Passer domesticus*). Gen. Comp. Endocrinol. 143 (1), 33–39.
Thomas, D.W., Blondel, J., Perret, P., Lambrechts, M.M., Speakman, J.R., 2001. Energetic and fitness costs of mismatching resource supply and demand in seasonally breeding birds. Science 291, 2598–2600.
Tramontin, A.D., Perfito, N., Wingfield, J.C., Brenowitz, E.A., 2001. Seasonal growth of song control nuclei precedes seasonal reproductive development in wild adult song sparrows. Gen. Comp. Endocrinol. 122, 1–9.
van Duyse, E., Pinxten, R., Eens, M., 2003. Seasonal fluctuations in plasma testosterone levels and diurnal song activity in free-living male great tits. Gen. Comp. Endocrinol. 134, 1–9.
van Gils, J., Absil, P., Grauwels, L., Moons, L., Vandesande, F., Balthazart, J., 1993. Distribution of luteinizing hormone-releasing hormones I and II (LHRH-I and-II) in the quail and chicken brain as demonstrated with antibodies directed against synthetic peptides. J. Comp. Neurol. 334, 304–323.
van Noordwijk, A.J., McCleery, R.H., Perrins, C.M., 1995. Selection for the timing of great tit breeding in relation to caterpillar growth and temperature. J. Anim. Ecol. 64, 451–458.
Visser, M.E., Lambrechts, M., 1999. Information constraints in the timing of reproduction in temperate zone birds: great and blue tits. In: Adams, N.J., Slotow, R.H. (Eds.), Proc. 22 Int. Ornithol. Congr., Durban. BirdLife, South Africa, Johannesburg, pp. 249–264.

Williams, T.D., Dawson, A., Nicholls, T.J., Goldsmith, A.R., 1987. Short days induce premature reproductive maturation in juvenile starlings, *Sturnus vulgaris*. J. Reprod. Fertil. 80, 327–333.

Wingfield, J.C., Moore, M.C., 1987. Hormonal, social, and environmental factors in the reproductive biology of free-living male birds. In: Crews, D. (Ed.), Psychology of Reproduction: An Evolutionary Perspective. Inc. Englewood Cliffs, New Jersey, Prentice-Hall, pp. 148–175.

Wingfield, J.C., Silverin, B., 2002. Ecophysiological studies of hormone-behavior relations in birds. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Brain and Behavior. Academic Press, San Diego, pp. 587–648.

Wingfield, J.C., Jacobs, J., Hillgarth, N., 1997. Ecological constraints and the evolution of hormone–behavior interrelationships. Integr. Neurobiol. Affil. 22–41.