

Social Context Affects Testosterone-Induced Singing and the Volume of Song Control Nuclei in Male Canaries (*Serinus canaria*)

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ABSTRACT: The contribution of social factors to seasonal plasticity in singing behavior and forebrain nuclei controlling song, and their interplay with gonadal steroid hormones are still poorly understood. In many songbird species, testosterone (T) enhances singing behavior but elevated plasma T concentrations are not absolutely required for singing to occur. Singing is generally produced either to defend a territory or to attract a mate and it is therefore not surprising that singing rate can be influenced by the sex and behavior of the social partner. We investigated, based on two independent experiments, the effect of the presence of a male or female partner on the rate of song produced by male canaries. In the first experiment, song rate was measured in dyads composed of one male and one female (M-F) or two males (M-M). Birds were implanted with T-filled Silastic capsules or with empty capsules as control. The number of complete song bouts produced by all males was recorded during 240 min on week 1, 2, 4, and 8 after implantation. On the day following each recording session, brains from approximately one-fourth of the birds were collected and the volumes of the song control nuclei HVC and RA were measured. T increased the singing rate and volume of HVC and RA but these effects were affected by the social context. Singing rates were higher in the M-M

than in the M-F dyads. Also, in the M-M dyads a dominance-subordination relationship soon became established and dominant males sang at higher rates than subordinates in T-treated but not in control pairs. The differences in song production were not reflected in the size of the song control nuclei: HVC was larger in M-F than in M-M males and within the M-M dyads, no difference in HVC or RA size could be detected between dominant and subordinate males. At the individual level, the song rate was positively correlated with RA and to a lower degree HVC volume, but this relationship was observed only in M-M dyads, specifically in dominant males. A second experiment, carried out with castrated males that were all treated with T and exposed either to another T-treated castrate or to an estradiol-implanted female, confirmed that song rate was higher in the M-M than in the M-F condition and that HVC volume was larger in heterosexual than in same-sex dyads. The effects of T on singing rate and on the volume of the song control nuclei are thus modulated by the social environment, including the presence/absence of a potential mate and dominance status among males. © 2006 Wiley Periodicals, Inc. *J Neurobiol* 66: 1044–1060, 2006

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INTRODUCTION

The main functions that have been ascribed to song behavior in passerine birds are territory defense (or spacing behavior) and mate attraction (Kroodsma and Byers, 1991; Collins, 2004). Song production is controlled by a specialized network of brain nuclei, called

the song control system, which can be divided into an anterior forebrain pathway involved in song learning during ontogeny and maintenance of song stability in adulthood and a posterior motor circuit that regulates song production (Nottebohm et al., 1976; Brenowitz et al., 1997; Doupe and Kuhl, 1999; Jarvis, 2004). The motor pathway consists of the telencephalic nucleus HVC (used as a proper name; see Reiner et al., 2004a,b for the recent revision of the avian brain nomenclature), which projects to the nucleus robustus of the arcopallium (RA). This nucleus then projects to several medullary components, including the tracheo-syringeal part of the nucleus of the XIIth cranial nerve that innervates the vocal production organ, the syrinx, as well as to nuclei that coordinate respiratory activity with song production (Wild, 1994, 2004).

Singing rates are, in many species, increased in the presence of high circulating levels of the gonadal hormone, testosterone (T) (Schlinger, 1997; Ball et al., 2003; Harding, 2004), but elevated concentrations of T are not absolutely required for singing to occur (Ball et al., 2003). For example, some species display high singing rates in the fall when T plasma concentrations are low or undetectable (Riters et al., 2000; Soma et al., 2002). One can therefore characterize song, like many other hormone-modulated behaviors, as being facilitated by T but not regulated by the steroid in an all or none fashion (Ball et al., 2003). Nevertheless, in many of the songbird species living in the temperate zone, the vernal increase in day length stimulates testicular activity and the resulting increase in plasma T results in an increase in singing activity (Ball, 1999). During the spring, there is also in parallel a marked increase in the volume of several song control nuclei such as HVC and RA (Nottebohm, 1981; see Ball, 1999; Brenowitz, 2004 for reviews). Based on this correlated vernal increase in singing rate and in the volume of song control nuclei and on the observation that treatment of castrated males increases both the volume of these brain nuclei and singing rates (Ball et al., 2003), it was originally assumed that the increase in plasma T directly causes an increase in HVC and RA size (through changes in neuronal spacing and branching and through increased incorporation and survival of new neurons in HVC; Brenowitz, 2004), which then leads as a consequence to changes in singing behavior.

Three types of observations indicate, however, that the increase in plasma T is not the only mechanism involved in the control of seasonal changes in HVC and RA volume:

1. High plasma levels of T are not always necessary to induce the growth of song control nuclei. In song sparrows (*Melospiza melodia*), for ex-

ample, HVC and RA are already developed in early spring when plasma T concentrations have shown a small increase but are still well below maximal concentrations that will occur later (Smith et al., 1997; Tramontin et al., 2001).

2. Long photoperiods have been shown to increase the size of song nuclei to varying degrees even in castrated birds with low to nondetectable T concentrations such as American tree sparrows (*Spizella arborea*) (Bernard et al., 1997), Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) (Smith et al., 1997), starlings (*Sturnus vulgaris*) (Bentley et al., 1999), and dark-eyed juncos (*Junco hyemalis*) (Dloniak and Deviche, 2001).
3. Social cues, such as those provided by a sexually receptive female, are also able to enhance the seasonal growth of song nuclei in white-crowned sparrows (Tramontin et al., 1999), again independently of T.

The respective roles of these different factors remain, however, unclear at present. This article focuses on the effects of social cues in the control of singing and of the growth of song control nuclei in canaries.

Recent work in European starlings (*S. vulgaris*) and canaries (*Serinus canaria*) points to one way by which effects of social cues on singing could regulate the development of song control nuclei. It was shown that not only treatment with T (Rasika et al., 1999) but the act of singing *per se* enhances the central production of brain-derived neurotrophic factor (BDNF) (Li et al., 2000), a protein that increases the recruitment and survival of new neurons in HVC (Li et al., 2000; Alvarez-Borda and Nottebohm, 2002; Alvarez-Borda et al., 2004) of canaries. In starlings maintained in male-male (M-M) dyads, males that sing at very high rates have larger volumes of HVC without apparent differences in T (Sartor and Ball, 2005). These sorts of findings suggest that high singing itself may promote HVC neuroplasticity independently of T (Ball et al., 2004).

Song is used in both intersexual (courtship, to attract mates) and intrasexual (competition, to declare and defend a territory) contexts. It is therefore expected that the singing rate of a male, potentially as a consequence of the size of its song control nuclei, varies as a function of the social environment a male is exposed to. Relatively few studies have investigated the role of social factors in this regard. In one significant study, it was shown in captive white-crowned sparrows that the song rate and song nuclei size were larger in males housed with a sexually

receptive female than in males kept alone or kept in M-M dyads (Tramontin et al., 1999), and this effect appears to be independent of T in that plasma concentrations of T did not differ between the two groups.

In male songbirds there is also clear evidence that social status can modulate song output. A positive correlation between male dominance status and different components of song behavior, such as song rate and repertoire size, has, for example, been reported in several species (chickadees *poecile atricapilla*, Otter et al., 1997; starlings, Riters et al., 2004; Sartor and Ball, 2005; Spencer et al., 2004), while field studies have showed that conspecific density, and therefore the male social environment, markedly affects vocal behavior (e.g., Galeotti et al., 1997). One experimental study specifically investigated the interaction between dominance, singing rate, and T. In white-throated sparrows (*Zonotrichia albicollis*), males with the highest rank sang most frequently in natural situations. When the birds were brought into aviaries and implanted with T, top ranking T-treated birds sang more frequently than lower ranking birds, while very low ranking birds with T implants never sang (Wiley et al., 1993). These findings indicate that in male flocks the highest rates of singing require high social status as well as high T concentrations in the plasma.

In this study, we investigated the potential interactions between endocrine state (presence/absence of exogenous T), social environment (presence of a male or a female), and hierarchical status (dominant/subordinate) in the control of singing output and growth of the song control nuclei, HVC and RA in domestic male canaries. Canary song functions in part as a mate attraction vocalization and clearly can serve as a cue for female choice (Vallet and Kreutzer, 1995; Leitner et al., 2001a; Leitner and Catchpole, 2002). Studies of wild canaries indicate that they do not defend large all-purpose territories but rather small areas around nest sites (Leitner et al., 2001a). However, the presence of another male can elicit increased rates of singing in male canaries (Kreutzer et al., 1999). The results of our study demonstrate that effects of T on singing and on song control nuclei are modulated by complex interactions between the social environment, including the presence/absence of a potential mate, and dominance status among males.

MATERIALS AND METHODS

Subjects and Housing

Two independent experiments were carried out with male and female adult canaries (*S. canaria*) that were obtained in

October 2003 (experiment 1) and June 2004 (experiment 2) from a local dealer in Liège (Belgium). Before their arrival in the laboratory, birds were housed in outdoor aviaries under natural photoperiod. Immediately after their arrival in the university animal facility, all birds were housed indoors in unisex flocks of three to four individuals at a stable temperature (20–24°C) under a 11:13 L/D day photoperiod. No male was housed with a female nor with his future cage-mate. All experimental procedures were in agreement with the Belgian laws on protection and welfare of animals and on the protection of experimental animals and the International Guiding Principles for Biomedical Research Involving Animals published by the Council for International Organizations of Medical Sciences. The protocols were approved by the Ethics Committee for the Use of Animals at the University of Liège.

Food, water, sand, and bathing water were always available *ad libitum*. All birds were yearlings and therefore presumably sexually naive before the beginning of these experiments. Specific aspects of the protocol of each experiment will first be described before considering methods common to both studies.

Experiment 1

Ten females and 36 males were used for this experiment. Preliminary observations carried out during the month that followed arrival in the laboratory while birds were in unisex groups of three to four subjects indicated that song production was very low or absent in all males, suggesting that they had at that time regressed testes, as could be expected for first-year birds in October. The regressed state of the testes (length smaller than 1 mm) was further confirmed by laparotomy in four subjects.

These birds were then randomly assigned to 13 M-M dyads and 10 male-female dyads (M-F) that were housed for the entire experiment in individual cages (29 × 33 × 40 cm) in a room still exposed to a photoperiod of 11:13 L/D. On the same day (day 0 of the experiment), all birds were implanted with one Silastic capsule filled with either crystalline T (Cat. # 86500; Fluka Chemika, Buchs, Switzerland) or left empty as control (C). Capsules were made of Silastic tubing (Cat. # 602-175; outer diameter: 1.65 mm, inner diameter: 0.76 mm; Degania Silicone, Degania Bet, Israel) cut at a length of 12 mm that was closed at both ends with Silastic glue (Silastic Medical Adhesive Silicone type A; Coventy, UK), leaving a length of 10 mm that was either filled with T or empty. This type of capsule has been shown previously to restore circulating levels of T to levels typical of sexually mature males in castrated male canaries (Nottebohm, 1980; Appeltants et al., 2003).

Both birds in each dyad received the same type of implant (T or C) and approximately one-half of all birds were submitted to each endocrine treatment, thus providing six T and seven C M-M dyads and six T and four C M-F dyads. One additional goal of the present study was originally to assess the effects of T on the singing behavior in females. However, no useful data were obtained on this

question given the small number of available female subjects, their low apparent singing rate (possibly as a consequence of social suppression by males), and, most importantly, because it was quickly realized that the singing activity of females, which consists of shorter, less intense songs than in males, could not be accurately recorded with the experimental procedures that had been selected. Females indeed never sang full songs even after T implantation, but they produced short vocalizations (trills) comprising a few notes only, which almost invariably were interrupted by the male they were housed with. These trills were not taken into account for the present study.

All birds were housed in the same room in visual but not acoustic isolation. Singing of all subjects was observed and quantified systematically by an observer (G.B.) who was blind to the endocrine condition of the subjects. Quantification was carried out four times per day during 60 min (8:00; 11:00; 14:00; 18:00) on day 6, 13, 27, and 55 (weeks 1, 2, 4, and 8).

The numbers of complete songs (longer than three phrases or trills, defined as the repetition of the same note or syllable; see Williams, 2004 for definitions) expressed by each male during the 60 min recording periods were counted by direct observation. Previous training during preliminary observations allowed the observer to rapidly identify each singing bird based on specific characteristics of its song. The identification of the singing subject was then confirmed visually.

This procedure allowed us to record songs from all birds in the group simultaneously for extended periods. Taping or actual recording with a stopwatch would have required separate observation of single individuals, which was not only time-consuming (hundreds of hours would have been required) but also would have resulted in songs from the different subjects being recorded at different times (it is impossible to score or tape that many birds at the same time), resulting in additional variance in the data set (song output varies enormously from one time to another). We therefore opted for this semiquantitative scoring system that was also used in many other previously published studies (see for example: Tramontin et al., 1999; Li et al., 2000; Alvarez-Borda and Nottebohm, 2002).

During each hour, each cage in turn was also sampled focally for 5 min and all songs produced in that cage were noted. This therefore generated for each observation period another measure of song rate during 5 min, which was more accurate than the song rate measured during the entire 60 min period (some vocalizations could potentially have been missed). Statistical analysis identified a high positive correlation between the total number of songs recorded on day 6 (during 4×60 min) and the number of songs recorded during these focal periods (4×5 min) of observation ($r = 0.824$, $n = 46$, $p < 0.0001$). This high degree of correspondence between the two scores provides evidence that the singing behavior of all subjects was accurately recorded by the observer who was previously trained to recognize individual song characteristics. This analysis was carried out on data collected on day 6 when all subjects ($n = 46$) were still present in the experimental room. The accuracy of recording could only increase when the number of experimental subjects decreased (see below) as the experiment progressed.

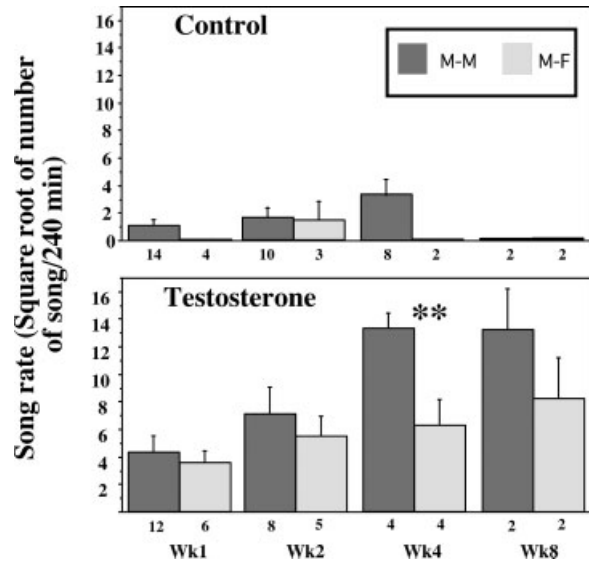


Figure 1 Effects of the presence of a female (M-F) or another male (M-M) on the song rate expressed by male canaries that were either treated with testosterone (T) or left intact as controls (C). Song recording took place on week 1, 2, 4, and 8 after the beginning of the treatment with testosterone. Average song rates (\pm SEM) measured in control (top panel) and T-treated (bottom panel) canaries are presented for all birds that were available on each given date. Data from each week were analyzed by a separate two-way ANOVA that indicated significant effects of T on each week and a significant effect to the dyad type (** $p < 0.01$) on week 4. The numbers below the bars indicate the numbers of subjects available for each specific set of data.

Within M-M dyads it readily became apparent that expression of aggressive interactions was leading in most cages to the establishment of a dominance-subordinate hierarchy. The hierarchy in these dyads was determined based on the most frequent position of each bird on the perches (high or low perch in the cage) as observed during nonsystematic spot checks of the bird's activity and during the systematic song recording sessions. This position on the perches corresponded with the more frequent expression of aggressive acts such as wing waves, attacks, and horizontal postures with open bill in the high perching bird.

Approximately one-fourth of the birds were killed during the day following each series of observations, that is, on days 7 (week 1), 14 (week 2), 28 (week 4), and 56 (week 8). The two birds housed in the same cage were always killed on the same day. Birds from all social and endocrine conditions were randomly distributed among the 4 days of sacrifice (see Fig. 1 for exact numbers of birds killed on each date).

Experiment 2

The 17 males and seven females used in this experiment were first maintained for 2 weeks in unisex groups of three

subjects under an 11:13 L/D photoperiod. The sex of all birds was then confirmed by laparotomy and all males were castrated at that time by methods that were previously described (Sartor et al., 2005). They were then allowed to recover for 2 weeks in the same social and environmental conditions and transferred to a short photoperiod (6:18 L/D) for 8 weeks to make sure they would be photosensitive at the beginning of the endocrine treatments (Nicholls and Storey, 1977).

At the beginning of the experiment (day 0), all males were implanted with one 10 mm long Silastic capsule filled with T (same characteristics as above) and all females with one 10 mm long Silastic capsule (same characteristics for tubing also) filled with estradiol-17 β (E2; Cat. #: E8875-5G; Sigma, St. Louis, MO). This dose of E2 was previously shown to induce sexual receptivity in female white-crowned sparrows held under short days (Tramontin et al., 1999) and in female canaries (Leboucher et al., 1994). At that time, birds were randomly assigned to five M-M dyads and seven M-F dyads that were kept in individual cages (same as above) held in the same room, in visual but not acoustic isolation (see above). On that day, photoperiod was also switched to 16:8 L/D.

The singing rates of all males were then quantified by the methods described above three times a day during 60 min (10:00; 14:00; 18:00) on day 4, 6, 8, 11, 13, 15, 18, and 20. All birds were killed on day 21.

Perfusion

Birds were first tranquilized by an injection of 25 μ L Medetomidin (Domitor; Pfizer, Louvain-la-Neuve, Belgium), which was followed 10 min later by a complete anesthesia induced by an injection of 25 μ L of a mixture of zolazepam and tiletamine, both at 50 mg/mL (Zoletil; Virbac, Louvain-la-Neuve, Belgium). The thoraco-abdominal cavity was then opened, 50 μ L of heparin (20 mg/mL) was injected into the left ventricle, and birds were perfused through the left ventricle with 50–75 mL saline followed by approximately 100 mL of fixative [4% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M phosphate buffered saline (PBS)]. The brain was removed from the skull, post-fixed in the same fixative without glutaraldehyde for 2 h, and then cryoprotected in a solution of sucrose (20%) in PBS. Brains were frozen on dry ice and stored at -80°C .

During experiment 1, the testes of all birds were then measured and weighed, and the presence of the implants was confirmed. After perfusion during experiment 2, all males were autopsied and it could be confirmed that they were all completely castrated (no testes regrowth) and their implants were still in place.

Histology and Brain Analyses

Frozen brains were cut in coronal sections on a cryostat at 30 μ m thickness from the level of the locus coeruleus (LoC) to the rostral extent of Area X. Every sixth section

was Nissl-stained with Toluidine Blue and analyzed under the microscope at a magnification of 4X.

All sections containing the song control nuclei, HVC, RA, and the nucleus rotundus (Rt), a reference nucleus that can serve as a control in that it is not part of the song control system and whose volume is not expected to vary seasonally, were identified and digitized with a CCD camera connected to a Macintosh computer. The area of each nucleus was traced on both sides of the brain and measured on the digitized image using the NIH Image software (version 1.52; Wayne Rasband, Bethesda, MD). Areas were summed and multiplied by the sampling interval (180 μ m) to provide an estimate of volumes. The volumes of HVC and RA (average of volumes on the left and right side) were analyzed as raw data or after being divided by the volume of Rt (HVC/Rt and RA/Rt) to correct for possible individual differences in brain volume.

Data Analysis

Behavioral and morphological data for all males were analyzed by one-, two-, and three-way analyses of variance (ANOVA) with repeated measures as appropriate. The data of all males were considered as independent so that the degrees of freedom in these ANOVA correspond to the numbers of subjects (two in each M-M dyad, one in each M-F dyad) not the number of dyads. However, we also analyzed some of these data using the number of dyads instead of the number of subjects as statistical units for the analysis. In this case, the average of the behavior rates of the two birds in a M-M dyad was entered in the analysis. As is generally recommended (Keppel, 1982) analyses always started with a full model including all factors that were investigated, but nonsignificant factors (except for the factor of interest, dyad type or dominance status) were removed stepwise when they were not associated with significant main effect or interactions.

These ANOVAs were followed when appropriate by Fisher protected least significant difference (PLSD) posthoc tests. Behavioral variables that were considered (in one or both experiments) included the total number of songs produced during the entire experiment (total number of songs) and the total number of songs produced during the last day preceding sacrifice (songs last day). These totals were always computed for the four (experiment 1) or three (experiment 2) 60 min recording periods of the day. A correlational approach (simple linear regression and Pearson's product moment correlation coefficient) was also used to test the association between song output and brain morphology data.

Because behavioral data contain a large number of zero scores and were thus approaching a Poisson distribution, they were transformed to their square root to normalize distributions (Dagnelie, 1970). All analyses of song rates presented here were performed on these transformed data. Differences were considered significant for $p < 0.05$. All data are presented in the text and figures as means \pm SEM.

RESULTS

Experiment 1

Effects of the Sex of the Partner on Singing. We analyzed by four separate two-way ANOVAs (treatment and type of dyad as factors) the singing rate of all subjects that had been recorded at each separate week. Separate analyses had to be carried out because some males had their brains collected for histological studies after each week of observation. These data are illustrated in Figure 1, which also indicates the number of males recorded at each separate date (with n decreasing as the experiment progressed).

These four ANOVAs all confirmed the presence of a significant effect of T [$F(1, 32) = 10.317, p = 0.0030$; $F(1, 22) = 8.157, p = 0.0092$; $F(1, 14) = 26.885, p = 0.0001$; and $F(1, 4) = 26.283, p = 0.0069$ on weeks 1, 2, 4, and 8 respectively]. No interaction between treatment and dyad type could be detected (all $p \geq 0.29$), but a significant effect of the dyad type was present at week 4 [$F(1, 14) = 11.401, p = 0.0045$]. As is clearly apparent in Figure 1, the difference in singing rate between males exposed to another male or to a female increased progressively as the experiment progressed and reached statistical significance on week 4. The magnitude of this difference remained approximately at the same level on week 8, but the decrease in the number of available subjects prevented this effect from being significant.

The same analyses using the number of dyads instead of the numbers of subjects as units to avoid the potential pseudo-replication in the data fully confirmed these results and identified a significant or nearly significant effect of the endocrine treatment at each separate date [respectively: $F(1, 19) = 13.270, p = 0.0017$; $F(1, 13) = 12.525, p = 0.0036$; $F(1, 8) = 17.877, p = 0.0029$; $F(1, 2) = 17.865, p = 0.0517$], as well as a significant effect of the dyad type at week 4 only [$F(1, 8) = 9.229, p = 0.0161$]. No interaction was significant in these analyses (all $p \geq 0.34$).

Effect of Social Dominance on Singing. A large number of aggressive interactions were observed in the M-M dyads included in this experiment and rapidly a clear dominance-subordination relationship was obvious in 11 out of 13 of the M-M dyads (five T and six C pairs), based on the position occupied by each bird in the cage. For two dyads it was not possible to establish unambiguously the dominance relationship and these four males were thus removed from the corresponding analyses. We wondered whether singing rates of males from these 11 M-M dyads varied as a function of the hierarchical status of each bird.

The total number of songs produced in the day preceding the bird's sacrifice was first analyzed by a three-way ANOVA with two independent factors [treatment (T vs. C) and weeks (four levels)] and one within-subjects factor (social status, two levels). All main factors and interactions were nonsignificant in this analysis with the exception of the endocrine condition that was associated with a significant effect [$F(1, 4) = 9.423, p = 0.0373$]. The power of this analysis for the factor week (four levels) was, however, weak, and because this factor was not associated with a significant effect and was not interacting significantly with other factor, it was removed from the model. The resulting two-way ANOVA with mixed design (endocrine treatment and social status as factors) revealed a significant effect of the social status on the singing rate [$F(1, 9) = 8.487, p = 0.0172$]. After pooling data from the different weeks, the endocrine treatment had no significant effect [$F(1, 9) = 1.769, p = 0.2161$] but interacted almost significantly with status [$F(1, 9) = 4.735, p = 0.0575$]. As illustrated in Figure 2(A), the effect of T was clearly more prominent in dominant than in subordinate birds, which was confirmed by two matched-pairs t tests comparing singing rates in dominants and subordinates (in T birds: $t = 4.965, df = 4, p = 0.0077$; in C birds: $t = 0.4560, df = 5, p = 0.6650$). The difference in T birds was significant even after a Bonferroni correction taking into account the number of tests performed.

Qualitative analysis of these data suggested that the effect of status was present throughout the experiment right from the start. Analyses focusing on week 1 or week 2 (when sample size was the largest) confirmed that the effect of social status on singing rate was already present and significant during the first 2 weeks of the experiment [$F(1, 9) = 6.687, p = 0.0294$ and $F(1, 6) = 16.594, p = 0.0065$ on days 6 and 13 respectively; see Fig. 2(B)]. The effect of the endocrine treatment was, however, significant only on week 2 [$F(1, 6) = 8.936, p = 0.023$] and it significantly interacted with the effect of status [$F(1, 6) = 6.346, p = 0.0453$; see Fig. 2].

Song Control Nuclei. The volumes of HVC and RA could be successfully reconstructed in all males except four: one T-treated and two control birds in week 1, and one control in week 4. Analysis of the HVC volumes by three-way ANOVA (treatments, dyad types, and sacrifice week as factors) produced a different pattern of results compared to the analysis of song rates. No effect of the endocrine treatment was detected in the analysis of HVC volumes [$F(1, 17) = 0.467, p = 0.5032$] but main effects of the dyad

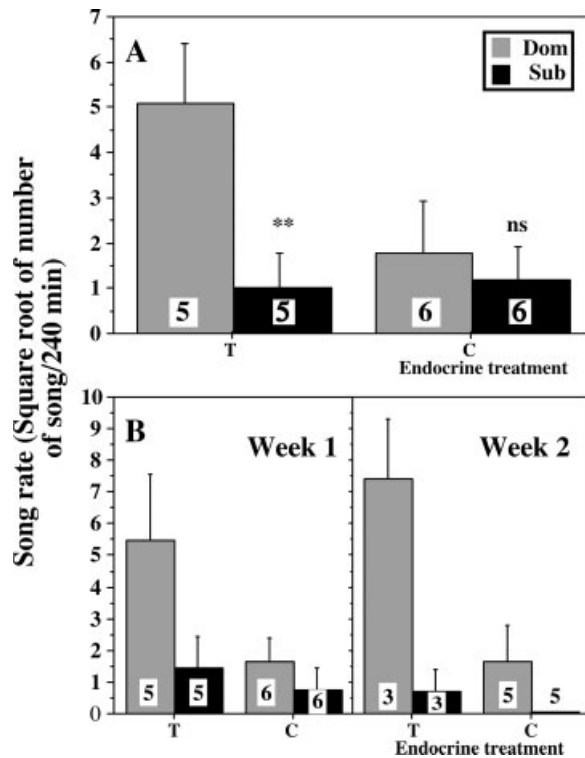


Figure 2 Effect of dominance (Dom) or subordination (Sub) on the song rates expressed by male canaries housed in same sex dyads and treated with testosterone (T) or left intact as controls (C). (A) Total number of songs produced on the day preceding sacrifice. Data recorded on weeks 1, 2, 4, and 8 are pooled because no effect of weeks and no interaction of weeks with other factors were detected in the ANOVA. (B) Song rates for all available males (dominant or subordinate, T-treated or control) on weeks 1 and 2. The numbers on the bars indicate the numbers of subjects available for each specific set of data.

type [$F(1, 17) = 17.783, p = 0.0006$] and of the week of sacrifice [$F(3, 17) = 3.956, p = 0.0262$] were observed. All primary and secondary interactions were nonsignificant (all $p > 0.17$). A two-way ANOVA excluding the nonsignificant factor (treatment) was therefore carried out, which confirmed the effect of the dyad type and week of sacrifice [respectively: $F(1, 24) = 18.309, p = 0.0003$ and $F(3, 24) = 4.449, p = 0.0127$; see Fig. 3(A)] and the absence of interaction [$F(3, 24) = 1.375, p = 0.2743$]. Posthoc tests indicated that HVC was significantly larger in M-F than in M-M dyads on weeks 2 and 4, and that within M-F dyads, HVC was larger on week 4 than on weeks 1 and 8. No significant change could be detected in the M-M dyads.

Analysis by three-way ANOVA of RA volumes failed to detect any significant main effect or interaction (all $p > 0.15$) except for the treatment factor for

which a statistical tendency was observed [$F(1, 17) = 4.237, p = 0.0552$]. A reanalysis of these data after exclusion of the treatment factor [see Fig. 3(B)] still detected no effect of the dyad type, the week of sacrifice, or their interaction (all $p > 0.18$).

Overall, the volumes of HVC and RA were numerically larger in the T-treated males than in controls (27.9 and 30.25%, respectively) and this difference was statistically significant for RA but not for HVC after combining different dyad types and sacrifice dates [HVC: $F(1, 33) = 2.822, p = 0.1024$; RA: $F(1, 33) = 4.596, p = 0.0395$].

A three-way ANOVA revealed no effect of the endocrine treatment, the dyad type, or the week of sacrifice on the Rt volumes (all $p > 0.22$). When Rt volumes were used to normalize the HVC and RA volumes, the same overall pattern as described above was observed except that the effect of T treatment now became more prominent. The three-way

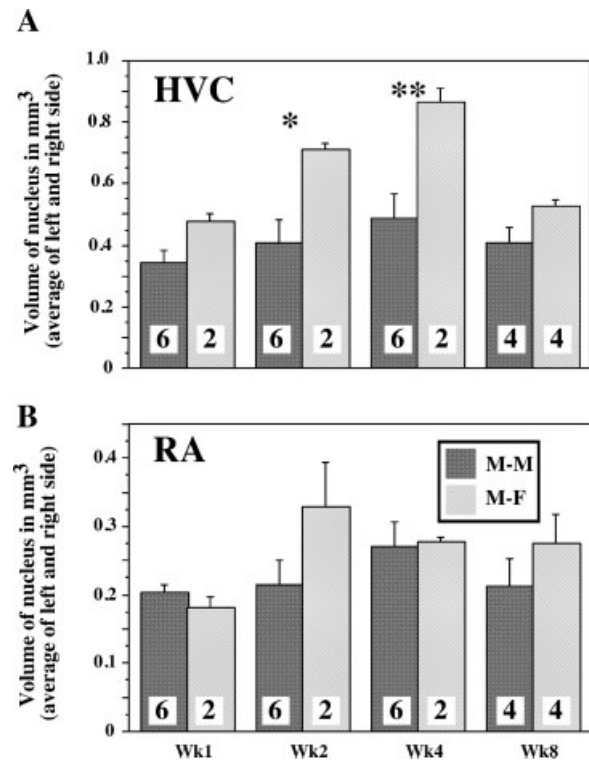


Figure 3 Volumes of HVC and RA measured after 1, 2, 4, or 8 weeks of treatment in male canaries that were housed in the presence of a female (M-F) or another male (M-M). Data for T-treated and control birds are pooled because no overall effect of the endocrine treatment was detected in the ANOVA (see text). ** $p < 0.01$ and * $p < 0.05$ for the comparison of HVC size in the two types of dyads by posthoc Fisher PLSD tests (see text). The numbers on the bars indicate the numbers of subjects available for each specific set of data.

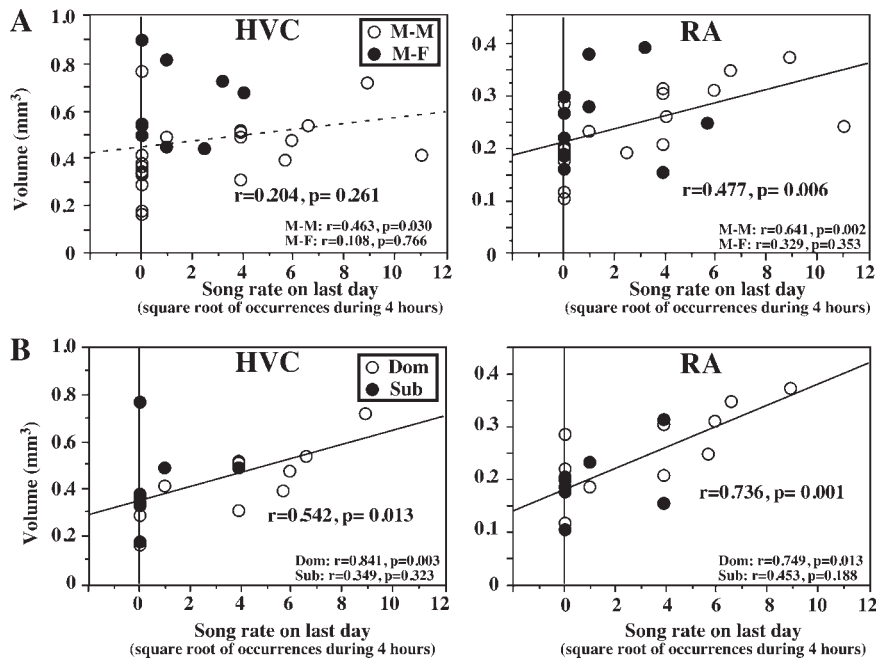


Figure 4 Correlation between the total song rates measured on the last day preceding sacrifice (4×60 min recording) and the volume of HVC or RA in the entire population of males used in this experiment [both M-M and M-F dyads (A)] or only in the males housed in same sex (M-M) pairs (B). In each case, males from the different subgroups [M-M, M-F, dominant (Dom), subordinate (Sub)] are indicated by different symbols. The corresponding correlation coefficients and probabilities are indicated in each case for the entire population concerned in the graph and for the two subpopulations. The general regression line is shown in each graph; it is indicated as a hatched line when nonsignificant.

ANOVA of HVC/Rt identified significant effects of the three main factors [treatment: $F(1, 17) = 4.451$, $p = 0.0500$; dyad type: $F(1, 17) = 20.787$, $p = 0.0003$; and week: $F(3, 17) = 4.756$, $p = 0.0138$] but no significant interaction. Analysis of RA volumes identified a significant effect of the endocrine treatment [$F(1, 17) = 6.908$, $p = 0.0176$] but the two other main factors (dyad type and week) and all interaction were nonsignificant.

The difference in singing rate produced as a function of the social status of the birds in the M-M dyads was not associated with differences in the volume of the two song control nuclei HVC and RA. The three-way ANOVA (endocrine treatment and weeks as independent and social status as repeated factor) of HVC and RA volumes detected no significant main effect and no interaction. Due to the small sample size, the power of this analysis was low and data were therefore also analyzed by two-way ANOVAs, pooling birds that had been killed at all dates (treatment as independent and social status as repeated factors). This also detected no effect of the social status or of treatment (and no interaction) on the size of the song control nuclei even after correction of the volumes by

the volume of nucleus rotundus (all $p > 0.10$; data not shown).

Correlations. In the entire population of males that was studied, no correlation could be detected between the volume of HVC and the total number of songs that had been produced by the birds during the day before sacrifice [$r = 0.204$, $p = 0.261$; Fig. 4(A)]. The volume of RA was in contrast positively correlated with the singing activity ($r = 0.477$, $p = 0.006$). The separate analysis of M-M and M-F dyads revealed, however, a significant correlation between song rate and the volume of HVC or of RA specifically in the M-M dyads. Significant correlations were not observed in the M-F dyads [see detail in Fig. 4(A)].

Data from the M-M dyads were therefore reanalyzed now taking into account the social status of the birds when it could be determined [Fig. 4(B)]. In the 22 birds (11 dyads) in which hierarchy had been identified and the volumes of song control nuclei could be reconstructed, the song rate on the last day was a good predictor of the volume of HVC ($r = 0.542$, $p = 0.013$) and of RA ($r = 0.736$, $p = 0.001$). This positive correlation was present only in dominant males;

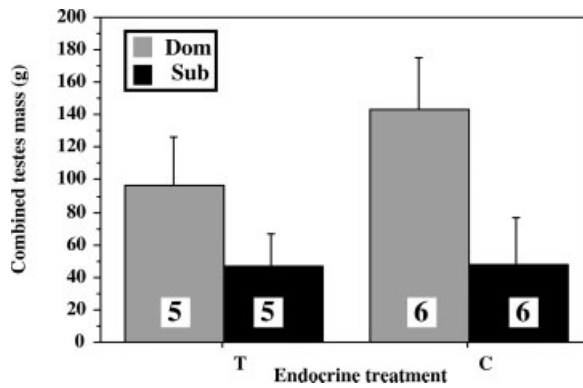


Figure 5 Combined testes mass in male canaries housed in same sex pairs (M-M) that were either treated with testosterone (T) or left intact as controls (C). Results are summarized in all males without taking into account the date of sacrifice, which had no overall significant effect in the ANOVA.

no relationship was identified in subordinates largely because many of them did not produce complete songs during the recording periods.

Testes Mass. In the entire population of males, the testes size (combined left + right testis mass) was not affected by the three experimental factors nor by their interactions (all $p > 0.22$ in the three-way ANOVA). The combined testis mass was approximately 30% smaller in T-treated than in control males (72.2 ± 14.3 vs. 100.8 ± 17.07 mg) but this difference was not significant [$F(1, 21) = 0.781$, $p = 0.3867$]. The type of dyad also had no detectable effect on the testes size [M-M: 87.16 ± 13.69 vs. M-F: 84.9 ± 20.4 mg, $F(1, 21) = 0.379$, $p = 0.5447$]. Partial analyses by two-way ANOVA after exclusion of either the factor week of sacrifice or the factor treatment similarly identified no main effect and no interaction (all $p \geq 0.15$, data not shown).

A subanalysis focusing on the M-M dyads in which a clear hierarchy had been identified was also carried out. A two-way ANOVA (endocrine treatment and social status as factors) identified a clear effect of social status, with dominant males having larger testes than subordinates both in the T and in the C group [status: $F(1, 9) = 5.888$, $p = 0.0382$; treatment: $F(1, 9) = 0.726$, $p = 0.4162$; and interaction: $F(1, 9) = 0.558$, $p = 0.4741$, see Fig. 5]. Qualitative inspection of these data indicated that this difference in testes mass between dominant and subordinates was already present with a similar magnitude at the beginning of the experiment (weeks 1 and 2), but the limited number of subjects available made it impossible to confirm this difference statistically (data not shown).

Experiment 2

The experiment described above indicated that the sex of the partner in a dyad had an opposite effect on the amounts of songs produced and on the HVC size of males: song rate was lower but HVC size was larger in males exposed to females than in males exposed to another male. However, females in this experiment were treated with T, which could have affected the behavior of their male partner in unexpected ways, and significant effects were detected only after 2 to 4 weeks of experiment when the sample size was markedly reduced. This experiment was thus replicated with a larger number of subjects that could be studied specifically after that latency. Furthermore, in the hope of maximizing the effect of the female partners, it was decided to treat them with estradiol to increase their sexual receptivity.

Behavior. All but one female showed extensive signs of sexual receptivity during observations performed between day 4 and 20. Six out of the seven females solicited copulation and copulation occurred on most observation days (as a median during five or three out of eight tests, respectively). Six out of the seven females also started building a nest in their feeder using their own feathers and paper that they were tearing from the cage floor. The last female never showed these behaviors and behaved in a very aggressive manner towards her mate throughout the experiment despite the fact that she was confirmed to have her estradiol implant at the end of the experiment. Data relative to this dyad were therefore excluded from the analysis. One M-M dyad was also excluded from the analysis because the two birds were observed to spend most of their time fighting aggressively and therefore almost never produced a full song.

The total numbers (sum of 3×60 min observations) of songs recorded on each experimental day in the other dyads (four M-M, i.e., eight males, and six M-F) were analyzed by a two-way ANOVA with the two dyad types as independent factor and successive days as repeated measure [Fig. 6(A)]. This analysis identified a significant effect of both the dyad type [$F(1, 12) = 5.395$, $p = 0.0386$] and of the repeated factor, the successive days [$F(7, 84) = 3.610$, $p = 0.0019$], but there was no interaction between these two factors [$F(7, 84) = 1.737$, $p = 0.1114$]. The effect of repetition resulted in all probability from the progressive development of the effects of the T implant. The drop in singing rate observed on day 15 (and to a lesser extent on day 18) relates to a period of lower than normal temperatures that took place at that time as a result of a failure of the climate control system. The changes in time are therefore of little in-

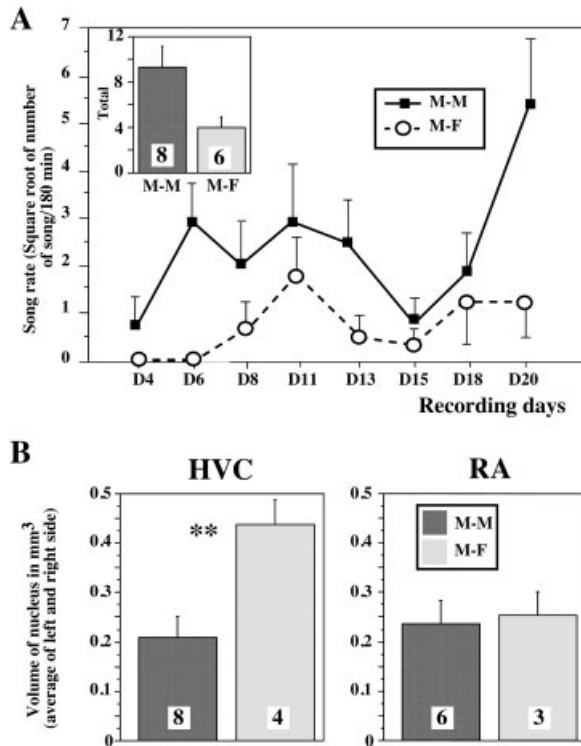


Figure 6 Effects of the presence of an estradiol-treated female (M-F) or another castrated T-treated male (M-M) on the song rate expressed by castrated male canaries that were treated with testosterone. (A) Song rates measured during the recording sessions (3×60 min) that took place on different days between day 4 and 20 after the beginning of the endocrine treatment. The insert illustrates the total numbers of songs produced as an average by the M-M or M-F males. (B) Volumes of HVC and RA measured in male canaries that were housed in M-F or M-M condition. $**p < 0.01$ for the comparison of HVC size in the two types of dyads (see text). The numbers on the bars indicate the numbers of subjects available for each specific set of data.

terest in the present context and because they do not interact with the partner effect, an additional analysis was performed comparing the total number of songs produced by the males in the two types of dyads. This one-way ANOVA confirmed the significantly larger number of songs produced by the males in the M-M compared to the M-F dyads [see insert of Fig. 6(A): $F(1, 12) = 5.151, p = 0.0425$]. This effect was still significant if the analysis was performed on the number of dyads (four M-M and six M-F dyads) rather than on the number of males (eight M-M and six M-F) to remove any possible effect of the pseudo-replication [$F(1, 8) = 7.347, p = 0.0266$].

Song Control Nuclei. HVC volume could be reconstructed in all but two female-mated males. As in experiment 1, this analysis revealed a substantially

higher HVC volume in the M-F than in the M-M males [see Fig. 6(B); $F(1, 10) = 10.612, p = 0.0086$]. The RA volumes did not exhibit this difference [see Fig. 6(B); $F(1, 7) = 0.061, p = 0.8117$]. As was the case in the first experiment, the volume of Rt was not affected by the type of partner [$F(1, 10) = 0.112, p = 0.9177$]. The volume of HVC corrected by Rt (HVC/Rt) was significantly larger in M-F than in M-M males [$F(1, 10) = 13.903, p = 0.0039$], but there was still no difference present for RA/Rt [$F(1, 7) = 0.034, p = 0.8579$].

DISCUSSION

Two experiments were carried out to analyze the interaction of T and social factors in the control of singing and of the development of related brain nuclei in canaries. T increased the singing rate and volume of HVC and RA but these effects were markedly affected by the social context. Males housed with another male displayed higher singing rates than males housed with a female. In addition, in same-sex male pairs, dominant males sang at higher rates than subordinates in T-treated but not in control pairs. These behavioral differences were reflected in the size of the song control nuclei in the following way: HVC was larger in M-F than in M-M pairs but was not affected by the dominance status in males. Song rate was positively correlated with RA and to a lesser extent with HVC volume, but this relationship was observed only in the dominant birds in M-M pairs. These effects of the sex of the partner in the dyad on the singing rate and on HVC size were replicated in a second experiment in which sexual receptivity of the females was optimized by a treatment with exogenous estradiol. These studies therefore demonstrate that the actions of T on singing and on song control nuclei are modulated by the interaction with the social environment, including the presence/absence of a potential mate and dominance status among males. Interestingly, the volume of the song control nuclei was not always affected in the same direction as the singing output, which raises interesting questions regarding the mode of action of T on song rates and song control nuclei. Based on various types of correlational and direct experimental evidence (see Schlinger, 1997; Ball et al., 2004; Brenowitz, 2004; Harding, 2004 for review) and on the presence of androgen receptors in several song control nuclei including HVC and RA (Arnold et al., 1976; Balthazart et al., 1992; Ball et al., 2003), it is often assumed that the seasonal changes (characterized by a vernal increase) in singing rates of songbirds result from a direct action of T on the song control nuclei, which

increase in size following exposure to the steroid leading in turn to an increased song output. The exact mechanisms by which the increased volume of the song control nuclei, reflecting the presence of larger numbers of neurons or a more extensive dendritic arborization (Tramontin and Brenowitz, 2000), could lead to an increase in song rate have not been identified. Recent experiments indicate that alternative causal links could exist between the correlated increases in plasma T, song control nuclei volumes, and song rates that are observed seasonally. It has indeed been noted that the T-induced increase in the volume of song control nuclei is significantly attenuated in birds that are prevented from singing, for example as a consequence of M-M interactions (Sartor et al., 2002; Sartor and Ball, 2005). This therefore suggests an alternative scenario in which T, acting in the brain but not directly in song control nuclei (e.g., in the preoptic area; see Riters and Ball, 1999; Riters et al., 2000; Ball et al., 2004), would increase the motivation to sing, which in turn would enhance the development of the song control nuclei. This scenario is supported by the observation that the expression of the BDNF, which increases neuronal recruitment in HVC (Li et al., 2000; Alvarez-Borda and Nottebohm, 2002; Alvarez-Borda et al., 2004), is enhanced by the act of singing in male canaries (Li et al., 2000). The present experiments identifying variable effects of T on behavior depending on the social conditions and demonstrating dissociated effects on singing rates and growth of the song control nuclei challenge some of these ideas and bring new information concerning the interactions between hormones, brain, and behavior.

A study of white-crowned sparrows has identified the presence of a female as being a powerful regulator of seasonal neuroplasticity in the song system independent of the possible effects of the female on endogenous T release (Tramontin et al., 1999). Investigations of white-crowned sparrows (Wiley et al., 1993) and European starlings (Sartor and Ball, 2005) indicate that dominance can modulate song production, again apparently independently of T. Thus many of the issues we address in this study have been addressed by other studies on other species. This is the first study in which these different modulatory factors on song behavior and song system plasticity have been investigated in a single study in a single species. We will discuss these different effects in turn.

Effects on Singing Behavior

As a Function of the Sex of the Cage Mate. In agreement with previous reports, we found here that

T markedly increased overall singing rates. This observation is in line with a vast body of literature indicating that treatment with exogenous T increases singing activity in a variety of songbirds species (e.g., for review: Ball, 1999; Schlinger, 1997; Schlinger and Brenowitz, 2002; Ball et al., 2003; Harding, 2004), including canaries (Nottebohm, 1980; DeVogd and Nottebohm, 1981; Sartor et al., 2005).

However, we also found in this study that the singing rate was significantly affected by the social context experienced by the bird. Specifically, singing rates were much higher in the M-M than in the M-F dyads, and within M-M dyads, singing was more frequent in dominants than in subordinates. Effects of the social context on song output have been reported previously (see Introduction). There are species differences in the degree to which a male or a female is a more potent stimulus in eliciting song, presumably related to species variation in whether song functions more for male territorial defense or female choice (Collins, 2004). For example, in white-crowned sparrows males sing more frequently when exposed to a female than to a male (Tramontin et al., 1999). The effects of the female on plasma hormone concentrations is one obvious way to explain this enhancement of song behavior. However, plasma androgen levels did not seem to differ between groups in the Tramontin et al. (1999) study. It is however possible that the presence of the female increased the activity of the hypothalamo-pituitary-gonadal axis and that subtle changes in the concentrations of plasma T were responsible for the change in singing behavior. For example, it was observed in previous studies that gonadally intact male white-crowned sparrows that were in contact with sexually receptive females implanted with estradiol had higher plasma levels of T than males housed with blank-implanted females (Moore, 1983), and similar findings have been reported in other species (Wingfield and Moore, 1987). In the two experiments reported in this article on canaries, plasma T levels were clamped at a high, relatively constant level by implantation of a T-filled Silastic capsule. Some variation within this high range of concentrations was certainly possible during experiment 1 because males were not castrated, but this was less likely during experiment 2, in which males were castrated. It is therefore unlikely that changes in circulating concentration of T could explain the behavioral effects that were observed because plasma T concentrations were maintained at relatively stable levels.

Our finding that the singing rate in M-M dyads is higher as compared to M-F dyads is generally consistent with previous reports on captive held canaries,

though the differential effects of males and females on song rate have not been systematically studied in this species previously. As noted in the Introduction it is well known that presenting a stimulus male can elicit high rates of singing in male canaries (Kreutzer et al., 1999). The difference in song rate we observed in the M-M pairs versus the M-F pairs can be considered from a consideration of both ultimate factors and proximate factors that shape the production of this effect. In proximate terms, the low singing rate in M-F pairs in the first experiment might potentially be explained by the fact that females were in this case submitted to the same endocrine treatment as the males (control empty or T-filled implant) and were therefore not sexually receptive. This argument, however, does not apply to the second experiment in which all females were treated with exogenous estrogens and were observed to show clear signs of sexual receptivity. In ultimate terms, canary song seems to function in both a M-M competitive context and as an attractant to females. In many species song used to attract a female decreases once the male is mated (e.g., European starlings: Cuthill and Hindmarsh, 1985; Cassin's finch: Sockman et al., 2005) while song used in a territorial context is enhanced by the presence of a challenging male. Our data on canaries are consistent with this sort of scenario applying to this species. However, there is no field evidence that male canaries decrease their song rate once they are mated (Leitner et al., 2001b; Voigt et al., 2003).

The experimental set-up should also be considered when interpreting these data. In the present experiments, males were constrained in a limited space that contained either a female or a male. From a functional point of view, males in the M-F pairs had therefore no reason to sing to attract a female, because she was already present all the time, but males in the M-M condition had a male conspecific that was also continuously present and could be considered as a potential rival. This difference provides one sort of functional explanation as to why song output was higher in the M-M than in the M-F condition. It was also the case that all pairs of birds were visually isolated but were kept in the same room and were therefore not acoustically isolated. It is possible that the high singing rate in the M-M pairs was directed at females that were known to be present in the room but were not visible. Similarly, the crowing rate of male quail is high when females are not directly accessible but drops to very low levels as soon as a female is introduced into the male's cage (Potash, 1974; Goodson and Adkins-Regan, 1997). The high singing rate in the M-M pairs could thus still be related to female attraction as well as to M-M competition.

Interestingly, the difference in singing rate between M-M and M-F pairs only developed progressively in the course of experiment 1 [see Fig. 1(B)] and became clearly established only during the fourth week of observation (day 27), which also provides some insight into the factors underlying this difference. This difference appeared due to an increase in the singing rate in M-M dyads, not to a change in M-F pairs that sang at a relatively constant rate throughout the experiment. The difference may therefore relate to the development of aggressive relationships in the M-M pairs that were expressed in one way via changes in song behavior. This interpretation is indirectly supported by the fact that the increase in singing in the M-M pairs leading to a difference with the M-F pairs was observed only in T-treated and not in control pairs.

As a Function of Position in the Dominance Hierarchy. Within the M-M dyads a higher rate of song production was observed in dominant birds as compared to subordinate males [Fig. 2(A)]. Such a difference is not surprising and has been reported before in other oscine species (e.g., Wiley et al., 1993; see also Introduction). The presence of a systematic difference in singing rate despite the fact that plasma T concentrations were probably high and similar in all birds due to the presence of a T-filled Silastic implant is also consistent with the fact that many bird species display territorial singing outside the breeding season that does not directly depend on androgen activation. For example, European robins (*Erythacus rubecula*) sing intensively and display agonistic behavior on their home range during winter without any relation to circulating T levels (Schwabl and Kriner, 1991). Nonmigratory populations of song sparrows in the western USA similarly display singing and agonistic behavior outside the breeding season when plasma T concentrations are basal (Soma et al., 2002). Singing rate therefore does not need to correlate with circulating T levels. The paired design used in the present experiment in which males submitted to the same endocrine treatment were housed in stable dyads may have enhanced the behavioral differences between members of a same pair.

The mechanisms that generated this difference in singing behavior related to social status cannot be identified with certainty. The difference developed quite quickly, especially in the T-treated birds, where it was apparent by the end of the first week after pairing [Fig. 2(B)]. Dominant males had larger testes than subordinates right from the start and this difference was detectable throughout the experiment except during the last week of the experiment [Fig. 5]. It seems

therefore likely that at the beginning of the experiment, the male with the larger testes and thus perhaps higher T concentrations became dominant in each dyad and then inhibited the singing behavior that should have developed in his cage mate following T treatment. Alternatively, the male with the larger testes was possibly singing more than his cage mate and this may have induced his dominant status. Subsequently, because both members of the pair were treated with the same amount of T, this treatment did not influence the dominance/subordination relationship and thus the singing rate that was pre-established before exogenous T could have its effects.

Singing inhibition in the subordinate could result from an effect comparable to social defeat, which induces, at least in some individuals, an activation of the HPA axis and impairment of motor activity in great tits (*Parus major*; Carere et al., 2001, 2003; see also Sartor et al., 2005). The present results thus corroborate the findings of Wiley and coworkers (1993), who reported that dominant white-throated sparrows sing more than subordinates even when T concentrations are clamped. As a consequence, T was only effective in stimulating singing in dominant males, indicating that T and dominance act cooperatively to regulate high rates of song behavior.

Dissociations between Effects on Song and on Song Control Nuclei

In this study, the volumes of HVC and RA did not always covary with the singing rates observed in different conditions. The higher song output in the dominant male of M-M pairs treated with T was not associated with an increased volume of the song control nuclei as was observed in starlings (Sartor and Ball, 2005). In both experiments, HVC volume was larger in M-F than in M-M dyads despite the fact that the latter sang more frequently than the former. This social enhancement of song system size by access to a female in a manner independent of T concentration constitutes a replication in canaries of a similar phenomenon observed by Tramontin et al. (1999) in white-crowned sparrows.

The effect of T on the volume of song control nuclei was not very prominent in experiment 1 because males were gonadally intact and were just given an additional T implant to insure that their T plasma concentrations would be in the high physiological range. The increase in T concentrations was thus sufficient to elevate singing activity but not to a large degree the volume of the song control nuclei [significant effect for RA only, statistical tendency

($p = 0.1024$) for HVC]. Additionally, effects of T were distributed in different groups that were killed after different durations of exposure to the steroid and kept in different social conditions. Because both factors affected the dependent variable, the variance in the ANOVA was distributed among multiple factors, making it less likely that an effect of T itself would be detected. Although many studies have demonstrated effects of T on HVC or RA volume, the correlation is by far not perfect and it was, for example, reported that, in wild canaries, song changes seasonally (in parallel with plasma T) while the volume of HVC is not affected (Leitner et al., 2001; see Ball et al., 2004 for review).

Initial studies correlating variation in the size of forebrain song nuclei with measures of song behavior indicated that in canaries the volume of HVC is positively correlated with the size of a male's song repertoire (Nottebohm et al., 1981; Nottebohm, 2004). More recently a study that took into account variation in age found that a positive correlation could be established between the volume of HVC and a particular aspect of the male's song repertoire (Leitner and Catchpole, 2004). Studies of song and choice in male canaries had identified phrases that were particularly attractive to females (Vallet and Kreutzer, 1995). The number of these particularly attractive phrases was found to be positively correlated with variation in HVC volume in male canaries, but only in birds over 2 years old (Leitner and Catchpole, 2004). These findings are consistent with studies in other species that continue to change their song behavior in adulthood, such as European starlings, where it was found that variation in HVC volume correlates with variation in song bout length, rather than repertoire size (Bernard et al., 1996; Sartor et al., 2005). Females prefer males who produce song consisting of long bouts in starlings (Eens, 1997), and this measure positively correlates with measures of high quality aspects of the male's phenotype such as immune function (Duffy and Ball, 2002). There is interesting species variation in the aspect of song that correlates best with variation in the volume of the song control nuclei (Garamszegi and Eens, 2004). Sometimes measures of song performance such as song duration provide a better predictor than a measure of song complexity such as repertoire. Recent work indicating that the neurotrophin BDNF that promotes the recruitment of new neurons in HVC can be released as a function of song activity (Alvarez-Borda and Nottebohm, 2002) has raised the possibility that song rate may promote growth of the song nuclei and lead to positive correlations between high rates of singing and large song control nuclei (Ball et al., 2004;

Adkins-Regan, 2005; Sartor and Ball, 2005). High rates of singing have been found to be positively correlated with large song nuclei in some cases (e.g., Sartor et al., 2005) but not in all cases. In the current study a positive correlation was observed here between HVC or RA volume and the singing rate but these correlation coefficients were significant only for dominant birds in the M-M pairs.

In this study, we did not identify a difference between the HVC (or RA) volumes in dominant (that sang a lot) and subordinate (that sang very little) birds in the M-M dyads. The difference in song production may have been insufficient to generate the difference in brain physiology (e.g., differential production of BDNF; Li et al., 2000; Alvarez-Borda and Nottebohm, 2002; Sartor and Ball, 2005) that resulted in a change in song nucleus volume, or the social suppression by dominants of singing in subordinates might have been associated with an increased degree of psychosocial stress in both members of the pair. Stress and the associated release of adrenal stress hormones are known to have deleterious effects on health and brain development (Sapolsky, 1996a,b, 2005). This could have resulted in a reduction of HVC in both the dominant and the subordinate. Interestingly, this explanation could also perhaps provide an explanation as to why HVC volumes were larger in M-F than in M-M dyads despite the fact that song rates were significantly higher in M-M pairs.

We observed indeed in both experiments that males exposed to a female had a larger HVC than males exposed to another male, despite the fact that singing rates were significantly higher in M-M than in M-F dyads. Because levels of T were clamped at high values in all males, female-induced changes in circulating levels of T probably cannot be invoked to explain the differential growth of the song control nucleus (especially in experiment 2 in which males were additionally castrated). Analysis of the time-course of this effect during experiment 1 (Fig. 3) also suggests that the difference between HVC volumes in M-M and M-F dyads progressively appeared as a result of the increase of HVC size in M-F pairs, while no major change was observed in M-M pairs despite the fact that many of these birds were treated with T. Social stress and detrimental effects of high circulating levels of corticosteroids on brain development and neuronal survival that are potentially associated with aggressive interactions between males could be responsible for the lack of development of HVC in the M-M pairs. Stress early in ontogeny inhibits HVC growth in zebra finches (Buchanan et al., 2004). It is possible that a similar inhibition of HVC volume as a result of psychosocial stress could occur in associa-

tion with seasonal changes in the song system of canaries. If differential brain activity that has been described in zebra finches associated with female-directed or undirected song (Jarvis et al., 1998; see above) also occurs in canaries this could also contribute to this differential HVC growth.

The difference in HVC size between the M-M and M-F dyads decreased and almost disappeared on week 8 for reasons that are difficult to identify. It is unlikely that birds became photorefractory, and thus potentially less sensitive to T, because they were kept throughout on 11:13 L/D, a photoperiod that is not known to induce photorefractoriness in canaries (Storey and Nicholls, 1976). It is also unlikely that the release of T from the Silastic implants decreased at that time because such implants have been shown to release T for many weeks in other avian species such as the Japanese quail (Desjardin and Turek, 1977). It seems most likely that this decrease resulted from a progressive adaptation of the subjects to their social condition (e.g., loss of stimulating value of the female) or alternatively just reflects random variation in the samples. The reliability and potential causes of this phenomenon should be investigated further.

RA volumes did not change in this study but it is interesting to note that the experiment identifying an effect of developmental stress on HVC size similarly found no effect on RA volume (Buchanan et al., 2004). Previous experiments have shown that changes in RA are not always detected when changes in HVC are observed. Differential mechanisms seem to control the development of both nuclei (e.g., see Grisham et al., 1994; Tramontin et al., 2000; Brenowitz, 2004) and changes in HVC size are known to occur more rapidly than for RA (see Tramontin et al., 2000; Sartor et al., 2005). Changes in RA volume appear to occur based on trans-synaptic inputs from HVC rather than via direct actions of steroids, which means that it is expected that a change in HVC would be observed before any change would occur in RA (Brenowitz and Lent, 2001, 2002).

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