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Age-dependent and age–independent effects of testosterone in male quail

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Abstract

Various studies in rodents recently concluded that puberty should be considered as a second period of organization of brain and behavior and that action of sex steroids at that time is long lasting and possibly permanent. We tested this notion in male Japanese quail that had been castrated before 3 weeks post-hatch by analyzing whether a similar treatment with exogenous testosterone initiated at 3, 5 or 7 weeks post-hatch has a differential influence on the development of testosterone-dependent morphological, behavioral and neural characteristics that are known to be sexually differentiated. The growth of the androgen-dependent cloacal gland was significantly faster when testosterone treatment was initiated later in life indicating that the target tissue is not ready to fully respond to androgens at 3 weeks post-hatch. The three groups of birds nevertheless developed a gland of the same size typical of intact sexually mature birds. When adults, all birds expressed copulatory behavior with the same frequencies and latencies and they displayed the same level of aromatase activity and of vasotocinergic innervation in the preoptic area as gonadally intact males despite the fact that they had been treated with testosterone for different durations starting at different ages. Surprisingly, the frequency of cloacal sphincter contractions, a measure of appetitive sexual behavior, was significantly higher when testosterone treatment had been initiated later. Together these data provide no clear evidence for an organizational action of testosterone during sexual maturation of male quail but additional experiments should investigate whether estrogens have such an action in females.

Keywords

Sexual differentiation; Puberty; Preoptic area; Sexual behavior; Aromatase; Vasotocin

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1. Introduction

Sex steroids actions have two distinct types of consequences on brain and behavior. During early ontogeny they organize brain and behavior during a more or less strictly delimited time window and these effects are considered irreversible (Phoenix et al., 1959). Later in life, they activate reproductive and other behaviors in parallel with the induction of related brain plasticity but these effects are transient and dissipate when the steroid is removed (McEwen, 1981). These two types of effects are classically considered completely independent (organization vs. activation) but a host of relatively recent studies have suggested that the activation of reproduction behaviors that takes place at the time of sexual maturation (puberty) could have long-lasting effects, a feature typical of organizing effects of steroids (Patlak, 2012; Schulz et al., 2009a; Sisk and Zehr, 2005).

In rodents, pre- or peri-natal testosterone (T), acting as such (an androgen) or via aromatization into an estrogen, masculinizes and defeminizes reproductive behaviors (mounts and intromissions, lordosis) and sexually differentiates multiple related brain structures (Arnold and Gorski, 1984; McCarthy, 2012; McCarthy et al., 2009). These behaviors will then be differentially activated by exposure to androgens or estrogens in adult males and females (Arnold and Gorski, 1984). It was however shown that deprivation of male Syrian hamsters (*Mesocricetus auratus*) of sex steroids during adolescence compromises activation by steroids of adult social behavior (Schulz et al., 2004). Furthermore in male hamsters gonadectomized on postnatal day 10, T treatment before or during, but not after, puberty facilitates mating behavior in adulthood (Schulz et al., 2009b). In parallel, the pre-pubertal treatments with T, although they did not induce sexual behaviors in the short-term, increased the volume of bed nucleus of the stria terminalis and of parts of the amygdala, two sexually dimorphic brain nuclei, to adult size (Schulz et al., 2009b). Studies in rats also showed that sex differences in sexually dimorphic nuclei of the brain such as the sexually dimorphic nucleus of the preoptic area, the anteroventral periventral nucleus of the hypothalamus and the medial amygdala are partly the result of a differential addition of new cells to these nuclei during puberty and that pre-pubertal gonadectomy eliminates these sex differences in cell addition (neurons but also other cell types) during puberty (Ahmed et al., 2008).

Puberty should in this context be considered as a second period of organization of brain and behavior since effects of steroids at that time are long lasting and possibly permanent (Schulz et al., 2009a; Sisk and Zehr, 2005). Although direct experimental data are lacking for obvious reasons, circumstantial evidence suggest that in humans, puberty could also be a period when sex steroids exert permanent organizational-like effects on sexually differentiated behaviors with behavioral traits potentially concerned ranging from gender identity to cognitive capacities through sexually differentiated forms of psychopathology (Beltz and Berenbaum, 2013; Berenbaum and Beltz, 2011).

Japanese quail (*Coturnix japonica*), a well-established model in neuroendocrine studies of sexual differentiation (Ball and Balthazart, 2011a; Balthazart and Ball, 1998), offers an interesting model to test the generality of these conclusions. Sexual behaviors and several neuroanatomical or neurochemical traits are sexually differentiated in this species with some

of the differences depending on embryonic organizational effects of steroids while others simply result from a differential activation by T or estradiol in adulthood (Balthazart et al., 2009). Specifically in quail, male-typical copulatory behavior is demasculinized in females, mostly before day 12 of incubation ((Adkins, 1979; Adkins-Regan, 1983; Balthazart et al., 1992), but see (Balthazart and Schumacher, 1984b; Hutchison, 1978; Schumacher and Balthazart, 1984)) by estrogens secreted by the embryonic ovary, while in contrast, female receptivity does not appear to be differentiated by organizational effects of steroids and can be activated in adults of both sexes by a proper treatment with estrogens (Adkins, 1975). The cloacal gland, an androgen-dependent structure located at the back of the cloaca, is larger in males than in females and this difference in size results in part from the differential activation by T in adults (Balthazart et al., 1983) but also from a demasculinizing action of embryonic estrogens (Balthazart et al., 1992; Schumacher and Balthazart, 1983).

Multiple brain features are also differentiated between males and females including the volume of the medial preoptic nucleus (POM;(Panzica et al., 1996b; Viglietti-Panzica et al., 1986)), the aromatase activity (AA) in the preoptic area (Schumacher and Balthazart, 1986) and more specifically the POM (Balthazart et al., 1990b; Cornil et al., 2011), and the vasotocinergic innervation of the POM (Panzica et al., 1997; Panzica et al., 1998). All these traits are T-dependent in adulthood (Balthazart and Ball, 2007; Panzica et al., 1996a; Panzica et al., 1996b) but clear evidence for an organizational action of embryonic estrogens on these sex differences is available only for vasotocinergic innervation of the POM (Panzica et al., 1998). Indeed, no effect of embryonic exposure to estrogens on POM volume could be detected (Aste et al., 1991; Panzica et al., 1987; Panzica et al., 1991). Likewise, variable results were obtained for the preoptic AA. Some experiments detected a significantly higher AA in males than in females when all subjects were gonadectomized and treated with a same dose of T (Schumacher and Balthazart, 1986) but several other studies failed to find such a difference or found a quantitatively much smaller difference that was not statistically significant (Balthazart, 1989; Balthazart et al., 1990b; Cornil et al., 2011).

Based on the information on long term effects of steroids at the time of mammalian puberty described just before, we wondered whether these variations from one experiment to another in the magnitude of the sex difference affecting brain AA could not be related to the age at which birds had been gonadectomized and when their treatment with T had been initiated. The age at gonadectomy was reasonably standardized (± 3 weeks post-hatch) between experiments for practical reasons (gonads must be removed before they start growing due to extensive vascularization). But since we had shown that T restores full copulatory behavior in birds gonadectomized before sexual maturity (Balthazart et al., 1990a; Balthazart et al., 1983), we had not controlled to the nearest week the specific age when T treatment was initiated. We had also serendipitously discovered that there is around 40-60 days post-hatch an increase in cell divisions as identified by BrdU labeling in the preoptic area of male and female quail (Mouriec and Balthazart, 2013). This suggested that, in quail also, the rise in sex steroid concentrations occurring at the time of sexual maturation (Ottinger and Brinkley, 1979a) could be associated with a remodeling of the preoptic area, a brain region critically implicated in the control of reproduction where multiple sex differences have been identified (Balthazart and Ball, 2007; Panzica et al., 1996b).

We therefore decided to test whether T could have long-lasting organizing effects in quail around the time of sexual maturation. More specifically we analyzed whether the age at which administration of T is initiated in male quail that have been castrated around 3 weeks post-hatch has an influence on the development of T-dependent morphological, behavioral and neural characteristics known to be affected by sex differences as a result of either a different organization or activation by steroids. We show here that the growth rate of the cloacal gland was markedly affected by the age when T was first administered (faster in older birds) but in contrast, no effect of the age of T treatment initiation was detected on the adult level of behavioral and neural T-dependent features that were considered with the possible exception of the measure of sexual motivation that was higher when T treatment had been initiated at a later age. This apparent discrepancy with the results of previous studies in rodents might relate to the mode of sexual differentiation in these two groups of vertebrates (masculinization by T of male rodents vs. demasculinization by estrogens of female quail) and future studies should test whether long-lasting peri-pubertal effects of estrogens would be present in female quail.

2. Methods

2.1. Subjects

This experiment was performed with a total of 49 male Japanese quail (*Coturnix japonica*) derived from our breeding colony at the University of Liège. Sexually mature females obtained from the same colony served as stimuli during the behavioral tests. From hatching, chicks were initially raised in groups in heated brooding cages. Temperature in these cages was progressively dropped from 40°C to room temperature (20-25°C) during the first three weeks of life. At 9 weeks of age, two days before the beginning of behavioral tests, all males were isolated in individual cages where they stayed until the end of the experiment. In these cages birds were in physical isolation but they could see and hear each other. Throughout the experiment all birds were maintained under a 16L:8D photoperiod simulating long summer days and they were provided with food and water *ad libitum*. All experimental procedures were in agreement with the Belgian laws on the protection of experimental animals and were approved by the local animal care committee.

2.2. Hormonal treatments

At the age of 17-18 days, 40 males were bilaterally castrated (CX) through a unilateral incision behind the last rib on the left side as described previously (Balthazart et al., 1998; Schumacher and Balthazart, 1984). The remaining 9 males were sham-operated: their testes were visualized through a similar incision but they were left in place (Intact birds).

At the age of 3 (n=14), 5 (n=13) or 7 (n=13) weeks each castrated bird then received two Silastic™ implants that were either filled with crystalline T (Sigma-Aldrich, Diegem Belgium; n=30) or left empty as a control (n=10). Sham-operated intact birds (n=9) all received two empty implants. These treatments thus defined 9 experimental groups of males implanted with T or with empty implants beginning at 3 (3 Intact, 4 CX and 10 CX+T), 5 (3 Intact, 3 CX and 10 CX+T) or 7 weeks (3 Intact, 3 CX and 10 CX+T) post-hatch. These ages were selected because they are positioned just before (3 weeks), during (5 weeks) and

just after (7 weeks) sexual maturation in quail. It is indeed demonstrated that testicular development and appearance of sexual behaviors take place between day 30 and day 50 post-hatch in male quail (Ottinger and Brinkley, 1978, 1979a,b). By selecting these ages, we were thus defining groups of birds that had been exposed to testosterone starting before, during or after the normal age of sexual maturation.

Each implant was made of a 20 mm long section of Silastic™ tubing (Silclear™ Tubing, Degania Silicone Ltd, Degania Bet, Israel; internal diameter 1.57 mm, external diameter 2.41 mm) and closed at both end with a Silastic™ glue plug for a length of about 1 mm. All implants were incubated overnight in 0.9% saline solution to initiate the steroid diffusion through the tube walls and then implanted subcutaneously in the neck region. This T treatment restores in castrated male quail physiological concentrations of T typically seen in sexually mature males (Balthazart et al., 1983).

2.3. Morphological measures

Starting on Week 5 post-hatch, all males were weighed once a week to the nearest gram. At the same time, the length and width of their cloacal gland was measured to the nearest 0.1 mm with a caliper and the cloacal gland area was defined as the product of these two measures in mm^2. This gland is an androgen-sensitive structure (Sachs, 1967), and its surface provides a sensitive measure of the endocrine state of the birds (Delville et al., 1984; Follett and Maung, 1978). These measures were collected weekly until the end of the experiment when birds were 12 weeks old.

2.4. Behavioral tests

All males were tested 5 times for consummatory sexual (copulatory) behavior (CSB) and once for appetitive sexual behavior (ASB) (Ball and Balthazart, 2011b). These tests were always performed in the afternoon over a 10 day period starting when birds were 9 weeks old. The ASB test was performed 5 days after the 5^{th} test for CSB. Males were tested in a different order each time to avoid any systematic bias between groups. These behavioral tests were described in detail previously (Ball and Balthazart, 2011a; Balthazart et al., 1998) and are thus only briefly presented here

2.4.1. Consummatory sexual behavior (CSB) test—the male was placed in a test arena (30 [width] × 70 [length] × 60 [height] cm) containing a sexually mature female and the two birds were allowed to interact freely for 5 min. During that time, the frequency and latency of the first occurrence of male sexual behaviors were recorded. The following behavior patterns were systematically noted: strut, neck grab (NG), mount attempt (MA), mount (M), and cloacal contact movements (CCM) [for a detailed description, see (Adkins and Adler, 1972; Hutchison, 1978)]. A latency of 300 sec (5 min) was assigned to behaviors that were not observed during a given test.

2.4.2. Appetitive sexual behavior (ASB) test—The male quail androgen-dependent cloacal gland produces, by repeated rhythmic contractions, a meringue-like foam that is transferred into the female's cloaca during copulation (Sachs, 1967; Seiwert and Adkins-Regan, 1998) and enhances male fertilization success (Cheng et al., 1989a; Cheng et al.,

1989b). These Rhythmic Cloacal Sphincter Movements (RCSM) are elicited in males by the view of a female (Balthazart et al., 1998; Seiwert and Adkins-Regan, 1998). The expression of RCSM strongly decreases after castration, but increases following a systemic treatment with exogenous T (Adkins-Regan and Leung, 2006; Balthazart et al., 1998; Cornil et al., 2011). The frequency of RCSM is a reliable measure of male ASB; they are produced in anticipation of copulation when the male comes into visual contact with the female (Ball and Balthazart, 2011a, b).

All males were tested here for RCSM frequency in response to the visual presentation of a female. The glass testing chamber (40 [width] × 20 [length] × 25 [height] cm) used to quantify RCSM was divided into two equal chambers by a glass partition. A mirror was located under the testing chamber at an angle of 45° providing the observer with an unobstructed view of the test male's cloacal gland (see (Absil et al., 2002) for description of the procedure and test chamber). The experimental male was placed for 2.5 min in one of the chambers while an opaque partition prevented him from viewing the female located in the other chamber, and the baseline RCSM frequency was recorded during this period. The opaque partition was then removed and the RCSM frequency was recorded for another 2.5 min period during which the experimental male had visual access to the female but could still not physically interact with her.

2.5. Brain collection and processing

After this last behavioral test, all males were killed by decapitation and brains were rapidly dissected out of the skull. The completeness of castration and presence of the 2 Silastic™ implants were also verified at that time. All subjects were found to be in the expected endocrine condition (complete castration and two implants present).

The two brain hemispheres were separated with a scalpel by a cut in the sagittal plane. The left hemisphere was then fixed by immersion for 150 min in 5% acrolein diluted in 0.01 M phosphate buffered saline (PBS). Samples were then rinsed twice for 30 min in PBS and then cryoprotected for two days in 30% sucrose before being frozen on dry ice and stored at -80°C in a freezer until processed for vasotocin immunostaining.

The hypothalamic-preoptic (HPOA) block was immediately dissected form the right hemisphere by two razor blade cuts placed at the level of the septopallio-mesencephalic tract (rostral end of the preoptic area), of the oculomotor nerves (caudal end of the hypothalamus) and then by a parasgittal cut located 2 mm from away from the midline and a final cut isolating the 2-mm-thick most ventral part of the sample. This block of tissue was homogenized in 2 ml of TEK buffer (150 mM KCl, 1 mM Na-EDTA, 10 mM Tris-HCl, pH 7.2), frozen on dry ice and stored at -80°C until assayed for aromatase activity.

2.6. Aromatase activity assay

The enzymatic aromatase activity (AA) was quantified in the measure of the amount of tritiated water produced during the conversion of [1β ^{3}H] androstenedione into estrone as previously described (Roselli and Resko, 1991). This procedure with slight modifications has been described in detail and validated for quail tissue (Baillien and Balthazart, 1997; Cornil et al., 2011; de Bournonville et al., 2012).

Briefly, duplicate aliquots of each sample were incubated in the presence of TEK buffer, ^{3}H-androstenedione (final concentration 25 nM, specific activity 26.3 Ci/mmol; Perkin-Elmer, Waltham MA USA), and NADPH (4.8 mM) at 37°C for 15 min. The reaction was stopped by adding 2% activated charcoal in 10% trichloroacetic acid. Samples were centrifuged to collect the supernatant (H_2O and $^{3}H_2O$) that was filtered through Dowex cation exchange columns. ^{3}H-water was then quantified by adding Optiphase "Highsafe" III (Perkin Elmer, Zaventem Belgium) and counting for 3 min on a Wallac Winspectral 1414 Liquid Scintillation. Enzymatic activity of samples was then expressed in pmol . h^{-1} after correction of the counts for quenching, recovery, blank values and percentage of tritium in β-position in the substrate (see (Baillien and Balthazart, 1997)).

2.7. Vasotocin immunohistochemistry

Left hemispheres were cut at 30 μm thickness on a cryostat at -20°C in a coronal plane adjusted to match as closely as possible the plane of the chicken brain atlas (Kuenzel and Masson, 1988). Sections were collected in four series from the level of the septopallio-mesencephalic tract to the level of the oculomotor nerves and stored in antifrost solution at -20°C.

One series of sections was stained by the avidin-biotin immunohistochemistry method with a primary rabbit antibody directed against vasotocin (gift of Dr. D.G. Gray, Max Planck Institute, Bad-Nauheim, Germany; see (Viglietti-Panzica et al., 1994) for validation of this antibody in quail brain tissue). Sections were rinsed 3 times for 5 min in 0.05 M Tris buffer-saline (TBS) between each step. Briefly, floating sections were first incubated in 0.1 % sodium borohydride (NaBH4) in TBS followed by a 20 min incubation in 0.6% hydrogen peroxide in TBS and a 30 min incubation in 5% normal goat serum in TBS plus 0.1 % Triton X-100 (TBST). Sections were then incubated for 36 hours at 4°C in the primary antibody diluted 1/2000 in TBST. Binding of the primary antibody was revealed by 120 min incubation in biotinylated goat anti-rabbit antibody (Dako A/S, Glostrup, Denmark) diluted 1/400 in TBST followed by 90 min incubation in the Vectastain ABC kit (Vector Laboratories, Burlingame CA, USA). The horseradish peroxidase (HRP) activity was finally visualized by 1.5 min incubation in 0.04 % diaminobenzidine (DAB) plus 0.012 % H_20_2 in TBS. Section were submitted to extensive final rinses, mounted on microscope slides and cover slipped in Eukitt Quick-hardening mounting medium. All sections were stained in a single batch to avoid inter-assay variance.

2.8. Quantification of vasotocin-imunoreactive fibers

Sections containing the medial preoptic area at the level of maximal extension of the anterior commissure were photographed though an Olympus BH-2 microscope at 20X objective magnification with a CCD camera (Model CFW-1612C, Scion Corporation, Frederick MD, USA) connected to a MacIntosh computer (iMAC running under 10.4 OS). Two quantification fields (460 × 350 μm) were photographed at standardized locations in this section. A dorsal field was placed in the corner formed by the edge of the third ventricle and the ventral border of the anterior commissure and a more ventral field was adjacent to the first one. Together these two fields (460 × 700 μm or 322,000 μm^2) cover the whole area of dense vastotocinergic innervation associated with the medial preoptic nucleus.

The surface covered by vasotocin-immunoreactive (VT-ir) in these fields was then quantified with the help of the Image J software (version 1.47, Wayne Rasband, NIH, Bethesda MD, USA) as described before (Barker et al., 2014). Briefly, images were converted to 8-bit grayscale, and the image converted to a binary (black and white) image. The area covered by labeled material (now black) was measured with Image J's measure function. The fractional area (FA) covered by VT-ir fibers as then calculated as the percentage of pixels covered by immunoreactive material divided by the total area quantified. In a few subjects, no data could be collected due to inadequate angle of sectioning or tissue tear thus reducing the number of data points to 8 in the CX+T3 group, 7 in the Intact group and 6 in the other groups.

2.9. Statistical analyses

All data were analyzed by one- or two-way analyses of variance (ANOVA) as appropriate followed when significant by Newman-Keuls post hoc tests. All analyses were performed with the SuperAnova, Prism 5 or Statistica 9 software running on MacIntosh computers. Differences were considered significant for $p<0.05$ and all data are presented by their mean and standard error of the mean (SEM).

In an initial step, we compared by one-way ANOVA for each dependent variable the results of the 3 subgroups of Intact and 3 subgroups of CX males in order to test whether the different ages when they had received an empty Silastic™ implant had any impact on the results. All these comparisons yielded non significant results so that data for the 3 subgroups were pooled in each case. All final analyses presented in this paper thus concern 5 experimental groups: Intact (n=9), CX (n= 10) and CX+T implanted at 3 weeks (CX+T3; n=10), at 5 weeks CX+T5; n=10) or at 7 weeks (CX+T7; n=10).

3. Results

3.1. Morphology

3.1.1. Body weight—Birds were initially matched between groups for body weight and there was therefore no group difference (overall mean and SEM: 171.2 ± 2.7 g; $F_{4,44}=0.347$, $p=0.844$). Body weight then progressively increased in all birds as typically observed at that age to reach an average of 220.1 ± 3.6 g at the end of the experiment. This increase was however not similar in all groups so that an overall two-way ANOVA of these data revealed an overall effect of groups ($F_{4,44}=3.201$, $p=0.022$), of time ($F_{7,308}=97.951$, $p<0.0001$) and also an interaction between these factors ($F_{28,308}=3.204$, $p<0.0001$). These effects were mostly linked to the faster and larger growth of CX males and a slightly limited growth in Intact birds as compared to the 3 CX+T groups. The group difference thus disappeared when the CX group was excluded from the analysis ($F_{3,35}=0.271$, $p=0.846$) although some interaction between groups and time was still present ($F_{21,245}=1.928$, $p=0.010$) due to a lower body weight increase in the Intact as compared to the CX+T birds. An ANOVA focusing on these 3 CX+T groups identified no group difference ($F_{2,27}=0.209$, $p=0.823$) and no group by time interaction ($F_{14,189}=1.424$, $p=0.145$) although the effect of time was highly significant ($F_{7,189}=57.972$, $p<0.0001$). Body weights on week 12 at the end of the experiment were thus different between groups ($F_{4,44}=4.077$, $p=0.007$) with CX birds being

significantly heavier (244.6±5.9 g) that the other 4 groups that all had similar not significantly different body weights (Intact: 207±5.8, CX+T3: 217.6±7.4, CX+T5: 216.8±10.2, CX+T7: 213.4±4.7 g).

3.1.2. Cloacal gland area—Analysis of the cloacal gland areas (CGA) measured throughout the experiment (Weeks 5 to 12) by two-Way ANOVA revealed the presence of large differences between groups ($F_{4,44}$=113.80, p<0.0001) and of major effects of time ($F_{7,308}$=286.34, p<0.0001) and of the time by groups interaction ($F_{28,308}$=28.25, p<0.0001). This was largely expected since CX birds receiving no T had completely non-developed glands and T treatment in the 3 CX+T groups had been initiated at different time points (see Fig. 1)

Qualitative inspection of these data suggested however that in the 3 CX+T groups, the gland grew at various rates after the initiation of the T treatment. For example, the CGA was still relatively small on week 5 in the CX+T3 groups (i.e., after two weeks of treatment) whereas after the same duration of exposure to T (i.e. on week 9), the gland had a nearly maximal size in the CX+T7 group. Data for these 3 groups were thus plotted again using the age of implantation as starting point (Weeks 3, 5 or 7 being week 0 of T treatment; see Insert of Fig. 1). Analysis of the corresponding data (weeks 2 to 5 of T treatment, no data available for weeks 0 and 1 of T treatment in the CX+T3 groups since measures were only started on week 5) indeed revealed the existence of significant group differences ($F_{2,27}$=25.15, p<0.0001), of the expected significant effect of time ($F_{3,81}$=23.73, p<0.0001) but also of an interesting interaction between time and group ($F_{6,81}$=4.35, p<0.001). Post-hoc Newman-Keuls tests indicated a progressive decrease in the differences between groups (CX+T3 different for CX+T7 from week 2 to 4 and different from CX+T5 only on weeks 2-3, CX +T5 different from CX+T7 on weeks 2 only: see detail in Fig. 1 inset).

3.2. Sexual Behavior

3.2.1. Consummatory sexual behavior—Five behavioral tests were performed over a period of 10 days when CX+T birds had been exposed to exogenous T for at least 2 weeks (2 weeks in CX+T7, 4 weeks in CX+T5 and 6 weeks in CX+T3), a duration normally sufficient for the steroid to exert its maximal effects (Balthazart et al., 1983). As a consequence, no obvious evolution in the behavior of these males was detected with the possible exception that behavior frequencies tended to be lower during the first test but this was associated with random variations and similar in all experimental groups. In order to simplify the presentation, we shall thus consider only here the average behavior frequencies and latencies across the 5 tests. Similar results were observed for the 4 behavior patterns of the copulatory sequence and to avoid redundancy, we shall just focus here on MA and CCM.

The frequency and latency of both behaviors was significantly affected by the experimental treatments (MA Frequency: $F_{4,48}$= 8.585, p<0.0001; CCM Frequency: $F_{4,48}$= 9.655, p<0.0001; MA Latency: $F_{4,48}$= 60.09, p<0.0001; CCM Latency: $F_{4,48}$= 22.18, p<0.0001). For each dependent variable, post-hoc Newman-Keuls tests indicated that the CX group was significantly different for the 4 other groups while no other difference was observed (see

detail in Figure 2). In particular no significant difference was detected between the 3 CX+T groups.

3.2.2. Appetitive sexual behavior—As observed previously males produced few (usually less than 10) Rhythmic Cloacal Sphincter Movements (RCSM) during the 2.5 min when the female was not visible and these contraction frequencies were similar in all groups ($F_{4,48}$=0.7828, p=0.542; data not shown). These frequencies then markedly increased in the 4 groups exposed to T (Intact and 3 CX+T groups) when given visual access to the female so that group differences became highly significant ($F_{4,48}$=29.97, p<0.0001; see Fig. 2E). Post hoc tests indicated that the CX group produced significantly fewer RCSM than each of the other 4 groups. There was in addition a significant difference between the CX+T3 and CX+T7 groups with the latter producing significantly more RCSM. The CX+T5 group was characterized by an intermediate RCSM frequency and was not different from the other two. A separate ANOVA focusing on the 3 CX+T groups that were the focus of this experiment confirmed that they were significantly different ($F_{2,29}$=3.638, p=0.0399) with the post-hoc Newman-Keuls test indicating significant difference between the two most extreme groups (CX+T3 and CX+T7).

3.3. Preoptic aromatase activity

Aromatase activity in the HPOA was significantly affected by the experimental treatments ($F_{4,48}$=15.16, p<0.0001; see Fig. 3A). As previously demonstrated and published multiple times, CX birds had a lower brain aromatase activity that males exposed to T and post hoc tests confirmed the presence of this difference between the CX and each of the 4 other groups. The ANOVA focused on the 3 CX+T groups identified no significant difference ($F_{2,29}$=1.782, p=0.1875).

3.4. Vasotocinergic innervation of the preoptic area

Similarly, treatments overall affected the density of the vasotocinergic innervation of the preoptic area ($F_{4,48}$=14.95, p<0.0001; see Fig. 3B) but the only significant differences concerned the comparisons of the CX groups with the 4 others. There was no difference between the 3 CX+T groups ($F_{2,19}$=1.514, p=0.2482).

4. Discussion

This experiment showed that multiple sexually differentiated variables (males > females) are restored by exogenous T in castrated male quail to levels typical of sexually mature gonadally intact males (included as a positive control). Importantly, no difference was detected in the magnitude of these effects as a function of the age when the treatment with T was initiated with one exception: there was a gradual increase in the frequency of RCSM, a measure of appetitive sexual behavior, as the beginning of the treatment shifted from 3 to 5 to 7 weeks post-hatch and the difference between these two extreme groups (CX+T3 and CX+T7) was statistically significant.

Surprisingly, this effect is in the opposite direction of what could have been anticipated based on the rat and hamster data reviewed in the introduction. It has indeed been shown that adult responses to T in rodents decrease as the age of peri-pubertal exposure to T

increases (Schulz et al., 2009b) leading to the suggestion that subjects entering puberty earlier should display stronger male characteristics than subjects with a late puberty (Schulz et al., 2009a; Sisk and Zehr, 2005) and correlative evidence suggests the existence of a similar phenomenon in humans (Beltz and Berenbaum, 2013; Berenbaum and Beltz, 2011). The higher frequency of RCSM in the CX+T7 birds as compared to the CX+T3 subjects thus appears at first sight counterintuitive but it must be noted that the protocol used in the present experiment does not fully reproduce the experiments performed in rats (Ahmed et al., 2008) and hamsters (Schulz et al., 2009b). As a consequence the present experiment does not permit in itself to fully identify the cause of the behavioral difference detected here.

Birds here were all tested at the same age (9 weeks post-hatch) with a T treatment that was initiated at 3, 5 or 7 weeks post-hatch and continued until testing. The duration of the treatment therefore represents a potential confound, as it does not permit to fully assign a difference to the age when T treatment was started. The alternative protocol that was used in the rodent studies is to perform at different peri-pubertal ages treatments with T of the same duration and then later in life to test either at the same age, but after various duration of treatment interruption, or at a specified time after the end of the exposure to T, but then at different ages, the effects of a standard activation by T. In both cases, additional control groups are needed to ensure that observed differences do not reflect the difference in age or in duration of T withdrawal but rather the age when T was initially administered. Such controls were not included here since this was a first experiment assessing whether age at first T treatment has any effect. However, this methodological limitation should not prevent us from drawing reasonably firm conclusions. Since we know that when quail have been exposed to Silastic™ implants of T for 2-3 weeks their behavior remains stable for many weeks if not months, it is unlikely that any putative effect of the age at initiation of T treatment could have been counterbalanced by the effect of the total duration of the treatment on copulatory behavior, AA and vasotocin . We therefore considered that performing additional experiments with large numbers of control subjects would be a waste of time, resources and animal lives.

The only significant difference between the CX+T3 and CX+T7 groups relates to a higher frequency of RCSM in the latter of these groups. It appears unlikely that the shorter 2-week-treatment with T (initiated at 7 weeks post-hatch) as compared to the longer 6-week-treatment (initiated at 3 weeks post-hatch) could be held responsible for the enhanced response. The opposite would rather be expected but this is also true if we try to relate this difference to the age when treatment was started: based on rodent studies an opposite pattern of response should have been observed (Schulz et al., 2009b). Silastic™ T implants such as those used here are known to establish stable plasma concentrations of T in quail for several weeks to months (Desjardins and Turek, 1977) so that a decrease in the activation by the exogenous steroid is also unlikely. There is thus at this time no really satisfactory mechanism to explain this difference and additional studies should be performed to test whether this difference in a measure of sexual motivation is reproducible.

The only effect of T that was clearly affected by the age at which the treatment was initiated concerns the growth rate of the cloacal gland even if the gland finally reached the same full size (on week 12) that is characteristic of sexually mature gonadally intact males. This

response is thus not different from the other T-dependent responses. The growth of the cloacal gland was significantly faster at 7 than at 3 weeks post-hatch (see insert of Fig. 1). The delayed growth in younger birds could relate to their lower body weight but this seems unlikely since at the time when the largest differences in cloacal gland areas was noted (week 2 of T treatment) birds were 5, 7 or 9 week old (in the CX+T3 CX+T5 and CX+T7 respectively) and they had already reached a nearly adult body weight (166.7± 3.6, 195.0±5.9 and 205.0±4.7 g respectively). The limited differences in body weight (maximum 39 g or 20-25% between the two extreme groups) cannot explain the 150% difference observed in cloacal gland area (difference in volume or weight would have an even larger amplitude). Other limiting factors for T action must therefore be invoked and two likely candidates are actually easily identifiable.

Firstly, cloacal gland growth is activated by T acting as an androgen since injections of androgen receptor blockers but not of estrogen receptor blockers markedly inhibit this process (Alexandre and Balthazart, 1986). Correlative evidence indicates that the affinity of androgen receptors is a limiting factor for gland growth. The regrowth of the gland in birds recovering from the exposure to short days is indeed closely paralleled by the increase in plasma T concentration. The half-maximal increase occurs for a plasma concentration of 0.69 nM that is close to the dissociation constant (Kd) of the androgen receptor suggesting that the affinity of the receptor and availability of this receptor and its substrate are the limiting factors (Delville et al., 1985). A sub-maximal androgen receptor expression at 3 or 5 week of age could thus explain the slower reaction to T when applied at that time.

Secondly, T metabolism in the gland could also be limiting its growth in young birds. The T metabolite 5α-dihydrotestosterone (5α-DHT) is more active than T itself for inducing the crowing vocalization and cloacal gland growth in young quail chick (Balthazart et al., 1984) presumably because the 5β-reductase activity (production of the inactive metabolite 5β-DHT) (Massa et al., 1980) is high in young chicks and inactivates T but not 5α-DHT trophic action (Balthazart and Schumacher, 1984a). This age-related difference in 5β-reductase activity was however not as prominent in the cloacal gland as it was in the brain even if there was a clear decline between one and five weeks of age (Balthazart and Schumacher, 1984a). Additional studies would thus be needed to precisely identify the mechanism(s) underlying the age-differentiated response to T of the cloacal gland.

Overall the present experiment thus suggests a discrepancy between results in rodents and possibly humans where peri-pubertal T seems to have long-term effects on brain structure and behavioral expression, and in quail where the age at which T treatment was initiated had little or no effect on adult behavior and on two sexually differentiated neurochemical characteristics. Whether this difference between mammals (two species so far, hamsters and rats) and birds (one species so far, quail) is generalizable remains to be established but if this proved to be the case, this would then point to an interesting generalization of the mechanisms underlying development of sexually differentiated characteristics. In mammals peri-natal T masculinizes and defeminizes behavior (McCarthy, 2012; McCarthy et al., 2009) and the peri-pubertal action of T can thus be seen as an extension of this earlier effect (Berenbaum and Beltz, 2011; Schulz et al., 2009b). Puberty has consequently been suggested to be a second period of organization by sex steroids. It must in this context be

recalled that in quail, sexual differentiation of copulatory behavior develops under the demasculinizing action of estrogens secreted by the embryonic ovary (Adkins, 1979; Adkins-Regan, 1983; Balthazart et al., 1992). T action during sexual maturation thus cannot be in any way considered as an extension of the early organizing effects of steroids. Testing in quail the equivalent of the peri-pubertal T actions described in mammals should thus in quail consist in testing the effects of estrogens during sexual maturation of females.

Previous studies showed that the demasculinizing effects of estradiol can only be demonstrated in male quail during a critical period ending on day 12 of incubation (Adkins, 1979; Balthazart et al., 1992). However, studies in females suggested that this critical period is not yet completely closed at hatching (16-17 days on incubation) since females that were ovariectomized during the first two weeks after hatching were still able when adults to display some T-induced male-typical copulatory behavior provided they were tested in suitable conditions (in their home cage) (Balthazart and Schumacher, 1984b; Hutchison, 1978; Schumacher and Balthazart, 1984). This suggested that the end of the critical period for demasculinization by estrogens is not equivalent in males and females (Schumacher and Balthazart, 1985) and that in females full demasculinization is only achieved around two weeks post-hatch (activation by T of male-typical copulatory behavior becomes impossible in females ovriectomized at a later stage) (Balthazart and Schumacher, 1984b; Schumacher and Balthazart, 1984). Identifying peri-pubertal effects of estrogens in females would definitely challenge this notion and this intriguing possibility is certainly worth exploring.

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Highlights

- Cloacal glands develop faster in response to testosterone in older quail
- Appetitive sexual behavior is enhanced more by a later testosterone implantation
- Consummatory sexual behavior is not affected by age at testosterone implantation
- Preoptic aromatase activity and vasotocinergic innervation are not affected
- Peri-pubertal testosterone does not seem to have organizational effects in male quail

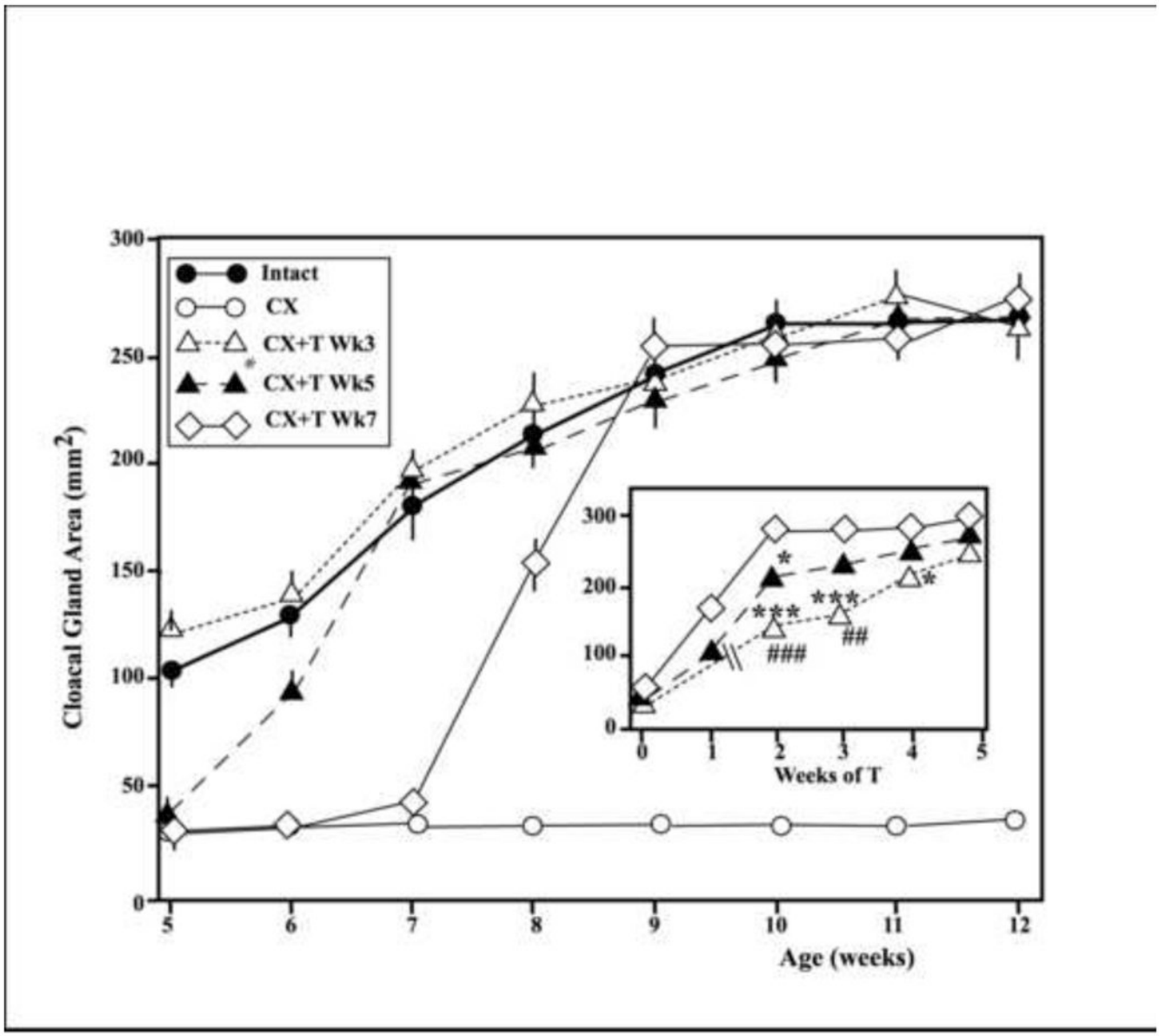

Fig. 1.

Changes in the androgen-dependent cloacal gland area as a function of age (5 to 12 weeks post-hatch) in groups of male quail that were either gonadally intact (Intact) or had been castrated at 17-18 days post-hatch and then received empty Silastic implants (CX) or implants filled with testosterone (T) at the age of 3 weeks (CX+T3), 5 week (CX+T5) or 7 weeks (CX+T7) post-hatch. Data in the insert were replotted in the three CX+T groups with the age at implantation set at zero. Symbols in this graph represent the probabilities of the differences between groups (* = differences vs. the CX+T7 group; # = differences vs. the CX+T5 group) as derived from post-hoc Newman-Keuls tests; see text for detail). * or # = $p<0.05$, ** or ## = $p<0.01$ and *** or ### = $p<0.001$.

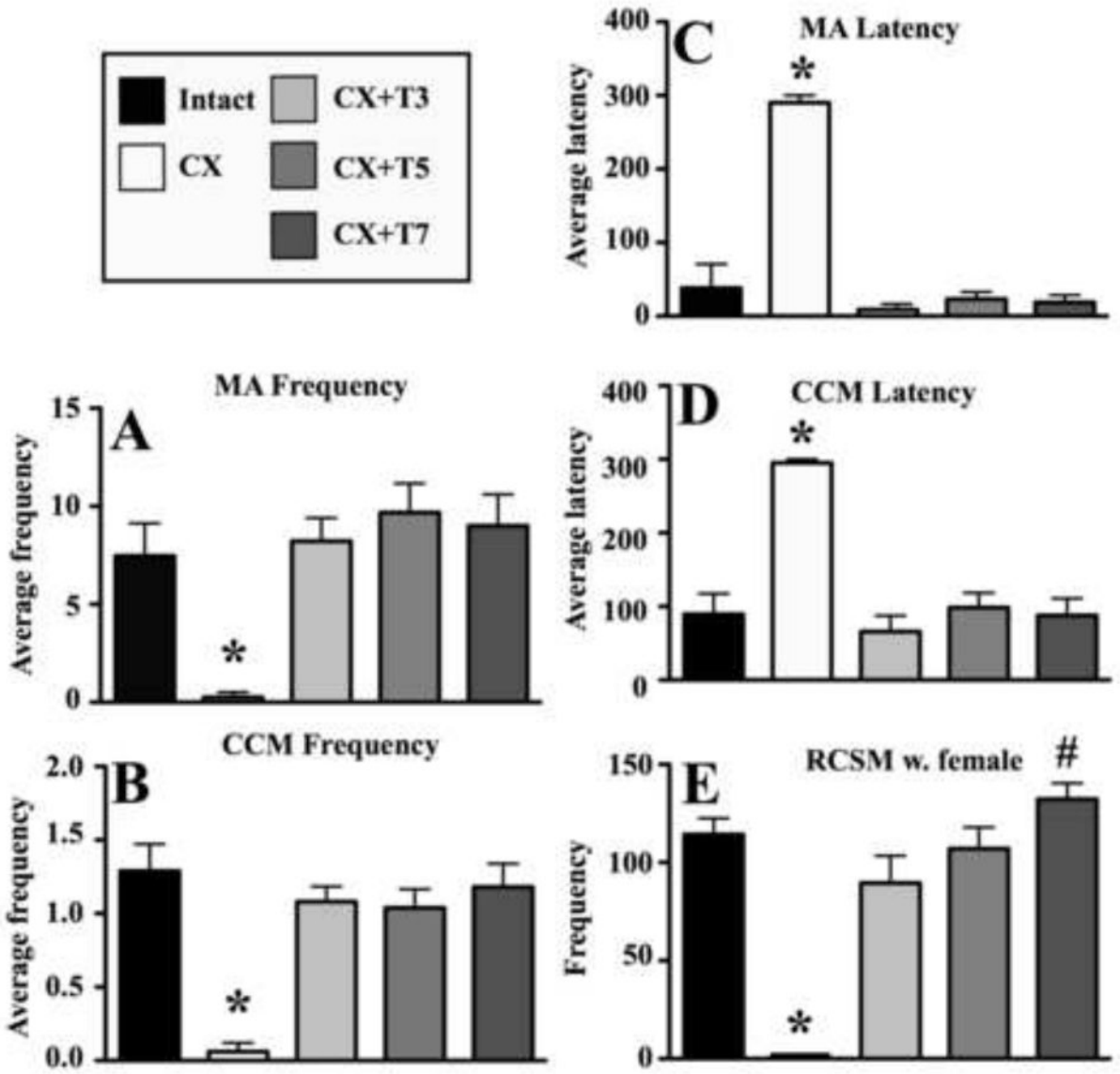

Fig. 2.

Average frequencies and latencies of mount attempts (MA) and cloacal contact movements (CCM) and frequencies Rhythmic Cloacal Sphincter Movements (RCSM w. female) when the stimulus female was in sight in groups of male quail that were either gonadally intact (Intact) or had been castrated at 17-18 days post-hatch and then received empty Silastic implants (CX) or implants filled with testosterone (T) at the age of 3 weeks (CX+T3), 5 week (CX+T5) or 7 weeks (CX+T7) post-hatch. *= $p<0.05$ vs. the 4 other groups; # = $p<0.05$ vs. the CX+T3 group.

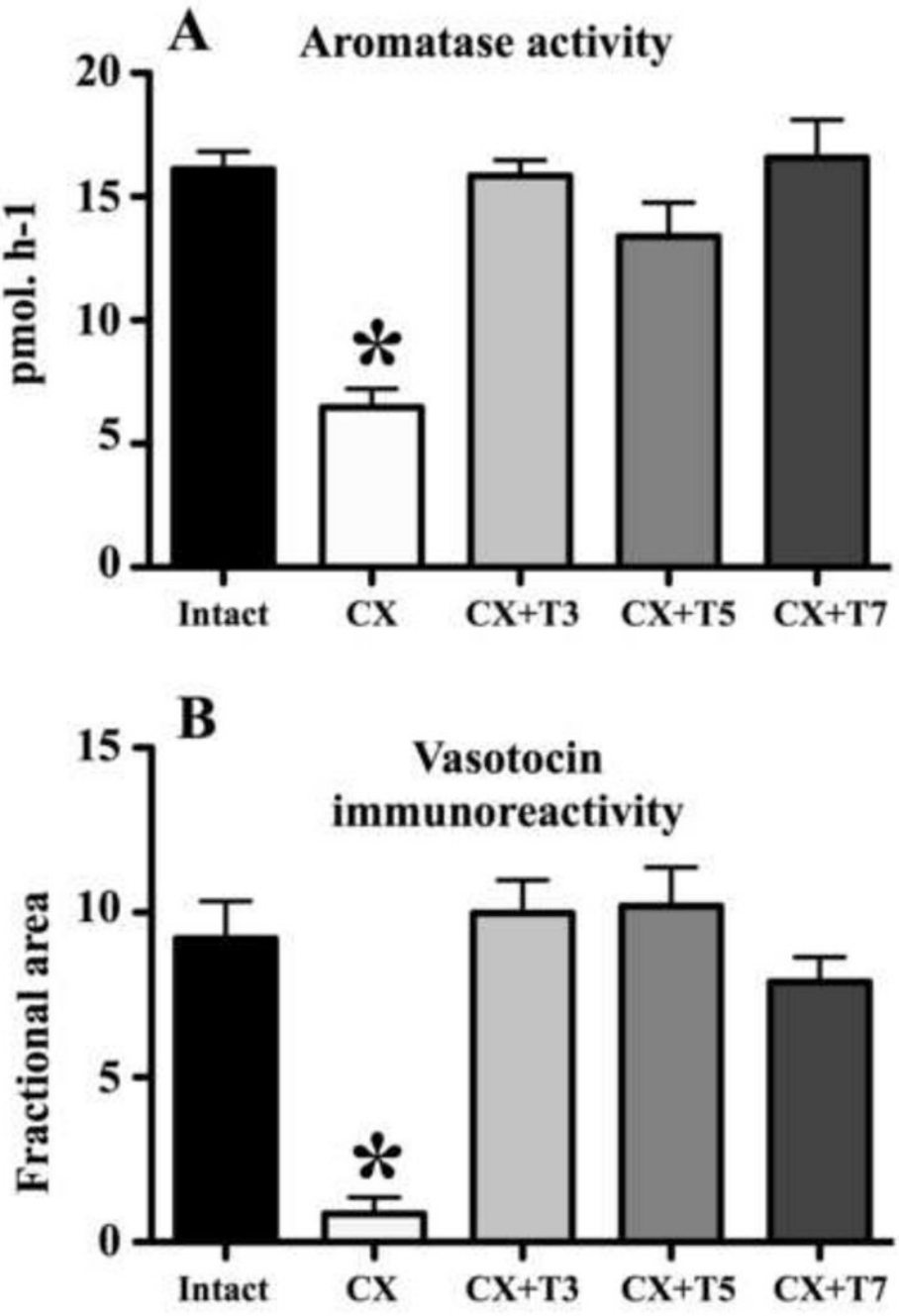

Fig. 3.

Aromatase activity (in pmol . h^{-1}) and density of the vasotocinergic innervation (fractional area in % covered by immunoreactive fibers) in groups of male quail that were either gonadally intact (Intact) or had been castrated at 17-18 days post-hatch and then received empty Silastic implants (CX) or implants filled with testosterone (T) at the age of 3 weeks (CX+T3), 5 week (CX+T5) or 7 weeks (CX+T7) post-hatch. Numbers of subject in which

quantification of VT could be performed is indicated in the bars. *= $p<0.05$ vs. the 4 other groups.