Neuroendocrine correlates of sex-role reversal in barred buttonquails

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Abstract

Sex differences in brain structure and behaviour are well-documented among vertebrates. An excellent model exploring the neural mechanisms of sex differences in behaviour represent sex-role reversed species. In the majority of bird species males compete over access to mates and resources more strongly than do females. It is thought that the responsible brain regions

are, therefore, more developed in males than in females. Because these behaviours and brain regions are activated by androgens, males usually have increased testosterone levels during breeding. Therefore, in species with sex-role reversal, certain areas of the female brain should be more developed or steroid hormone profiles should be sexually reversed. Here I studied circulating hormone levels and gene expression of steroid hormone receptors and aromatase in a captive population of barred buttonquails (*Turnix suscitator*). While females performed courtship and agonistic behaviours, there was no evidence for sexually reversed hormone profiles. However, I found female-biased sex differences in gene expression of androgen receptors in several hypothalamic and limbic brain regions that were already in place at hatching. Such sex differences are not known from non-sex-role reversed species. These data suggest that increased neural sensitivity to androgens could be involved in the mechanisms mediating sex-role reversed behaviours.

Keywords: sex-role reversal, testosterone, barred buttonquail, steroid hormone receptor, sex differences

1. Background

Among vertebrates, neural sex differences are abundant. Such influences of sex can concern anatomy, neurochemistry and neuronal activity of the brain (Cahill 2006; DeVries and Soedersten 2009; McCarthy et al. 2012). Functionally well-defined neural sexual dimorphisms constitute for example the sexually dimorphic nucleus of the medial preoptic area in Japanese Quail (*Coturnix japonica*), which is about 40% larger in males than in females and mediates copulatory behaviour (Viglietti-Panzica et al. 1986) and the song control system of songbirds. The latter represents a network of forebrain areas, responsible for song learning and song production, which exhibits extreme sex differences in size and neuron

number in some species. In zebra finches (*Taeniopygia guttata*), where only the male sings, the volumes of song control nuclei RA and HVC are about 5 times larger in males than in females (Nottebohm and Arnold 1976).

Sexual differentiation of brain and behaviour is no longer thought to be solely driven by the action of gonadal steroid hormones but accumulating evidence suggests that non-steroidal and genetic mechanisms are crucial (for review, see McCarthy and Arnold 2011). Classically, gonadal steroid hormones act on neurons via the presence of androgen and estrogen receptors (McEwen et al. 1979). The area-specific expression of these steroid hormone receptors in the brain is assumed to be controlled by brain-intrinsic genetic factors (Gahr and Balaban 1996; Gahr and Metzdorf 1999). Then, gonadal hormones determine the development of sexually differentiated brain areas, which is called organizational action. Later in life, gonadal hormones are thought to modify those brain areas such as the medial preoptic nucleus in male quail to mediate certain types of behaviours, which is called activational action (Arnold and Gorski, 1984).

In the majority of bird species males compete over females. The responsible brain areas mediating courtship and aggressive behaviour are, therefore, thought to be more developed in males than in females. Because these behaviours and brain regions are activated either directly by the androgen testosterone or indirectly following aromatization in the brain to 17β-estradiol, males usually show increased testosterone levels during breeding (Adkins et al. 1980, Steimer and Hutchison 1981, Balthazart 1983, Wingfield et al. 1987). However, in approximately 1% of all avian species, called classically polyandrous species, sex-roles are reversed; females compete over males and/or resources. Beyond that, they are often much larger in body size and more conspicuously coloured than males. The males, on the other hand, have the sole responsibility for all parental care, including nestbuilding and incubation (Emlen and Oring 1977; Ridley 1978; Andersson 2005). For example, female American

jacanas (*Jacana spinosa*) are about 75% larger than males and defend super-territories that include the territories of up to four mates (Jenni and Collier 1972). Thus, according to the concept of sexual differentiation of brain and behaviour in sex-role reversed species, certain areas of the female brain that control what in other species are typical masculine behaviours should be more developed than those of males.

The few studies that have tackled the physiological basis of sex-role reversal, have failed to uncover reversal in circulating steroid hormone levels. In all species studied so far, males have been found to have either higher levels of testosterone than females, resembling the pattern of non-sex-role reversed birds (Rissman and Wingfield, 1984; Fivizzani et al., 1986; Fivizzani and Oring, 1986; Oring et al., 1988; Gratto-Trevor et al., 1990; Goymann and Wingfield, 2004) or testosterone levels were similar between sexes (Muck and Goymann 2011). To date, only two species have been subject to neuroendocrine studies. In the sex-role reversed Wilson's phalarope (*Phalaropus tricolor*), Schlinger et al. (1989) measured the activity of three enzymes metabolizing androgens in the brain, aromatase, 5α- and 5ß-reductase. While for the latter no sex differences were found, aromatase activity was male-biased in the hypothalamic-preoptic area, matching results from non-sex-role reversed species (Schumacher and Balthazart 1986; Roselli and Resko 1993). A more recent study by Voigt and Goymann (2007) on sex-role reversed black coucals (*Centropus grillii*) reported for the first time a neural sex difference in favour of females, concerning the expression level of androgen receptors in nucleus taeniae of the amygdala, a region of the arcopallium that is known to be involved in agonistic and sexual behaviour in other bird species (Akerman 1966; Maley 1969; Phillips and Youngren 1971). Taken together, these data do not support the view that sexually dimorphic levels of circulating steroid hormones directly activate sex-role reversed behaviour. Rather, they suggest organizational action of gonadal hormones at the responsible brain regions or direct genetic effects.

Barred buttonquails (*Turnix suscitator*) are a classically polyandrous species of the order Charadriiformes, with a marked sexual dimorphism in size and plumage colouration. Females are larger than males and have a black throat and breast patch, which is lacking in males. Moreover, while females engage in territory defence and mate attraction, the latter through producing a booming vocalization, males are responsible for parental care including incubation (Starck 1991, Debus 1996). The purpose of my study was to identify whether male and female buttonquails show differential steroid hormone sensitivity in particular target regions of the brain. Owing to its short incubation period of 14 days and the short generation time of 3 months, barred buttonquails constitute an ideal model species. By means of *in situ* hybridization, I analysed the mRNA expression levels of androgen receptor (AR), estrogen receptor alpha and beta (ERα and ERβ) and the estrogen-forming enzyme aromatase in hypothalamic and limbic brain regions involved in the regulation of vertebrate social behaviour (for review, see Goodson 2005). Additionally, I included a midbrain area, the nucleus intercollicularis, implicated in avian vocal behaviour (Potash 1969; Brown 1970). In order to distinguish between neural sex differences that are driven by activational effects of gonadal hormones in adulthood and those that are either genetic or organized early in life, I studied both adult and hatchling (posthatching day 0) buttonquails.

2. Materials and Methods

(a) Animals

This study was carried out with sexually mature and newly hatched male and female barred buttonquails (*Turnix suscitator*) between 2009 and 2011. Hatchlings were sexed by means of DNA sexing using the P2/P8 primers from Griffiths et al. (1998). Animals were either purchased from local breeders or raised in the breeding stock of the Max Planck Institute for Ornithology, Germany. Birds were kept pairwise in cages of 1.25 x 0.8 x 0.6 m at a light-dark

cycle of 13:11 (L/D) simulating the photoperiod of their natural environment. The cages were equipped with artificial plants to provide shelter and a bowl of warmed sand for dust bathing. Hay was given as nesting material. Food and water were available *ad libitum*. For individual recognition, animals received a numbered aluminium ring. Hatchling birds were obtained by incubating eggs in an Incubator (J. Hemel Brutgeräte) at 37.3°C and 69% humidity. Eggs hatched after 13-14 days.

(b) Behaviour observations

Behavioural observations were carried out on 10 pairs by using a digital Handycam DCR-TRV8E (Sony Electronic Industries) mounted on a tripod. Pairs were videotaped for an hour in the morning and in the afternoon, respectively. In total, 14 hours of observation were recorded from each pair. The following behaviours were recorded: *Calling*: The individual resumes a "booming" position and produces an uninterrupted booming sound (Video S1). *Chasing*: The individual rushes towards the conspecific while it runs away. Each burst of locomotion towards the fleeing individual was termed a chase (Video S2). *Courtship feeding (Tidbitting)*: The individual picks up a food item (e.g. buffalo worm) and holds it in its bill until the other individual has picked it up. It utters a soft call at the same time (Video S3). *Nestbuilding*: The individual carries grass stems towards the nest site or the individual arranges the pieces of grass inside the nest. Each bout of grass carrying was counted as one event. Behavioural frequencies were calculated as total number of observations during 14 hours of recording.

(c) Blood sampling

From adult birds (N=14 males, N=14 females), blood samples were obtained between June and August in 2009 and 2010 when pairs were in breeding condition which was confirmed by

egg-laying. A blood sample (approx. 200 µl) was taken within 5 min of opening the cage by puncturing the wing vein and blood was collected into heparinized capillaries. From birds at hatching day (N=7 males, N=8 females) the trunk blood (65-100µl) was collected at the time of sacrifice. Because hatchlings yielded very small amounts of blood, samples from two individuals from each sex were pooled, respectively. All blood samples were centrifuged immediately at 10000 rpm for 10 min and plasma was stored at -80°C until hormone analysis.

(d) Hormone analysis

The steroids progesterone (P4), testosterone (T), 5α-dihydrotestosterone (DHT), and 17β-estradiol (E2) were measured by radioimmunoassay (RIA) after extraction with dichlormethane and partial purification on diatomaceous earth microcolumns following previously described protocols (Fusani et al. 2000, Goymann et al. 2001) with a modification of the method described by Wingfield and Farner (1975). Due to chromatographical separation of the steroids it is unlikely that the antisera have cross-reacted with other steroids. Plasma samples of adults and hatchlings were analysed in the same assays, respectively. The detection limits (pg/ml plasma) were 20.8 for P4, 4.1 for T, 4.7 for DHT, and 2.1 for E2. Water blanks were always below the lower detection limit. The average recoveries were between 60.0 and 85.1%. The intra-assay variation was 9.4% for P4, 6.2% for DHT, 7.7% for T and 2.9% for E2.

(e) Brain histology

Birds (adults: N=6 for each sex; hatchlings: N=7 for each sex) were killed by decapitation, brains were dissected out of the skull, frozen on dry ice and stored at -80°C until used. Frozen brains were cut on a cryostat into 30 µm coronal sections. The plane of the sections was adjusted to match as closely as possible the plane of the quail brain atlas (Baylé et al. 1974).

Sections were mounted onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) in five different series. *In situ* hybridization for androgen receptor (*AR*), aromatase (*ARO*), estrogen receptor α (*ERα*) and estrogen receptor β (*ERβ*) mRNA was carried out on adjacent series of sections. There was no sex difference in brain weight either between adults (males: 0.59 ± 0.02g, females: 0.62 ± 0.02g, t=1.28, df=10, P=0.23) or hatchlings (males: 0.22 ± 0.02g, females: 0.19 ± 0.01g, t=1.32, df=12, P=0.21).

(f) Cloning of cDNA probes

Based on sequence information available from Japanese quail, PCR was used to amplify fragments of the *AR*, *ARO*, *ERα* and *ERβ* genes from buttonquails. Total RNA was extracted from buttonquail hypothalamus by using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The synthesis of first-strand cDNA was done with SUPERSCRIPT III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and random primers. The resulting RNA-DNA hybrids were subsequently used in PCR to generate pieces of the appropriate genes (see Table S1 for primer sequences). The cloned *AR* sequence [GenBank: KJ689378] is 618 bp in length, the *ARO* sequence [GenBank: KJ689380] is 490 bp in length, the *ERα* sequence [GenBank: KJ689379] is 924 bp in length and the *ERβ* sequence [GenBank: KJ689377] is 1032 bp in length. Homology to the respective genes from quail is 89-94%. PCR was carried out for 40 cycles by using the following parameters: 94°C for 1 minute, 54°C (*ERα* and *ARO*) or 58°C (AR and *ERβ*) for 45 seconds, 72°C for 1 minute. Amplified fragments were purified and cloned into the pCRII TOPO vector using the TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany). Resultant clones were sequenced to verify the authenticity and fidelity of the amplification.

(g) *In situ* hybridization

The expression of *AR*, *ARO*, *ERα* and *ERβ* in brain sections was detected with antisense RNA probes labeled with ^{35}S-CTP. Labeling of the probes with ^{35}S-CTP (1250 Ci/mmol; Perkin Elmer, Rodgau, Germany) was performed using the Riboprobe System (Promega). Our *in situ* hybridization procedure followed a standard protocol with modifications (Whitfield et al. 1990). For signal detection, sections were exposed to autoradiographic film (Kodak Biomax MR, Rochester, NY, USA) for different durations (*AR*, *ARO* 9 weeks; *ERα*, 10 weeks; *ERβ*, 7 weeks). Male and female brains were run through the entire procedure at the same time and sections from both sexes were placed on each autoradiographic film to avoid any possible effect of small differences in procedures on the observed sex differences.

(h) Data analysis

Images from autoradiograms were scanned with an Epson Perfection V750 Pro scanner connected to a PC running the image analysis software Image J 1.43u (NIH, USA; see http://rsb.info.nih.gov/ij/). The system was calibrated by using a calibrated optical density step tablet (T2115CC; Stouffer Industries, Inc., Mishawaka, IN, USA) and a calibration curve was fitted with the Rodbard function of Image J [y = d + (a - d) / (1 + (x/c)^b)]. This calibration was applied to all images and extended beyond the darkest spot to be measured in the autoradiograms so that the signals that were measured did never reach saturation. Regions of interest in each section were delineated on screen with the computer mouse and their average optical density (OD) was calculated by the built-in function of the software. Background optical density of the film was measured in a rectangular area (2 mm^2) in the same image immediately ventral to the brain section of interest. Final OD measurements were obtained by subtracting the film background OD value from the OD value of the region of

interest. Brain regions were identified using the atlas of the quail and the chicken (Baylé et al. 1974; Kuenzel and Masson 1988).

(i) Statistical analysis

Statistical analyses were carried out using JMP software. Data are presented as means ± SEM. Sex differences in body mass of adults and hatchlings were analyzed with t-tests. For analysis of the posthatching growth pattern, a REML-model was employed with sex and posthatching day as fixed factors and bird ID as random factor. Behavioural data and plasma hormone levels were analysed using nonparametric statistics. For analysis of gene expression, a REML-model was employed with sex and brain region as fixed factors and bird ID as random factor. Posthoc analyses were performed using the 'test slices' comparison in JMP. All tests were two-tailed and the significance level was fixed at $p<0.05$.

3. Results

(a) Body mass of adult and hatchling buttonquails

Adult female buttonquails were about 30% heavier than males (75.4 ± 2.2g vs. 56.2 ± 1.1g, t=7.91, df=26, P=0.0001). Hatchling males and females did not differ in body mass (3.16 ± 0.19g vs. 3.17 ± 0.16g, t=0.06, df=12, P=0.952). The sex difference emerged at posthatching day 19 (sex: $F_{1,13.64}$=0.006, P=0.93; day: $F_{10,100}$=1510.3, P<0.0001; interaction: $F_{10,100}$=3.41, P=0.0007).

(b) Sex differences in behaviour

In adult buttonquails, four different types of behaviours were recorded (Fig. 1). Significant differences were found in the frequency of *calling* (Wilcoxon test, W=0, N=10, P=0.009), *chasing* (Wilcoxon test, W=0, N=10, P=0.022) and *courtship feeding* (Wilcoxon test, W=1,

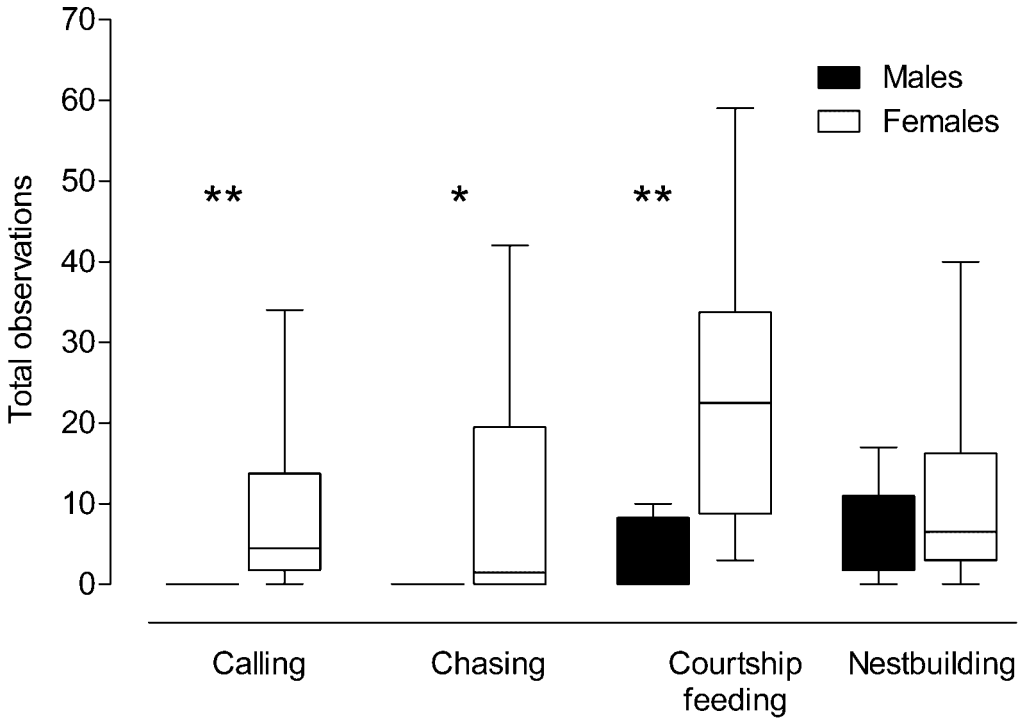

Figure 1. Sex differences in behaviours associated with breeding of male and female barred buttonquails (median, 1st, 3rd quartile, range). *$p < 0.05$, **$p < 0.01$.

N=10, P=0.004) with higher occurrence in females than in males. In fact, *calling* and *chasing* were exclusively performed by females. *Nestbuilding* frequency did not differ between sexes (Wilcoxon test, W=10, P>0.05). The female call constitutes a booming sound, which is produced by inflating the anterior air sacs and the trachea (Fig. 2 a, b, Video S1). The call rate increased significantly when females were kept on their own compared to being paired to a male, which supports the view that this call type functions in mate attraction (Wilcoxon test, N=7, W=0, P=0.031, Fig. 2c).

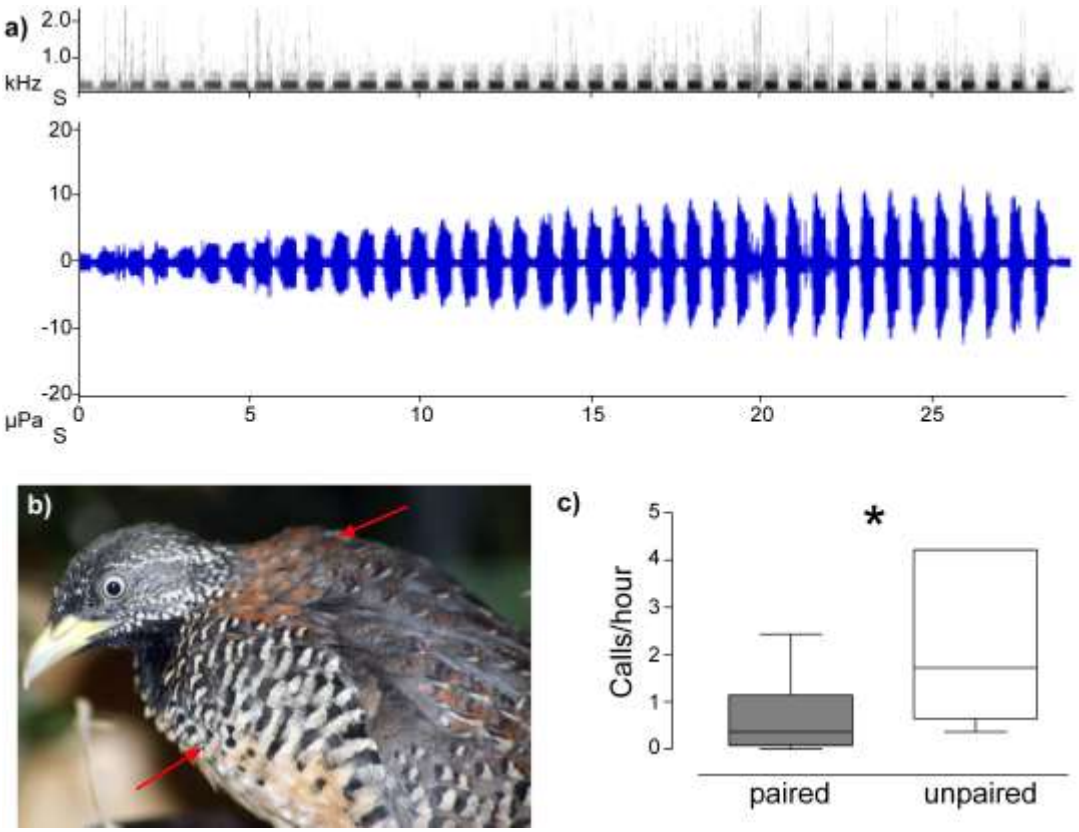

Figure 2. (*a*) Wavelength and spectrogram of the female booming call. (*b*) Photograph showing a female in 'booming' position. Arrows indicate the inflated trachea and anterior air sacs. (*c*) Call rate of paired and unpaired females (median, 1st, 3rd quartile, range; *$p < 0.05$).

(c) Circulating steroid hormone levels of adult and hatchling buttonquails

Adult male and female buttonquails did not differ significantly in circulating T levels (males: 0.5 ± 0.1 ng/ml, females: 0.2 ± 0.1 ng/ml, Mann-Whitney U-test, U=57, P=0.06). P4 levels showed considerable variation among individuals in both sexes but no significant difference was found (males: 1.1 ± 0.5 ng/ml, females: 0.6 ± 0.2 ng/ml, Mann-Whitney U-test, U=81, P=0.45). Concentrations of DHT and E2 were close to or below the detection limit and not significantly different between sexes (DHT: males: 0.03 ± 0.008 ng/ml, females: 0.03 ± 0.007 ng/ml, Mann-Whitney U-test, U=93.5, P=0.84; E2: males: 0.002 ± 0.0 ng/ml, females: 0.003 ± 0.0008 ng/ml, Mann-Whitney U-test, U=57, P=0.76). In hatchling buttonquails, levels of E2 and DHT were below the detection limit. T levels were above the detection limit only in two females (0.033 ng/ml, 0.038 ng/ml) and none of the males (males: 0.004 ± 0.0 ng/ml, females: 0.012 ± 0.005 ng/ml, Mann-Whitney U-test, U=21, P=0.17). Similar to adult birds, P4 levels

were highly variable among individuals but not significantly different between males and females (males: 1.06 ± 1.79 ng/ml, females: 0.91 ± 0.21 ng/ml, Mann-Whitney U-test, U=20, P=0.39).

(d) Sex differences in gene expression levels in adults and hatchlings

I quantified the mRNA expression levels of AR, ERα, ERβ and ARO in several areas of the hypothalamic and limbic system that are implicated in the control of agonistic and sexual behaviour in birds and mammals and in an area of the midbrain involved in vocal behaviour (Potash 1969, Newman 1999; Goodson 2005). Those regions were the medial preoptic nucleus (POM), the bed nucleus of the stria terminalis (BSTM), the lateral septum (LS), the mediobasal hypothalamus (MBH), the nucleus taeniae of the amygdala (TnA) and the nucleus intercollicularis (ICo). The four genes were expressed in a specific manner in those nuclei and the overall distribution was found to be similar in adult and hatchling buttonquails (Fig. 3; electronic supplementary Fig. S1). Measurement of the average optical density in these different cell groups revealed significant sex differences, both in adults and hatchlings (Table 1). In adults, AR expression was female-biased in LS, TnA and MBH (Fig. 3A, E, I vs. M, Q, U, Table 2). Concerning ERα expression, a female-bias was found in MBH (Fig. 3J vs. V), while in the POM the female-biased difference failed to be significant (p=0.05). ERβ expression was not significantly different between the sexes in any region. ARO expression showed a strong male-bias in POM and MBH (Fig. 3D, L vs. T, X). In hatchlings, a strong female-bias in AR expression was found in POM, LS, TnA and MBH, and to a lesser extent in ICo (Fig. S1 A, E, I vs. M, Q, U, Table 2). ERα expression showed the same female-bias in POM and MBH as in adults (Fig. S1 B, J vs. N, V). No sex differences existed regarding ERβ and ARO expression.

Table 1: Linear mixed model results for the effects of sex and brain region on gene expression and mean expression levels of males and females in different brain regions of hatchling and adult buttonquails (* p<0.05, ** p<0.01, *** p<0.001; - no expression detected)

		AR		ERα		ERβ		ARO	
		hatchlings	adults	hatchlings	adults	hatchlings	adults	hatchlings	adults
sex		$F_{1,12}$=105.60 p<0.001	$F_{1,10}$=3.01 p=0.11	$F_{1,12}$=22.46 p<0.001	$F_{1,10}$=4.97 p=0.049	$F_{1,12}$=0.16 p=0.695	$F_{1,10}$=0.115 p=0.742	$F_{1,12}$=0.46 p=0.513	$F_{1,10}$=41.54 p<0.001
region		$F_{4,48}$=20.55 p<0.001	$F_{4,40}$=21.16 p<0.001	$F_{4,48}$=31.65 p<0.001	$F_{4,40}$=192.31 p<0.001	$F_{4,48}$=87.57 p<0.001	$F_{4,40}$=108.54 p<0.001	$F_{2,24}$=19.93 p<0.001	$F_{2,20}$=31.56 p<0.001
sex x region		$F_{4,48}$=1.98 p=0.112	$F_{4,40}$=8.22 p<0.001	$F_{4,48}$=4.59 p=0.003	$F_{4,40}$=6.45 p<0.001	$F_{4,40}$=0.85 p=0.503	$F_{4,40}$=1.01 p=0.412	$F_{2,24}$=1.09 p=0.357	$F_{2,20}$=2.70 p=0.092
POM	♀ ♂	0.51±0.04 0.32±0.02 ***	0.46±0.03 0.49±0.06	0.40±0.05 0.21±0.03 ***	0.44±0.04 0.37±0.03 p=0.05	0.57±0.03 0.52±0.05	0.64±0.04 0.57±0.05	0.29±0.02 0.33±0.04	0.36±0.04 0.57±0.02 ***
MBH	♀ ♂	0.47±0.03 0.31±0.03 ***	0.51±0.03 0.39±0.05 *	0.32±0.02 0.19±0.02 ***	0.54±0.04 0.38±0.03 ***	0.53±0.03 0.50±0.05	0.62±0.02 0.63±0.03	0.48±0.02 0.46±0.02	0.40±0.04 0.62±0.03 ***
LS	♀ ♂	0.53±0.01 0.33±0.02 ***	0.54±0.03 0.40±0.03 *	0.15±0.01 0.09±0.01	0.11±0.01 0.07±0.01	-	-	-	-
TnA	♀ ♂	0.60±0.03 0.40±0.02 ***	0.51±0.03 0.35±0.04 **	0.11±0.01 0.08±0.02	0.05±0.004 0.05±0.004	0.33±0.05 0.30±0.04	0.34±0.02 0.38±0.03	-	-
BSTM	♀ ♂	-	-	-	-	0.34±0.02 0.37±0.04	0.42±0.05 0.40±0.04	0.38±0.04 0.42±0.04	0.23±0.04 0.31±0.03
ICo	♀ ♂	0.31±0.02 0.23±0.03 *	0.29±0.02 0.30±0.03	0.24±0.02 0.18±0.02	0.21±0.01 0.23±0.02	0.17±0.02 0.16±0.03	0.15±0.03 0.14±0.02	-	-

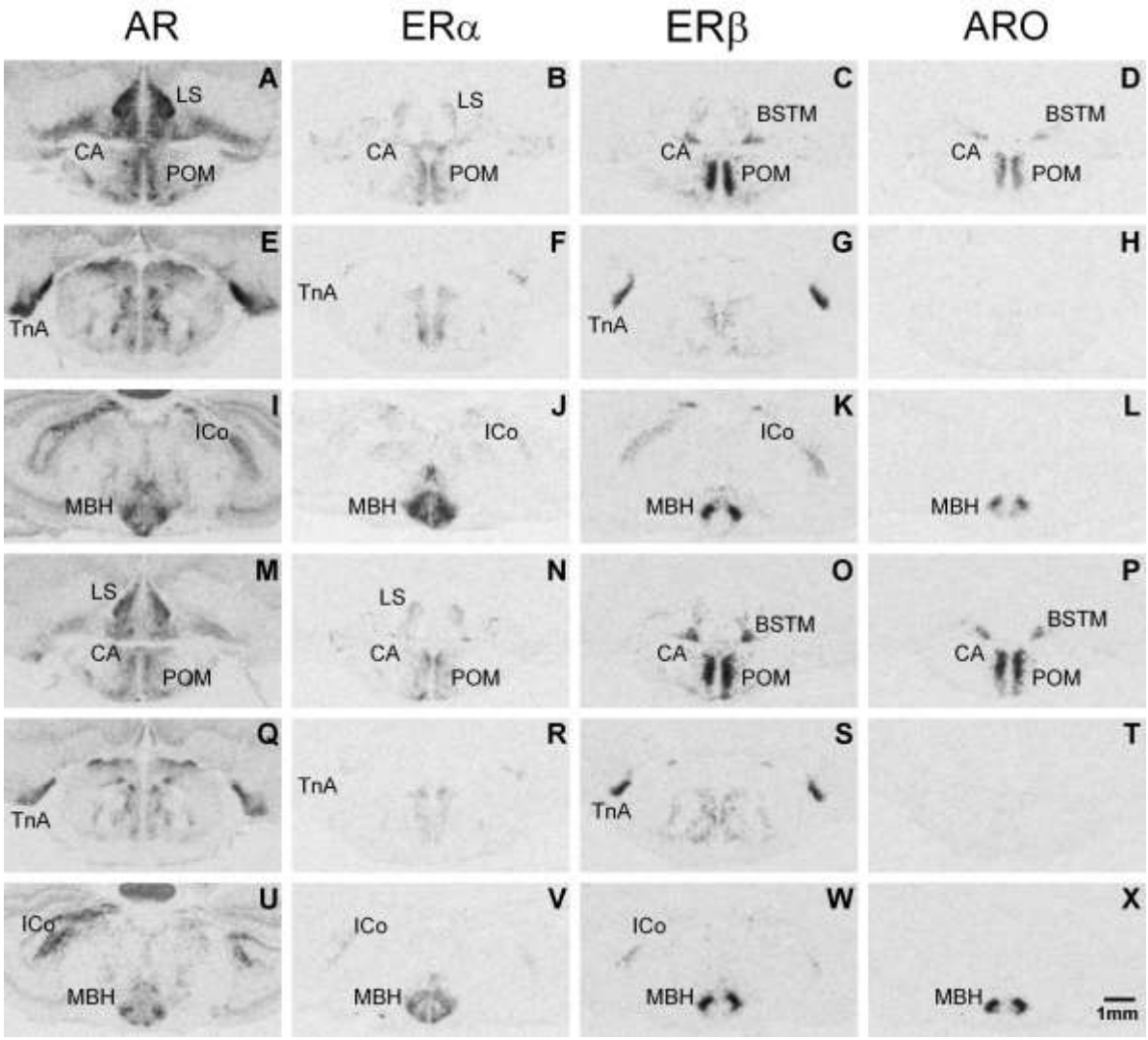

Figure 3. Autoradiograms of coronal sections through the brain of (*a–l*) an adult female and (*m–x*) an adult male buttonquail illustrating the expression of AR, ERα, ERβ and ARO mRNA visualized by *in situ* hybridization. For each gene, sections are presented in a rostral to caudal order. Panels (*a–d*) and (*m–p*) are at the level of the anterior commissure. Panels (*i–l*) and (*u–x*) are at the level of the caudal hypothalamus. Abbreviations: BSTM, bed nucleus of the stria terminalis; CA, commisura anterior; ICO, nucleus intercollicularis; LS, lateral septum; MBH, mediobasal hypothalamus; POM, medial preoptic nucleus; TnA, nucleus taeniae of the amygdala.

Table 2: Comparison of the mean AR mRNA expression levels in the brain of barred buttonquails with those in a non-sex-role reversed species

	AR			
	sex-role reversed		non-sex-role reversed[1]	
	hatchlings	adults	hatchlings	adults
POM	♀>>>♂	♀=♂	unknown	♀=♂
MBH	♀>>>♂	♀>♂		♀=♂
LS	♀>>>♂	♀>♂		♀=♂
TnA	♀>>>♂	♀>>♂		♀=♂
ICo	♀>♂	♀=♂		♀=♂

[1]Voigt et al. 2009

4. Discussion

The present study provides for the first time a detailed insight into the neuroendocrine characteristics of a species with sex-role reversed behaviours. In the barred buttonquail, I demonstrate a female-biased sexual dimorphism in body size and in courtship and agonistic behaviours. Furthermore, while I do not find evidence for sexually reversed steroid hormone profiles, the study identifies increased neural androgen sensitivity in females, which is already in place at hatching.

(a) Steroid hormone profiles in adults and hatchlings

The testosterone levels of buttonquails are in contrast to those of all other sex-role reversed species studied so far (all but one from the order Charadriiformes), in which males were found to have significantly higher levels of testosterone than females prior to the onset of incubation (reviewed in Ketterson et al. 2005). Such a pattern is also known from non-sex-role reversed charadriiform species. Similar testosterone levels between sexes exist, for

example, in a procellariiform species, the black-browed albatross (*Diomedea melanophris*), which is, however, a monogamous and monomorphic species (Ketterson et al. 2005). The finding that female buttonquails do not have higher testosterone levels than males makes it unlikely, that the agonistic and courtship behaviours of females result from purely activational effects of gonadal hormones. Nevertheless, a recent study in the same species shows that female testosterone levels, although being generally low, were positively correlated with body size and condition as well as with the size and darkness of the black throat patch (Muck and Goymann 2011). These data indicate that small increases in circulating testosterone levels in females could be sufficient to activate secondary sexual characters such as plumage colouration. Experimental evidence from sex-role reversed Wilson's phalaropes shows that the growth of the colourful female nuptial plumage can be induced by exogenous testosterone (Johns 1964). Manipulating adult androgen levels in barred buttonquails should reveal their importance in maintaining courtship and agonistic behaviours in females.

The radioimmunoassay was not sensitive enough to detect circulating levels of testosterone and estradiol in hatchling buttonquails except for two female hatchlings where testosterone levels were above the detection limit. In Japanese quail, steroid hormone levels are detectable from around day 9 of incubation onwards and differ between sexes at certain stages (Schumacher et al. 1988, Ottinger et al. 2001). Likewise, it seems possible that male and female buttonquails are exposed to different steroid hormone levels during embryonic development. Assuming that courtship and agonistic behaviours of female buttonquails are under the control of estrogens and that females are exposed to higher levels in the egg than males, this would mean that females are "masculinised" in terms of these behaviours. In this scenario, the lower estrogen levels in male eggs lead to "demasculinisation" and hence, absence of these behaviours in adult males. This hypothesis could be tested by treating female eggs with an aromatase inhibitor. Such masculinising action of estrogen is known from

songbirds, where in female zebra finches that normally never sing, early estrogen treatment leads to differentiation of the male-typical song control system and singing (Gurney and Konishi 1980). Interestingly, progesterone levels, when normalized for body weight, were about 4-5 times higher in hatchlings of buttonquails than Japanese quails (Schumacher et al. 1988). Progesterone has a protective function from androgen action in mammals and birds (Diamond 1966; Erickson et al. 1967; Erpino 1973; Bottoni et al. 1985), which could in the case of the buttonquails, explain the elevated levels found in males.

(b) Functional significance of the gene expression of steroid hormone receptors and aromatase

Several brain regions that are implicated in the regulation of reproductive behaviours showed strong mRNA expression of steroid hormone receptors and aromatase in buttonquails. The male-biased sex difference in ARO expression in the POM and in the MBH of adults but not hatchlings indicates an activational effect of gonadal hormones that develops later in life due to an increase in circulating testosterone levels in males. In relation, in Japanese quail, the strong male-biased sex difference in volume, ARO activity and ARO expression level of the POM develops postnatal and therefore seems to be activational in nature (Thompson and Adkins-Regan 1992; Balthazart et al. 2000). High levels of aromatase activity in the POM of males, which is up-regulated by circulating testosterone, are thought to be critical for the activation of male copulatory behaviour (Schumacher & Balthazart, 1986, Panzica et al. 1996). Moreover, recent data show that castrated male Japanese quail treated with testosterone, which restores physiological hormone levels and fully activates male sexual behaviour, have significantly increased ARO expression in POM and MBH compared to untreated controls (Voigt et al. 2011). Therefore, the high levels of ARO expression found in

the POM of male buttonquails suggest that the mechanism of activation of copulatory behaviour might be similar in the two species.

Female-biased sex differences in the expression of estrogen receptor α were found in the MBH and POM at hatching and in the latter they disappeared in adulthood, which could be related to an increase in ERα levels in males. The higher expression level of ERα found in the MBH of female buttonquails compared to males probably relates to the role of estradiol in this region in activating female receptive behaviour as was suggested in different vertebrates (Pfaff & Sakuma 1979; Gibson and Cheng 1979; Wade and Crews 1991). Such a sex difference also exists in Japanese quail (Voigt et al. 2009). In male buttonquails, the pronounced increase (76%) in ERα levels in the POM of adults compared to hatchlings most likely reflects the key role of locally produced estrogens (catalyzed by the preoptic aromatase) in the activation of male copulatory behaviour, which is mediated through activation of estrogen receptor α (for review, see Balthazart et al. 2009). As no quantitative data on steroid hormone receptor expression are available for quails or other non-sex-role reversed birds at hatching day, no direct comparisons can be made.

A strong female-bias in AR expression was found in hatchling buttonquails in all regions investigated such as the POM, MBH, LS, TnA and ICo. These sex differences were still present in adults, except for POM and ICo. In the latter, AR expression in adult males had increased to levels similar to females, suggesting that these regions are sensitive to activational effects of gonadal hormones in males. The present data confirm our previous findings in another sex-role reversed species, the black coucal (*Centropus grillii*), where females had significantly higher AR expression than males in TnA (Voigt & Goymann 2007). To my knowledge, no such overall sexually dimorphic AR gene expression in hypothalamic and limbic regions is known from non-sex-role reversed bird species, neither in hatchlings nor in adults. A study in adult Japanese quail, which investigated the same brain regions as in the

current study, found that mean AR expression levels did not differ between sexes (Table 2; Voigt et al. 2009). From previous studies in other species investigating AR level in regions involved in reproductive behaviour by means of different techniques limited data are available, which relate to the findings in Japanese quail. In adult zebra finches, androgen binding activity was measured in brain extracts containing the diencephalon and was found to be similar in both sexes (Siegel et al. 1986). Immunocytochemical detection of androgen receptors (AR-ir) revealed an increased number of AR-ir cells in the TnA of male compared to female domestic fowl (*Gallus domesticus*, Jurkevich and Grossmann, 1998). With the same technique, Belle et al. (2005) reports similar numbers of AR-ir cells in hypothalamic nuclei of male and female ring doves (*Streptopelia risoria*). Finally, a recent study in dark-eyed juncos (*Junco hyemalis*) using qPCR did not detect a sex difference in AR transcript abundance in brain extracts from the hypothalamus and caudal telencephalon including TnA (Rosvall et al. 2012). Likewise, in adult rats, AR levels in hypothalamic and limbic brain regions are reported to be either similar between sexes or male-biased (Roselli 1991). In the light of these data, the finding of an overall sex difference in AR expression favouring female buttonquails is unusual.

In the present study, two brain regions, the LS and TnA were devoid of the testosterone-metabolising enzyme aromatase, thus, direct action of androgen through its receptor can be assumed. Both areas have been implicated in the regulation of sexual behaviour and aggression (Goodson et al. 1997; Absil et al. 2002). In relation, in POM and MBH, females had significantly lower ARO levels compared to males, while no such sex difference exists in Japanese quail (Voigt et al. 2007). These data suggest that aromatization plays only a minor role in female buttonquails and instead confirm the importance of androgen receptors in these regions. Maintaining a higher number of androgen receptors in those areas would allow females to bind more hormone compared to males. Besides hormone

receptors, several other cellular components could be involved and thus differentially regulated in females versus males. Gene expression profiles from these brain regions could uncover more such candidate genes. Taken together, since circulating androgen levels were not sexually reversed, other endocrine mechanisms are likely to be at work to restrict aggression and courtship behaviours to females. Enhanced neural androgen sensitivity of females, as found in the present study, could constitute such a mechanism. Moreover, in males, the relatively low testosterone levels found may be linked to the reduced expression of male-typical behaviours. Further experiments involving the manipulation of embryonic hormone levels are necessary to determine the organizational effects of gonadal hormones in controlling sex-role reversed behaviours.

Ethics statement

Experimental procedures were in agreement with the Italian and German laws on "Protection and Welfare of Animals" and on the "Protection of experimental animals". Blood samples from adult birds were obtained at the Max Planck Institute for Ornithology, Germany under the licence of the Government of Upper Bavaria, Germany.

Data accessibility

Data are available at Dryad: doi:10.5061/dryad.f5vn5

Competing interests

I declare no competing interests.

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