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Brain aromatase mRNA expression in two populations of the peacock blenny *Salaria pavo* with divergent mating systems

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ABSTRACT

Aromatase, the key enzyme in the conversion of androgens to estrogens, regulates the availability of these hormones in tissues and controls many physiological and behavioral processes. In fish and other vertebrates, the regulation of aromatase expression in the brain has been implicated in the modulation of male sexual and aggressive behaviors. Here, the pattern of mRNA expression of the brain aromatase isoform (encoded by the *CYP19A2* gene also referred as *CYP19b*) was quantified at the peak of spawning season in brain macroareas from males and females of the blenny *Salaria pavo* originated from two populations displaying male alternative reproductive tactics but differing in their mating systems. In Trieste (Adriatic) nesting males aggressively defend nests and take the initiative in courtship and perform sexual displays more often than females while in Ria Formosa (Southern Portugal) the pattern is reversed as a result of shortage of appropriate nesting sites. Nesting males from Ria Formosa had overall higher levels of brain aromatase mRNA expression than nesting males from Trieste, suggesting a higher brain estrogen synthesis in these males. Since in some fish species exogenous estradiol administration has been shown to decrease sexual and agonistic behaviors, the higher levels of brain aromatase in Ria Formosa nesting males may explain their reduced expression of sexual and aggressive displays when compared with nesting males from Trieste. Alternatively, the higher brain aromatase levels in nesting males from Ria Formosa could be a mechanism to decrease the putative androgen-induced activation of aggressive and sexual displays by reducing the local availability of androgens through their metabolization into estrogens. Although females and parasitic female-like males also differ in their displays between populations, the interpopulational pattern of brain aromatase mRNA expression was similar, suggesting that other neuroendocrine agents mediate the expression of female and female-like behaviors. In conclusion, brain aromatase availability seems like a probable mechanism to regulate the effects of steroids on the brain circuits underlying the expression of sexual and agonistic displays in *S. pavo*.

Introduction

The activation of male sexual displays in adult mammals and birds depends largely on brain aromatization of androgens into estrogens (reviewed by Ball and Balthazart, 2004; Baum, 2003; see Oliveira, 2004 for a historical overview). In fishes, the role of brain aromatization in the regulation of sexual displays has been poorly investigated. This is surprising as fishes have the highest levels of brain aromatase across all vertebrate classes (Callard et al., 1990) and aromatase has been shown to occur in brain areas related to the control of reproduction (e.g., Forlano et al., 2001; Menuet et al., 2003; Menuet et al., 2005) and to peak during the reproductive period in seasonal breeders (Forlano and Bass, 2005; Gelinas et al., 1998; Gonzalez and Piferrer, 2003). Studies conducted so far investigating the role of aromatization in male fish reproductive behavior have produced conflicting results (Munakata and Kobayashi, 2009). For example, in a study on guppies, aromatase activity was pharmacologically inhibited with the drug fadrozole, administered in the water, and the frequency of two of three male sexual displays decreased (Hallgren et al., 2006). Also, in the goldfish testosterone (T) and estradiol (E_2) injections in males stimulated approach behavior toward females within minutes, while fadrozole blocked the stimulatory effect of T (Lord et al., 2009). Finally, in the plainfin midshipman intramuscular injections of both 11KT and E_2 to courting males facilitated the duration of fictive vocalizations (similar to natural vocalizations produced in an agonistic and sexual context) measured in a neurophysiological brain preparation (Remage-Healey and Bass, 2004). These examples would suggest that, similarly to what has been found for birds and mammals, E_2 locally synthesized in the brain from testosterone (T) facilitates some aspects of male sexual behavior in fishes. However, in five fish species for which exogenous E_2 was

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administered to males sexual displays were reduced in four cases and remained unchanged in one case (see Table 3.1 in Oliveira and Gonçalves, 2008), and an inhibitory effect of estrogenic endocrine disruptors in male sexual behavior has been generally reported (e.g., Bayley et al., 1999; Bjerselius et al., 2001). Several issues limit the interpretation of these studies, in particular the fact that endocrine manipulations were systemic and thus it is possible that some of the behavioral effects were due to peripheral rather than central action of hormones. These findings show that more data from carefully controlled experiments are necessary in order to understand the role played by androgens and estrogens in the regulation of male sexual displays in fishes.

Brain aromatase has also been implicated in intrasexual behavioral differences in species with male alternative reproductive tactics (ART). In the plainfin midshipman type I males reproduce by courting females into their nests and type II males try to approach the nests of type I males unnoticed and achieve parasitic fertilizations of eggs (Brantley and Bass, 1994). Type I males emit "hum" vocalizations in order to attract females, while both females and type II males do not emit these vocalizations (Bass and McKibben, 2003; Brantley and Bass, 1994). When compared with type I males, both females and type II males have high levels of aromatase activity in the hindbrain sonic motor nucleus that controls vocalizations (Schlinger et al., 1999). The high levels of aromatase activity in females and type II males were interpreted as a possible mechanism to avoid the testosterone-induced masculinization of the vocal motor nucleus through an increase in androgen to estrogen conversion (Schlinger et al., 1999).

Finally, aromatase has also been implicated in the regulation of aggression in fish. Brain aromatase activity was found to be negatively correlated with the expression of aggression in a sex-changing goby (Black et al., 2005), and in general male fish exposed to estrogens or estrogen-like substances show reduced aggression (e.g., Clotfelter and Rodriguez, 2006; Colman et al., 2009). These examples point towards a suppressive effect of estrogens on fish aggressive behavior.

Taken together the available data suggests that brain aromatase plays a significant role in the regulation of both sexual and aggressive displays in fish.

In the peacock blenny *Salaria pavo* male ART and interpopulation variability in the mating system have been described. A population at the Adriatic, in Trieste, occurs in an area with a rocky bottom where nests are available in abundance. Males establish nests in rock crevices or holes and aggressively defend a territory around the nest. Males take the initiative in courtship and the frequency of male courtship displays is higher than the frequency of female courtship displays (Saraiva et al., unpublished data). In contrast, a population at the Ria Formosa (southern Portugal) occurs in a mudflat area where the only substrates available for nesting are artificial materials such as bricks and tiles used by clam culturists to delimit concessions. The scarcity of nest sites promotes a strong male–male competition for nests and only large competitive males are able to acquire a nest (Almada et al., 1994). After the breeding season starts males seldom leave their nests and do not defend any area around the nest, and it is common to observe males nesting in adjacent holes of the same brick (Almada et al., 1994). At the peak of the breeding season most nests are filled with eggs and nest space may become a limiting factor for female reproduction (Almada et al., 1994). Females compete for the access to nests and the sex-roles are reversed with females taking the initiative and displaying courtship more often than males (Almada et al., 1995). Small males are unable to acquire nests and reproduce by mimicking the females' appearance and courtship displays in order to approach nesting males and parasitically fertilize eggs (Gonçalves et al., 2005; Gonçalves et al., 1996). Parasitic males switch into nesting males from their second breeding season onwards (Fagundes et al., unpublished data), thus undergoing major morphologic and behavioral modifications. Parasitic males have also been described for the Trieste population but in a much lower frequency (Saraiva et al., unpublished data), suggesting that nest availability mediates the frequency of male ART. Interestingly, parasitic males seem to adjust the frequency of female-like displays to the frequency of female courtship behavior in the population. In the Ria Formosa, where females are most actively engaged in courting, female-like displays towards nesting males by parasitic males are very common. In Trieste, where males take the initiative in courtship and females assume a more passive role, parasitic males do not court males and for now it is unclear how they reproduce (Saraiva et al., unpublished data).

Sex steroids have been shown to influence sexual displays in *S. pavo*. Administration of both T and 11KT to parasitic males decreases the frequency of the female-like sexual displays and promotes the development of male secondary sexual characters (Gonçalves et al., 2007; Oliveira et al., 2001). This is consistent with the fact that circulating levels of both T and 11KT are higher in nesting males than in parasitic males (Gonçalves et al., 2008), and in fish species with male ART higher levels of 11KT (but not T) have consistently been found in nesting males (Brantley et al., 1993; Oliveira, 2006). Brain aromatase activity is lower in parasitic than in nesting males (Gonçalves et al., 2008), and from the above it seems possible that this difference is related to the divergent sexual displays exhibited by the two male morphs.

Here, it was hypothesized that aromatase mediates the above described differences in the reproductive and aggressive behavior of females, nesting males and parasitic males across the Ria Formosa and the Trieste population. Unlike most mammals, for which only one aromatase coding gene has been described (Conley and Hinshelwood, 2001), two aromatase isoforms have been found in fish, one preferentially expressed in the ovary and encoded by the *CYP19A1* (or *CYP19a*) gene and another preferentially expressed in the brain and encoded by the *CYP19A2* (or *CYP19b*) gene (e.g., Chang et al., 1997; Tchoudakova and Callard, 1998). In this study the *CYP19A2* mRNA levels were compared in brain macroareas of females and parasitic, transitional and nesting males of *S. pavo* captured at Trieste and in the Ria Formosa.

Methods

Fish collection

Fish were collected during the peak of the breeding season at Culatra island, southern Portugal, 36°59′N;7°51′W (11 females, 12 nesting males, 12 parasitic males and 10 transitional males) and Trieste, northern Adriatic Sea, 45°40′N;13°35′ E (9 females, 10 nesting males, 5 parasitic males and 5 transitional males). The criteria to discriminate the various morphotypes were as follows: nesting males had fully developed male secondary sexual characters and were defending a nest with eggs; females had swollen abdomens, an indicator of ripeness, and after dissection their ovaries were confirmed to have fully developed oocytes; parasitic males lacked male secondary sexual characters, sperm could be easily extruded from their vas deferens by gently pressing the abdomen and had enlarged testes in comparison with nesting males (confirmed during dissection), as described by Gonçalves et al., 1996); transitional males were beginning to develop secondary sexual characters. These males have never been observed guarding eggs or courting nesting males with female-like displays and most likely they do not reproduce. Their testes are usually very small when compared with both parasitic and nesting males and this was confirmed during dissection (see Gonçalves et al., 2008). In Ria Formosa, fish were collected during low-tide with a hand-net from bricks used as nests. Animals were immediately lightly anaesthetized with MS222 (Sigma-Aldrich, dilution 1:10,000) and euthanized by sectioning the spinal cord. Fish were kept in isolated plastic bags in a mixture of ice and water (approximately 0 °C) until dissection, which took place in a field

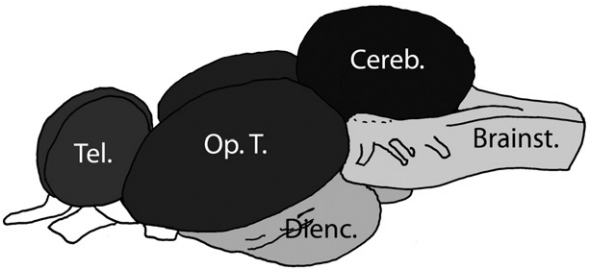

Fig. 1. Division of the brain into five macroareas for the quantification of *CYP19b* mRNA expression. Tel.: telencephalon; Op.T.: optic tectum; Dienc.: diencephalon; Cereb.: cerebellum; Brainst.: brainstem.

station within 1–2 h. In the field station, animals were measured, weighed and dissected. Brains were divided into five macroareas during dissection: telencephalon, optic tectum, cerebellum, diencephalon (excluding the pituitary) and brainstem (Fig. 1).

The brain macroareas were first briefly homogenized in a fixed volume (20–50 µl, depending on the macroarea) of chilled RNAse free 0.1 M, phosphate buffer pH 7.5. Half of this volume was transferred to a tube with 300 µl of Tri reagent (Sigma, Spain) for RNA extraction and the other half to another tube for an enzymatic aromatase activity assay. Aromatase activity data has been published elsewhere (Gonçalves et al., 2008).

In Trieste fish were caught in food traps or fish nets while snorkeling and brains were preserved in RNAlater (Ambion, UK) at −20 °C until RNA extraction. Macroareas were transferred into 300 µl of chilled Tri reagent (Sigma, Spain) and homogenized as above.

During the experimental procedure the "ASAB guidelines for the use of animals in research" were followed.

CYP19A2 and 18S sequences

The *CYP19A2* and *18S* cDNAs were obtained by reverse transcription polymerase chain reaction (RT-PCR) of brain total RNA pooled from nesting males, females and parasitic males and extracted with TRI reagent (Sigma, Spain) following the manufacturer's instructions. RNA was treated with DNAse I (Ambion, Portugal). For cDNA synthesis 5 µg of total RNA was reverse transcribed using oligo-dT primers and M-MLV reverse transcriptase (Promega, Spain). For *CYP19A2*, a 450 bp fragment was amplified using primers designed from the tilapia *Oreochromis mossambicus* ortholog sequence (forward: 5′-TCTTAGCAGGACTCGGTCCAAT- 3′; reverse: 5′-AGATGTCCAACACGATGGCTCT- 3′). The PCR mix contained 1× reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 20 pmol of each primer, 1 U of *Taq* DNA polymerase (Promega) and 2 µl of first-strand cDNA. Cycling conditions were 2 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 59 °C and 1 min at 72 °C, followed by 5 min at 72 °C. RT-PCR products were run on a 2% agarose gel and the band corresponding to the predicted fragment size was cut from the gel, eluted (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences, Piscataway) and cloned into pGem-T-easy vector (Promega). After sequencing in both directions (Macrogen, Inc., South Korea) a cDNA with highest similarity to other teleosts' *CYP19A2* sequences was identified and received accession number FN356970. A 18S cDNA of 407 bp was obtained from total RNA using primers designed for conserved regions of other teleosts (forward: 5′-GTTCCGACCATAAACGATGC-3′; reverse: 5′-CTCAATCTCGTGTGGCTGAA-3′). The 18S sequence received accession number FN356969.

CYP19A2 relative mRNA expression

For quantitative real-time PCR (QPCR) specific primers were designed based on the above sequences (*CYP19A2*, forward: 5′-TATGGCAGCATTACCAGGGT-3′, reverse: 5′-GCCGAATCTTGACGTGT-ACTG-3′, fragment size: 111 bp; 18S, forward: 5′-GCATGGCCGTTCT-TAGTTGGT-3′, reverse: 5′-TTAGCAAGCCGGAGTCTCGTT-3′, fragment size: 73 bp). The identity of the resulting PCR products was confirmed by DNA sequencing of PCR products.

Expression of *CYP19A2* was normalized to the expression of 18S to account for variation in total RNA levels between samples and in reverse transcription reaction efficiencies. Total RNA from each brain macroareas was extracted, DNAse treated and reversed transcribed (1 µg) into cDNA as described above except that random hexamers (Promega, Spain) were used during reverse transcription. QPCR reactions (25 µl) were run in a Stratagene MX3000p thermocycler with Stratagene's Brilliant SYBR green QRT-PCR Master Mix (Stratagene, Spain) and primers at 0.5 µM. Thermocycling conditions were equal for both reactions and were as follows: 10 min at 95 °C, 40 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. After PCR, a melting curve program from 55 to 95 °C with 0.5 °C change in 10-s intervals was applied and the presence of a single reaction product in each tube was confirmed. For the same animal, reactions for each macroarea and for both 18S and *CYP19A2* were run in duplicate in a single PCR. Animals from the several morphotypes and from the two populations were randomly assigned to QPCR reactions. Controls without template were included for both primer sets and a set of samples were included in all reactions to determine interassay variation. Raw fluorescence data was submitted to PCR Miner (http://miner.ewindup.info/miner; Zhao and Fernald, 2005) to calculate reaction efficiencies and cycle thresholds from individual wells during the reaction. The average reaction efficiencies (E) were 1.8 for 18S and 1.9 for aromatase and the average intrassay and interassay coefficient of variation in cycle threshold (CT) were 1.7% and 5.3%, respectively. For each sample, the mean CT of 18S and aromatase was calculated and the relative initial template concentration (R0) of both genes determined from 1/1(1 + E)^CT (Zhao and Fernald, 2005). The relative aromatase mRNA expression was thus given by the ratio between the aromatase and 18S R0s. The 18S average R0s did not differ between macroareas, morphotypes or populations (data not presented), suggesting that this is an appropriate reference gene.

Statistical analyses

A three-way ANOVA with factors morphotype (four levels), population (two levels) and brain macroarea (five levels) was applied to test differences in *CYP19A2* relative mRNA expression. Data was

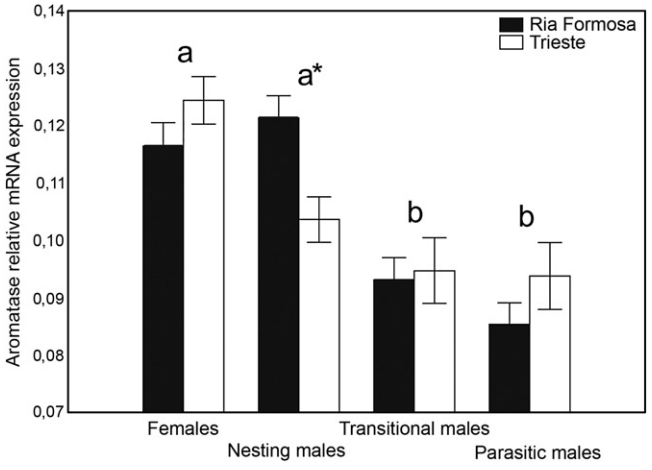

Fig. 2. Average ± SE aromatase (*CYP19A2*) relative mRNA expression in the brain of females and nesting, transitional and parasitic males of *S. pavo* collected in the Ria Formosa and in Trieste. Different letters indicate significant differences ($P < 0.05$) between morphotypes (average of both populations). Significant differences between populations for the same morphotype are marked with *.

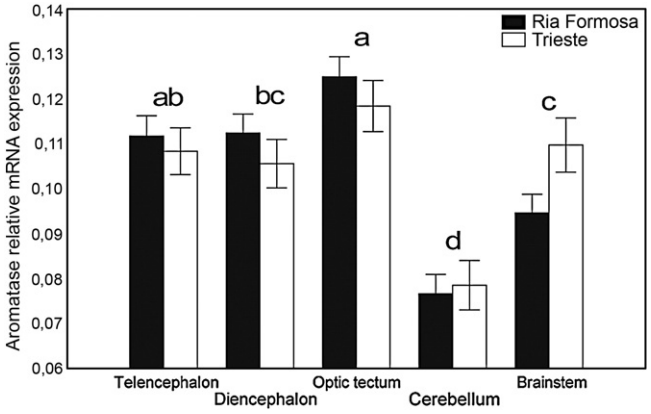

Fig. 3. Average ± SE aromatase (*CYP19A2*) relative mRNA expression measured in the five brain macroareas in Ria Formosa and in Trieste. Different letters indicate significant differences ($P< 0.05$) between macroareas (average of all morphotypes).

squared-root transformed to comply with normality and homocedasticity assumptions. When the ANOVA results were significant, relevant differences were tested with contrast analysis.

Results

For the Ria Formosa samples, both aromatase activity and *CYP19A2* mRNA expression were measured in each macroarea and it was possible to correlate these different measures. *CYP19A2* mRNA expression levels in the brain macroareas were positively and significantly correlated with aromatase activity measured by Gonçalves et al. (2008)—Pearson correlation coefficient: telencephalon, $r= 0.71$, $n= 32$, $P< 0.001$; optic tectum, $r= 0.54$, $n= 32$, $P= 0.001$; diencephalon, $r= 0.54$, $n= 36$, $P= 0.001$; cerebellum, $r= 0.57$, $n= 35$, $P<0.001$; brainstem, $r= 0.72$, $n= 38$, $P< 0.001$.

Overall, *CYP19A2* mRNA expression differed between the morphotypes ($F_{3, 255}= 21.6$, $P< 0.001$). Females and nesting males had higher values than both transitional and parasitic males ($P< 0.001$). The remaining comparisons were non-significant ($P> 0.14$). The overall pattern of *CYP19A2* mRNA expression was similar between populations ($F_{1,255}= 0.00$, $P= 0.984$). However, there was a significant location * morphotype interaction ($F_{3, 255}= 4.5$, $P= 0.004$, Fig. 2). In Ria Formosa, males had overall higher aromatase mRNA expression values than in Trieste ($P= 0.001$). Females, transitional males and parasitic males did not differ between locations ($P>0.17$).

Aromatase mRNA expression differed between macroareas ($F_{4, 255}= 21.6$, $P< 0.001$, Fig. 3). The cerebellum had the lowest expression ($P< 0.001$ in the comparison with the other macroareas) followed by the brainstem (marginally different from the diencephalon, $P= 0.078$, and significantly different from the other macroareas, $P< 0.03$). The optic tectum had significantly higher levels than the diencephalon ($P= 0.025$) and marginally than the telencephalon ($P= 0.066$). The diencephalon and telencephalon did not differ ($P= 0.99$). Differences between macroareas were similar between locations (location * macroarea interaction: $F_{4, 255}= 1.6$, $P= 0.117$, Fig. 3).

The morphotype * macroarea interaction ($F_{12, 255}= 1.6$, $P= 0.076$) and the morphotype * macroarea * location interaction ($F_{12, 255}= 1.1$, $P= 0.372$) were not significant. However, because the power to detect small interaction effects of this statistical approach is low with the current n (Murphy and Myors, 2004), differences in

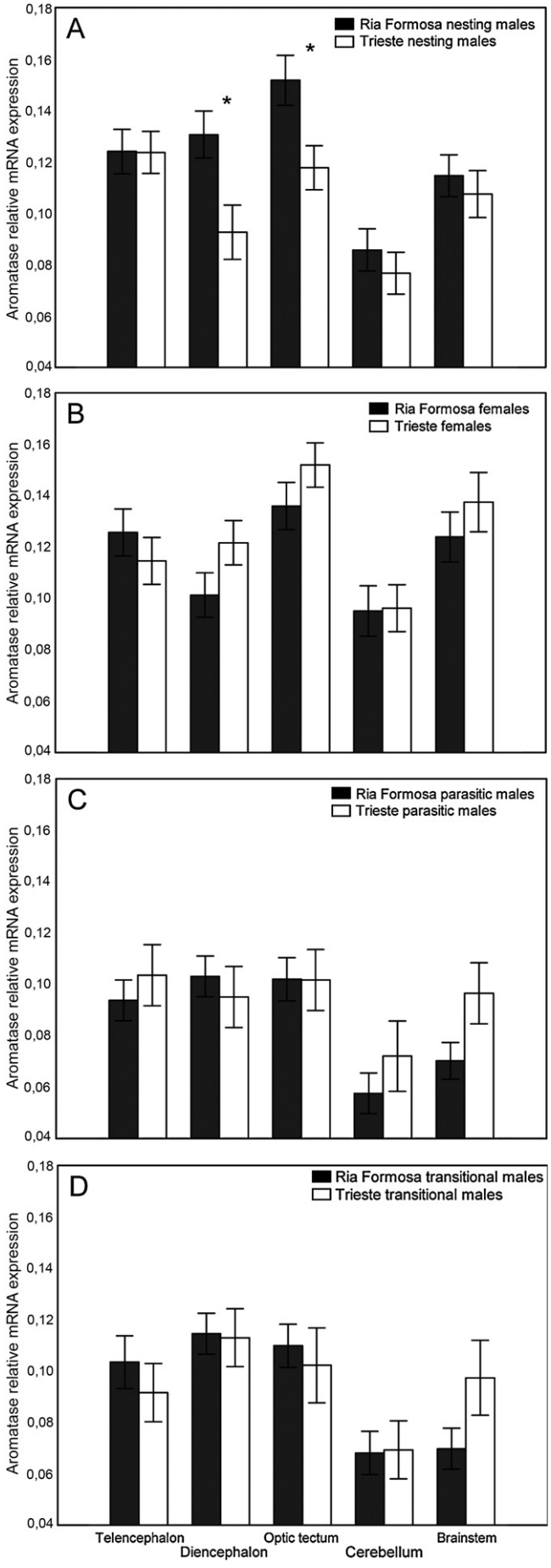

Fig. 4. Average ± SE aromatase (*CYP19b*) relative mRNA expression measured in the five brain macroareas of Ria Formosa and Trieste (A) nesting males, (B) females, (C) parasitic males and (D) transitional males. Significant differences between populations for the same macroarea are marked with * ($P< 0.05$).

aromatase expression between populations in the same macroareas of the same morphotypes were also tested with t-tests. Aromatase expression in the same macroarea did not differ in females, transitional males or sneakers from the two populations ($P > 0.07$). In males, differences were significant for the diencephalon ($P = 0.043$) and the optic tectum ($P = 0.043$) but not for the remaining macroareas ($P > 0.35$, Fig. 4).

Discussion

Correlation between aromatase activity and mRNA expression

In this study the normalized *CYP19A2* mRNA expression was positively and significantly correlated with aromatase activity measured in a previous study in the same brain samples (Gonçalves et al., 2008). In fish, significant positive correlations between aromatase activity and normalized mRNA levels of both the *CYP19A1* (e.g., Gen et al., 2001; Villeneuve et al., 2006) and *CYP19A2* (Villeneuve et al., 2006) genes have been reported for ovarian tissue. However, the relationship between aromatase activity and *CYP19A1* or *CYP19A2* mRNA expression in the same brain samples had not been previously tested in fish. The positive correlation between brain *CYP19A2* mRNA expression and aromatase activity found in this study, together with the fact that in the fish brain the *CYP19A2* gene expresses much more than the *CYP19A1* gene (e.g., Hinfray et al., 2006; Kishida and Callard, 2001; Villeneuve et al., 2006), suggests that *CYP19A2* mRNA expression levels provide a good indication of the potential for aromatization in the *S. pavo* brain.

Differences between populations

The pattern of aromatase (*CYP19A2* isoform) mRNA expression in the brain was similar between the two populations, with the exception of nesting males which presented higher levels in Ria Formosa. Aromatase has been implicated in the regulation of sexual behaviors in vertebrates (Ball and Balthazart, 2004; Baum, 2003) and thus it seems possible that the measured interpopulational difference in brain aromatase expression relates to the divergence in the nesting males' behavior. Previous studies have shown that the reproductive behavior of nesting males, but also of females and parasitic males, differs between populations. Field observations carried out during the species breeding season in Trieste and in the Ria Formosa revealed a 10-fold higher frequency of nesting male courtship displays in Trieste than at Ria Formosa and a 10-fold higher frequency of female courtship displays in the Ria Formosa than at Trieste (Saraiva et al., unpublished data). Sex-roles are thus reversed at Ria Formosa with females displaying most of courtship behavior while at Trieste males are the ones leading the role in courtship.

In fish, little is known about the central effects of sex steroids in male reproductive behavior. An experimental study testing the effects of aromatization in male guppy sexual displays found a significant decrease of these behaviors with aromatase blockage (Hallgren et al., 2006), and E_2 promoted within minutes male approach behavior towards females in the goldfish (Lord et al., 2009). On the contrary, in five studies where E_2 was administered to males, sexual displays were reduced in four cases and remained unchanged in one (Oliveira and Gonçalves, 2008) and similar findings have been described for fish exposed to other estrogen and estrogenic-like compounds (e.g., Colman et al., 2009). The interpretation of these results warrants some caution as in all these studies substances were administered peripherally and the observed behavioral effects could also have been the consequence of non-central effects. For example, E_2 administration to males has a suppressive effect on testicular development and plasma androgen levels (e.g., Yamaguchi et al., 2006) and these could have accounted for the reported decrease in sexual displays of males treated with E_2. Additionally, estrogen manipulation in many of these studies seems to have resulted in supraphysiological levels.

In the plainfin midshipman, a species that also presents male alternative reproductive phenotypes, injections of both 11KT and E_2 facilitated the duration of fictive vocalizations in courting males measured in a neurophysiological brain preparation (Remage-Healey and Bass, 2004). However, the fact that courting males presented much lower levels of aromatase in brain regions implicated in the control of vocal courtship displays when compared with parasitic males and females (Schlinger et al., 1999) suggests that T conversion into E_2 is not a main mechanism facilitating male vocal courtship behavior in this species. In *S. pavo*, differences in aromatase mRNA levels between males of the two populations were significant in two brain macroareas presumably containing important nuclei for the regulation of reproductive behavior, the diencephalon and the optic tectum (Fig. 4, see below). The higher brain aromatase levels in these areas in males from Ria Formosa will induce a higher local T to E_2 synthesis, assuming a similar availability of T in these regions in males from both populations. However, although plasma T levels did not differ between males of both populations (Saraiva et al., 2010), it was not possible to quantify the local availability of T in brain macroareas. With this limitation in mind, the fact that males from Ria Formosa exhibit a lower expression of sexual displays and have higher brain aromatase mRNA levels than males from Trieste points to a central inhibitory effect of E_2 in male sexual displays. Experiments testing the effects of T and E2 in *S. pavo* male sexual displays are ongoing.

A second not exclusive hypothesis relates to the expression of aggressive behavior. In Trieste males aggressively defend an area around the nest from other competitors (Saraiva et al., unpublished data), while in the Ria Formosa males do not defend territories and very often have one or more males nesting in adjacent brick holes (Almada et al., 1994). Estrogens have been shown to reduce aggression in fish (e.g., Bell, 2001; Munro and Pitcher, 1983) and increases in aggression were negatively correlated with brain aromatase activity in a sex-changing goby (Black et al., 2005). Importantly, E_2 administration to parasitic males of *S. pavo* from the Ria Formosa population was also shown to inhibit aggression (Gonçalves et al., 2007). Thus, the higher brain aromatase levels in males from Ria Formosa probably increase local E_2 synthesis and this may induce the necessary decrease in aggression due to the scarceness and aggregated nature of the available nests.

Although females and parasitic males exhibit notorious quantitative differences in sexual displays between these populations, the pattern of brain aromatase mRNA expression was similar. Females from Ria Formosa court more often nesting males than females from Trieste (Saraiva et al., unpublished data) and laboratory observations show that parasitic males follow the same pattern, courting very often nesting males in the Ria Formosa and not courting at all in Trieste (Saraiva et al., unpublished data). For now it is unclear how parasitic males reproduce in Trieste as field observations in this area focusing on the parasitic males' behavior are lacking. The absence of interpopulational differences in brain aromatase levels is more unexpected for females than for parasitic males. Estradiol administration to parasitic males from Ria Formosa had no effect in the female-like displays (Gonçalves et al., 2007) and thus interpopulational differences are likely to be regulated by other neuroendocrine agents. Also, both female and parasitic female-like courtship behaviors in *S. pavo* have been shown to depend on the action of the brain neuropeptide arginine vasotocin (AVT), the fish homologous of the mammalian arginine vasopressin (AVP). Females and parasitic males have higher preoptic levels of AVT mRNA than nesting males (Grober et al., 2002) and exogenous AVT administration to females and parasitic males promoted female courtship displays (Carneiro et al., 2003). This suggests that AVT generally promotes female and female-like displays, and interpopulational differences in female and parasitic

male sexual behaviour may be more related to variations in central AVT levels than to the direct action of steroids in the CNS.

Differences between morphotypes

Females and nesting males had higher brain aromatase mRNA levels than parasitic and transitional males. Transitional males had intermediate values between parasitic and nesting males, although the difference was only significant for nesting males. This is in accordance with what has been described in a previous study measuring brain aromatase activity in the same samples (Gonçalves et al., 2008). In that study, the increase in brain aromatase activity during the transition suggested a role for estrogen activation of male sexual displays, as demonstrated for other vertebrates. However, the data in this study argues against that hypothesis as males from Trieste, expressing more courtship behavior, had lower brain aromatase mRNA expression. One hypothesis is that male sexual displays are modulated by the direct action of androgens and not by estrogens. Androgens have been shown to promote male sexual displays in fish (reviewed by Borg, 1994; Liley and Stacey, 1983). In *S. pavo*, levels of both 11KT and T are higher in nesting males (Gonçalves et al., 2008), and, in general, in species with male ART nesting males have higher levels of 11KT, a non-aromatizable androgen, but not of T when compared with parasitic males (Brantley et al., 1993; Oliveira, 2006). The administration of both T and 11KT to parasitic males inhibited the expression of female-like displays, although it did not induce male-like sexual behaviors (Gonçalves et al., 2007; Oliveira et al., 2001). Those experiments only lasted for 8 days and it is possible that a longer time frame would be necessary for the putative masculinizing behavioral effects of androgens to be observed. Under that scenario, the higher aromatase levels in males of the Ria Formosa population, where sex-roles are reversed, could be a mechanism to locally reduce androgen levels and the androgen-induced activation of male sexual displays. A similar mechanism was proposed for the plainfin midshipman where the higher levels of aromatase in the brains of females and parasitic males in comparison with nesting males was suggested to prevent the masculinization by androgens of brain regions implicated in the production of mating calls (Schlinger et al., 1999). Experiments are ongoing to test the effects of androgens in *S. pavo* male sexual behavior.

Differences between brain macroareas

Aromatase levels were highest in telencephalon, diencephalon and optic tectum and lower in the cerebellum and brainstem. This is in agreement with the results reported for fish where aromatase levels have been reported to be higher in forebrain regions known to regulate reproductive behaviors, particularly the preoptic and hypothalamic periventricular nuclei, central telencephalon and optic tectum (e.g., Forlano et al., 2001; Gelinas and Callard, 1993; Gelinas and Callard, 1997; Melo and Ramsdell, 2001). In the Gonçalves et al. (2008) study, the pattern of aromatase activity across brain macroareas differed between morphotypes, with females presenting higher levels in posterior brain regions when compared to males, a pattern also described for the medaka *Oryzias latipes* (Melo and Ramsdell, 2001). In this study the interaction between the macroarea and morphotype factor was marginally non-significant ($P = 0.07$), but females also presented somewhat higher values of aromatase mRNA expression in posterior brain regions, in particular in the cerebellum and brainstem, when compared with the other morphotypes (Fig. 4).

In conclusion, the correlational data presented in this study are consistent with a major role for brain aromatase in the regulation of sexual displays phenotype in *S. pavo*. High aromatase mRNA expression levels were recorded in brain macroareas containing nuclei associated with the control of sexual displays. Regulation of aromatase levels may be used as a mechanism to control the availability of both estrogens and androgens in local brain regions and, consequently, the effects of these steroids in the brain circuits underlying the expression of sexual and also aggressive behaviors. The described differences in the pattern of aromatase mRNA expression across the two populations and across morphotypes raises a number of hypotheses on the effects of estrogens and androgens in the regulation of behaviors in *S. pavo* that can be experimentally tested.

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