



Triploidy in a sexually dimorphic passerine provides new evidence for the effect of the W chromosome on secondary sexual traits in birds

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In birds, there are two main models for the determination of sex: the 'Z Dosage' model in which the number, or dose, of Z chromosomes determines sex, and the 'Dominant W' model which argues that a specific gene in the W chromosome may influence Z gene expression and determine sex. The best evidence for W determination of sex comes from birds with 2 copies of the Z chromosome paired with a single W (e.g. ZZW) which are nonetheless females. Here, we expand the species where such a mechanism may operate by reporting a case of a triploid Neotropical passerine bird with sexually dimorphic plumage, the São Paulo marsh antwren *Formicivora paludicola*. Evidence from 17 autosomal unlinked microsatellite loci, and CHD1 sex-linked locus, indicate that this individual is a 3n ZZW triploid with intermediate plumage pattern. This example expands our knowledge of sex determination mechanisms in birds by demonstrating that both the W and the two Z chromosomes affect the expression of morphological secondary sexual traits in a non-galliform bird.

In birds, the exact role of the Z and W chromosomes in sex determination is not fully understood (Ayers et al. 2013, Graves 2014). There are two main models proposed for this process: first, the 'Z Dosage' model which proposes that the number, or dose, of Z chromosomes influences the determination of sex (Clinton and Haines 1999, Smith et al. 2009), and second, the 'Dominant W' model which argues that a specific gene in the W chromosome may play an important role on the regulation of Z gene expression (Teranishi et al. 2001, Ellegren 2002, Arlt et al. 2004, Küpper et al. 2012). The presence on the Z chromosome of the genes DMRT1 and HEMOGEN involved in chicken embryo testes development has provided evidence for the effect of this chromosome in the determination of sex (Smith 2007, Smith et al. 2009), but the role of the W cannot be ruled out as recent transcriptome analyses have revealed that at least 26 genes are expressed on chicken W chromosome although their role in sex determination is unclear (Ayers et al. 2013).

Despite the recent advances on gene expression techniques, attempts to identify feminizing genes in the avian W chromosome have been inconclusive (reviewed by Ayers et al. 2013 and Graves 2014). The best evidence for a feminizing role of the W comes from cases of triploid birds with a single W and two Z chromosomes which are nonetheless female

(Graves 2003, 2014). These include cases of a triploid ZZW kentish plover *Charadrius alexandrinus*, and a diploid ZZW great reed warbler *Acrocephalus arundinaceus*, each of which were reproductive adult females (Arlt et al. 2004, Küpper et al. 2012), demonstrating the presence of active development of the ovary while the birds were adults.

Bilaterally gynandromorph chickens and zebra finches, with male phenotype in one side of the body and female phenotype in the other, were shown to have ZZ chromosomes in one half of the body, and ZW in the other half (Agate et al. 2003, Zhao et al. 2010). As both sides of the body were exposed to common gonadal hormones, the lateral differences suggest that the somatic sexual characteristics are not determined by gonadal hormones, but by autonomous mechanisms in ZZ and ZW somatic cells (Agate et al. 2003, Zhao et al. 2010, Graves 2014). Agate et al. (2003) also analyzed the sexual phenotype of the brain of a bilaterally gynandromorphic zebra finch, and observed that the neural song circuit had a more masculine phenotype in the male side of the body, indicating that the genetic sex of brain cells, and not only hormonal environment, has important implications in brain sexual characteristics, which ultimately might affect behavioral secondary sexual traits. As these gynandromorphs are ZZ on one side and ZW on the other, they do not provide evidence for 'Z Dosage' or

'Dominant W' in the determination of the somatic sexual characteristics, and again, triploids could shed light to this issue. In chickens ZZW triploids are initially sexed as females based on morphology, and at ages between 25 to 40 weeks they develop male morphological secondary sexual characteristics, such as spurs, pronounced combs, tail plumage, and exhibit male behavior (Abdel-Hameed and Shoffner 1971, De Boer et al. 1984). Although the formation of these masculine characteristics is easy to observe it is difficult to evaluate if female sexual secondary traits are also present, or if they are suppressed, since most studies have analyzed chicken races without sexually dimorphic plumage (De Boer et al. 1984, Bonaminio and Fehheimer 1993). In both the kentish plover and the great reed warbler, although ZZW individuals have behaved like females, sexual dimorphism is slight (Dyrcz 2006, Wiersma and Boesman 2013, Argüelles-Ticó et al. 2016), which makes it difficult to infer about the W effect on morphological secondary sexual traits. Another known case of avian triploidy is reported for a captive blue and yellow macaw *Ara ararauna*, based on karyotype. However, there is no sexual dimorphism in this species (Tiersch et al. 1991). Thus, how the W chromosome affects somatic sexual characteristics in birds such as Passeriformes with extreme sexual dimorphism in plumage remains a mystery.

Here, we report a case of triploidy in a Neotropical passerine with significant sexual dimorphism, the São Paulo marsh antwren *Formicivora paludicola*. Data from 17 autosomal unlinked microsatellite loci, and CHD1 sexing gene identified it as a 3n ZZW triploid. Our observation that this bird had an intermediate plumage pattern provides the first evidence for both the W and the Z chromosome effects in the expression of morphological secondary sexual traits in a non-galliform bird.

Methods

São Paulo marsh antwren (Thamnophilidae), is a small insectivorous passerine endemic to Atlantic Forest marshes of São Paulo state, Brazil. It is highly sexually dimorphic: males are distinguished by the presence of black throat and underparts, while the females are white with black stripes in these areas (Buzzetti et al. 2013). Juveniles resemble females, but they are gray, and present smaller black stripes in the lower parts.

To assay genetic variability in these birds, we mist-netted and genotyped 58 individuals (see Camargo et al. 2015a for details) that were screened with 17 autosomal unlinked microsatellite markers. Primers, PCR conditions, and genotyping procedures are described in Camargo et al. (2015b). Genomic locations of each loci using the zebra finch *Taeniopygia guttata* genome as a reference were assessed by blasting at least 200bp of one of the flanking regions of each loci in NCBI's BLAST tool, using the Blastn algorithm. Since young males exhibit a female-like plumage, all individuals were photographed in the field and were sexed by amplifying the homologous copies of the CHD1 (chromohelicase-DNA-binding) gene using the primers P2/P8 (Griffiths et al. 1998). CHD1-Z and CHD1-W sequences are located in sex chromosomes and differ in length. When used in amplifications, this set of primers generates one band for males and two bands for females. We also included the

primer (P0) developed by Han et al. (2009) in the same PCR reaction. This primer anneals to a unique sequence of the W chromosome, and results in a third band for females, usually 100 bp longer than CHD1-W, improving the resolution of sex identification. PCR reactions followed the protocol described by Anciães and Del Lama (2002), and the amplification products stained with GelRed™ (Biotium) were run on 3% agarose gels.

The sex chromosome composition was assessed to determine if this putative triploid individual exhibited a ZZW or ZWW genotype. We used a simplified semi-quantitative PCR procedure followed by capillary electrophoresis (LaFountain et al. 1998) of the sex-specific CHD1 gene amplicon. First, DNA samples of the putative triploid and of four regular individuals of each sex, used as controls, were quantified using a Qubit 2.0 Fluorometer, and standardized to a concentration of 20 ng μl^{-1} . Next, the CHD1 gene was amplified using only primers P2 and P8, following the same procedures as described above. Finally, the intensity of the bands resulted from the PCR were estimated by fluorescence using the capillary electrophoresis device QIAxcel Advanced System (Qiagen), using the QIAxcel DNA Fast Analysis Kit. This system provides electropherograms depicting product sizes (bp) on the x-axis, whereas the height of a peak corresponds to its intensity, represented in y-axis by a relative fluorescent unit (RFU) which is positively correlated to DNA concentration. We hypothesized that if the triploid individual is ZZW it should show: 1) a peak of the CHD1-Z corresponding in intensity to typical males (i.e. two copies); 2) a CHD1-W peak that is half the intensity of the CHD1-Z peak, and similar in intensity to peaks found in typical females.

Results

Of the 58 analyzed animals, one individual captured near the city of Salesópolis (23°34'S, 45°49'W) (SL14) on 1 November 2013 showed an unusual pattern of three alleles in eight of the 17 microsatellite loci (47.1%) (Table 1),

Table 1. Genotypes of a triploid São Paulo marsh antwren *Formicivora paludicola*, based on 17 microsatellite loci, and loci correspondent chromosomes in the zebra finch genome.

Loci	Alleles	Chromosome
Fpa11	395/443	19
Fpa13	393	4
Fpa14	588	1
Fpa15	216/262	5
Fpa17	456/460/464	10
Fpa18	368/372/376	4
Fpa23	231/243/249	3
Fpa24	444/459	2
Fpa25	268	5
MyEx19	298/307/313	unknown
MyEx41	247/255/259	unknown
MyEx46	298/322/330	19
Mex034	259/267/271	17
Mex120	227/443	7
Mex140	204/220/232	6
Mex162	236/252	2
Mex176	226/234	unknown

suggesting that it was a triploid. We are confident that the presence of the extra alleles did not result from sample contamination or other methodological artifacts because in the field the birds were kept in individual bags and were then processed one at a time, using sterilized disposable materials. In the laboratory, DNA was re-extracted and PCR reactions were repeated three times. Molecular sexing amplification revealed three bands in the agarose gel, corresponding to the expected genetic pattern for a female (Fig. 1). Peaks of fluorescence obtained in our semi-quantitative PCR procedure revealed a CHD1-Z corresponding in intensity to the pattern of regular males (around 6000 RFU), with the CHD1-W half the intensity of the CHD1-Z and corresponding in intensity to typical females (about 2000 to 4000

RFU) (Fig. 2). This suggests that the triploid individual had three copies of the sex-specific CDH1 gene, consistent with a ZZW genotype.

The triploid individual showed an intermediate sexual plumage pattern. It could not be confused with a juvenile because its throat and the background color of its underparts were pure white, resembling an adult female, instead of the grayish background color present in the juveniles (Fig. 1). However, the black stripes were deeper than those found in regular females, and black areas, typical of males, were present in the flanks and in the neck. It did not show any evidence of color pattern lateralization (Fig. 1) typical of many gynandromorph birds (Agate et al. 2003). Marsh antwrens exhibit adult plumage at the age of about seven months



Figure 1. Plumage patterns of São Paulo marsh antwrens: (A) diploid adult male, (B) diploid adult female, (C) ZZW triploid, and (D) diploid juvenile male. In the bottom right of each picture, the band patterns of molecular sexing are depicted. The lower band represents the CHD1-Z, and the two upper bands represent the different CHD1-W amplicons generated by the combination of three primers in the same PCR reaction. Images (B) and (C) evidence that the triploid bird had a band pattern similar to that of a regular female due to the presence of both Z and W chromosomes.

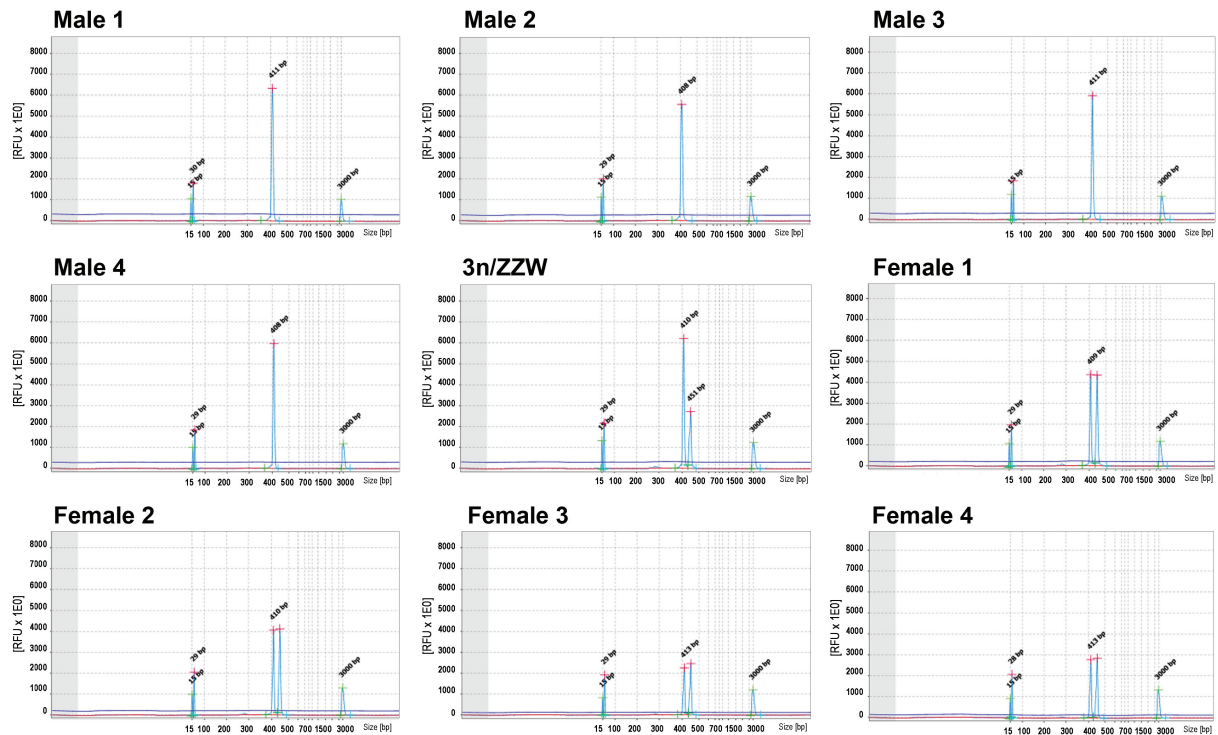


Figure 2. Electropherograms resulting from a semiquantitative PCR of the homologous copies of the CHD1-Z (around 410 bp) and CHD1-W (451 bp) genes used for molecular sexing of São Paulo marsh antwrens. Height of the peaks represent fluorescence intensity measured by a relative fluorescent unit (RFU). Peaks of 15, 28–29, and 3000 bp are resulted from the markers used to standardize the analyses. The pattern of fragments observed in the potential triploid, and its respective RFUs, are consistent with a genotype ZZW.

(Reinert 2008), and the triploid individual was re-captured in the same territory about three months later on 22 January 2014 and its color pattern had not changed. At least for time of the first capture, we were able to confirm that it was together with an adult male which was also caught and marked in the same occasion. Although it may suggest that the triploid was an adult animal, behaving like a female, we were unable to confirm if the triploid São Paulo marsh antwren was a male with feminized plumage, or a female with masculinized plumage, as we could not locate a nest attended by this bird and it was not collected for gonadal analysis.

Discussion

Triploidy often leads to the death of the affected individuals during embryonic development (Forstmeier and Ellegren 2010), and studies reporting triploid birds living in the nature are rare (Arlt et al. 2004, Küpper et al. 2012). Triploid chickens with a ZWW genotype are not viable, whereas triploid ZZZ animals develop a male phenotype (Thorne et al. 1991, Graves 2003). Our results indicate that the São Paulo marsh antwren reported here is ZZW, similar to the three other reported cases of non-galliforme adult birds (kentish plover, great reed warbler, and blue and yellow macaw) (Tiersch et al. 1991, Arlt et al. 2004, Küpper et al. 2012). We do not believe that the genetic pattern detected for this bird is due to mosaicism or chimaerism. Mosaics present two or more cell lines with distinct genotypes originated from a single zygote (Biesecker and Spinner 2013). As such, they

are expected to show up to two different alleles in most loci unlike the three alleles we observed in eight of the 17 loci we analyzed, and it seems unlikely that post-zygotic mutations could account for this additional allele. In a chimaera the different cell lines result from the union of two or more fertilized eggs, which can lead to intersex characteristics if the sex of the zygotes are different (reviewed by Biesecker and Spinner 2013). In this case, part of the cells (i.e. in the blood) would be ZZ and part ZW, and a deficit on the concentration of Z chromosomes would be expected when compared to regular males that present two copies of the Z chromosome in all of the cells. Thus, our best evidence against chimaerism in our studied specimen is the fact that it shows a pattern of CHD1-Z concentration consistent with the presence of two Z chromosomes in all of the cells.

The intermediate sexual plumage characteristics that we observed are likely due to the fact that W chromosome could not balance the effect of duplicated Z chromosomes. On the other hand, this plumage pattern suggests that the presence of the W has impeded complete masculinization, and that this chromosome has a feminizing effect that is also expressed in plumage secondary sexual traits governed by autonomous cellular mechanisms (Agate et al. 2003). As such our findings support a feminizing influence of the W chromosome, although the specific molecular mechanisms involved are unclear (Ayers et al. 2013, Graves 2014). Recent gene expression studies in chicken embryos have demonstrated at least 26 transcriptionally active W-linked genes, and a set of small W-linked RNAs (Ayers et al. 2013). Most of these genes have identical or similar Z-linked homologues

and encode proteins involved in general cell functions, making them unlikely candidates for female-determining genes (Ayers et al. 2013). Thus, the two main candidates as determinants of femaleness in the W chromosome are HINTW, whose sequence differs from its Z homologue, and FAF, which has no Z homologue, but their sex-determining functions still have to be better investigated (Ayers et al. 2013). As non-coding RNAs can regulate the expression of other genes, i.e. sex determining genes, the findings of W-linked small RNAs has opened another field of investigation for the feminizing role of the W (Ayers et al. 2013, Graves 2014). On the other hand, the ‘Z Dosage’ mechanism cannot be ruled out, as ZZ chicken embryos for which the Z-linked DMRT1 gene expression was reduced by knockout technique developed ovaries instead of testes, while the over-expression of this gene induced male pathway genes and counteracted the female pathway in embryonic chicken gonads, proving the role of the DMRT1 dosage for avian testicular development (Smith et al. 2009, Graves 2014, Lambeth et al. 2014). These results indicate that the ‘Z Dosage’ versus ‘Dominant W’ paradigm may be overly simplistic (see also Küpper et al. 2012), and both mechanisms may operate in avian sex determination through complex interaction pathways. In summary, our findings expand our knowledge of sex determination mechanisms in birds by demonstrating that the presence of a single W has a feminizing effect expressed in plumage secondary sexual traits governed by autonomous cellular mechanisms, despite the presence of multiple Z chromosomes.

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