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1 **Triploid plover female provides support for a role of the W**
2 **chromosome in avian sex determination**

3

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18

19

20 Abstract

21 Two models, *Z Dosage* and *Dominant W*, have been proposed to explain sex determination in birds
22 where males are characterized by the presence of two Z chromosomes and females are hemizygous
23 with a Z and a W chromosome. According to the *Z Dosage* model, high dosage of a Z-linked gene is
24 needed to trigger male development, whereas the *Dominant W* model postulates that female
25 development is triggered by a still unknown W-linked gene. Using 33 polymorphic microsatellite
26 markers we describe a female triploid Kentish plover *Charadrius alexandrinus* identified by
27 characteristic ‘three allele’ genotypes at 14 autosomal markers that produced viable diploid offspring.
28 Peak ratio analysis showed that the sex chromosome composition of this female was ZZW. Our
29 results suggest a prominent role for a female determining gene on the W chromosome. In light of this
30 result, we propose that avian sex determination is more dynamic and complex than currently
31 envisioned.

32

33 Introduction

34 Birds show striking sexual dimorphism with pronounced phenotypic differences between males and
35 females. Sex in birds is determined genetically; males are ZZ and females are ZW. However,
36 precisely how the phenotypic sexual dimorphism is initiated, is debated (Teranishi et al. 2001; Smith
37 et al. 2009; Ellegren 2011). Two models have been proposed to explain sex determination in birds
38 (Clinton 1998). The *Z Dosage* model postulates that the main determinant for sex is located on the Z
39 chromosome. This sex determinant interacts with an autosomal gene and, depending on the ratio
40 between copies of Z chromosomes and autosomes (Z:A ratio), the embryo will develop as male or
41 female. If Z:A = 1 the embryo will develop into a male and, if Z:A = 0.5 into a female. *Z Dosage* is
42 based on the observed ineffective dosage compensation for Z genes, i.e. their expression is
43 proportional to the copy number (Itoh et al. 2007; but see Mank, Ellegren 2009). The model is
44 supported by experimental RNA inhibition of *DMRT1* a major sex determining gene in vertebrates
45 which is located on the Z chromosome (Smith et al. 2009). When *DMRT1* was inhibited early in

46 development, ZZ chicken *Gallus gallus* embryos subsequently developed ovaries but no testes. By
47 contrast, the *Dominant W* model postulates that the main determinant for females is located on the W
48 chromosome. For example, the presence of a gene located on the W chromosome may
49 antagonistically interact with *DMRT1* by altering methylation of the male hypermethylated region
50 (MHM) adjacent to *DMRT1* in chicken (Teranishi et al. 2001). However, such a ‘female gene’,
51 potentially located upstream of *DMRT1* in the sex determination cascade, has yet to be described in
52 birds.

53

54 Chromosomal aberrations such as aneuploidy can help to clarify the sex determination mechanism
55 although they are often already lethal at the embryonic stage in birds (Forstmeier, Ellegren 2010).
56 Triploid chickens with a ZWW genotype are not viable whereas triploid ZZZ chickens develop a male
57 phenotype but produce only abnormal sperm. Triploid ZZW chickens initially develop female
58 phenotypes but before sexual maturity they develop male phenotypes (Lin et al. 1995). Their right
59 gonad develops into a testis whereas the left gonad develops into an ovotestis that degenerates shortly
60 after hatching. Importantly, these intersexual chickens fail to produce viable gametes (Lin et al. 1995).

61

62 Here we present a case of a female triploid Kentish plover *Charadrius alexandrinus* that reproduced
63 successfully in a natural population. We explore the type of sex chromosome aneuploidy exhibited by
64 this bird and discuss the implications of this case for models of avian sex determination.

65

66 Material and Methods

67 The female was a regular breeder in a large Kentish plover population at Tuzla, Turkey (36°42' N,
68 35°03' W), and captured during incubation in both 1997 and 1999. In 1999 this female, her mate and
69 their three chicks were sampled for blood. Twenty-five µl of blood were taken from either brachial
70 vein (adults) or metatarsal vein (chicks) and stored in Queen's lysis buffer (Seutin, White, Boag

71 1991). The female and her mate were initially sexed in the field based on plumage characteristics and
72 sex-specific pattern of incubation in this species (Cramp, Simmons 1983; Kosztolányi, Székely 2002;
73 AlRashidi et al. 2010). Molecular sexing (described in (Küpper et al. 2009) confirmed the field
74 observation by the presence of Z and W fragments in the female and Z fragments only in her mate.
75 Sexing of the offspring showed that all three chicks were male. Subsequently the family was
76 genotyped using 33 microsatellite markers including two Z-linked and one W-linked locus (Küpper et
77 al. 2007; Küpper et al. 2008; Dawson et al. 2010). Genotypes were checked for consistency across
78 two runs. Because no shorebird genome is yet available we mapped the location of the microsatellites
79 to the chicken (WSHUC2) and zebra finch *Taeniopygia guttata* (taeGut3.2.4) genome data bases
80 following the methodology described in (Küpper et al. 2008).

81

82 The sex-linked markers had low polymorphism and the female genetic profile was monomorphic for
83 all three sex chromosomal markers (Supplementary Material). Therefore we performed a peak height
84 ratio analysis to establish the composition and number of sex chromosomes (Young et al. 2001). We
85 amplified products for the W-linked marker *Calex-31* and one Z-linked marker *Calex-26* together in a
86 single PCR with 35 cycles and established the W/Z peak height ratio of the triploid female and 22
87 females from the same population that had the same genetic profiles at the sex-linked markers. We
88 then compared the value of the triploid female to the distribution of W/Z peak height ratios of the
89 control females.

90

91 Results

92 All alleles of the chicks could be assigned to either the triploid mother or the diploid father. None of
93 the chicks nor the male showed a three-allele genotype. For 17 of the 33 markers we identified
94 homologues on nine zebra finch and nine chicken chromosomes (Table S1). The female had three
95 allelic genotypes at 14 markers and all three female alleles were represented in the offspring for six of

96 these 14 markers (Table 1, for a genotype profile example see Figure S2). Eight three allelic markers
97 could be mapped to six zebra finch and eight chicken autosomes (Table 1). The peak height ratio
98 analysis revealed that the triploid female differed from the mean peak height ratio of the 22 control
99 females by 4.47 standard deviations. The Z product was overrepresented in the triploid female by a
100 factor of 1.5 to 2.2 in comparison with the control females suggesting a ZZW sex chromosome
101 aneuploidy (Figure 1).

102

103 Discussion

104 We have demonstrated that a triploid ZZW Kentish plover produced viable diploid offspring.
105 Triploidy is usually lethal at the embryonic stage in birds (Forstmeier, Ellegren 2010). However, it
106 may occur more frequently than presently thought because the presence of three alleles at a single
107 locus is easily confused with contamination.

108

109 The Z:A ratio is an important feature of the *Z Dosage* model (Clinton 1998). Triploid ZZW chickens
110 that have an intermediate Z:A ratios of 2:3 are sex changers that start as females but assume
111 phenotypic characteristics of males before sexual maturity. Importantly, these sex changers do not
112 produce viable gametes (Lin et al. 1995) whereas the triploid Kentish plover we studied produced
113 viable female gametes. The Kentish plover female was observed twice over a period of three years
114 and we noted two attempts of reproduction with the same male. The age of the female was at least
115 three years when it reproduced successfully and last seen alive. We consider it unlikely that she
116 changed her sex subsequently, long after onset of sexual maturity and successful reproduction.

117

118 The observation of a reproducing ZZW female has implications for avian sex determination. Despite
119 the recent support for an important role for *DMRT1* in the sex determination cascade in a bird, an
120 effect of a W-linked gene that triggers femaleness should not be discarded (Ellegren 2011). This still

121 unknown gene could antagonistically interact with *DMRT1*, for example through changes of
122 methylation patterns (Teranishi et al. 2001). In amphibians with a ZW sex determination system, *DM-*
123 *W*, a recently identified truncated paralogue of *DMRT1*, interacts antagonistically with *DMRT1* and is
124 known to trigger femaleness (Yoshimoto et al. 2010). *DM-W* has no known homologue in chicken
125 although current lack of sequence information for the W chromosome from other birds does not rule
126 out the presence of a *DMRT1* paralogue or other potentially female-determining genes in other avian
127 lineages.

128

129 We suggest that more than one sex determination mechanism may have evolved in birds and that the
130 current description of *DMRT1*-driven male determination in birds is incomplete or overly simplistic.
131 In most vertebrate groups the mechanism of sex determination is not fully conserved (Graves, Peichel
132 2010). For example, switches between environmental and genetic sex determination (ZW or XY) have
133 occurred frequently during the evolutionary history of reptiles (Ezaz et al. 2006; Janes, Organ,
134 Edwards 2009). Previously, adult ZZW females have been reported in blue-and-yellow macaw *Ara*
135 *ararauna* and great reed warbler *Acrocephalus arundinaceus* (Tiersch, Beck, Douglass 1991; Arlt et
136 al. 2004). However, the evidence of both cases was less conclusive than in our case. In both previous
137 studies the aneuploidy was established only for blood cells. The females either did not have offspring
138 (in case of the macaw) or transmitted only alleles of one Z chromosome to her 17 offspring (in case of
139 the warbler). Therefore alternative explanations such as a tissue-restricted mosaicism cannot be ruled
140 out (Fechheimer, Jaap 1980). By contrast, we observed triploidy in blood cells and found all three
141 alleles for a number of chromosomes in the offspring profiles. Nevertheless, taken together the three
142 ZZW cases suggest the intriguing possibility that non-galliform birds may have evolved a different
143 sex determination mechanism different from chicken. This is further supported by the large extent of
144 size variation in bird sex chromosomes (Stiglec, Ezaz, Graves 2007), and the observation that the
145 expression of Z-linked genes, including the region where major sex determination factors such as
146 *DMRT1* and MHM are located, differs between Galliform and non-galliform birds (Itoh et al. 2010).
147 Taken together, our findings suggest that avian sex determination is more complex and dynamic than

148 currently recognized. We suggest that future studies should focus not only on chicken but include a
149 phylogenetically broad range of bird species to fully understand the sex determination pathway in
150 birds.

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156

157 **Table 1.** Genotypes of 14 diagnostic loci of the triploid female Kentish plover, her mate and their
 158 offspring.

Marker	# Chr Chicken / Zebra Finch	Female	Male	Chick1	Chick2	Chick3
Calex-02	1 / ?	148/152/158	150/156	156/158	148/150	148/150
Calex-04 ^a	2 / 2	213/217/221	211/219	217/219	219/221	211/213
Calex-14 ^a	14 / 14	204/206/218	206/208	204/206	208/218	206/208
Calex-18 ^a	17 / ?	155/159/163	157/159	155/159	157/159	157/163
Calex-19	20 / 20	303/306/308	301/303	303/308	303/306	303/306
Calex-39	? / ?	145/147/153	139/141	139/153	141/153	139/147
C201	na	129/133/139	131/137	137/139	129/131	129/131
C203	na	183/185/187	183/187	183/187	187/187	183/187
C205 ^a	na	179/183/187	177/185	183/185	185/187	177/179
Hru2	? / ?	144/146/148	146/148	144/148	163/167	146/146
Calex-35	? / ?	127/141/147	141/143	141/143	143/143	143/147
Tgu04-004 ^a	4 / 4	161/163/169	161/167	167/169	163/167	161/167
Tgu03-002	3 / 3	120/122/124	122/122	122/122	122/124	122/124
RGB18 ^a	9 / 9	260/266/270	264/266	266/270	260/264	266/266

159
 160 #Chr, Chromosome number of hit in Chicken (*Gga*) / Zebra Finch (*Tgu*)

161 ^aall three female alleles are represented in offspring

162 ?, no conclusive hit to genome map

163 na, microsatellite flanking region sequence not available

164

165 **Figure 1.** Peak height ratio of one triploid (black circle) and 22 diploid (open circles) females for
166 *Calex-26* (Z-linked) and *Calex-31* (W-linked).

167

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