Genetic patterns of Black-tailed Godwit populations and their implications for conservation

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CHAPTER

3

Intronic variation on the CHD1-Z gene in Black-tailed Godwits *Limosa limosa limosa*: correlations with fitness components revisited

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Abstract

Recently, Schroeder *et al.* suggested that intronic variation in the CHD1-Z gene of Black-tailed Godwits (*Limosa limosa limosa*) breeding in southwest Friesland, The Netherlands, correlated with fitness components. Here we re-examine this surprising result using a much expanded dataset (on 2088 birds from 2004-2010 rather than 284 birds from 2004-2007). We find that the presence of the Z* allele (9% of the birds) does not associate with breeding habitat type, nor with egg size, adult survival, adult body mass or adult body condition. The results presented here, when used in synergy with the previously reported findings, suggest that there might be a tendency towards female adults with the Z* allele laying earlier clutches than adult females without the Z* allele. The occurrence of the Z* allele was associated with a higher chick body mass and return rate. Chicks with the Z* allele that had hatched early in the breeding season were heavier at birth compared to chicks without the Z* allele and chicks with the Z* allele that had hatched late. The present results suggest that neutral variation in the CHD1-Z gene may indeed have arisen as a byproduct of selection in females and during the egg and chick-phases.

Keywords CHD1-Z*, molecular sexing, neutral variation, sample size issues, selection

Introduction

In species where sexes are morphologically difficult to distinguish, molecular intronic markers have been used to assign sex (Griffiths *et al.* 1996; 1998, Ellrich *et al.* 2010, Saino *et al.* 2010). The Chromo-Helicase-DNA Binding (CHD1) gene, present on both the Z (CDH1-Z) and W-chromosome (CDH1-W) in birds, is widely used for this purpose. By simultaneous Polymerase Chain Reaction (PCR), the CDH1-Z and CDH1-W loci are amplified and the length difference between the resulting allele fragments is used to distinguish male and female genotypes. Males will show two allele fragments of the same length (ZZ), whereas females will show allele fragments of different size (ZW). There are an increasing number of studies reporting length variation in the PCR-amplified fragments of the CHD1-Z locus (Dawson *et al.* 2001, Montell *et al.* 2001, Lee *et al.* 2002, Agate *et al.* 2004, Jarvi and Farias 2006, Casey *et al.* 2009, Schroeder *et al.* 2010), variation that is expected to be selectively neutral.

Nevertheless, other studies have reported correlations between intraspecific variation in the CHD1-Z gene and fitness components. In Common Moorhens (*Gallinula chloropus*) a polymorphism in the CHD1-Z gene was associated with increased mortality in heterozygous male chicks (Lee *et al.* 2002). In Ovenbirds a polymorphism in the CHD1-Z gene was found to correlate with male body mass (Toms *et al.* 2012). In Black-tailed Godwits (*Limosa limosa limosa*), normally amplified CHD1-W and CHD1-Z alleles fragment have lengths of 393 and 378 bp, respectively, whereas a polymorphism on the CHD1-Z gene resulted in a third possible allele fragment of 374 bp (Z*) (Schroeder *et al.* 2010). Accordingly, Black-tailed Godwit males have three different genotypes (ZZ, ZZ* or Z*Z*) and females two (ZW, Z*W). Based on a sample of 284 Black-tailed Godwits from Friesland, The Netherlands, the rare Z* allele appeared to be positively associated with several fitness components, but homozygote Z*Z*genotypes were not found. Males and females with the Z* allele had less extensive breeding plumage, and females bred earlier and had larger eggs than birds without the Z* allele. There was also an association with breeding habitat type, such that in areas managed as meadowbird reserves (extensively agricultural land) the Z* allele was found in 14% of the birds whereas no birds with the Z*allele were found in modern, intensively managed agricultural land.

Schroeder *et al.* (2010) proposed three explanations for such intriguing functional correlations with supposedly neutral genetic variation. First, the polymorphism could reflect underlying population structure. Second, the polymorphism might have been directly affecting phenotypic variation. Schroeder *et al.* (2010) indicated that this is not very likely as the CHD1-Z gene has a role in chromatin structure mediation and organization during transcription and should therefore be conserved (Stokes and Perry 1995, Schroeder *et al.* 2010). The third possibility was that this polymorphism is linked to other, non-neutral, loci on the same chromosome.

Here we revisit the story of Schroeder *et al.* (2010) with a much larger sample of 2088 individuals collected in 2004-2010 in the same study area and including the earlier data. Not only was sample size higher, we were also able to look at a greater range of fitness correlates, including chick survival in addition to habitat selection, timing of breeding, egg size and adult survival.

Methods

In late May-June 2004 to 2010 blood samples of individual Black-tailed Godwits were collected in southwest Friesland in a study area that from 2007 onward comprised 8340 hectare of intensively and extensively managed grasslands (see Groen *et al.* 2012 for more details on the study area). Upon finding a nest during incubation, the floating method of Liebezeit *et al.* (2007) was used to predict hatch date, so that around the day of predicted hatch adults and chicks could be captured on the nest. Each adult bird received an individual combination of four colour-rings plus a flag on their tibia for individual recognition. Since 2008 hatchlings (chicks still in the nest) were given an engraved leg-flag: a flag with a written three letter/number combination. Subsequently, different biometric measurements (bill length, total head, wing length, tarsus length, tarsus toe length) and body weight were taken from each individual. In addition to ringing the birds and taking their biometrics, we obtained 30 µl whole blood samples from the brachial vein of adults or the leg vein of chicks. Blood was stored in individual 1.5 ml Eppendorf tubes containing 97% alcohol buffer, after which the individual tubes were frozen at -80 ˚C.

DNA was extracted from 6-10 µl of blood using the Ammonium Acetate method as described by Richardson *et al.* (2001) or by using Qiagen Dneasy Tissue Kit (Qiagen 2003), with minor modifications as described by Trimbos *et al.* (2009). DNA quality and quantity were checked twice, using the NanoDrop ND-1000 (Thermo Scientific) for 260/280 ratios and concentration values. For optimal PCR amplification, blood samples were diluted to concentrations below 10 ng/µl. We used fluorescently labeled P8 and P2 primers and the PCR amplification protocol as described by Griffiths *et al.* (1998). The PCR products were separated on an ABI 377 automatic sequencer (data from 2004 - 2007) or AB3730 DNA analyzer (data from 2008 - 2010). Subsequently their fragment length was determined using Genescan 3.1 software or Genemapper 4.0 software, respectively.

A total of 2088 individuals, comprising 618 adults and 1470 chicks, were typed for sex and the presence of the Z*allele (Table 1). To be able to compare results between Schroeder *et al.* (2010) and this study, most of the fitness calculations of Schroeder *et al.* (2010) were repeated for more extensive samples that included the ones used by Schroeder *et al*. (2010). We excluded plumage traits from the fitness analyses because of the impossibility to score this in all birds (especially hatchlings).

Table 1. The number of individuals used for Hardy-Weinberg equilibrium and habitat selection calculations are displayed below. The number of Individuals with and without the Z* allele are subdivided by sex, age and habitat type and totals for every subcategory are given.

	Male adult	Male chick	Total	Female adult	Female chick	Total	Intensively managed meadowland	Extensively managed meadowland	Moved between management types	Total
Z	266	658	924	296	683	979	98	363	74	535
Z*	32	92	124	24	37	61	8	34	13	55
Total	298	750	1048	320	720	1040	106	397	87	590

When the Z* allele is indeed positively correlated with different fitness variables, or when the homozygote Z*Z*genotype is lethal, the amount of different genotypes present in the populations might deviate from Hardy-Weinberg expectations (Frankham *et al.* 2009). The sex ratio is expected to be 1:1. However, possible correlates between the CHD1-Z* allele variant and fitness components might cause skew, and we thus verified if sex ratio deviated from expected values. Chi-Square tests were performed to test if the differences between observed and expected values for the number of genotypes, sex ratio, and males and females carrying the Z* allele were significant.

We tested if the occurrence of the rare Z*allele was restricted to individuals breeding on meadows managed for meadow birds (herb-rich meadows', Schroeder *et al.* 2010, Groen *et al*. 2012). Hatchlings and larger chicks were excluded from this analysis. Because some adults were captured in multiple years, we used a generalized linear mixed model (GLMM) with breeding habitat as response variable, individual as random effect, and genotype as fixed effect. In total 708 observations of 590 different adults were included in this analysis (Table 1).

To analyze if the Z*allele had an effect on yearly apparent survival, marked godwits were recaptured and/or resighted with binoculars or spotting telescopes (Lourenço *et al.* 2011). Adults with known genotypes marked in 2004-2009, and resighted from 2005 to 2010 were selected for an analysis of annual apparent survival using a Cormack Jolly Seber (CJS) mark-recapture model in the program MARK (White and Burnham 1999). Of the 461 individuals included in this analysis, 42 carried the Z*allele. An individual was reported alive if it was seen at least twice and on different dates within the breeding season March to June. The full model, was the model in which the survival probability was dependent on year, presence or absence of the Z*allele, and the interaction between year and Z*allele. The resighting probability was able to vary only between years because we think it is unlikely that the Z*allele would influence resighting probabilities. The goodness-of-fit of the global model was tested with the program U-CARE (Choquet *et al.* 2009) and the model fitted the data well (χ^2 =25.7945, df = 26, p = 0.47). Model selection was based on Akaike's Information Criterion (AIC) to select the most parsimonious model (Burnham and Andersson 2002).

Black-tailed Godwits vary greatly in size, so in addition to body mass which is body size dependent, we used a body size independent mass termed 'condition' (van der Meer and Piersma 1994). Of 603 adults body size measurements (bill, total head, wing, tarsus, tarsus toe length), the body mass, molecular sex and CHD1-Z genotype were known. For 'condition' the size parameters were collapsed in a Principal Components Analysis to extract a single size parameter, representing structural body size. The first principal component (PC1) explained 80.8% of the variation. The residuals of the linear regression of body mass and PC1 ($R^2 = 0.70$, $F_{1,601} = 1429$, $p < 0.001$) were taken as a size independent condition measure.

To test for correlations between presence or absence of the CHD1-Z*and body mass and body condition, we used a linear mixed effect model, with sex, the interaction sex and CHD1-Z genotype, habitat quality (modern or herb-rich agricultural land), and date of capture as fixed effects. Because some individuals were captured in multiple years, we used individual as random factor. Additionally, year of capture was used as random effect.

Egg volumes and lay dates (the day that the first egg was layed) of nests with at least one of the parents carrying the Z*allele were compared to egg volumes and lay dates of nests where none of the parents carried the Z*allele. For this analysis we used only nests of which we knew both parents. Hereafter, we analyzed if there was an effect of the occurrence of Z*on egg volume in males and females separately. Similarly, a mixed effect model was used here, with breeding pairs or individuals (pairs are monogamous and no divorces were observed) and year as random effect. For the egg volume analysis we used, lay date, habitat quality and the interaction of lay date with CHD1-Z genotype as fixed effects. The egg volume analysis for two parents per nest resulted in a too small dataset to perform the analysis with year as a random factor, therefore year was included as fixed factor. For the analysis on lay date, habitat quality, the interaction CHD1-Z genotype with habitat quality and the interaction of CHD1-Z genotype with year as fixed effects.

From 2008 to 2010, 914 freshly hatched chicks were marked with an engraved leg flag, enabling us to investigate if there was a difference in return rates of chicks with and without the Z* allele. We used observations of chicks in subsequent years (in either breeding area or on the migration route to increase resightings). Since the quality of the hatching habitat (modern or herb-rich agricultural land) and hatching year might influence chick survival, we included these factors in the analysis. Genotype, year, habitat quality, hatching date, body mass, the interaction genotype and hatching date, the interaction genotype and body mass and the interaction genotype and habitat quality were included in the analyses as fixed effect. We could not assign year as a random factor, as there were only two years included. Because of the low number of resightings and observation years, we used a Generalized Linear Model for binomial data with a logit link, instead of doing a mark-recapture analysis. We also analysed if the CHD1-Z genotype of the chick itself had an influence on its body mass. For this analysis we used the body mass data from 1470 chicks that were caught between 2004 and 2010. To prevent pseudo replication of chicks that were born within the same nest, we analyzed this with a mixed model with nest and year as a random effect. Genotype, sex, habitat quality and hatching date, the interaction genotype and hatching date and the interaction genotype and sex were included in the analyses as fixed effect. We included hatching date as it has been suggested to be correlated with reproductive success (Kruk *et al.* 1997, Arnold *et al.* 2006, Schroeder *et al.* 2012) and might be an important explanatory factor. Differences in sample size between different analyses were caused by missing values of some variables.

Statistics were done in R.2.11.1 (R Development Core Team, 2008) unless stated otherwise. Non-normal variables were transformed. The function lm() was used for the general linear model, lme() for linear mixed effect models, lmer() for the generalized linear mixed effect model and glm() for generalized linear models. For the linear mixed models we performed a manual backward selection using the anova() function for models fitted with the ML method, while the estimates were given after refitting the model with the REML method. The PCA was done with the prcomp() function, using a correlation matrix. Chi square tests were conducted using chisq.test().

Results

Of the 2088 individuals 1048 were male (298 adults and 750 chicks) and 1040 were female (320 adults and 720 chicks), which did not deviate from an expected sex ratio of 1 (X^2 = 0.03, df = 1, P = 0.86) (Table 1). 9% of the reported genotypes carried the Z* allele (Table 1). This corresponded to a Z*allele frequency of 6% (q = 0.06, and p = 0.94). We expected 4 males out of 1040 to be Z*Z* given the Z*allele frequency. We encountered one chick with the homozygous Z*Z* genotype; its biometrics were comparable with other chicks (mass 29 gram, bill 17.6 mm). The number of observed genotypes did not differ significantly from the number of genotypes expected under Hardy-Weinberg expectations (Fisher's exact test P = 0.78). Eight out of 106 godwits caught on intensive and 34 out of397 godwits caught on herb-rich meadows carried the Z*allele. Additionally, 13 out of 87 godwits that were caught on meadows of both management types throughout the study period, carried the Z* allele (Table 1). There was no difference between the occurrence of the Z* allele in birds in intensively and herb-rich agricultural land (z = 0.82, P = 0.4).

The model where apparent survival varied per year and resighting probability was constant was the best supported (Table 2). There was no support that the Z* allele had an effect on yearly apparent survival. The most parsimonious model including Z* was the model with an interaction between Z* and year and had a ΔAICc of 7.6. Resighting probability was high (P = 0.89, SE=0.01), as was average yearly apparent survival (*phi* = 0.89, SE = 0.004).

Table 2. Model results for the apparent survival of Black-tailed Godwits and its relationship with the Z* allele. Apparent survival (phi) can be year dependent (t), dependent on genotype (Z) or dependent on the interaction Z*t, or not vary over years and genotype (.). Resighting probability (p) can be year dependent (t) or not (.).

Model	AICc	Δ AICc	AICc Weights	Model Likelihood	Num. Par	Deviance
Phi(t) p(.)	**1510.24**	**0.00**	**0.92**	**1.00**	**7**	**168.99**
Phi(t) p(t)	1515.85	5.61	0.06	0.06	11	166.48
Phi(Z*t) p(.)	1517.81	7.58	0.02	0.02	13	164.35
Phi(Z*t) p(t)	1523.44	13.20	0.00	0.00	17	161.76
Phi(.) p(.)	1533.54	23.31	0.00	0.00	2	202.38
Phi(Z) p(.)	1534.85	24.61	0.00	0.00	3	201.68
Phi(.) p(t)	1537.32	27.08	0.00	0.00	7	196.07
Phi(Z) p(t)	1538.63	28.40	0.00	0.00	8	195.36

In the model describing adult body mass (Table 3), only sex remained as a significant term after model selection (t_{595} = -34.97, P < 0.001), with males being 56 g lighter than females. Adults with a Z* allele were not heavier than adults without the Z* allele (t_{594} = 0.46, P > 0.5). The interaction term sex and genotype was not significant according to the 5% criterion (t_{593} = -1.77, p = 0.08). This interaction compares females without the Z-allele (the intercept) to males with the Z* allele. If the interaction between sex and Z* presence was kept in the model, females with a Z* allele were not

significantly heavier (estimate = 6.02 ± 3.82 SE gram, p = 0.11) than females without a Z* allele, and males with a Z* also did not differ in mass to males without a Z* allele; they were 3.31 ± 4.47 SE gram lighter. Sex was also the only remaining term in the best model for condition (estimate = -7.95 ± 1.49, t_{595} = -5.33, P <0.001), suggesting that females had a higher size-independent mass. Genotype was not maintained in this model (t_{592} = 0.40, P > 0.5). Leaving capture date (t_{594} = -1.80, P = 0.07) in the model did not change the effect of genotype (t_{593} = 1.00, P > 0.5).

Table 3. Model results for the general linear mixed model of CHD1-Z genotype related to body mass and condition of adult male and female Black-tailed Godwits. Terms left in the model are shown in bold/italic, and the terms are shown in reversed order of exclusion from the model. Estimates and statistics were derived from the last model before exclusion. Reference category was: [1] female, [2] Z allele, [3] intensively agriculturally managed.

	Estimate	SE	t value	df	P
Body Mass					
Intercept	***311.07***	***1.28***	***243.47***	***595***	***<0.001***
Sex[1]	***-56.10***	***1.60***	***-34.97***	***595***	***<0.001***
Z[2]	1.21	2.64	0.46	594	>0.5
Z*Sex	-9.33	5.27	-1.77	593	0.08
Habitat quality[3]	2.44	2.12	1.15	591	0.25
Capture date	-0.11	0.09	-1.23	592	0.22
Random effects					
$\sigma 2_{year}$	***1.68***				
$\sigma 2_{individual}$	***18.37***				
$\sigma 2_{residual}$	***6.83***				
Condition					
Intercept	***3.60***	***1.12***	***3.21***	***595***	***0.001***
Sex[1]	***-7.95***	***1.49***	***-5.33***	***595***	***<0.001***
Z[2]	0.98	2.45	0.40	592	>0.5
Capture date	-0.15	0.08	-1.80	594	0.07
Z*Sex	-6.03	4.91	-1.23	591	0.22
Habitat quality[3]	-2.29	1.93	-1.19	593	0.24
Random effects					
$\sigma 2_{year}$	***1.20***				
$\sigma 2_{individual}$	***17.19***				
$\sigma 2_{residual}$	***6.08***				

Table 4. Model results of the general linear mixed model of CHD1-Z genotype related to egg size and lay dates for male and female Black-tailed Godwits (lay date is measured as day since 1st of April). Terms left in the model are shown in bold/italic, and the terms are shown in reversed order of exclusion from the model. Estimates and statistics were derived from the last model before exclusion. Reference categories were: [1] 2004, [2] intensively agriculturally managed, [3] Z allele.

Laydate						Egg size					
	Estimate	**SE**	**t-value**	**df**	**P**		**Estimate**	**SE**	**t-value**	**df**	**P**
Both						**Both**					
Intercept	***5.07***	***0.20***	***25.30***	***115***	***<0.001***	***Intercept***	***40.90***	***0.31***	***131.98***	***97***	***<0.001***
Z[3]	-0.23	0.17	-1.36	114	0.18	Year[1]					
Habitat quality[2]	0.17	0.26	0.65	113	0.5	2005	-1.09	1.05	1.03	20	0.3
Z * habitat quality	-1.26	0.90	-1.40	112	0.16	2006	0.83	0.69	1.19	20	0.2
						2007	-0.71	0.75	-0.95	20	0.4
						2008	0.50	0.82	0.61	20	>0.5
						2009	-1.48	0.94	1.58	20	0.13
						2010	0.71	0.87	0.82	20	0.4
						Z[2]	0.78	0.72	1.08	95	0.3
						Sqrt laydate	-0.01	0.26	-0.04	19	>0.5
						Z * sqrt(laydate)	-0.55	0.57	-0.55	18	>0.5
						Habitat quality[2]	-0.0	0.98	0	94	>0.5
Random effects						**Random effects**					
$\sigma 2_{year}$	***0.48***					$\sigma 2_{intercept}$	***2,65***				
$\sigma 2_{individual}$	***0.00***					$\sigma 2_{residuals}$	***1,61***				
$\sigma 2_{residuals}$	***0.84***										
Male						**Male**					
Intercept	***5.43***	***0.15***	***35.53***	***354***	***<0.001***	***Intercept***	***41.13***	***0.42***	***98.97***	***354***	***<0.001***
Habitat quality[2]	***-0.38***	***0.12***	***-3.28***	***8***	***0,01***	Z[3]	0.77	0.43	1.79	353	0.08
Z[3]	-0.06	0.13	-0.48	353	>0.5	Habitat quality[2]	0.41	0.39	1.04	8	0.3
Z * habitat quality	0.28	0.33	0.86	352	0.4	Sqrt laydate	0.02	0.18	0.10	7	>0.5
						Z * sqrt(laydate)	-0.28	0.51	-0.56	6	>0.5
Random effects						**Random effects**					
$\sigma 2_{year}$	***0.28***					$\sigma 2_{year}$	***1.01***				
$\sigma 2_{individual}$	***0.47***					$\sigma 2_{individual}$	***1.40***				
$\sigma 2_{residuals}$	***0.67***					$\sigma 2_{residuals}$	***2.40***				
Female						**Female**					
Intercept	***5.38***	***0.16***	***33.97***	***423***	***<0.001***	***Intercept***	***42.73***	***0.66***	***64.67***	***423***	***<0.001***
Habitat quality [2]	***-0.36***	***0.11***	***-3.23***	***21***	***0.004***	***Sqrt laydate***	***-0.37***	***0.13***	***-2.92***	***21***	***0.01***
Z[3]	-0.28	0.15	-1.93	422	0.05	Habitat quality[2]	-0.06	0.27	-0.22	20	>0.5
Z * habitat quality	-0.86	0.65	-1.32	400	0.19	Z[3]	-0.74	0.53	1.40	422	0.16
						Z * sqrt(laydate)	0.34	0.68	0.50	421	>0.5
Random effects						**Random effects**					
$\sigma 2_{year}$	***0.31***					$\sigma 2_{year}$	***0.14***				
$\sigma 2_{individual}$	***0.36***					$\sigma 2_{individual}$	***2.92***				
$\sigma 2_{residuals}$	***0.80***					$\sigma 2_{residuals}$	***0.89***				

The eggs in nests of which one parent had the Z*allele did not differ in volume compared with eggs in nests where neither of the parents had the Z*allele (t_{95} = 1.08, P = 0.3) (Table 4). The occurrence of the CHD1-Z* genotype (Table 4) was not correlated with average egg volume in a separate analysis for male and female (male: t_{353} = 1.79, P = 0.08, female: t_{353} = -0.48, P > 0.5), while for males eggs differed in volume between years and for females eggs differed between years and were smaller later in the season (Table 4).

Laying dates for nests where only one parent had the Z*allele were not significantly different from nests where neither of the parents carried the Z*allele (t_{114} = -0.23, P = 0.18) (Table 4). When the effect of Z*allele on males and females was tested separately, the term habitat quality remained in the best model (Table 4) describing lay date. In one of the sexes the CHD1-Z* genotype had an almost significant statistical effect on lay date (males: t_{353} = -0.48, P >0.5, females: t_{422} = -1.93, P = 0.05). Males and females started breeding earlier on herb-rich agricultural land than in intensively managed meadows (males: t_{8} = -3.28, P = 0.01, females: t_{21} = -3.23, P = 0.004).

Table 5. Model results of the general linear mixed model of CHD1-Z genotype related to Black-tailed Godwit chick body mass (hatching date is measured as day since 1st of April) on the right and to Black-tailed Godwit chick return rate on the left. Terms left in the model are shown in bold/italic, and the terms are shown in reversed order of exclusion from the model. Estimates and statistics were derived from the last model before exclusion. Reference categories: [1] Z allele, [2] female, [3] 2008, [4] intensively agriculturally managed.

	Estimate	SE	z-value	P		Estimate	SE	t-value	df	P
Return Rates					**Chick Body Mass**					
Intercept	***-3.28***	***0.37***	***-8.88***	***<0.001***	***Intercept***	***28.17***	***1.96***	***14.67***	***803***	***<0.001***
Z[1]	***1.08***	***0.37***	***2.93***	***0.003***	***Z[1]***	***13.08***	***4.14***	***3.16***	***803***	***0.002***
Habitat quality[4]	***-1.36***	***0.44***	***-3.07***	***0.002***	***Z* log(hatching date)***	***-3.49***	***1.089***	***-3.22***	***803***	***0.001***
Year[3]					***log(hatching date)***	***-0.10***	***0.50***	***-0.19***	***803***	***>0.5***
2009	***0.66***	***0.45***	***1.47***	***0.14***	Sex[2]	-0.14	0.09	-1.51	802	0.13
2010	***0.92***	***0.41***	***2.26***	***0.02***	Sex*Z	0.01	0.38	0.02	800	>0.5
Body mass	0.00	0.06	0.01	>0.5	Habitat quality[4]	-0.09	0.21	-0.46	801	>0.5
Z* Body mass	-0.26	0.17	-1.57	0.12						
Z*Habitat quality	1.30	0.96	1.36	0.18	**Random effects**					
Hatching date	-0.01	0.02	-0.61	>0.5	$\sigma 2_{year}$	***0.26***				
Z*Hatching date	0.03	0.04	0.86	0.4	$\sigma 2_{individual}$	***1.99***				
					$\sigma 2_{residuals}$	***1.44***				

Chicks with a Z* genotype had a higher return rate than chicks with a Z genotype (z = 2.93, P = 0.003). Additionally, chicks that had hatched on intensively managed meadows had a lower return rate (z = -3.07, P = 0.002). Also the year of hatching influenced return rates of the chicks in consecutive years

(2009: $z = 1.47$, $P = 0.14$, 2010: $z = 2.26$, $P = 0.02$). Hatching date and the interactions of the terms genotype x habitat quality and genotype x hatching date did not remain in the model (Table 5). We had information on body mass of 1470 chicks from 664 nests, of which 129 had a Z* allele. Chicks carrying the Z* allele were heavier if they had hatched early in the season, and lighter if they had hatched late in the breeding season compared with chicks without the Z* allele (Table 5 and Fig. 1). At the mean hatching date of 17 May, chicks were equally heavy.

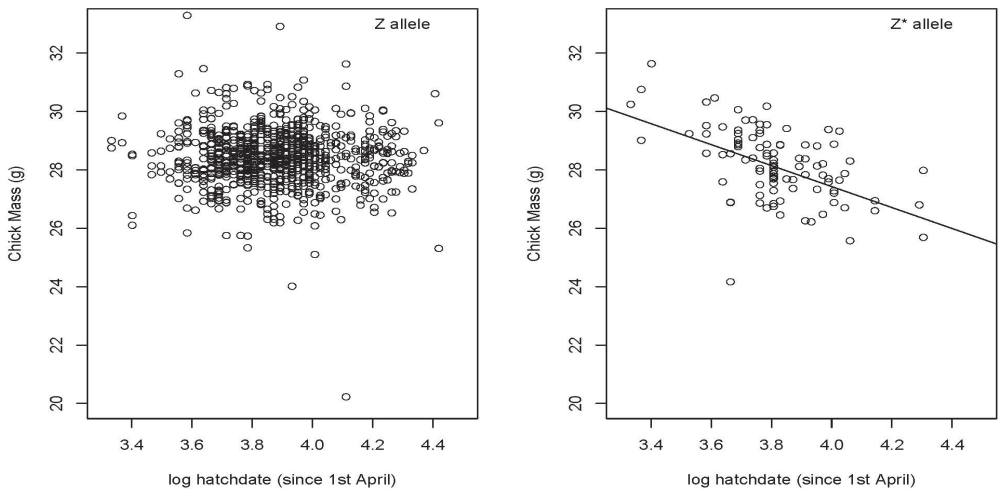

Figure 1. Relationship between hatchdate and chick mass for chicks witout (left) and with the Z* allele (right). The data shown here is corrected for random effects.

Discussion

As pointed out by Benedict *et al.* (2010), links between fitness components and supposedly neutral variation on the sex-chromosome are both surprising and very interesting. Although there were clear correlations between the occurrence of the Z* allele with apparent chick fitness correlates, on the basis of a strongly enlarged sample size we could not confirm all the correlations between Z-allele variants and fitness components in the adults as reported by Schroeder *et al.* (2010).

Our data indicate that the polymorphism does not reflect underlying population structure, a suggestion consistent with a recent study that showed no population structure between Black-tailed Godwits breeding in different parts of The Netherlands (Trimbos *et al.* 2011). Genotype numbers were not significantly different from Hardy-Weinberg or sex ratio expectations, indicating that the

heterozygous genotype did not introduce a selective advantage or disadvantage as compared to the other genotypes. Only one homozygous Z* individual was found, and the biometrics of this chick fell within the population range. Note that on the basis of the rarity of the Z* allele only 4 individuals were expected in the dataset used here.

The fitness calculations in the adults showed one significant, according to the 5% significance criterion, (negative) correlation, between the occurrence of Z* and lay date. Neither adult males nor females carrying the Z* allele showed a higher survival rate, had larger eggs and had a higher body mass or condition than birds without the Z* allele. Schroeder *et al.* (2010) indicated an effect of Z* females breeding earlier than females without the Z* allele (effect size = -4.38 ± 2.15, $P = 0.04$). However, in their analysis they corrected for year only, while we additionally corrected for habitat quality and found a slight effect of Z* on lay date which was almost significant, next to a strong effect of habitat quality. This suggests that the reported correlation in Schroeder *et al.* (2010) may have been partially explained by habitat quality, but also that there might be a tendency that females carrying the Z* allele lay their clutch earlier.

Additionally, we found apparent fitness correlates between the presence of the Z* allele with chick body mass and with chick return rate. The Z* allele had a positive effect on body mass of chicks that had hatched early in the breeding season and a negative effect on chicks that hatched later in the breeding season, compared to chicks without the Z* allele. It is often suggested that early-hatching is related to a higher chick body mass (Arnold *et al.* 2006, Schekkerman *et al.* 2008; 2009, Schroeder *et al.* 2012), and both hatch date and chick body mass often correlate positively with chick survival and should therefore positively influence chick return rates (Arnold *et al.* 2006, Schekkerman *et al.* 2008; 2009, Schroeder *et al.* 2012, Kentie *et al*. 2013). However, we did not find a correlation of chick body mass nor hatching date with return rates, whilst chicks without the Z* allele, the majority of the chicks, did not show a correlation of hatching date with body mass. Return rates were, however, higher for chicks with the Z* allele. Interestingly, our data indicate that birds breeding on herb-rich agricultural land started breeding earlier than birds breeding on intensively managed meadows. Additionally, return rates of chicks that had hatched on herb-rich agricultural land were higher than return rates of chicks that had hatched on intensively managed meadows. This reflects the importance of extensive grassland management for the survival of Black-tailed Godwit chicks, which is the factor responsible for the ongoing annual decline in Black-tailed Godwit breeding populations in northwestern Europe (Teunissen and Soldaat 2005, Schekkerman and Beintema 2007, Schekkerman *et al.* 2008, Zwarts *et al.* 2009).

Whilst not all previous reported associations of Z* with fitness correlates presented by Schroeder *et al.* (2010) could be confirmed here, we did find that Z* females laid earlier and laid eggs which, when hatching a chick with the Z*allele, produced chicks that were also heavier and survived better compared with individuals without the Z* allele. Moreover, given the strong correlations between Z* and fitness in chicks, combined with the notion that Z* chicks have at least one Z* parent, the lay date pattern found in adult females is likely to be a biological reality. Taken together, the results presented here and by Schroeder *et al.* 2010 indeed indicate an association of the Z* allele with fitness.

Under positive selection, the allele frequency of the allele(s) expressing this intronic (and therefore supposedly neutral) variation should become higher through time. Schroeder *et al.* (2010) demonstrated that the Z* allele was present in European Black-tailed Godwits 80 years ago. Although the frequency of the Z* allele could have increased since that time, at present the frequency of the Z* allele is still low. Possibly, because of the weak associations between the Z* allele and the fitness benefits this frequency has not increased noticeably over time. Another explanation might be found through a historical line of reasoning. In response to agricultural intensification, the recruitment of Black-tailed Godwit chicks has been decreasing steadily after the 1960s, resulting in population declines (Schekkerman *et al.* 2008). Between 1900 and the 1960s Black-tailed Godwits were thriving on wet herb-rich grasslands created for dairy farming in northwestern Europe (Haverschmidt 1963, Beintema *et al.* 1995). During this time, due to high chick recruitment, variance of fitness was low throughout the population. As a result the presence of Z*, and the positive effects it has on fitness, probably did not have a selective advantage then. With chick recruitment decreasing since 1967, selective pressure of the Z* allele on chick body mass and return rate might have a more prominent positive effect at present. If so, Z* allele frequencies might still be relatively low because positive selection for this allele only started to become effective recently.

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