Individual fitness correlates in the black-tailed godwit

Schröder, Julia

Box C

Effects of diet

Julia Schroeder, Francisco Santiago-Quesada, José A Masero,
Juan M Sánchez-Guzmán, Christiaan Both & Theunis Piersma

Introduction

BLACK-TAILED GODWITS *Limosa l. limosa* are temperate breeding migratory waders (Beintema *et al.*, 1995). Their wintering areas lie in West Africa (Lourenço & Piersma, 2008b). From December onwards, godwits start their northward migration to the breeding grounds. Large numbers of black-tailed godwits of the nominate race arrive in Spain and Portugal on the rice fields in late December and early January, where they forage on rice seeds (Sánchez-Guzmán *et al.*, 2007; Lourenço & Piersma, 2008a). A small part of the population does not migrate via South-western Europe, but uses stop-over sites in Tunisia, Morocco, France, Greece and Italy, where invertebrates are most likely their main prey items (Kuijper *et al.*, 2006, Lourenço & Piersma, 2008b). From here on, the last part of spring migration continues, and the first birds arrive in their Dutch breeding areas in the beginning of March (Chapter 2). During the breeding season, godwits forage mainly on earthworms and tipulid larvae. However, when, during dry periods, the soil becomes too hard to be penetrated, godwits are also known to feed on small insects living in the grass strata (Beintema *et al.*, 1995).

Not much is known about how black-tailed godwits cope with this variety of diet. In this box, we take the first step and examine how different diets affect body mass gain and breeding plumage development of black-tailed godwits during their last stop-over before reaching the breeding grounds.

Methods

THE LAST MAJOR STOP-OVER sites before the breeding grounds for black-tailed godwits breeding in The Netherlands are the rice fields of the Iberian Penninsula (Lourenço & Piersma, 2008a, Sánchez-Guzmán *et al.*, 2007). Godwits spend here nearly two month before continuing their journey to the breeding grounds (F. Santiago Quesada & J. A. Masero, pers. comm.). This is where they molt into breeding plumage (per. obs.). Godwits were caught on the rice fields in Iberia close to the village Hernán Cortéz in Extremadura, Spain (30°01'N, 5°55'W, for a detailed description of the study area see Sánchez-Guzmán *et al.*, 2007). These birds were weighed on capture, and a blood sample was taken for molecular sexing and genotyping with respect to CHD1-Z (Chapters 4, 8). One male in the rice group was found to be of the rare Z* genotype. Birds were housed in large outdoor cages (5 × 2.5 × 2 m), provided with fresh water ad libitum and grit for gastroliths.

We created two groups. The first group consisted of two cages with 6 male and 5 female godwits that were fed fly larvae *ad libitum*. The second group consisted of two cages with 7 male and 5 female godwits fed rice *ad libitum*. For the first week, birds were fed a mixed diet of rice and fly larvae for a week to habituate to captivity and feeding trays. Fly larvae were bought at a local store selling equipment for fishermen. The rice came from the rice fields in Extremadura that godwits use for staging. The treatment took place for five weeks, during which all birds were weight weekly to the

nearest gram. On the same occasions, digital pictures were taken of the birds. Because birds would not moult into a full breeding plumage over the duration of the experiment and to verify that it would model the real world to a satisfactory degree, we took another set of digital photos from all experimental birds 6 weeks after the experiment was finished, assuming that by then the breeding plumage was fully expressed. Excluding this data did not change the outcome of our analyses.

We used the plumage scores described in Chapter 4. White in head, white in neck and black in neck could not easily be distinguished from the predominant grey winter plumage. We therefore decided to only use scores that changed noticeably over the course of the experiment, and that were orange, bars, back and feathers. These scores were collapsed in a principle component analysis that resulted in one component with an eigenvalue of 2.77. This principal component explained 72% of the variation in plumage ornamentation. Birds that score high on PC1 were more colorful with respect to all plumage scores than those with a lower PC1 score.

We calculated body mass gain as the difference between body mass at the start of the treatment (after a week of habituation) and body mass after four weeks of treatment. To test whether body mass gain differed between the treatments, we employed a linear mixed model, with treatment as fixed factor and cage as random effect. Since diet may have a different effect on the sexes (Santiago-Quesada *et al.*, 2009), we included sex (females were coded as 0, males as 1) as a fixed factor and the interaction between sex and treatment in the model. Genotype (CHD1-Z) affects female, but not male body condition (Chapter 8). Since we only had one bird of the rare genotype we ignored it in the main analysis and ran another one on the dataset with that male excluded.

We analyzed plumage (PC1) as dependent variable in a linear mixed model (LMM); individuals nested in cages were modeled as random effects, treatment and sex as fixed factors and week as covariate. We expected godwits to get more colorful over time. Further, it may be that the sexes differ in their reaction to the treatments (Chapter 9). Therefore, we also tested for the two-way interactions between week and treatment and sex and treatment. We coded females as 0 and males as 1. Alike, fly larvae diet was used as the reference category. Since we know that genotype (CHD1-Z) affects plumage expression in males (Chapters 4, 8), we again ran two models, one with that male in the dataset, and an additional model with it excluded. We used R and the lme() function of the nlme package to compute LMMs (R Development Core Team 2008). We selected the most parsimonious model by AIC (Burnham & Anderson, 2002).

Results

ALL CAPTIVE BLACK-TAILED GODWITS LOST BODY MASS during the first week of habituating to captivity (Fig. C.1A, B). Since we were only interested in the effect of diet, we excluded the measurements from the day of capture for the analysis of body

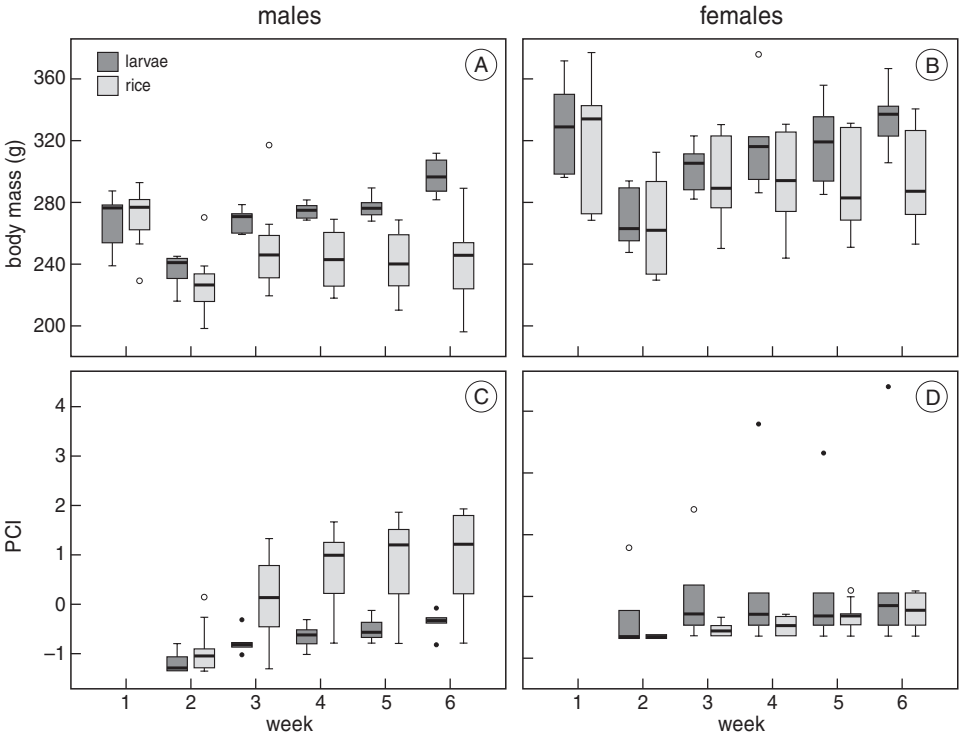

Figure C.1: The effect of diet (rice and fly larvae) on body mass (A, B) and plumage ornamentation (PC1, C, D) in male and female black-tailed godwits in captivity. Week 1 represents measurements taken at capture, treatment started after measurements were taken at week 2.

Table C.1: Results of linear mixed models on the effect of a diet (fly lavae or rice grains) on plumage ornamentation (PC1) in captive black-tailed godwits. Observations (N = 132/126) on individual birds (N = 22/21) nested in cages (N = 4) were modeled as random effects. Female was the reference category for sex, fly larvae for treatment.

Effects in the final model	dataset: full			dataset: without Z*		
	estimate±SE	t	P	estimate±SE	t	P
Week	0.15 ±0.02	6.61	<0.001	0.15 ±0.02	6.56	<0.001
Treatment (rice)	-0.11 ±0.52	-0.20	0.86	-0.08 ±0.46	-0.17	0.88
Sex (male)	0.88 ±0.29	3.09	0.007	0.98 ±0.30	3.27	0.005
Week x treatment	0.06 ±0.03	1.78	0.08	0.07 ±0.03	2.07	0.04
Rejected effect						
Treatment x sex	0.22 ±0.59	0.37	0.72	0.49 ±0.61	0.80	0.43

mass change. We found that birds feeding on rice did not fatten up as the birds that were fed fly larvae did (Fig. C.1A, B). Total body mass gain in the rice group was 30.8g ±6.84SE in females and 6.17g ±8.78SE in males. In the fly larvae group, females gained on average 69.8g ±9.23SE, males 59.33g ±2.96SE. This difference was significant between treatments and between sexes (LMM, fly larvae and female as reference groups: $\beta_{rice} = -46.73 \pm 7.25$, $t_{treatment} = -6.49$, $P_{treatment} = 0.02$; $\beta_{males} = -17.56 \pm 7.28$, $t_{sex} = -2.41$, $P_{sex} = 0.03$; N = 22 observations in 4 groups). We removed the interaction between treatment and sex from the most parsimonious model ($\beta_{rice \times males} = -13.18 \pm 14.36$, $t_{treatment \times sex} = -0.92$, $P_{treatment \times sex} = 0.37$). The results did not change quantitatively, and only little qualitatively, when excluding the Z* male (same model structure: $\beta_{rice} = -48.67 \pm 7.35$, $t_{treatment} = -6.62$, $P_{treatment} = 0.02$; $\beta_{males} = -19.33 \pm 7.35$, $t_{sex} = -2.63$, $P_{sex} = 0.02$; N = 21 observations in 4 groups).

Godwits of both sexes became more colorful over the course of time; this was more pronounced in males (Fig. C.1C, D; Table C.1). When excluding the Z* male, we found that the birds feeding on rice molted a more colorful and ornamented breeding plumage than the birds feeding on fly larvae (Fig. C.1C, D; Table C.1). For both datasets, we removed the interaction between treatment and sex from the most parsimonious model (Table C.1).

Conclusion

WE FOUND THAT, DURING SPRING STOP-OVER, captive black-tailed godwits feeding on fly larvae gained more body mass and molted a less ornamented and colorful breeding plumage than godwits feeding on rice. The little gain of body mass in the rice groups was surprising, since rice is the major diet of black-tailed godwits for up to two month during spring stop-over in Iberia before they engage on a 3000km flight to their breeding grounds (Sánchez-Guzmán *et al.*, 2007; Lourenço & Piersma, 2008a). Further, godwits caught on the rice fields in Extremadura during spring stop-over did not exhibit conspicuously low body masses (own observations).

One study recently analyzed godwit faeces collected on rice fields in Portugal, when godwits were foraging there (Lourenço & Piersma, 2008a). Here, rice grains were the most common food representing 94% of all identified prey items (Lourenço & Piersma, 2008a). Santiago-Quesada *et al.* (2009) found a high assimilation efficiency of rice grains in captive black-tailed godwits (90.0%), and this, together with the energetic value of the food indicates that rice *ad libitum* should be more than sufficient for godwits to fatten up in captivity (P. Lourenço, pers. comm.).

Only a few anecdotal studies describe the effects of plant eating in black-tailed godwits. Lange (1968) noted that his two birds would start eating grains after five days of captivity, preferring them above offered invertebrates. A stomach analysis of godwits from Hungarian rice fields showed a large fraction of rice, but one week later, when the rice fields where dried out, stomachs of godwits caught from the same area contained mainly chironomidae larvae (Sterbetz, 1962). One study on stomach

contents of godwits from Kazakhstan reports from a situation without rice fields, where, even though 58% of the collected stomachs contained grains, the majority of prey items (>70%) in terms of mass and volume were arthropods and insects (Rjabow & Mosalowa, 1967; for an overview see Glutz von Blotzheim *et al.*, 1985 and references within). M. Kersten (pers. comm.) showed that, when forced to switch diets between two different kinds of animal food sources, black-tailed godwits directly lost weight and it took them several days to adjust to the new diet.

In our experiments, females were less affected and gained half of what females gained feeding on fly larvae, while males feeding on rice gained only a tenth of what they gained when feeding on animal prey. One explanation for this could be the sexual size dimorphism; larger females with longer and larger intestines may be better in digesting plant material than males (Santiago-Quesada *et al.*, 2009). However, the difference in body mass gain of birds feeding on rice between the sexes is larger than expected, based on a small difference in assimilation efficiency (91.4% in females, 88.8% in males, Santiago-Quesada *et al.*, 2009). Another explanation may be found in behavioral differences between males that were fed rice and those fed animal prey items. It could be that godwits that were fed rice, and especially males, participated in activities that are costly energy and time wise. We noted that birds in the rice groups were more aggressive towards each other, defending the food trays against each other. It may be that more colorful and ornamented males were more prone to participate in aggressive interactions (McGraw *et al.*, 2003), which in turn may have prevented birds from feeding, which may have led to them gaining less mass than birds feeding on fly larvae (see Chapter 10 for an elaboration of this idea).

This explanation is not sufficient, however, because we do not know why the birds feeding on fly larvae, being in excellent body condition, did not molt into a more colorful breeding plumage, while the birds from the rice group did so. One could argue that birds that are more aggressive towards each other may have higher levels of circulating steroids (i.e. testosterone), which in turn may affect the expression of sexually selected breeding plumage (see also Chapter 7 and 10; Rubenstein & Hauber, 2008). However, this is a circular argument, since we do not know the initial trigger. It may be that the low body mass triggered monopolizing food behavior and exhibiting aggressiveness which in turn may, via an endocrine pathway, led to a more ornamented plumage (Safran *et al.*, 2008). But it also may be the other way around (Safran *et al.*, 2008, Rubenstein & Hauber, 2008). We need more experiments, preferably where testosterone levels and aggressive interactions are quantified, to solve this problem.

In conclusion, although we could not unravel the mechanism, our experiment suggests that diet choice during spring migration is related to body mass gain and plumage acquisition in black-tailed godwit. There also may be a link between plumage and body mass gain in the Black-tailed godwit that deserves our future attention.

Eight

Linking intronic polymorphism on the CHD1-Z gene with fitness correlates in black-tailed godwits

Julia Schroeder, Rosemarie Kentie, Marco van der Velde, Jos C.E.W. Hooijmeijer, Christiaan Both, Oliver Haddrath, Allen J. Baker & Theunis Piersma

Abstract

We report an intronic length polymorphism in the CHD1-Z gene in Black-tailed Godwits (*Limosa limosa limosa*). The Z* allele was found in 14% of 251 adult birds from nature reserves, while Z* was not found among 33 birds breeding in intensively managed agricultural lands. Males and females with the Z* allele expressed less breeding plumage, had higher body mass, bred earlier and had bigger eggs. There were no significant differences in annual survival. DNA harvested from museum skins showed that this polymorphism was present at low frequency in 1929. Strong asymmetrical overdominance may explain the low frequency of the Z* allele. Genetic linkage to causal genes might be an explanation for the phenotypic correlations. Our findings suggest a degree of cryptic genetic population structuring in the Dutch godwit population.

Ibis, in press

Introduction

MOLECULAR METHODS OF AVIAN SEX ASSIGNMENT make use of intronic DNA (Griffiths *et al.*, 1996; Ellegren & Sheldon, 1997; Griffiths *et al.*, 1998; Fridolfsson & Ellegren, 1999). In birds, males are the homogametic sex (ZZ), while females are heterogametic (ZW). The sexing methods use PCR amplification of a noncoding, supposedly neutral fragment of an intron on the conservative CHD1 gene located on both sex chromosomes, labelled CHD1-Z and CHD1-W, which conveniently differ in base pair length. Males have two fragments of the same length (ZZ genotype), whereas females have two fragments of unequal length (genotype ZW).

However, studies on five auklet species, one rail and three shorebird species report length variation in this locus. In some cases, this complicates band interpretation and can lead to wrong sex assignment (Dawson *et al.*, 2001; Lee & Griffiths, 2003; Robertson & Gemmel, 2006; Schroeder *et al.*, 2008a; Casey *et al.*, 2009; AJB, unpublished data). One study examines fitness correlates of this polymorphism: in Moorhens (*Gallinula chloropus*), Lee *et al.*, (2002) reported increased mortality in male chicks with the polymorphism on CHD1-Z. The authors proposed that CHD1-Z may have hitchhiked with the causal gene(s). In Black-tailed Godwits (*Limosa limosa limosa*), PCR products originating from the Z chromosome are either 374 (the rare type CHD1-Z*) or 378 (CHD1-Z) basepairs (bp) in length. Male godwits could come in three genotypes: 378/378 bp (CHD1-Z/CHD1-Z, hereafter abbreviated as ZZ), 378/374 bp (ZZ*), or 374/374 bp (Z*Z*). The PCR product of the W chromosome is 393 bp long and females could have two different genotypes: 378/393 bp (ZW) or 374/393 bp (Z*W). Schroeder *et al.*, (2008a) found 29% of 70 sexed male godwits to be of genotype ZZ*, none had the Z*Z* genotype, and 9% of 64 females had the Z*W genotype. Further, ZZ* males had paler breeding plumage than homozygous ZZ males, and this genetic polymorphism is correlated with phenotypic differences (Schroeder *et al.*, 2008a).

Here, we present an analysis of correlations between length variation in an intronic amplicon used for molecular sexing and fitness-related traits in Black-tailed Godwits. Because type I statistical errors can never be excluded, we repeated the analysis of Schroeder *et al.*, (2008a) on covariation of CHD1-Z with plumage traits with a larger sample size. We then test for covariation of CHD1-Z with the fitness related variables of presumed quality of the breeding site, body mass, condition, correlates of reproductive success (egg volume and laying date) and adult survival. We additionally test for the occurrence of this variation in archived DNA from museum specimen from the beginning of the 20th century and discuss possible explanations of the observed patterns.

Materials and Methods

FROM 2004 TO 2007, WE CAPTURED 121 ADULT male and 163 female godwits on their nests in southwest Friesland, The Netherlands. Of these birds 203 came from our core-study area, the Workumerwaard (52°59'N, 5°24'E), which is described in detail by Schroeder *et al.*, (2008a) and by van den Brink *et al.*, (2008). The other 81 individuals were caught on surrounding farmlands and in nature reserves. Overall, 251 individuals (109 males and 142 females) were from nature reserves with restricted agricultural management schemes and 33 (12 males and 21 females) from intensively managed agricultural land. Birds were captured at the end of incubation (Schroeder *et al.*, 2008a), were weighed to the nearest g, and tarsus + toe length (±1 mm) was measured. Each individual bird received an individual combination of four color rings plus a flag on their tibia.

To quantify plumage, digital pictures were taken of each captured bird with a resolution of 2272×1704 pixels using Nikon Cool Pix 4500 digital cameras. Seven plumage variables were scored by visual inspection of the pictures: (1) Bars score describes the extent of black bars on the belly on a scale from one to five. (2) Orange score is the intensity of orange a bird displays on the breast. (3) White in the head is the percentage of white feathers covering the head in side profile, with an accuracy of five percent. (4) White spots score is the percentage of the neck covered with white feathers, with an accuracy of ten percent. (5) Black spots score is the percentage of the neck covered with black spots with ten percent accuracy. (6) Back score is the extent of breeding feathers covering the back of a bird, on a scale from one to five. (7) The absolute number of breeding feathers on the back of a bird. For a more detailed description of these scores and their repeatability see Schroeder *et al.*, (2008a).

Length and width of all eggs in the nests were measured (± 1 mm), and egg volume was calculated by the formula 0.52 * length * width^2 (Romanoff & Romanoff, 1949). Black-tailed Godwits have an invariant clutch size of four eggs (Cramp & Simmons, 1983). Hence, if a female decides for a high investment in a clutch she has to increase the volume of the eggs. The chicks are precocial and for the first few days of their lives they rely on energy stores left at hatching, which also affects chick survival during the first weeks after hatching (Bolton, 1991; Blomqvist *et al.*, 1997; Schekkerman *et al.*, 2008; our own data). Once godwit chicks fledge, annual survival is relatively high (0.70, unpublished data). Therefore, we consider egg volume as an indication of chick survival and therefore reproductive success. We do not have a more direct measure of reproductive success, because individual fledging success can only be determined reliably with radio transmitters in Black-tailed Godwit (see Roodbergen & Klok, 2008; Schekkerman *et al.*, 2008). Recruiting individuals were too few to be used in a statistical analysis. The start of incubation was estimated by measuring the degree of buoyancy of the eggs in water, as this is related to incubation stage (van Paassen *et al.*, 1984; Liebezeit *et al.*, 2007).

A blood sample of 20 µl was drawn from each bird from the brachial vein with a sterilized microcapillary tube. The sample was stored in 96% ethanol at –20°C for the

first four to six weeks and at –80˚C thereafter. DNA was extracted using the chelex extraction method of Walsh *et al.*, (1991). Birds were sexed following the amplification protocol of Griffiths *et al.*, (1998). Fluorescently labeled PCR products were separated on an ABI 377 automatic sequencer and subsequently their exact length was determined using Genescan 3.1 software (Schroeder *et al.*, 2008a).

We collected small (≈1mm^3) skin samples from toe-pads of museum skins of 34 godwits from the years 1901–1931 from the Zoological Museum in Copenhagen. The skins were all collected at sites in Denmark. DNA from the skin samples was extracted with DNeasy TISSUE Kits from QIAGEN following the manufacturers' protocol in an archive-DNA clean laboratory at the Royal Ontario Museum (see e.g. Baker *et al.*, 2005). Birds were sexed with the primers M5 (Bantock *et al.*, 2008) and P8 (Griffiths *et al.*, 1998), which prime for a shorter amplicon of the intron than the combination P2 and P8 (Bantock *et al.*, 2008). The benefit of this method is that it has a higher success chance in partially degraded museum DNA. More importantly, it was shown to contain the same genetic polymorphism of the CHD1-Z in moorhens (Bantock *et al.*, 2008). We ran negative controls in both the DNA extraction and PCR to exclude artifacts. To verify that the genetic polymorphism observed with this new primer is the same as the one measured with the method of Griffiths *et al.*, (1998), we additionally genotyped seven female (two with the Z* allele) and six male (three of them with the Z* allele) contemporary DNA samples with known genotypes as controls with this method.

For each contemporary bird, only data of one capture occasion was used to prevent pseudoreplication. Body mass variation can result from variation in size or variation in nutritional stores (van der Meer & Piersma, 1994), and to differentiate between these two possibilities we estimated size-corrected body mass (hereafter called 'condition'). Step-wise linear regression was carried out with body mass as dependent variable and tarsus-toe length as predictor variable and sex as fixed factor. We used the standardized residuals of this analysis as an index of condition ($F_{2,275} = 300.2$, $R^2 = 0.69$, $P < 0.001$). Data on all plumage traits (bars score, orange score, white head, white spots, black spots, back score, breeding feathers) were combined in a principal component analysis. We extracted only factors with eigenvalues >1. The first two principal components (PC1, PC2) explained 63% of the variation in plumage traits (PCA: KMO = 0.74, $\chi^2 = 631.96$, $P < 0.001$). Birds that scored high on PC1 had more breeding feathers on their back, were more orange and had a larger extent of black bars on their belly; they also had less white plumage in head and neck. Birds that scored high on PC2 had more black spots on their neck. Principal component scores were normally distributed. We found no significant effects of PC2 and therefore do not report on this component from here onwards.

To confirm the results from Schroeder *et al.*, (2008a), we first tested univariately for differences in the plumage traits separately for both sexes with nonparametric Mann-Whitney U tests. We then performed GLMs on PC1, body mass and condition. Sex and Z* were modeled as explanatory factors, and the interaction between them was used to detect differences between the sexes. Birds carrying the more frequent Z allele were coded as 0, and birds with genotype including Z* as 1. Females were

coded as 0 and males as 1. As plumage may fade over the course of the season and nutritional status may change over time, we included date of capture as a covariate in the models.

We tested whether average egg volume per nest and laying date differed between nests of which at least one parent had a Z* allele and nests of which none of the parents had the Z* allele. As the genotype of both partners may influence the reproductive parameters, for the control group of that analysis we only used nests for which the genotypes of both parents were known to not contain the Z* allele. This considerably reduced sample size, and to account for all birds, we additionally performed analysis on individual birds (ignoring the genotype of the partner) to determine whether average egg volume per nest and laying date differed between the sexes and genotypes. A GLM was performed with male and female genotype as explanatory factors (ZZ or ZW was coded as 0, ZZ* or Z*W as 1). For two males of the ZZ* genotype, we had only data on one variable of reproductive output, which explains differences in sample sizes. Egg volume may decline over the course of the season, and egg volume and laying date also may vary between years (unpublished data). Therefore, laying date was modeled as a covariate with egg volume, and year as a fixed factor in both models. Laying date, season and year were not significant in any model and we therefore do not report statistics for these variables.

To determine the likelihood of missing a homozygous male (Z*Z*) in a sample the size of our data set we used a simple randomization model. Genotypes for 121 male birds (respective 92 for the core study area only) were drawn with the expected frequencies for being homozygous Z* or not, and iterated 1000 times.

For the survival analysis, we assembled resighting histories of 190 individuals ringed as adults on the breeding grounds between 2004-2008. Individuals were recorded as being alive if caught or observed at least twice during the breeding period from February until July. Model notation follows Lebreton *et al.*, (1992). We first set up an a-priori global model with the parameters that were deemed important (sex, time). Goodness of fit (GOF) of this global model was tested with bootstrap procedures. We calculated the variance inflation factor by dividing the model deviance by the bootstrapped deviance. The model fitted the data well ($P = 0.20$). We used AIC to select the most parsimonious model (Akaike, 1973). As there was no evidence for strong overdispersion (c-hat = 1.08), we adjusted AIC values to allow for the extent of overdispersion measured by c-hat, using quasi-likelihood (QAIC). Preference for one model over another was based on ΔQAIC larger than two (Burnham & Anderson, 2002). To test for the effect of genotype on annual apparent survival (φ), we changed the most parsimonious model and made survival probability dependent on genotype and genotype * sex and report the change in ΔQAIC.

We used R.2.7.1 statistical software (R Development Core Team, 2008) to compute statistics. We used the lm() function for constructing models and the step() function (both base package) to select the most parsimonious model by AIC (Akaike, 1973; Burnham & Anderson, 2002). We report parameter estimates ±SE for all effects that remained significant in the most parsimonious model, with covariates for correction

(year, date of season, laying date) included in the model, and F-statistics for each presented parameter and the final model. For the survival analysis, we used the program MARK (White & Burnham, 1992).

Results

eighteen (9%) Black-tailed Godwits carried the Z* allele. We found no homozygous males with the Z*Z* genotype. No deviation from Hardy-Weinberg Equilibrium was detected in the dataset ($\chi^2_{males} = 0.78$, $P_{males} = 0.93$, $\chi^2_{females} = 0.38$, $P_{females} = 0.95$). Given the frequency of the Z* allele in the population (8% of Z-alleles were Z*), we expected 0.7% of all males being of the Z*Z* genotype, which of our 121 genotyped males would be less than one individual. The chance to miss a homozygous male by chance in a dataset of this size is 0.38.

We had data on reproductive success at 37 nests where both adult birds were of the more frequent ZZ or ZW genotypes, and of 38 nests where one bird was ZZ* or Z*W. No nest was incubated by two birds with the Z* allele. All adult birds with the Z* allele were caught breeding in nature reserve areas (33 out of 251), whereas none of them were caught on intensively managed agricultural land (33 birds). This difference is statistically significant (P=0.02, Fisher's Exact Test).

In general ZZ* males had a paler breeding plumage compared with ZZ males. ZZ* males had significantly fewer black bars on the breast and more white in the neck plumage than ZZ males, consistent with our earlier results (Table 8.1). There was no such effect of the Z* allele in female godwits (Table 8.1). The first principal component (PC1) of male plumage traits differed between ZZ* and ZZ males, the latter being more ornamented (Fig. 8.1). The interaction of sex*genotype was removed from the final model (Table 8.2, parameter estimate when in model:

Table 8.1: Univariate analyses of the effect of genotypic variation (ZZ, ZZ*, ZW, Z*W) on breeding plumage in male and female black-tailed godwits breeding in The Netherlands.

	males			females		
	N_{ZZ}/N_{ZZ*}	Z	P	N_{ZW}/N_{Z*W}	Z	P
bars	81/14	–2.49	0.01	111/14	–0.26	0.79
orange	85/15	–0.06	0.96	114/14	–1.74	0.08
white head	85/15	–0.30	0.77	115/14	–0.01	0.99
white	85/15	–2.91	0.004	116/14	–0.08	0.94
black	85/15	–1.33	0.18	116/14	–0.80	0.42
back	82/15	–0.45	0.65	113/14	–0.28	0.78
feathers	71/14	–1.04	0.30	109/11	–0.43	0.67

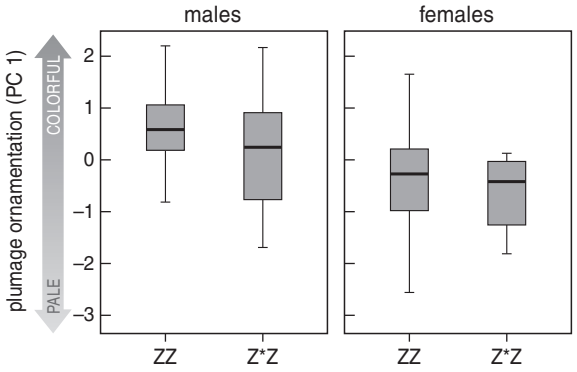

Figure 8.1: Phenotypic variation in plumage ornamentation of black-tailed godwit males and females with different genotypes on the CHD1-gene: ZZ, ZZ*, ZW and Z*W. Plumage ornamentation is presented as PC1 scores; birds scoring higher on PC1 are more ornamented than birds scoring low. Boxes depict the lowest and highest quartiles, lines through the Boxes indicate the median and whiskers the range of the observations.

b $\pm SE_{sex*genotype}$ = –0.21 ±0.39, $F_{1,187}$ = 0.28, P = 0.60): thus although the effect seemed more prominent in males than in females (Fig. 8.1), we could not show this sexual difference statistically.

Z*W females were on average 13 g heavier than the more frequent ZW females (Fig. 8.2, Table 8.2). This was not the case in males; the interaction between sex*genotype remained in the most parsimonious model (Table 8.2). There was a trend for Z*W females to be heavier in relation to their size as evidenced by their higher condition (Fig. 8.2, Table 8.2), while we found no effect in males. Although the effect was not significant, the interaction of sex*genotype remained in the final model explaining condition (Table 8.2). We did not find a difference in body dimensions (tarsus+toe length) between the different genotypes. In a model, the interaction between sex and genotype, and genotype got removed from the final model and only sex remained (parameter estimates when in model: $\beta \pm SE_{sex*genotype}$ =0.41 ±1.82, $F_{1,278}$ = 0.05, P = 0.82; without interaction: $\beta \pm SE_{genotype}$ = 0.77 ±0.91, $F_{1,279}$ = 0.14, P = 0.75).

Nests with one Z* bird had a higher average egg volume compared with nests in which both of the incubating birds only had the Z allele (t = –2.09, P = 0.04; N_Z = 31, N_{Z*} = 36). This was mainly due to an effect of ZZ* males incubating at nests that contained larger eggs than those of other males (t = –2.33, P = 0.03; N_Z = 103, N_{Z*} = 18), whereas we did not find such an effect in Z*W females (t = –0.44, P = 0.66; N_Z = 148, N_{Z*} = 15, Fig. 8.3). A nest-independent GLM of individual genotype confirmed that eggs incubated by ZZ* males were 2 cm^3 larger than eggs in incubated in nests by ZZ males, and female genotype did not remain in the most parsimonious model (Table 8.3). There was no effect of nests with at least one parent having the Z* allele on timing of breeding (t = 0.75; P = 0.46; N_{ZZ} = 36, N_{ZZ*} = 31). However, on the individual level, Z*W females initiated their clutches earlier (t = 2.52; P = 0.02; N_{ZZ} = 148,

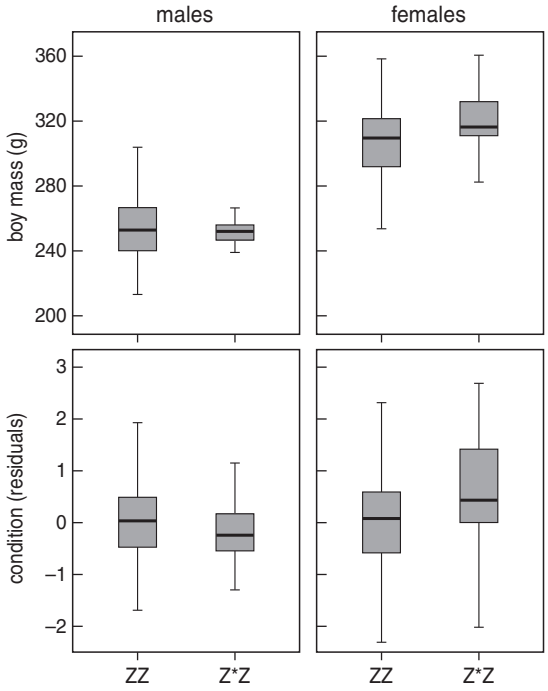

Figure 8.2: Body mass (g) and condition (residuals of a linear regression of body mass on tarsus-toe length, see text for statistics) of male and female black-tailed godwits in relation to genotypic variation on the CHD1-gene. Boxes depict the lowest and highest quartiles, lines through the Boxes indicate the median and whiskers extend to the range of the observations.

Table 8.2: Model results of the final GLM explaining black-tailed godwit breeding plumage ornamentation (measured as PC1), body mass and condition by genotypic variation on the CHD1-gene during late incubation. Date in the season was added to the most parsimonious model as a covariate. The F-statistics are for the final model including the (non-significant) date covariate (not presented). Coding: females = 0, males = 1; Z = 0; Z* = 1. PC1: $R^2 = 0.20$; $F_{3,188} = 17.27$; $P < 0.001$. Body mass: $R^2 = 0.62$; $F_{4,277} = 111.2$; $P < 0.001$. Condition: $R^2 = 0.03$; $F_{4,268} = 2.93$; $P = 0.02$.

	β ±SE	F	p
plumage ornamentation (PC1)			
Genotype	-0.39 ±0.20	3.81	0.05
Sex	0.91 ±0.13	55.51	<0.001
body mass (g)			
Genotype	11.64 ±2.75	1.34	0.25
Sex	–52.69 ±2.75	440.53	<0.001
Genotype x sex	–13.48 ±8.07	2.80	0.09
condition (residuals)			
Genotype	0.43 ±0.27	0.61	0.44
Sex	–0.31 ±0.13	9.03	0.003
Genotype x sex	–0.54 ±0.38	2.06	0.15

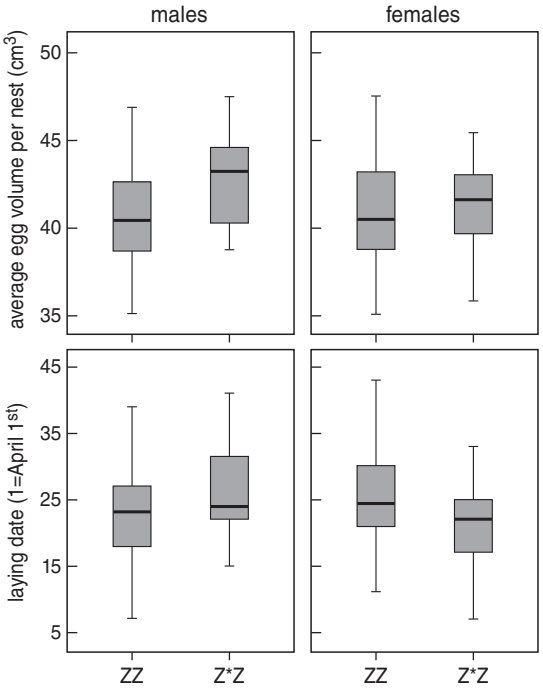

Figure 8.3: Average egg volume (cm^3) and laying date in relation to the genotypic variation at the CHD1-gene of male and female black-tailed godwits. Boxes depict the lowest and highest quartiles, lines through the Boxes indicate the median and whiskers extend to the range of the observations.

Table 8.3: Results of the final model explaining black-tailed godwit average egg volume per nest and laying date as a function of genotypic variation on the CHD1-gene of the parents (whether or not a parent carries the Z* allele). Year was added to the most parsimonious model as fixed factor and, in the model with egg volume, laying date as covariate. The F-statistics are of the final model including (non-significant) year and laying date as main effects (not presented). Egg volume: $R^2 = 0.14$; $F_{5,67} = 2.26$; $P = 0.06$; Laying date: $R^2 = 0.11$; $F_{4,70} = 2.06$; $P = 0.09$.

	β ±SE	F	p
Average egg volume			
Male genotype	1.68 ± 0.87	6.05	0.02
Laying date			
Female genotype	-4.38 ± 2.15	4.27	0.04

$N_{ZZ*} = 15$), but there was no effect of male genotype on timing of breeding ($t = –0.40$; $P = 0.69$; $N_{ZZ} = 103$, $N_{ZZ*} = 18$, Fig. 8.3). The GLM on the individual genotypes confirmed that Z*W females initiated their clutches on average four days earlier than ZW females, and male genotype was removed in the most parsimonious model (Table 8.3). Since in this model we did not distinguish between nature reserves and regular agricultural habitat, we repeated all above analyses (plumage, body mass, condition,

egg volume and laying date as response variables) on birds caught only in the core study area, the nature reserve with the highest sample size (N_{ZZ} = 75, N_{ZZ*} = 17, N_{ZW} = 102, N_{Z*W} = 9). These analyses gave qualitatively the same results as the full dataset, with lower significance values (all <0.05). Similar results were obtained when using all nature reserves, including the core study area, but for laying date we detected no significant effect (N_{ZZ} = 94, N_{ZZ*} = 18, N_{ZW} = 127, N_{Z*W} = 15). This indicates that the links between genotype and fitness correlates do not arise due to a bias of the Z* allele occurring only in nature reserves where fitness is higher (R. Kentie *et al.*, unpublished data).

In the most parsimonious survival model, adult survival was time and sex independent (Table 8.4). Resighting probability was high and independent of year (0.90±0.02SE). Annual adult survival estimated over the four years was relatively high (φ = 0.95). We found no support (ΔQAIC<2) for a statistical difference between this model and a model including genotype or a model including sex (Table 8.4, model 1 vs. model 2 vs. model 3). In the model that includes genotype, birds carrying the Z* allele had statistically non-significant higher survival by 0.02 than birds with the more frequent allele (Table 8.5).

Table 8.4: Summary of model statistics of sex and genotypic variation on the CHD1-gene (Z*) effects on adult survival of black-tailed Godwits breeding in The Netherlands.

No.	Model	No. Par.	DQAIC	Q deviance	QAIC weight
1	Φ(.)P(.)	2	0	70.45	0.38
2	Φ(Z*)P(.)	3	1.75	70.17	0.16
3	Φ(sex)P(.)	3	1.96	70.38	0.14
4	Φ(.)P(t)	5	2.24	66.57	0.13
5	Φ(Z*)P(t)	6	3.82	66.10	0.15
6	Φ(t)P(t)	7	4.04	64.25	0.05
7	Φ(sex)P(t)	6	4.18	66.46	0.05
8	Φ(sex x Z*)P(.)	5	5.41	69.75	0.07
9	Φ(sex x Z*)P(t)	8	7.45	65.58	0.01

Table 8.5: Survival estimates for black-tailed godwits breeding in The Netherlands for the three best supported survival models (Table 7.4).

model	group	F	SE	95%CI
(1)	all adults	0.950	0.019	0.894–0.976
(2)	birds with Z* allele	0.968	0.034	0.778–0.996
	birds without Z* allele	0.946	0.014	0.907–0.969
(3)	males	0.945	0.019	0.892–0.973
	females	0.952	0.019	0.894–0.976

In the DNA from the museum skin samples, the Z fragment of the M5-P8 method was 266 bp long, the Z* fragment was 262 bp and the W amplicon was 282 bp long and indicated the same length polymorphism than the P5-P8 primers. We successfully sexed 23 of the 34 museum samples (68% success rate). However, most likely due to PCR allelic dropout, the Z amplicon of three females could not be detected. We found the Z* allele to be present in one female (from the year 1929) among the remaining 20 samples of known genotype (59% success rate for determining genotype with respect to Z* allele). We found no correlation between genotyping success and age of the skin; successfully genotyped skins came from a range of years between 1901–1931.

Discussion

WE REPORT CORRELATIONS BETWEEN INTRONIC VARIATION on CHD1-Z and fitness correlates in male and female adult Black-tailed Godwits. This is the second species with a report on variation at this locus being linked with fitness-correlated traits (Lee *et al.*, 2002).

Earlier we showed that in Black-tailed Godwits, paler males pair with females that lay larger eggs, and are in better condition themselves (Schroeder *et al.*, 2009). Here we add that part of this effect may be associated with genetic variation of the Z-allele, too: Z* males are also paler and indeed paired to females producing larger eggs, and we detected a correlation with female body mass. Our estimate for annual survival is comparable to previous ones (Roodbergen *et al.*, 2008). There was a trend for birds of both sexes with the Z* allele to have a higher survival probability than birds with the more frequent allele. That this pattern was not statistically significant at the 5% level may be due to limited statistical power. As Black-tailed Godwits are long-lived, a slight increase in survival probability can mean a rather large increase in reproductive life. Moreover, lower survival in moorhen chicks with the Z* allele were reported by Lee *et al.*, (2002), suggesting that CHD-Z variation is linked to genes affecting survival in moorhens and possibly birds in general. It is therefore conceivable that such a correlation will eventually be shown to exist in Black-tailed Godwits. Despite the low frequency of the Z* allele, and consequently small sample sizes for the ZZ* and Z*W genotypes, the effect sizes were usually large, and the consistency of the patterns supports the notion that the correlation of genetic variation with fitness is real. All effects are in the same direction, lowering the chance that our conclusion is based on a type I error.

We found evidence that the genetic variation on CHDZ-1 was already present in the godwit population 80 years ago, indicating that the Z* allele is not a new mutation. This notion is supported by the fact that this mutation was found in a number of other bird species, which means that it is either old, or has arisen independently in many bird lineages. However, despite its apparent association with fitness, the frequency of the allele is relatively low. As the sample size of the historic data is small, we are unable to say whether the allele is changing in frequency.

We did not find assortative mating by birds with the Z* allele. This is puzzling, given that Z* females are of high quality and Z* males are able to attract females of high quality, and thus assortative mating by genotype might be expected. We also did not find any homozygous Z*Z* males, which might be due to chance. It may also suggest that the fitness consequences of this variation are strongly asymmetric with respect to the different genotypes: a slight heterozygous advantage over the homozygous ZZ, but strong selection against the Z*Z*.

Associations between genetic and phenotypic variation mainly arises due to one of three reasons. (1) The polymorphism indeed affects phenotypic variation directly, (2) the polymorphism is linked (and in linkage disequilibrium) with other loci on the same chromosome which causally affect the phenotype or (3) the polymorphism reflects underlying, probably cryptic, population structure. (1) Since the observed CHD1-Z variation is expected to be neutral (located in a non-coding intron), we do not favour a direct causal relationship as an explanation. (2) However, the CHD1-Z locus may be physically linked with a gene(s) coding for or affecting the studied fitness correlates in godwits, resulting in the observed correlation between CHD1-Z variation and fitness. Even though genes influencing the expression of male plumage traits are most likely located on the Z sex chromosome (Sætre *et al.*, 2003; Gunnarsson *et al.*, 2007), it is unlikely that the CHD1-Z gene itself is responsible for this effect. This gene is known to have a role in transcription and gene expression, and therefore is expected to be very conservative and most likely not related to plumage (Stokes & Perry, 1995). It supposedly mediates chromatin structure and organization during transcription and is involved in interactions with DNA and RNA (Ellegren, 1996). Because all these are involved in basic protein synthesis CHD1 is considered a very conservative gene and should not have a fast mutation rate.

The genetic polymorphism may be linked to a different set of genes responsible for the fitness effects, by genetic linkage or epistasis (Lee *et al.*, 2002). Genetic linkage and epistasis occur more frequently when the linked alleles are on the same chromosome. This is even more likely if there is only one causal gene that affects a whole suite of traits including plumage ornamentation and body mass change, as recently suggest by Ducrest *et al.*, (2008). The differences between the sexes can also be explained by the fact that the Z* polymorphism (including a linked causal gene) is on a sex chromosome. For example for body mass, the causal allele associated with Z* is recessive, and therefore maybe only visible in females. Likewise, the causal allele for plumage might not be expressed in Z*W females or suppressed by genes on the W chromosome. However, since data from families is, due to the low recruitment rates in godwits, not available, we can neither support nor exclude the possibilities that variation on CHD1-Z may directly or indirectly linked with genes affecting fitness.

The differential occurrence of the Z* allele in breeding habitats of different quality indicates some degree of population structuring (3). Population structure is highly likely in Black-tailed Godwits as adult birds are highly faithful to their previous nest-site and in the relatively rare cases where they do change nest sites, dispersal distances are relatively short (Groen, 1993; van den Brink *et al.*, 2008; but see Schroeder *et al.*,

2008b). In a closely related subspecies, the Icelandic Black-tailed Godwit (*L. l. islandica*), it has been shown that nesting birds are partitioned by habitat quality: birds wintering on high quality foraging grounds are known to also breed in high quality breeding grounds and have a higher reproductive success (Gunnarsson *et al.*, 2005). In our case, this may mean that high quality birds – including those with the Z* allele – are more likely to be found on high quality breeding areas, and their offspring with the inherited Z* allele are likely to breed there, too. Using mitochondrial DNA control region sequences, Höglund *et al.*, (2009) did not detect any population structure in godwits breeding in The Netherlands. While it is currently not possible to distinguish between the three alternative explanations, we suggest that more extensive studies are required to detect cryptic population structure in the Dutch Black-tailed godwit population.

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