On the behaviour and ecology of the Black-tailed Godwit

Verhoeven, Mo; Loonstra, Jelle

DOI:
10.33612/diss.147165577

5 Genetic parentage of the socially monogamous Black-tailed Godwit *Limosa limosa limosa*

A.H. Jelle Loonstra, Mo A. Verhoeven, Julia I. Camacho García,
Joslyn C.E.W. Hooijmeijer, Marco van der Velde, Yvonne I. Verkuil & Theunis Piersma

Submitted Manuscript

ABSTRACT

Variation in reproductive systems is ubiquitous among socially monogamous bird species, but parentage analysis with genetic markers are still lacking in many natural populations. Here we used 10 unique genetic markers to examine the occurrence of extra-pair offspring in broods of the Black-tailed Godwit (*Limosa limosa limosa*), a socially monogamous wader. Most of the social offspring (94%) could be unambiguously assigned to their social parents as they shared all alleles, we did not find evidence of extra-pair paternity or quasi-parasitism, however we found evidence for conspecific brood parasitism in seven parent-offspring combinations from five broods. Once again our results stress the importance of parentage studies with genetic markers when determining the mating system of a socially monogamous species and raises questions about the occurrence of these parasitic female Black-tailed Godwits.

INTRODUCTION

In the past decade, parentage studies with molecular markers transformed our view on the reproductive strategies of various bird species, but also stressed the importance of genetic markers to show whether social parents are also the biological parents of the offspring they take care of (Thomas *et al.* 2007, Yom-Tov 2008). For example, studies on socially monogamous bird species have shown that siblings from the same nest are not necessarily related to either of the social parents (egg-dumping or conspecific brood parasitism), their social father (extra-pair paternity) or their social mother (quasi-parasitism) (Griffith *et al.* 2004). While a surprising high diversity of reproductive strategies has been uncovered, both among and within different species, we still lack descriptive knowledge on the reproductive behaviour of most species (Pitelka *et al.* 1974, Yom-Tov 2008, Reneerkens *et al.* 2014).

The Continental Black-tailed Godwit (*Limosa limosa limosa*; hereafter 'godwit') has been the subject of many studies concerning its reproductive biology, migration and conservation (van Balen 1959, Haverschmidt 1963, Kentie *et al.* 2018, Verhoeven *et al.* 2018, Loonstra *et al.* 2019). Based on behavioural observations, godwits are assumed to maintain long-lasting socially monogamous pair bounds (Haverschmidt 1963, Kentie *et al.* 2014). And even though the relatedness between social parents and offspring has never been investigated with genetic markers, it has been assumed that social parents are also the biological parents. Nevertheless, both de Bont (1945) and Haverschmidt (1963), confronted with clutches of more than the usual four eggs (Verhoeven *et al.* 2019b), already wondered whether this is always true. As studies on godwits are becoming more individual-based (Kentie *et al.* 2018, Verhoeven *et al.* 2018), there is an increasing need to pin the answer down. Therefore we performed a parentage analysis with 10 microsatellite loci, and successfully examined the genetic relatedness between social parents and chicks in 37 families across 12 years.

METHODS

Study species

Godwits in the East-Atlantic Flyway are long-distance migratory birds that breed across much of Western Europe, but mostly in The Netherlands (Kentie *et al.*

2016). While approximately 25% of the population in the East-Atlantic Flyway remains north of the Sahara in the Mediterranean region for the entire non-breeding season, the other 75% crosses the Sahara desert twice a year to spend part of the non-breeding season in sub-Saharan Africa (Márquez- Ferrando *et al.* 2014, Kentie *et al.* 2017, Verhoeven *et al. under review*). Adult godwits in our Dutch study area (centered at 52°55'N; 05°25'E) arrive between late February and can be present until late August (Senner *et al.* 2015, Howison *et al.* 2018, Verhoeven *et al.* 2019a). After arrival, godwits establish territories and lay an invariant clutch of four eggs (however clutches of 5–6 eggs are possible; de Bont 1945, Haverschmidt 1963, Verhoeven *et al.* 2019b). Eggs are incubated for an average of 24.5 days, range: 22–27 days, and the precocial chicks take on average 25 days to fledge (Haverschmidt 1963, Verhoeven *et al.* 2020).

Data collection

In this study we aimed to assess the genetic relatedness of 50 social godwit families (Table 5.1). All families were breeding within our study area in southwest Friesland from 2004–2015 (Howison *et al.* 2018). Godwit nests were located by our field crew, landowners and volunteers. Once a nest was found, we used the egg flotation method to predict its hatching date in order to ring the chicks before they left the nest (Liebezeit *et al.* 2007). To ensure the correct assignment of the social parents, we only used families on which both parents were caught while incubating. Adults were caught using a walk-in-trap, drop-cage, or occasionally picked up by hand. Blood samples, from which DNA was extracted, were obtained by puncturing the leg vein of a one day old chick or the wing vein from an adult. Blood was stored in individually labeled 1.5 ml Eppendorf tubes containing 96% alcohol buffer and frozen at –20°C as soon as possible. Samples were stored long-term at –80°C.

Molecular methods and parentage analysis

The extraction of DNA from whole blood was performed by the standard ammonium acetate method described in Richardson *et al.* (2001). All individuals were genotyped at 10 polymorphic microsatellite loci (LIM3, LIM10, LIM11, LIM12A, LIM14, LIM22, LIM24, LIM26, LIM32, LIM33), which were developed by Verkuil *et al.* (2009). To prevent overlapping allele size ranges of loci, polymerase chain reactions (PCR) were carried out in two multiplex PCRs (Multiplex 1: LIM3, LIM11, LIM12a, LIM32, LIM33. Multiplex 2: LIM10, LIM14, LIM22, LIM24, LIM26). PCRs were carried out in 10 μL volume containing 5 μL QMPM (QIAGEN Multiplex PCR Mastermix), 0.5 μL primer mix (originating from: 1 μL of each forward and 1 μL of each reverse primer in 10 μL of H_2O (stock 10 μM)), 2.5 μL H_2O, and 2 μL DNA template. The PCRs were run on a Thermal Cycler (Applied Biosystems, Foster City, California, USA) or a Mastercycler (Eppendorf, Hamburg, Germany). The PCR amplification profile for Multiplex 1 was: 15 min 95°C, 35 cycles of 94°C for 30 s, 55°C for 90 s, 72°C for 90 s, and a final elongation time of 30 min at 60°C. For Multiplex 2 we used the following PCR profile: 15 min 95°C, 35 cycles of 94°C for 30 s, 57°C for 90 s, 72°C for 90 s, and a final elongation time of 30 min at 60°C. All fluorescently labeled PCR products were analysed on a 3730 DNA Analyzer (Applied Biosystems) and allele sizes were scored using GeneMapper version 4.0 (Applied Biosystems). Evidence for null alleles at microsatellite loci in the adult population was examined using the allele frequency analysis in Cervus 3.0 (Kalinowski *et al.* 2007).

To assess the genetic relatedness between social parents and offspring we compared all microsatellite genotypes using a strict set of rules. We only compared the genetic relationship between parent and offspring when more than 8 loci successfully amplified. Because of the relatively high estimation of null allele frequencies for LIM32, LIM3, LIM14 and LIM22 we disre-

Table 5.1. Total number of families that were collected per family size and number of families (per family size) that could be genotyped for more than 8 loci. Because of our predefined set of rules and the amplification failure of some individuals the number of families decreased after genotyping.

Family size (number of chicks)	# Families collected	# Families after genotyping	Families of which offspring was related to social parents	Families with ≥1 case of egg-dumping
1 chick	0	1	1	0
2 chicks	1	4	3	1
3 chicks	18	21	18	3
4 chicks	31	11	10	1

garded null alleles at these loci as mismatches (Table 5.2). A genetic relationship between social parent and chick was confirmed when at least all but one of the loci matched between social parent and chick. If only the social father and chick differed at more than one of the loci we considered this as a case of extra-pair paternity. When only the social mother and chick mismatched at more than one of the loci we called this quasi- parasitism. In cases were a young was unrelated to both the social father and mother we called this conspecific brood parasitism. To check for genotyping errors, we randomly genotyped 33 individuals for a second time and found no deviations.

RESULTS

Out of 100 sampled adult individuals, 81% were genotyped in all 10 loci, 5% at 9 loci, 3% at 8 loci, 1% at 7 loci, 1% at 6 loci, 6% at 5 loci, 1% at 3 loci, 2% at 0 loci. Of all sampled chicks (n = 169), we could genotype 82.2% for all 10 loci, 4.1% at 9 loci, 1.2% at 8 loci, 1.8% at 7 loci, 1.8% at 6 loci, 2.4% at 5 loci, 0.6% at 4 loci, 1.8% at 3 loci, 0.6% at 1 loci, 3.6% at 0 loci.

Altogether, we could successfully determine the genetic relationship between parents and offspring of 37 families (Table 5.1). Most of the social offspring (94%) could be unambiguously assigned to their genetic parents as they shared all alleles (Table 5.1). However, among all 116 parent-offspring combinations we found seven cases of conspecific brood parasitism, no evidence was found for extra-pair paternity or quasi-parasitism. The number of eggs that were dumped in a single nest ranged from one (3 clutches) to two (2 clutches).

DISCUSSION

Using a microsatellite-based parentage analysis we were able to distinguish between parasitic and host eggs of the Continental Black-tailed Godwit. Among the 116 sampled parent-offspring combinations from 37 social families, we found no evidence of extrapair copulations, but seven cases (five social families) of conspecific brood parasitism.

Despite our ability to identify a second breeding strategy among godwits, we are cautious to exclude other breeding strategies among godwits. Also, we suggest that the actual frequency of egg-dumping might be higher than found in this study. This follows because our relatively high rate of amplification failures and initial sample of nests that only included eleven complete families resulted in a rather low number of complete families off which all chicks could be genotyped (Table 5.1). Furthermore, our initial dataset was selected to only include successfully hatched chicks. As hatching-asynchrony is expected to be a high cost of conspecific brood parasitism in precocial bird-species (Hauber 2003), unhatched godwit eggs might have a higher chance to be parasitic and the subsequent inclusion of these eggs could increase the occurrence of conspecific brood parasitism among godwits.

Studies on the evolution of mating strategies found that polyandrous mating systems are associated with populations that have a male-biased adult sex ratios (Székely *et al.* 2014, Eberhart-Phillips *et al.* 2018). Despite the male-biased adult sex ratio in the godwit population studied here (Loonstra *et al.* 2019), we did not find evidence for a polyandrous mating-system. Therefore, one may ask whether the male-skewed adult sex ratio is so recent that mating patterns have not

Table 5.2. Microsatellite loci used for genetic parentage analysis in adult Godwits (N_a = number of alleles), N_t = number of adult individuals genotyped, H_o = observed heterozygosity, H_e = expected heterozygosity, $Freq_{null}$ = estimated null allele frequency, Sig(*P*) = significance of H_o vs. H_e, and P_{ID} = probability of identity ($P_{ID\text{-}combined} = 2.97 \cdot 10^{-13}$).

Locus	N_t	N_a	H_o	H_e	$Freq_{null}$	Sig (*P*)	P_{ID}
LIM3	73	12	0.60	0.83	0.163	<0.001	0.05
LIM10	69	9	0.80	0.82	0.011	0.56	0.06
LIM11	73	8	0.85	0.81	–0.026	0.87	0.07
LIM12A	72	11	0.81	0.85	0.022	0.91	0.04
LIM14	73	17	0.80	0.92	0.067	–	0.01
LIM22	72	6	0.43	0.49	0.083	0.10	0.29
LIM24	73	9	0.71	0.68	–0.028	0.89	0.14
LIM26	73	11	0.85	0.86	0.0034	0.21	0.04
LIM32	72	15	0.47	0.89	0.30	0.09	0.03
LIM33	71	10	0.69	0.79	0.031	0.90	0.07

caught up yet, or whether our sampling scheme failed to identify these cases?

Waders are well known for their diversity in mating and breeding strategies, including conspecific nest parasitism (Pitelka *et al.* 1974, Yom-Tov 2008). While it is unclear why some female godwits have adopted this reproductive strategy, causes of this nest parasitism could be induced by a lack of suitable nest sites, a response to nest predation during egg- laying (best-of-bad-job strategy), the life-long strategy of an individual female and kin selection (Lyon & Eadie 2008). As a first step towards a further illumination on the causes of egg-dumping in godwits it would be insightful to establish the identity of the egg-dumping females and to uncover the relationship between these parasitic females and hosts (Lyon & Eadie 2008). However, in a breeding species which relies on crypsis, and where so much reproductive events remain unrecorded even in a tightly observed population (Verhoeven *et al.* 2020), this will be challenging research goal.

ACKNOWLEDGEMENTS

We thank the crew of all the 'Black-tailed Godwit field-teams' that contributed to this study, as well as the many students and volunteers who assisted along the way. Nature conservation organizations "It Fryske Gea", "Staatsbosbeheer" and private land owners generously issued permission to access their properties. This study was funded by the Netherlands Ministry of Agriculture, Nature Management, the Province of Friesland and the Spinoza Premium 2014 awarded to TP by the Netherlands Organization for Scientific Research (NWO). Additional financial support came from the Prins Bernard Cultuurfonds, the Van der Hucht de Beukelaar Stichting, the University of Groningen, Birdlife-Netherlands, WWF-Netherlands, Gieskes Strijbis Fonds and an anonymous donor. This work was done under license number 6350A following the Dutch Animal Welfare Act Articles 9 and 11. All data needed to evaluate the conclusions in the paper are present in the paper and will become available from the Figshare Data Repository.