FINE SCALE GENETIC STRUCTURE AND EXTRA-PAIR PARENTAGE IN THE SOCIALLY MONOGAMOUS UPLAND SANDPIPER

by

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Abstract

In birds, the offspring of females in socially monogamous species can be sired not only by their social partner (within-pair mating) but also by other males (extra-pair mating), resulting in broods of mixed paternity. Several hypotheses have been proposed which attempt to explain the adaptive significance of this behavior, including the *genetic diversity* hypothesis, the *good genes* hypothesis, the *genetic compatibility* hypothesis and the *fertility insurance* hypothesis. I report results of a 5 year population study of the Upland Sandpiper (*Bartramia longicauda*) at Konza Prairie Biological Station in northeast Kansas. My objective was to determine the genetic mating system of this socially monogamous shorebird, and determine which of the genetic hypotheses best explains the patterns of extra-pair paternity (EPP) in the population. As part of the analysis, I optimized laboratory protocols for genetic sexing of our monomorphic study species. Potential errors in molecular sexing have been previously described but usually result in females being misidentified as males. Here, I report evidence that events in PCR reactions can lead to the opposite error, with males misidentified as females. I recommend the use of multiple primer sets and large samples of known-sex birds for validation when designing protocols for molecular sex analysis.

I genotyped birds and tested for the existence of EPP in 58 family groups of Upland Sandpipers. I found 15% of chicks and 30% of broods were the result of extra-pair paternity in this population, which is high in comparison to other socially monogamous shorebirds. Only 2% of chicks and 2% of broods were attended by females unrelated to the young. I tested ecological covariates known to influence EPP in other birds including relatedness of mated pairs,

morphology of the within-pair male, and nest initiation date, as well as variables which signify genetic benefits, including morphology of the offspring and offspring heterozygosity, but found no significant relationships. None of the prevailing genetic hypotheses can fully explain the high rates of EPP in this population of Upland Sandpipers. However, the discovery of fine-scale genetic structure in female birds, but not in males, suggests female natal philopatry or male-biased dispersal. This sex-specific genetic structure could be a mechanism of inbreeding avoidance, thereby eliminating the need for females to choose mates based on relatedness.

This study provides the first estimates of EPP for the socially monogamous Upland Sandpiper, and provides evidence that the inbreeding avoidance mechanism of engaging in extra-pair copulations does not seem to be as important in Upland Sandpipers as in other socially monogamous shorebirds. Future research should include the identification of extra-pair males and the determination of offspring fitness after departure from the nest.

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CHAPTER 1 - Introduction

Field applications of molecular tools have led to substantial revisions in our understanding of the behavioral ecology of birds. While it was once thought that the majority of birds were completely monogamous (Lack 1968), the discovery of extra-pair mating behaviors has changed this view, and ornithologists must now distinguish between the behavioral and genetic strategies of avian mating systems ('social' vs. 'genetic' monogamy; Neudorf 2004). The current understanding of extra-pair mating and estimates of extra-pair paternity have been primarily based upon studies of passerine songbirds (>60%, n = 131 species, Griffith et al. 2002).

Shorebirds (Order Charadriiformes: Suborder Charadrii) are a diverse lineage of birds that includes curlews, phalaropes, plovers, sandpipers, and their allies. Shorebirds exhibit an array of mating systems and parental care that nearly encompasses the entire range of social systems found in birds worldwide, including promiscuity (including lek-mating), monogamy, polygyny and polyandry (Jönsson and Alerstam 1990), and parental care can be biparental, uniparental by females or uniparental by males (Reynolds and Székely 1997). Despite the remarkable variation in shorebird mating systems, estimates of extra-pair paternity are currently available for only 16 of 155 shorebird species.

One important aspect to studies of parentage is the determination of sex of the parent birds. It is vital to know which birds are the fathers and which birds are the mothers in order to conduct studies of extra-pair paternity. Many species of birds are difficult to sex in the field due to monomorphic body size and plumage. Consequently, molecular markers are

widely used to sex monomorphic avian species (Griffiths *et al.* 1998). While these molecular approaches are usually reliable, a certain level of caution should be taken when designing a sexing protocol because errors are possible.

The goals of my research were to determine the genetic mating system of the Upland Sandpiper, a socially monogamous shorebird. More specifically, I aimed to 1) determine the mating habits of this relatively unknown species, 2) make comparisons to other shorebirds and other socially monogamous species, and 3) determine the ecological and genetic drivers behind the mating behavior in the species.

My thesis is organized into four main chapters. Here, I introduce the study. In Chapter 2, I report on a new potential source of error for avian ecologists using molecular methods to determine the sex of individual birds. I evaluate the reliability of a widely used protocol that we applied to our study of Upland Sandpipers and make recommendations to avoid potential errors in molecular sexing in the future. In Chapter 3, I evaluate the genetic mating system of the Upland Sandpiper and examine ecological and genetic correlates associated with extra-pair mating behavior. Additionally, I examine the genetic structure of the population to determine how the structure may affect extra-pair mating activities. Chapter 4 is a synthesis of the main conclusions from my research.

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CHAPTER 2 - Heteroduplex molecules cause sexing errors in a standard molecular protocol for avian sexing*

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Abstract

Molecular methods are a necessary tool for sexing monomorphic birds. These molecular approaches are usually reliable, but sexing protocols should be evaluated carefully because biochemical interactions may lead to errors. We optimized laboratory protocols for genetic sexing of a monomorphic shorebird, the Upland Sandpiper (*Bartramia longicauda),* using two independent sets of primers, P2/P8 and 2550F/2718R, to amplify regions of the sex-linked *CHD-Z* and *CHD-W* genes. We discovered polymorphisms in the region of the *CHD-Z* intron amplified by the primers P2/P8 which caused four males to be misidentified as females (n = 90 mated pairs). We cloned and sequenced one *CHD-W* allele (370 bp) and three *CHD-Z* alleles in our population: Z^0 (335 bp), Z' (331 bp) and Z'' (330 bp). Normal (Z^0Z^0) males showed one band in agarose gel analysis and were easily differentiated from females (Z^0W), which showed two bands. However, males heterozygous for *CHD-Z* alleles ($Z'Z''$) unexpectedly showed two bands in a pattern similar to females. While the Z' and Z'' fragments contained only short deletions, they annealed together during the PCR process and formed heteroduplex molecules that were similar in size to the *W*

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fragment. Errors previously reported for molecular sex-assignment have usually been due to allelic dropout, causing females to be misidentified as males. Here, we report evidence that events in PCR reactions can lead to the opposite error, with males misidentified as females. We recommend use of multiple primer sets and large samples of known-sex birds for validation when designing protocols for molecular sex analysis.

Introduction

Reliable methods for determining the sex of birds in laboratory and field studies provide the essential demographic information that is required for many questions in ecology and evolutionary biology. In birds, the sex of neonates can rarely be determined during early development, and more than 50% of bird species are sexually monomorphic in body size and plumage as adults (Jensen *et al.* 2003). Molecular methods for sex identification are a useful tool and have been widely applied to studies of brood sex ratios (Andersson *et al.* 2003; Szekely *et al.* 2004), sex-specific life history characteristics (Merila *et al.* 1997; Lopes *et al.* 2006; Remisiewicz & Wennerberg 2006), and captive breeding programs (Griffiths & Tiwari 1995; Jarvi & Banko 2000).

At least three universal primer sets for avian molecular sexing have been developed, the most commonly used being P2/P8 (Griffiths *et al.* 1998), 1237L/1272H (Kahn *et al.* 1998), and 2550F/2718R (Fridolfsson & Ellegren 1999). All three methods are based on the co-amplification of homologous, sex-linked *CHD-Z* and *CHD-W* genes and generally work for all birds, except ratites (Ellegren 1996). Sex in birds is determined genetically by sex chromosomes, where males are the homogametic sex (*ZZ*) and females are the heterogametic sex (*ZW*). The P2/P8 and 1237L/1272H primers amplify an intron within the

CHD gene, where size differences between the *Z* and *W* homologues can be used to determine the sex of male and female birds (Griffiths *et al.* 1998). The 2550F/2718R primers operate in a similar way, except they amplify a different *CHD* intron. Thus, after electrophoresis, homozygous males (*ZZ*) and heterozygous females (*ZW*) are identified by one or two bands, respectively. In the case of the P2/P8 primers, the homologues of *Z* and *W* are sometimes similar sized fragments, and digestion with restriction enzymes specific to one of the homologues may be used to increase separation on a gel (Griffiths *et al.* 1998).

Despite greater assurance of accuracy of sex assignment when compared to morphological approaches, errors are still possible with molecular techniques. To date, molecular sexing errors detected in birds have been primarily due to allelic dropout, where females are missexed as males (Arnold *et al.* 2003; Robertson & Gemmell 2006). A second possible source of error could be due to *Z*-chromosome polymorphisms. *Z*-chromosome polymorphisms have been reported with use of the P2/P8 primers and occur in at least 18 species of birds, encompassing 5 families and 3 orders (Dawson *et al.* 2001; Lee *et al.* 2002; Dawson 2005). Sexing errors could occur if *Z*-polymorphisms were the result of large insertions or deletions, but known *Z* indels are generally small (4 to 40 bp, Dawson *et al*. 2001; Lee *et al.* 2002). Nevertheless, *Z*-polymorphisms might be a concern if mutations generate different alleles, if those alleles interact together during the PCR process, and if interactions create heteroduplex molecules, which are double-stranded DNA molecules composed of two different alleles (Nagamine *et al.* 1989). Although never previously reported for birds, heteroduplex molecules could lead to errors in avian sexing by disrupting the normal gel banding patterns for males and females.

The Upland Sandpiper (*Bartramia longicauda*) is a sexually monomorphic shorebird that breeds in the grasslands of North America and winters in the pampas of South America (Houston & Bowen 2001). This species is socially monogamous; both sexes take part in incubation and males provide uniparental care during brood-rearing. During a six-year study of Upland Sandpiper population biology, we used the P2/P8 protocol to determine the sex of adults in our population. Here, we report the first evidence for PCR-based sexing errors in birds due to the formation of heteroduplex DNA molecules in polymorphic males.

Materials and Methods

Field methods

We sampled mated pairs of Upland Sandpipers at Konza Prairie Biological Station, Manhattan, Kansas from 2001-2006. Adult sandpipers were captured at night with the use of spotlights and a long handled net. We attached radio transmitters (PD-2, Holohil Systems Ltd., ON, Canada) to each sandpiper, and tracked each radio-marked bird until we located its nest (Mong & Sandercock 2007). We used mist nets to capture the mate of each radio-marked bird at the nest site. At first capture, we banded each sandpiper with a numbered metal band, a unique combination of colored plastic bands, and collected a 200 µl blood sample from the brachial wing vein. Blood was stored in Queen's Lysis Buffer in a refrigerator until DNA extractions were completed (Seutin *et al.* 1991).

Molecular sexing

DNA was extracted using the DNeasy Extraction Kit (Qiagen Inc., Valencia, CA) and used in polymerase chain reaction (PCR) with primers P2 and P8 to amplify introns from the *CHD-Z* and *CHD-W* genes, following the protocols of Griffiths *et al*. (1998). Each 20 μL reaction contained 4 μL of genomic DNA, 1.5 μM of each primer, and 1 Unit of *Taq* DNA Polymerase (Promega Corp., Madison, WI) in the manufacturer's buffer, including 1.75 mM $MgCl_2$ and 0.2 mM of each dNTP. PCR was performed in an Eppendorf Mastercycler ep gradient thermal cycler (Eppendorf, Westbury, NY). In the reaction profile for the P2/P8 primers, an initial denaturing step at 94°C for 2 min was followed by 45 cycles of 94°C for 30 s, 48°C for 45 s and 68°C for 45 s, then a final step at 68°C for 10 min. Agarose gels did not provide sufficient resolution to differentiate between the resulting *CHD-Z* and *W* fragments for Upland Sandpipers. Thus, we used a *CHD-Z* specific restriction enzyme, *HaeIII*, to selectively digest the *CHD-Z* fragments. PCR products were visualized on 3% agarose gels stained with GelStar (Lonza Group Ltd., Switzerland). If the P2/P8 primers gave questionable results, we used an independent set of primers to reevaluate our molecular-based sex determination (2550F/2718R, Fridolfsson & Ellegren 1999). For the second set of primers, each 10 μL reaction contained 2 μL of genomic DNA, 2 μM of each primer, and 0.5 Units of *Taq* DNA Polymerase in the manufacturer's buffer, including 1.75 mM $MgCl_2$ and 0.2 mM of each dNTP. The reaction profile used with the 2550F/2718 primers was 94°C for 2 min, then 10 cycles of 94°C for 30s, 54°C for 15 s and 72°C for 30s, followed by 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30s, and finally 72°C for 5 min.

Cloning and sequencing

We amplified *CHD-Z* and *CHD-W* alleles using the P2/P8 primer pair for six individuals: two males and two females whose sex determination matched using both primer sets, and two unknown individuals who were sexed differently by the two independent primer sets. PCR products were sequenced directly from the first two males. Both females and two unknown individuals produced gels with lanes containing different sized bands (i.e., heterozygotes). Thus, we isolated the PCR products by running them on a 3% agarose gel and by excising the resulting DNA fragments directly from the gel. The isolated fragments were purified using a QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA) and cloned using XL-1 Blue Supercompetent Cells and pBluescript II Phagemid Vectors (Stratagene, La Jolla, CA). We sequenced all DNA fragments with an ABI 3730 DNA Analyzer, using Applied Biosystems Big Dye chemistries for sequencing. Sequences were aligned using Program Sequencher (Gene Codes Corp., Ann Arbor, MI) and deposited in GenBank (Accession numbers: EU784665-EU784668).

Assessing formation of heteroduplex molecules

To test for the possible formation of heteroduplex DNA molecules, we amplified each *CHD-Z* allele variant from our clone library with a 20 µl P2/P8 PCR reaction. Using these PCR products, we created synthetic combinations of the *Z* alleles by mixing them in equal proportions of 5 µl each. Each combination was produced in duplicate for further experimentation. One tube of each combination was left at room temperature as a control, while the second tube was subjected to five PCR temperature cycles to recreate the original P2/P8 thermal kinetics within the experimental samples. We then visualized the resulting

products on a 3% high resolution 3:1 (low:high gelling temperature) agarose gel, formulated for separation of small DNA fragments (Sigma-Aldrich, St. Louis, MO).

Results

Gel electrophoresis of P2/P8 products

We determined the sex of 90 mated pairs of Upland Sandpipers using the P2/P8 primer set, and discovered 86 male-female pairs and four putative female-female pairs. We reanalyzed the eight birds in the four female-female pairs with an independent set of primers and found that the 2550F/2718R primers identified four of the putative females as males. Upon closer inspection of our PCR products at the P2/P8 locus, we found three distinct genotypes in our population of Upland Sandpipers, both before and after digestion with the restriction enzyme, *HaeIII* (Fig. 2-1). Without digestion with *HaeIII*, there was a thick single band for normal homozygous males (Z^0Z^0), two similar sized bands for females (Z^0W), and a different double band pattern with a greater size difference for an individual who had conflicting sex identification (Fig. 2-1). Digestion with *HaeIII* reduced the size of both fragments in the normal male and the individual of unknown sex, but the bands had similar relative positions on the agarose gel. The digested products for the female were smaller at *Z* but not *W,* as expected (Fig. 2-1). We initially inferred that the individuals of unknown sex were males heterozygous for the *CHD-Z* intron, with an approximately 70 base pair (bp) insertion in one of the *CHD*-Z alleles, according to mobility of fragments on our agarose gels when compared to 100 bp size standard (Promega Corporation, Madison, WI).

Cloning and sequencing P2/P8 products

The size of the PCR products based on sequence analysis did not agree with the size expected based on separation of the fragments on agarose gel. We cloned and sequenced one *W* allele (370 bp) from the two females we examined. From the normal males and unknown individuals, we cloned and sequenced three different *CHD-Z* alleles: Z^0, Z' and Z''. The Z^0 allele was 335 bp in length, and was found in the two females and the two males whose sex was determined correctly with both independent sets of primers. The sequences from the two unknown individuals contained both the Z' and Z'' alleles. Unexpectedly, neither fragment contained a large insertion. Instead, the Z' allele contained a 4 bp deletion with respect to Z^0, whereas the Z'' allele contained a different 5 bp deletion (Table 2-1).

Determination of heteroduplex formation

To explain the discrepancy between the results of our gel mobility and sequence analyses, we visualized the three *CHD-Z* alleles independently and in synthetic mixtures on an agarose gel. We exposed mixtures to one of two conditions: room temperature or five temperature cycles of denaturing and reannealing. When run independently and in synthetic mixtures at room temperature, all three *CHD-Z* alleles appeared to be approximately the same size (Fig. 2-2A). However, after mixing the alleles and then cycling synthetic mixtures through a PCR thermal regime, we obtained the same double band pattern that lead to sexing errors in $Z'Z''$ males (Fig. 2-2B).

Discussion

PCR-based sexing errors are a concern for researchers using molecular methods to sex young or monomorphic species of birds. The most common direction of error has been females misidentified as males, which has been reported in eight species of birds (Robertson & Gemmell 2006). Here, we present the first report of avian molecular sexing errors where males can be misidentified as females due to the formation of heteroduplex DNA molecules. This phenomenon was identified in Upland Sandpipers, but our results are potentially relevant to many species of birds due to the universality and widespread use of the P2/P8 primers.

The small differences in fragment size among the three *CHD-Z* alleles (4-5 bp) in Upland Sandpipers should not have caused sexing errors when compared to a *CHD-W* fragment that was 40 bp larger. However, we have characterized an interaction between different *Z* alleles which annealed to form heteroduplexes. When the hybrid molecules were run on agarose gels, their mobility on the gel appeared to be that of a much larger fragment, presumably because of looping of mismatched areas. Heteroduplex molecules have not been documented previously with primers for molecular sexing, but *Z*-chromosome polymorphisms have been reported in auklets and moorhens with the P2/P8 primers, and in Red Knots (*Calidris canutus*) with the 1237L/1272H primers (Baker & Piersma 1999; Dawson *et al.* 2001; Lee *et al.* 2002). Given the widespread occurence of *CHD-Z* polymorphisms in birds (20 species), *Z*-polymorphisms may cause undetected sexing errors in other species. For example, Gunnarson *et al*. (2006) found two unexplained errors of males missexed as females in a study of Black-tailed Godwits (*Limosa limosa*), which the authors attributed to possible masculinisation of genotypic females (Jacob & Mather 2004).

Our results suggest that low-frequency *Z*-chromosome polymorphisms could be an alternative explanation. Errors due to heteroduplex formation would not occur if individuals were analyzed through automated fragment analysis, because fragments are denatured before analysis, and small differences in fragment length (< 5 bp) are easily identified. Nevertheless, agarose gel analysis is the most widely used method for molecular sexing because the equipment is widely available and the cost is comparatively low.

The *Z*-chromosome polymorphisms reported here were in low frequency in Upland Sandpipers, but higher frequencies have been found in other species. In auklet species, 3% to 50% of individuals carried a polymorphic *CHD-Z* allele and 32% of Common Moorhen (*Gallinula chloropus*) males carried a *Z*-polymorphism (Dawson *et al.* 2001; Lee *et al.* 2002). To ensure continued reliability of molecular sexing methods, we make three recommendations for future studies. First, the low frequency polymorphisms we have reported will be difficult to detect if validation samples of known sex birds are small. We were only able to detect the sexing errors because we were working with mated pairs of sandpipers. To reduce uncertainty in molecular sexing protocols it will be desirable to have large validation samples of known-sex birds based on morphology, behavioral observations, laparoscopy, or necropsy. Second, we advocate the use of independent primer sets as a first step in optimizing a molecular sexing protocol for untested species of birds. Last, if independent primer sets yield conflicting results, comparison of fragment mobility with and without restriction enzymes, and on agarose gels versus a capillary sequencing system will be useful in testing for the presence of heteroduplex molecules.

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Figure 2-1. Comparison of sexing results with P2/P8 primers under standard PCR conditions on high resolution agarose. In the original protocol, *HaeIII* was used with P2/P8 primers to increase differences in *CHD-Z* and *CHD-W* alleles. The difference between misdiagnosed males and females was negligible in low resolution gels leading to misidentification of some males as females (not shown). Sample 1 is a normal (Z^0Z^0) male (M), sample 2 is a normal (Z^0W) female (F), and sample 3 is a male whose sex assignment differed between the two primer sets. The left three lanes include the PCR products before digestion with *HaeIII*, whereas the right three lanes include the same samples after digestion with *HaeIII*. M1: 100 bp size standard (Promega Corp., Madison, WI).

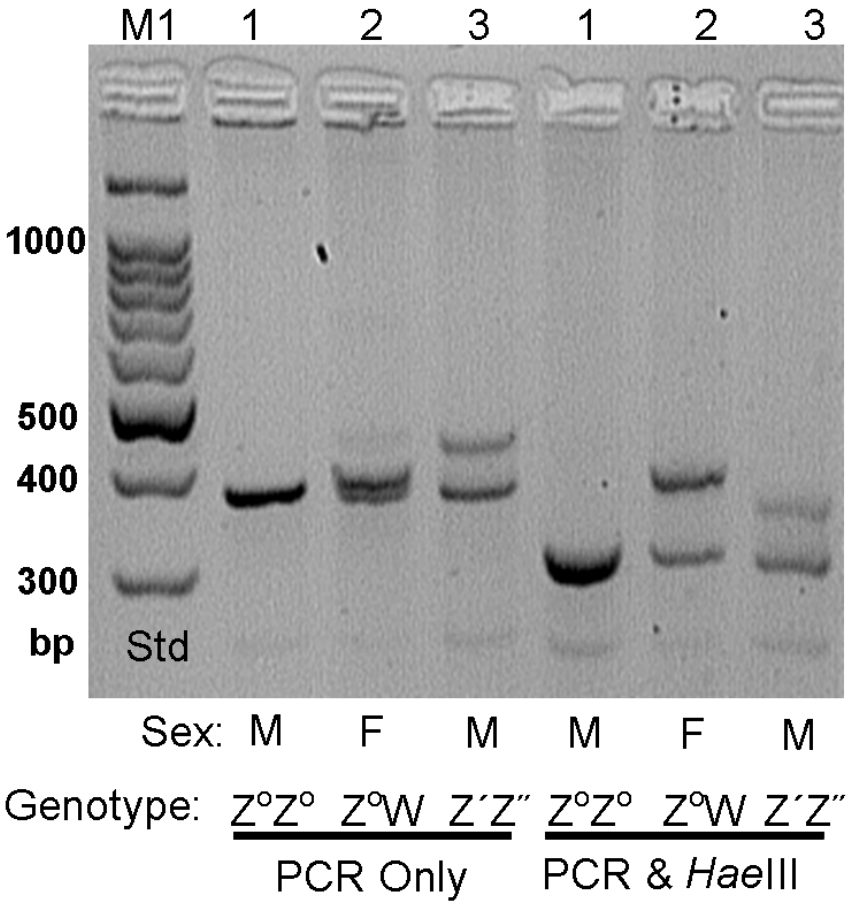

Figure 2-2. Formation of heteroduplex molecules in synthetic mixtures of three CHD-Z alleles. (a) PCR product of a Z′Z″ male (lane 1), isolates of the three CHD-Z alleles (lanes 2-4), and synthetic mixtures of the alleles after incubating them at room temperature (lanes 5-6); (b) PCR product of a Z′Z″ male (lane 1), and synthetic mixtures of the alleles after treating them with five cycles of denaturing and annealing (lanes 2-4). M1: 100 bp size standard (Promega Corp., Madison, WI).

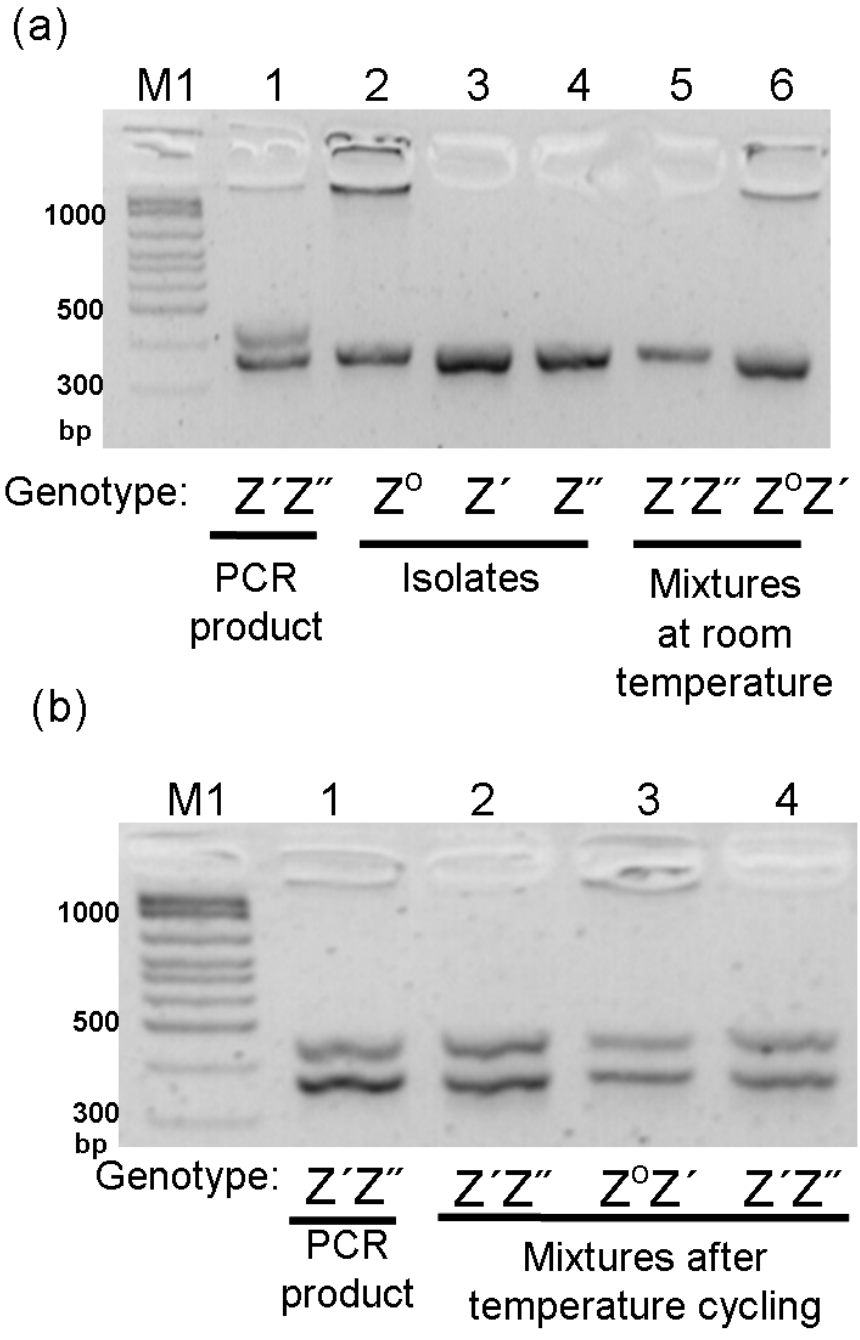

Table 2-1. Nucleotide sequence alignment of the three *CHD-Z* alleles. Nucleotide position is indicated relative to the 335 bp *CHD-Z*^{0} PCR product of the primers P2 and P8. Absent bases are represented by dashes. The restriction enzyme *HaeIII* cuts the *CHD-Z*^{0} fragment at restriction sites centered on positions 290 and 308.

Accession Number	*Total Fragment Length (bp)*	*Allele*	64	65	66	67	68	69	70	71	72	73	...	146	147	148	149	150	151	152	153	154	155	156
EU784665	335	*CHD-Z*^{0}	G	T	T	T	G	T	T	G	G	G	...	A	A	A	C	T	T	T	A	C	T	T
EU784666	331	*CHD-Z′*	G	T	T	T	G	T	T	G	G	G	...	A	A	A	-	-	-	-	-	C	T	T
EU784667	330	*CHD-Z″*	G	T	T	-	-	-	-	G	G	G	...	A	A	A	C	T	T	T	A	C	T	T

CHAPTER 3 - Genetic parentage and local population structure in the Upland Sandpiper

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Abstract

In birds, the offspring of females in socially monogamous species can be sired not only by their social partner (within-pair mating) but also by other males (extra-pair mating), resulting in broods of mixed paternity. The majority of studies on extra-pair paternity (EPP) have been conducted on songbirds, and the other lineages of birds have received less attention. We conducted a population study of the Upland Sandpiper (*Bartramia longicauda*), at Konza Prairie Biological Station in northeast Kansas from 2003-2007. Our objective was to determine the genetic mating system of this socially monogamous shorebird and to test four main genetic hypotheses proposed to explain variation in extra-pair mating behavior. We used six microsatellite markers to genotype birds and test for the existence of EPP in 58 family groups of sandpipers (n = 107 parents, n = 184 offspring). We found that 15% of chicks and 30% of broods contained extra-pair offspring. Only 2% of chicks and 2% of broods were attended by a female unrelated to the young. We found non-random settling of female birds, suggesting female settlement near kin or natal philopatry. Relatedness between mated pairs, morphology of the within-pair male, morphology of chicks, and offspring heterozygosity did not differ with respect to EPP. We conclude that none of the genetic hypotheses are explanations for the high level of extra-pair paternity in

this population of Upland Sandpipers. However, semi-colonial breeding behavior, along with the female natal philopatry could allow for high rates of EPP with minimal inbreeding consequences.

Introduction

The recent application of molecular genetic techniques to ecological studies has transformed our view of avian mating systems. In birds, monogamy is the most common mating system, where a male and a female form an exclusive pair bond that lasts for at least one breeding attempt. While it was once thought that the majority of birds were completely monogamous (Lack 1968), molecular tools have shown this assumption to be incorrect. Birds may form a social pair bond that persists for the breeding attempt, but extra-pair mating activities often occur. Ornithologists must now distinguish between genetic monogamy, where there is an exclusive mating relationship between a male and female, and social monogamy, where there is a pair-bond between a male and female but the mating relationship is not exclusive (Neudorf 2004). True genetic monogamy occurs in less than 25% of the socially monogamous species studied to date, and extra-pair paternity (EPP) occurs regularly in the remaining 75% of species (Griffith *et al.* 2002). EPP is defined as the proportion of fertilizations resulting from mating outside the bonds of the social mating system (Westneat *et al.* 1990). Another less common type of extra-pair parentage is quasi-parasitism (QP), or extra-pair maternity, in which a female mates with the same male and lays her eggs in the nest of an already mated pair of the same species. Finally, intraspecific nest parasitism, where a female lays eggs parasitically in nests of other broods, can lead to inclusion of young that are unrelated to either parent.

Two main demographic factors have been proposed to explain variation in rates of EPP. Increased *breeding synchrony* is often correlated with increased rates of EPP. This hypothesis proposes that birds which breed on the same schedule have greater opportunity to find extra-pair mates (Stutchbury & Morton 1995). Increased need for *paternal care* is often correlated with decreased rates of EPP. This hypothesis predicts that it is risky for females to engage in extra-pair mating because the risk of abandonment or reduced paternal care by males increases when males have less certainty of paternity. Thus, females should be less likely to seek extra-pair copulations when they must rely on their male partner for parental care (Griffith *et al.* 2002).

In addition to demographic predictors of EPP, a question remains as to the adaptive function of extra-pair mating. Males benefit by increasing their reproductive success at little cost, by fertilizing a female at the expense of another male who will provide the care. For females, however, the benefits are not as clear. Several hypotheses have been developed to explain why it may be adaptively advantageous for female birds to engage in extra-pair mating activities, and they fall into the main categories of direct ecological benefits, and indirect genetic benefits. The ecological hypotheses refer to immediate benefits, such as acquiring food from courtship, or acquiring extra help with brood rearing. However, there is little evidence to support these hypotheses of direct benefits, and the direct benefits do not usually apply to birds that produce precocial self-feeding young (Charmantier *et al.* 2004).

Four main indirect genetic hypotheses have received more support. First, the *genetic diversity* hypothesis predicts that females should seek extra-pair mates to increase the genetic diversity within their brood, and thus the fitness of their offspring in variable environmental conditions (Westneat *et al.* 1990). Second, the *good genes* hypothesis

predicts that females should seek extra-pair mates by assessing their genetic quality based on phenotypes, such as body size, color, or behavior. If there is enough variation in these traits, females should choose the male with the highest genetic quality (Petrie & Kempenaers 1998). Third, the *genetic compatibility* hypothesis predicts that females should seek extra-pair mates that complement their genotypes, thus increasing the heterozygosity of their offspring. This behavior should be especially important when females are mated with closely related males because it reduces the amount of inbreeding in the population (Amos *et al.* 2001). Finally, the *fertility insurance* hypothesis predicts that females should seek extra-pair mates simply to guard against a mate's infertility and guarantee that all of her eggs are fertilized (Wetton & Parkin 1991). Since females are not basing choices on male quality, there should be no differences in quality between extra-pair and within-pair offspring.

Studies of EPP have largely been focused on songbirds (61%, $n = 131$ species) but levels of EPP may be different in other Orders of birds (Griffith et al. 2002). Shorebirds (Order Charadriiformes: Suborder Charadrii) are a diverse group of birds that are found in a wide variety of habitats. Almost all species lay a clutch size of 3 or 4 eggs, and they produce precocial, usually self-feeding young (Sandercock 1997). In precocial species, one parent may be capable of incubating or raising the brood alone, which may leave the other parent with the option of deserting and possibly remating or producing another clutch (Szekely *et al.* 1996). The costs of caring for the young lead to a conflict between males and females over who should care for the offspring, and to what degree. As a result, shorebirds have evolved a remarkable array of social mating systems, which include examples of polygyny, polyandry, and monogamy, as well as lek-mating and double-clutching. In

polyandrous species there are instances of sex role reversal, where females are the larger, more colorful sex, and fight each other for access to males in the population (Szekely *et al.* 2006). Additionally, within monogamous species, parental care can be biparental or uniparental by either sex (Reynolds & Szekely 1997). Despite this immense variability in social mating systems, few studies have been done to examine the genetic mating systems in this diverse group (16 of 155 species).

We studied the population genetics and mating system in the Upland Sandpiper (*Bartramia longicauda*). Upland Sandpipers are a socially monogamous shorebird that breed in the grasslands of North America and winter in the pampas of South America. Unlike most shorebirds, the species is completely terrestrial and is rarely found along coastal areas. The species is listed as threatened or endangered by 13 states, and is a species of special concern in 5 additional states due to widespread declines in population numbers (Houston & Bowen 2001). The population declines are primarily due to habitat loss and fragmentation (Webb 1985; Dechant *et al.* 1999; Winter & Faaborg 1999). Mated pairs usually nest in loose colonies and raise one clutch of 4 eggs per season. Both sexes incubate, and males are responsible for parental care after hatching.

Our study objective was to determine the rate of EPP in our population of Upland Sandpipers. Because males contribute to parental care both during incubation and for brood rearing, and because of low levels of EPP found in other socially monogamous shorebirds, we predicted we would find low levels of EPP in our population. We also set out to determine the drivers of EPP in Upland Sandpipers, and to determine which genetic hypotheses best explained the patterns we found in the population. We examined the morphology, heterozygosity and pairwise relatedness among putative family members.

Finally, we examined how the nesting and mating patterns of individual birds contributed to the genetic structure of the population.

Methods

Field methods

We sampled mated pairs of Upland Sandpipers at Konza Prairie Biological Station, Manhattan, Kansas, in a 5-year period (2003-2007). Adult sandpipers were captured at night in early spring with the use of spotlights and a long handled net. We attached a radio transmitter (PD-2, Holohil Systems Ltd., ON, Canada) to each sandpiper, and tracked each radio-marked bird until we located its nest (Mong & Sandercock 2007). We then used mist nets to capture the mate of each radio-marked bird during incubation. At first capture, we banded each sandpiper with a numbered metal band, a unique combination of colored plastic bands, and collected a 200 µl blood sample from the brachial wing vein. We measured the total body mass to the nearest gram, and total head, tarsus, wing, and tail length of each bird to the nearest millimeter.

We estimated the hatch date of each nest by egg floatation methods (Liebezeit *et al.* 2007), and captured chicks at the nest on the day of hatch. We recorded the same morphological measures on chicks as adults, except for tail length. To increase our sample of successful nests, we removed three clutches in 2007 and replaced the eggs with clay dummy eggs, painted to resemble real eggs. Eggs were placed in an incubator (model D-2362-N Hova-Bator; G.Q.F. Mfg. Co., Savannah, GA) and monitored until hatching. Eggs were automatically turned throughout incubation. At hatching, we measured the chicks,

collected a blood sample, and returned the chicks to the original nest. In all cases, the attending parent accepted the brood. Blood (~20 µl) was collected from all chicks by clipping the toenail. All blood was stored in Queen's Lysis Buffer in the dark at 5 °C until DNA extractions were completed (Seutin *et al.* 1991).

Genetic Analysis

DNA was extracted using the DNeasy Extraction Kit (Qiagen Inc., Valencia, CA) from the blood of adults (n = 130) and chicks (n = 177), or from samples of brain tissue collected from unhatched embryos (n = 4). All individuals were sexed based on molecular markers with the primers P2/P8 or 2550/2718R (Griffiths *et al.* 1998; Fridolfsson & Ellegren 1999; Casey *et al.* 2008). Microsatellite markers were amplified by polymerase chain reaction (PCR) in an Eppendorf Mastercycler ep gradient thermal cycler (Eppendorf, Westbury, NY) and run on an ABI Prism 3730 Genetic Analyzer using fluorescently labeled primers (Schuelke 2000). Allele sizes were visualized using GeneMarker software version 1.6 (SoftGenetics, LLC, State College, PA). We genotyped each bird at six microsatellite loci developed for three other species of shorebirds: 4A11, 49F6 (Van Treuren *et al.* 1999), Calp2 (Wennerberg & Bensch 2001), Ruff1, Ruff6 and Ruff8 (Thuman *et al.*). Homozygous individuals were run twice to control for potential allelic dropout.

Parentage analysis

We suspected that a high rate of allelic dropout occurred at the Ruff8 locus, so we conducted a null allele analysis using Program Micro-Checker (Van Oosterhout *et al.* 2004*)*. The combined exclusion power of our six loci was calculated using program Cervus (Kalinowski *et al.* 2007) and was >0.996 with one parent known. Parentage was assessed at

each nest by comparing the genotypes of offspring and the presumed parents who attended the nest. Offspring were considered to be the result of extrapair matings when they mismatched at two or more loci with their putative father or mother.

Genetic similarity and heterozygosity

As a measure of genetic similarity between different individuals, pairwise relatedness estimates were calculated with Program Relatedness (Queller & Goodnight). Relatedness estimates depend on accurate knowledge of the background allele frequencies, so we excluded the Ruff8 locus because we suspected high allelic dropout rates.

We calculated standardized individual heterozygosity (hereafter, 'heterozygosity') for each individual as the proportion of heterozygous loci per average observed heterozygosity for all loci typed. By use of the standardized measure, we eliminate bias toward individuals that were genotyped at all loci compared to those that failed to amplify at one or more loci (Coltman *et al.* 1999).

Genetic structure among nesting birds

We analyzed the relationship between pairwise relatedness and distance among nests across all 5 years (2003-2007) for adult males and females using Program Spagedi (Hardy & Vekemans 2002). To increase our precision, we genotyped and included an additional 23 adults, which encompassed all of the birds we found nesting in 2006, regardless of whether the nest hatched. Individuals were grouped by year and all calculations were made within group. The mean distance to the nearest nest in our study was 347 m (± 25 m SE, range: 2 m – 2478 m) so we grouped all possible pairs of birds into distance classes of 500 m to account for the nearest neighbor, then into intervals of 1000 m, beginning with 1000 m.

Since sample sizes at larger distances decreased, we pooled all pairs of birds that nested at distances >3000 m. In this analysis we excluded all birds that renested in each year by using only their first nesting attempt. We used program SPAGEDI to compute average values of relatedness for each distance interval, jackknifing over loci to obtain standard errors for each relatedness estimate. We tested for a trend in relatedness vs. pairwise nesting distance, and we used permutation tests to determine the significance of the relatedness estimate in each distance class. The permutation procedure is analogous to performing a Mantel test between matrices of genetic and geographic distances (Hardy & Vekemans 2002).

Statistics

We examined three correlates of extra-pair paternity: timing of clutch initiation, morphology and degree of relatedness within mated pairs. We used logistic regression to calculate estimates of the relationship of mate relatedness and nest inititation date on probability of extra-pair fertilization occurrence. Regression analyses were performed in JMP 4.0.4. All further analyses were performed with procedures of SAS 8.0.2. We compared the morphological characteristics (body mass, head length, tarsus length, wing length, and tail length) of males attending nests with and without extra-pair young using t-tests (Proc ttest). We also used t-tests to compare mean relatedness of mated pairs and mean relatedness of siblings in nests and without extra-pair young. We completed the analyses with broods that were completely genotyped (i.e., all 4 chicks were genotyped), and for all broods combined (1-4 chicks genotyped). Paired t-tests were used to determine the significance of differences in heterozygosity and morphological characteristics between within-pair and extra-pair half-siblings (Proc Univariate).

Results

Microsatellites

The six microsatellite markers showed high levels of heterozygosity (range: 0.628-0.861), and allelic diversity ranged from 8-21 alleles per locus (Table 3-1). The Ruff8 locus found to contain null alleles, so we used half-genotypes for the parentage analysis (Van Treuren, 1998 #152). Analyses of the observed level of heterozygosity at each locus showed deviation from Hardy-Weinberg expectations at two of the loci, with Ruff1 showing heterozygote excess and Ruff6 showing heterozygote deficit. We verified the accuracy of the two loci that were out of HWE by re-visualizing all mismatches between known parent-offspring pairs (Hoffman & Amos 2005). Extra-pair parentage was considered after confirming that the offspring mismatched at two or more loci. The mean number of loci mismatching between extra-pair offspring and their excluded parents was 3.25 (± 0.21) of six loci tested. In every case where exclusion was based on two mismatches, at least one of the loci was in HWE. Additionally, a lack of mismatches between mothers and offspring leads us to conclude that despite the deviation from HWE in two of the loci, our parentage assignments are reliable (Marshall *et al.* 1998).

Social Mating System

We examined parentage in 57 family groups (108 adults and 181 chicks). The number of chicks analyzed constitutes 79% of the potential number of chicks produced in these family groups (n = 228 chicks, assuming all pairs produced 4 eggs). Missing young were due to partial clutch predation, failure to hatch and failure to catch chicks that had departed the nest. In total, we had 50 family groups for which both parents were genotyped,

one family group for which we only obtained the genotype of the female parent, and six family groups for which we only obtained the genotype of the male parent. Across all 5 years of the study, nearly all pairs formed socially monogamous pair bonds and jointly incubated the nest (n = 86). We found three cases where pairings diverged from the traditional male-female bond. In 2004, one nest was attended by three birds, but we were only able to obtain blood samples for one male and one female attending. The female did not match the offspring at 4 out of 6 loci and the third bird may have been the true genetic mother. Additionally, in each of the years 2006 and 2007 there was one nest attended by two females. In total, we sexed 89 mated pairs and only three groupings (3.4 %) deviated from social monogamy. Although we found a few instances of unusual breeding patterns, social monogamy was the predominant mating system in our study population.

Genetic mating system

Across all five years of the study, 30.4% (17/56) of broods contained at least one offspring sired by an extra-pair male, and 14.7% (26/177) of chicks were sired by an extra-pair male (Table 3-2). Frequency of extra-pair fertilizations did not vary by year for chicks ($Chi^2 = 3.53$, $P = 0.47$) or broods ($X^2 = 1.18$, $P = 0.88$). Only 2% (1/51) of broods were attended by an unrelated female, which constituted 2% (4/164) of chicks. The nest with the extra-pair female was the nest for which three birds were caught attending in 2004. Fourteen broods contained both within-pair and extra-pair offspring (i.e. mixed broods), which were used for comparisons of half-siblings. Sample sizes varied because complete information was not available for all nests.

Genetic similarity

Pairwise relatedness estimates for first order relatives (expected $r = 0.5$) identified through parentage assignment averaged 0.48 (± 0.01 SE, n = 155). Half-siblings (expected $r = 0.25$) as identified by parentage assignment averaged lower relatedness (mean $r = 0.31 \pm 0.03$, n = 14), and mated pairs (expected $r = 0$) were the least closely related group (mean $r = 0.002 \pm 0.03$, n = 50; Figure 3-1). Sex-specific estimates of the loss in certainty of parentage (an individual's assessment of its relatedness to offspring) can be determined by calculating the difference between the mean relatedness of parents to within-pair offspring and the mean relatedness of parents to all offspring. This difference was 12% greater for males than for females (Figure 3-1).

Morphological comparisons

Males with and without extra-pair young did not differ in any morphological measures (Table 3-3). Extra-pair chicks and within-pair chicks did not differ in any morphological measures at the time of hatching (Table 3-4).

Timing of nest initiation

Nest initiation date had no significant relationship with the presence of extra-pair offspring (logistic regression, $Chi^2 = 0.53$, $R^2 = 0.008$, $P = 0.46$).

Mated pair comparisons

We found no indication that females were choosing extra-pair mates based on relatedness. There was no relationship between the degree of relatedness of mated pairs and the presence of extra-pair offspring (logistic regression, $Chi^2 = 0.41$, $P = 0.52$). Furthermore, mean relatedness between mated pairs with complete 4-chick broods grouped

by presence of extra-pair young did not differ (t-test, t = -1.46, $P = 0.16$; Fig. 3-2). Additionally, when we included data from both complete and incomplete broods, mean relatedness between mated pairs did not differ (t = -0.87, $P = 0.39$; Fig. 3-2).

Offspring comparisons

As expected, mean relatedness differed between chicks in complete 4-chick broods as a function of extra-pair mating, with broods containing no extra-pair young being more highly related than broods with extra-pair young (t-test, t = 4.49, $P < 0.001$; Fig. 3-3). When we included both complete and incomplete broods, those without extra-pair young were again more related than those containing extra-pair young (t-test, t = 3.54, P $P < 0.001$; Fig. 3-3).

Extra-pair young had higher heterozygosity than within-pair young per brood, but the difference (0.07 ± 0.06, n = 14 broods) was not significant (paired t-test, t = 0.67, $P = 0.51$). Furthermore, within-pair offspring from broods that contained no extrapair young (0.98 ± 0.02) did not differ in heterozygosity from within-pair offspring from broods of mixed paternity (1.00 ± 0.06, t = -0.44, $P = 0.65$).

Genetic structure

When we tested for deviations from an expected random distribution of genotypes, we found evidence of non-random settling of the breeding females. Females nesting nearest to each other were significantly more related than expected by chance in class '500' ($P = 0.015$) and in class '1000' ($P = 0.040$). Females at an intermediate distance (class '2000') were less related than expected ($P = 0.049$; Fig. 3-4A). When we examined males, we found no significant deviations from a random distribution of genotypes (Fig. 3-4B).

Discussion

We report relatively high rates of extra-pair paternity in Upland Sandpipers breeding on Konza Prairie compared to rates found in other socially monogamous shorebird species (Table 3-5). The rate of EPP in Upland Sandpipers is intermediate to shorebirds with lekking or polyandrous mating systems where we would expect higher rates of multiple mating, which range from 0% of chicks and broods in the sequentially polyandrous Wilson's Phalarope (Delehanty *et al.* 1998) to 59% of broods in the lek-mating Ruff (Lank *et al.* 2002).

The rate of extra-pair female nest attendance we observed (2% of broods) is consistent with rates found in other studies of Upland Sandpipers. In south-central North Dakota, Bowen and Kruse (1993) found that 6/342 (1.8%) of nests contained more than the modal clutch of 4 eggs (range: 5-7), and hypothesized that the clutches could be the result of two laying females. We also observed one nest that contained 5 eggs in our population. Unusual female behaviors have also been reported in other species of shorebirds, where rates of quasi-parasitism range from 3.1% in the Kentish Plover (*Charadrius alexandrinus*) to 13.3% in the Common Sandpiper (Blomqvist *et al*. 2002). Interestingly, in none of these instances did quasi-parasitism increase clutch size.

Since the loss in certainty of parentage was greater for males than for females, we might expect that males would invest less in care of the offspring. But in Upland Sandpipers males invest equally during incubation, and much more than females after the brood has hatched (Houston & Bowen 2001). One possible explanation for this discrepancy is that males are more capable than females at providing parental care for the brood. In a study of Kentish Plovers (*Charadrius alexandrinus*), a shorebird species in which either parent may

desert the brood, Szekely (1996) found that broods attended by males had higher rates of survival than female-attended broods. Thus, it is possible that the increase in offspring survival may outweigh the costs of caring for extra-pair young for male Upland Sandpipers.

We attempted to distinguish between the four main genetic hypotheses explaining why females benefit from extra-pair mating behavior. It was difficult to test the *genetic diversity* hypothesis given the study system in which we worked. If the benefits of having higher genetic diversity within each brood do play a role in promoting EPP, the only way to detect the effect would be to determine the long-term fitness differences between broods with and without extra-pair offspring. While we detected no difference in offspring characteristics upon hatch (Table 4), measurements may have been recorded too early to detect any fitness differences due to genetic diversity. Offspring phenotypes are largely due to maternal effects, given that maternal condition determines egg size, which is the main predictor of chick growth and development during the early life stages (Rubolini *et al.* 2006). Since we were unable to track the survival of chicks in our population, we have no evidence to confirm or refute the *genetic diversity* hypothesis.

We found no evidence in support of the *good genes* hypothesis based on the traits we measured. However, the lack of morphological differences between males is not surprising due to the fact that Upland Sandpipers are socially monogamous and sexually monomorphic. The *good genes* hypothesis may not apply to the Upland Sandpiper breeding system, or females could be using other phenotypic traits than those we measured to make their choices. For example, ultraviolet reflectance has been confirmed as an important aspect of female mate choice in some passerines (Delhey et al. 2003, Johnson et al. 2003).

We also have no evidence to support the *genetic compatibility* hypothesis, which has been the most widely supported of the genetic hypotheses (Kempenaers *et al.* 1999; Blomqvist *et al.* 2002; Foerster *et al.* 2003). The main predictions of this hypothesis are that extra-pair offspring will be more heterozygous, and more closely related pairs will engage in higher rates of EPP. We determined that relatedness did not differ between mated pairs with and without extra-pair young, and heterozygosity did not differ between within-pair and extra-pair young. However, since we were unable to assign paternity to extra-pair fathers, the possibility remains that extra-pair males could have been less related to the females than the within-pair males (Griffith *et al.* 2002). Additionally, coefficients of relatedness based on neutral microsatellite markers may not reflect differences between males at important functional and regulatory genes, so females could be choosing males based on other genetic factors (Reed & Frankham 2001).

Perhaps the most likely explanation, given our results, is the *fertility insurance* hypothesis. In order to support this hypothesis, the distribution of extra-pair young must be random among broods, and EPP should not depend on male quality. We showed that there were minimal morphological differences between males with and without extra-pair young, but we did not determine the distribution of extra-pair young among broods. While we have no direct evidence to support this hypothesis, it cannot be ruled out based on any of our results.

In socially monogamous species it is generally thought that the direct benefits of EPP are minimal, so variation in mating behavior is considered to be a balance between the costs of the extra-pair activities to the adult, and the genetic benefits to the offspring (Lindstedt *et al.* 2007). Increased breeding density and increased breeding synchrony can reduce the

costs of extra-pair mating and facilitate higher frequencies of extra-pair copulations (Stutchbury & Morton 1995; Westneat & Sherman 1997). Reduced costs of finding extra-pair mates then make it beneficial for females to cluster in their nesting territories, and it is thought that these benefits can lead to colony formation as an adaptive advantage (Wagner 1993). The genetic structure we observed for female Upland Sandpipers, in combination with the semi-colonial nesting habits of the species, suggests that females may cluster together with relatives to form these colonial nesting areas. This clustering can explain the higher rates of EPP we observed in comparison to other socially monogamous shorebirds.

The local population structure we observed also suggests male-biased dispersal in Upland Sandpipers. Females are likely returning more closely to their site of birth, and males are dispersing further. While male-biased dispersal is less common than female-biased dispersal in birds, 22 species, including three other species of shorebird and three species of colonial breeders, exhibit a male-biased pattern (Clarke *et al.* 1997). Sex-biased natal dispersal has been suggested as an inbreeding avoidance mechanism (Waser & Jones 1983), and appears to be the mechanism at play in Upland Sandpipers.

In conclusion, we have reported the first estimates of EPP for Upland Sandpipers. The rate of EPP we discovered is relatively high in comparison to other socially monogamous shorebirds, and our sample size was larger than what has been used in many studies of EPP. While we were unable to fully support any of the genetic hypotheses explaining EPP, we noted some important differences in Upland Sandpipers in comparison to other shorebirds. Relatedness has been found to be a main driving factor for other shorebirds, but does not seem to be affecting EPP in Upland Sandpipers. This suggests there may be other factors influencing EPP. While our study was somewhat limited by the

extensive nesting area and ground nesting habits of the species, future studies could benefit from more intensive nest-searching and tracking of brood success after departure from the nest. Additional monitoring would allow the addition of more data, such as the identity of the extra-pair mates and the fitness of offspring after nest departure, which could provide valuable information to further identify the adaptive advantages of EPP in Upland Sandpipers.

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Figure 3-1. Average relatedness (± SE) between known first-order relatives (n = 105), known half-siblings (n = 14), and mated pairs (n = 50). Relatedness (r) is Queller and Goodnight's relatedness coefficient. Numbers above the x-axis indicate the number of pairwise comparisons.

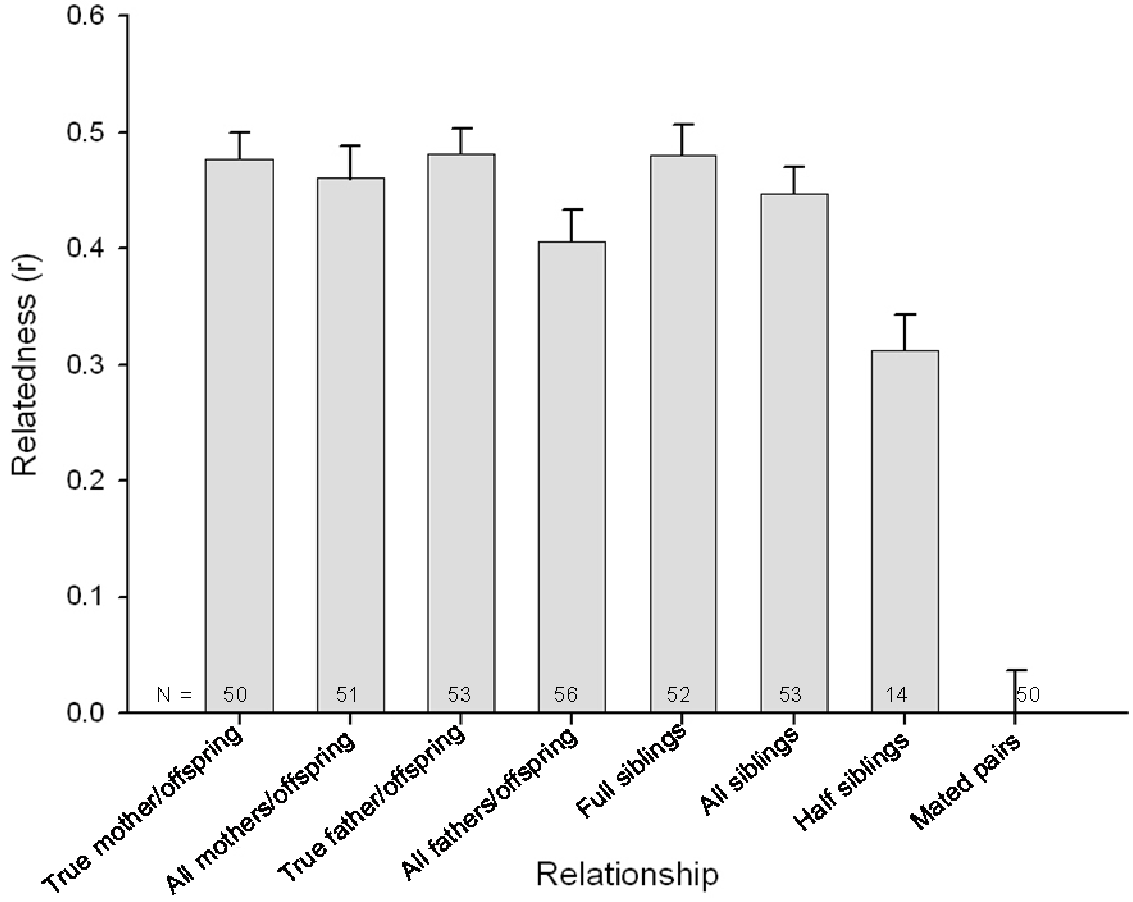

Figure 3-2. Genetic relatedness between mated pairs attending broods with and without extra-pair offspring. Analyses were run separately for complete 4-chick clutches and all clutches combined. Box plots show 25th and 75th percentiles (box), median (line within box), 10th and 90th percentiles (whiskers), and data points outside the 10th and 90th percentiles. Relatedness (r) is Queller and Goodnight's relatedness coefficient. Numbers above the x-axis indicate the number of mated pairs included.

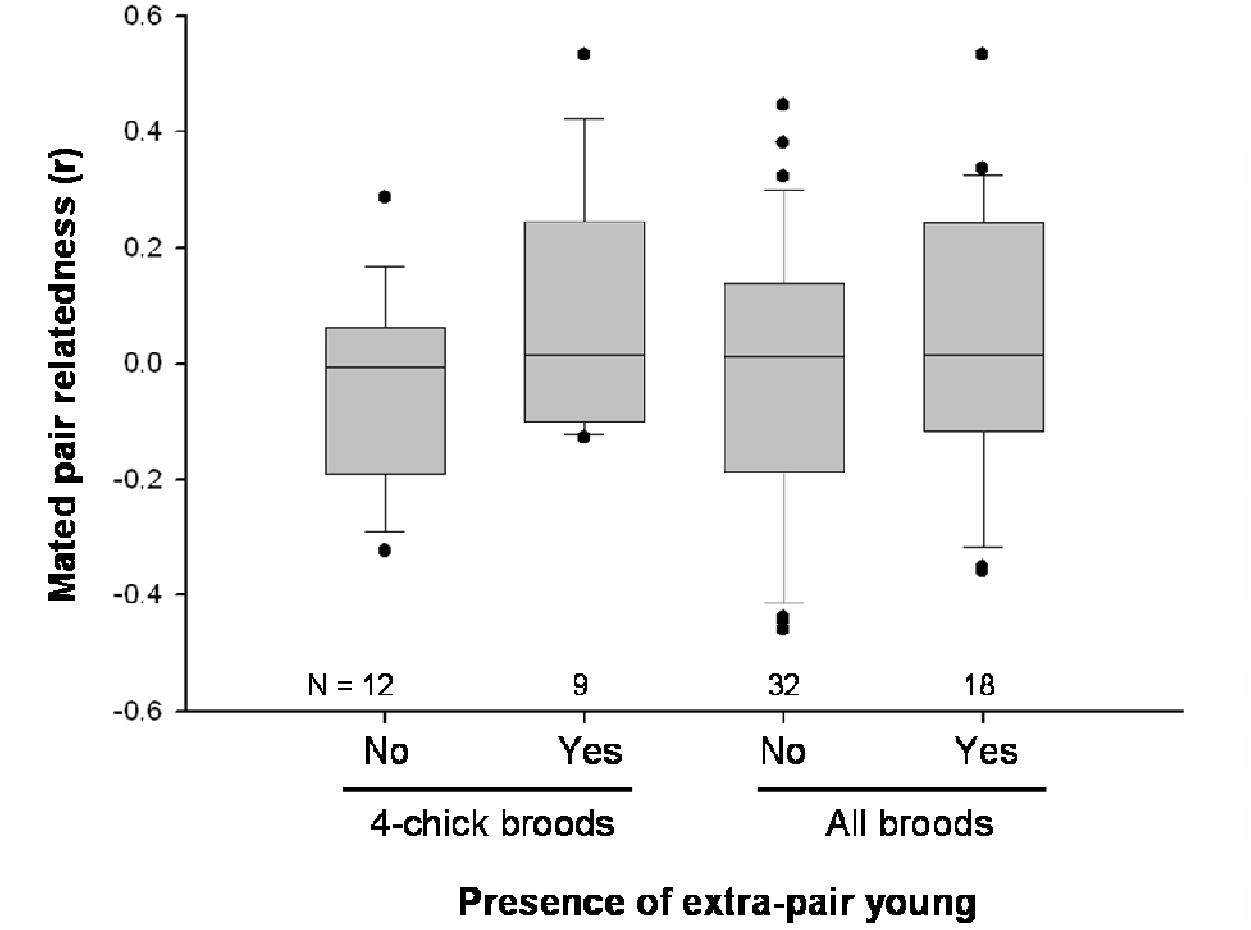

Figure 3-3. Genetic relatedness between all siblings in broods with and without extra-pair offspring. Analyses were run separately for complete 4-chick clutches and for all clutches combined. Box plots show 25th and 75th percentiles (box), median (line within box), 10th and 90th percentiles (whiskers), and data points outside the 10th and 90th percentiles. Relatedness (r) is Queller and Goodnight's relatedness coefficient. Numbers above the x-axis indicate the number of broods included.

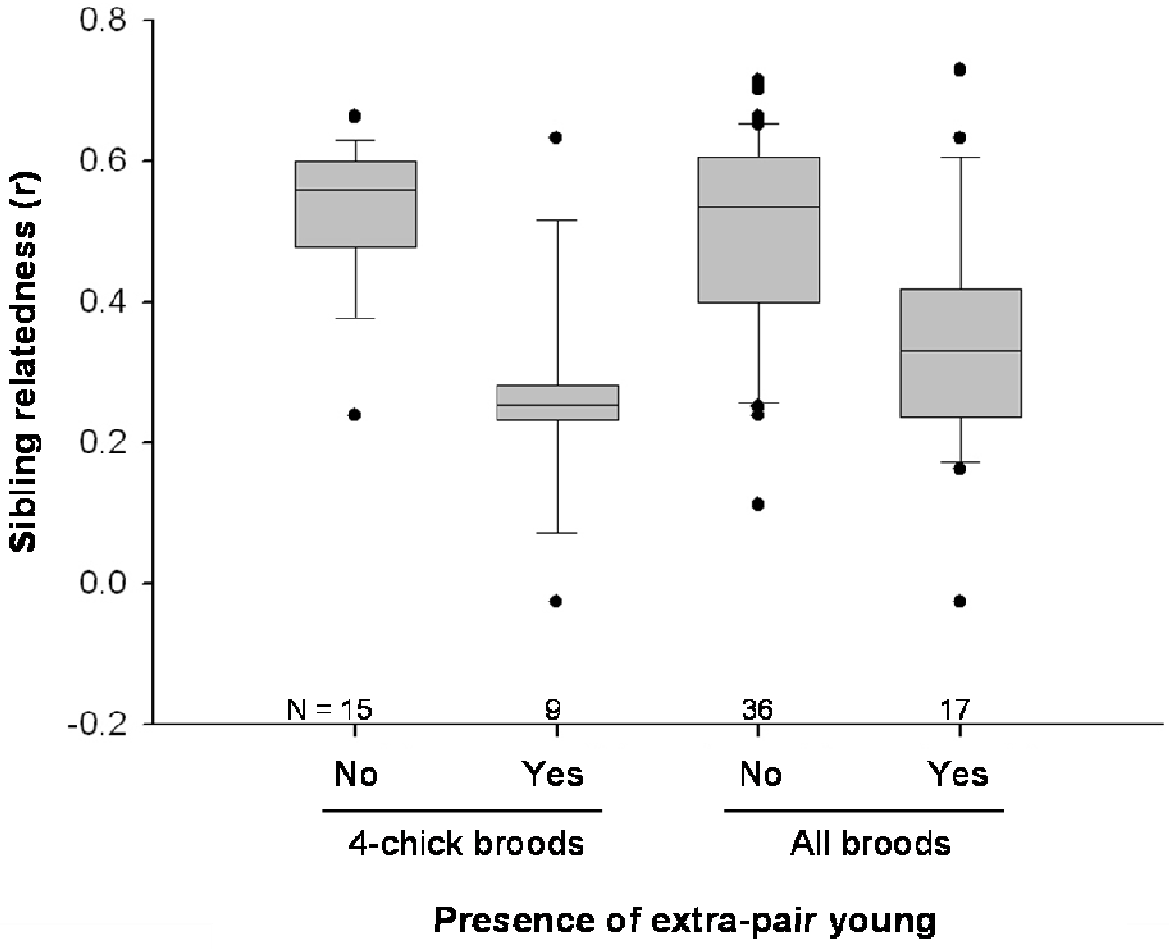

Figure 3-4. Average pairwise relatedness among Upland Sandpipers across all years in relation to the distance between their nests. The observed mean relatedness was compared to the expected mean relatedness for each distance class calculated from 10,000 random permutations of individual locations among individuals within each year of the study. Asterisks indicate observations that were significantly different from the expected value (P < 0.05). Error bars show standard errors obtained by jackknifing over loci. Numbers above the x-axis indicate the number of pairwise comparisons for each distance class.

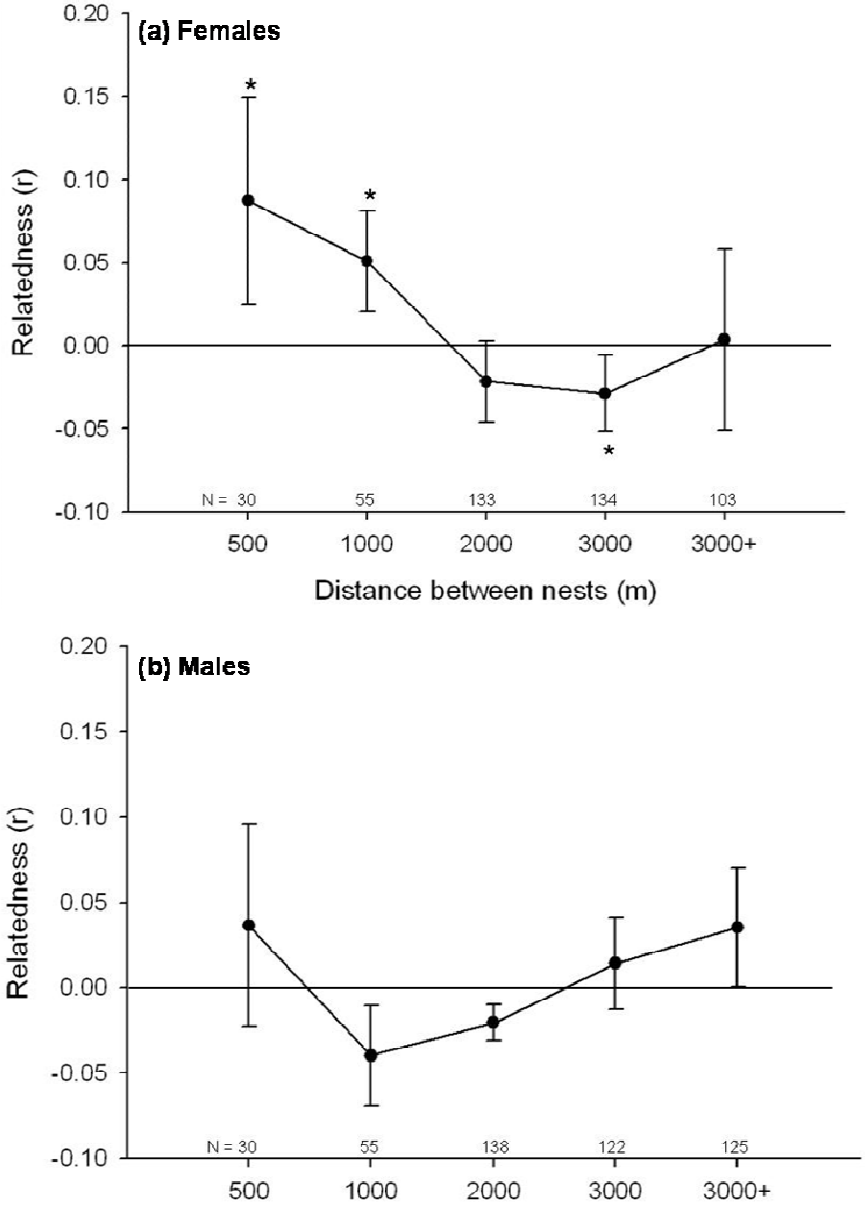

Table 3-1. Summary of six microsatellite markers optimized for use with Upland Sandpipers.

Species	*Locus*	H_E	H_O	*Allelic Diversity*	T_a (°C)	*Source*
Eurasian Oystercatcher *Haematopus ostralegus*	4A11	0.860	0.861	14	60	Treuren et al. 1999
Eurasian Oystercatcher *Haematopus ostralegus*	49F6	0.733	0.704	11	55	Treuren et al. 1999
Dunlin *Calidris alpina*	Calp2	0.665	0.639	8	53	Wennerberg & Bensch 2001
Ruff *Philomachus pugnax*	Ruff1	0.619	0.763*	8	52	Thuman et al. 2002
Ruff *Philomachus pugnax*	Ruff6	0.900	0.628*	21	52	Thuman et al. 2002
Ruff *Philomachus pugnax*	Ruff8	--	--	12	48	Thuman et al. 2002

Notes*: Species*, shorebird species for which the primers were originally developed; *Locus*, marker name; H_E, expected heterozygosity; H_O, observed heterozygosity; *Allelic Diversity*, number of alleles found at each locus; * Significant departure from Hardy-Weinberg equilibrium; -- due to high levels of allelic dropout we determined half-genotypes for most individuals at the Ruff8 locus, and were unable to calculate estimates of heterozygosity; T_a, optimum annealing temperature.

Table 3-2. Annual variation in rates of extrapair paternity in Upland Sandpipers at Konza Prairie Biological Station, 2003-2007.

Year	*Chicks*		*Broods*	
2003	9/48	18.8%	6/17	18.8%
2004	5/36	13.9%	3/11	13.9%
2005	1/26	3.8%	1/7	3.8%
2006	7/38	18.4%	4/12	18.4%
2007	4/29	13.8%	3/9	13.8%
Total	**26/177**	**14.7%**	**17/56**	**14.7%**

Table 3-3. Comparison of morphometrics between males attending nests with or without extra-pair young (EPY). Significance values were Bonferroni corrected ($\alpha = 0.008$).

Characteristic	Mean (SE) of cuckolded males (n=17)	Mean (SE) of non-cuckolded males (n=39)	Comparison of cuckolded and non-cuckolded males	
			t-value	*P*-value
Body mass (g)	139.35 (2.51)	139.97 (1.34)	-0.24	0.812
Head length (mm)	62.44 (0.53)	62.38 (0.29)	0.10	0.922
Tarsus length (mm)	50.42 (0.39)	50.76 (0.23)	-0.79	0.434
Wing length (mm)	168.18 (0.92)	166.90 (0.62)	1.15	0.256
Tail length (mm)	90.29 (0.68)	87.84 (0.59)	2.43	0.018
Relatedness to female (R)	0.0322 (0.06)	-0.0141 (0.04)	-0.63	0.532

Table 3-4. Comparison of morphometrics between extrapair young (EPY) and within pair young (WPY) for broods of mixed paternity (n=13). The mean difference was calculated as the average difference per brood in each characteristic.

Offspring Characteristic	Mean (SE) of EPY(n=13)	Mean (SE) of WPY (n=13)	Mean difference EPY-WPY	Comparison of EPY and WPY	
				t-value	*P*-value
Body mass (g)	17.50 (0.36)	18.25 (0.48)	-0.45 (0.29)	-1.53	0.15
Head length (mm)	33.54 (0.28)	33.45 (0.22)	0.22 (0.28)	0.82	0.43
Tarsus length (mm)	27.79 (0.34)	28.31 (0.28)	-0.27 (0.34)	-0.78	0.45
Wing length (mm)	16.47 (0.42)	16.20 (0.56)	0.29 (0.31)	0.95	0.36

Table 3-5. A summary of extra-pair paternity rates in relation to mating system in wild populations of shorebirds.

Mating system and shorebird species	% Extra-pair paternity		Parental Care		Source
	Young (n)	Broods (n)	Eggs	Young	
Monogamous					
Upland Sandpiper *Bartramia longicauda*	14.7 (177)	30.4 (56)	MF	M	This study
Purple Sandpiper *Calidris maritama*	1.2 (82)	3.7 (27)	MF	M	Pierce & Lijfield 1998
Common Sandpiper *Actitis hypoleucos*	15.7 (83)	18.5 (27)	MF	M(F)	Mee et al. 2004
Western Sandpiper *Calidris mauri*	5 (98)	8 (40)	MF	M(F)	Blomqvist et al. 2002b
Kentish Plover *Charadrius alexandrinus*	0.6 (170)	1.5 (65)	MF	M(F)	Blomqvist et al. 2002a
Ringed Plover *Charadrius hiaticula*	0 (57)	0 (21)	MF	MF	Wallander et al. 2001
Semipalmated Plover *Charadrius semipalmatus*	4.7 (85)	4.0 (24)	MF	MF	Zharikov and Nol 2000
Eurasian Oystercatcher *Haematopus ostralagus*	1.5 (65)	3.8 (26)	MF	MF	Heg et al. 1993
Promiscuous/Lekking					
Buff-breasted Sandpiper *Tryngites subruficollis*	---	40.4 (47)	F	F	Lanctot et al. 1997
Ruff	---	59.0 (34)	F	F	Lank et al. 2002
Philomachus pugnax	---	44.1 (34)			Thuman & Griffith 2005
Polyandrous – Sequential					
Eurasian Dotterel *Charadrius morinellus*	4.5 (44)	9.1 (22)	M	M	Owens et al. 1995
Red-necked Phalarope *Phalaropus lobatus*	1.7 (226)	6.0 (63)	M	M	Schamel et al. 2004
Red Phalarope *Phalaropus fulicaria*	8.6 (70) 4.3 (70)	33.3 (18)	M	M	Dale et al. 1999
Wilson's Phalarope *Phalaropus wilsonia*	0.0 (43)	0.0 (15)	M	M	Delehanty et al. 1998
Polyandrous – Simultaneous					
Spotted Sandpiper *Actitis macularia*	10.8 (111)	20.6 (34)	M	M	Oring et al. 1992
Comb-crested Jacana *Irediparra gallinacea*	2.8 (36)	10.0 (10)	M	M	Haig et al. 2003
Wattled Jacana *Jacana jacana*	7.5 (235)	17.9 (74)	M	M	Emlen et al. 1998

M = male only, F = female only, MF = biparental, M(F) = biparental but predominantly male.

Chapter 4 - Conclusions

The discovery that it is common for birds to mate with individuals besides their own social partner, resulting in extra-pair paternity (EPP), has been declared as one of the most important developments in the field of avian mating systems over the last thirty years. However, we still have only a rudimentary understanding of the function of these extra-pair mating behaviors or the reason for the considerable variation in the frequency of EPP both between and within different species. One of the leading factors proposed to explain the adaptive advantage of EPP is the genetic benefits for offspring. Using a population of Upland Sandpipers in northeast Kansas, our objective was to determine the first rates of EPP for the species, and to determine whether the hypotheses of genetic benefits explained the observed mating behaviors. In the process of carrying out our study, we discovered a potential source of error in avian molecular sexing analyses. We emphasize that it is critical that researchers designing molecular sexing protocols understand the potential sources of error and take the proper precautions to avoid sexing errors.

Contrary to our predictions, we found a relatively high rate of extra-pair paternity among Upland Sandpipers in this study, compared to other socially monogamous shorebirds. Furthermore, unlike many species of birds, we observed no relationship between EPP and the relatedness of mated pairs, the morphology of males, the morphology of offspring, or the heterozygosity of offspring in Upland Sandpipers. Mated pairs of sandpipers exhibited relatedness values close to zero, suggesting a fully outbred population. None of the genetic hypotheses could fully explain our observations. However, we did find fine-scale genetic structure among the female adults nesting on Konza Prairie, suggesting either female natal

philopatry, or female preferential settlement near kin. Taken in combination with the semi-colonial nesting habits of the species, this pattern suggests the colonies may be serving as both a means for females to gain more extra-pair copulations, and a means of inbreeding avoidance.

Overall, this study provides valuable information on the mating behaviors for the Upland Sandpiper, and the insights gained shed further light on the adaptive significance and importance of EPP in avian mating systems.