CONSPECIFIC BROOD PARASITISM IN RUDDY DUCKS

(*OXYURA JAMAICENSIS*)

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of LETITIA MARIE REICHART find it satisfactory and recommend that it be accepted.

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(*OXYURA JAMAICENSIS*)

Abstract

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Conspecific brood parasitism (CBP) is a reproductive strategy in which females lay eggs in the nests of other conspecific females. This behavior occurs in many species of birds, fishes, amphibians, and insects. CBP is intriguing because females laying eggs parasitically do not incur the costs associated with parental care; instead hosts (recipients of parasitic eggs) incur the costs of raising these parasitic offspring. The factors that influence females to lay eggs parasitically are unclear, and few studies have examined the role of maternal effects in CBP (parasitic eggs may contain substances increasing offspring survival). Here I investigate CBP in ruddy ducks (*Oxyura jamaicensis*), an over-water nesting species, where females lay large expensive eggs to produce highly precocial offspring (requiring minimal parental care).

First I identify parasitic offspring and the females that produce them using a combination of two molecular techniques – egg albumin protein fingerprinting (a maternal marker) and a large number of nuclear microsatellite loci. CBP was common in ruddy ducks (64% of nests contain parasitic offspring) and females used a mixed reproductive strategy (laying eggs parasitically in addition to nesting).

Next, I investigate whether kin selection might play a role in the evolution of CBP in ruddy ducks, given hosts and parasites may be genetic relatives (via high female natal philopatry). I use molecular markers to estimate relatedness among all females and then specifically between hosts and parasites. I found that female ruddy ducks showed no relatedness structure (neighbors were unrelated). Relatedness among host-parasite pairs was relatively low and not significantly different than background levels of relatedness in the population, thus kin selection is not a likely explanation for the evolution of CBP in ruddy ducks.

Third, I investigate possible parasite adaptations (via maternal effects), realized through egg characteristics. Steroid hormone content and egg size between parasitic and non-parasitic eggs did not differ; however, parasitic eggs were more likely to be male.

Finally, I discuss broad conclusions for the patterns of CBP in ruddy ducks with respect to other systems. In addition I suggest possible questions for further investigation of CBP as reproductive strategy in populations.

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Dedication

In loving memory of my grandfather, Sidney Claude Hawkins, thank you for always sharing your endless wonder of nature's grandeur.

GENERAL INTRODUCTION

Since the initial work of ethologists, such as Karl von Frisch, Konrad Lorenz and Niko Tinbergen, understanding the factors influencing animal social behavior has been the focus of behavioral ecologists for several decades. Specifically, the study of animal reproductive behavior has led to discoveries of various cryptic behaviors within species (e.g., extra-pair mating; existence of alternative reproductive phenotypes including sneaker males, and female mimics), where individuals use multiple methods to obtain reproductive fitness. With the advent of molecular genetic techniques, researchers have investigated kinship and parent-offspring relationships, leading to a better understanding of both animal mating systems and social behavior. Most previous studies have focused on timing and location of breeding, parental care, mate choice and more recently, extra-pair mating and its potential benefits.

For my dissertation research, I focus on a relatively less studied aspect of female reproductive behavior, conspecific brood parasitism (CBP), a reproductive strategy in which females lay eggs in the nests of other conspecific females. This behavior occurs in over 236 species of birds, many fishes, amphibians, and various species of insects. In particular CBP is intriguing because females laying eggs parasitically do not incur the costs associated with parental care, instead hosts (recipients of parasitic eggs) incur the costs of raising these parasitic offspring.

Although CBP has been studied in various species of birds the factors that influence females to lay eggs parasitically are unclear. In addition, few studies have examined the possible role of maternal effects in conspecific brood parasitism, where parasitic females may deposit substances in eggs (e.g., steroid hormones, nutrients) possibly increasing the probability of

survival for her parasitic offspring. Here I investigate CBP in ruddy ducks (*Oxyura jamaicensis*), an emergent nesting species, where large expensive eggs produce highly precocial offspring, requiring minimal parental care after hatch. For this research, I integrated animal behavior, multiple molecular genetic techniques and physiology. In chapter one, I identify parasitic offspring and also the females that produce them in a wild population of ruddy ducks using molecular markers. I used a combination of two molecular techniques – egg albumin protein fingerprinting (a maternal marker) and a large number of nuclear microsatellite loci. Next, hosts and parasites may be genetic relatives, given waterfowl exhibit high female natal philopatry. Therefore, in chapter two I investigate whether kin selection might play a role in the evolution of CBP in ruddy ducks. I used molecular markers to estimate relatedness among females in the population and then specifically between hosts and parasites. In chapter three I investigated possible parasite adaptations (via maternal effects), realized through egg characteristics. Specifically I tested for differences in steroid hormones, egg size, and offspring sex between parasitic and non-parasitic eggs. Then, I conclude with a broad discussion of the patterns of CBP found in ruddy ducks with respect to other study systems and suggest possible questions for further investigation to clarify the role of CBP as female reproductive strategy in populations.

In addition the research questions addressed, data collection in the field, laboratory work, data analysis, and interpretations are entirely my own, with a few exceptions. For all three chapters, Mike Webster provided much discussion and critical feedback for all hypotheses tested. Specifically in chapter one, Mike Webster provided advice, to help me develop criteria used to identify parasitic offspring and the females who produced them using microsatellite data. John Eadie also generously contributed numerous discussions for all three chapters, providing insight

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CHAPTER ONE

MOLECULAR IDENTIFICATION OF BROOD PARASITIC FEMALE REVEALS A MIXED REPRODUCTIVE STRATEGY IN RUDDY DUCKS

INTRODUCTION

Conspecific brood parasitism (CBP) is a reproductive strategy in which females lay eggs in the nests of other conspecific females. This behavior occurs in over 236 species of birds (Yom-Tov 2001), many fishes and amphibians (Harris *et al.* 1995, Wisenden 1999), and various species of insects (Brockman 1993). Brood parasitism is intriguing because females who lay parasitically do not provide parental care to their parasitic offspring; instead, host females incur the parental care costs of raising these parasitic offspring. However, although CBP is likely an adaptive behavior that enhances female reproductive success, frequency of CBP varies both within and across species (Sayler 1992), and the factors that favor parasitism remain unclear. Several hypotheses have been proposed to explain why females lay eggs parasitically.

First, parasitism may be a specialist strategy, such that parasitic females only lay eggs parasitically and do not have their own nests. Under this hypothesis parasitic females would depend entirely on non-parasitic nesting females for incubation and care of their offspring, similar to females of obligate brood parasitic species (i.e., species in which females only lay eggs parasitically in the nest of another species). Specialist parasitic females could arise via a genetic polymorphism (as a part of a mixed evolutionary stable strategy), analogous to alternative male reproductive strategies (e.g., in Ruff, *Philomachus pugnax*, Lank *et al.* 1995), and possibly maintained in the population by frequency-dependent selection as part of a mixed evolutionary

stable strategy (ESS, Maynard Smith 1982). However, to date there is no evidence from any species that some females only specialize on conspecific parasitism (Lyon & Eadie 2008).

Second, brood parasitism may be a conditional strategy, such that any female is capable of laying parasitic eggs in response to extrinsic or intrinsic factors that limit independent breeding. One extrinsic factor likely to lead a female to lay eggs parasitically, rather than nesting independently, is nest site limitation, which may be particularly important in cavity nesting species where suitable cavities are often limiting (e.g., goldeneye, Andersson & Eriksson1982; Eadie 1989, 1991; wood duck, Semel & Sherman 2001). Similarly, females who lose nests (e.g., via predation; Feare 1991; Jackson 1993) may respond by laying eggs parasitically instead of renesting or foregoing reproduction (Haramis *et al.* 1983). CBP might also be a conditional strategy arising from intrinsic factors. For example, females who are inexperienced (Sorenson 1991) or in poor physiological condition (Sayler 1992) may lay eggs parasitically because they do not have the resources to nest on their own. Some studies have found floater females (females without nests) laying eggs parasitically in a single season, but given an opportunity those females nest either later in the season or during the following breeding season (Forslund & Larsson 1995; Sandell & Diemer 1999). In more general terms, the conditional strategy hypothesis suggests that females nest independently under some conditions but switch to brood parasitism, and do not nest independently, under other conditions.

Finally, brood parasitism could arise as a mixed reproductive strategy, where individual females have their own nests and also lay eggs parasitically. Under this hypothesis, females do not lay parasitically due to extrinsic or intrinsic factors that limit their own ability to nest independently, but rather do so whenever the opportunity arises. A mixed reproductive strategy may allow individuals to increase overall fitness by laying additional eggs in another female's

nest (Trivers 1972) or when parasitic eggs yield higher fitness than additional eggs in the female's own nest (Lyon 1998). The opportunity to lay parasitically could be affected by a number of factors, including nest density (Brown 1984; Eadie & Fryxell 1992; Sayler 1992; Møller 1998) and/or nesting synchrony among conspecific females (Lyon 1993a, b; McRae & Burke 1996).

To distinguish among these hypotheses it is necessary to identify both parasitic offspring and the females who produced them. Previous studies of CBP have identified parasitic offspring using a multitude of non-genetic techniques, such as differences in egg shape, size, and color or discrepancies in egg laying intervals (e.g., Yom-Tov 1980; Pinxten *et al*. 1991; Jackson 1992; Lyon 1993a,b; Lahti & Lahti 2002). However, these indirect, non-genetic methods rely on a number of assumptions and may not accurately quantify parasitic behaviors in most systems (Andersson & Åhlund 2001; Grønstøl *et al*. 2002). Accordingly, many studies have incorporated the use of molecular methods to identify parasitic young/eggs by comparing the genotypes of young in a brood to that of the attending female (reviewed by Arnold & Owens 2002). These studies have successfully used a number of genetic markers, including microsatellites (Heckel & Von Helversen 2003), yolk proteins *(*Cariello *et al.* 2002), AFLPs (Questiau *et al.* 1999), and egg albumen protein fingerprinting (Andersson & Ahlund 2000; 2001), to identify parasitic young/eggs.

Although a number of methods have been used to identify brood parasitic offspring, it has proven much more challenging to identify the females who produced them. Some previous studies have identified parasitic females by using intensive behavioral observations of marked individuals at nests (Eadie 1989; Sorenson 1991, 1993), or by discriminating between individuals using variation in egg shell coloration (Jackson 1992; Lyon 1993a,b). Unfortunately, for many

species detailed behavioral observations are very difficult to obtain and egg shell patterns are not sufficiently variable. Molecular techniques offer an alternative approach that should potentially be applicable to a wide variety of species, and indeed have been used to identify individual parasitic females in studies of obligate brood parasites (Martinez *et al*. 1998; Langmore *et al.* 2007). However, molecular identification of parasitic females can be difficult because typically neither parent is known, and thus a number of females in the population could possess alleles compatible with a parasitic egg. This problem is particularly acute if markers show low levels of polymorphism, if a large number of females are candidate parasites, and/or if some females in the population are closely related to each other (Richardson *et al.* 2001). Some studies have used molecular markers to identify parasites when the potential parasites could be limited to a small group of females, for example through intensive behavioral observations at nests (e.g. Andersson & Åhlund 2001; Nielsen *et al.* 2006a,b; Andersson & Waldeck 2007; Waldeck *et al.* 2008). However, in many study systems intensive behavioral observations at nests will not be possible and any female in the population could be a potential parasite. As a consequence, the ability to examine individual female reproductive strategies has been limited, and a somewhat different approach is needed.

Here we report the first study to identify parasitic offspring and also the females that produce them in a wild population of birds using molecular markers alone. We used a combination of two molecular techniques – egg albumen protein fingerprinting (a maternal marker) and a large number of nuclear microsatellite loci – to investigate CBP in the ruddy duck (*Oxyura jamaicensis*). Ruddy ducks are an ideal species to test these hypotheses because of their propensity to participate in conspecific brood parasitism: previous studies have estimated that 8 - 38% of clutches were parasitized by conspecifics (Joyner, 1983; Siegfried, 1976a; Brua, pers.

comm.). In addition, ruddy duck eggs appear to be unusually costly to produce (see below), but the offspring are highly precocial and able to forage on their own immediately after hatching (Sommerville 1985). Thus, for this species the costs of producing parasitic eggs are high but the costs (to the host) of being parasitized may be low. Finally, although the mating system of ruddy ducks is thought to be highly polygynous (Oring and Sayler 1992), this has not been verified empirically. Because our molecular approaches have allowed us to determine the females who produced parasitic eggs, we are able to examine the reproductive strategies of female ruddy ducks, including the mating patterns of individual females, strategies of conspecific brood parasitism, and the factors that might influence whether a female produces parasitic eggs.

MATERIALS AND METHODS

Study Species

Ruddy ducks are members of the stiff-tail subfamily (Oxyurinae). Little is known about social structures of stiff-tails, but most species are thought to have polygynous mating systems (Oring & Sayler 1992) and engage in brood parasitism. In contrast to most waterfowl species (Oring & Sayler 1992), ruddy duck males and females do not associate with each other until arrival on the breeding grounds (Gray 1980).

Egg-laying appears to be more costly for female ruddy ducks than for most other waterfowl: female ruddy ducks typically lay a clutch of 6 – 8 eggs (Joyner 1975), with each egg weighing approximately 14% of a female's body weight (Gray 1980; Alisauskas & Ankney 1992; 1994). Maximum daily cost (584kJ) of egg production is 280% of basal metabolic rate (Alisauskas & Ankney 1994b). Females incubate the eggs for approximately 24 days (Bellrose 1980; Brua 1998). Resource requirements during egg laying and incubation are high, with

females consuming up to 6,000 midge larvae (*Chironomidae*) per hour during this period (Gray 1980; Carbonell 1983; Tome 1987). Ruddy ducks are single brooded and few females renest after nest loss, likely due to high nutrient demand of egg formation and lateness of nest initiation (Brua 2001).

Ruddy duck offspring are highly precocial and able to forage on their own immediately after hatching (Sommerville 1985). Adult females stay with a brood from 28 to 42 days after hatching to provide parental care, mainly in the form of vigilance for predators (Joyner 1975; Gray 1980). Females do not feed their offspring and some brood amalgamation behavior has also been noted (Joyner 1975). Offspring survival is high (Bellrose 1980), and ducklings from larger eggs have a higher probability of survival (Pelyo & Clark 2002; 2003).

Field data and sample collection

This research was carried out during the ruddy duck breeding season (mid-May to early August) each year from 2003-2005 in the prairie pothole region of Minnedosa, Manitoba, Canada (50º10'N, 99 º47'W). This area is characterized by numerous permanent and ephemeral wetlands ranging in size from 0.1 to >4.0 ha (Pelayo & Clark 2003). Wetland margins contain various types of emergent vegetation including cattail (*Typha* spp.), hardstem bulrush (*Scirpus acutus*), cane (*Phragmites communis*), and whitetop rivergrass (*Scholochloa festucacea*). Nesting habitat for ruddy ducks is abundant in this region because females build nests overwater in the emergent vegetation.

We trapped adult ruddy ducks prior to nesting using floating mist nets (technique described in Breault & Cheng 1989). Once nesting began we also trapped adult females during late incubation (eggs approximately 18-20 d) using drop door nest traps (Weller 1957). At the

time of capture adults were weighed to the nearest gram using a Pesola spring scale and standard morphological measures were taken (e.g. tarsus length, culmen length, bill width, skull, wing length). We marked females using a temporary, modified nasal disc (Pelayo & Clark 2000), and USFWS/CWS leg bands. Approximately 100 µl of blood was collected from the tarsus vein from each trapped individual using a 25-gauge sterile needle and a heparinized capillary tube, then transferred to 1.5mL eppendorf tubes containing 1mL of lysis buffer (White and Densmore 1992).

Ruddy duck nests were found by systematically searching the emergent vegetation around wetlands from mid-May to late July. When nests were found, nest location was marked using a numbered stake placed on land 5 to 15 meters away. We never created a direct path from land to nests to reduce the risk of increased nest predation. Embryonic development (incubation stage) was measured using an egg floatation method specifically developed for ruddy ducks (Brua & Machin 2000). We estimated nest initiation date by back-dating from the stage of embryonic development. Nests were rechecked every 7 days to evaluate development stage and/or nest fate.

To reduce loss of samples – i.e., due to predation and/or because the precocial young leave the nest very soon after hatching – we trapped females during late incubation (see above) and replaced their clutches with an identical number of non-viable chicken eggs (Pelayo & Clark 2003). The removed ruddy duck eggs were artificially incubated in air-circulating cabinet incubators at 37ºC, 67-70% humidity. When the incubated eggs hatched, we recorded the egg from which individuals hatched and kept ducklings in brooders until dry (approximately 3- 4 h). All ducklings were then banded with a federal duckling band (wood duck size B, prepared with a clay mixture of 50% Chavant DaVinci soft and 50% Roma Plastilina #1; Blums *et al*. 1999), and

weighed to the nearest .01 g. We also collected blood samples from all ducklings using the same methods above, with the exception that we used 27.5 gauge needles. Ducklings were then returned to nests of origin; nest females incubated chicken eggs for <5 days before their ducklings were returned.

We collected blood samples from 162 adults and 516 ducklings from 80 nests for genetic analysis. We also collected egg albumin from nests found during laying or early incubation (1-3 days). To collect albumin we punctured a small hole through the egg shell, removed approximately 200-300 µl of egg albumin with a syringe then sealed the hole with super glue. Albumin samples were immediately stored on ice, and then placed at -20ºC until protein fingerprint analysis (see below). We collected albumin samples from 148 eggs from 29 nests.

Protein Fingerprinting

Protein fingerprinting uses isoelectric focusing (IEF) to separate proteins across an electric field applied over a fixed pH gradient in precast gels; proteins are visualized as narrow bands on a gel representing their isoelectric points (Rhigetti 1990). Egg albumin is solely of maternal origin, therefore representing the genotype of the female that laid the egg rather than the developing embryo. Andersson & Åhlund (2001) demonstrated that protein bands are both reproducible and genetically polymorphic across females, and accordingly has resolution sufficient to detect parasitic eggs and sometimes also the females who laid those eggs.

All protein fingerprint analyses were conducted in Malte Andersson's lab at Göteborg University, Sweden, from November to December 2005. To resolve a sufficient number of protein variants, albumin samples were run on 4 gel types, 3 dehydrated gel types (Amersham Pharmacia Biotech, Uppsala, Sweden;), Immobiline DryPlates pH 4-7 code no. 1824-400, and

pH4.5-5.4 code no. 1824-450 (run with two recipes) and 1 hydrated gel type (Proteios, Zwaagdijk-Oost, Netherlands), code no. ProtEwi012. Amersham gel types and the Proteios gel type have slightly different protocols for running samples, thus we present the protocol used for Amersham gels first.

We rehydrated Amersham gels for 2 h with recipes modified from Andersson & Åhlund (2001) (Table 1). Electrophoresis was conducted with Amersham Pharmacia Biotech Multiphor II System and power supply EPS 3501, with a cooling temperature of 10ºC at 3000V, 1mA and max power of 3 W. Five µl of each sample was applied to an IEF sample application piece (Amersham Pharmacia Biotech) placed along the cathode. We did not dilute or buffer samples. Up to 30 samples were run on one gel, where we ran all eggs from each clutch beside each other on all gel types. We used de-ionized water as the electrode solution and gel type B was run overnight for approximately 12 hours and gel types C and D were run during the day for approximately 6 hours.

After electrophoresis gels were fixed in a solution of trichloroacetic acid and 5-sulphosalicyclic-dihydrate acid for 30 - 60 min. Before staining, gels were rinsed 5 min in destaining solution (25% v/v ethanol and 8% v/v acetic acid in de-ionized water), and then stained 6 min in Coomassie 250-R w/v in destaining solution heated to 60ºC. After staining, gels were kept in destaining solution for approximately 24 h. Before drying, gels were wiped with a soft tissue to remove excessive dye, and then washed 2 x 2 min in destaining solution. The gels were air dried for approximately 24 h before being read.

For Proteios gels, electrophoresis was conducted with Amersham Pharmacia Biotech Multiphor II System and power supply EPS 3501, with a cooling temperature of 10ºC. Pre-focusing was completed at 750 V, 25 mA, with a maximum of 10 W, for 15 min to establish the

pH gradient (3-7pH). We used an anode-buffer (no. 42984) and a cathode buffer (no. 42986), purchased from Proteios, as our electrode solutions. Samples were diluted and buffered in an extraction buffer [10% (v/v) glycerol de-ionized water solution with 1,5% (v/v) 3-10 carrier ampholyte] by adding 20 µl of egg albumin to 140 µl of extraction buffer. We loaded 7 µl of each diluted albumin sample into wells on an applicator strip (Proteios) placed directly on the gel near the cathode. Up to 48 samples were run on each gel, where we ran all eggs from each clutch beside each other. Gels were then run for 30 min at 200 V, for 24 min at 500 V, then for 2 h 40 min at 3000V (all at 25 mA, 10 W).

After electrophoresis, gels were fixed in a solution (20% Trichloroacetic acid in de-ionized water) for 30 min (15 min with no movement, then 15 min gently shaking). Gels were stained for 30 min in Coomassie 250-R w/v in destaining solution (see above). After staining, gels were kept in destaining solution for approximately 24 hours. Before drying, gels were wiped with a de-ionized water soaked soft tissue to remove excessive dye, and then washed 3 x 5 min in de-ionized water. The gels were air dried for approximately 24 hours before being read.

We scored all bands identifiable as present or absent for each individual. When one or more eggs from a nest had differing band patterns on at least one gel type the nest was scored as parasitized (see Results). The most common albumin pattern among eggs in a nest was assumed to represent the nesting (host) female, as demonstrated by Andersson & Åhlund (2001).

DNA Microsatellite Methods

We extracted genomic DNA from blood samples using standard phenol-chloroform methods after digestion with proteinase-K solution (Westneat 1990). We used variation at 10 microsatellite loci to identify parasitic ducklings and maternity for a subset of those individuals

(Table 2). Eight of these loci (all *Oxy* loci) were developed specifically for ruddy ducks (Muñoz-fuentes *et al.* 2005), and two (*Blm*5 and 12) were developed for the closely related Musk duck (*Biziura lobata*; Guay & Mulder 2005). Initially an eleventh locus (*Oxy*4) was included in the analysis, but it showed a high frequency of null alleles (0.287) and we discarded it from analyses.

To determine microsatellite genotypes for all individuals, we amplified genomic DNA from each individual in 10µl PCR reactions. To avoid labeling individual primers with a fluorescent label, we added an M13 reverse or CAG tag to the 5' end of the forward primer, and added a labeled M13 reverse or CAG tag in the PCR reactions (Hauswaldt & Glenn 2003). We used the following PCR mix for all *Oxy* primers: approximately 25 ng of genomic DNA, sterile H_2O, 1.00 X PCR buffer, 2.00 – 2.50 mM $MgCl_2$, 0.05 µM of forward primer (end labeled with either M13 reverse or CAG tag), 0.5 µM of reverse primer, 0.45 µM fluorescent labeled primer (M13 reverse or CAG tag labeled with one of four ABI PRISM ® fluorescent tags: 6FAM™, NED™, VIC®, PET®), 0.15 mM of each dNTP, and 2 units of Taq polymerase. PCR conditions for all *Oxy* primers included initial heating at 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing (X°C) for 20 sec, extension at 72°C for 30 sec, then a final extension at 72°C for 10 min. We used the following mix for both *Blm* primers: approximately 25 ng of genomic DNA, sterile H_2O, 1.00 X 10X PCR buffer, 2.50 mM $MgCl_2$, 0.33 µM of forward primer (end labeled with M13 reverse), 0.34 µM of reverse primer, 0.33 µM fluorescent labeled primer (M13 reverse or CAG tag labeled with one of two ABI PRISM ® fluorescent tags: VIC®, PET®), 0.15 mM of each dNTP, and 2 units of Taq polymerase. PCR conditions for both *Blm* primers included: initial heating at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing (X°C) for 1 min, extension at 72°C for 45

sec, then a final extension at 72°C for 5 min. Annealing temperatures and concentration of $MgCl_2$ depended on the primer used in the reaction (Table 2).

PCR products were visualized on an ABI 3730 automated capillary sequencer, and were analyzed using GeneMapper Version 3.5 (© 2003 Applied Biosystems). We assigned allele sizes for each individual at each locus, analyzed allelic mismatches between nesting females and offspring in their nests, and assessed maternity using the software package CERVUS 3.0.3 (Kalinowski *et al.* 2007), which uses a maximum likelihood approach to identify parents. However, because parentage can be difficult to assess genetically when neither parent is known, we also developed a set of conservative maternity assignment rules (see Results). In this paper we rely on these maternity rules and compare these results to the output from CERVUS.

Estimates of mating patterns for individual females

We also used microsatellite genotypes to analyze female mating patterns by determining paternal alleles from comparisons between ducklings and their biological mothers (using only non-parasitic ducklings that matched their nesting female, verified as described above). To do this we used GERUD 2.0 (Jones 2005) to estimate the minimum number of males contributing to offspring of nesting females (again, parasitic offspring were excluded). GERUD 2.0 estimates the minimum number of male mates using genotype reconstruction based on microsatellite genotypes from offspring and a known parent.

Statistical Analyses

To assess the possibility of pooling data from different years, we tested for a year effect on the frequency of parasitic ducklings in the population and the frequency of parasitic nests

using chi-square analyses. To assess whether we could pool parasitism data (frequency of parasitic nests only) from our two molecular data sets, we used a chi-square analysis with Yates Correction (conservative correction useful for small sample sizes) to test for differences in parasite detection between molecular techniques. Multiple linear regression was used to test if the frequency of parasitism was affected by the percent nests depredated on wetlands, where we also included year in the model. To obtain a measure of female condition, we regressed female weight on tarsus length, and used the residuals from this analysis as our measure of condition, thus controlling for female size. We then used multiple linear regression to test female egg laying behavior (females laying parasitic vs. females who did not) differed in condition, where we also included julian capture date and year in the model. Logistic regression was used to assess whether the probability a nest is parasitized is affected by the number of nests on a wetland, year was also included in the model. A two-tailed t-test was used to test if there was a significant difference in distance from a nesting parasite's nearest active neighbor and the nest where she laid a parasitic egg. ANOVA was used to test for differences in average nest initiation date and average number of female egg laying days across years. To test for possible effects of synchrony on the frequency of parasitism, we first generated a random population of host-parasite pairs, where we created 100 host-parasite pairs by pairing an observed nesting parasite with a randomly chosen host (not the observed host) and compared these to observed host-parasite pairs. We calculated the difference (in number of days) between the first laid egg in the parasite's nest and the first laid egg in the host's nest, for both the observed and random populations. We used ANOVA to test for differences between years in the average number of days between egg laying for observed host-parasite pairs. Two-tailed t-tests were used to test for

differences between the observed and random populations, in the average number of days between egg laying, for each year.

RESULTS

Protein fingerprint identification of parasitic ducklings

We scored 86 variable protein bands using the 4 different gel types. Among these bands, 45 were uninformative, because they were common among individuals and/or correlated (redundant) with other bands. These uninformative bands were omitted from the analysis. Among the remaining 41 variable bands, the number of bands per individual was 12.8 ± 2.41 (mean ± SD), and the mean band frequency was 0.312 ± 0.258. Based on protein banding patterns (Figure 1), we identified 20 parasitic eggs out of the 126 eggs analyzed [16% ± 0.06 parasitic (± 95% CI)]. Parasitic eggs occurred in 9 of 20 nests analyzed; thus 45% ± 0.22 (± 95% CI) of nests contained parasitic offspring.

Microsatellite identification of Parasitic Ducklings

All 10 microsatellite loci proved to be polymorphic, with numbers of alleles ranging from 3 to 19 per locus and all showing relatively high levels of heterozygosity (Table 2). The combined probability that an arbitrarily chosen adult would not match the duckling at all ten loci was 0.993 when both parents are unknown (Table 2). Only one locus, *Oxy*14, showed a significant deficiency of heterozygotes, suggesting a relatively high frequency of null alleles at this locus (Table 2). This is unlikely to have biased our results as we accounted for possible null alleles when creating our scoring criterion (below).

To identify parasitic offspring, we first constructed a distribution of locus mismatches for the population, by comparing genotypes of nesting females to offspring in their nests. To determine the number of locus mismatches indicative of a parasitic duckling, we created a simulated data set of 100 comparisons, for which we compared the genotype of a duckling to that of a randomly chosen female (not the putative parent). We then contrasted the distribution of locus mismatches for the simulation data set (representing locus mismatches for comparisons of unrelated individuals) with the distribution of nesting female/offspring mismatches observed in the population (Figure 2).

Comparison of these distributions indicated that unrelated pairings rarely result in zero locus mismatches, but zero mismatches were common between observed nesting females and offspring in their nests. Thus, we concluded that cases with zero locus mismatches are indicative of true parent/offspring comparisons ($N = 334$ cases). Similarly, the simulation indicated unrelated pairings often have two or more locus mismatches, whereas these were relatively rare in female/offspring comparisons and most likely represent parasitic offspring ($N = 84$ cases of parasitic offspring). Cases of single mismatches were prevalent in both distributions. In some cases the single mismatch between a nesting female and a duckling in her nest could be attributed to the presence of a null allele, and in these cases we accepted the female as the biological mother of the duckling ($N = 18$ out of 98 cases of single mismatches). All other cases of a single mismatch between the nesting female and a duckling were considered ambiguous cases for which we could not accurately discriminate parasitic ducklings from true parent/offspring matches.

To obtain further resolution for these remaining ambiguous cases ($N = 80$ single locus mismatches not attributed to a null allele), we examined distributions of likelihood scores

(LODs) calculated by the program CERVUS for both true parent/offspring comparisons and the simulation data set (Figure 3). The two likelihood distributions overlapped from 0.14 – 1.87 (Figure 3); thus we determined that adult offspring comparisons must have an LOD score greater than 1.87 (two standard deviations above the mean LOD for unrelated individual comparisons), to represent a true parent/offspring relationship. Using this criterion we could distinguish between parasitic ducklings and biological offspring for an additional 69 out of 80 ambiguous cases (3 biological offspring and 66 parasitic young), 11 cases remained ambiguous and were excluded from further analysis.

Microsatellite analyses detected 150 parasitic offspring and 355 biological offspring out of 516 offspring analyzed (Table 3). The frequency of parasitic offspring differed significantly across years ($\chi^2 = 13.37$, d.f. = 2, $P = 0.001$), with 2005 having fewer parasitic ducklings than the other two years (Table 3). However, there was no significant difference in the percent parasitic nests across years ($\chi^2 = 5.139$, d.f. = 2, $P = 0.077$). Thus we only pooled data for percent nests across years and found that 67% ± 0.10 (± 95% CI) of nests contained parasitic offspring identified with microsatellites ($N = 55$ out of 82 nests).

We found that the two molecular techniques appeared to be equally successful in detecting parasitic eggs, using a side by side comparison of 18 eggs from 5 nests (cases where we collected both egg albumen and blood). In 17 cases, both albumen protein fingerprint and microsatellite egg identification agreed (identifying 14 eggs from nesting females and 3 parasitic eggs). In just one case an egg identified as parasitic by protein fingerprinting was not detected as such using our stringent microsatellite criterion. In this case CERVUS identified the nesting female as an unlikely candidate, but we had assigned the duckling to the nesting female because the nesting female and the duckling had zero locus mismatches.

We found no significant difference in the number of parasitic nests detected by each technique (chi-square with Yates correction, χ^2=2.474, d.f. = 1, P=0.116). Thus we pooled the two molecular data sets across years and found 64% ± 0.10 (± 95% CI) of nests contained parasitic offspring (61 out of 96 nests analyzed).

Maternity assignment

Maternity was assigned to the nesting female when the nesting female and an offspring in her nest had zero locus mismatches and in a few cases of single mismatches when the single mismatch could be due to a null allele (above). Parasitic ducklings were assigned to females when candidate female/offspring comparisons had zero locus mismatches and an LOD score greater than 1.87 (see above). Using these criteria we assigned maternity to a total of 448 offspring out of 505 analyzed (11 ambiguous cases excluded): 355 offspring were assigned to the nesting female incubating the clutch and 93 out of 150 parasitic offspring were assigned to other females. CERVUS agreed with assignments of females to offspring 81% of the time (N = 365 out of 448 offspring assigned). Disagreement occurred because CERVUS did not assign nesting females to offspring in their nests 83 out of 355 cases, when the nesting female/offspring comparison had zero locus mismatches or a single mismatch that could be explained by the presence of a null allele. In theses cases CERVUS assigned 19% (16 out of 83) of offspring to another female with high confidence, 71% (59 out of 83) with low confidence, and 10% were not assigned to another female (8 out of 83). In addition, we assigned parasitic females to 14 parasitic offspring when CERVUS did not assign the candidate female with high confidence, because the candidate fit our other assignment criteria above.

Parasitism as a specialist, conditional, or mixed strategy

Maternity assignment revealed that 50% ± 0.09 (± 95% CI) of females in the population (56 of 112 females analyzed) laid eggs parasitically. On average parasitic females laid 1.67 ± 0.81 eggs (mean ± SD) parasitically in 56 nests and laid parasitic eggs in 1.55 ± 0.66 nests. Of the females sampled, 86 were known to nest on the study site, and of these 50% ± 0.11 (± 95% CI) also laid eggs parasitically (N = 43 nesting females laid eggs parasitically in addition to nesting). No nests were detected for 13 females that laid eggs parasitically, but at least some of these females are likely to have nested off the study site (see below). Similarly, nesting females for which we did not identify any parasitic young may have laid parasitic eggs in unsampled nests off of the main study site. Thus, we found that 77% ± 0.11 (± 95% CI) of identified parasites were females with nests of their own (43 of 56 parasitic females identified), and this is likely to be a minimum estimate for the proportion of females that lay parasitic eggs in addition to nesting on their own.

We found no evidence that female ruddy ducks lay parasitic eggs as part of a conditional strategy. If, for example, females lay parasitically in response to nest loss, then most parasitic eggs should be laid relatively late, yet we found that nesting females laid parasitic eggs prior to, during and after initiating their own clutch (Figure 4). Moreover, 7 females who lost their nests also laid parasitic eggs, but in all cases these females laid their parasitic eggs prior to nest loss. Both year and the percent nests depredated on wetlands were not related to parasitism frequency on wetlands [multiple linear regression: $R = 0.021 \pm 0.450$ (± SE), $F_{2,75} = 0.814$, $N = 78$, $P = 0.447$]. Model parameters are summarized in Table 4.

Differences in female egg laying behavior (females laying parasitically vs. females who did not) were not related to female condition and there was no year effect on female condition.

However, female condition was negatively related to capture date, where females captured late in the season had the lowest condition scores [multiple linear regression: $R = 0.411 \pm 80.291$ (± SE), $F_{4,104} = 18.159$, $N = 109$, $P < 0.0001$]. Model parameters are summarized in Table 5.

What ecological factors influence the likelihood of parasitism?

Logistic regression revealed the probability that a nest was parasitized was unaffected by the number of nests on a wetland, or by year (χ^2=3.213, d.f. = 3, P=0.360; Figure 5). Model parameters are summarized in Table 6.

For nesting parasites ($N = 39$), we also tested whether females were more likely to parasitize their nearest active neighbor and found only 8% ± 0.08 (± 95% CI) of females parasitized their nearest neighbor. Another 10% ± 0.10 (± 95% CI) of females laid eggs parasitically in nests less than 70 meters from their nearest neighbor, and 82% ± 0.12 (± 95% CI) laid eggs parasitically in nests over 200 meters beyond their nearest neighbor. On average, the nests of parasitic females were seven times further way from the nest they parasitized (1680.89 ± 1359.82 m, mean ± SD) than they were from their nearest neighbor (237.30 ± 176.40 m; Figure 6; two-tailed t-test: $T = -8.356$, d.f. = 38, $P < 0.0001$). Overall, nesting females laying eggs parasitically do so within a large range of distances from their own nests (e.g. See Figure 7), and are unlikely to parasitize their nearest neighbors.

Across years nest initiation ranged from 24 May to 10 July (144-191 julian date). There was no difference in average nest initiation dates in 2003 and 2005 (165.96 ± 8.47, mean ± SD); however, the average nest initiation date for 2004 (177.43 ± 9.51, mean ± SD) was significantly later than the other two years (ANOVA: $F_{2,82} = 14.465$, $N = 85$, $P < 0.0001$). There was no difference in the number of days spent egg laying across years (ANOVA: $F_{2,39} = 2.268$, $N = 42$,

$P = 0.117$, for females) where females averaged 7.02 ± 2.12 (mean ± SD) days of egg laying per clutch across years (means are for complete nests with parasitic eggs removed).

To test if parasites were more likely to parasitize synchronous hosts (cases when egg laying of the nesting parasitic female overlapped that of the female she parasitized), we compared observed differences in egg laying for 63 host-parasite pairs to differences in egg laying for randomly selected pairs of females. The average number of days between observed host- parasite pairs was 8.43 ± 5.79 (mean ± SD), with no significant difference between years (ANOVA: $F_{2, 60} = 2.130$, $N = 63$, $P = 0.128$). The average number of days between randomly generated host-parasite pairs was 9.97 ±7.39 (mean ± SD) days. We found no significant difference in the average number of days between egg laying for observed host-parasite pairs and random host-parasite pairs for 2003 (two tailed t-test: $T = 0.938$, d.f. = 28, $P = 0.356$) and 2005 (two-tailed t-test: $T = 0.694$, d.f. = 55, $P = 0.491$). Although in 2004 observed host-parasite pairs were separated by 5.17 ± 1.45 (mean difference ± SE) fewer days than host-parasite pairs in the random population (two-tailed t-test: $T = -3.563$, d.f. = 73.14, $P < 0.001$). Thus in 2004 host-parasite pairs were more synchronous than random.

Mating patterns in the population

Nesting females mated with two or more males in 18% ± 0.09 (± 95% C.I.) of nests ($N =$ 13 out of 73 nests), as a single male could not account for genotypes observed in those nests (parasitic offspring removed prior to running nests on GERUD 2.0). On average females mated with 1.19 ± 0.43 (mean ± SD) males, suggesting low levels of polygynous mating.

DISCUSSION

Identification of parasitic females

This is the first study to identify parasitic offspring and the females producing them in a wild population using molecular markers alone, and also the first study to demonstrate that protein fingerprinting and microsatellites can similarly estimate the frequency of CBP. Each method has its own advantages. First, protein fingerprinting is useful for identifying parasitic offspring in nests because nests are sampled at laying and nest predation is unlikely to bias estimates. Protein fingerprinting is less useful for identifying parasitic females because fingerprinting patterns of eggs must somehow be matched to the females who produced them (e.g., behavioral observations; see Andersson & Åhlund 2000, 2001; Waldeck & Andersson 2006 and Andersson & Waldeck 2007). In contrast, microsatellites are powerful for matching females to parasitic eggs, but samples cannot be collected at laying (introducing problems of nest predation) and typically a large number of loci will be needed for adequate resolution when behavioral observations are difficult to obtain. Moreover, it is necessary to develop accurate assignment criteria (see above) to identify individuals and avoid false assignments. Our results indicate ambiguous cases may commonly occur in analyses of CBP because neither parent is known, thus increasing the need for multiple loci.

Reproductive strategies in ruddy ducks

At least 18% of ruddy duck females engage in extra-pair mating, confirming that ruddy ducks are polygynous; however, not as highly polygynous as previously thought. CBP was common in this population of ruddy ducks, where 64% of nests were parasitized. These estimates are similar to those obtained for other overwater nesting waterfowl (e.g., redheads, 61% of nests; Sorenson 1991; canvasbacks, 65% of nests; Sorenson 1993). Most parasites in this

population (77% of 56 parasitic females identified) laid parasitic eggs in addition to nesting on their own. For the remaining parasitic females, we did not detect nests, but they likely had nests off the study site, given that ruddy duck females lay parasitic eggs relatively far from their own nesting site (Figure 7). Thus, our results do not support the hypothesis that parasitic ruddy ducks are specialists who only lay eggs parasitically.

In addition, parasitism did not occur in response to nest loss (as observed in other systems; e.g., masked weavers; Jackson 1993) or predation on wetlands. We also found no differences in physiological condition between females who laid eggs parasitically and females who did not. Thus, we find no support for a conditional reproductive strategy for parasitic ruddy ducks. This contrasts with some other waterfowl species (e.g., redhead, goldeneyes, and american coots) showing high levels of CBP, where females often use a conditional strategy influenced by ecological constraints, age and/or physiological condition (e.g. Sayler 1985; Eadie 1989; Sorenson1991; 1993; Lyon 1993a). Although we identified a mixed reproductive strategy for parasitism in ruddy ducks, given only the costs of egg production, one might assume a conditional strategy best describes parasitic egg laying in ruddy ducks. We found no significant difference in female condition for females laying eggs parasitically and females who appeared to nest only. This may indicate that breeding females in this population are only females in good breeding condition, another study found a proportion of non-breeding females exist in the population (20% of 50 females, Siegfreid 1976), possibly representing females in poor condition. In addition female ruddy ducks nest relatively later in the season than most other waterfowl (Brua 1998) with few cases of renesting (Tome 1987). Thus, if females are not ready to breed early enough in a season, they become more likely to forgo breeding. In this population of ruddy ducks, nesting females are responsible for the majority of parasitic egg laying, similar to patterns

observed in cliff swallows (Brown & Brown 1998). Therefore we have shown evidence to support that CBP in this population is primarily due to a mixed reproductive strategy, not an alternative reproductive strategy or a conditional strategy.

Ecological factors affecting the probability of parasitism

Nest density did not appear to enhance the occurrence of parasitism in ruddy ducks nests in this species, as it does in other species (Brown 1984; Sayler 1992; Møller 1998). We found that females rarely lay parasitic eggs in nests of their nearest neighbors. In fact ruddy ducks lay eggs parasitically in nests relatively far from their own nests, seven times further away from the nest they parasitized (see above) than they were from their nearest neighbor. This is surprising given ruddy ducks are considered the least mobile of all North American waterfowl (Evans et al. 1952) and are rarely seen flying during the breeding season (Siegfried 1972; Brua 2001). Ruddy ducks are primarily aquatic; however, our research suggests females are capable of flying relatively long distances even during egg laying, when females are heaviest.

Overall, nesting synchrony is relatively high in this population of ruddy ducks and most nesting parasites lay eggs in nests synchronous with their own, or in nests initiated just prior to their own (see above). Although some nesting parasites laid parasitic eggs after completing their own egg laying sequence, most laid eggs immediately or soon after completing their own egg laying sequence. Interestingly, in 2004 we found that nesting parasites and their hosts were more synchronous in egg laying sequence than random host-parasite pairs (at least 5 days closer), even though this did not occur in 2003 or 2005. Perhaps nesting synchrony plays a role in a parasite's placement of parasitic eggs; however, discriminating between this possibility and chance are difficult because of high nesting synchrony in the population. In general, high nesting synchrony

for ruddy ducks in this population is likely due to high nutrient demand of egg formation and lateness of nest initiation relative to other species nesting in this region (Brua 2001). In addition, females are single brooded and few renest after nest loss (Tome 1987). Therefore, any lack of an obvious effect of nesting synchrony on CBP may be an artifact of many ruddy ducks initiating nests around the same time. Perhaps there is something more complex involved with host choice, such as host quality or relatedness among individuals (e.g., Andersson & Åhlund 2000; Nielsen *et al.* 2006b; Andersson & Waldeck 2007). Further investigation of the host/parasite relationship and an analysis of the costs and benefits associated with parasitic egg laying must be addressed to attempt to reveal underlying mechanisms of CBP in ruddy ducks.

In summary, we show that CBP is common in ruddy ducks, a species where egg laying appears to be costly (Alisauskas & Ankney 1994), and that females likely use a mixed reproductive strategy where they lay eggs parasitically in addition to nesting. Although for many other waterfowl species, cases of CBP can be attributed to a conditional reproductive strategy, this does not appear to be the case for ruddy ducks, perhaps reflecting differences in life history patterns and possibly a different mechanism by which parasitism evolved in this species.

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Table 1. Recipes for gel rehydration mix, for different gel types

Rehydration – Chemicals/gel	Gel Type (pH range of Immobiline Dry Plates)		
	B (4.5-5.4)	C (4-7)	D (4.5-5.4)
Tris, 2mM (ml)	20.0	15.0	15.0
Urea (g)	8.0	8.0	8.0
Glycerol 99.5% w/v, (ml)	0	5.0	5.0
Dithiothreitol, DTT (mg)	200	200	0
Carrier Ampholyt/Pharmalyte pH 3-10 (ul)		150	
Carrier Ampholyt/Pharmalyte pH 4.5-5.4 (ul)	150		150
Sample volume (ul)	5	5	5
Destaining duration (hours)	24	24	24

Table 2. Locus specific PCR conditions and variability of ten microsatellite loci for adult ruddy ducks sampled 2003-2005

					Heterozygosity			
Locus	n[a]	No. Alleles	Annealing Temperature XºC	$MgCl_2$ Concentration	Expected (H_e)	Observed (H_o)	Frequency of Null Allele	$P_{exclusion}$[c]
*Oxy*3	158	4	62	2.00	0.115	0.108	0.026	0.007
*Oxy*6	161	3	62	2.00	0.457	0.447	0.012	0.104
*Oxy*10	161	11	62	2.00	0.816	0.783	0.021	0.463
*Oxy*13	160	19	62	2.00	0.868	0.863	0.004	0.580
*Oxy*14	161	14	60	2.50	0.759	0.602[b]	0.118	0.379
*Oxy*15	162	6	60	2.50	0.162	0.173	0.037	0.013
*Oxy*17	162	6	60	2.50	0.695	0.685	0.001	0.275
*Oxy*19	159	3	60	2.00	0.551	0.560	0.009	0.151
*Blm*5	156	3	50	2.50	0.075	0.064	0.072	0.003
*Blm*12	149	7	60	2.50	0.479	0.483	0.008	0.119
					[d]Total Exclusionary Power = 0.933			

[a] Number of unrelated adults genotyped at a locus

[b]Significantly fewer heterozygotes observed than expected under Hardy-Weinberg equilibrium(χ^2 test, df=1, $p<0.05$)

[c] $P_{exclusion}$ = probability of excluding a randomly chosen parent of an arbitrary offspring given only the offspring's genotype

[d]Total exclusionary power = combined power of the 10 loci to exclude a candidate parent of an arbitrary offspring assuming both parents are unknown

Table 3. Ducklings separated into maternity assignment categories. Values in cells are listed as N (% ducklings sampled).

Maternity Assignment Category	2003	2004	2005	All years
Matched nesting female	58 (65)	128 (62)	169 (76)	355 (70)
Did not match nesting female (Parasitic ducklings)	29 (33)	73 (35)	48 (22)	150 (30)
Matched another female	12 (13)	49 (24)	31 (14)	92 (18)
Matched no female	17 (19)	24 (12)	17 (8)	58 (11)
Ambiguous*	2 (2)	5 (2)	4 (2)	11 (2)

*Cases where nesting female and offspring had a single mismatch;
N = 516 total ducklings analyzed

Table 4. Results of a multiple regression analysis testing for an effect of nest predation on the frequency of parasitism on a wetland. Parasitism frequency on a wetland was the response variable ($N = 78$ wetlands)

Parameter	Parameter Coefficient	Standard Error	*t* Ratio	p
Intercept	0.531	0.057	9.30	< 0.0001
% nests depredated on a wetland	0.022	0.190	0.12	0.907
Year	-0.068	0.053	-1.25	0.216

Table 5. Results of a multiple regression analysis testing for an effect of female condition on female egg laying behavior. Female condition was the response variable ($N = 109$ females)

Parameter	Parameter Coefficient	Standard Error	*t* Ratio	p
Intercept	663.972	82.086	8.09	< 0.0001
Julian capture date	-3.505	0.427	-8.21	< 0.0001
Female type (Parasite or non-parasite)	8.369	7.814	1.07	0.287
Year (2003)	-21.001	13.836	-1.52	0.132
Year (2004)	17.590	11.108	1.58	0.116

Table 6. Results of a logistic regression analysis testing for an effect of nesting density on parasitism frequency on a wetland. Frequency of nests parasitized on a wetland was the response variable (N = 78 nests, selected at random for each wetland)

Parameter	Parameter Coefficient	Standard Error	*Chi-Square*	p
Intercept	0.600	0.292	4.22	0.040*
# nests on wetland (single nest and multiple nest wetlands)	-0.364	0.266	1.88	0.171
Year (2003)	0.176	0.450	0.15	0.696
Year (2004)	0.161	0.355	0.20	0.651

* = significant effect

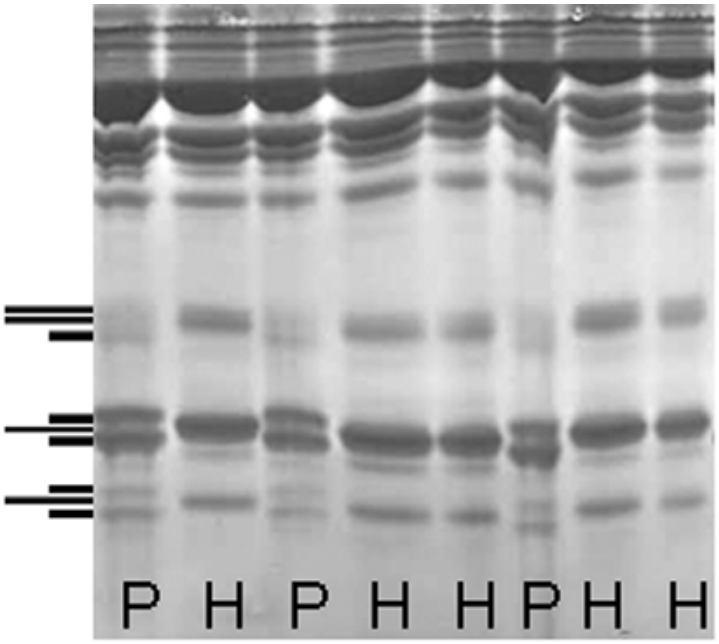

Figure 1. Electrophoretic protein fingerprint gel (Gel type D in Table 1) where 8 eggs from a single nest were run together; each lane is a sample from an individual egg. There are 3 parasitic eggs (P) and 5 host eggs (H). Diagnostic bands useful for distinguishing eggs within this clutch are highlighted on the left of the gel; short lines indicate bands found in parasitic eggs, long lines indicate bands found in host eggs.

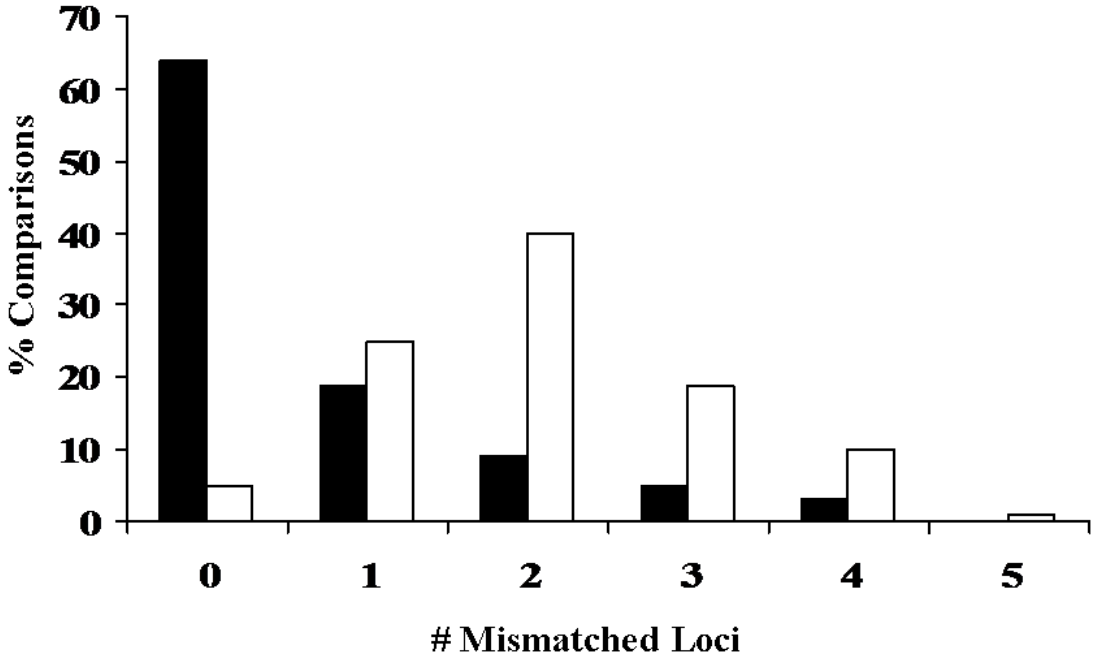

Figure 2. Distribution of number of locus mismatches for comparisons between (1) nesting females and offspring in the nest (dark bars, $N = 100$ comparisons between females and the ducklings in their nests), and (2) randomly chosen females and offspring (white bars, $N = 100$ simulated pairs).

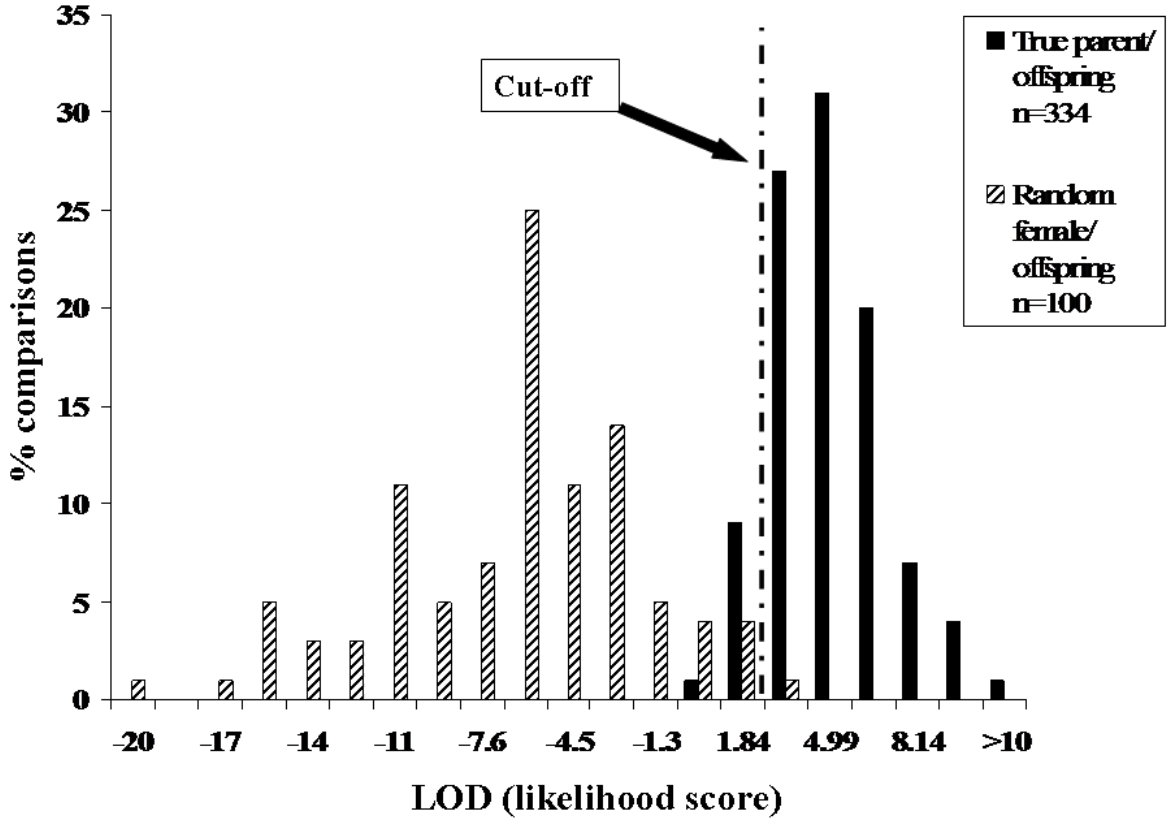

Figure 3. LOD distribution for comparisons of true parent/offspring and random female/offspring. LOD scores for nesting female/offspring comparisons with single mismatches were used to discriminate between host and parasitic ducklings. The dashed line indicates the lowest LOD score allowable for single mismatch cases where offspring in a nest were assigned to the nesting female. (Cut-off is two standard deviations from the mean LOD score for random parent/offspring comparisons).

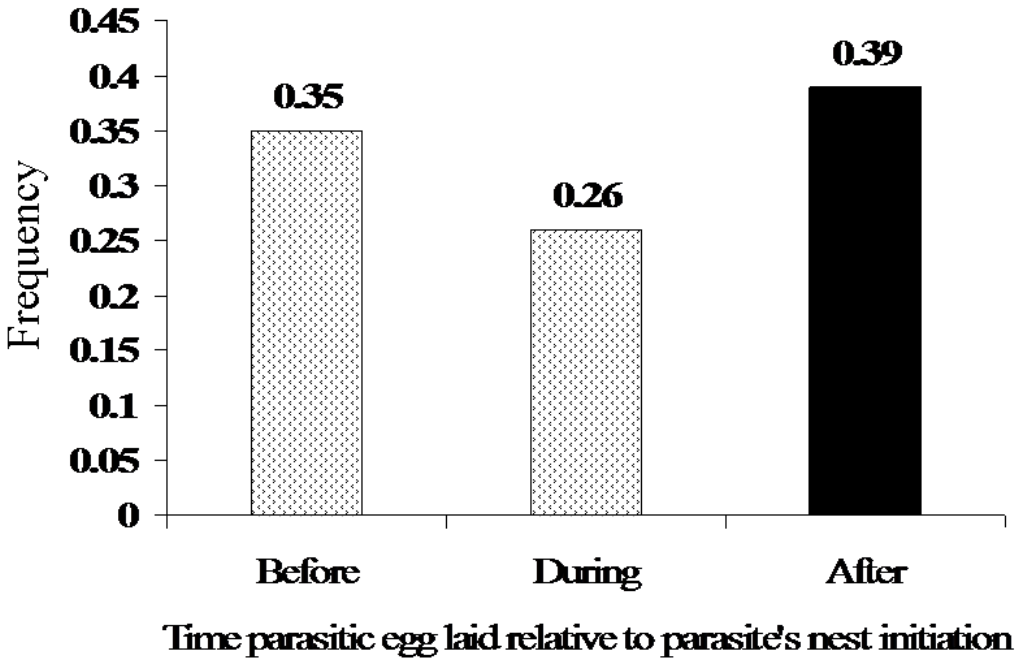

Figure 4. Estimated time when parasitic egg was laid relative to the parasite's nest initiation (*N* = 66 females) 39% of females laid eggs after completing their own clutch; however in all cases where a nest was loss, the parasitic egg was laid prior to nest loss

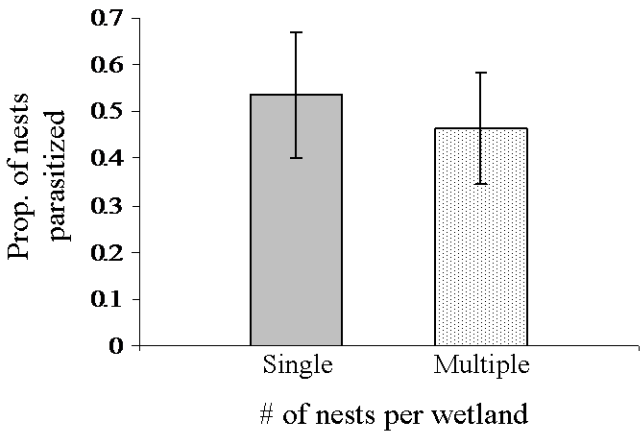

Figure 5. Parasitism on wetlands with a single ruddy duck nest ($N = 54$) compared to wetlands with multiple ruddy duck nests (2 -5 nests per wetland; $N = 24$). Error bars represent 95% confidence intervals.

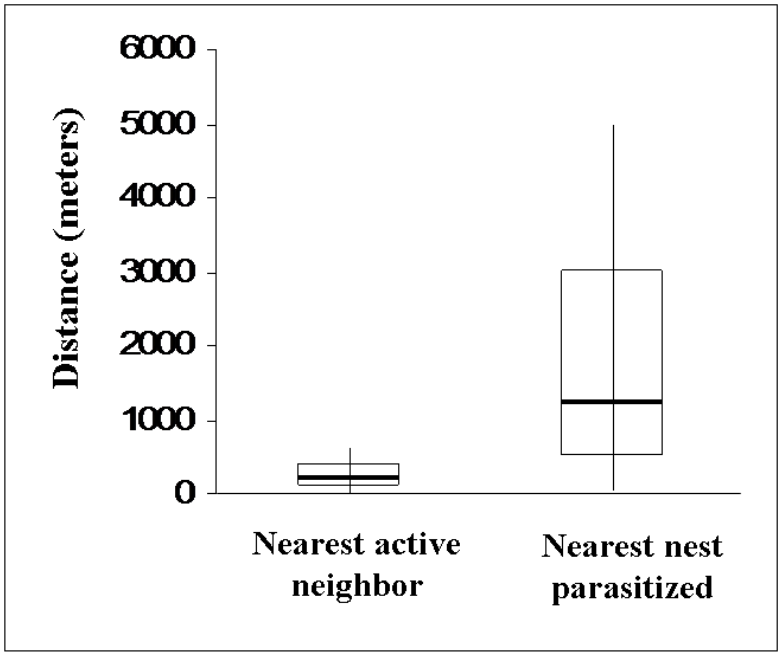

Figure 6. Average distance of nearest active neighbor (237.30 ± 176.40 m; mean ± SD) compared to the average distance for the nearest nest parasitized for nesting parasites (1931.51 ± 1269.99 m). Nesting females rarely parasitize nearest neighbors. (*N* = 39 nesting females laying eggs parasitically)

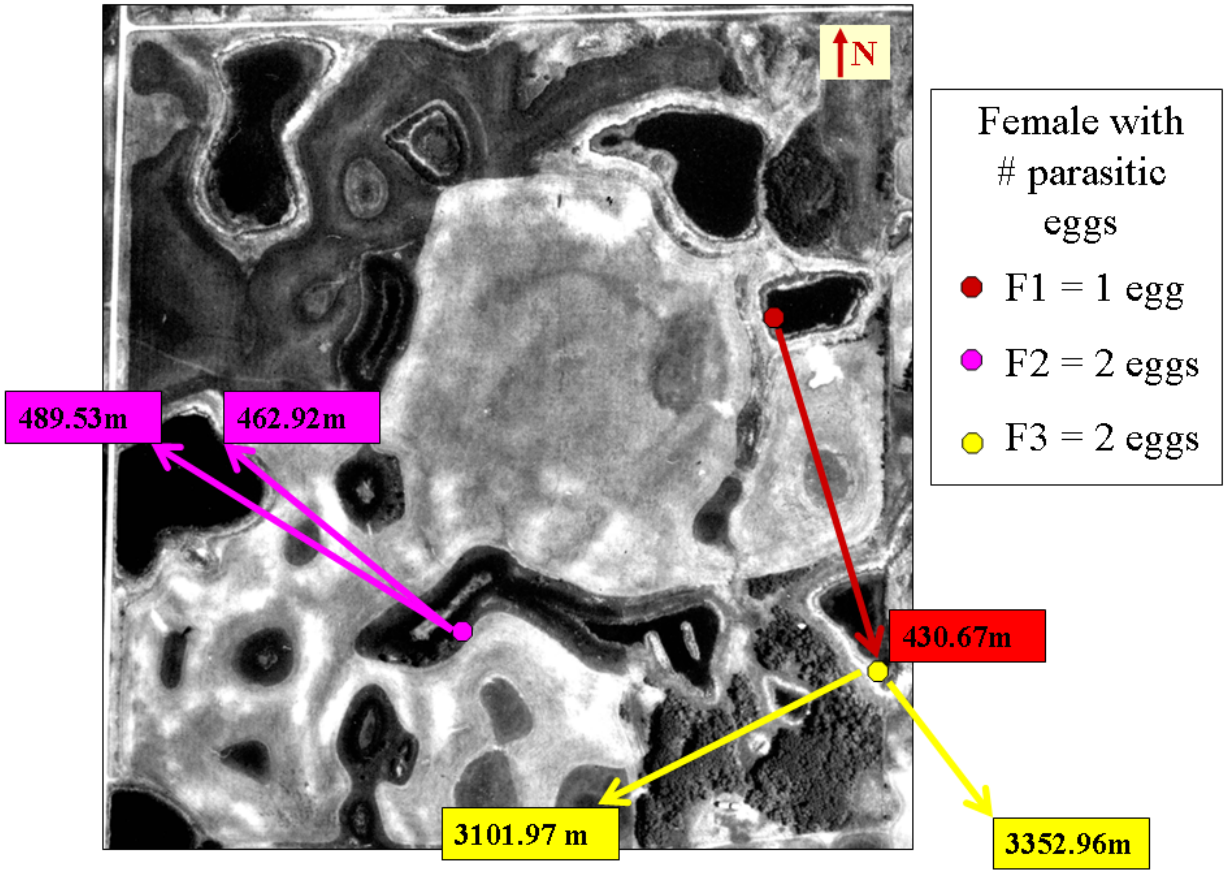

Figure 7. Map indicating where three nesting females also laid eggs parasitically various distances from their own nest. Parasitic female nests are indicated by individually colored circles. Corresponding colored arrows identify the location of nests those females parasitized, where distance (in meters) from parasite's nest to the nests parasitized are indicated in color coded boxes.

CHAPTER TWO

RELATEDNESS DOES NOT EXPLAIN HOST CHOICE BY CONSPECIFIC BROOD PARASITIC RUDDY DUCKS

INTRODUCTION

Females of some species sometimes lay eggs in nests of other conspecific females as a reproductive strategy termed "conspecific brood parasitism" (CBP). This behavior occurs in various taxa including: fishes (1995), amphibians (Wisenden 1999), various species of insects (Brockman 1993; Tallamy 2005) and over 236 species of birds (Yom-Tov 1980; 2001). Females laying such eggs do not provide parental care to their parasitic offspring; instead the nest-tending female (the "host") incurs any costs of raising parasitic offspring. CBP is particularly common in waterfowl (Rohwer & Freeman 1989; Sayler 1992), where the costs of raising offspring are likely reduced given the relatively precocial nature of offspring (Andersson 1984; 2001). In addition female waterfowl tend to be natally philopatric (reviewed in Anderson *et al.* 1992), and populations will likely exhibit local female relatedness structure (e.g., Ruusila *et al.* 2000; van der Jeugd *et al.* 2002; Fowler 2005; McKinnon *et al.* 2006; Waldeck *et al.* 2008). Thus hosts and parasites may be genetic relatives, providing an opportunity for kin selection (Hamilton 1964) to play a role in the evolution of CBP.

Andersson (1984) first suggested that if host parasite pairs are related, the cost of parasitism to the host (e.g. increased amount of parental care due to additional offspring) would be reduced because of inclusive fitness benefits gained by the host through relatedness to the parasite. Andersson (2001) further clarified this hypothesis to include both a component of kin

recognition and kin discrimination where hosts are selected to preferentially allow relatives over non-relatives (e.g., by egg ejection after the parasite lays an egg, or by nest defense) to lay eggs parasitically when the cost of parasitism to hosts is low. In addition, costs due to predation and/or the risk of mortality for normal nesting must be relatively high, such that hosts would gain inclusive fitness benefits through increased survival of related offspring (Andersson 2001). Similarly, López-Sepulcre & Kokko (2002) showed that when kin recognition is perfect or when hosts do not detect parasitic eggs, parasitic females are selected to preferentially parasitize relatives over non-relatives, but only when hosts benefit directly from having "parasitic" eggs in their nests and/or when costs of parasitism to the host are very low. Several studies have directly examined the relatedness between hosts and parasites in populations with high levels of female philopatry and frequent CBP, and lend some support to this hypothesis (McRae & Burke 1996; Andersson & Åhlund 2000; Nielsen *et al.* 2006; Andersson & Waldeck 2007; Waldeck *et al.* 2008), but the role of relatedness in CBP remains unclear.

Alternatively, parasites may be selected to preferentially avoid parasitizing kin, especially when costs of raising parasitic offspring are high (Zink 2000; Andersson 2001; López-Sepulcre & Kokko 2002). Thus, potential benefits to the parasite would decrease when they parasitize close relatives, because costs to the related host would decrease or eliminate any indirect fitness benefits a parasite may have gained (Lyon & Eadie 2000). A few studies of CBP have shown that parasites avoid parasitizing kin (Semel & Sherman 2001; Pöysä 2004), and a study in American coots (*Fulica americana*; Lyon *et al.* 2002) showed that raising a parasitic chick results in a direct fitness cost to the host.

Here we use molecular genetics to investigate relatedness between hosts and parasites in ruddy ducks (*Oxyura jamaicensis*). Ruddy ducks are an ideal species to test these hypotheses

given that they readily participate in CBP, where depending on population and year, 8 - 67% of clutches are parasitized by conspecifics (Siegfried 1976; Joyner 1983; Lyon *et al.* 2002; Reichart *et al.* in prep.). Parasites in our population are predominantly nesting parasites (i.e., females who lay both parasitic and non-parasitic eggs) and do not preferentially parasitize the closest active nest (Reichart *et al.* in prep.). Resource requirements during egg laying and incubation are high (see below) and ruddy ducks are single brooded. Few females renest after nest loss, likely due to high nutrient demand of egg formation and a relatively late breeding season (Brua 2001). Although egg production in ruddy ducks is costly (Alisauskas & Ankney 1994a), offspring are highly precocial, requiring minimal parental care after hatch (Sommerville 1985). Thus, the potential costs of parasitism in terms of parental care are relatively low in this species, although hosts may suffer energetic costs during incubation due to increased clutch sizes resulting from parasitism (e.g., Thomson *et al.* 1998). Based on the above characteristics of ruddy ducks, we predict that parasites would preferentially avoid parasitizing relatives. We also examine female relatedness with respect to spatial distance to determine whether relatives nest in close proximity to one another, as might be expected under female natal philopatry. Such a spatial pattern might facilitate the ability of females to parasitize, or avoid parasitizing, close kin.

MATERIALS AND METHODS

Study Species

Ruddy ducks are members of the stiff-tail subfamily (Oxyurinae). Egg-laying appears to be more costly for female ruddy ducks than for most other waterfowl: female ruddy ducks typically lay a clutch of 6 – 8 eggs (Joyner 1975), with each egg weighing approximately 14% of a female's body weight (Gray 1980; Alisauskas & Ankney 1992; 1994a). Maximum daily cost

(584kJ) of egg production is 280% of basal metabolic rate (Alisauskas & Ankney 1994a). Females incubate the eggs for approximately 24 days (Bellrose 1980; Brua 1998). Resource requirements during egg laying and incubation are high, with females consuming up to 6,000 midge larvae (*Chironomidae*) per hour during this period (Gray 1980; Carbonell 1983; Tome 1987).

Ruddy duck offspring are highly precocial and able to forage on their own immediately after hatching (Sommerville 1985). Adult females stay with a brood from 28 to 42 days after hatching to provide parental care, mainly in the form of vigilance for predators (Joyner 1975; Gray 1980). Females do not feed their offspring and some brood amalgamation behavior has also been noted (Joyner 1975). Offspring survival is high (Bellrose 1980), and ducklings from larger eggs have a higher probability of survival (Pelayo 2001; Pelayo & Clark 2003).

Field data and sample collection

This research was carried out during the ruddy duck breeding season (mid-May to early August) each year from 2004-2005 in the prairie pothole region of Minnedosa, Manitoba, Canada (50º10'N, 99 º47'W). This area is characterized by numerous permanent and ephemeral wetlands ranging in size from 0.1 to >4.0 ha (Pelayo & Clark 2003). Wetland margins contain various types of emergent vegetation including cattail (*Typha* spp.), hardstem bulrush (*Scirpus acutus*), cane (*Phragmites communis*), and whitetop rivergrass (*Scholochloa festucacea*). Nesting habitat for ruddy ducks is abundant in this region because females build nests overwater in the emergent vegetation.

We trapped adult ruddy ducks prior to nesting using floating mist nets (technique described in Breault & Cheng 1989). Once nesting began we also trapped adult females during

late incubation (eggs approximately 18-20d) using drop door nest traps (Weller 1957). At the time of capture adults were weighed to the nearest gram using a Pesola spring scale and standard morphological measures were taken (e.g. tarsus length, culmen length, bill width, skull, wing length). We marked females using a temporary, modified nasal disc (Pelayo & Clark 2000), and USFWS/CWS leg bands. Approximately 100 µl of blood was collected from the tarsus vein from each trapped individual using a 25-gauge sterile needle and a heparinized capillary tube, then transferred to 1.5mL eppendorf tubes containing 1mL of lysis buffer (White & Densmore 1992).

Ruddy duck nests were found by systematically searching the emergent vegetation around wetlands from mid-May to late July. When nests were found, nest location was marked using a numbered stake placed on land 5 to 15 meters away. We never created a direct path from land to nests to reduce the risk of increased nest predation. Embryonic development (incubation stage) was measured using an egg floatation method specifically developed for ruddy ducks (Brua & Machin 2000). We estimated nest initiation date by back-dating from the stage of embryonic development. Nests were rechecked every 7 days to evaluate development stage and/or nest fate.

To reduce loss of samples – i.e., due to predation and/or because the precocial young leave the nest very soon after hatching – we trapped females during late incubation (see above) and replaced their clutches with an identical number of non-viable chicken eggs (Pelayo & Clark 2003). The removed ruddy duck eggs were artificially incubated in air-circulating cabinet incubators at 37ºC, 67-70% humidity. When the incubated eggs hatched, we recorded the egg from which individuals hatched and kept ducklings in brooders until dry (approximately 3- 4 h). All ducklings were then banded with a federal duckling band (wood duck size B, prepared with a

clay mixture of 50% Chavant DaVinci soft and 50% Roma Plastilina #1; Blums *et al*. 1999), and weighed to the nearest .01 g. We also collected 100 µl blood samples from all ducklings using the methods above, with the exception that we used 27.5 gauge needles. Ducklings were then returned to nests of origin; nest females incubated chicken eggs for < 5 days before their ducklings were returned. We collected blood samples from 162 adults and 516 ducklings from 80 nests for genetic analysis.

DNA Microsatellite Methods

We extracted genomic DNA from blood samples using standard phenol-chloroform methods after digestion with proteinase-K solution (Westneat 1990). We used variation at 10 microsatellite loci to identify parasitic ducklings, assign maternity for a subset of those ducklings, and for estimates of relatedness among individuals. Eight of these loci (*Oxy3, 6, 10, 13, 14, 15, 17,* and *19*) were developed specifically for ruddy ducks (Munoz-fuentes *et al.* 2005), and two (*Blm*5 and *12*) were developed for the closely related Musk duck (*Biziura lobata*; Guay & Mulder 2005). Initially an eleventh locus (*Oxy*4) was included in the analysis, but it showed a high frequency of null alleles (0.287) and we discarded it from analyses.

To determine microsatellite genotypes for all individuals, we amplified genomic DNA from each individual in 10µl PCR reactions. Detailed methods for PCR amplification of microsatellites and criteria used to identify parasitic ducklings and assign maternity are described in Reichart *et al.* (in prep.). In this paper we rely on host and parasites identified previously (Reichart *et al.* in prep.).

Estimation of Relatedness

Relatedness coefficients for all possible pairwise comparisons of females (46 females from 2004 and 45 females from 2005) were calculated separately for each year using SPAGeDi (Hardy & Vekemans 2002). In SPAGeDi, we used the option to calculate relatedness using a method (based on comparisons of two homologous genes) estimated according to Queller & Goodnight's (1989) relatedness estimator, which utilizes likelihood methods applied to codominant genotype data. Relatedness estimates can range from $r = -1$ to $r = 1$, where negative one and positive one represent values at either extreme (maximally dissimilar and clones, respectively), and zero represents average relatedness of two randomly chosen individuals in the population.

In addition we also used SPAGeDi to test for a pattern of relatedness with respect to pairwise nest distances for nests of nesting females (those with estimates of relatedness, see above). We created a distance matrix including all pairwise comparisons for these nests. Pairwise distances between nests were entered as Euclidean distances calculated from UTM coordinates collected in the field. We specified distance intervals for analysis because SPAGeDi requires specific distance intervals that include both the minimum and maximum distance estimated, and at least 50% of all individuals in the population must be represented at least once in the interval (see SPAGeDi manual; Hardy & Vekemans 2002). We also used the option for permutation tests (option in SPAGeDi that permutes locations using a method equivalent to a Mantel test; Hardy & Vekemans 2002) which allowed us to test for a trend in relatedness vs. pairwise distance, and for a difference between observed and randomly permutated relatedness estimates for each distance interval. SPAGeDi then uses a regression of pairwise relatedness estimates on pairwise nest distances to test for differences in relatedness with respect to varying nest distance (across specific distance intervals). The output from this analysis was also used to

test if relatedness was highest for pairwise comparisons of females nesting in close proximity. Nests located between 1 – 500 m were considered in close proximity to one another, because this range included nests on the same wetland.

To specifically compare related estimates for host-parasite pairs, a distance of zero was entered in the nest distance matrix for all host-parasite comparisons, thus allowing SPAGeDi to recognize host-parasite pairs as a distinct group. We then compared relatedness estimates from host-parasite pairs to relatedness estimates of all other pairwise comparisons in the population.

For relatedness analyses, SPAGeDi generated multilocus estimates of relatedness for all pairwise comparisons. In addition for pairwise relatedness comparisons with respect to distance, SPAGeDi calculates an approximate standard error for each estimate using a method of jackknifing over all loci (Hardy & Vekemans 2002).

Statistical Analyses

We used linear regression to test if host-parasite relatedness varied across the breeding season by regressing pairwise relatedness of host-parasite pairs on date when host began incubation. We also tested for a pattern of relatedness of host-nesting parasite pairs with respect to timing of parasitic egg laying compared to the parasite's own nest initiation (categorized as: prior to nest initiation, during egg laying, after completing egg laying), using a Kruskal-Wallis test for multiple comparisons.

RESULTS

Spatial trend analysis and population relatedness

Overall population relatedness between nesting females was -0.033 ± 0.01 (mean ± SE) in 2004 and -0.032 ± 0.01 in 2005. Pairwise nest distances in 2004 ranged 9 m – 5 km and in 2005 ranged 6 m – 5 km. We found no spatial pattern of genetic relatedness, as pairwise relatedness coefficients between females were not significantly related to the distance between their nests (Figure 1a; 2004: $y = 0.000017 – 0.0000156x$, $P = 0.113$, 10 000 permutations, N = 46 females; Figure 1b; 2005: $y = -0.03994 + 0.00000404x$, $P = 0.757$, 10 000 permutations, N = 45 females). In addition, there was no difference in average relatedness for females nesting in closest proximity [2004: $r = 0.015 \pm 0.065$ (mean ± SE); 2005: $r = -0.019 \pm 0.033$ (mean ± SE)] compared to a randomly permuted value of relatedness in the population [2004: $r = - 0.023 \pm 0.059$ (mean ± SD), $P = 0.558$; 2005: $r = -0.023 \pm 0.037$ (mean ± SD), $P = 0.882$; 10 000 permutations, 2-sided test].

Host-parasite relatedness

Using genotypic data from ten microsatellite loci, we identified 46 host-parasite pairs (31 host-nesting parasite pairs and 15 pairs where we did not detect a nest for the parasite) in 2004, and 29 host-parasite pairs (22 host-nesting parasite pairs and 7 pairs where the parasite did not have a nest of her own) in 2005 (detailed methods in Reichart *et al.* in prep.). Relatedness among host-parasite pairs [2004: $r = 0.029 \pm 0.047$ (mean ± SE); 2005: $r = 0.055 \pm 0.077$ (mean ± SE)] was not significantly different compared to randomly permuted values of relatedness in the population for either year [2004: $r = - 0.023 \pm 0.037$ (mean ± SD), $P = 0.1179$; 2005: $r = 0.024 \pm 0.047$ (mean ± SD), $P = 0.078$; 2-sided permutation tests, Figures 1a, 1b].

We also examined whether host-parasite relatedness varied across the breeding season. Relatedness of host-parasite pairs was not correlated to the time when hosts began incubation

(simple linear regression: $R^2 = 0.002$, d.f. = 74, P = 0.530; Figure 2). In addition, there were no patterns of relatedness associated with timing of parasitic egg laying compared to the parasite's own nest initiation (Kruskal-Wallis test: $F_{2,50} = 1.773$, $P = 0.180$, N = 54 comparisons; Figure 3).

DISCUSSION

Spatial relatedness

We found no pattern of relatedness relative to distance between nests, as average relatedness between females nesting in close proximity did not differ from that of randomly chosen female pairs. Although female natal philopatry is common among waterfowl (Anderson *et al.* 1992), our data are not indicative of locally clustered kin groups facilitating CBP. Timing of settlement (e.g., with younger females arriving on the breeding grounds later than older individuals; Anderson *et al.* 1992), and ecological factors (e.g., availability of resources, reviewed in Anderson & Titman 1992) may influence settlement patterns more so than relatedness among individuals. Ruddy ducks are an over-water nesting species, and highly dependent on water conditions in wetlands each year (Bellrose 1980), thus females may not return to the exact location of hatch (as in other species such as mallards, D. Coulton pers.comm.) due to changes on the landscape each year. Thus, the lack of local female relatedness structure in ruddy ducks, indicates that parasites cannot preferentially parasitize (or avoid) close relatives by merely parasitizing (or avoiding) neighboring nests. The lack of spatial structure observed in ruddy ducks is consistent with some other studies (Nielsen *et al.* 2006; Andersson & Waldeck 2007), where female philopatry alone, can not explain the presence of CBP.

Host-parasite relatedness

Our analysis revealed that overall, host-parasite pairs are not any more or less related to each other than they are to a randomly selected pair. Although there are some cases where host-parasite pairs were more related to each other than a randomly chosen pair, our data indicate the role of relatedness is quite variable among individual pairs. For female ruddy ducks, given the lack of female relatedness structure, it is not surprising that overall host-parasite pairs are unrelated. Females do not appear to have a mechanism of detecting parasitic eggs (and possibly no mechanism of kin recognition) as ruddy ducks did not eject eggs laid parasitically by redheads and readily incubated non-viable chicken eggs (see methods above; Reichart pers. obs.). The only direct response to parasitism seemed to occur when clutch sizes were greater than 13 eggs, and females abandoned nests (Reichart pers. obs.), a similar response observed in common goldeneye nests (Eadie *et al.* 1995).

In general, the role of relatedness is variable across brood parasitic species. To date several studies have found high levels of relatedness among host-parasite pairs (e.g., Andersson & Åhlund 2000; Andersson & Waldeck 2007), and Nielsen *et al.* (2006) found high levels of relatedness in a subset of host-parasite pairs. In contrast, three other studies have found different patterns of relatedness between host-parasite pairs: one study found that parasites appeared to avoid kin (Semel & Sherman 2001), whereas two others found that parasites do not discriminate between related or unrelated hosts (Pöysä 2004; and an unpublished study (J.M. Eadie & R. Fernando) referenced in Lyon & Eadie (2000) of barrow's goldeneye (*Bucephala islandica*)).

One possible explanation for the conflicting patterns of relatedness between host-parasite pairs seen across species is that perhaps the degree of kin recognition is highly variable among

species. Both models presented by Andersson (2001) and López-Sepulcre & Kokko (2002) showed the importance of kin recognition for conspecific brood parasites. An accurate method of kin recognition seems incredibly important when potential costs of parasitism to the host are high, and parasites should avoid parasitizing relatives (López-Sepulcre & Kokko 2002). Pöysä (2004) presented a compelling model using data collected from a Finnish population of common goldeneyes (an extension of the model presented by López-Sepulcre & Kokko 2002), and found no support for selection favoring parasites who preferentially parasitize relatives. Although Pöysä (2004) found CBP should not be favored even among mother-daughter and sister relationships (assuming perfect kin recognition) actual relatedness estimates between host-parasite pairs were never measured. Pedigree analysis for this marked population (Ruusila *et al.* 2000) can provide reliable estimates of mother-daughter relationships, but it seems less likely to provide accurate estimates for sisters, as relatedness could be diluted via extra-pair copulations (males defend females during egg laying in this species, but extra-pair copulations have not been documented, Eadie *et al.* 1995). Nonetheless, results from Pöysä (2004), emphasize the importance of a kin recognition system for kin selection to promote the evolution of CBP (Andersson 2001; López-Sepulcre & Kokko 2002).

Another possible explanation for the conflicting patterns of relatedness between host-parasite pairs seen across species may be that kin selection is a relatively weak selective force compared to other ecological factors influencing the evolution of CBP. For example nest predation in various populations may have a stronger selective effect on CBP than kin selection (perhaps explaining different patterns of expected host-parasites relatedness for multiple populations of common goldeneye). The idea of risk spreading (a tactic to minimize predation risk and ensure at least one offspring successfully survives) was theoretically refuted (Bulmer

1984) soon after it was proposed. However, a recent paper by Pöysä & Pesonen (2007) show risk assessment of predation risk (a derivative of the risk spreading hypothesis) may provide a mechanism by which CBP could evolve. In this study, nests were not depredated at random and parasitic common goldeneyes (*Bucephala clangula*) preferentially laid parasitic eggs in safe nests. Risk assessment by parasitic goldeneyes is thought to occur via nest site prospecting, occurring after offspring hatch from nests (Pöysä 2006). In ruddy ducks, risk assessment could occur prior to females initiating nests, because ruddy ducks nest late in the season after most other waterfowl species have hatched (Brua 2001; Reichart pers.obs.). Previously we found the percent nests depredated on wetlands was not correlated to parasitism on wetlands (Reichart *et al.* in prep.). However, females may still be able to assess predation risk for nest sites and nest predation could play a role in the evolution of CBP in ruddy ducks. Although we do not have strong evidence that females use a mechanism of habitat assessment, we observed at least 2 cases where nesting females successfully hatched a clutch in 2004, then returned to the same wetland to lay a second clutch in 2005. Therefore nesting female ruddy ducks return to an area where they were previously successful, perhaps hinting that females could assess the relative safety of a nesting site, thus providing an alternative mechanism by which CBP could evolve in ruddy ducks.

One other explanation for the differences in patterns of relatedness among species and populations of conspecific brood parasites may be due to variation in the costs and benefits of parasitism to hosts and parasites (Lyon & Eadie 2000). In brief, host-parasite relatedness is most likely to occur when costs of parasitism to the host are low and related parasites are allowed to lay eggs parasitically, without resistance from the host (Andersson 2001). This implies that hosts will resist unrelated parasites, but the cost of resisting parasites may vary depending on the life

history characteristics of the species involved. For example cavity nesters will likely have an easier time defending a nest against unwanted females, because they can simply block the entrance to the cavity, but defending an overwater nest is more difficult because hosts may have to expend more energy in nest defense (Andersson 2001). Additionally, hosts could gain a direct benefit from parasitism (e.g., dilution of predation risk by having more offspring in the nest) and may not be able to discriminate between their own eggs and foreign eggs, thus relatives and non-relatives could lay eggs parasitically (López-Sepulcre & Kokko 2002). For ruddy ducks, parasitism may not be very costly to hosts, except in a few cases when females abandon nests with more than 13 eggs, although the costs and benefits of CBP have not been thoroughly evaluated. Also they do not readily eject parasitic eggs from the nest (including heterospecific eggs, see above), perhaps implying that parasitic eggs do not pose a significant cost when there are only a few additional eggs.

Finally one last explanation could be that ruddy duck females deposit their parasitic eggs indiscriminately according to opportunity (i.e., similar to the pattern observed in moorhens, *Gallinula chloropus*, McRae 1998). These results are consistent with our previous finding that CBP occurs as a mixed reproductive strategy in ruddy ducks, where most females lay eggs parasitically and almost all parasites are nesting females (Reichart *et al.* in prep.); however it does not exclude the possible influence of other explanations mentioned above. In addition, although overall, host-parasite pairs were unrelated, there were a few cases where host-parasite pairs were more related than a randomly chosen pair. Variation in relatedness among host-parasite pairs was predominant later in the breeding season, perhaps indicating some association among kin. Some females could arrive on the breeding grounds in kin groups, potentially increasing the possibility for interactions among kin.

Conclusions

Ruddy ducks in our study population showed no spatial pattern of relatedness. Overall relatedness among host-parasite pairs was relatively low and not significantly different than background levels of relatedness in the population, thus kin selection is not a likely explanation for the evolution of CBP in ruddy ducks. Our results further emphasize that the role of relatedness among populations of conspecific brood parasites remains variable across species, perhaps suggesting kin selection may be a weak selective force influencing CBP in some species more than others. In addition, our results suggest costs of CBP to hosts in ruddy ducks may not be high, however, a thorough examination of the costs and benefits of CBP in ruddy ducks may reveal additional factors influencing a female's decision to lay eggs parasitically.

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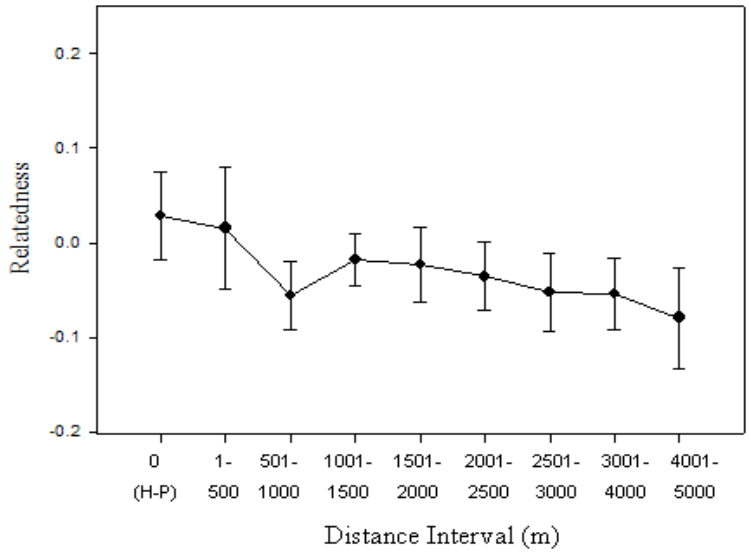
a)



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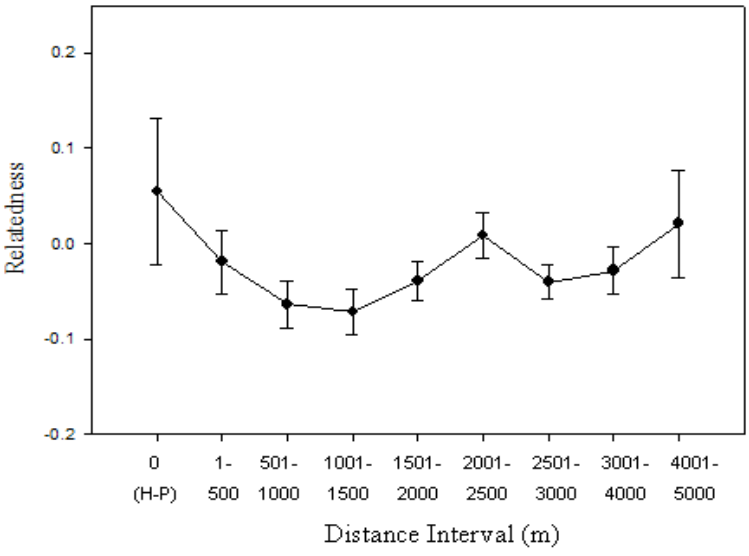

Figure 1. Pairwise relatedness estimates relative to pairwise distance estimates for female ruddy ducks captured in (a) 2004 and (b) 2005. Distance zero represents host-parasite comparisons in a nest. Error bars represent standard errors for each mean multi-locus relatedness estimate. The pattern indicates no spatial trend in relatedness.

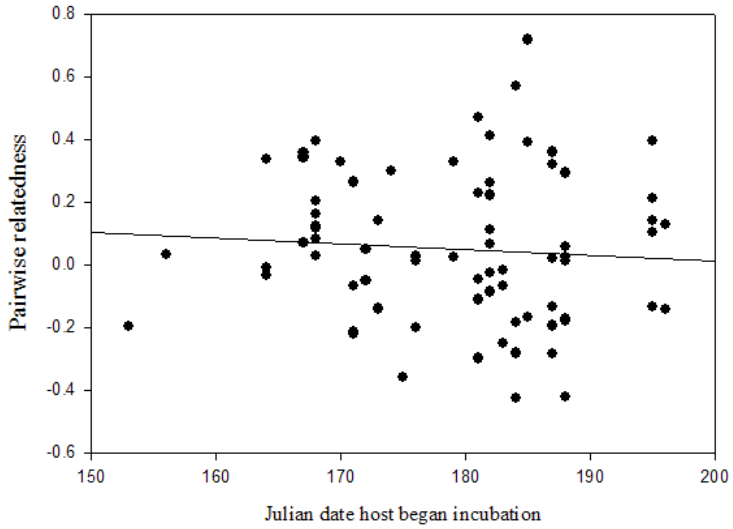

Figure 2. Pairwise relatedness of host parasite pairs was not correlated to time when the host began incubation. (Simple linear regression: $R^2 = 0.005$, d.f. = 74, $P = 0.53$)

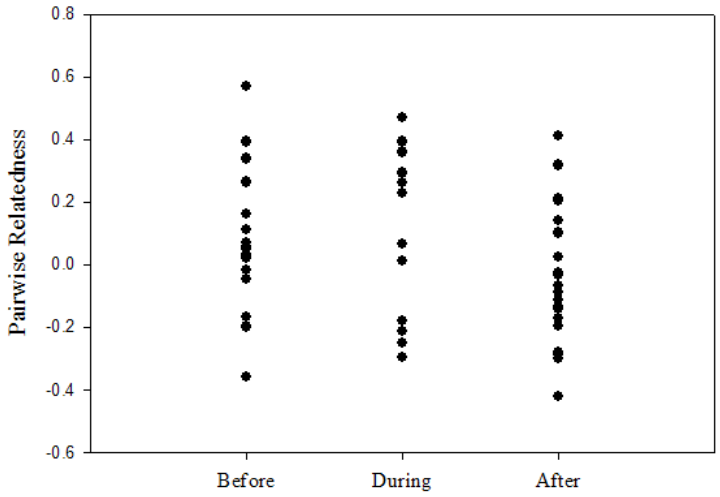

Figure 3: Pairwise relatedness of host parasite pairs (host nesting parasite) compared to the time a parasitic egg was laid, relative to the parasite's nest initiation. Each dot represents a single host parasite pair. Before ($N = 20$), During ($N = 12$), After ($N = 22$) represents parasitic eggs laid before, during or after a parasite's nest initiation.

CHAPTER THREE

NO DIFFERENCE IN THE CHARACTERISTICS OF PARASITIC AND NON-PARASITIC EGGS IN RUDDY DUCKS, BUT PARASITIC YOUNG ARE MORE LIKELY TO BE MALE

INTRODUCTION

Conspecific brood parasitism (CBP) occurs when a female lays an egg in the nest of a conspecific female, and is a female reproductive strategy that occurs in over 236 species of birds (e.g., Yom-Tov 1980; Yom-Tov 2001). Conspecific brood parasites provide no parental care to their parasitic offspring; instead host females incur the costs of raising those offspring. Because parasitic females completely surrender care of their young to another female, successful parasitism likely requires proper host choice, synchronization with the host's laying cycle, and possibly offspring traits that will allow successful competition with the host's own young.

Most previous studies of parasite adaptations have focused on obligate (interspecific) brood parasites, where females reproduce only via parasitism and adaptations are expected to be well developed. Results from these studies have revealed an array of adaptations that allow them to successfully parasitize heterospecifics (Hauber & Dearborn 2003), including behavioral strategies (e.g., timing and frequency of egg laying, chick begging intensity, shorter incubation time), physiological traits (e.g., rapid embryo and chick development), and morphological features (e.g. stronger egg shells, mimicry of host eggs or nestlings) (Friedmann 1929; Rothstein 1990; Hauber *et al.* 2000; Hauber & Sherman 2001; Dearborn & Lichtenstein 2002).

In contrast, it is unclear whether conspecific parasites employ tactics, comparable to the adaptations seen in obligate parasites, that might increase the success of their parasitic eggs, as these have been rarely investigated (Reed & Vleck 2001; Lyon 2003a; b). For example, parasitic females might invest differentially in parasitic versus non-parasitic eggs based on their relative likelihood of success. It is possible that such differential investment in eggs is particularly important in highly precocial species, as in these species offspring are independent immediately after hatch and survival can depend heavily on egg characteristics (Pelayo 2001; Pelayo & Clark 2003). However, the possible relationship between parasitism and egg characteristics has not been examined in any precocial species. There are several possible ways in which parasitic eggs may differ from non-parasitic eggs.

First, research over the past decade has made it clear that the eggs of females from many species contain androgenic hormones in their eggs (Schwabl 1993; Gil *et al.* 1999; Groothuis *et al.* 2005), and the amount of these hormones in eggs varies both across and within clutches (Schwabl 1993; Groothius & Schwabl 2002). Most research testing the adaptive significance of maternally-derived yolk hormones has been conducted for altricial or semi-precocial birds (Gil 2003; Andersson *et al.* 2004). This research has indicated that maternal androgenic hormones are important for embryonic development, and that higher hormone levels are correlated with shorter development time (Gorman & Williams 2005; Schwabl *et al.* 2007). Testosterone (T) is assumed to be the most influential hormone in offspring development for altricial birds, but little is known about effects of other androgens and androgenic hormones in precocial species such as waterfowl (Reed & Vleck 2001; Andersson *et al.* 2004). If androgenic hormones accelerate embryo development, then it may be beneficial for females lay parasitic eggs with higher levels of androgen, as this would increase the probability of parasitic eggs hatching with or even before

host eggs (Davies 2000). To date only two studies have explored this hypothesis (Pilz *et al.* 2005; Vedder *et al.* 2007). These studies found no association between yolk hormones and CBP, both of which focused on species with altricial offspring that require post-hatch parental care for survival. Yet yolk hormones that accelerate development may be particularly important for highly precocial species in which young leave the nest soon after hatching, as late-hatching eggs are likely to be abandoned. Therefore, we hypothesized that parasitic eggs will contain higher amounts of androgenic hormones than host eggs within a nest.

Second, egg size is a critical trait that can affect hatching success, growth and survival of offspring in many species. For example, young hatched from larger eggs have a higher probability of survival, (Williams 1994; Pelayo 2001; Christians 2002; Pelayo & Clark 2003). Accordingly, females may lay larger eggs to compensate for other factors that may reduce offspring survival. For example female collared flycatchers (*Ficedula albicollis*) lay larger eggs at the end of the laying sequence helping to mitigate costs of offspring survival created via hatching asynchrony (Rosivall *et al.* 2005). Given that the likelihood of successful hatch differs substantially between parasitic and non-parasitic eggs (Lyon 1993; McRae 1998), we hypothesized that parasitic eggs produced by a female would be larger than her non-parasitic eggs, to increase the likelihood of success for the parasitic eggs.

Finally, in many species differences in egg quality may be associated with offspring sex, a factor that could vary with the reproductive value of an individual offspring (Mock & Parker 1997). The reproductive value of each sex is expected to be equal (in diploid populations), and females should produce an equal number of sons and daughters (Fisher 1958). Alternatively, the reproductive value for each sex may differ. For example, local resource competition in species with sex biased natal dispersal would favor production of the dispersing sex, to minimize future

competition between parents and offspring (Gowaty 1993; Wild 2006). In waterfowl, male offspring disperse from the natal area (Anderson *et al.* 1992), thus females should produce male biased clutches if resources are limited. The overall offspring sex ratio (parasitic and non-parasitic eggs) produced by nesting parasites would be male biased. Even in this case, we predict no difference in the sex of parasitic and non-parasitic eggs laid by nesting parasites, given parasitic and non-parasitic eggs have an equal likelihood of successful hatch. However given the likelihood of successful hatch differs between parasitic and non-parasitic eggs (Lyon 1993; McRae 1998), if parasitic offspring are less likely to hatch, then in this case parasitic offspring should be female biased to support the local resource competition hypothesis in waterfowl. To date no study has investigated offspring sex among parasitic and non-parasitic offspring produced by individual females.

Here we present the first study to investigate possible parasitic adaptations, realized through egg characteristics, for a highly precocial conspecific brood parasite, the ruddy duck (*Oxyura jamaicensis*). This is among the first studies to examine physiological differences between parasitic and non-parasitic eggs (Pilz *et al.* 2005; Vedder *et al.* 2007). Specifically, we tested for differences in hormone concentrations, egg size and offspring sex between parasitic and non-parasitic ruddy duck eggs. Ruddy ducks are an ideal species to test for such differences between parasitic and non-parasitic eggs because they produce extremely expensive eggs (see below), ducklings are highly precocial at hatch (see below), and ruddy duck females readily lay eggs parasitically in the nests of both conspecifics and heterospecifics (Misterek 1974; Siegfried 1976; Joyner 1983; Reichart *et al.* in prep.).

MATERIALS AND METHODS

Study Species

Ruddy ducks are members of the stiff-tail subfamily (Oxyurinae). Ruddy duck eggs are the largest relative to body size among all waterfowl species (Lack 1967). Egg-laying appears to be more costly for female ruddy ducks than for most other waterfowl: female ruddy ducks typically lay a clutch of 6 – 8 eggs (Joyner 1975), with each egg weighing approximately 14% of a female's body weight (Gray 1980; Alisauskas & Ankney 1992; 1994a; Pelayo & Clark 2002). Maximum daily cost (584kJ) of egg production is 280% of basal metabolic rate (Alisauskas & Ankney 1994b). Females incubate the eggs for approximately 24 days (Bellrose 1980; Brua 1998). Resource requirements during egg laying and incubation are high, with females consuming up to 6,000 midge larvae (*Chironomidae*) per hour during this period (Gray 1980; Carbonell 1983; Tome 1987). Ruddy ducks are single brooded and few females renest after nest loss, likely due to high nutrient demand of egg formation and the relatively late breeding season (Brua 2001).

Ruddy ducks have highly precocial offspring, who hatch with good fat reserves for insulation allowing them to dive and forage on their own immediately after hatching (Lack 1967; Sommerville 1985). Adult females stay with a brood from 28 to 42 days after hatching to provide minimal parental care, mainly in the form of vigilance for predators (Joyner 1975; Gray 1980). Females do not feed their offspring and some brood amalgamation behavior has also been noted (Joyner 1975). Offspring survival is high (Bellrose 1980), and ducklings from larger eggs have a higher probability of survival (Pelayo 2001; Pelayo & Clark 2003).

Field data and sample collection

This research was carried out during the ruddy duck breeding season (mid-May to early August) each year from 2003-2005 in the prairie pothole region of Minnedosa, Manitoba, Canada (50º10'N, 99 º47'W). This area is characterized by numerous permanent and ephemeral wetlands ranging in size from 0.1 to >4.0 ha (Pelayo & Clark 2003). Wetland margins contain various types of emergent vegetation including cattail (*Typha* spp.), hardstem bulrush (*Scirpus acutus*), cane (*Phragmites communis*), and whitetop rivergrass (*Scholochloa festucacea*). Nesting habitat for ruddy ducks is abundant in this region because females build nests overwater in the emergent vegetation.

We trapped adult ruddy ducks prior to nesting using floating mist nets (Breault & Cheng 1989). Once nesting began we also trapped adult females during late incubation (eggs incubated approximately 18-20 d) using drop door nest traps (Weller 1957). At the time of capture adults were weighed to the nearest gram using a Pesola spring scale and standard morphological measures were taken (e.g. tarsus length, culmen length, bill width, skull, wing length). We marked females using a temporary, modified nasal disc (Pelayo & Clark 2000), and USFWS/CWS leg bands. Approximately 100 µl of blood was collected from the tarsus vein from each trapped individual using a 25-gauge sterile needle and a heparinized capillary tube, then transferred to 1.5mL eppendorf tubes containing 1 mL of lysis buffer (White & Densmore 1992).

Ruddy duck nests were found by systematically searching the emergent vegetation around wetlands from mid-May to late July. When nests were found, nest location was marked using a numbered stake placed on land 5 to 15 meters away. We never created a direct path from land to nests to reduce the risk of increased nest predation. Individual eggs in nests were numbered, using a felt tipped marker and we measured both the length and breadth of each egg

in the nest to the nearest 0.01 mm. Egg volume was calculated using Hoyt's formula (1979): Volume = 0.515*length*breadth^2. Embryonic development (identified via incubation stage) was assessed using an egg floatation method specifically developed for ruddy ducks (Brua & Machin 2000). We estimated lay initiation date by back-dating from the stage of embryonic development. Nests were rechecked every 7 days to evaluate development stage and/or nest fate.

To reduce loss of samples – i.e., due to predation and/or because the precocial young leave the nest very soon after hatching – we trapped females during late incubation (see above) and replaced their clutches with an identical number of non-viable chicken eggs (Pelayo & Clark 2003). The removed ruddy duck eggs were artificially incubated in air-circulating cabinet incubators at 37ºC, 67-70% humidity. When the incubated eggs hatched, we recorded the egg from which individuals hatched and kept ducklings in brooders until dry (approximately 3- 4 h). All ducklings were then banded with a federal duckling band (wood duck size B, prepared with a clay mixture of 50% Chavant DaVinci soft and 50% Roma Plastilina #1; Blums *et al*. 1999), and weighed to the nearest .01 g. We also collected blood samples from all ducklings using the methods above, with the exception that we used 27.5 gauge needles. Ducklings were then returned to nests of origin; nest females incubated chicken eggs for <5 days before their ducklings were returned.

We collected blood samples from 162 adults and 516 ducklings from 80 nests for genetic analysis. We also collected egg albumin and egg yolk from nests found during laying (eggs not yet incubated). To collect albumin and yolk we punctured a small hole through the egg shell, removed approximately 200-300 µl of egg albumin with a syringe, inserted a second syringe and removed approximately 20 -30 mg of yolk, then sealed the hole with super glue. Yolk samples were placed in numbered, pre-weighed (to the 0.001 g) eppendorf tubes containing 500 µl of

dH_2O. Yolk samples were vortexed to create a water/yolk homogenate, then reweighed to obtain the exact yolk mass collected (to the 0.001 g). Both albumin samples and yolk samples were then placed at -20ºC until protein fingerprint and yolk hormone analyses (see below). We collected albumin samples from 148 eggs in 29 nests and yolk samples from 113 eggs in 28 nests. For some eggs, only albumin was sampled, because it can be sampled 0 – 2 d after laying (Andersson & Åhlund 2001), but yolk samples must be collected when eggs are fresh (i.e. the day of laying) to accurately assess hormones attributed to the egg laying female, not endogenous steroid produced by the developing embryo.

Yolk steroid extraction and radioimmunoassay

Yolk steroids were extracted from eggs following methods in Schwabl (1993). Briefly, we extracted yolk steroids from yolk homogenates with diethyl ether/ petroleum ether. Lipids were removed from samples, then steroids were separated using diatomaceous earth column chromatography. We used a standard radioimmunoassay protocol following Schwabl (1993) to measure steroid concentrations in ruddy duck eggs for the following measurable yolk hormones: androstenedione (A_4), testosterone (T), 5α-dihydrotestosterone (5α-DHT). We used the following antibodies in the assay: T 3003 (Wien Laboratories) for T and 5α-DHT, and A 1707 (Wien Laboratories) for A_4. The following ^{3}H-labeled steroids were also used for the assay: NET 553 (T), NET 544 (5α-DHT), and NET 469 (A_4) were obtained from PerkinElmer Life and Analytical Sciences. Recoveries averaged 40.8 ± 0.1 % (mean ± SD) for A_4, 41 ± 0.1 % for T and 24 ± 0.1 % for 5α-DHT. Intra-assay variation was 8.3 % for A_4, 13.9 % for T, and 14.2 % for 5α-DHT. Inter-assay variation was 29 % for A_4, 20.7 % for T, and 20.5 % for 5α-DHT.

Protein Fingerprinting

All protein fingerprint analyses were conducted in Malte Andersson's lab at Göteborg University, Sweden, from November to December 2005. Protein fingerprinting uses isoelectric focusing (IEF) to separate proteins across an electric field applied over a fixed pH gradient in precast gels; proteins are visualized as narrow bands on a gel representing their isoelectric points (Righetti 1990). Egg albumin is solely of maternal origin, therefore representing the genotype of the female that laid the egg rather than the developing embryo. Andersson & Åhlund (2001) demonstrated that protein bands are both reproducible and genetically polymorphic across females, and accordingly has resolution sufficient to detect parasitic eggs and sometimes also the females who laid those eggs.

Detailed methods for protein fingerprinting are described in Reichart *et al.* (in prep). Briefly, we ran all individuals from each nest together on four different gel types, and scored all protein bands identifiable as present or absent for each individual. When one or more eggs from a nest had differing band patterns on at least one gel type the nest was scored as parasitized (details in Reichart *et al.* in prep). The most common albumin pattern among eggs in a nest was assumed to represent the nesting (host) female, as demonstrated by Andersson & Åhlund (2001).

DNA Microsatellite Methods

We extracted genomic DNA from blood samples using standard phenol-chloroform methods after digestion with proteinase-K solution (Westneat 1990). We used variation at 10 microsatellite loci to identify parasitic ducklings and maternity for a subset of those individuals (Reichart *et al.* in prep). Eight of these loci (*Oxy3, 6, 10, 13, 14, 15, 17,* and *19*) were developed specifically for ruddy ducks (Munoz-fuentes *et al.* 2005), and two (*Blm*5 and *12*) were developed

for the closely related Musk duck (*Biziura lobata*; Guay & Mulder 2005). Initially an eleventh locus (*Oxy*4) was included in the analysis, but it showed a high frequency of null alleles (0.287) and we discarded it from analyses.

To determine microsatellite genotypes for all individuals, we amplified genomic DNA from each individual in 10µl PCR reactions. Detailed methods for PCR amplification of microsatellites and criteria used to identify parasitic ducklings and assign maternity are described in Reichart *et al.* (in prep).

Molecular sexing

We determined the sex of 207 ducklings from 30 complete nests (all offspring sampled) using a molecular sexing technique that amplifies an intron in the *CHD* gene on the avian sex chromosomes (Kahn *et al.* 1998). We used the primer pair 1237L/ 1272H (Kahn *et al.* 1998) which produces one band in males (2 copies of the Z-chromosome) and two bands in females (one each from the W- and Z-chromosomes). The following PCR mix (10 µl total volume) was used for amplification with the 1237L/ 1272H primer pair: approximately 25 ng of genomic DNA, sterile H_2O, 1.00 X PCR buffer, 2.00 mM $MgCl_2$, 0.5 µM of each primer, 0.15 mM of each dNTP, and 2.5 units of *Taq* polymerase. PCR conditions included initial heating at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing 57°C for 1 min, extension at 72°C for 45 sec, then a final extension at 72°C for 5 min.

PCR products were visualized using agarose gel electrophoresis on 3 % agarose gels stained with EtBr. We ran gels at 100 V for approximately 1 h, and photographed them under UV light. For controls, we included PCR products of known different sex individuals on each gel (2 per gel) and compared band patterns produced from known sex individuals to band

patterns observed in ducklings. All individuals were visually scored, where females have two bands and males have a single band (Figure 1).

Statistical analyses

For yolk androgens found in ruddy duck eggs, we report raw data (pg / mg yolk) as mean ± SE for each androgen. Prior to statistical analyses, yolk androgen concentrations were log transformed [log(yolk androgen value +1)] to normalize the data. To test for a pattern of yolk androgens across laying sequence, we used repeated measures ANOVA for known laying sequence nests. Specifically we used PROC MIXED (SAS System for Windows, Version 9.1) a mixed-model repeated measures ANOVA, with eggs within a clutch as the repeated measure and female as a random effect. Mixed-model ANOVA was used over Model I (fixed effects) ANOVA because of the unbalanced and nested structure of our data. We also used mixed-model ANOVA to test for androgen variation among females, using eggs genetically assigned to individual females (to eliminate differences attributed to parasitic eggs). To test for androgen variation between parasitic and non-parasitic eggs laid by females (nesting parasites), we also used a mixed-model repeated measures ANOVA, where eggs laid by females (identified as parasitic or non-parasitic) were the repeated measure and female was a random effect.

Egg volume was not normally distributed; thus we used log transformation [log(egg volume)], which successfully normalized the data. Similar to yolk androgen statistical analyses (above), we used mixed-model repeated measures ANOVA to test for variation of egg volume among females and also for variation within a females's laying sequence (known laying sequence eggs, excluding parasitic eggs). To test for differences in egg volume between parasitic and non-parasitic eggs laid by females (nesting parasites) we also used mixed-model repeated measures

ANOVA similar to the analysis for yolk androgens (above). Mixed-model ANOVA was also used to test for differences between male and females eggs laid by females (nesting parasites only). We used simple linear regression to test for a linear relationship between egg volume and duckling mass at hatch.

We used Neuhauser's (2004) optimally weighted estimator test designed for clustered binomial data to test if the primary sex ratio (i.e., the proportion of male offspring per brood) for the overall population varied significantly from unity. We used this test instead of a chi-squared test because the data was clustered and the sex of ducklings from the same brood cannot be considered independent. We used this estimator to measure the primary sex ratio of all offspring in nests (parasitic and non-parasitic), non-parasitic offspring in nests, and for parasitic offspring in nests. We used chi-squared tests to test for differences in primary sex ratio between years, and also between non-parasitic nest types (non-parasitic nests that were parasitized and non-parasitic nests that were not parasitized) to assess whether data sets could be pooled.

In ruddy ducks, most parasitic females also nest independently (i.e., produce a non-parasitic brood). Accordingly, we also tested the primary sex ratio produced by nesting parasites (females laying both non-parasitic and parasitic eggs). We identified both parasitic and non-parasitic eggs for 14 nesting parasites (Reichart *et al.* in prep). Again, we used Neuhauser (2004) to identify the primary offspring sex ratio among all eggs, non-parasitic eggs, and parasitic eggs laid by nesting parasites.

In addition, we tested for possible effects of laying order on offspring sex. First we calculated the interval between laying of a parasitic egg and initiation of the parasitic female's own nest (i.e., nesting parasite's nest initiation date minus the laying date of her parasitic egg). One-way ANOVA was used to test for differences in egg laying intervals between male and

female parasitic offspring. To further assess possible effects that could be attributed to laying order, we used a chi-square test to examine differences in offspring sex for parasitic eggs laid prior to, during, and after a parasite produced her own (non-parasitic) brood.

Overall, samples sizes vary among tests because it was not possible to collect data on all variables from each individual.

RESULTS

Yolk Hormones

Ruddy duck eggs contained 23.62 ± 2.01 (Mean ± SE) pg/mg yolk A_4 (N = 121 eggs); 1.76 ± 0.30 pg/mg yolk T (N = 116 eggs); and 1.77 ± 0.14 pg/mg yolk 5α-DHT (N = 118 eggs). Yolk androgen concentrations of eggs in nests were not associated with laying order (Repeated measures ANOVA; A_4: $F_{5,31} = 1.42$, $P = 0.245$; T: $F_{5,30} = 0.44$, $P = 0.814$; 5α-DHT: $F_{5,30} = 0.78$, $P = 0.574$; N = 10 nests). We found significant variation of yolk androgen concentrations among females (parasitic eggs removed) for A_4, but not for T or 5α-DHT (Repeated measures ANOVA; A_4: $F_{9,31} = 2.65$, $P = 0.021$; T: $F_{9,30} = 1.38$, $P = 0.239$; 5α-DHT: $F_{9,30} = 1.03$, $P = 0.439$; N = 10 nests). However, non-parasitic and parasitic eggs within a nest (N = 7 nests) did not differ in androgen concentrations (Repeated measures ANOVA; A_4: $F_{1,6} = 0.02$, $P = 0.900$; T: $F_{1,6} = 5.60$, $P = 0.056$; 5α-DHT: $F_{1,6} = 1.35$, $P = 0.290$).

Egg volume

Egg volume varied significantly between females, but there was no pattern associated with laying order and no significant difference in egg size for eggs within a female's nest (analysis restricted to eggs laid by the female incubating the nest, N= 13 nests of known laying

sequence; repeated measures ANOVA; between nests: $F_{12,58} = 5.18$, $P < 0.001$; within nests: $F_{7,58} = 1.87$, $P = 0.092$).

To test for differences in egg volume between parasitic and non-parasitic eggs, we identified eggs laid by 13 nesting parasites (females who laid both parasitic and non-parasitic eggs). We found no significant difference in egg volume for parasitic and non-parasitic eggs laid by the same female (Repeated measures ANOVA: $F_{1,12} = 1.03$, $P = 0.331$). In addition, there were no differences in egg volume for male and female eggs laid by nesting parasites (Repeated measures ANOVA: $F_{1,12} = 0.24$, $P = 0.632$).

Although we found no significant differences in egg volume between parasitic and non-parasitic eggs or male and female eggs, egg volume was significant predictor of duckling mass at hatch (range: 37.5 – 59.6 g; $N = 429$, $r^2 = 0.64$, $F_{1,427} = 771.79$, $P < 0.0001$; Figure 2).

Offspring sex

We identified the sex of 207 offspring from 30 broods. The overall population offspring sex ratio did not differ from parity (Figure 3, 48.7% males; $z = -0.36$, $P = 0.7181$), with no significant difference between years ($\chi^2 = 0.173$, d.f. = 1, $P = 0.677$). However, the offspring sex ratio for non-parasitic offspring ($N = 29$ nests; where 13 were not parasitized and 17 were parasitized) was significantly female biased (Figure 3, 40.0% males; $z = -2.32$, $P = 0.020$); the offspring sex ratio of non-parasitic eggs did not differ significantly between nests that were parasitized and nests that were not ($\chi^2 = 0.004$, d.f. = 1, $P = 0.949$). Balancing this, the overall sex ratio for parasitic offspring was significantly male-biased (Figure 3, 71.3% males; $z = 4.81$, $P = 0.0001$, $N = 17$ nests containing 53 parasitic offspring).

In addition, we restricted analyses to include only nests of nesting parasitic females (Figure 4). Overall offspring sex ratio of nesting parasites was not significantly different from parity (51% males, $z = 0.30$, $P = 0.382$, $N = 14$ nests containing 86 offspring). The offspring sex ratio of non-parasitic eggs laid by nesting parasites was not significantly different from parity (46.1% males, $z = -0.62$, $P = 0.268$, $N = 14$ nests containing 63 offspring). In addition, although the offspring sex ratio of parasitic eggs laid by nesting parasites trended toward male bias, it was not significantly different from parity (62.8% males, $z = 1.36$, $P = 0.09$, $N = 14$ nests containing 24 offspring).

The male bias of parasitic eggs found across all nests (above) might be due to a laying order effect if early-laid eggs are more likely to be male. To test this possibility we first calculated the interval between the nesting parasite's initiation date and laying date of her parasitic egg ($N = 53$ comparisons). On average female parasitic eggs were laid 1.84 ± 1.64 (mean ± SE) days prior to parasites' own nest initiation and male parasitic eggs were laid 3.82 ± 1.89 days prior to parasites' own nest initiation (Figure 5). There were no significant differences between egg laying intervals between male and female parasitic offspring (one-way ANOVA: $F_{1,51} = 0.494$, $P = 0.485$, $N = 53$ parasitic offspring). In addition we found that male and female parasitic eggs were equally likely to be ($N = 53$) laid prior to, during, and after a parasite's own egg laying sequence ($\chi^2 = 0.486$, d.f. = 2, $P = 0.784$).

DISCUSSION

Egg characteristics

We found that ruddy duck eggs contained varying amounts of all three yolk androgens, but A_4 occurred in the highest concentration. Yolk androgens did not vary across female laying

sequence and only A_4 varied significantly among females. In general concentrations of yolk androgens in ruddy ducks are relatively similar to those found in other species (e.g., Schwabl 1997; Eising *et al.* 2003; Göth *et al.* 2008). In addition an analysis across avian developmental modes showed no differences in yolk T and A_4 concentrations between the two extreme developmental modes (altricial vs. precocial) when controlling for body mass and phylogeny (Gil *et al.* 2007).

Although previous research with altricial and semi-precocial species have shown that higher concentrations of androgenic hormones can provide beneficial developmental effects for individual offspring (e.g. earlier hatching, increased begging, increased growth; reviewed in Groothuis *et al.* 2005), it is unclear whether yolk androgens have similar effects in precocial species (but see Andersson *et al.* 2004; Göth *et al.* 2008). Moreover, we found no differences in yolk androgen content between parasitic and non-parasitic eggs within a nest, indicating that deposition of egg yolk androgens do not play a role in CBP in ruddy ducks.

Egg volume in ruddy ducks is a significant predictor of duckling size at hatch, where larger eggs produce larger ducklings, and in this same population larger ducklings have a higher probability of survival (Pelayo & Clark 2003). These patterns are also consistent with those found in some other species, including lesser scaup (*Aytha affinis*, Dawson & Clark 1996), snow geese (*Chen caerulescens*, Ankney 1980), and king eiders (*Somateria spectabilis*, Anderson & Alisauskas 2002). However, additional physiological characteristics of eggs (e.g., egg yolk mass) may explain more of the variation associated with offspring survival (Cunningham & Russell 2000; Nager *et al.* 2000).

We also found significant variation in egg volume among females, suggesting that some females are capable of laying larger eggs than those laid by other females. Overall, egg size did

not vary across the laying sequence and multiple eggs (parasitic and non-parasitic) laid by nesting parasites were not significantly variable in size. In addition there were no differences in egg size between male and female eggs within a nest. These patterns are consistent with Christians (2002), who found that although egg size varies among females, intra-clutch egg size varies relatively little in most avian species.

Overall, we found that parasitic and non-parasitic eggs laid by nesting parasites do not differ in any of the egg characteristics that we measured. Our results are consistent with two previous studies addressing a possible role of yolk androgens and egg size in altricial species (Pilz *et al.* 2005; Vedder *et al.* 2007). CBP is common in ruddy ducks and females participate in a mixed reproductive strategy, where females lay eggs parasitically in addition to nesting (Reichart *et al.* in prep). Pilz *et al.* (2005) studied the European starling (*Sturnis vulgaris*), where parasitic eggs tend to be laid by low quality females. Vedder *et al.* (2007) studied blue tits (*Cyanistes caeruleus*) where CBP is reportedly rare, and seems to occur as a result of limited nest site availability. Both studies suggested that a lack of an effect could be due to the high costs of investing in eggs, where low quality females would be unable to do so. High costs of egg production is a plausible hypothesis for female ruddy ducks, but this does not appear to be due to low quality females laying parasitic eggs (parasites and non-parasites do not differ in female condition in ruddy ducks; Reichart *et al.* in prep). Another possibility is a lack of competition-based benefits in offspring. Ruddy ducks are highly precocial at hatch and parental care is limited, occurring only in the form of vigilance behavior by the female (Joyner 1975; Gray 1980). Thus offspring do not compete for resources in the nest, as seen in altricial species. Finally, females may not have the ability to vary investment in parasitic and non-parasitic eggs,

as the amount of female control for various physiological characteristics in eggs (e.g., yolk hormones) has yet to be determined (Groothuis & Schwabl 2008).

Offspring sex

We found the overall population sex ratio did not differ from parity. This was also true for nesting parasites, because the sex ratio of all offspring produced (parasitic plus non-parasitic) did not differ from parity. In contrast, we found that parasitic eggs were significantly more likely to be male, and non-parasitic young were more likely to be female (Figure 4). We found a similar pattern for offspring sex ratios when analyses were restricted to nesting parasites, but were not statistically significant, possibly due to small sample sizes.

The tendency for parasitic offspring to be male might be due to a laying order effect, as has been shown in some other species (e.g., Badyaev *et al.* 2002a; Badyaev *et al.* 2002b), for example if male offspring are laid early in the laying sequence and parasitic eggs are laid prior to the female laying her own clutch. However, our data do not support this hypothesis, as we found no significant difference in egg laying interval for male and female parasitic eggs (Figure 6), and male and female parasitic eggs were equally likely to be laid prior to, during, or after parasites initiated their own nests.

Although we can not rule out that the observed differences in offspring sex ratio are due to some unknown artifact, we provide two alternative hypotheses to explain these patterns (but further research is necessary to elucidate factors influencing these patterns). One possible reason male eggs are more likely to be laid parasitically may be due to differences in embryonic development time between the sexes (reviewed in Uller 2006). For example, females may bias their parasitic eggs toward males because development time is shorter than for female eggs, and

thus may have a higher probability of hatching synchronously with the host's offspring. Another possibility may be that the observed bias is attributed to fitness benefits gained via kin selection (Hamilton 1964). Alternative to the local resource competition hypothesis, the local resource enhancement hypothesis predicts that when one sex (e.g. natally philopatric sex) enhances parental fitness (e.g., via cooperation; Komdeur *et al.* 1997), females should bias offspring production toward that sex. In waterfowl, females tend to be natally philopatric (Anderson *et al.* 1992), and several studies have found local female relatedness structure in waterfowl populations (e.g., Ruusila *et al.* 2001; Fowler 2005; McKinnon *et al.* 2006). Perhaps females produce female biased clutches if related offspring can enhance fitness of their mothers due to some form of cooperation. Several studies of CBP have found patterns of relatedness between hosts and parasites (Andersson & Åhlund 2000; Ruusila *et al.* 2001; Nielsen *et al.* 2006) and in some cases show a higher probability of success for parasitic eggs laid in relatives' nests (Nielsen *et al.* 2006).

Conclusions

Overall we found that parasitic and non-parasitic eggs do not differ in yolk androgens or egg size, suggesting these measures of egg quality are not important to the success of parasitic eggs in ruddy ducks, or that females do not have sufficient control over these characteristics to vary them accordingly. Female parasites appear to invest equally in parasitic and non-parasitic eggs laid. This further supports our previous finding that CBP in ruddy ducks is a mixed reproductive strategy, where females lay eggs parasitically when given the opportunity. Perhaps successful hatch of parasitic ruddy ducks is achieved by proper host choice (i.e., laying eggs parasitically in nests during the host's egg laying sequence; Reichart *et al.* in prep). The pattern

of female biased offspring for non-parasitic eggs may be the result of some unknown artifact, or may be explained by differences in embryonic development or benefits obtained via kin selection. In order to elucidate observed patterns of offspring sex ratio, further study of costs and benefits of producing male and female offspring as well as studies of the relatedness structure within the population are needed.

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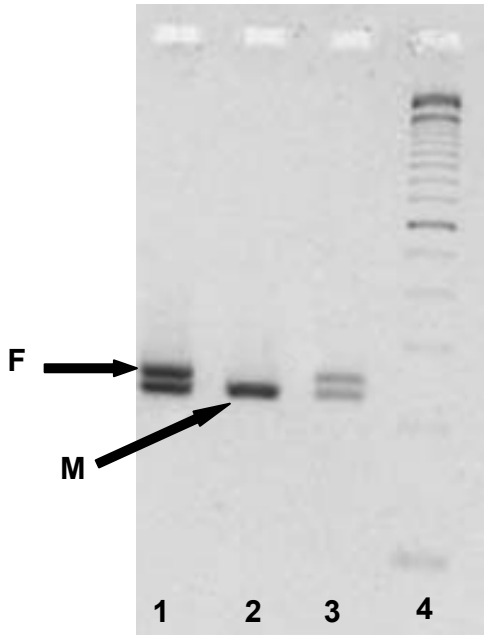

Figure 1. Electrophoretic agarose gel with four lanes. Lanes one and three contain females. Lane two contains a male. Lane four contains a 100bp size standard. The arrows indicate the diagnostic bands, where two bands indicate a female and one band indicates a male.

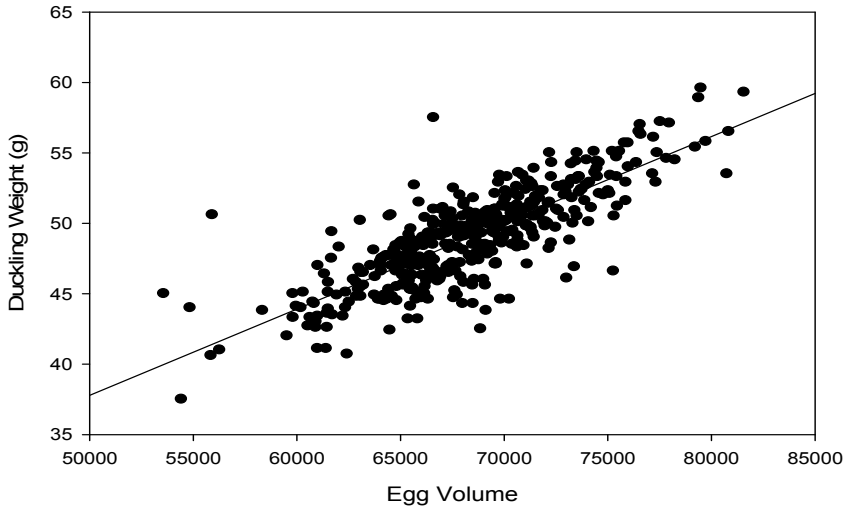

Figure 2. Egg volume predicted duckling weight at hatch ($r^2 = 0.64$). Ducklings from larger eggs are larger at hatch.

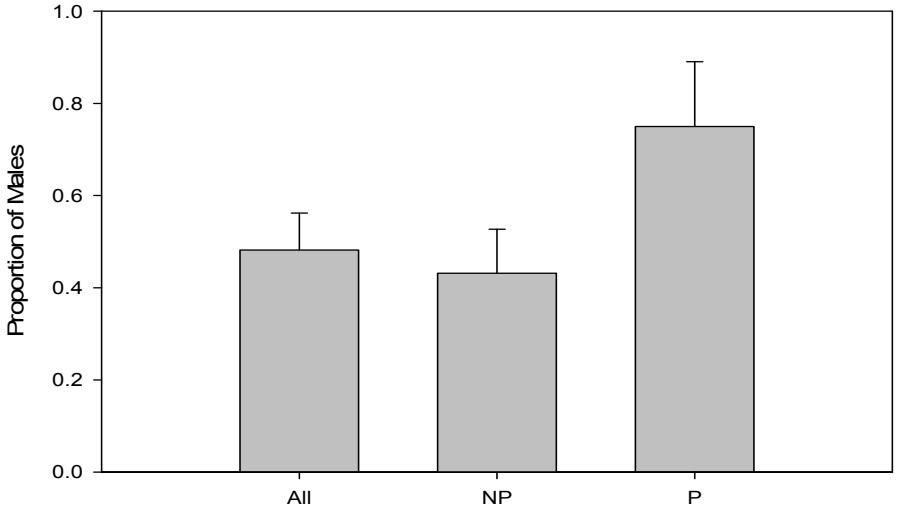

Figure 3. Proportion of male offspring from all broods sampled. All = proportion of all male offspring in broods (non-parasitic and parasitic combined). NP = proportion of non-parasitic male offspring in broods. P = proportion of parasitic male offspring in broods. Error bars represent 95% CI.

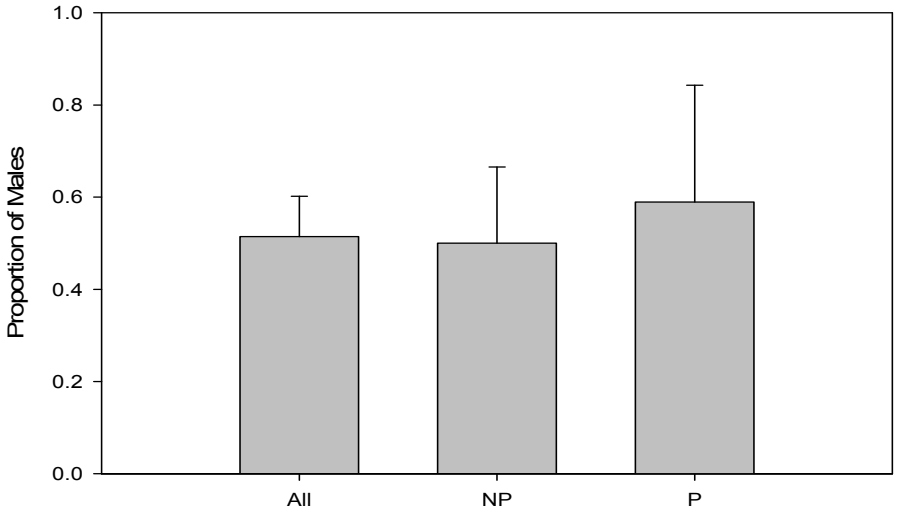

Figure 4. Proportion of male offspring produced by nesting parasites only. All = proportion of all male offspring produced by nesting parasites (non-parasitic and parasitic). NP = proportion of non-parasitic male offspring produced by nesting parasites. P = proportion of parasitic male offspring produced by nesting parasites. Error bars represent 95% CI.

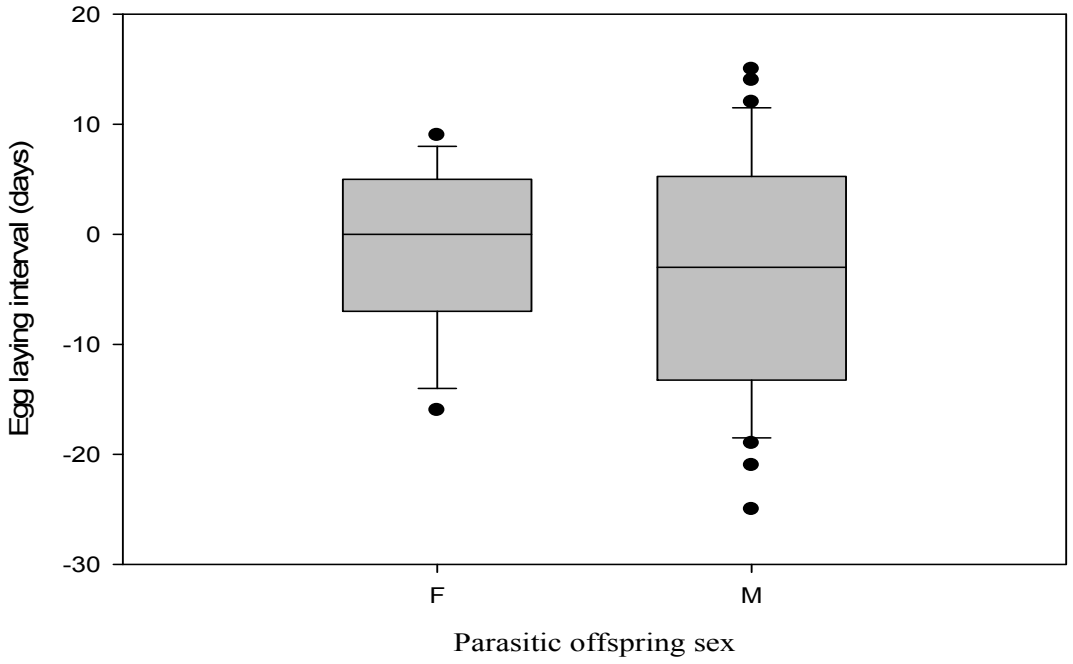

Figure 5. Egg laying interval (parasite nest initiation date – parasitic egg laying date) for female (N = 19) and male (N = 34) parasitic offspring. Black circles indicate outliers.

CONCLUSIONS AND SYNTHESIS

Conspecific brood parasitism (CBP) occurs in a multitude of species and is particularly common among waterfowl species (Yom-Tov 1980; Rohwer & Freeman 1989; Yom-Tov 2001), perhaps because the costs of raising precocial offspring are relatively minimal. Currently, studies of CBP indicate that CBP within populations and among species is facilitated by various ecological and social factors, whereby no single pattern can explain the occurrence of this behavior. Most likely variation in the frequency and use of conspecific brood parasitic behavior between species is attributed to differences in life histories, where CBP has likely evolved independently several times (Lyon & Eadie 2000; Arnold & Owens 2002; reviewed in Lyon & Eadie 2008). Variation between populations and individuals may be more likely attributed to differences in varying ecological conditions or fitness costs and benefits (Arnold & Owens 2002). However, depending on a parasite's phenotype (e.g., nesting parasites, non-nesting parasites) the importance of ecological and social conditions as well as the relative fitness costs and benefits can vary with overall lifetime reproductive success.

For example, CBP in some waterfowl species occurs as a conditional reproductive strategy, where the parasite's phenotype likely differs given various ecological and social conditions (e.g., Sorenson 1991; 1993). However, we found ruddy ducks use a mixed reproductive strategy, where most females nest and also lay eggs parasitically. In addition, the ecological and social factors influencing parasitism in other species (e.g., nest predation, nesting density, kin selection) do not explain the existence of CBP in ruddy ducks. Although, nesting synchrony among females in this population may enhance CBP because females exhibit high levels of nesting synchrony likely due to a short reproductive season. Thus we were unable to

discriminate between parasites choosing to lay eggs in a synchronous nest vs. laying eggs randomly in a synchronous nest.

Another possible component contributing to the success of parasitic egg laying behavior could be attributed to maternal effects (realized through physiological differences between eggs), where parasitic eggs contain substances that can affect offspring survival. We found no evidence of physiological differences (steroid hormones and egg size) between parasitic and non-parasitic eggs laid by nesting parasites; however, parasitic eggs were more likely to be male. The lack of physiological differences between egg types may indicate that parasites do not vary egg contents between parasitic and non-parasitic eggs, or that females lack control of this physiological mechanism. This research is among the first studies to investigate physiological differences among parasitic and non-parasitic eggs, and although there were no differences found in ruddy duck eggs, it does not negate the possibility that physiological differences can occur in other species. Physiological differences in eggs may be much more likely to occur in species participating in CBP as a conditional reproductive strategy, because physiological condition is more likely to vary among females using different reproductive tactics.

Finally future research on species participating in CBP should focus on estimating the actual fitness costs and benefits of parasitism to both hosts and parasites (as also suggested by Lyon & Eadie 2008). This may be facilitated by using study systems where ecological and social factors influencing parasitic behavior have been identified. More research (using experimentation) examining fitness costs and benefits for emergent nesting species may help identify some key life history differences influencing the evolution of CBP. Continued work in cavity nesting systems will provide more controlled study systems in which costs and benefits of parasitic behavior may be easier to investigate. In addition future research should also examine

hypotheses concerning optimal clutch size in brood parasitic species. Ruddy ducks may be an ideal species to test hypotheses associated with egg allocation trade-offs, such as those suggested by Lyon (1998), because nesting females lay eggs parasitically, eggs are costly, and renesting attempts are minimal. Therefore females are likely limited to a single reproductive opportunity per breeding season.

In conclusion, to further investigate variation in the occurrence of CBP (including variation mentioned above) and to understand how this behavior evolved, long term studies incorporating demographic information would be useful. Studies of survival, fertility, and dispersal of individuals at different life stages may be more likely to explain behavioral variation observed within and across species. Investigation of this type will not only advance the study of CBP, it will also provide a better platform to study animal social behavior in general.

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