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Who's Your Daddy? A Study of Extra-Pair Copulation and Mating Behaviors of *Protontaria citrea.*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Abstract:

Mating behavior has a profound impact on reproductive success and the resulting genetic structure of offspring. Extra-pair copulation is a widely observed behavior within avian species. This study explored the genetic effects of mating behaviors of Prothonotary warblers, *Protonotaria citrea* (Parulidae), using co-dominant microsatellite markers. Prothonotary warblers are migratory songbirds that build nests in cavities, commonly found in wetland habitats. A set of artificial nest boxes were initiated by Dr Robert Reilly in 2002 in Dutch Gap, Chesterfield County Virginia, USA, a tidal tributary off the James River. From this population, 28 nest boxes were surveyed yielding 47 adults and 110 offspring. All individuals were genotyped and the multilocus genotypes were used to identify parentage. Using paternity exclusion, 27.2% of offspring were identified as resulting from extra-pair mating. Surprisingly, an additional 11.8% of offspring were classified as genetically unrelated to the resident female, being presumably the result of an egg dumping from a female not caught at the nest box. The vast majority of nest boxes, 82.1% in this study, had at least one offspring that was a result of a mating behavior outside of the social pair. There was also positive spatial autocorrelation in extra-pair paternity suggesting that these matings are not randomly distributed across the sampling landscape. These data show that *Protonotaria citrea* engage in both extra-pair copulations and nest parasitism. Whether this is normal mating behavior for the species, or something that is unique to this population is unknown.

Introduction

Choosing one's mate is one of the most important decisions an individual will make in their lifetime. The consequences of that decision dictate whether the offspring will be healthy, hungry or survive into adulthood. The process by which a mate is selected varies among species. Originally, monogamy was thought to be widespread in avian species (Lack 1968), but recent molecular work (Avise 1996) has shown that only thirty percent (Griffith *et al.* 2002) of bird species are monogamous. These results show that the vast majority of avian species do participate in extra-pair copulation (hereafter EPC) and mating behaviors that have significant effects on the survival of their young, average population-level fitness, and the stability of population genetic structure.

Engaging in extra-pair copulations can have both positive and negative consequences for bird species. Males commonly protect paternity at their home nest by guarding their mate and copulating frequently (Mota & Hoi-Leitner 2003; Westneat & Stewart 2003; Birkhead &Moeller 1992; Lifjeld *et al.,* 1994). If males have higher paternity at the home nest, they are often more likely to increase paternal care (Moeller 2000), which in turn leads to a net increase in offspring survival. The most widely accepted consequence of a female engaging in EPC is reduced paternal care by her pair mate (reviewed by Westneat & Stewart 2003; Westneat *et al.,* 1990). Consequently, a male giving subpar care would increase the pursuit of an extra mate for both sexes (Bennett & Owens 2002). In species where the young are precocial, such a consequence is, however, irrelevant. By engaging in EPC, a female can influence the genetic quality of her own offspring (Trivers 1972). Offspring from monogamous mates are all full siblings with all of their genes coming from the same set of parents and an average relatedness of 50%. Genetic diversity is lower in families were fertilization is restricted to the 'resident pair' relative to

families where monogamy is not the rule. Overall, this may be a net detriment for families where one of the parents has genotypes that contribute to traits of lower overall fitness. Conversely, if species engage in EPC, individuals may be able to choose from a much larger population of potential mates, improving the opportunity for selecting traits of perceived greater fitness.

The potential fitness benefits of EPCs come at a cost that may be prohibitive. First, adults must expel both time and energy finding primary and secondary mates. If the fitness consequences of EPC do not offset the time and energy necessary to find additional mates, then EPC is not expected to be an advantageous behavior. For example, Byers *et al.* (2004) showed that more than half of male chestnut-sided warblers (*Dendoica pensylvanica)* that gained an extra-pair fertilization lost paternity at the home nest. As a consequence, the cost of EPC may not always be beneficial to individual males. Female blue tits, however, increase their fitness and reproductive success by selecting putatively more fit males as extra mates and thereby improving the potential fitness of their offspring (Foerster *et al.,* 2003). Offspring of female western red-winged blackbirds who copulated with extra mates had a higher hatching and fledging success (Gray 1997) with no recordable cost to the female. Populations with a high variance in reproductive success have reductions in both the effective population size and genetic diversity (Castro *et al.,* 2004). Extra-pair copulations could reduce this variation by allowing single males to gain paternity, or increase it if sexually selected males sire a disproportionate number of offspring (Byers *et al.,* 2004).

The mechanisms by which mates are chosen vary widely across avian taxa. Extra-pair mates can be chosen by plumage brightness (Kempenaers *et al.* 1992; Saino *et al.,* 1997; Johnsen *et al.,* 1998), quality of song (Poesel *et al., 2006; Kempenaers et al.,* 1997; Griffith *et al.,* 2002),

dominance (Mennill *et al., 2004*), age (Poesel *et al.,* 2006; Wagner *et al.,* 1996) body size(Petrie *et al.,* 1998, Yezerinac & Weatherhead 1997), mask size (Thusius *et al.,* 2001), speed (Hoi & Hoi-Leitner 1997), vocalizing when fertile (Neudorf *et al.,* 1997), or a combination of several factors (Webster *et al.,* 1995). Male Prothonotary warblers prefer to mate with older females with more tail spots (Smith 2008) because they have greater reproductive success. If their social mate is younger, males may be more likely to solicit an older female as an extra-pair mate to increase their own reproductive success. Prothonotary warblers with the most vibrate plumage (Beck 2013) and males that sang earlier in the day and more frequently (Clarkson 2007) acquired the most desirable territory and nest sites. Females looking to improve the genetic quality of her offspring by finding an extra mate (Trivers 1972) would then be more likely to choose a male that can acquire the best territory and safest nest box.

Mate choice is not solely driven by sexual selection; the proximity to the home nest of a potential extra-pair mate can also influence the occurrence of EPC (Smith 1988; Kempenaers 1992; Sheldon 1994; Byers *et al.,* 2004), if the home ranges of individuals are spatially restricted. After finding a primary mate, the convenience of not wasting energy to find an additional extra mate with the nearest neighbor can influence mate selection. Spatial proximity of an extra-pair mate can influence the genetic quality of the resulting offspring. In a population that has site fidelity, it is important to maintain genetic diversity in mate selection. The genetic relatedness of the parent's affects the survival of their offspring (Bean *et al.,* 2004; Spottiswoode & Moeller 2003) and their own reproductive success (Amos *et al.,* 2001). One of the ways a female gains an EPC is through foray (actively entering another's territory) or waiting for a male to enter hers. Dominant males—those who aggressively defend their territory—are the most likely to secure the best breeding sites with the best resources (Clarkson 2007; Petit 1989). By

foraying in that territory she can obtain more valuable resources and increase the genetic value of her offspring. *Protonotaria citrea* has an estimated home range of 3.7 acres (Schoener 1968). In a nesting site that uses nest boxes that are only 32 m a part, individual territories overlap. The likelihood of a female entering into a male's territory for extra copulations, or for other reasons, such as nest material or foraging, is therefore greater in this close proximity. Numerous species have documented females actively seeking out extra mates via foray (Kempenaers *et al.,* 1992; Smith 1988; Sheldon 1994; Gray 1996; Wagner 1991; Hoi & Hoi-Leitner 1997). Even if a female's intention was not seeking an extra mate, her presence in another male's territory increases the rate of extra-pair copulation because the male may attempt to mount her, as in boat-tailed grackles (*Quiscalus major,* Poston 1997). Proximity to adjacent male territories would then serve as a means of increasing encounters with extra mates.

Offspring resulting from EPC have a potentially different set of genotypes than their half siblings which most likely result in an increase in heterozygosity (e.g., Foerster *et al.* 2003). Increasing, genetic diversity at the level of the individual has direct consequences for putatively adaptive traits such as susceptibility to parasite loading (Coltman *et al.,* 1999) and increased hatching potential (Hansson 2004). Individual adults that engage in EPC, in turn, benefit by increased reproductive fitness in two potential ways. First, EPC may result in a net increase in the number of offspring sired; more offspring result in a greater contribution to the next generations' gene pool (Castro *et al.,* 2004). Second, EPC may also provide additional fitness benefits to individuals with less fit phenotypes as they seek out more fit mates in the population. From the perspective of the less fit individual, this is a net gain allowing more of their own genetics to be left for subsequent generations. It, in essence, allows an individual to "not put all of their eggs in one basket".

Post mating behaviors may also have a net effect on individual fitness and the genetic identity of individual nestlings. Intraspecific and interspecific nest parasitism, or egg dumping, is where individuals leave eggs in another nest outside of their own thereby turning over parenting and provisioning to another mating pair (Hoover 2003a; Hoover 2003b, Hoover *et al.*, 2006b; Nielson *et al.*, 2006a; Nielson *et al.*, 2006b; Forman 2005). Intraspecific parasitism is a common behavior in both waterfowl and colonial birds where young do not need large amounts of parental care. Wood ducks, *Aix sponsa*, are notorious intraspecific parasites (Nielsen *et al.*, 2006a) along with others belonging to Anseriformes. Interspecific parasites, such as cowbirds, place the entire burden of offspring rearing onto their host. This can be a dangerous means of reproduction, as the selected host can reject the parasite egg, in turn provoking the parasitic parent to destroy the nest. Intraspecific parasites may spread their eggs among multiple nests thus reducing the overall burden of offspring rearing. In many species, brood parasites will remove a host's egg before depositing its own, decreasing the host's fitness immediately (Andersson 1984). In a southern Illinois population of Prothonotary warblers, cowbirds laid in 50% of nest boxes and 41% of natural cavity hosts (Hoover 2001). As a result of the brood parasitism, host clutch size, hatching success and the survival rate of the host nestling all decreased (Hoover 2003a). Prothonotary warblers do not appear to have an adequate response mechanism to eject parasitic eggs (Hoover *et al.*, 2006b). From the perspective of the egg dumping female, this behavior can increase an offspring's chance of survival if the new nest environment is of higher general quality. This behavior may also act as a mechanism to reduce the burden of additional mouths to feed at the home nest (Nielsen *et al.*, 2006a) for particularly fecund individuals. No previous studies have reported intraspecific parasitism in Prothonotary populations.

Protonotaria citrea has the longest migration of any small cavity nesting passerine in the eastern US (Blem *et al.,* 1999a). They winter primarily in Central and South American coastal mangrove forests, where they form mobile flocks (Warkentin & Morton 2000; Warkentin 1996). The species is mainly insectivorous, foraging in trees (Petit *et al.,* 1990). Prothonotary warblers have a propensity for site fidelity at both their wintering site (Warkentin 1996) and summer breeding sites (Hoover 2003b). In a study conducted by Blem in 1999(b), it was reported that 47.9% of females were recaptured in following years. They summer in the coastal-plain riparian or levee forests, where they have the most breeding success (Lyons 2005). Males migrate and arrive first at the breeding site in early to mid-April (Blem & Blem 1992). Males compete for nest sites prior to the females' arrival (Petit 1989). Males that sang early in the season, and more frequently, acquire the most valuable territory (Clarkson 2007). Individuals with the brightest plumage (Beck 2013) are able to secure the most desirable nest boxes over deep water (Hoover 2006a). *Protonotaria citrea* weigh an average of 16.1 grams with an estimated territory of 3.7 acres (Schoener 1968), however, more recent research at Dutch Gap revealed males establish an average territory of 0.26 ± 0.04 ha in size (Clarkson 2007). Males begin construction of the nest by laying a base of moss, topped with a nest cup constructed of drier materials (Blem & Blem 1992). Females lay dry grasses and rootlets on top completing a nest about 7 to 15 cm deep and high enough for them to peer out of the nesting hole (Blem *et al.,* 1999a). No mate permanence has been shown, with new resident pairs chosen each year (Reilly, unpub). Factors influencing nest box selection include predation, competition by other species, and the environment surrounding the box (Blem & Blem 1991, 1994). Based on banding data, females have shown high site fidelity, some even returning to the same nest box (Reilly, unpub). Females lay between 2 to 8 eggs varying between years (Blem & Blem 1992) with an average of 5 eggs per

clutch (Schoener 1968; Walkinshaw 1953; Petit 1989). They typically lay two clutches per season depending on environmental conditions, with clutch size diminishing as breeding season progresses with 5 eggs per clutch in the spring to 4 in later summer (Blem & Blem 1992). Nestlings have higher growth rates and a 51% chance of fledging from first broods, and a 55% chance of fledging from second broods (Podlesak & Blem 2001). Older females lay between 0.4 - 1.0 more eggs per clutch and earlier in the season than first time layers (Blem *et al.,* 1999b). Eggs hatch after an average incubation period of 12 days, followed by young fledging after 10 days (Podlesak & Blem 2002). Banding data over twelve years, revealed warblers have a life span up to 8 years (Blem *et al.*, 1999b). Brown-headed cowbirds, *Molothrus ater*, are a known brood parasite for Prothonotary warblers. Host clutch size, hatching success and mass of host nestlings all decrease with the presence of a parasitic egg (Hoover 2003a, Hoover 2003b). Native predators for Prothonotary warblers include the black rat snack and raccoons, though the introduction of nest boxes in these study areas has greatly reduced predation and brood parasitism (Blem & Blem 1991).

This thesis explores the mating behaviors of *Protonotaria citrae* to examine the occurrences of extra-pair mating and potential egg dumping behaviors. Since 2002, the Dutch Gap Conservation Area, adjacent to Henricus Historical Park (Chesterfield County, Virginia) has been established as a breeding site by inserting nest boxes for *Protonotaria citrae*. This population has undergoing annual censuses by Dr. Bob Reilly (Virginia Commonwealth University). Previous work at this site has shown a high level of female site fidelity, with females returning not only to the site, but some returning to the same nest box (Reilly, unpub). Using molecular markers and blood collected from families at this Dutch Gap population, the following questions were asked: A) Is there evidence of egg dumping? If so, are eggs dumped

into nests spatially proximate to maternal individuals or are they randomly distributed across the study site? B) For the set of non-dumped offspring surveyed, what is the rate of extra-pair copulation in *P. citrae* and is the occurrence of EPC in this population spatially restricted? These questions will allow a broader understanding of mating behaviors of *P. citrea* and potentially provide insights into dumping behavior salient to a broader range of species.

Methods

Study Site: Sampling occurred at the Dutch Gap Conservation Area (37°22'52.42'N, 77°22'49.16"W) Chesterfield County, Virginia adjacent to Henricus Historical Park. The target site was a tidal swamp forest within the James River. Nest boxes were established in 2002 (Dr. Robert Reilly). The tidal flux ranged between 1 m or greater from high to low tide. The 131 nest boxes on site are on poles 1-2m above the water spaced 32 m apart (Figure 1). Since the nest boxes inception, *Protonotaria citrea* have shifted nesting in natural cavities to these boxes. In 2005, the nest box's entrances were reduced from the original 3.8 cm in diameter to prevent use by tree swallows, and other species, as well as to prevent nest parasitism by cowbirds, *Molothrus ater*. Boxes were subsequently modified to accommodate a radio receiver that actuates a trap-door on the front of the box (developed by Dr. Robert Reilly).

Field Sampling: Samples were taken April-July of the 2006 mating season in three stages. The first arrivals, usually males establishing a nesting site and territory, arrived in late April. The first of these males was captured April 15th using mist nets, a male decoy and a song recording. The birds were assigned to the nearest nest box. Before the second sampling stage, nest boxes with nesting material were monitored and targeted. Females were sampled by visiting the targeted nest boxes via canoe, while they incubated eggs between late April and early May. The females were captured by placing a small net over the opening of the nest box and tapping lightly to encourage the bird to fly into the net. The final sampling of males and offspring occurred in early May. Offspring were sampled at one week old by simply taking and replacing them from the nest. Males were captured by activating the trapdoor via remote control (Dr. Robert Reilly) when

they visited their nest box to feed offspring. Second clutches were sampled via canoe using the same methods from mid-May to early June.

Blood Collection: Blood was collected from the tarsal vein using a 27 (adults) or 30 (offspring) gauge needle. The needle was used to pierce the vein, then removed and the talon was pinched to create a drop of blood between 5 and 50μL to be collected and stabilized on Whatman FTA mini-cards and stored in multi-barrier pouches (Whatman: WB100036). The samples were stored at room temperature as per the manufacturer's instructions. All birds sampled were immediately released. All procedures followed IACUC Study protocol #AM10230. There was a 0% mortality rate after sampling. Genotypes were assayed using blood sampled from each bird.

Genetic Analysis: Parentage was assayed using four modified microsatellite loci (*Lsw5, Lsw12, Lsw14, Lsw18*), which were originally developed for Lucy's warbler (Winker *et al.*, 1999) though found to be assayable and polymorphic for P*rotonotaria citrea.* Locus *Lsw19* was applicable to *P. citrea,* but did not have significant amplification to warrant use in this study. Amplification protocols for these loci followed Winker *et al.* (1999). DNA was extracted from the FTA mini-card using a 1.2 mm micro-punch (Whatman) following the manufacturer's instructions. The DNA present on a 1.2 mm disc was used in a 25μL polymerase chain reaction (PCR). Fluorescently tagged primer was used to assay genotypes using a MegaBace 1000 (Amersham Biosciences). For loci *Lsw5, Lsw12* and *Lsw14*, a three-primer combination was used (Table 1) to tag primers with fluorescence following the methods outlined in Boutin-Ganache *et al.,* (2001). The PCR cocktail consisted of a 1.2mm disc of blood extracted from the Whatman FTA card, 4.63μL of both reverse and fluorescent dye primers, 3.24μL of forward

tailed primer and 12.5μL of JumpStart Ready Mix REDTaq (Sigma). For the *Lsw18* primer mix, a 5' fluorescent tag fluorescence was added to the forward primer directly and the cocktail consisted of 12.5 μL of JumpStart ReadyMix REDTaq (Sigma) and 6.25 μL of both forward and reverse primer. The PCR thermal regime for both sets of primers was: 3 min 45s at 94◦C; then 35 cycles of 94◦C for 1 min, primer-specific annealing temperature for 30s, and 72◦C for 30s. Genotypes were determined using the MegaBace 1000 (Amersham). PCR products for all loci were combined in a 1:1 ratio in each well for genotyping. They were desalted using 0.1% Tween 20 and run with an ET400-Rox550 size standard (GE Healthcare), allele sizes estimated using the program Fragment Profiler (Amersham Biosciences). Individual genotypes were formatted for analysis in R (R Development Team, 2008) using the gstudio package (Dyer 2009).

Genetic Assignment: Offspring and maternal individuals were all grouped by nest box. The multilocus genotypes of all individuals were used to identify offspring as either *dumped, EPC,* or *resident.* Dumped individuals are those offspring whose genotypes do not contain any of the alleles present in the sampled mother. For autosomal genetic markers in a diploid species, offspring must have at least one of the maternal individuals' alleles. Mismatches between maternal and offspring genotypes can only occur if the egg was dumped or if the putative maternal individual captured at the nest box is not actually the mother of the offspring. For classification of *dumped* individuals, a strict exclusion criterion was used.

Of the individuals who were not classified as *dumped*, paternity analysis was used to differentiate between *EPC* and *resident* offspring. As in the classification of *dumped*, a strict exclusion criteria was used for all complete mother, offspring, and putative father triplets.

Individuals were classified as *EPC* if the combination of maternal and paternal genotypes could not produce the observed offspring genotype.

Of the individuals that could be categorized as *resident* (e.g., the offspring genotype is a possible genotype for given both sampled parents), a further step was taken to examine the likelihood of paternity using the fractional method (Devlin *et al.*, (1988). This method uses multilocus genetic transition probabilities to estimate the relative likelihood of paternity among all sampled males. Fractional paternity is a more conservative approach than assigning paternity to the most, probabilistically, likely father in studies like this for two reasons. First, the fraction of sampled male individuals was relatively small given the number of potential fathers in the population. As a result, the real father may not have been sampled and therefore cannot be examined. Second, even with a 'most-likely' father designation, if there is/are more than one potential male, we cannot accept the probabilistically most likely, only with strict exclusion (e.g., the exclusion of all putative males except one), can we be certain of paternity. The fractional approach allows us to examine population-level patterns of paternity, and in particular, to estimate spatial correlations with parentage.

Spatial Analyses of Mating Behaviors: Given that the location of nest boxes and the overlap of home ranges may influence mating behavior, a two level analysis of behavior was performed to elucidate any spatial components that may exist. In these analyses, the proportion of individuals classified as *dumped*, *EPC*, or *resident* was used as the sampling stratum. At the larger spatial scale, the correlation between egg dumping and EPC at each nest box with spatial (Euclidean) separation was determined. This distance analysis covered all pairs of nest boxes across all

distance classes and was conducted using the Mantel Test (Mantel 1967), a permutation approach for estimating matrix correlation as implemented in the vegan (Oksanen *et al.* 2013) library of R. Spatial components of this behavior may not be spread across the entirety of the sampling landscape in a linear fashion (e.g., they may be localized). To identify any localized correlation, both egg dumping and EPC were examined using spatial autocorrelation. Here the correlations are estimated based upon samples in discrete distance bins rather than across all distance classes. Each bin is quantified for statistical correlation. For this analysis the autocorrelation approach from Smouse & Peakall (1999) was used.

Results

The total number of sampled individuals was 205 at the conclusion of the mating season. Only those individuals associated with a family group were included in parentage analysis. A total of 28 of the 131 nest boxes on site were sampled (Figure 1). Both parents were collected from 75% of the boxes and in 25% of the boxes only maternal individuals and offspring were collected. It was assumed that parentage was putative and assigned initially by the origin of collection. In addition to adults caught at the boxes, one additional male was also collected. The total sample size was 47 Adults (21 males and 26 females) and 110 offspring.

Using the microsatellite loci developed for the Lucy's warbler (*Vermivora luciae;* Winker *et al.,* 1999), five loci were assayable for the Prothonotary warbler: *Lsw5*, *Lsw12*, *Lsw14*, *Lsw18* and *Lsw19,* however, *Lsw19* did not have adequate amplification and was removed. If an individual failed to amplify at two loci, they were removed from paternity analysis and considered as missing data. Each locus had between 5 and 14 alleles (Figure 2), with effective allele sizes ranging between 1.49 and 9.25 (Table 3) when rare alleles were factored. The multilocus exclusion probability across all loci is $P_{excl} = 0.961$ (Table 3) meaning that, on average, if this were a randomly mating population, one should be able to exclude 96% of the potential males. After removing individuals with partial multilocus genotypes, the dataset consisted of 157 individual birds. Genotypes were available for 21 complete (male, female, offspring) triplets and 7 incomplete triplets (female, offspring only). Since the inception of nest boxes, the warblers have switched from natural cavity nests to almost exclusively nesting in boxes. To estimate the relative fraction of the overall population that was sampled at this site, the number of nest boxes that were assayed was compared to the overall number of available nest

boxes. Using this method, 21% of all breeding *Protonotaria citrea* in the population were sampled.

Offspring were analyzed one at a time using transition probability to determine if they were genetically related to the female collected at their home box. If they were not, the offspring was classified as *dumped*. Box K5 was the second nest and clutch of the mated pair originally in box P23. Two clutches were sampled from box K8 from the same resident pair. L1 had only a single male sampled and was excluded, leaving 26 boxes for analysis (Table 2). The fraction of offspring excluded in each box as a result of genetic incompatibility to the female varied between 0.08 and 0.35 for each nest box. As a conservative measure, only offspring whose genotypes amplified at two loci or more were considered, resulting in 13 *dumped* eggs and 94 whose genotypes are compatible with the sampled maternal individual (Table 4). Spatially, the proportion of *dumped* eggs per box (Figure 3), was not correlated with the physical separation of boxes across the entire sampling landscape (Mantel Correlation $r = 0.034$, $P = 0.322$; Figure SUPP-1) or at any of the observed distance classes (largest autocorrelation $r = 0.36$; $P > 0.05$, Figure 4). While egg dumping appears to be present, it appears to be randomly distributed across the sampling landscape.

Paternity analysis was applied to boxes that had both mother and father genotypes, which reduced the number of potential offspring to 84 individuals in 21 nest boxes. Using a two-locus exclusion model as above, 27.2% of those offspring were determined as the product of EPC. The likelihood of EPC at each box was not significantly correlated with spatial separation in total or at any distance class (Mantel test, $r = 0.0574$; $P = 0.414$). Overall fractional paternity, the relative likelihood of male paternity, was spatially autocorrelated (Figure 5) at the smallest

spatial distance classes. This means that for offspring that were genetically compatible with the adults sampled at that box, the most likely father was, probabilistically, the resident one.

Across all nest boxes sampled, 92.8% contained at least one offspring from the social mate (Figure 6), but only seven of the boxes sampled contained nestlings that were genetically full siblings. Offspring that were the result of *dumping* represented 11.8% of all the chicks in the study and 35.7% of all sampled nests had at least one dumped egg present. Extra-pair copulation was prevalent, accounting for 27.2% of the offspring sampled and occurring at least once in the majority (53.5%) of nests sampled.

Discussion

These data suggest *Protonotaria citrea* are not monogamous, their mating behaviors create offspring resulting from pair-bonded individuals, extra-pair copulation, as well as intraspecific nest parasitism. Over a quarter of the sampled offspring, and representing over half of the sampled nest boxes, resulted from extra- copulation occurring outside the home nest. In over a third of the sampled nests, at least one offspring was not genetically related to the female. Although, EPC may be expected in *P. citrea* given the prevalence of this behavior in birds in general, an especially interesting result of this study, was the prevalence of egg dumping, a behavior that has not previously been reported prior in *P. citrea*. Nielsen *et al.* (2006b) suggested that egg dumping might be a response to overburdening of females in provisioning, though what is driving this behavior in this particular population is not evident. Anecdotal evidence was attained from volunteers on the warbler project, who after hearing these results, commented that they had on occasion seen eggs that appeared anomalous and that on occasions, a single nest observed to have two eggs deposited on a single day, a feat that is most likely physiologically impossible for a single mother. Researchers at nearby site also claimed to witness more than one female tending a box, and more than one male provisioning a single box. The current genetic data provide convincing support for the notion that such eggs are not offspring of the adults tending the nest box.

Intraspecific nest parasitism, the act of placing an egg into another's nest, is most common among colony birds where the nearest nest is within pecking distance and young are born with little need for parental care. Populations that breed in nest boxes have a higher rate of intraspecific nest parasitism, but clutches are smaller in natural cavity populations (Nielson *et al.*,

2006a, Nielson *et al.*, 2006b). Females who lay in other's nests do not diminish their own parental care at the home nest (Forman 2005). The drawbacks of using such a mating behavior is reduced hatching success and nest abandonment (Nielson *et al.,* 2006a), a need for increased incubation time with each added parasitic egg and a reduction of eggs produced by the social pair at the home nest (Nielson *et al.,* 2006b).

The act of extra-pair copulation can be potentially beneficial to all parties involved. In a sexually selective population, extra-pair copulation can drive competition and improve the gene pool of future generations. The most desirable males are older (*Wagner et al.,* 1996) with bright plumage (Saino *et al.,* 1997; Johnsen *et al.,* 1998), high song quality (Griffith *et al.,* 2002) and are the most likely to be selected for EPC. However, proximity to the home nest can also influence an EPC mate (Smith 1988; Kempenaers 1992; Sheldon 1994; Westneat 1992; Gray 1996; Byers *et al.,* 2004). Although the Mantel tests did not show a spatial relationship to genetic relatedness versus distance, there was an autocorrelation in paternity suggesting that when parentage is applied, it is most likely the resident individual. If a female simply chooses an extra mate via proximity, does it positively affect the genetic quality of her offspring? Studies have shown that EPC increases an offspring's likelihood of being heterozygous (Foerster *et al.,* 2003). Extra-pair fertilization also increases the parent's own genetic fitness in this, and potential future generations (Amos *et al.,* 2001). In this population, where females inhabit the same nest box year after year (Reilly, unpub), EPC could be a means of seeking a mate via sexual selection instead of settling for the male that chooses your box. Other studies have shown that EPC can be a mechanism for preventing incest (Amos *et al.,* 2001; Bean *et al.,* 2004; Keane 1990; Peacock & Smith 1997). If two individuals are related, their resulting eggs may be unfertilized (Hansson 2004). EPC could then be a mechanism to further the population without

the risk of contaminating the gene pool with fixed alleles. Integrating measures of relatedness into this system would begin to address these kinds of hypotheses, though unfortunately the data in this work do not have sufficient resolution.

Extra-pair copulations are common in passerines (reviewed by Westneat & Stewart 2003). *Protonotaria citrea's* behavior at this site is consistent with those of other species in its own family. The bright yellow plumage and territory displays (Clarkson 2007) are indicative of a sexually selective population, which would engage in extra-pair copulation. Based on the banding data of past years, there is no mate permanence between seasons. The introduction of nest boxes—along with the removal of predators, nest competition by tree swallows, and the reduction of brood parasitism by brown-headed cowbirds— may have created a colony like environment. *P. citrea* are natural cavity nest builders and build nests in whatever cavity they can find (i.e., milk jugs, tires). By creating a safe and reliable environment for them to nest in year after year, has the establishment of this population created a colony effect? As of yet, there have been no other documented cases of egg dumping at natural cavity Prothonotary warbler sites. More observational and comparative studies would further confirm these mating behaviors across the species.

Multiple factors may have contributed to this *Protonotaria citrea* population's mating behaviors that differ from other nest box and natural cavity Prothonotary warbler populations. Despite their size, they have the longest migration of any songbird (Blem *et al.,* 1999a). It has been suggested that dominant birds travel the shortest distances, wintering in Mexico and breeding in southeastern states (Warkentin 1996). Sites where birds have reproductive success have high rates of site fidelity (Hoover 2003b). Blem and Blem (1999b) captured one female every year for eight years in a nearby nesting site at Presquile, Virginia. Females at this study

site are not only loyal to the location but some to a particular box (Reilly, unpub). Since the inception of the nest boxes, the population has switched to almost exclusively using them to rear their broods. They have created a renewable and safe nest site, year after year, that does not require males to hunt for a suitable nest cavity allowing them to focus more attention to territory battles and song. In a population that is dense with high quality males, high EPC rates driven by sexual selection would be expected.

The prevalence of nest box availability and proximity may have influenced the mating behaviors of *Protonotaria citrea.* Populations that have high breeding density have been correlated with high rates of extra-pair fertilization, as in the American robin (Rowe & Weatherhead 2007), eastern bluebirds (Stewart *et al.*, 2010) and barn swallows (Moeller 1991). The relative proximity of nests to potential extra-mates increases the probability of extra-pair copulation. This notion is supported in this study, where the most likely extra-mate is autocorrelated at the smallest distance class. Breeding density is not the only variable in predicting the rate of extra-pair copulation and can be a contributing factor to extra-pair fertilizations in conjunction with breeding synchrony (Stewart *et al.*, 2010). Male house sparrows alter their copulating frequency in high-density populations and mate more frequently during fertile periods to protect paternity at the home nest (Hoi *et al.*, 2011). The abundance of nest boxes and their spacing may have increased the occurrence of extra-pair copulations by increasing the breeding density of the population.

Populations of *P. citrea* are commonly parasitized by brown-headed cowbirds who can lay one or more parasitic egg(s) in a host nest (Hoover 2003a; Hoover *et al.*, 2006b). Unfortunately, *P. citrea* has not adapted an adequate coping mechanism and accepts the parasitic eggs with great detriment to their own clutch's survival (Hoover 2003a). It is unknown if the

birds can distinguish between their own egg and one of a conspecific parasitic egg. If *P. citrea* does not react to a cowbird's parasitic egg, they would not be expected to react to a parasitic egg of their own species. Intraspecific nest parasitism may be an adaptive trait that has gone undetected in *Protonotaria citrea* until now. Since predation by cowbirds has been eliminated with the use of next boxes, the occurrence of intraspecific parasitism can more easily observed. Intraspecific parasitism could be the cause of clutch sizes variations between *P. citrea* Michigan (Walkinshaw 1953), Tennessee, (Petit 1989) and Virginia (Blem & Blem 1992) populations. Blem & Blem (1992) observed 8 eggs in a single nest, when the average clutch size of a single female is five. An alternative hypothesis to egg dumping, is the theory that unpaired females returning to the nesting site for the first time are unable to secure a nest box and dump their offspring into established nests. It has been observed by Blem (unpub) that second year females do not secure a nest box the year following their hatching. In a study of common eiders, parasitic females laid eggs in nests where they were related to the host (Andersson & Waldeck 2007). The act of laying a parasitic egg in a relative's nest potentially reduces the fitness cost to the host, making parasitism a possible adaptive behavior (Andersson 2001). In a population engaging site fidelity, unpaired females may target relatives for egg dumping, however, further observational studies would be able to evaluate such a claim.

Extra-pair copulations can be highly advantageous to an individual by increasing their overall reproductive fitness. However, the population may suffer because genetic variability would be reduced if a disproportionate amount of offspring were sired by a small group of dominant individuals (Castro *et al.,* 2004; Byers *et al.,* 2004). If a male were able to distinguish between his own genetic offspring versus unrelated offspring and kill them, there would be no benefit for EPC and the population would favor monogamy (Kempenaers & Sheldon 1996).

Males of several species do not discriminate against offspring resulting from extra-pair fertilizations (Kempenaers *et al.,* 1998; Kempenaers & Sheldon 1996) and do not alter their behavior after extra-pair copulating (Rutz 2005). Extra-pair copulations are then driving sexual selection (Byers *et al.,* 2004) for desired phenotypes, with limited effects on male reactions to unrelated offspring in their home nest thus increasing female fitness.

This study generated thought-provoking questions on *Protonotaria citrea*'s mating behaviors. More observational studies from both cavity and nest box populations should be conducted to determine if egg dumping is a reoccurring behavior. Ideally more polymorphic genetic loci need to be developed to allow for more powerful statistical analyses. The contributions of genetic studies, such as this one, will continue to provide insights into the compelling mating behaviors of birds.

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Tables

Table 1-Primers: Genetic primers derived for Lucys wood warbler that are applicable to *Protonotaria citrea* from (Winker *et al.*, 1999) and modified following Boutin-Ganache *et al.*, (2001) for primers *Lsw* 5, 12 and 14. Primer *Lsw 18* has added fluorescence.

Primer	Sequence (5' – 3')	Allele Range	**# of Alleles**
Lsw 5F	CTTGCATCAGTTTTTAGTGCT	234 to 330 bp	**5**
Lsw 5R tailed	CAGTCGGGCGTCATCAGCTGGGTGCACACACACA		
*Lsw 12*F tailed	CAGTCGGGCGTCATCAATCCCATTGAGGACTTTCTTG	422 to 464 bp	**14**
Lsw 12R	TTCCCTGAAGGARATCAACATC		
Lsw 18F	TTGCTGAAAGAAGTACTAAGA	218 to 270 bp	**13**
Lsw 18R	CTGKTTGCAGGATATGTATAC		
Lsw 14F tailed	CAGTCGGGCGTCATCAGTTATGCTCCAACAAAATAGATA	149 to 275 bp	**7**
Lsw 14R	AGGTTTTRAAGGARAGATTTATA		

Table 2- Collections: Collection of samples including box id, spatial location and the indication of *Protonotaria citrea* adults (Male M & Female F) and offspring sampled. * indicates double clutch

Box	Longitude	Latitude	Adults	Offspring
C1	W077:22’20.66”	N37:21’50.86”	M & F	2
C10	W077:23’09.79”	N37:21’57.58”	M & F	5
C11	W077:23’23.97”	N37:22’02.75”	M & F	4
C7	W077:22’07.07”	N37:21’43.13”	M & F	4
K15	W077:23’06.39”	N37:22’20.00”	M & F	5
K27	W077:22’29.68”	N37:21’40.83”	M & F	5
K28	W077:22’23.43”	N37:21’40.95”	M & F	3
K6	W077:22’29.34”	N37:21’59.33”	M & F	1
K7	W077:22’37.64”	N37:22’07.00”	M & F	5
***K8**	W077:22’42.20”	N37:22’14.05”	M & F	9
L2	W077:22’41.23”	N37:21’47.07”	M & F	5
M1	W077:22’29.37”	N37:21’52.50”	M & F	3
M5	W077:22’16.45”	N37:21’52.30”	M & F	3
P23	W077:22’39.18”	N37:21’41.08”	M & F	5
P25	W077:22’36.06”	N37:21’46.28”	M & F	4
C4	W077:22’15.74”	N37:21’47.84”	F	4
K12	W077:22’39.47”	N37:21’52.84”	M & F	4
K2	W077:22’55.08”	N37:21’41.41”	F	4
***K5**	W077:22’35.40”	N37:21’43.50”	M & F of P23	5
K9	W077:23’01.90”	N37:22’10.03”	F	2
L1	W077:22’40.21”	N37:21’50.66”	M	0
P14	W077:22’53.96”	N37:21’36.76”	F	4
P16	W077:22’42.33”	N37:21’37.86”	F	5
R1	W077:22’56.31”	N37:21’44.17”	M & F	4
R11	W077:23’00.76”	N37:21’34.38”	M & F	4
R2	W077:23’03.19”	N37:21’46.45”	F	2
T26	W077:22’42.16”	N37:22’11.00”	M & F	4
T8	W077:22’31.34”	N37:22’01.67”	M & F	5

Table 3- Genetic Diversity: Genetic diversity in *Protonotaria citrea* adults and offspring. A represents the number of alleles, Ae is the number of effective alleles, Fis the expected heterozygosity, and the relative probability of exclusion P(excl) for each locus.

Locus	A	Ae	Fis	P(excl)
Lsw 5	5	1.49	0.33	0.18
Lsw 12	14	7.38	0.86	0.74
Lsw 14	7	1.35	0.26	0.15
Lsw 18	13	9.25	0.89	0.78

Table 4-OffspringAfterDumping: Number of *Protonotaria citrea* offspring left in each box separated by genetic similarity to the maternal individual and classified as 'Dumped' (e.g., maternal individual sampled cannot be the genetic mother) and 'Resident' (sampled mother genotype consistent with being genetic mother)

Box	Dumped	Resident
C1	1	2
C10	1	1
C11	0	4
C7	0	4
K15	0	5
K27	1	4
K28	0	3
K6	0	1
K7	1	4
K8	0	9
L2	1	4
M1	0	3
M5	0	3
P23	0	5
P25	0	4
C4	1	3
K12	0	4
K2	0	4
K5	0	5
K9	1	1
P14	2	2
P16	0	5
R1	0	4
R11	2	2
R2	2	0
T26	0	4
T8	0	5

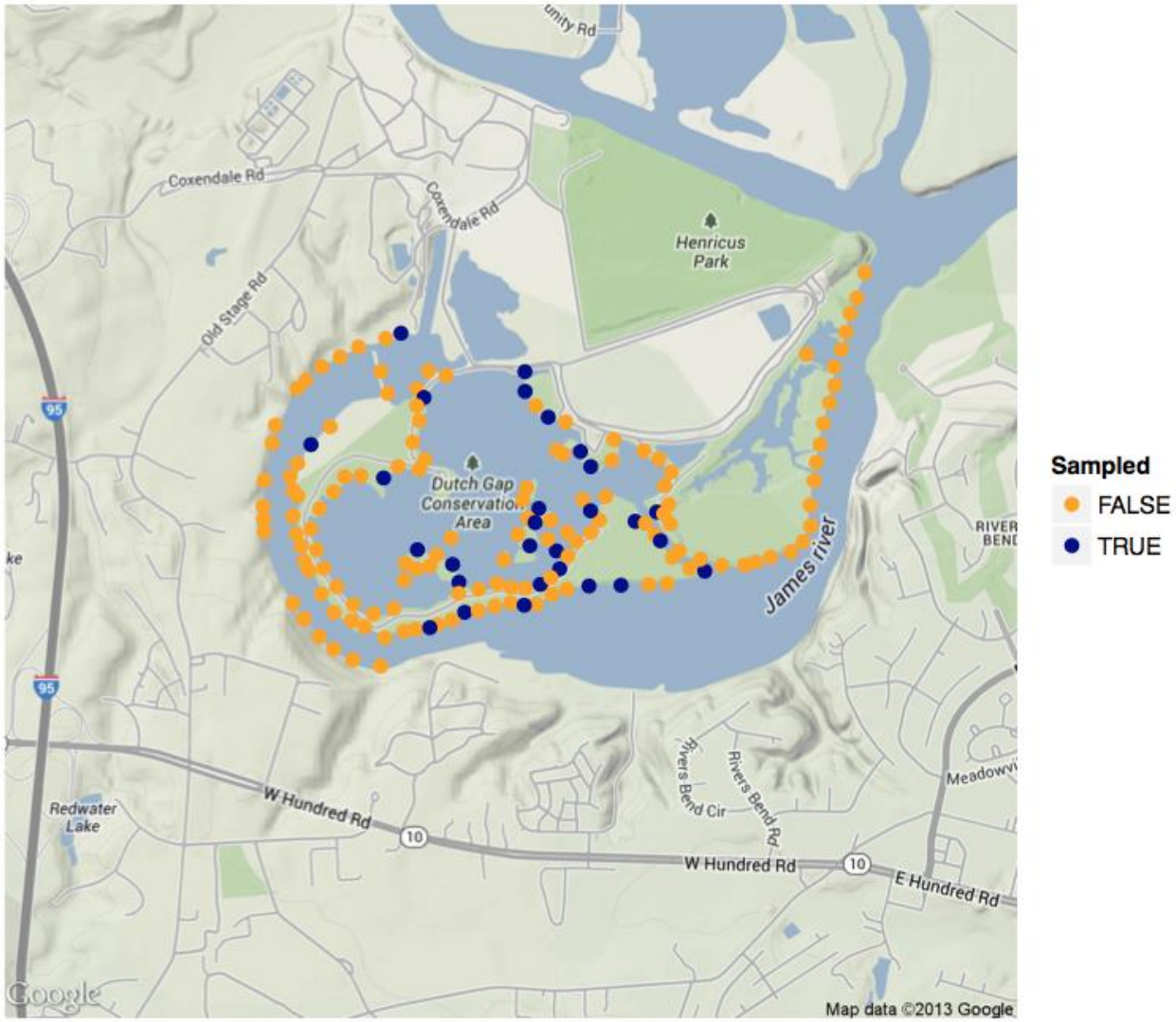


Figure 1-Map. Location of Prothonotary warbler nest boxes and indication of which boxes were sampled for this study.

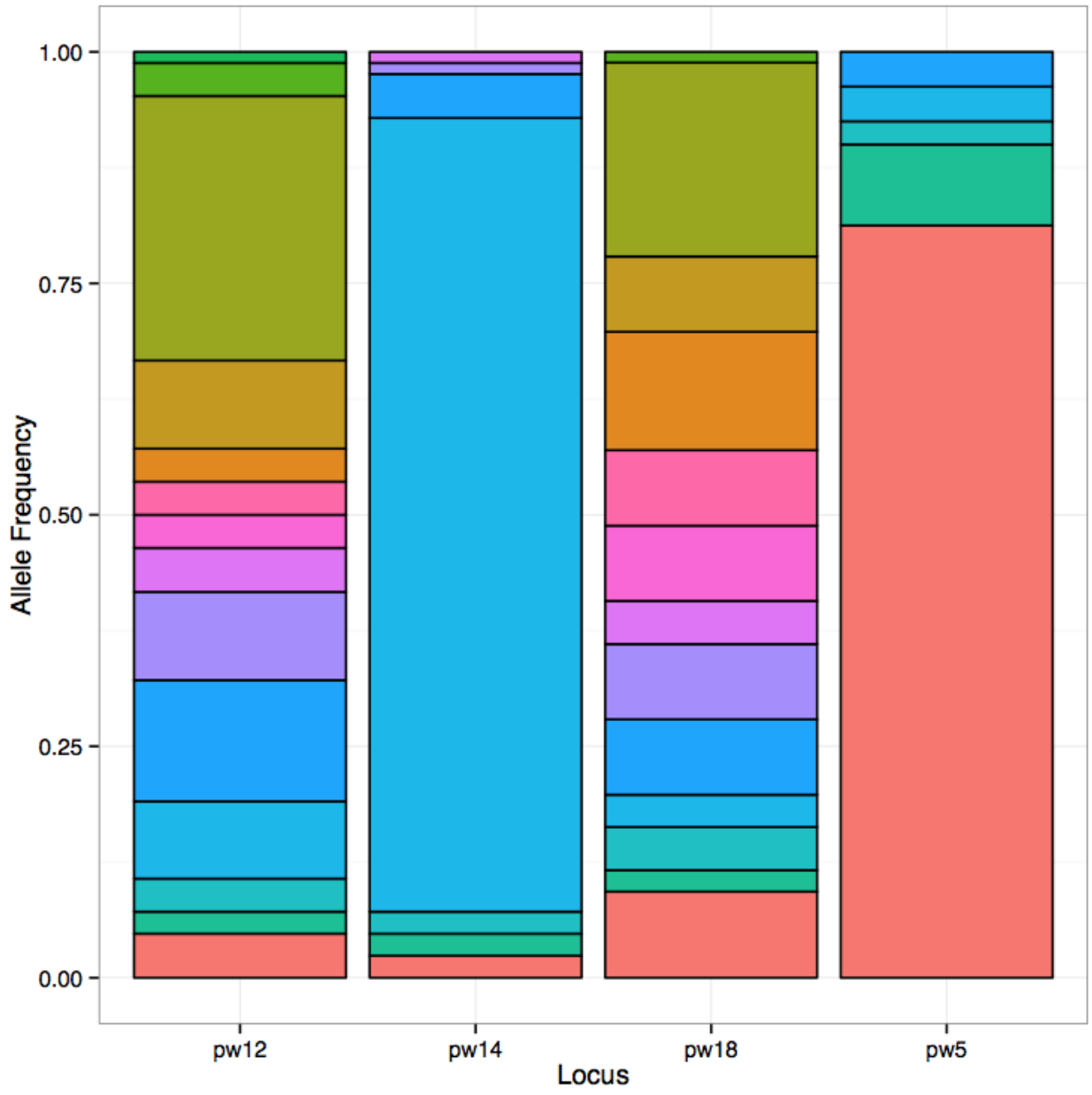


Figure 2-Allele: Frequency distribution of sampled *Protonotaria citrea* microsatellite alleles.

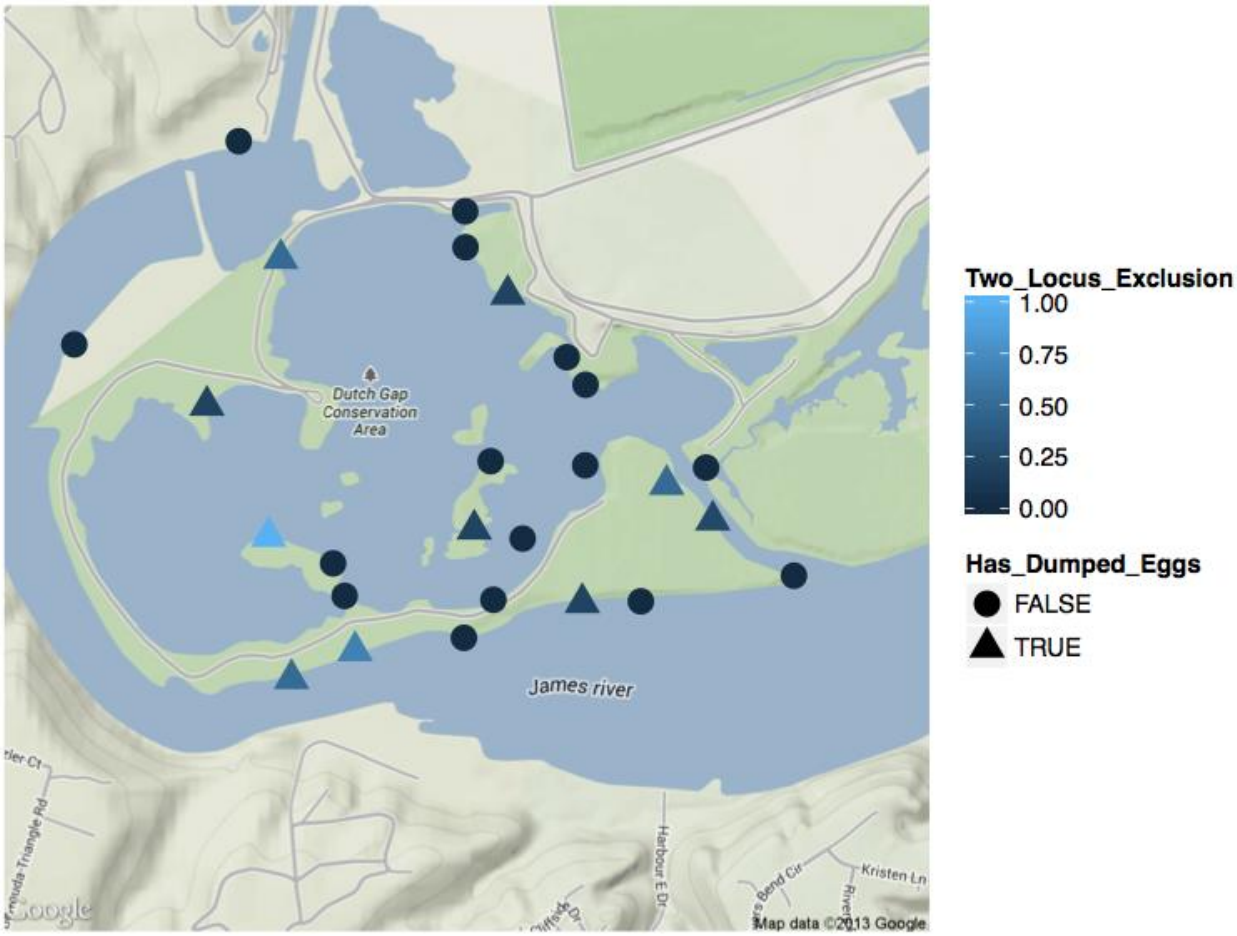


Figure 3-Dumped: Map of *Protonotaria citrea* dumped egg probabilities. True indicates a nest with a minimum of one dumped egg.

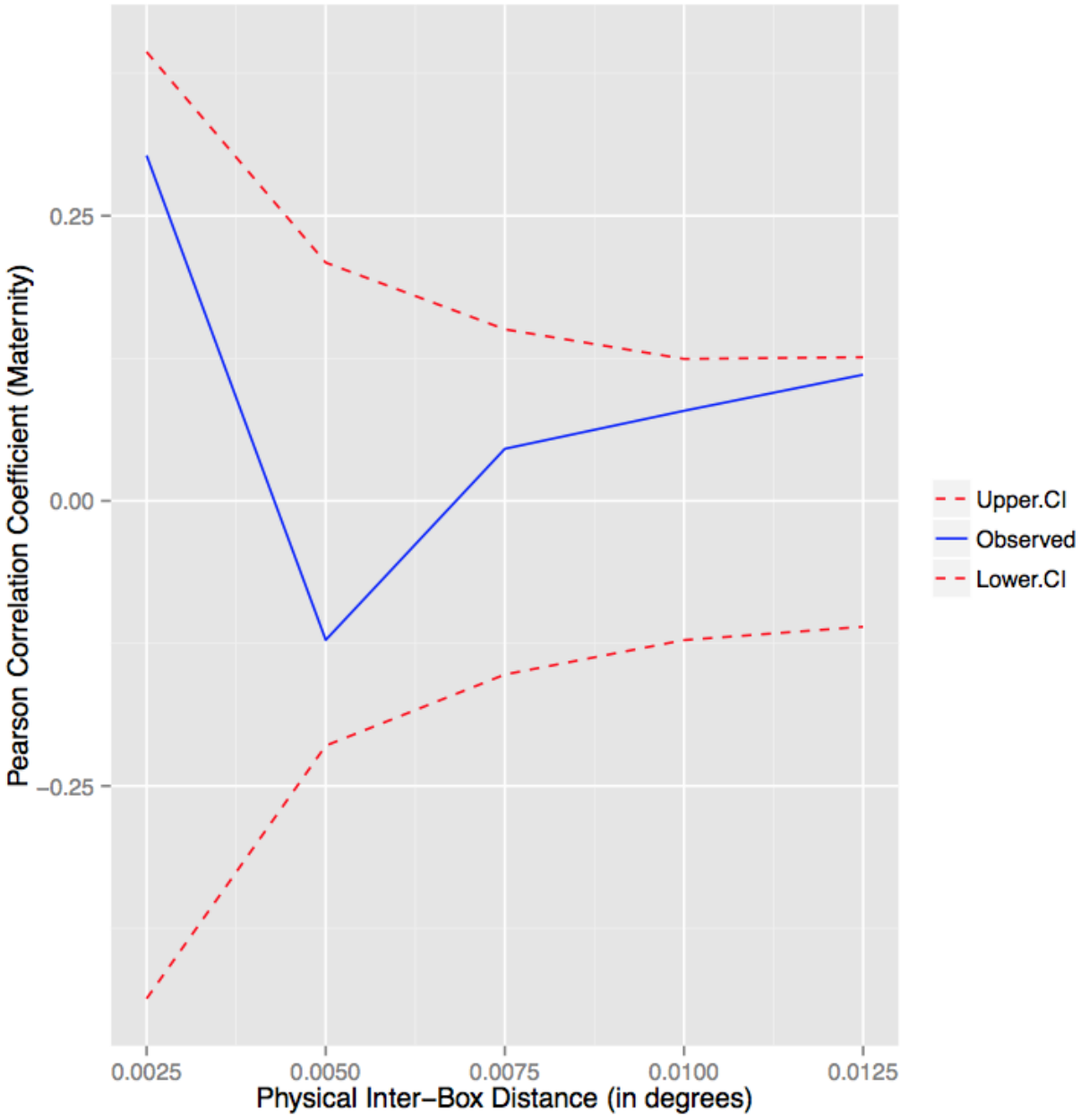


Figure 4-Spatial-Autocorrelation-Dumping. Spatial autocorrelation of *Protonotaria citrea* egg dumping using Smouse & Peakall's (1999) method. Dashed lines represent 95% confidence intervals on the regression coefficient (determined via permutation).

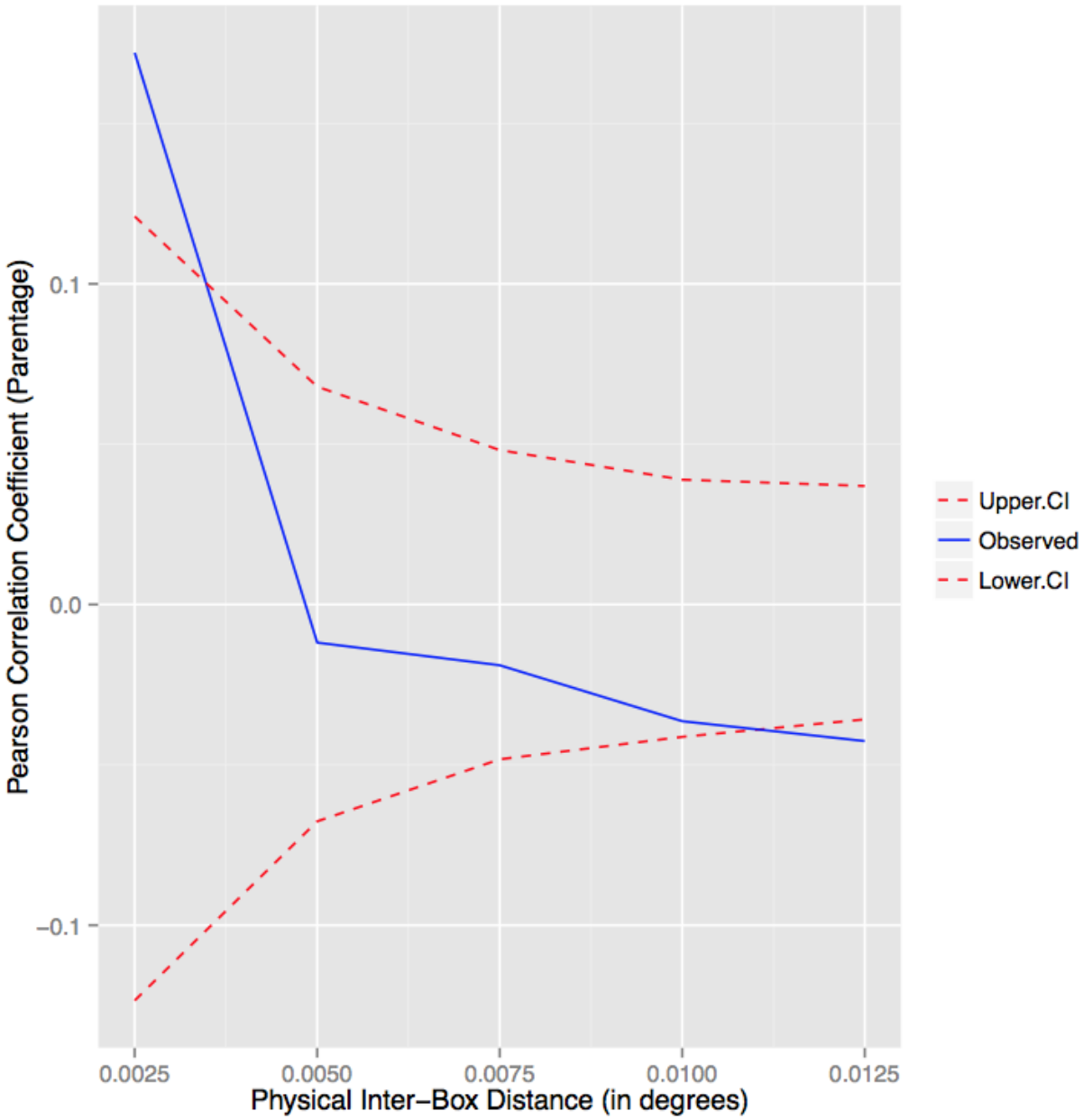


Figure 5-Spatial-Autocorrelation-Paternity. Spatial autocorrelation of *Protonotaria citrea* fractional paternity using Smouse & Peakall's (1999) method. Dashed lines represent 95% confidence intervals on the regression coefficient (determined via permutation).

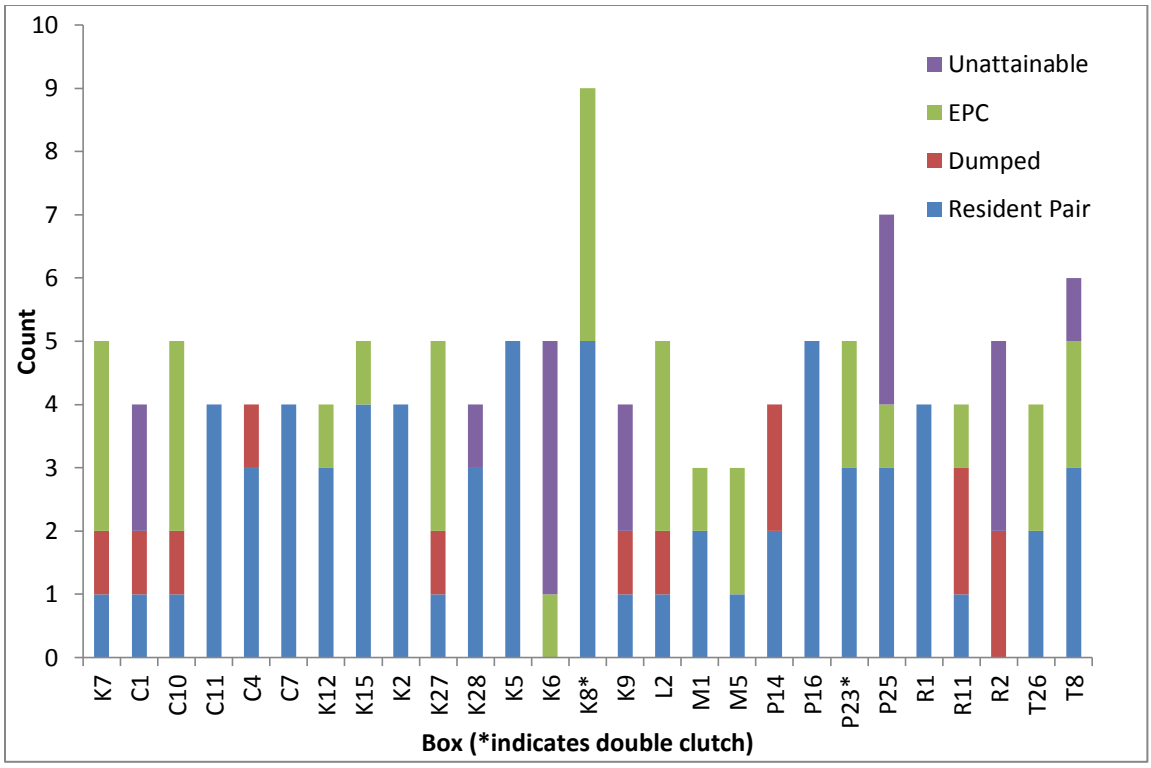


Figure 6: Distribution of *Protonotaria citrea* offspring based on mating behavior. Unattainable offspring are those who failed to amplify at a minimum of two loci.

Supplementary Materials

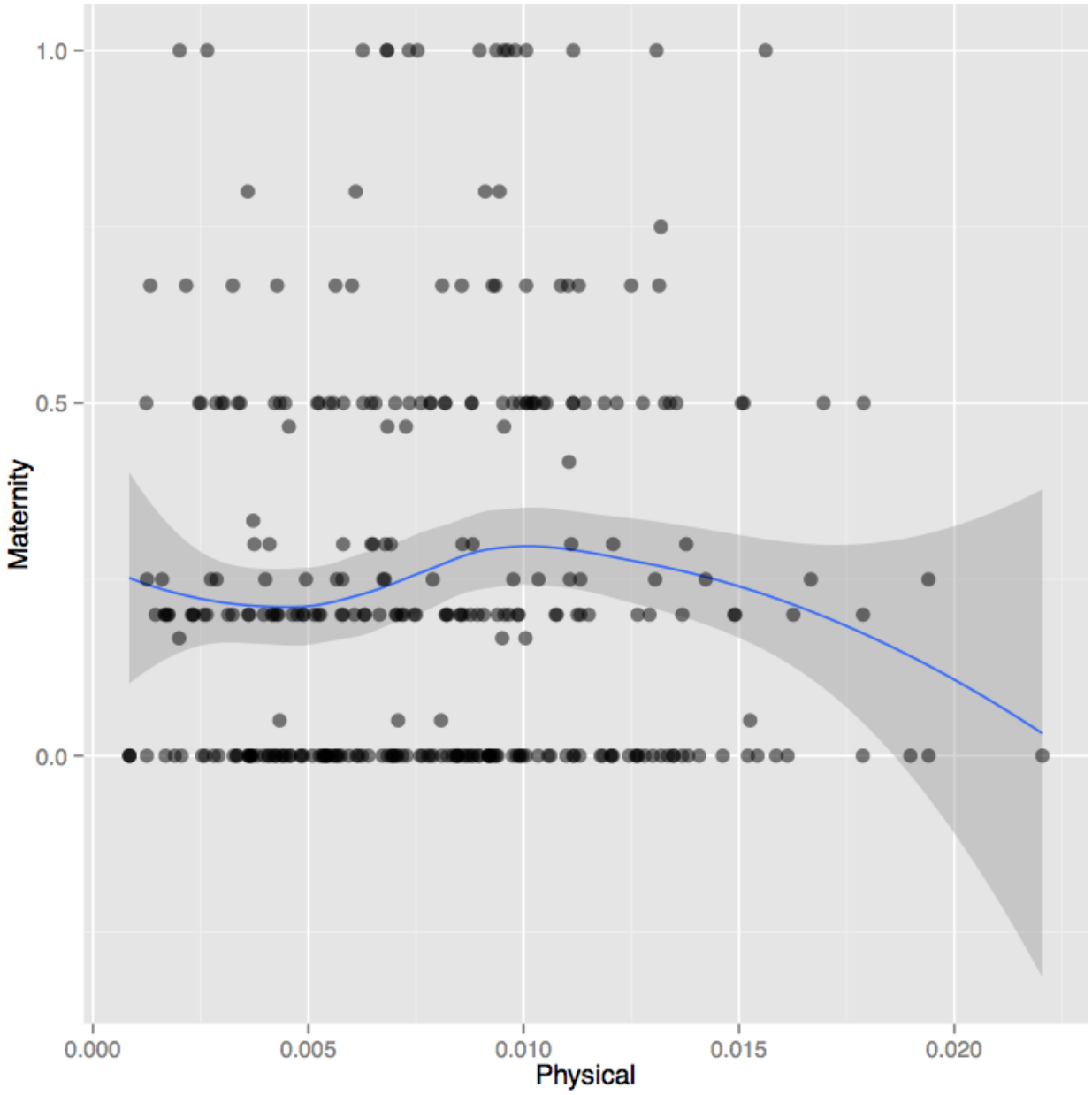


Figure Supp-1-MantelMaternity: Correlation between physical distance and fraction of *Protonotaria citrea* dumped eggs. Fitted line is polynomial regression with shaded 95% confidence intervals. r=0.034, P=0.322