



## Mating system in a gopher tortoise population established through multiple translocations: Apparent advantage of prior residence

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### ABSTRACT

Population manipulations such as translocation are becoming increasingly important tools in the management of rare and declining species. Evaluating the effectiveness of such manipulations requires comprehensive monitoring of population processes, including dispersal, survivorship, and reproduction. We investigated the mating system of a translocated population of gopher tortoises (*Gopherus polyphemus*) established through multiple releases, which occurred primarily during 1987–1994. During 2006–2007, we sampled and genotyped 27 candidate males (candidate sires), 34 candidate females (candidate dams), and 121 offspring from 19 clutches at five polymorphic microsatellite loci to determine the relative frequency of multiple paternity and to estimate individual reproductive success. Multiple paternity was detected in 57% of clutches genotyped, and females of single-sire clutches and females of multiple-sire clutches were of similar size. Reproductive success varied among male tortoises, and successful sires were significantly larger than males to which no offspring were attributed. Among successful sires, previously established males sired a disproportionate number of the offspring sampled, despite being significantly smaller than subsequently released males. The high variance in individual reproductive success and the apparent reproductive advantage associated with prior residence observed in this gopher tortoise population has important implications for the design of future translocation projects.

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### 1. Introduction

As ecosystems become more and more impacted by human activities, natural processes become increasingly disrupted and the ability of populations to compensate for these perturbations diminishes. As a result, species become more vulnerable to local extirpations and even range-wide extinction. Although non-intrusive management approaches such as habitat protection, restoration, and maintenance are critical to maintaining biodiversity and ecosystem function, some species will require more intensive management, including population manipulations. With reptiles experiencing world-wide population declines (Gibbons et al., 2000; Todd et al., 2010) and at least two-thirds of the world's turtle spe-

cies considered threatened with extinction by the International Union for Conservation of Nature, population manipulations such as translocation are increasingly employed to manage chelonians (Seigel and Dodd, 2000; Turtle Conservation Fund, 2002).

One of the main hindrances to evaluating the utility of translocation as a management tool for turtle species is the lack of explicit criteria for defining success, although recent work by Armstrong and Seddon (2008) and Bertolero and Oro (2009) provide a conceptual framework for *a priori* identification of key research questions and specific assessment criteria. A common definition for success is that the translocation results in a self-sustaining population (Griffith et al., 1989; but see Seddon, 1999), but this definition falls short of elaborating on how populations can be determined to be self-sustaining. A basic tenet of population ecology is that for populations to be stable (self-sustaining), four basic population processes must be in equilibrium, such that individuals added to the population as a result of births and immigrations replace individuals lost through death or emigration (Cohen, 1969; Pulliam, 1988). Most post-translocation monitoring focuses on a single population process (e.g., fidelity to the release area or evidence of successful reproduction) when evaluating project success. However, when determining whether translocated populations are self-sustaining,

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all four population processes are important, as Bertolero and Oro (2009) have demonstrated. Because translocations are basically managed immigrations, the remaining three processes (births, deaths, and emigrations) are of primary interest. Therefore, a conceptual framework for evaluating population viability—and hence the success of individual translocation projects—should consider post-release site fidelity, survivorship and reproduction (Bertolero and Oro, 2009; Tuberville, 2008).

Few studies have employed molecular tools to investigate mating system parameters in translocated populations, including reproductive success of individual animals (Keogh, 2009; but see Miller et al., 2009; Sigg et al., 2005). There are several reasons why mating systems might be expected to differ in translocated populations relative to naturally-occurring populations. First, the stress associated with translocation could potentially suppress or interfere with reproduction, at least temporarily (Dickens et al., 2010; Teixeira et al., 2007; Wingfield and Sapolsky, 2003). In addition, the increased movement behavior often observed during the “settling” phase immediately following release (Cook, 2004; Reinert and Rupert, 1999; Tuberville et al., 2005) could be so energetically costly that insufficient stores remain to devote to reproduction. Animals released in an unfamiliar environment also may not be able to locate important resources related to reproduction, including nest sites or even potential mates, particularly if the release animals are solitary, occur at low densities, or fail to establish home ranges or territories (Courchamp et al., 1999; Stephens and Sutherland, 1999). Finally, augmentation of an existing population could result in competitive interactions that affect the relative reproductive success of newly released animals compared to previously established residents (Berry, 1986).

Among reptiles, tortoises are the taxonomic group that has most frequently been managed through translocations (Seigel and Dodd, 2000). To evaluate the potential effects of translocation on the mating system of tortoises, we investigated multiple paternity and individual reproductive success in a translocated population of gopher tortoises (*Gopherus polyphemus*). The population was established through multiple releases over a 20-year period (1987–2007). Although several studies have described the behavioral and genetic mating system of gopher tortoises in naturally-occurring populations (Boglioli et al., 2003; Johnson et al., 2007, 2009; Moon et al., 2006), none has characterized the mating system in translocated populations. Our study addressed the following questions: What is the relative occurrence of single vs. multiple paternity within individual clutches? Does reproductive success vary among males? What proportion of translocated males sire offspring? Does order of establishment at the release site influence reproductive success?

## 2. Methods

### 2.1. Study species

Gopher tortoises (*G. polyphemus*) are long-lived terrestrial turtles, attaining sexually maturity at 220–255 mm carapace length (CL) and 10–21 years of age (Iverson, 1980; Landers et al., 1980), with individuals from populations at higher latitudes or in lower quality habitats taking longer to reach maturity. Gopher tortoises are restricted to the Coastal Plain of the southeastern United States, where they occur in local ‘colonies’ and construct large burrows in deep sandy soils. Individuals will use multiple burrows throughout the activity season, and several tortoises may sequentially occupy a given burrow (Eubanks et al., 2003). Although they tend to occupy burrows singly, gopher tortoises frequently visit each other at their respective burrows, with most social interactions occurring on the mound of sand (‘apron’) outside the burrow entrance (Boglioli

et al., 2003). The mating system of gopher tortoises was originally characterized as female defense polygyny (Douglass, 1976) in which dominant males were thought to control access to small groups or “harems” of females. More recent data provide compelling evidence that gopher tortoises have a mating system more consistent with scramble competition polygyny (Boglioli et al., 2003; Johnson et al., 2009).

Behavioral observations indicate that both sexes breed with multiple mates (Boglioli et al., 2003), with individual females experiencing an average of 26 mating attempts by 6–14 individual males in a single year (Johnson et al., 2007). Hormonal, morphological, and behavioral data suggest that female gopher tortoises can store sperm from the fall peak mating season to fertilize eggs laid the following spring (Gist and Congdon, 1998; Johnson et al., 2007; Ott et al., 2000). Sperm storage from multiple matings also provides the opportunity for multiple males to sire a single clutch, a phenomenon that has been corroborated with genetic analysis of clutches collected from known females (Moon et al., 2006). An adult female produces only a single small clutch of eggs (typically 3–9) during the reproductive season, often depositing them in the burrow apron, but may not reproduce every year (Diemer and Moore, 1994; Rostal and Jones, 2002).

### 2.2. Study population

We conducted our study on St. Catherines Island, a privately owned 5670 ha barrier island 6.4 km off the coast of Liberty Co., Georgia, USA. Gopher tortoises are not native to the island but approximately 105–115 tortoises from multiple source populations have been released at an abandoned pasture (162 ha; <3% of island area) at the north end of the island. The release site, which served as the study area for this research, is described in greater detail in Tuberville et al. (2008). Prior to 1994, approximately 25–30 tortoises (“previously established tortoises”) had been introduced but the wild populations from which they originated are not known. In spring 1994, a population of 74 wild-captured tortoises (“newly released tortoises”) was translocated to the island from a development site in Bulloch Co., GA (approximately 110 km away) and represented the largest release of tortoises on the island. Since 1994, at least 13 formerly captive or rehabilitated tortoises (“waifs”) have also been released. All tortoises were permanently marked prior to release except previously established tortoises, which were marked upon first recapture. Mark-recapture with variable sampling effort was conducted during 1994–2007 to monitor survivorship, reproduction, health status, and recruitment in the study population. Mark-recapture histories of individual tortoises allowed us to estimate the current size of the breeding pool (Tuberville et al., 2008).

### 2.3. Sample collection

We intensively trapped burrows during 2004, 2006 and 2007 to capture sexually mature adults. We weighed (to nearest 0.01 kg) and measured (mid-line carapace length [CL] to the nearest mm) each individual and classified tortoises at least 220 mm CL in size as adults. We classified adults with concave plastrons and elongated gular scutes as males and adults lacking these secondary sexual characteristics as females.

During the nesting seasons (May–early July) of 2006 and 2007, we radiographed adult females to detect the presence and number of shelled eggs (Gibbons and Greene, 1979). We injected females having fully calcified eggs with oxytocin to induce oviposition prior to their release (Ewert and Legler, 1978). We incubated eggs collected directly from females at 28–30 °C until hatching, approximately 85–100 days later. Gopher tortoises exhibit temperature-dependent sex determination (Burke et al., 1996; Demuth, 2001).

Therefore, we selected incubation temperatures to encompass the pivotal temperature for sex determination so as to produce clutches with approximately a 1:1 sex ratio. We collected additional clutches by searching burrow aprons for natural nests during May–September. In 2006, we completely excavated nests and placed them in incubators until hatching. In 2007, we protected nests from predators with wire cages and allowed nests to incubate in the field until 21 August, shortly before hatching.

We collected blood from the brachial vein of adults and from the subcarapacial vein of hatchlings (Hernandez-Divers et al., 2002). We stored blood samples in lysis buffer (100 mM Tris pH 8.0, 100 mM EDTA, 150 mM NaCl, 1% SDS) until extracting DNA using a protocol modified from Carter and Milton (1993; modified protocol available upon request). We dissected eggs that were damaged during nest excavation or that failed to hatch and collected tissue from partially developed embryos. We did not dissect unhatched eggs until after the normal hatchling emergence period (mid-October) and due to tissue deterioration, were not always able to obtain high quality DNA.

#### 2.4. Microsatellite genotyping

We developed five polymorphic microsatellite loci for *G. polyphemus* using the protocol by Glenn and Schable (2005). We optimized polymerase chain reaction (PCR) conditions for each locus using genomic DNA from 24 individuals originating from McIntosh Co., GA (Tuberville, unpublished data). The basic properties and PCR conditions for each locus, based on samples collected from St. Catherines Island, are presented in Table 1. We modified one primer in each pair at the 5' end with an engineered sequence (CAG tag 5'-CAGTCGGGCGTCATCA-3'; see Schable et al., 2002) to allow use in the PCR of a third primer that was fluorescently labeled for detection on the ABI 3130xl sequencer. Four bases (a GTTT pigtail) were added to the 5' end of three of the reverse primers (Gopo-2, Gopo-5, Gopo-12) to drive amplicons to +A to help produce more clear genotypes (Brownstein et al., 1996).

We performed single-locus PCR amplifications in a 11.5 µL volume (10 mM Tris pH 8.4, 50 mM KCl, 25.0 µg/ml BSA, 0.4 µM unlabeled primer, 0.04 µM tag labeled primer, 0.36 µM universal dye-labeled primer, 2 mM MgCl<sub>2</sub>, 0.15 mM dNTPs, 0.5 units Jump-Start Taq DNA polymerase (Sigma), and 5–50 ng DNA template) using an Applied Biosystems (GeneAmp PCR System 9700) or Eppendorf Mastercycler Gradient thermal cycler. For amplification, we used touchdown thermal cycling programs (Don et al. 1991) encompassing a 10° span of annealing temperatures ranging between 65–55 °C (TD65) or 60–50 °C (TD60). Our cycling parameters were 95 °C for 3 min, 21 cycles of 96 °C for 20 s, highest annealing temperature (decreased 0.5 °C per cycle) for 20 s, and

72 °C for 30 s; and 15 cycles of 94 °C for 20 s, lowest annealing temperature for 20 s, and 72 °C for 30 s.

We pooled PCR products from Gopo-2, Gopo-5, and Gopo-14 in a single well that also contained a Naurox size standard prepared as described in DeWoody et al. (2004). We similarly pooled PCR products from Gopo-1 and Gopo-12. We ran the pooled PCR products and internal size standard on an ABI-3130x automated DNA sequencer and analyzed results using GENEMAPPER version 4.0 (Applied Biosystems).

#### 2.5. Statistical analyses

Using CERVUS 3.0 (Kalinowski et al., 2007), we calculated allele frequencies for the study population based on genotypes of sexually mature adults. Because the tortoises studied here originated from multiple populations, it was expected that the markers should not be in Hardy–Weinberg equilibrium (HWE) among the translocated animals. Therefore, we did not calculate deviations from HWE or the frequency of null alleles. We used CERVUS to calculate single- and multi-locus probabilities of identity (probability that two unrelated individuals share the same genotype) and probabilities of exclusion when neither parent is known and when one parent is known (Table 2). We tested for linkage disequilibrium between loci using Genepop 1.2 (Raymond and Rousset, 1995; <http://genepop.curtin.edu.au/>).

We performed parentage analysis on each clutch using both categorical allocation and parental reconstruction. Parental reconstruction uses genotypes of full-sib or half-sib progeny arrays (in this case, clutches collected directly from females or from natural nests) to reconstruct all possible combinations of paternal and maternal genotypes that could explain the observed offspring genotypes (Jones and Ardren, 2003). When more than one combination of maternal and paternal genotypes is possible, the solutions are ranked based on allele frequencies in the population and the rules of Mendelian inheritance. GERUD 2.0 (Jones, 2005) allows reconstruction of parental genotypes from progeny arrays, whether or not the maternal genotypes are known. Parental reconstruction is possible even when candidates of one or both sexes are unsampled in the population, provided that allele frequencies have been estimated. If candidate parents have been sampled, the reconstructed genotypes can be compared to genotypes from the pool of candidate parents (Jones and Ardren, 2003). Although the program will not always generate a multi-locus genotype that exactly matches the genotypes of one of the candidate fathers, GERUD is useful for detecting multiple paternity within progeny arrays, determining the minimum number of fathers necessary to explain the offspring genotypes, and estimating reproductive skew among males contributing to multiply-sired clutches.

**Table 1**

Characterization of five polymorphic microsatellite loci for *Gopherus polyphemus*. The portion of the primer sequence corresponding to the engineered CAG tag and the GTTT pigtail is indicated in italics. TD is the initial annealing temperature for amplifications. Size indicates the range of allele sizes in bp observed among adult breeders in the St. Catherines Island population. Polymorphic information content (PIC) was estimated using CERVUS 3.0.

Primer	Sequence 5'–3'	Dye	TD	Repeat motif	Size (bp)	PIC
Gopo-01 F	<i>CAGTCGGGCGTCATC</i> AGGTCCTTTCAACCCCTAATCTT	FAM	65	(AAAG) <sub>21</sub>	250–326	0.893
Gopo-01 R	GCAGCCAATTTCTTAACTAA					
Gopo-02 F	<i>CAGTCGGGCGTCATC</i> AGGCAGCAGAGAATAGAT	VIC	60	(AGAT) <sub>23</sub>	244–340	0.909
Gopo-02 R	<i>GTTTTATCAGCTATCCCGTGTA</i>					
Gopo-05 F	<i>CAGTCGGGCGTCATCATCTGTAATGCCTAGAAATCAA</i>	FAM	60	(AAAG) <sub>10</sub> (ACTC) <sub>6</sub>	331–383	0.834
Gopo-05 R	<i>GTTTTGCCATTCTGTTAAAGTTC</i>					
Gopo-12 F	<i>CAGTCGGGCGTCATCACTTTGGAAGCCATTGTAATA</i>	NED	65	(AAAC) <sub>13</sub>	348–368	0.714
Gopo-12 R	<i>GTTTCATTTGCACCACTTAACTA</i>					
Gopo-14 F	GTCCTGGGATTACAATCAAT	NED	60	(ATCC) <sub>13</sub>	161–211	0.715
Gopo-14 R	<i>CAGTCGGGCGTCATCA</i> CCAATCTTTTCGTAATGTAT					

**Table 2**  
Basic properties of five microsatellite loci for *G. polyphemus*, as observed in the St. Catherines Island mixed population. The number of alleles ( $k$ ), observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities, probability of identity and probabilities of exclusion were calculated in CERVUS 3.0 for each individual locus and for all loci combined.

Locus	$k$	$H_O$	$H_E$	Probability of identity	Exclusion probabilities		
					First parent (when neither parent known)	Second parent (when first parent known)	Parent pair
Gopo-01	16	0.900	0.909	0.018216663	0.6680	0.8015	0.9374
Gopo-02	18	0.883	0.922	0.005164358	0.7067	0.8281	0.9520
Gopo-05	14	0.800	0.857	0.035555298	0.5430	0.7054	0.8750
Gopo-12	6	0.678	0.755	0.100322442	0.3533	0.5342	0.7240
Gopo-14	11	0.650	0.741	0.091321642	0.3680	0.5567	0.7693
Multi-locus				$3.06 \times 10^{-8}$	0.9818	0.9979	0.9999

Categorical allocation is a likelihood based approach to assigning parentage from a pool of candidate parents when the identity of one or both parents is unknown (Jones and Ardren, 2003). For each offspring, the genotypes of candidate parents are searched to identify all parents with compatible genotypes. For each offspring and candidate parent (if one parent known) or parent-pair (if neither parent known) combination, the  $\log_e$  likelihood ratio (LOD score) is calculated comparing the likelihood that a candidate parent (or parent-pair) is the true parent (or parent-pair) of the offspring to the likelihood that they are unrelated (Jones and Ardren, 2003). Offspring are assigned to the parent or parent-pair with the highest LOD score. CERVUS 3.0 (Kalinowski et al., 2007) can be used to identify the most likely parent or parent-pair for individual offspring, even when parent-offspring mismatches occur due to mutations, null alleles or genotyping error. However, CERVUS requires at least partial sampling of candidate parents and an estimate of the proportion of breeders sampled to calculate meaningful LOD scores. Offspring whose true parent was unsampled may be assigned to an unrelated candidate parent with the most compatible genotype, resulting in a false positive, or remain unassigned, but LOD scores will be low for these offspring-parent pairs. CERVUS is also useful for calculating reproductive success of candidate sires.

For clutches from known females (i.e., hatched from eggs collected directly from females), we visually inspected offspring genotypes to verify their compatibility with the maternal genotype. We analyzed the hatchling and maternal genotypes of each clutch in GERUD 2.0 to calculate the minimum number of sires necessary to explain the observed offspring genotypes. Next, we performed paternity analysis in CERVUS with all sampled males included in the list of candidate fathers. We then determined the number of sires contributing to the clutch by compiling the list of fathers assigned in CERVUS to individual offspring comprising the clutch. Finally, we compared the minimum number of sires calculated by CERVUS and GERUD for each clutch. When CERVUS and GERUD differed in the number of sires assigned to individual clutches, we visually inspected the genotypes of the offspring, mother, and putative fathers assigned to the clutch. We chose the more conservative estimate of number of fathers when the additional sire was assigned to a single offspring but was not needed to explain that offspring's genotype (i.e., the offspring was compatible with the other father(s) assigned to the clutch).

For field-collected nests for which identity of the mother was unknown, we analyzed hatchling genotypes in GERUD 2.0 to calculate the minimum number of sires. Next, we performed parentage analysis in CERVUS to identify the most likely parent-pair for each offspring. We included all sampled males in the list of candidate fathers. However, we created a separate list of candidate mothers for each clutch by excluding any female that was hormonally-induced to lay eggs in captivity or whose clutch size determined from radiographs did not match clutch size of the field-collected nest. We calculated the number of sires contributing to each clutch by comparing fathers assigned by CERVUS to individual offspring

comprising the clutch. We compared the number of sires estimated by GERUD and CERVUS as previously described. Similarly, we compared the female assigned to individual offspring comprising the clutch. For those clutches in which a single female was consistently assigned as the most likely mother of all offspring, we reanalyzed offspring genotypes in both GERUD and CERVUS with that female designated as the known mother.

### 3. Results

We captured 27 candidate sires (248–350 mm CL, mean = 309 mm CL) and 34 candidate dams (255–344 mm CL, mean = 316 mm CL), each representing 57% of the estimated total number of each sex released on St. Catherines Island. However, after excluding animals not captured in the past 10 years (i.e., since 1996), we estimate that we sampled at least 84% of the adult males and 75% of the adult females still residing at the release site.

The five microsatellite loci had 6–18 alleles per locus with observed heterozygosity ranging from 0.65 to 0.90 (Table 2) in the mixed breeding pool, which consisted of previously established tortoises, newly released tortoises, and waifs. We did not detect any linkage disequilibrium among the 10 paired loci comparisons.

The five markers combined to yield high power. The probability of two unrelated tortoises sharing the same genotype (probability of identity) was  $3.06 \times 10^{-8}$  (Table 2). Single-locus probabilities of detecting multiple paternity ranged from 0.353 to 0.707 when the mother's genotype was unknown and 0.534–0.828 when the mother's genotype was known. The multi-locus probability of detecting multiple paternity was 0.982 when the mother's genotype was unknown and 0.998 when the mother's genotype was known.

We collected a total of 192 eggs from 24 clutches – 71 eggs from eight clutches in 2006 and 121 eggs from 16 clutches in 2007. Thirty-three eggs from five clutches in 2006 and 83 eggs from 14 clutches in 2007 successfully hatched. In addition, we were able to extract DNA from four partially developed embryos in 2006 and one embryo in 2007, for a total of 121 offspring from 19 clutches genotyped during the study (Table 3). Some eggs failed to hatch due to damage during oviposition or nest excavation, incubator overheating, early embryonic death, or unknown causes. Five clutches (totaling 42 eggs), including both lab- and field-incubated nests, experienced complete hatching failure.

We classified clutches represented by at least four genotyped offspring as having either single or multiple sires and calculated the proportion of offspring within a clutch assigned to each male (Table 3). Females with multiply-sired clutches were not significantly different in size (mm in CL) than females of singly-sired clutches ( $t_{df=7} = -1.233, P = 0.257$ ). Even though multiple paternity was detected in clutches with as few as three offspring (clutch 2006-03), clutches with fewer than four offspring ( $n = 5$ ) were excluded from calculations because only a small proportion of the original clutch was genotyped (<40%). Of the remaining 14

**Table 3**

Characteristics of clutch, mother, and father for single and multiply-sired clutches. Number of offspring genotyped includes both live hatchlings and embryos salvaged from unhatched eggs. Clutches with fewer than four genotyped offspring were excluded from calculations of number of sires and reproductive skew among males within multiply-sired clutches.

Clutch ID	No. eggs	No. hatched	% Hatched	No. offspring genotyped (%)	Mother ID	Mother's origin	Mother, CL (mm)	Min. # sires	Father ID	No. assigned offspring (%)	Father's origin	Father, CL (mm)
<i>Single sire</i>												
2006-01	8	8	100.0	8 (100.0)	423 <sup>a</sup>	Established	282	1	213	8 (100.0)	Established	314
2006-08	11	9	81.8	10 (90.9)	152 <sup>c</sup>	New release	334	1	221	10 (100.0)	Established	304
2007-03	12	12	100.0	12 (100.0)	152 <sup>c</sup>	New release	334	1	221	12 (100.0)	Established	304
2007-10	8	6	75.0	6 (75.0)	214 <sup>b</sup>	Established	300	1	213	6 (100.0)	Established	314
2007-14	9	9	100.0	9 (100.0)	410 <sup>b</sup>	Established	284	1	305	9 (100.0)	Established	322
2007-16	7	6	85.7	6 (85.7)	129 <sup>a</sup>	New release	328	1 <sup>A</sup>	127	6 (100.0)	New release	311
<i>Multiple sires</i>												
2006-02	9	8	88.9	9 (100.0)	154 <sup>c</sup>	New release	344	2 <sup>A</sup>	118 221	6 (66.6) 3 (33.3)	New release Established	324 304
2006-04	8	6	75.0	7 (87.5)	119 <sup>c</sup>	New release	329	2	221 NS <sup>d</sup>	6 (85.7) 1 (14.3)	Established	304
2007-04	6	3	50.0	4 (66.7)	159 <sup>a</sup>	New release	329	2	156 NS	3 (75.0) 1 (25.0)	New release	328
2007-06	7	7	100.0	7 (100.0)	174 <sup>b</sup>	New release	298	2 <sup>A</sup>	143 157	4 (57.1) 3 (42.9)	New release New release	332 350
2007-08	10	10	100.0	10 (100.0)	145 <sup>b</sup>	New release	334	2	151 221	6 (60.0) 4 (40.0)	New release Established	344 304
2007-09	6	6	100.0	6 (100.0)	142 <sup>b</sup>	New release	323	2 <sup>A</sup>	156 103	5 (83.3) 1 (16.7)	New release New release	328 293
2007-12	8	7	87.5	8 (100.0)	NS <sup>d</sup>			2	305 NS	7 (87.5) 1 (12.5)	Established	322
2007-15	6	6	100.0	6 (100.0)	414 <sup>a</sup>	Established	294	2	305 203	5 (83.3) 1 (16.7)	Established Established	322 294
<i>Excluded</i>												
2006-03	8	2	25.0	3 (37.5)	116 <sup>c</sup>	New release	332	2	203 NS	2 1	Established	294
2007-13	8	3	37.5	3 (37.5)	212 <sup>b</sup>	Established	NR <sup>D</sup>	1	NS	3		
2007-11	7 <sup>B</sup>	3		3	215 <sup>a</sup>	Established	329	1	157	3	New release	350
2007-17	7	2	28.6	2 (28.6)	168 <sup>a</sup>	New release	312	1	NS	2		
2007-02	9	2	22.2	2 (22.2)	502 <sup>c</sup>	Waif <sup>c</sup>	320	1	118	2	New release	324

<sup>a</sup> Maternity was known because eggs were collected directly from female.

<sup>b</sup> Maternity was assigned by CERVUS.

<sup>c</sup> Maternity was assigned by CERVUS and corroborated by field observations.

<sup>d</sup> Parent was not sampled.

<sup>A</sup> CERVUS assigned an additional sire to a single offspring but the additional sire was not necessary to explain observed offspring genotypes.

<sup>B</sup> Complete clutch size not known because female laid some eggs in trap and eggs were too damaged to count.

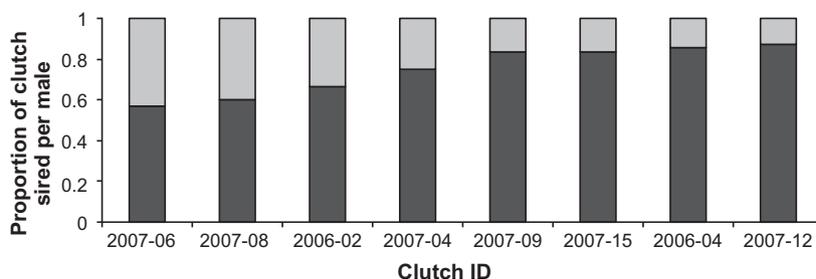
<sup>C</sup> First released on island in May 2006.

<sup>D</sup> Not recorded.

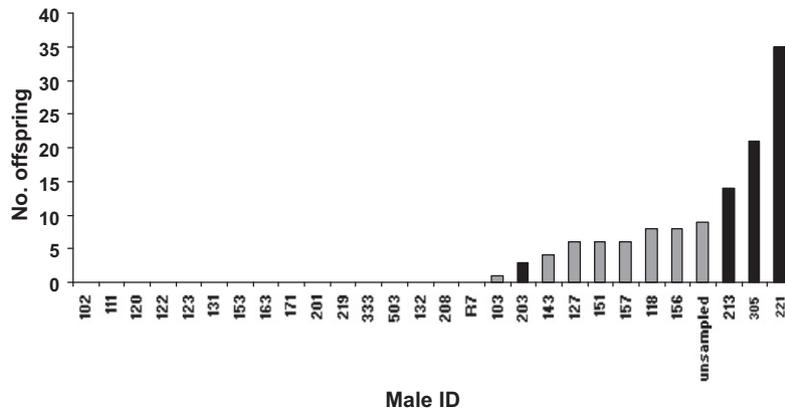
clutches, six (42.9%) were sired by a single male. In one of the six clutches, CERVUS assigned a second sire, but both GERUD analysis and visual inspection indicated that all offspring genotypes could be explained by a single candidate sire. Eight clutches (57.1%) were fathered by multiple sires. CERVUS assigned a third sire to a single offspring in three of the eight multiply-sired clutches. However, GERUD analysis and visual inspection revealed that the third sire was not necessary to explain the offspring genotypes, leading us to conclude that the eight multiply-sired clutches were each likely fathered by only two males. An average of 74.5% (range 57.1–87.5%) of offspring from multiply-sired clutches were assigned to

the primary male (i.e., the male siring the most offspring; Fig. 1). For each multiply-sired clutch, the primary male tended to be larger in CL than the other male assigned to the clutch, but the trend was not statistically significant ( $t_{df=8} = 1.872$ ,  $P = 0.098$ ).

The 19 clutches producing offspring for analyses (including those with fewer than four offspring genotyped) were attributed to 18 females, with only female 152 having clutches represented in both years of sampling. Male 221 was assigned as the sole sire to both of female 152's clutches. The 18 assigned females included six previously established (33.3%), 10 newly released (55.6%), one waif (5.5%), and one unsampled female (5.5%). Fifty percent of



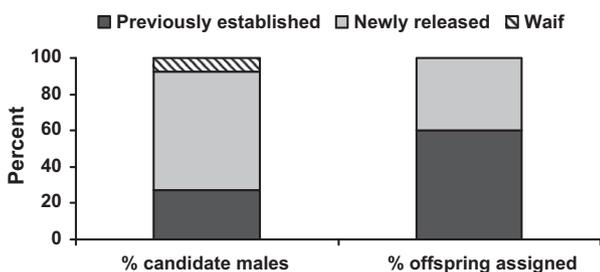
**Fig. 1.** Reproductive skew among males of multiply-sired clutches collected from a translocated gopher tortoise population at St. Catherines Island, GA during 2006–2007.



**Fig. 2.** Distribution of reproductive success (i.e., number of offspring sired) among candidate sire gopher tortoises based on 121 offspring from 19 clutches collected from St. Catherines Island, GA during 2006–2007, including offspring attributed to one or more unsampled males. Gray bars represent newly release sires, black bars indicate previously established sires.

the candidate dams were represented in clutches we sampled during 2006–2007.

Eleven males (40.7% of the candidate sires we sampled) were attributed to the 19 clutches with genotyped offspring. Successful males (sires) were larger in CL than non-sires, and this trend was nearly significant ( $t_{df=23} = 2.059, P = 0.051$ ). All of the 11 sires were represented in the 14 clutches from 2007, but only four of those sires were also represented in the five clutches from 2006, probably due in part to the reduced sampling effort in 2006. Among the 11 sires were four previously established males (36.4%) and seven newly released (63.6%) males. One or more unsampled males were also assigned to nine offspring from six clutches. Two (7.4%) candidate sires accounted for 46.3% of all offspring sampled – male 221 (a previously established male) sired 35 offspring from five clutches and male 305 (also a previously established male) sired 21 offspring from three clutches. In contrast, only a single offspring (0.8%) was assigned to male 103 (Fig. 2). Of the total candidate sire breeding pool, previously established males represented 26.9% of the breeders but sired 60.3% of all offspring whereas newly released males represented 65.4% of breeders but sired 32.2% of offspring, and waif males represented 7.7% of breeders but sired no offspring (Fig. 3). Thus, significantly more offspring were attributed to previously established males than expected when compared to subsequently released males based on their relative abundance in the pool of candidate breeders ( $\chi^2 = 70.6, 2 \text{ df}, P < 0.0001$ ). Newly released males comprising the pool of candidate breeders were significantly larger (mm CL) than previously established males ( $t_{df=7} = 2.501, P = 0.041$ ).



**Fig. 3.** A comparison of the relative abundance of previously established males (black bars) to newly released (gray bars) and waif male gopher tortoises (hatched bars) subsequently released on St. Catherines Island and the proportion of 2006–2007 offspring they were known to have sired. Candidate sires sampled during our study ( $n = 27$ ) represented approximately 84% of estimated males comprising the resident breeding pool. Offspring ( $n = 121$ ) were assigned to candidate sires using CERVUS.

**Table 4**

Number of offspring resulting from mating interactions among previously established and newly released males and females, as documented through parentage analysis. Number of clutches from which offspring originated is given in parentheses.

Female parent	Male parent	
	Previously established	Newly released
Previously established	39 (5)	0 (0)
Newly released	35 (5)	34 (7)
Total	74	34

We documented successful matings between previously established and newly released tortoises of both sexes, although none of the genotyped offspring resulted from matings between previously established females and newly released males (Table 4). Only a single waif (female 502) was known to have successfully reproduced, but only six of the 13 released waifs were sexually mature and all were released during our 2006–2007 study. Female 502 was released during May 2006 and was not gravid at the time of her release. The following year, she successfully nested and the resulting offspring were assigned to male 118 (a newly released male).

#### 4. Discussion

The markers developed in the study worked well to determine parentage in this population. The large number of alleles per locus yielded high power for parentage determination. Because some microsatellite loci are conserved among divergent turtle species (FitzSimmons et al., 1995), these loci should be a valuable resource for future studies in these and other tortoises.

Multiple paternity occurred in at least eight of 14 clutches (57.1%) and potentially occurred in as many as nine clutches (64.3%) from the population of translocated gopher tortoises established on St. Catherines Island, Georgia. Multiple paternity was observed in only 28.6% of clutches from a naturally-occurring population in Florida (Moon et al., 2006). The lower incidence of multiple paternity reported by Moon and colleagues may be an artifact of a smaller sample size ( $n = 7$  clutches) and the reduced power of their markers to detect multiple paternity (0.876 when the mother's genotype was known vs. 0.998 in our study). Both studies, however, demonstrate that multiply-sired clutches are a common occurrence in gopher tortoise populations, a finding consistent with behavioral observations that individual females experience numerous mating attempts by multiple males in a single year (Johnson et al., 2007). Multiple paternity appears to

be a common phenomenon among turtles and is facilitated by the ability of females to store sperm within and between mating seasons (Galbraith, 1993; Pearse and Avise, 2001), resulting in temporal polyandry (Moon et al., 2006).

For each clutch we classified as multiply-sired, two sires were sufficient to explain the observed offspring genotypes, although a third male potentially sired a single offspring in three of the eight clutches. Assuming that multiply-sired clutches were each sired by only two males, the contribution of the primary male (i.e., the male siring more offspring) ranged from 57.1% to 87.5% of genotyped offspring within the clutch. The primary male tended to be larger (~20 mm CL) than the secondary male. Overall, successful sires—whether of singly- or multiply-sired clutches—also tended to be larger than males to which no offspring were attributed, corroborating similar findings by Moon et al. (2006). Although these size trends did not quite reach statistically significant levels, we nonetheless consider them biologically meaningful. In addition, more than 60% of successful sires fertilized eggs produced by more than one female, demonstrating that males also mate with multiple females. Without taking order of establishment into account (i.e., whether sire was a previously established or subsequently released tortoise), greater reproductive success by larger male tortoises suggests that larger males may have a competitive advantage over smaller males (in attaining copulations, sperm quality, or both), are more attractive to receptive females, or females choose their sperm preferentially. Size has been documented as an important determinant of male social rank in snapping turtles (Galbraith et al., 1987). In wood turtles rank was positively correlated with weight and age, and higher ranking males obtained more copulations with females (Kaufmann, 1992) and successfully sired more offspring than lower ranking males (Galbraith, 1991). We did not observe a relationship between female size and number of sires, as has been reported by Moon et al. (2006).

As had been observed in a naturally-occurring population of gopher tortoises (Moon et al., 2006), overall reproductive success was highly variable among sires, but especially among candidate translocated sires, with the two most successful males siring 46.3% of all genotyped offspring. Interestingly, the three most successful males (in terms of number of known offspring sired) were all previously established tortoises. In fact, previously established males, even though significantly smaller (~30 mm CL) than newly released males, were more than twice as likely to sire offspring than expected based solely on their relative abundance in the population (previously established males were assigned to 60.3% of all offspring but only represented 26.9% of the candidate sires sampled). “Incumbent advantage” in terms of increased survivorship of previously established animals over newly released animals has been documented for common lizards (*Lacerta vivipera*; Massot et al., 1994) and Hermann’s tortoises (*Testudo hermanni*; Bertolero et al., 2007). In addition, prior residence has been shown to influence dominance interactions and social rank in birds (Cristof et al., 1990; Eden, 1987; Holberton et al., 1990; Wiley, 1990). Our findings suggest that order of establishment or residency time may also be an important factor influencing reproductive success of male translocated gopher tortoises, even more than a decade after release.

There are alternative explanations that could produce the patterns of reproductive success observed in our study and which must be considered. First, disproportionate reproductive success of previously established males could be due to those males being older or having more experience than subsequently released males (e.g., Limmer and Becker, 2010). Unfortunately, ages of the breeding adults in our study were not known, as individual turtles can typically only be aged if first captured as still-growing juveniles with distinct growth annuli (Aresco and Guyer, 1998) and most tortoises translocated to St. Catherines Island were released as

adults. Because age and size (carapace length) tend to be correlated in turtles – at least within a population—size is frequently used as a proxy for age. If we assume that individual size is correlated with age in gopher tortoises at our study site and that previously established tortoises are older than newly released tortoises, we would expect that previously established males would also be larger than newly released males, when in fact the opposite pattern was observed. In addition, previously established and newly released tortoises (and even individual previously established tortoises themselves) originated from different source populations, and body size and growth rates are known to vary among populations (Aresco and Guyer, 1999; Ashton et al., 2007). These factors complicate any attempt to evaluate the influence of age on male reproductive success.

Another potential explanation is that mate choice and/or reproductive success in gopher tortoises translocated to St. Catherines Island was affected by genetic relatedness of potential breeding pairs. Genetic relatedness has been shown to influence both mate preference and reproductive success in other species (Johnson et al., 2010; Lihoreau et al., 2008; Miller et al., 2010), presumably serving as a mechanism of inbreeding avoidance. In our study system, newly released tortoises were comprised of a single wild population of tortoises, whereas the previously established tortoises originated from multiple source populations and were presumably unrelated to newly released tortoises and to each other. Previously established males did sire a disproportionate number of offspring genotyped during our 2 year study, as would be predicted if reproductive success varied inversely as a function of relatedness. However, in our study system, the potential effects of genetic relatedness cannot be distinguished from potential effects of release order, as the two are confounding factors that simultaneously differed between release groups. In addition, an examination of the number of offspring resulting from different possible breeding combinations revealed a more complex pattern (Table 4). If reproductive success increases with decreasing genetic relatedness of breeding pairs, previously established male and female gopher tortoises should be equally likely to produce offspring with a newly released tortoise as with a previously established tortoise of the opposite sex, but newly released tortoises should be more likely to produce offspring with a previously established tortoise than another newly released tortoise. In contrast to these predicted patterns, newly released females were equally likely to produce offspring with newly released males as previously established males. And although previously established males were equally likely to sire offspring from previously established females as newly released females, previously established females only produced offspring sired by previously established males. None of the genotyped offspring resulted from matings between a previously established female and a newly released (and presumably unrelated) male, suggesting that—at least in our study system—social factors may have had a greater influence on reproductive success than did genetic relatedness.

Despite a possible reproductive advantage of previously established over newly released males and the apparent lack of offspring resulting from previously established females and newly released males, males and females of both release groups were represented among genotyped offspring. In addition, one waif female (#502) successfully mated with a newly released male within the first year of her release. An apparent lack of dominance hierarchy in females may allow female tortoises to be integrated into an existing breeding population more quickly than males.

Our study investigated the mating system of translocated gopher tortoises more than a decade following their initial release, presumably long after most tortoises had become established in the release area and incorporated into the breeding population. However, male–female and same-sex social interactions and how

they shape mating success may differ markedly during the so-called settling phase immediately following release. In contrast to tortoises from naturally-occurring populations or to previously-established translocated tortoises, recently released tortoises are unfamiliar with their surrounding environment and the location of important resources, including potential mates. In addition, burrow fidelity may be lower in female gopher tortoises from translocated populations than in females from naturally-occurring populations (Tuberville, unpublished data), perhaps further hindering the ability of recently released males to locate females. Consequently, mating opportunities may be temporarily disrupted in translocated populations. Parentage analysis of offspring from recently translocated gopher tortoises would provide valuable insight into the factors affecting mating success during the settling phase of reintroductions.

Paternity data reported by Moon et al. (2006) for a naturally-occurring population provide a basis of comparison for our results from the translocated population on St. Catherines Island, but additional parentage studies of both naturally-occurring and translocated tortoise populations are needed to fully characterize the potential factors influencing the mating system of translocated populations. Additionally, multi-year studies could reveal how mating system dynamics and individual reproductive success vary from year to year, by addressing the following questions: Do individual females consistently produce either singly- or multiply-sired clutches? Are successive clutches of an individual female sired by the same male or set of males, as has been reported for painted turtles (Pearse et al., 2002) and alligators (Lance et al., 2009)? Do males that sire a large proportion of offspring in 1 year do so every year or does relative reproductive success of individual males change from year to year?

Nonetheless, even with limited sampling, we observed several patterns that are important to consider when designing future translocations of gopher tortoises or other social or territorial turtle species. First, reproductive success was highly variable among candidate sires, with only a few males contributing to a large proportion of the offspring sampled. Such pronounced variation in reproductive success, if sustained, could significantly reduce effective population size of the translocated population (Milinkovitch et al., 2004). To maximize genetic diversity in a reintroduced population, decisions regarding the number of animals targeted for release and the individuals selected for release should consider the likelihood of large variance among individuals in their genetic contribution to the next generation (Sigg et al., 2005). At least some representative translocated populations should be genetically monitored for loss of heterozygosity or other signs of inbreeding.

Second, many of the offspring resulted from successful matings by both previously established and newly released tortoises, demonstrating the integration of adult gopher tortoises from multiple source populations into the St. Catherines Island breeding pool. However, males with prior residence appeared to have a reproductive advantage over subsequently released males, even though both groups had been established on the island for more than a decade. Although alternative explanations cannot be definitively excluded based on data currently available, this apparent trend nonetheless has important implications for translocation projects designed to augment existing populations or to establish new populations through multiple releases. When only a few animals are introduced into small, fragmented population to increase genetic diversity or to simulate metapopulation structure, releasing females may be more effective than releasing males, both in terms of increasing offspring production and in quickly incorporating newly released animals into an existing breeding pool. Behavioral studies of social interactions between previously established and recently released tortoises would help clarify the role of social

structure in mating system and population dynamics in these manipulated populations (Berry, 1986).

Finally, translocated populations provide an excellent opportunity to investigate mating systems and conservation genetics of free-ranging turtle populations. Prior to release, animals can be permanently and uniquely marked for future identification, and tissue or blood samples can be collected for genetic analysis. Populations established in previously unoccupied habitat can be completely catalogued, with the identity, size, and life stage of each animal known. Post-release monitoring can provide detailed histories on health, survivorship and breeding status of individual animals—information useful for interpreting results of genetic analyses. Translocations can also be designed to experimentally test specific hypotheses regarding mating systems, by manipulating release conditions such as number of animals released, order of release, sex ratios, or use of captive-reared vs. wild-born stock. As translocations and other population manipulations play an increasingly important role in turtle conservation, such studies will help guide development of effective strategies for the establishment and management of translocated populations.

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