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Common-garden experiment reveals outbreeding depression and region-of-origin effects on reproductive success in a frequently translocated tortoise

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Abstract

Human-mediated animal movement can expose wildlife populations to novel environments. Phenotypic plasticity can buffer against the challenges presented by novel environments, while adaptation to local ecosystems may limit resilience in novel ecosystems. Outbreeding depression during the mixing of disparate gene pools can also reduce reproductive success after long-distance movement. Here, we use a 'common-garden' population of gopher tortoises (*Gopherus polyphemus*), translocated from numerous sites across the state of Florida, USA, to a mitigation site in the north-west (panhandle) region to assess whether geographic origin, outbreeding effects, and behavioral plasticity influence reproductive success in this threatened keystone species. We found that females from north-east Florida produced clutches with lower hatching success than females from other regions. We detected regional differentiation in nest site selection behavior in the common environment of the translocation site, though these differences did not mediate the regional effect on hatching success. We also found evidence for outbreeding depression: hatching success declined with increasing parental geographic and genetic distances, dropping from 93% to 67% across the range of observed parental genetic distances. Together, these results suggest that newly admixed populations may suffer reproductive costs due to historical population differentiation, and that undetected outbreeding depression could significantly hamper conservation efforts for this species and others undergoing a variety of human-mediated movements.

Introduction

Plant and animal populations exist in a dynamic world. Human activities both deliberately and unintentionally accelerate the long-distance movement of wildlife, exposing individuals to the stress of novel environments and mixing previously distinct populations (Bullock *et al.*, 2018). Humans deliberately move plants and animals, often long distances, for population management, (e.g. population augmentation), ecosystem restoration (e.g. repatriation), managed relocation (Aitken & Whitlock, 2013), and translocation to partially mitigate the loss of habitat or minimize human-wildlife conflict (Germano *et al.*, 2015). Inadvertent long-distance movements can result from the pet trade (Da Silva *et al.*, 2010), hitchhiking on vehicles and transported goods (Johansson *et al.*, 2018; Gippet *et al.*, 2019), and changes in land use (Amaral *et al.*, 2016). Beyond human-mediated movement, climate change exposes wild populations to stressful abiotic conditions *in situ*, and could prompt range shifts resulting in the mixing of formerly disparate gene pools (Krehenwinkel & Tautz, 2013). Given the magnitude of anthropogenic movement and environmental change, it is critical to measure the capacity of populations to weather those changes (Refsnider & Janzen, 2012; Valladares *et al.*, 2014; Beever *et al.*, 2016; Telemeco *et al.*, 2017). Understanding population resilience is important to identify vulnerable species, determine the scope of management solutions that could be used to ensure species persistence, and determine whether conservation and recovery objectives are achievable.

One primary mechanism allowing species to persist in a new environment is the individual adjustment of behaviors (e.g. to reduce thermal stress (Refsnider & Janzen, 2012) or accommodate diet changes (Peers *et al.*, 2014)) or physiological acclimation (e.g. increasing tolerance of hotter or drier conditions; Stillman, 2003). This functional-trait plasticity, where applicable, provides the most immediate and effective response to novel environmental conditions because it confers upon populations a latent ability to persist and even thrive in novel or altered environments (Dawson *et al.*, 2011; Refsnider & Janzen, 2012; Sandoval-Castillo *et al.*, 2020). In contrast, a lack of plasticity can lead to negative fitness consequences upon exposure to novel environments (Hereford, 2009; Telemeco *et al.*, 2017). Quantifying the degree of phenotypic plasticity and the geographic extent of local adaptation can inform translocation efforts to avoid environmental mismatch (Hereford, 2009), and delineate priority conservation areas to maintain functional diversity and species-level resilience in the face of global change (Fraser & Bernatchez, 2001).

Long-distance movement, including translocation, can result in the accelerated admixture of differentiated gene pools. In some contexts the introduction of novel alleles can result in higher fitness – a process known as heterosis (Weeks *et al.*, 2011). However, admixture can also reduce offspring fitness by producing offspring with intermediate, maladaptive phenotypes, or by disrupting coadapted gene complexes, in a process called outbreeding depression (Edmands, 2007; Frankham *et al.*, 2011; Weeks *et al.*, 2011). Outbreeding depression is most likely to occur between populations segregated for longer periods, that inhabit different environments, and that possess fixed chromosomal differences (Frankham *et al.*, 2011). Although recent arguments suggest that the risk of outbreeding has been overstated (Frankham *et al.*, 2011), outbreeding depression has been documented in several conservation contexts (e.g. Sagvik, Uller, & Olsson, 2005; Huff *et al.*, 2011). Quantifying the degree to which genetic mixing benefits or harms survival and reproduction can reveal the genetic and geographic scale over which conservation strategies can manipulate populations without reducing population fitness.

Experiments to determine the tolerance of threatened populations to environmental and genetic perturbation could be risky, expensive and difficult, particularly for large vertebrates. However, mitigation translocations (wherein large numbers of individuals from a variety of locations are moved to a limited number of protected recipient sites) can serve as (semi-)natural common-garden experiments for investigating individual resilience to altered biotic and abiotic conditions, testing for local adaptation, and for assessing the effects of genetic admixture such as heterosis or outbreeding depression (Kawecki & Ebert, 2004; Huff *et al.*, 2011; Cooper *et al.*, 2019). Site-of-origin effects on behavioral traits or reproductive outcomes, when expressed in a common environment, would suggest both geographic differentiation and limited behavioral or physiological plasticity in response to novel conditions.

Here, we use a major recipient site for mitigation translocations to test for site-of-origin effects and impacts of genetic admixture on reproductive behavior and success in the gopher tortoise (*Gopherus polyphemus*), a keystone species and ecosystem engineer of the coastal plain of the south-eastern United States. This species exhibits substantial population structure across its range (Gaillard *et al.*, 2017), including neutral (Krohn *et al.*, 2024) and possibly adaptive (Elbers, Brown, & Taylor, 2018) genetic differentiation on relatively small scales. The gopher tortoise inhabits fire-maintained habitats, and its burrows promote diversity by serving as critical thermal and fire refugia for hundreds of species of commensals (Catano & Stout, 2015). This species is declining steeply on protected areas (McCoy, Mushinsky, & Lindzey, 2006), although the greatest threat likely comes from development-driven habitat loss (Smith *et al.*, 2006). In the state of Florida, a stronghold for the species (Folt *et al.*, 2022), the number of tortoises translocated annually from development sites has exceeded 10,000 in recent years, and over 130,000 have been translocated since 1989 (Germano *et al.*, 2015; Loope *et al.*, 2024).

Climate differences can play a major role in determining translocation project outcomes for a variety of species (Bellis *et al.*, 2020), and other translocation features such as translocation distance, source population genetic diversity and disease history are also important (Waugh *et al.*, 2016; Scott *et al.*, 2020; Choquette *et al.*, 2023). Despite the scale of mitigation translocation, factors influencing translocation outcomes remain poorly understood for gopher tortoises. A recent long-term study of adult survival of translocated tortoises at a recipient site in Florida found little support for the ‘climate difference’ hypothesis that the degree of climatic difference between source and translocation site explains differences in translocation outcomes (Loope *et al.*, 2024). However, Loope *et al.* (2024) did find support for the ‘intrinsic regional differences’ hypothesis, which predicts that individual region of origin is associated with translocation outcomes in a way not correlated with climate differences, supporting the view that population characteristics or environments vary across regions in a way that predisposes individuals from some regions to better tolerate translocation to a new environment. Some possible explanations for intrinsic regional differences could be spatial genetic heterogeneity across the range reflecting differential resiliency to environmental features other than climate, such as disease (Elbers *et al.*, 2018). In the context of reproduction post-translocation, there could be local adaptation or behavioral acclimation at sites of origin creating geographic heterogeneity in maternal investment strategies, reproductive phenology or burrow or nest site selection in a common environment. Because regions differ in climate, if regional differences are observed but also correspond to regional differences in climate, and if climate predictors are found to better explain reproductive outcomes, then results would support the climate difference hypothesis over the intrinsic regional differences hypothesis, which exclusively concerns geographic effects other than climate.

In this study, our primary goals were to determine how gopher tortoise reproduction responds to a novel climatic and genetic environment in a common-garden translocation site. Specifically, we tested how hatching success is affected by: 1) climate mismatch between mother’s origin site and translocation site (climate difference hypothesis), 2) mother’s region of origin (intrinsic regional differences hypothesis), and 3) genetic and geographic distance between parents (outbreeding depression hypothesis). Because we observed female region-of-origin effects on hatching success, we also tested whether this effect was mediated by associated lack of plasticity in female nest site selection behavior, which is one possible mechanism linking regional variation to hatching success by affecting nest temperature.

Materials and methods

Study site

Nokuse is a 22,040-hectare private nature preserve located in the panhandle of Florida (Fig. 1), and is dedicated to restoring the longleaf pine (*Pinus palustris*) savanna ecosystem and the species that depend upon it, including the gopher tortoise. From 2006 to 2022, Nokuse received and released over 5,000 translocated tortoises from across the state of Florida. Before release, all tortoises were given unique marginal scute markings to allow individual identification. We studied translocated tortoise reproduction at two release sites, separated by ~1.5 kilometers, located in xeric sandhill areas with a longleaf pine overstory and a grass-dominated understory. Native tortoises are virtually absent (Cozad *et al.*, 2020). The Magnolia site initially comprised 207 tortoises largely from the panhandle and north Florida. In contrast, the Wolf site comprised 192 tortoises from throughout peninsular Florida (Fig. 1; Table S1). Tortoises had at least one year, and typically multiple years (range: 1–7; see Results), to acclimate to their new surroundings before this study was conducted (releases occurred at Magnolia in 2013–2017, and in Wolf at 2016–2017). We located and monitored nests at Magnolia in 2019–2020 and Wolf in 2018–2020. Sites were temporarily fenced, creating pens to prevent tortoise dispersal immediately following release (Tuberville *et al.*, 2005). These fences were removed in 2020 (Wolf) and 2015 and 2019 (for the two sections of Magnolia). For more details of the site and translocation methodology, see (Cozad *et al.*, 2020; Loope *et al.*, 2024).

Nest data collection

All gopher tortoise burrows within each site were searched for nests at least twice throughout each nesting season (May–July) by carefully excavating all burrows and aprons encountered to a depth of 30 cm. Eggs were delicately extracted to measure nest depth (vertical distance from surface to bottom of nest cavity in cm), and all eggs were carefully replaced in their original configuration. We placed a temperature data-logger in the center of each nest (iButton DS1921G-F5, Embedded Data Systems, Lawrenceburg, KY), then measured nest horizontal distance to burrow mouth (in cm; negative values indicate nests within burrows), and a shade index (the number of grid cells, out of 90, obscured by vegetation using a hemispherical densiometer at ground level facing south). To minimize predation risk, nests were protected with hardware cloth. A small percentage of eggs (3.3%) were damaged during nest searching and removed from analyses.

Because lay dates were unknown and nests were not found at the same time, we calculated nest temperature statistics using data from the dates in which all nests in a given year had temperature dataloggers in place ('period of overlap'). Periods of overlap were June 23-Aug 21 2018 (59 days), July 4-Aug 5 2019 (32 days), and June 26-Aug

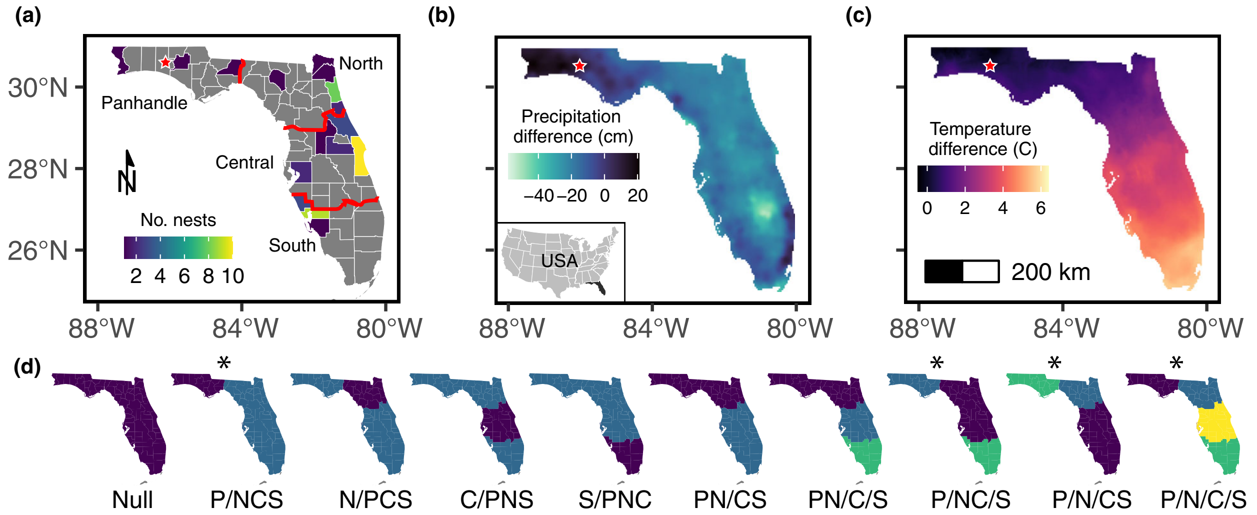

Figure 1 (a) Source counties for identified mothers of 47 gopher tortoise (*Gopherus polyphemus*) nests studied 2018–2020 at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA (red star). Red lines delineate regions used in analysis. (b) Statewide difference in 30-year normal mean annual precipitation (PRISM Climate Group), relative to Nokuse (red star). (c) Statewide difference in 30-year normal mean annual temperature (PRISM Climate Group), relative to Nokuse (red star). (d) Ten alternative regional groupings used in testing the intrinsic regional differences hypothesis. Groupings marked with an asterisk were omitted from hatching success models because all Panhandle nests had 100% hatching success, and so estimates of that region effect could not be made due to lack of variance.

14 2020 (49 days). Temperatures were collected in 90-min increments until excavation. For each nest, we calculated mean and standard deviation of nest temperature during the year's period of overlap. Temperature data were standardized and mean-centered within years.

In early August, ~1 week before the first nest typically hatches, eggs were excavated and incubated in the laboratory for the remainder of development. Hatchlings were bled and released at the nest site within 3 days of hatching (see Supplemental Methods for details). At the end of the season, a subset of unhatched eggs, often decomposed, were dissected and assigned to three developmental categories: no visible development, early-stage (stages 1–19), or late-stage embryo (stage >19; Yntema, 1968).

Parentage, parental genetic distance and geographic distance

We captured adult tortoises with wire traps (2018–2022) and sampled DNA to genetically assign parentage to hatchlings using microsatellite genotypes (Supplemental Methods; Table S1). We genotyped blood from hatchlings and potential parents to determine clutch parentage and parental genetic distance. We genotyped a total of 273 hatchlings from 47 clutches, 53 candidate fathers and 53 candidate mothers in Wolf, and 36 hatchlings from 9 clutches, 22 candidate fathers and 24 candidate mothers in Magnolia. We obtained hatchling genotype data for 57 of 61 clutches (mean = 5.4 hatchlings per clutch; range = 1–10). Both adults and hatchlings were genotyped using 12 microsatellite markers that have previously been effectively used for parentage assignment (White *et al.*, 2018). Parentage was assigned within each site using the program Colony (v2.0.6.8), a likelihood-based tool for parentage assignment and parental genotype reconstruction (Jones & Wang, 2010). For details, see Supplemental Methods.

We estimated the pairwise genetic distance between parents as an index of outbreeding (Kosman & Leonard, 2005; Fig. S1; for details, see supplementary methods; supplementary code). Seven of the 57 clutches had multiple paternity (five cases of two fathers, two cases of three fathers). In these cases, we used the average of the genetic distance between the mother and each father as the estimate for each clutch. We also calculated geographic distance between female and male source sites for 23 females for which all male mates were identified as a second index of outbreeding depression potential. None of these clutches had multiple paternity.

Climate data

To estimate climate differences between females' sites of origin and the translocation site, we obtained climate data from the PRISM Climate Group online database (PRISM Climate Group, 2014). Temperature and precipitation were selected as environmental variables because of their known effects on gopher tortoise reproduction (Hunter *et al.*, 2021). We used 30-year normals that describe average annual climate conditions from 1981 to 2010. The climate difference was calculated as the difference between the origin and translocation sites (Fig. 1).

Regions

To test the intrinsic regional differences hypothesis that female region-of-origin affects hatching success and nest site selection, we partitioned Florida counties into four geographic regions: panhandle (i.e. far north-west), north, central, and south (Fig. 1a). The boundaries between panhandle, north and central Florida regions delineated here roughly correspond to different genetic clusters in a recent population genetic analysis (Gaillard *et al.*, 2017), and the boundary between central and north Florida also corresponds roughly with the east–west line 100 miles of latitude south of Nokuse, currently of management significance because Florida law prohibits most translocations beyond this distance (Loope *et al.*, 2024). Because these four regions are necessarily somewhat arbitrary, we generated 10 different grouping scenarios with various combinations of these regions (Fig. 1d), including the null scenario (all regions the same). The purpose of these groupings is to look for regional variation that does not involve all four of the regions (e.g. if one region is distinct from the others) and includes nearly all logical combinations of these four regions (i.e. each region alone and adjacent pairings of regions).

Analysis

We created generalized linear models with binomial error and logit link to estimate the effects of female origin, parental genetic and geographic distance, years since release (accounting for variation in acclimation) and nest temperature on hatching success. Hatching success was measured as the number of hatchlings out of the total number of eggs that could have hatched (excluding eggs damaged during excavation). All predictor variables were standardized and zero-centered. All statistics were performed in program R v.4.2.3 (R Core Team, 2020). For each set of model comparisons, the full set of models is reported in the supplementary tables.

Because data were collected over three nesting seasons, some mothers had multiple nests in the dataset ($n = 10$ mothers). Inference about the effects of female origin is at the female level (not the nest level), so to avoid pseudoreplication, we averaged each female's nest data across years (Table S1). One nest produced by a female with nests in multiple years was omitted as an outlier because it was not in a burrow or apron, and thus did not have an appropriate distance from the apron value. For each set of models, we only included covariates that were not correlated ($|r| < 0.60$). Mean and standard deviation of nest temperatures were correlated, and given the stronger mechanistic understanding of mean nest temperature on hatching success, we removed the standard deviation of nest temperature from all analyses. We included a quadratic term on the effect of mean temperature to allow for effects of 'optimal' nest temperatures.

Geographic and genetic drivers of hatching success

To assess whether female origin effects on hatching success were better described by climate differences or intrinsic regional differences, we created two model sets. For the climate difference model set, we examined all possible linear combinations of annual temperature difference, annual precipitation difference, parental genetic distance, years since release and nest temperature. For the regions model set, we examined all possible linear combinations of each region grouping with parental genetic distance, nest temperature and years since release. Region groupings in which the Panhandle was a separate region (indicated with an asterisk in Fig. 1d) were omitted, as all Panhandle nests had 100% hatching success, and thus lacked variance. We compared all models from the combination of both sets ($n = 108$ total) using Akaike's information criterion corrected for small sample sizes (*AIC*c). As a secondary test of the outbreeding depression hypothesis, we examined the effect of geographic distance between parental source sites in place of parental genetic distance using data from the subset of females ($n = 23$) with identified mates with known source locations. We excluded models with region terms, as female region of origin necessarily constrains geographic distance between parents ($n = 48$ models). Because we detected an effect of region of origin, we tested whether this same pattern was present when examining male, instead of female, region of origin. We similarly calculated mean hatching success for the nests sired by each of the 16 fathers of known origin (excluding 7 clutches with multiple paternity) and compared the hatching success of fathers from north Florida to fathers from other regions (the regional difference observed in the female-aggregated analysis) with a binomial generalized linear model (GLM).

Nest site selection and nest temperature

Because we observed a region-of-origin effect on hatching success, we tested whether this effect was mediated by region-of-origin variation in nest site selection characteristics (depth, distance from burrow entrance, and shade), which could influence nest environment and, therefore, hatching success. We compared *AIC*c for models representing 10 alternative regional groupings (Fig. 1d) and three climate difference models (i.e. effects of mean temperature difference, mean precipitation difference, or both) to determine whether region-of-origin or origin climate predicted these three nest site characteristics and nest temperature. We used negative binomial regression to model shade index and linear models for all other nest site variables. We then compared *AIC*c for models from all possible linear combinations of the three nest site characteristics predicting hatching success (8 models), and similarly tested for nest site effects on mean nest temperature.

Results

We located 14 nests in 2018, 23 nests in 2019, and 24 nests in 2020. Clutch size varied between study sites (mean: 5.83 ± 1.52 standard deviation [SD]; range 4–9 eggs for Magnolia, mean: 7.18 ± 1.98 SD; range 2–12 eggs for Wolf). Average hatching success was 74% ± 30% (mean ± 1 SD) and was similar between study sites and across years. Dissection of 61 unhatched eggs found that 38 (62%) had no visible development, 15 (25%) contained an early-stage embryo (stages 1–19), and 8 (13%) contained a late-stage embryo (stage 20+).

Parentage analysis connected 47 nests (out of a total of 56 with hatchling genotypes) to 33 identified females translocated from 16 Florida counties (Fig. 1; Table S1). Of these 33, we were able to identify all male mates for 23 females. The nests of most identified females (22 of 33) were studied 2–3 years after release (range: 1–7; Table S1). Genetic distance between adults increased with geographic distance up to *c*. 350 km (Fig. S1), suggesting isolation by distance.

The top hatching success model for these 33 females included genetic distance between parents and the regional grouping with north Florida separated from the other three regions (Table 1). Among the five models within 4 *AIC*c units of the top model, genetic distance was present in all models, and the top three models were intrinsic regional differences models with the same regional combination as the top model. Nest temperature and years-since-release effects were not consistently present in top models (Table 1).

Table 1 Top binomial regression models to assess the effects of parental genetic distance and female site of origin on gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018–2020 nesting seasons at Nokuse, a gopher tortoise translocation site located in Florida, USA

Precipitation difference	Temperature difference	Parental genetic distance	Nest temperature	Years post-release	Region (PCS vs. N)	K	Δ*AIC*c	Weight
		−0.47			0.97	4	0	0.26
		−0.47		−0.03	0.95	5	2.58	0.07
		−0.47	−0.02		0.96	5	2.59	0.07
0.47	0.33	−0.46				5	2.75	0.07
0.50		−0.43				4	3.51	0.05

Standardized effect sizes are reported below each predictor. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c). *K* = parameter number; Δ*AIC*c is the difference between a model and the top model. Only models within 4 Δ*AIC*c of the top model are shown. For a list of all models, see Table S2.

All top models had negative effects of genetic distance on hatching success (Fig. 2, Table 1; Table S2). The top intrinsic regional difference model estimated lower hatching success for females originated from north Florida compared to all other regions (Fig. 2; Table S3). The top climate difference model estimated lower hatching success for females from origins with less precipitation, but higher hatching success for females from origins with higher mean annual temperatures, though confidence intervals for climate difference effect sizes overlapped zero (Fig. S2; Table S4). Aggregating hatching success by father instead of by mother indicated a similar regional pattern, with lower hatching success for fathers originating from north Florida relative to fathers from other regions (Fig. S3; $n = 16$ fathers; Binomial GLM: Est. = −1.28; S.E. = 0.48; $z = -2.69$; $P = 0.007$).

The top model using geographic distance between parent source sites, rather than genetic distance, similarly indicated outbreeding depression: parents from more distant sites produced clutches with lower hatching success for the 23 females with identified male mates (Fig. 3; Tables S5 and S6).

Some nest site attributes varied with female region-of-origin (Fig. 4; Tables S7–S12; Δ*AIC*c of null models were 9.49 and 5.90 for burrow distance and nest depth; $n = 33$ or 32, see Fig. 4), though the regional groupings present in the top models varied across nest site attributes, top models had competing models with Δ*AIC*c < 3, and adjusted R^2 values were generally low, suggesting that regional effects may be present but not well captured by our groupings. Nest temperatures decreased with increasing temperature difference between maternal origin and translocation sites, supporting the climate difference hypothesis for nest site selection (null model Δ*AIC*c = 3.05; top region-only model Δ*AIC*c = 2.33; Fig. 4a; Tables S13 and S14). Nest site attributes predicted mean nest temperature (Tables S15 and S16); temperature increased with distance from the burrow and decreased with increasing shade (Table S16). Nest site attributes did not predict hatching success (Table S17).

Discussion

Our central aim was to use a common-garden translocation site to assess whether geographic and genetic background can influence reproductive outcomes in a novel environment. Our most important finding was a negative effect of parental genetic and geographic distance on hatching success, suggesting that outbreeding depression may hamper reproduction in manipulated populations of gopher tortoises, including ongoing translocation projects. The gold standard for demonstrating outbreeding depression is controlled crosses (e.g. (Sagvik *et al.*, 2005; Tymchuk, Sundström, & Devlin, 2007)), but breeding tortoises in captivity at the scale necessary to test for real-world implications of outbreeding depression could be challenging and expensive and could affect

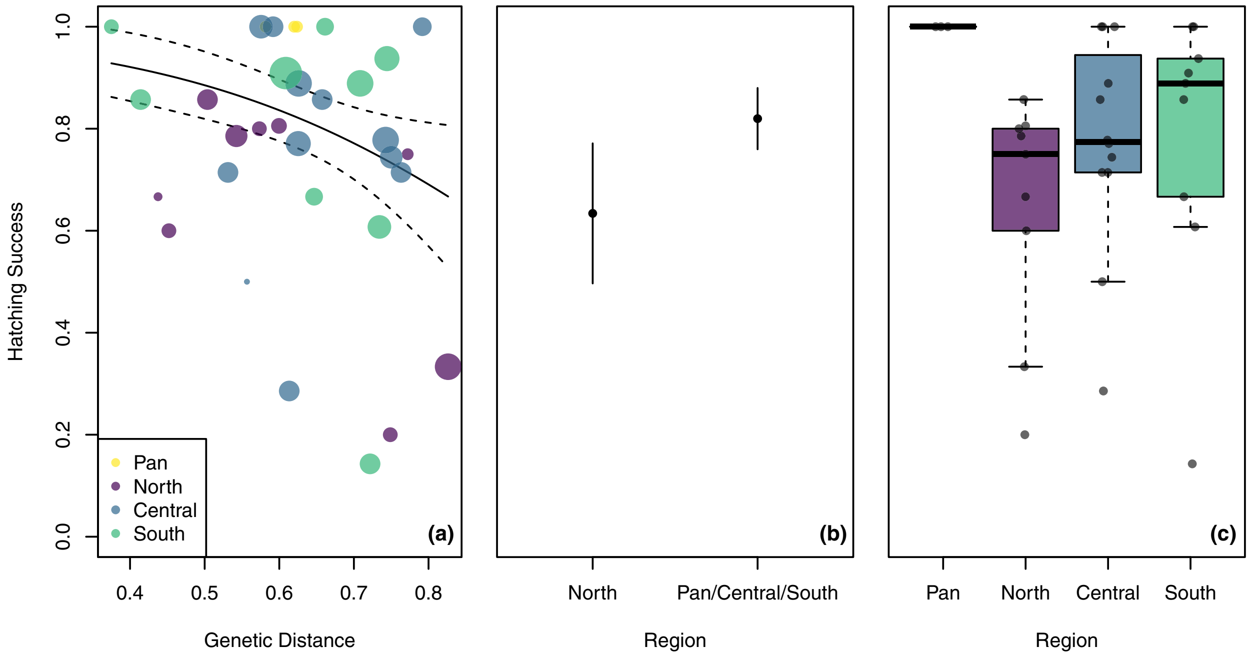

Figure 2 Estimates of the top intrinsic regional difference model of gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018–2020 nesting seasons at Nokuse, a translocation site located in Florida, USA. In panel (a), the solid line is the median effect and dashed lines are 95% confidence intervals for predictions from the model over the observed values for genetic distance for the grouped Panhandle/Central/South region; points are raw data aggregated to the female level, circle size is proportional to clutch size ($n = 33$). In panel (b), points are medians and lines are 95% confidence intervals for predictions from the model at the mean genetic distance value. Raw data of hatching success by region is shown in panel (c). The Panhandle had 100% hatching success for all three nests; otherwise, heavy lines are medians, boxes are 1st and 3rd quartiles, and whiskers are minima and maxima excluding outliers.

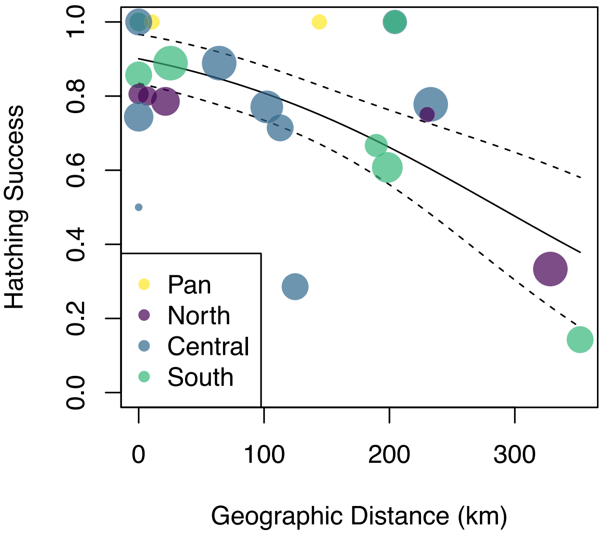

Figure 3 Geographic distance between maternal and paternal source sites predicts gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018–2020 nesting seasons at Nokuse, a translocation site located in Florida, USA. Solid line is the median effect and dashed lines are 95% confidence intervals for predictions from the top model over the observed values at mean values for other predictors. Points are raw data mean-aggregated to the female level across clutches, circle size is proportional to clutch size (*n* = 23). Pan = panhandle.

recovery probability for a protected species. Our common-garden approach using a translocated population eliminates many of the confounding factors that might otherwise muddle a correlative demonstration of this effect, and suggests that outbreeding depression likely has real-world effects in ongoing tortoise translocation projects. However, one limitation of our approach is that we were able to determine parental identity only by genotyping offspring and thus could not obtain parentage information for four clutches that had no eggs hatch; alternative parentage assignment methods would be necessary to determine whether these clutches failed completely as a result of outbreeding depression or some other cause such as fertilization failure. Outbreeding depression can result from disruption of coadapted gene complexes, or from phenotypes maladapted to the environment (Edmands, 2007). We have little information on the underlying mechanism, though our findings are consistent with genetic incompatibility, as the majority of failed embryos died before any visible development had occurred. Outbreeding depression may pose only temporary costs to populations, as natural selection on the hybrid population can eliminate the negative outbreeding effects within a few generations (Edmands *et al.*, 2005). However, for populations of species like the gopher tortoise, with intrinsically low reproductive rates (Folt *et al.*, 2022) and adult survival possibly already reduced by translocation (Cozad *et al.*, 2020; Loope *et al.*, 2024), even a temporary drop in reproduction could increase the population extinction risk. Given the slow maturation of young tortoises and great longevity of adult tortoises, the fitness costs of outbreeding by first-generation individuals are likely to be borne for decades. Our linear model suggests that, at the limits of observed outbreeding at our site, hatching success declines from 93% to 67% (Fig. 1). This may underestimate the true effect: outbreeding depression may be most pronounced in later life-history stages (Edmands, 2007), and can be expressed through reduced immunity (Goldberg *et al.*, 2005) and predator avoidance (Tymchuk *et al.*, 2007).

Our analysis also bears on absolute effects of female origin, beyond the interaction between maternal and paternal origin. Although hatching success differed among

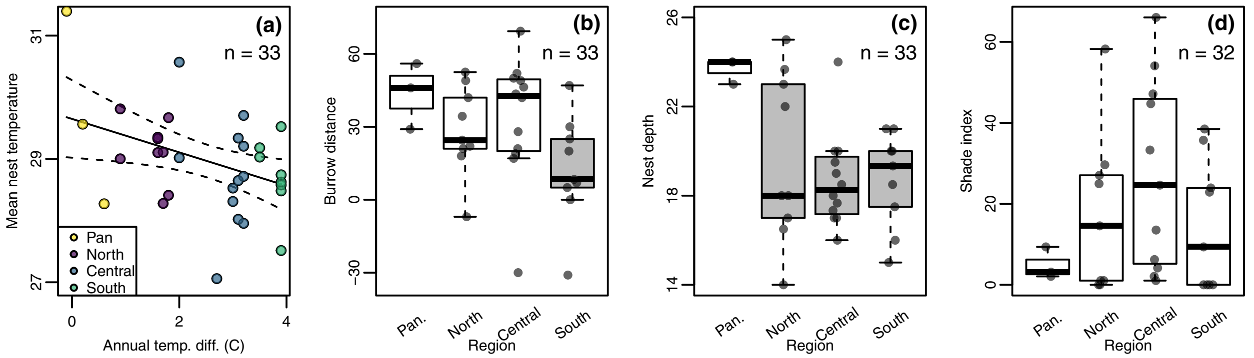

Figure 4 Nest site attributes and mean nest temperature are affected by mother's geographic origin for gopher tortoise (*Gopherus polyphemus*) nests during the 2018–2020 nesting seasons at Nokuse, a translocation site located in Walton County, Florida, USA. Data are averaged to the female level for multi-clutch females. (a) Mean nest temperature (late June – early August) is predicted by the difference in annual temperature between mother source site and our study site. (b) Distance from nest to burrow (cm; negative values represent nests inside of burrows). (c) Depth is the vertical distance in cm between surface and the bottom of the nest cavity. (d) An index of nest shade ranging from 0 (complete sun) to 90 (maximum shade). For panels (b–d), box fill color indicates regions that are grouped together (as in Fig. 1d) in the top model for response variables with evidence for regional effects ($\Delta AICc$ of null model >2). For boxplots, heavy lines are medians, boxes are 1st and 3rd quartiles, and whiskers are minima and maxima excluding outliers. Sample sizes are indicated on each figure. Pan = panhandle.

translocated female gopher tortoises from differing climates (climate difference hypothesis), variation in hatching success was better explained by a region-based model, with lower hatching success for clutches laid by females from north Florida relative to females from the other three regions, supporting the intrinsic regional differences hypothesis. Further, although we had data from only three females translocated from the panhandle region where our study took place, all three had 100% hatching success, consistent with a 'local' advantage. The observed regional signature in hatching success was consistent with some degree of regional or local differentiation, and with imperfect female behavioral (or nestling physiological) plasticity in response to novel conditions. Local adaptation is one possible explanation, and would not be surprising across the scale considered, given the variation in habitats and climates, existing genetic structure across the state (Gaillard *et al*., 2017), and evidence that genetic differentiation in this species is on the scale of ~100 km (Elbers *et al*., 2018; Krohn *et al*., 2024). Gaillard *et al*. (2017), in a range-wide analysis using microsatellites, delineated five subpopulations, three of which exist in the state of Florida, roughly corresponding to our panhandle, north, and a combined central + south region. Local adaptation of thermal tolerance in green turtle (*Chelonia mydas*) embryos has been demonstrated for two nesting sites separated by only 6 km (Weber *et al*., 2011), and genomic analyses suggest local adaptation in bog turtle (*Glyptemys muhlenbergii*) populations separated by distances similar to those in our study (Dresser, Pierson, & Fitzpatrick, 2018). We were not able to rule out maternal effects as an alternative explanation for this regional differentiation (Kawecki & Ebert, 2004; Weber *et al*., 2011). However, the detection of a parallel disadvantage to clutches sired by fathers from north Florida (Fig. S3) does suggest a genetic effect on offspring rather than a maternal effect mediated by female behavior, though the small sample size renders this finding tentative. Because we were unable to study the original source sites, we cannot determine if the observed lower hatching success for north Florida parents results only in a translocation context or if it is also present in residents of this region that are not translocated, perhaps as a result of an evolved response to tradeoffs between maternal investment in reproduction and survival.

Regional differentiation and imperfect maternal behavioral plasticity are also supported by the intrinsic regional differences in female nest site selection and a climate difference effect on nest temperature in the common environment at our study site. Females from warmer regions (central and south) appear to have alternative nesting strategies in the common environment: south-region tortoises place nests closer to burrows, while central-region tortoises (non-significantly) tend to place nests in shadier locations. Both behaviors decrease nest temperature (Table S16), and nests from these warmer regions were cooler than nests of females from cooler regions, suggesting that plasticity in female nesting behavior does not completely compensate for the novel environment (Fig. 4a). Numerous studies have found geographic variation in reptile nest site selection across species' ranges (e.g. Ewert, Lang, & Nelson, 2005; Doody *et al*., 2006; Bodensteiner *et al*., 2023); here we show such differences can persist in a common environment. A similar common-garden experiment in painted turtles (*Chrysemys picta*) found partial plasticity in nesting behaviors, with sites of origin predicting nest depth but not canopy cover (Refsnider & Janzen, 2012). Paralleling findings from two desert lizard species (*Elgaria* spp., Telemeco *et al*., 2017), our results suggest limited plasticity in key nest site selection behaviors influencing nest temperature that could have consequences for reproductive outcomes when expressed in novel environments.

The observed regional variation in female nest site selection at our field site did not explain the reduced hatching success of clutches from north Florida females. Nest temperature did not consistently predict hatching success across top models, suggesting that nest site selection resulted in suitable nest temperatures in the years studied. Because the gopher tortoise exhibits temperature-dependent sex determination (Demuth, 2001), geographic differentiation in nest site selection behavior could also result in maladaptive sex ratios in novel environments if there is insufficient behavioral plasticity in nest site selection to adjust to changing conditions (Janzen, 1994; Telemeco *et al*., 2017).

The combined evidence of region-of-origin effects and reduced hatching success for crosses from populations only ~100–300 km distant (Fig. 3) suggest that some gopher tortoise populations are sufficiently differentiated and insufficiently plastic to avoid reproductive costs in novel environments, resulting from translocation or climate change. Similar to adult survival (Loope *et al*., 2024), region-of-origin differences in hatching success (rather than a simple linear effect of environmental gradients or geographic distances) present a challenge for managers delineating translocation practices. However, the strong pattern of reduced hatching success with increasing parental distance does suggest that limits on translocation distance could prevent reduced hatching success at translocation sites with multiple source populations. Current Florida state regulations on translocation distance (<100 miles of latitudinal distance) permit long-distance east–west movements and may still result in substantial outbreeding depression. These negative effects are likely to be context-dependent, reflecting the environment and genetic background of the affected populations (Tymchuk *et al*., 2007; Frankham *et al*., 2011), but both effects also suggest that it may be important to preserve geographic variation in functional diversity across the range (Fraser & Bernatchez, 2001). Although natural selection could render these costs temporary on the timescale of generations, it is also possible that they could worsen in subsequent generations (Tymchuk *et al*., 2007), and thus the benefits of long-distance tortoise translocations may be negated by reduced reproduction in the novel environment and the disruption of local adaptations. Given the increasing use of translocation for conservation and conflict mitigation aims (Germano *et al*., 2015), including in related tortoises of conservation concern (e.g. Field *et al*., 2007; Bertolero, Pretus, & Oro, 2018), we suggest that outbreeding depression and local adaptation in reproductive traits be tested for in other translocation contexts, as monitoring programs focused on adult survival and evidence of reproduction may overlook more nuanced effects that could influence long-term persistence. In many cases, the benefits of alleviating inbreeding depression may outweigh the risks of outbreeding depression (Frankham *et al*., 2011). However, for the numerous situations where dispersal is artificially increased or translocation occurs in the absence of observed inbreeding depression, quantifying the reproductive costs of novel environments and outbreeding depression may be important to maintain recruitment and to prevent the erosion of local adaptive diversity.

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Author contributions

EAH, KTS, KJL, JND designed the study; JND, KJL and EAH collected the data; KJL, JND and EAH performed data analysis; KJL wrote the manuscript with assistance from all authors.

Data availability statement

Data and code available from the Virginia Tech Digital Repository (https://doi.org/10.7294/26023951; Loope, 2024).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Genetic and geographic distances between source sites for all pairwise comparisons of genotyped adult gopher tortoise (*Gopherus polyphemus*) translocated to Nokuse from known source sites (n = 134). These individuals are the candidate parents used in parentage assignment. Genetic distance in (A) calculated using the infinite alleles model (IAM) based on allelic identity (Kosman & Leonard 2005); Genetic distance in (B) calculated using stepwise mutation model (SMM), incorporating information about repeat number in addition to allele identity (Kosman & Leonard 2019). All reported analyses in main text use genetic distance based on IAM, as this better correlates with geographic distance, though results are qualitatively the same with SMM-based distances. Blue lines are loess-smoothed splines.

Figure S2. Estimates of the top climate difference model of gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Solid lines are the median effect and dashed lines are 95% confidence intervals for predictions from the model over the observed values for each variable while holding other variables at their mean value. Points are raw data aggregated to the female level (n = 33).

Figure S3. Hatching success mean-aggregated to 16 gopher tortoise (*Gopherus polyphemus*) fathers of known origin across 39 single-paternity clutches across father region of origin after translocation to Nokuse, a translocation site located in Walton County, Florida, USA. Each point is the mean hatching success for a given father across all observed clutches from 2018 to 2020 (n = 1 – 6 clutches per father). Heavy lines are medians, boxes are 1st and 3rd quartiles, and whiskers are minima and maxima excluding outliers.

Table S1. Summary data from gopher tortoise (*Gopherus polyphemus*) nests, mean-aggregated to female level. “Eggs” is the mean number of eggs per clutch after removing eggs damaged during excavation. “Temp. diff” is the difference in mean annual temperature (degrees C) between the female’s source site and Nokuse. “Precip. diff” is the difference in mean annual precipitation (in mm) between the female’s source site and Nokuse. “Nest temp.” is the measured mean nest temperature (degrees C). “Genetic distance” is the mean genetic distance (*sensu* Kosman & Leonard 2005). “Burrow distance” is the horizontal distance in cm between the nest and the burrow mouth (negative values are nests placed inside the burrow). “Nest depth” is the depth in cm at the bottom of the nest cavity. “Shade index” is the densiometer reading (lower numbers indicate less shade) taken with a hemispherical densiometer facing south approximately 10cm above the nest site. “ND” indicates a nest without a shade reading. “Unk.” Indicates females with unidentified male mates

Table S2. Results of a binomial regression analysis to assess the effects of genetic distance between parents, region of origin, climate differences and nest temperature on gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model. Genetic. dist. is the genetic distance between parents, NT is mean nest temperature, Precip. diff. is difference in mean annual precipitation between female source site and Nokuse, and Temp. diff. is difference in mean annual temperature between female source site and Nokuse

Table S3. Top intrinsic regional differences model from model selection of a binomial regression analysis to assess the effects of climate differences, female site of origin and parental genetic distance on gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA

Table S4. Top climate difference model from model selection of a binomial regression analysis to assess the effects of climate differences, female site of origin and parental genetic distance on gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA

Table S5. Results of a binomial regression analysis to assess the effects of geographic distance between parents’ sites of origin, climate differences and nest temperature on gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model. Geog. dist. is the geographic distance between parent’s source sites, NT is mean nest temperature, Precip is difference in mean annual precipitation between female source site and Nokuse, and Temp is difference in mean annual temperature between female source site and Nokuse

Table S6. Top model from model selection of a binomial regression analysis to assess the effects of geographic distance between parents’ sites of origin on gopher tortoise (*Gopherus polyphemus*) hatching success during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA

Table S7. Results of model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) female region and climate of origin on nest distance from the burrow entrance during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were of two types: intrinsic regional difference in which origin effects were categorical region groups (regions: N = North, P = Panhandle, C = Central, S = South, see Figure 1), and climatic difference (CD) in which origin effects were continuous climate difference covariates. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model. Precip. diff. is difference in mean annual precipitation between female source site and Nokuse, and Temp. diff. is difference in mean annual temperature between female source site and Nokuse

Table S8. Top model from model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) site of origin and climate difference from origin site on nest distance from the burrow mouth during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Estimates are for standardized and zero-centered predictors

Table S9. Results of model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) female region and climate of origin on nest depth during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were of two types: intrinsic regional difference in which origin effects were categorical region groups (regions: N = North, P = Panhandle, C = Central, S = South, see Figure 1), and climatic difference (CD) in which origin effects were continuous climate difference covariates. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model. Precip. diff. is difference in mean annual precipitation between female source site and Nokuse, and Temp. diff. is difference in mean annual temperature between female source site and Nokuse

Table S10. Top model from model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) site of origin and climate difference from origin site on nest depth during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Estimates are for standardized and zero-centered predictors

Table S11. Results of model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) female region and climate of origin on nest shade during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were of two types: intrinsic regional difference in which origin effects were categorical region groups (regions: N = North, P = Panhandle, C = Central, S = South, see Figure 1), and climatic difference (CD) in which origin effects were continuous climate difference covariates. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model. Precip. diff. is difference in mean annual precipitation between female source site and Nokuse, and Temp. diff. is difference in mean annual temperature between female source site and Nokuse

Table S12. Top model from model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) site of origin and climate difference from origin site on nest shade during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Estimates are for standardized and zero-centered predictors

Table S13. Results of model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) female region and climate of origin on mean nest temperature during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were of two types: intrinsic regional difference in which origin effects were categorical region groups (regions: N=North, P=Panhandle, C=Central, S=South, see Figure 1), and climatic difference (CD) in which origin effects were continuous climate difference covariates. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model. Precip. diff. is difference in mean annual precipitation between female source site and Nokuse, and Temp. diff. is difference in mean annual temperature between female source site and Nokuse

Table S14. Top model from model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) site of origin and climate difference from origin site on mean nest temperature during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Estimates are for standardized and zero-centered predictors

Table S15. Results of model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) nest site characteristics on mean nest temperature during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model

Table S16. Top model from model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) nest site characteristics on mean nest temperature during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Estimates are for standardized and zero-centered predictors

Table S17. Results of model selection to assess the effects of translocated gopher tortoise (*Gopherus polyphemus*) nest site characteristics on hatching success during the 2018-2020 nesting season at Nokuse, a gopher tortoise translocation site located in Walton County, Florida, USA. Models were compared with Akaike Information Criterion corrected for small sample sizes (*AIC*c); *K* is the number of parameters and Δ*AIC*c is the difference between a model and the top model