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Close-kin mating, but not inbred parents, reduces hatching rates and offspring quality in a threatened tortoise

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Abstract

Inbreeding depression, the reduction in fitness due to mating of related individuals, is of particular conservation concern in species with small, isolated populations. Although inbreeding depression is widespread in natural populations, long-lived species may be buffered from its effects during population declines due to long generation times and thus are less likely to have evolved mechanisms of inbreeding avoidance than species with shorter generation times. However, empirical evidence of the consequences of inbreeding in threatened, long-lived species is limited. In this study, we leverage a well-studied population of gopher tortoises, *Gopherus polyphemus*, to examine the role of inbreeding depression and the potential for behavioural inbreeding avoidance in a natural population of a long-lived species. We tested the hypothesis that increased parental inbreeding leads to reduced hatching rates and offspring quality. Additionally, we tested for evidence of inbreeding avoidance. We found that high parental relatedness results in offspring with lower quality and that high parental relatedness is correlated with reduced hatching success. However, we found that hatching success and offspring quality increase with maternal inbreeding, likely due to highly inbred females mating with more distantly related males. We did not find evidence for inbreeding avoidance in males and outbred females, suggesting sex-specific evolutionary trade-offs may have driven the evolution of mating behaviour. Our results demonstrate inbreeding depression in a long-lived species and that the evolution of inbreeding avoidance is shaped by multiple selective forces.

KEYWORDS
conservation genetics, gopher tortoise, *Gopherus polyphemus*, inbreeding avoidance, mating system, microsatellite, relatedness, reproductive success

1 | INTRODUCTION

Inbreeding depression occurs when mating of related individuals generates offspring of reduced fitness compared with random mating. This phenomenon is of central conservation concern because it can lead to reduced reproductive success and lowered offspring quality, increasing the risk of demographic decline especially in small, isolated populations (Charlesworth & Charlesworth, 1987; Frankham, 2010; Keller & Waller, 2002). Compared to population-level loss of genetic diversity, the effects of inbreeding depression can occur more rapidly following initial decline if overall relatedness increases (Amos & Balmford, 2001). Highly inbred individuals may also be less fit under environmental stress, when the expression of inbreeding depression is most severe (Armbruster & Reed, 2005;

Keller, Grant, Grant, Petren, & Merilä, 2002). Thus, the effects of inbreeding depression can exacerbate population declines initially caused by other factors (Frankham, 1995; O'Grady et al., 2006). However, increased homozygosity from inbreeding can also purge deleterious alleles, particularly lethal recessives, thereby increasing mean fitness of the population over generations (García-Dorado, 2012; Hedrick, 1994, 2002; Kirkpatrick & Jarne, 2000; Nei, 1968). Conversely, demographic declines can eventually lead to fixation of otherwise mildly or moderately deleterious alleles through drift (Bataillon & Kirkpatrick, 2000; Glémin, Ronfort, & Bataillon, 2003; Lynch, Conery, & Burger, 1995), at which point inbreeding depression *sensu stricto* ceases to act despite retention of a high genetic load (Lohr & Haag, 2015).

Empirical evidence shows that inbreeding depression occurs broadly in natural populations (Frankham, 2010) and that species have evolved mechanisms to reduce inbreeding risk, including sex-biased dispersal (Perrin & Mazalov, 2000; Pusey, 1987), polygyny (Cornell & Tregenza, 2007; Pusey & Wolf, 1996) and behavioural kin avoidance (Archie et al., 2007; Lehmann & Perrin, 2003; Pusey & Wolf, 1996; but see Waser, Austad, & Keane, 1986). Yet, we still do not have a general understanding of the evolutionary response to inbreeding among animal lineages. Particularly, we do not know the specific consequences of inbreeding depression and mechanisms of inbreeding avoidance across different demographics and life histories (but see Archie et al., 2007; Kuo & Janzen, 2004; Slate, Kruuk, Marshall, Pemberton, & Clutton-Brock, 2000; Taylor et al., 2017).

Theory predicts that in long-lived species, the effects of inbreeding following population declines will be delayed due to their longer generation times (Amos & Balmford, 2001), and empirical evidence has generally supported this hypothesis (Keane, Creel, & Waser, 1996; Kuo & Janzen, 2004; Lippé, Dumont, & Bernatchez, 2006). Additionally, the evolution of behavioural inbreeding avoidance is rare in long-lived species (Keane et al., 1996), likely because of relaxed selection against inbreeding relative to the cost of inbreeding avoidance (Kokko & Ots, 2006; Waser et al., 1986). Long-lived species often have smaller effective population sizes and longer generation times limiting individual reproductive opportunities; thus, kin avoidance may limit otherwise valuable mating opportunities. Still, examples of pronounced inbreeding depression and the evolution of behavioural inbreeding avoidance have been observed in some long-lived species such as the little spotted kiwi (*Apteryx owenii*) (Taylor et al., 2017) and the African elephant (*Loxodonta africana*) (Archie et al., 2007). In this study, we take advantage of a well-studied, semi-isolated population of gopher tortoises (*Gopherus polyphemus*) to examine the role of inbreeding depression in natural populations of a long-lived species.

Gopherus polyphemus is a large, fossorial tortoise native to the south-eastern United States. The species is of conservation concern owing to ongoing population declines, particularly in the western portion of its range (Diemer, 1986; McCoy, Mushinsky, & Lindzey, 2006). A phylogeographic break separating the western and eastern populations occurs at the Apalachicola–Chattahoochee Rivers (Ennen et al., 2012; Gaillard et al., 2017).

Previous studies have demonstrated that poor recruitment in western populations relative to eastern populations is largely attributed to lower hatching success even under controlled laboratory conditions (Epperson & Heise, 2003; Noel, Qualls, & Ennen, 2012). Population genetic studies have also shown that western populations have substantially lower genetic diversity (Ennen, Kreiser, & Qualls, 2010; Gaillard et al., 2017), which has led to the hypothesis that low hatching success is caused by inbreeding depression or the loss of genetic diversity more broadly (Noel et al., 2012). Because gopher tortoises exhibit low genetic diversity throughout the western part of their range, deleterious alleles associated with innate hatching rates are likely to be fixed in those populations. Thus, we can no longer effectively assess the effects of inbreeding on reproductive success during initial declines in western populations. However, several populations in the more genetically diverse eastern part of the range have been the focus of long-term studies (e.g. Ashton, Engelhardt, & Branciforte, 2008; Guyer, Johnson, & Hermann, 2012; Tuberville, Norton, Todd, & Spratt, 2008), providing an opportunity to test the hypothesis that inbreeding depression leads to reduced recruitment in *G. polyphemus*.

We studied an eastern population of gopher tortoises located at Archbold Biological Station (ABS) in south-central Florida, which displays sufficient population-level genetic diversity for calculation of individual inbreeding and relatedness between mate pairs (Yuan et al., 2015). Our goals are to determine (a) whether inbreeding, measured as high parental relatedness, leads to reduced reproductive success, (b) whether inbreeding leads to reduced offspring quality as measured by size, body condition and locomotor performance, (c) whether maternal inbreeding and paternal inbreeding differentially affect male and female reproductive success and offspring quality, and (d) whether mate choice is a potential mechanism of inbreeding avoidance in gopher tortoises. We discuss our results in the context of the current understanding of mechanisms of inbreeding depression and evolution of inbreeding avoidance and highlight the relevance of our findings for conserving populations of long-lived species.

2 | MATERIALS AND METHODS

2.1 | Study population and sample collection

From 2012 to 2016, we annually surveyed a population of *G. polyphemus* at ABS, which is located at the southern end of the Lake Wales Ridge in Highlands County, Florida. Our study population inhabits a site within ABS known as Red Hill (RH), which consists of 7 ha of old-field habitat surrounded by approximately 90 ha of southern ridge sandhill in varying stages of restoration from historical fire suppression (Ashton et al., 2008). The RH gopher tortoise population is the focus of an ongoing mark–recapture study initiated in 1967 (Layne, 1989). All adult tortoises in the RH population are uniquely marked by drilling marginal scutes to allow for individual identification (Ernst, Hershey, & Barbour, 1974).

We captured individuals by hand or by using Havahart live animal traps (Woodstream Corporation) placed at burrow entrances. At first capture, we nondestructively collected 0.5–1.0 ml of blood in lysis buffer (100 mM Tris Base, 100 mM EDTA, 150 mM NaCl, 1% SDS) from all adult tortoises (N = 114) via the subcarapacial vein (Hernandez-Divers, Hernandez-Divers, & Wyneken, 2002). We sexed each individual using secondary sexual characteristics (McRae, Landers, & Cleveland, 1981) or penis extrusion. Additionally, we measured mass (kg), straight carapace length (mm), straight carapace width (mm), and shell height (mm) at each capture.

During 2015–2016, we surveyed burrow aprons for nests and protected them *in situ* until late in development (White, Rothermel, Zamudio, & Tuberville, 2018). We then excavated all detected nests (N = 31), noted the total number of eggs in each clutch and artificially incubated all clutches at 29 ± 1°C and ~ 80% humidity for the remainder of their development. Incubation allowed us to examine the innate viability of each clutch while preventing total clutch failure due to predation and controlling for potential effects of environmental conditions in later stages. We collected blood samples from hatchlings and tissue from inviable eggs for genotyping (N = 220). We measured the mass (g), straight carapace length (mm) and straight carapace width (mm) of each hatchling (N = 194) following full absorption of the yolk sac. Hatchlings were typically kept in the laboratory for 1–2 days following completion of data collection, then released either at their natal burrow, if it was unoccupied by an adult tortoise or potential predators, or at the nearest unoccupied burrow if their natal burrow was unsuitable.

2.2 | Hatchling locomotor performance

In 2016, we housed hatchlings for up to 10 days post-hatching to conduct locomotor trials as an offspring fitness proxy. Locomotor performance trials have been used in several reptile systems including *G. polyphemus* as a fitness proxy (Demuth, 2001; Elphick & Shine, 1998). We conducted three locomotor performance trials for each hatchling (N = 87) on days 4, 5 and 6 after yolk absorption. Performance tracks consisted of a metre-long, sand-covered wooden track terminating in a cardboard shelter. We mounted a camcorder (JVC HD Everio, GZ-HM440) directly above the track to videorecord each trial. Hatchlings were acclimated to ambient temperature (29°–34°C) for at least 30 min prior to performance trials. We placed hatchlings on the wooden track and gently tapped the posterior carapace at regular intervals to motivate them to move along the track. We re-randomized the order in which hatchlings were tested on each day of trials. For each trial, we recorded latency time (the time from release to first forward movement), split times for each 0.1-m interval, and sprint speed (calculated from the average of an individual's 0.1 m split times) from videos using Kinovea (https://www.kinovea.org/). All times were recorded to the nearest 0.1 second and averaged across successful trials. We discarded all trials in which the tortoise ceased forward progress for longer than 5 s or turned around.

2.3 | Microsatellite analysis

We extracted whole genomic DNA using the DNeasy Blood and Tissue Kit (Qiagen). For each individual (i.e. adult, hatchling and inviable egg), we PCR-amplified 15 previously published microsatellite markers (Kreiser, Ennen, Gaillard, & Qualls, 2013; Schwartz, Osentoski, Lamb, & Karl, 2003; Tuberville, Norton, Waffa, Hagen, & Glenn, 2011) modified following Yuan et al. (2015) (Table S1). We conducted PCRs using a three-primer system (Waldbieser, Quiniou, & Karsi, 2003) consisting of locus-specific primer pairs and a third fluorescently tagged universal primer. In brief, we attached a 5′-CGAGTTTTCCCAGTCACGAC-3′ tag to the 3′ end of one microsatellite primer in each pair to allow for annealing of a fluorescently tagged universal primer. Fluorescently tagged universal primers were 5′-tagged with either VIC, NED, PET or 6FAM. We also attached a 5′-GTTT-3′ tag to the 5′ end of each untagged primer to reduce stutter.

We performed all PCRs in 10 μl reactions including 1 μl template DNA (1–10 ng), 1X PCR buffer, 1.5 mM MgCl$_2$, 0.1 μM dNTPS, 0.2 μM each of untagged and universal fluorescent primers, 0.4 μM tagged primer and 0.25 units of *Taq* polymerase (Roche). PCRs consisted of an initial denaturing temperature of 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 1 min, locus-specific annealing temperature for 1 min (Table S1), extension at 72°C for 1 min and a final extension at 72°C for 5 min. We conducted genotyping in pools by mixing 1 μl of pooled PCR product with 18.85 μl Hi-Di™ formamide and 0.15 μl GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems). We genotyped samples using an ABI 3730xl DNA Analyzer (Applied Biosystems) and called alleles using GeneMarker v2.4.0 (SoftGenetics).

2.4 | Parentage assignment and inbreeding estimation

For each pair of tortoises, we calculated Wang's relatedness estimator (W) in COANCESTRY (Wang, 2011). Wang's relatedness estimator is robust to smaller sample sizes, highly polymorphic loci, and sampling bias in reference populations compared to alternative relatedness metrics (Wang, 2002). We also estimated inbreeding coefficients (F) for all adult individuals and hatchlings using the Ritland moment estimator (Ritland, 1996). Variance in inbreeding is necessary for heterozygosity-fitness correlations to arise (Szulkin, Bierne, & David, 2010). Therefore, we tested for significant disequilibrium (g_2), a measure of variance in inbreeding, following David, Pujol, Viard, Castella, and Goudet (2007). For each clutch, we assigned parentage from offspring genotypes using a maximum-likelihood approach in COLONY (Jones & Wang, 2010) and a pairwise likelihood approach in CERVUS (Kalinowski, Taper, & Marshall, 2007; see White et al., 2018). We provided all genotyped adult tortoises as potential parents and allowed for assignment of unsampled individuals as parents.

2.5 | Data analyses

We defined innate clutch viability as the proportion of incubated eggs that successfully hatched. We excluded all clutches in which

FIGURE 1 (a) Relationship between pairwise parental relatedness and clutch viability. (b) Relationship between maternal inbreeding and clutch viability. In both a and b, lines depict predicted values from the fitted GLM with a quasibinomial distribution
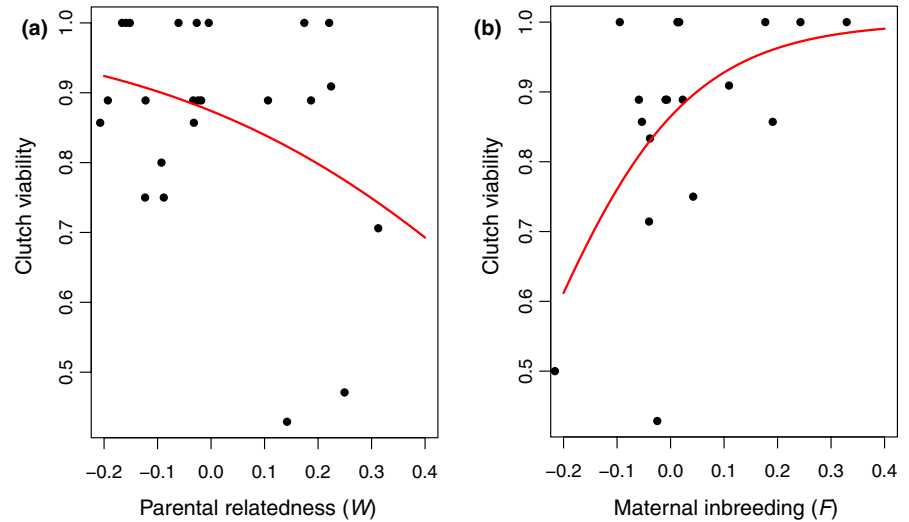
one or both parents were assigned to an unsampled individual or in which less than 70% of eggs/hatchlings were successfully genotyped. In clutches with greater than 70%, but less than 100%, genotyping success, we assigned fathers to individuals that failed to genotype based on the sire or sires attributed to the remainder of the clutch. If a single sire was detected, we assigned all unknown eggs to that sire. In clutches with multiple paternity, we assigned partial paternity to each father based on the proportion of the remainder of the clutch sired (i.e. if a father sired 50% of a clutch, he was assigned 0.5 of the un-genotyped individual).

For parental W analyses, we divided clutches by unique mating pairs. For clutches in which assigned parents were identical between years, we averaged clutch size and clutch viability between years (after testing for significant differences in clutch viability and clutch size between years) to avoid pseudoreplication. For paternal and maternal F, we combined all assigned offspring across clutches for each parent to generate an overall clutch viability. To test for overdispersion in clutch size and clutch viability, we compared the observed and theoretical variances to a chi-square distribution (Cameron & Trivedi, 1990). We tested the correlation of clutch viability with parental relatedness as well as maternal and paternal inbreeding coefficients using general linear models (GLM) with quasibinomial distributions to account for overdispersion.

Because clutch size is known to correlate with female body size (Ashton, Burke, & Layne, 2007; Landers, Garner, & McRae, 1980; Rothermel & Castellón, 2014), we included straight carapace length as a covariate when examining the relationship between clutch size and maternal inbreeding coefficient using a GLM with a Poisson distribution. To determine whether tortoises used mate choice to mitigate inbreeding, we tested the relationship between maternal and paternal inbreeding coefficients and parental pairwise relatedness across breeding pairs by simple linear regression. We also tested whether pairwise relatedness in our sample of inbred ($W < 0$) and outbred ($W > 0$) breeding pairs was statistically different than the average population-level relatedness. Due to the pairwise nature of relatedness, we tested for significance using permutation tests in

which a null distribution was generated by permuting relatedness indices across all possible dyads 999 times.

We used residuals from a regression of log mass on log carapace length as the measure of hatchling body condition, a proxy for physiological state (Lagarde et al., 2001). Additionally, we corrected sprint speed for body size by retaining residuals after regressing log sprint speed on log carapace length. We used linear mixed models to test for the influence of inbreeding on our measures of offspring quality. For each model, we tested one of four dependent variables (log carapace length, body condition, log latency time, and average residual sprint speed of hatchlings) against all independent variables (parental relatedness and offspring, paternal, and maternal inbreeding coefficients). Parent IDs were included as crossed random factors to account for the effect of shared parentage. To assess potential multicollinearity, we calculated variance inflation factors (VIFs) for all independent variables. All data analyses were conducted in R v3.5.1 (R Core Team, 2018).

3 | RESULTS

We located clutches for 15 females in 2015 and 16 females in 2016, with nine females represented in both years. Clutches from seven females were entirely sired by the same male in both years. Two females produced multiply sired clutches in both years; one female's clutches were sired by the same two males in both years, whereas the other female's clutches only shared one sire between years. Thus, for repeated multiply sired clutches produced by the same female, at least one father was identical between years. The remaining 13 females (six in 2015 and seven in 2016) for which we recovered nests were unique between years. Four clutches (two in 2015 and two in 2016) were excluded from analysis due to low genotyping coverage.

Clutch viability ranged from 0.33 to 1.0 with a median of 0.9, and clutch size ranged from 1 to 15 eggs with a median of seven eggs across
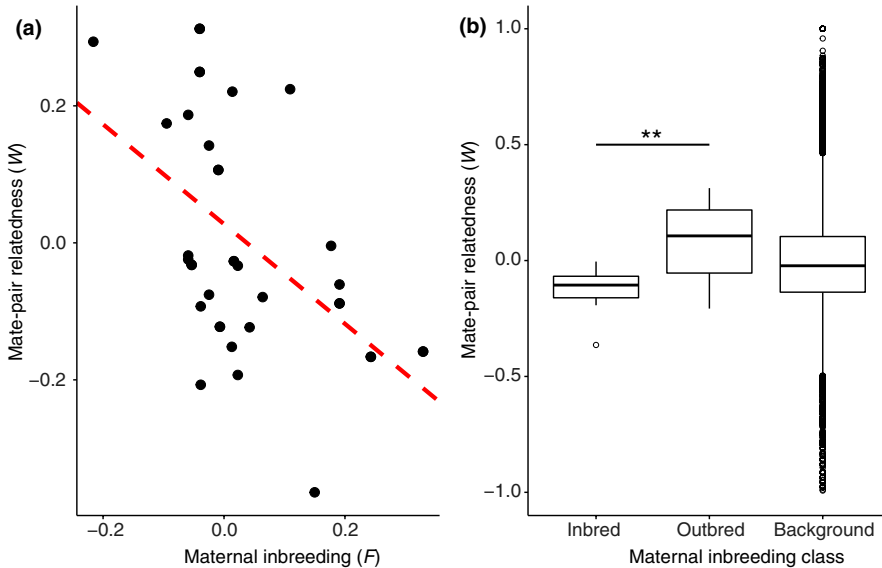
FIGURE 2 (a) Relationship between maternal Ritland moment estimator of inbreeding (F) and Wang's relatedness (W) for all mating pairs in which both parents were sampled. Linear regression is depicted as a hashed line ($R^2 = .276$, $p < .001$). (b) Box plots of pairwise relatedness for mate pairs with inbred females (maternal $F < 0$), mate pairs with outbred females (maternal $F > 0$) and all possible other pairs. Significantly different mean pairwise relatedness is indicated ($p < .05^*$, $p < .01^{**}$, $p < .001^{***}$)

unique mate pairs. Overall clutch viability was 81.7% for incubated eggs. Clutch viability (chi-squared test: all $p > .05$) and size (pairwise t test: all $p > .05$) did not differ between years in clutches with identical parents. We found that innate clutch viability was overdispersed ($p < .001$), whereas clutch size was not ($p = .135$). We found a significant positive correlation between log clutch size and log maternal body size ($F = 19.037$, $R^2 = .515$, $p < .001$). Additionally, we found that identity disequilibrium was significantly greater than zero ($\hat{g}_2 = 0.011$, expected bias = 3.43×10^{-5}, $SD = 0.004$, $p < .001$), indicating that heterozygosity-fitness correlation can arise in this system. We then detected a significantly positive correlation between maternal inbreeding and clutch viability (deviance = 1.200, $df = 17$, $p = .007$), as well as a marginally significant negative correlation between parental relatedness and clutch viability (deviance = 0.548, $df = 22$, $p = .072$; Figure 1). However, paternal inbreeding was not correlated with clutch viability, nor were maternal inbreeding coefficients correlated with clutch size ($p > .05$).

We found that maternal inbreeding was negatively correlated with parental relatedness across clutches ($F_{1,36} = 13.754$, $R^2 = .276$, $p < .001$; Figure 2a), whereas paternal inbreeding was not correlated with parental relatedness ($F_{1,35} = 0.120$, $R^2 = .005$, $p = .682$). The relatedness of mating pairs did not differ from average population-level relatedness (permutations = 999, $p = .850$). However, relatedness of

mating pairs with inbred females was significantly lower than relatedness of mating pairs with outbred females (permutations = 999, $p = .006$; Figure 2b).

We discarded 16 failed locomotor trials. Only a single individual failed to complete at least one successful trial and was completely removed from the dataset. Six additional hatchlings were excluded because either they or their parents could not be genotyped. All independent variables had VIFs less than 2; thus, bias due to multicollinearity was minimal in our analyses (Table S2). We found that mean latency time from locomotor trials was negatively correlated with maternal inbreeding, but was not correlated with paternal inbreeding, offspring inbreeding or parental relatedness (Figures 3 and S1; Table 1). Mean sprint speed was positively correlated with maternal inbreeding indicating hatchlings with more inbred mothers moved faster in addition to having faster reaction times. Hatchling body condition was also positively correlated with maternal inbreeding and parental relatedness. Additionally, hatchling size (i.e. log hatchling straight carapace length) was negatively correlated with parental relatedness. Finally, we found that neither offspring inbreeding nor paternal inbreeding was correlated with any metric of individual offspring quality or locomotor performance (Figure S2; Tables 1 and S3).

TABLE 1 The chi-square statistics, degrees of freedom and p-values from linear mixed models testing the correlation of offspring inbreeding, parental inbreeding and parental relatedness on hatchling quality and locomotor performance metrics

		Offspring inbreeding			Paternal inbreeding (F)			Maternal inbreeding (F)			Parental relatedness (W)		
	N	χ^2	df	p	χ^2	df	p	χ^2	df	p	χ^2	df	p
Body condition	188	0.246	4, 183	.620	0.005	4, 183	.945	6.385	4, 183	*.039* (+)	4.242	4, 183	*.012* (+)
Carapace length	188	0.361	4, 183	.548	1.752	4, 183	.186	0.512	4, 183	.474	7.692	4, 183	*.006* (−)
Sprint speed	86	0.749	4, 81	.387	0.057	4, 81	.811	5.247	4, 81	*.022* (+)	1.203	4, 81	.273
Latency time	86	0.478	4, 81	.489	0.656	4, 81	.418	5.288	4, 81	*.021* (−)	0.115	4, 81	.735

Note: F is the Ritland moment estimator of inbreeding, and W is Wang's relatedness estimator. Significant p-values are denoted by bolded italics with the direction of effect in parentheses.

4 | DISCUSSION

4.1 | Maternal inbreeding effects on offspring quality

Contrary to expectations, highly inbred females in our population had greater innate clutch viability and more robust offspring with greater locomotor performance and higher body condition (Figure 1 and 3; Table 1). We propose that this pattern may be driven by mate choice to mitigate inbreeding because parental pairwise relatedness was inversely related to maternal inbreeding (Figure 2) and parental relatedness was a major predictor of inbreeding depression for both reproductive and offspring traits (Figures 1 and 3e; Table 1). Individual inbreeding effects are often reduced in offspring if parents are distantly related (Charlesworth & Charlesworth, 1987). This is because deleterious recessive alleles are likely to become masked in offspring in these instances. Therefore, mate choice can strongly mitigate the risk of inbreeding depression (Brown, 1997). Mate choice plays an important role in the breeding system of gopher tortoises. Females are not universally receptive to male courtship, and forced copulation is unlikely given female-biased sexual size dimorphism (McRae et al., 1981). Additionally, females are courted regularly by multiple males (Boglioli, Guyer, Michener, & Douglas, 2003) and initiate social interactions with males, including moving to co-occupy burrows with males (Guyer, Hermann, & Johnson, 2014; Johnson, Guyer, Hermann, Eubanks, & Michener, 2009). Female gopher tortoises in a translocated population also preferred males with longer-term residency over males introduced to the population more recently, indicating individual-level recognition (Tuberville et al., 2011). Finally, tortoises within the genus *Gopherus* have chin glands whose secretions are used in conspecific recognition by both male and female tortoises (Alberts, Rostal, & Lance, 1994; Landers et al., 1980), which could also potentially facilitate kin avoidance during courtship.

The apparent kin avoidance in our study population was limited to female tortoises. These results are consistent with theory, because the costs of inbreeding and subsequent selection for inbreeding avoidance are greater in females than males, as females invest more in reproduction per offspring (Brown, 1997; Lehmann & Perrin, 2003; Pizzari, Lø, & Cornwallis, 2004; Waser et al., 1986). Although the relative costs of inbreeding between sexes may be lessened by the occurrence of polyandry (Newcomer, Zeh, & Zeh, 1999; Stockley, Searle, MacDonald, & Jones, 1993), in tortoises offspring production is still severely limited in females relative to polygynous males (Landers et al., 1980; White et al., 2018) and per-gamete resource allocation remains greater in eggs than sperm (Bateman, 1948; Hayward & Gillooly, 2011). Thus, the fitness costs of inbreeding should still be higher in females despite polyandry.

Apparent kin avoidance in female gopher tortoises also appeared to be limited to more inbred females. In general, inbred individuals would be expected to share fewer alleles with a partner selected at random. However, this alone does not adequately explain our observed mating patterns because this expectation should hold for both sexes, yet we found no evidence of inbred males partnering with less-related females. Likewise, although mate choice against outbreeding depression could lead to the kind of correlation we observed (Luo et al., 2015; Palmer & Edmands, 2000), we did not see evidence of outbreeding depression in traits measured for this study. We hypothesize the observed relationship between maternal inbreeding and parental pairwise relatedness may result from trade-offs between kin avoidance and inbreeding tolerance. Kin avoidance has been hypothesized to be costly due to reduced availability of potential mates and increased energetic costs required for reproduction (Waser et al., 1986). If trade-offs between kin avoidance and inbreeding tolerance exist, then in highly inbred individuals the cost of inbreeding tolerance may outweigh the cost of kin avoidance. This hypothesis would require the ability for individuals to determine their degree of inbreeding. Whether such a mechanism exists is unclear.
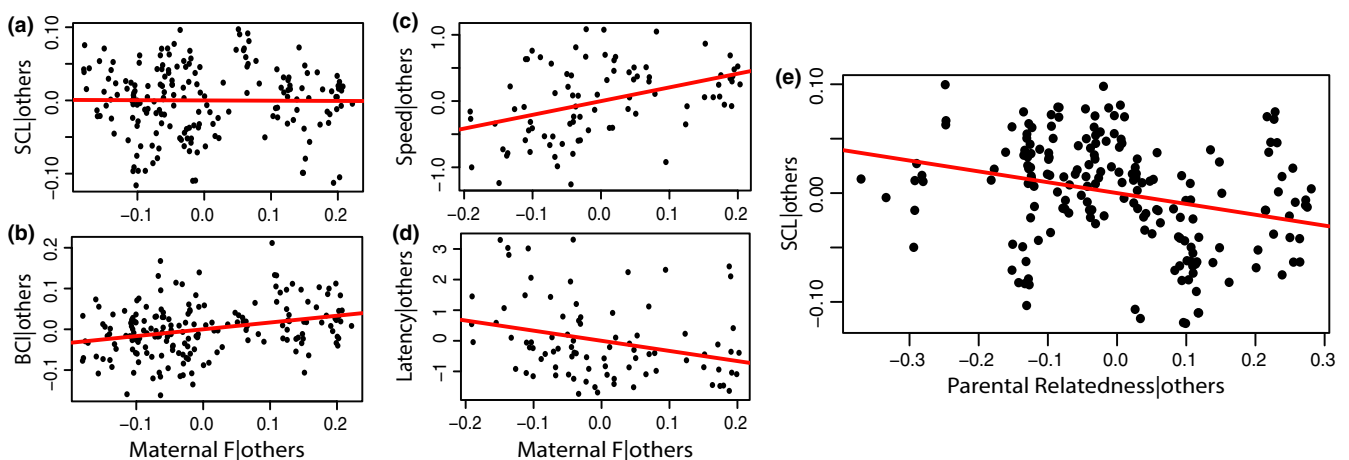
FIGURE 3 Partial regression plots for the effect of maternal inbreeding on (a) straight carapace length, (b) body condition index, (c) average sprint speed and (d) latency time. (e) Partial regression for the effect of parental relatedness on straight carapace length. In all plots, *Y*-axes are residuals of each model with the independent variable of interest removed and *X*-axes are residuals of the independent variable of interest regressed against all other independent variables

The potential role of genetic purging should also be considered as an alternative explanation for the observed correlation between maternal inbreeding and reproductive fitness. That is, more inbred females might have greater clutch viability and higher offspring quality because individuals with deleterious alleles are less likely to survive to reproductive maturity. Stronger selection against inbred individuals relative to noninbred individuals could lead to those inbred individuals that reach reproductive age being fitter overall (García-Dorado, 2012; Hedrick, 1994; Kirkpatrick & Jarne, 2000). However, genetic purging does not explain why highly inbred females, but not highly inbred males, tend to mate with more unrelated partners. Nor does genetic purging directly account for the relationship between mate-pair relatedness and clutch viability. Still, we cannot fully discount genetic purging as a potential mechanism. Further studies will be required to fully differentiate between these alternative hypotheses.

We have shown a cost to inbreeding in both innate clutch viability (Figure 1a) and offspring quality in controlled environments (Table 1). Previous work has also demonstrated mechanisms for conspecific recognition in *Gopherus* tortoises (Alberts et al., 1994; Guyer et al., 2014; Landers et al., 1980; Tuberville et al., 2011). Yet, because our findings are based on genotyping of resulting offspring, they are influenced by both mate choice behaviour and post-copulatory mechanisms affecting fertilization. Direct studies of mating behaviours, sperm storage, and sperm use are needed to clarify the role of kin avoidance in female mate choice. Still, our data are consistent with female mate choice as a potential driver of inbreeding avoidance in gopher tortoises, contrary to the observation that long generation times often reduce selection for kin avoidance (Keane et al., 1996; Lippé et al., 2006; Waser et al., 1986).

4.2 | Inbreeding depression in a declining, long-lived species

Although inbreeding can have morphological consequences, in most cases inbreeding depression exerts stronger selection on life-history traits directly related to fitness compared with indirectly related morphological traits (DeRose & Roff, 1999). However, while we did observe evidence for reduced clutch viability due to inbreeding (Figure 1), our data also show a strong signal of inbreeding depression in hatchling carapace length. This reduction in hatchling body size potentially has negative fitness consequences. Mortality is highest in juvenile gopher tortoises, with studies reporting upwards of 94% mortality during the first year (Alford, 1980). Due to low adult mortality, the consequences of inbreeding in adulthood are likely to be primarily reproductive. Thus, fitness consequences of decreased body size and condition are likely more pronounced in hatchlings than adults (O'Brien, Robert, & Tiandray, 2005). Body size in particular is a major predictor of predation pressure in hatchling turtles. Larger bodied individuals are better able to avoid predation and survive to adulthood (Janzen, 1993; Janzen, Tucker, & Paukstis, 2000). In the closely related desert tortoise, *Gopherus agassizii*, hatchlings of smaller size and decreased body condition also have increased

mortality attributed to reduced water and energy reserves (Nafus, Todd, Buhlmann, & Tuberville, 2015). Therefore, resource storage, as it relates to body size and condition, may also be important for survival of hatchling *G. polyphemus*, particularly during the southern Florida dry season.

Because juvenile tortoises are vulnerable to a wider range of predators than adults, due to their smaller and structurally weaker shells, their primary mechanism of predator avoidance is crypsis or sheltering in burrows (Butler & Sowell, 1996; Epperson & Heise, 2003; Smith, Steen, Conner, & Rutledge, 2013). Therefore, locomotor performance trials are pertinent to the ability of hatchling tortoises to escape predation by fleeing into their burrows. Additionally, gopher tortoises utilize their burrows for thermoregulation (Douglass & Layne, 1978). If poor locomotor performance is linked to poor digging performance, tortoises may not only be at greater risk of predation, but also reduced thermoregulatory efficiency due to inefficient burrow construction. We did not find strong evidence for inbreeding depression in locomotor traits (Table 1). However, as with other traits, the relationship with maternal inbreeding was contrary to expectations. More inbred mothers had faster and more responsive offspring. Nevertheless, the relationship between laboratory-measured performance and survival in natural populations is not always straightforward (Husak, Fox, & Schwenk, 2006; Irschick & Losos, 1998; Miles, 2004) and has not yet been adequately assessed in this species. Beyond the reduction in clutch viability and hatchling size demonstrated in this study, inbreeding has also been previously linked to changes in gut microbial community composition (Yuan et al., 2015) and shell deformities (Velo-Antón, Becker, & Cordero-Rivera, 2011) in tortoises.

The broad fitness consequences of inbreeding depression in *G. polyphemus* have significant conservation implications. Our data are consistent with the hypothesis that inbreeding depression can lead to low recruitment in *G. polyphemus* (Ennen et al., 2010; Noel et al., 2012) (Figure 1). Notably, our results support the hypothesis that western populations with low genetic diversity may be experiencing low hatching success in part due to highly related mating pairs. Although western populations may not be currently experiencing inbreeding depression *sensu stricto* if deleterious alleles associated with reduced clutch viability are now fixed, our demonstration of inbreeding depression in a more genetically diverse population suggests inbreeding depression may have exacerbated initial population declines. Nest protection and controlled incubation of late-stage eggs did not allow us to fully examine the potential context dependence of inbreeding effects under natural conditions. However, our approach likely provided a conservative test of the negative effects of high parental relatedness, given the influence of inbreeding is often stronger during environmental stress (Jiménez, Hughes, Alaks, Graham, & Lacy, 1994).

Despite potential behavioural inbreeding avoidance in our Red Hill population and multiple paternity in gopher tortoises (Moon, McCoy, Mushinsky, & Karl, 2006; Tuberville et al., 2011; White et al., 2018), uniformly low genetic diversity likely means mating of related individuals is unavoidable in western populations compared to larger,

more diverse eastern populations (Ennen et al., 2010). In the light of continued population declines throughout their range (Diemer, 1986; McCoy & Mushinsky, 1992; McCoy et al., 2006), our results suggest that the long-term viability of western gopher tortoise populations will likely require intervention to increase genetic diversity (e.g. Whiteley, Fitzpatrick, Funk, & Tallmon, 2015). However, genetic rescue efforts may be dampened by lower reproductive success in recently translocated compared to previously established males (Mulder et al., 2017; Tuberville et al., 2011). Additionally, the western populations of *G. polyphemus* have a unique phylogeographic history (Gaillard et al., 2017; Osentoski & Lamb, 1995), and it may be desirable to maintain this lineage independent from eastern populations (Crandall, Bininda-Emonds, Mace, & Wayne, 2000; Petit, El Mousadik, & Pons, 1998).

4.3 | Conclusion

We show that inbreeding depression occurs in *G. polyphemus* and may explain poor recruitment observed in low-diversity populations compared with high-diversity populations. However, highly inbred parents do not inevitably produce reduced-quality offspring. Rather, our data suggest that highly inbred female tortoises in our study population chose less-related mates, which may counteract inbreeding depression. In fact, distantly related pairs had higher clutch viability and more robust offspring even when females were highly inbred. We found no evidence of inbreeding avoidance through mate choice in males or in outbred females, suggesting evolutionary trade-offs and sex-specific selective pressures have driven the evolution of mating behaviour. Our results show that the consequences of inbreeding depression can be multifaceted, and the evolution of inbreeding avoidance is driven by the interplay of differing selective pressures.

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SUPPORTING INFORMATION

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