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Does sex-ratio selection influence nest-site choice in a reptile with temperature-dependent sex determination?

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Evolutionary theory predicts that dioecious species should produce a balanced primary sex ratio maintained by frequency-dependent selection. Organisms with environmental sex determination, however, are vulnerable to maladaptive sex ratios, because environmental conditions vary spatio-temporally. For reptiles with temperature-dependent sex determination, nest-site choice is a behavioural maternal effect that could respond to sex-ratio selection, as mothers could adjust offspring sex ratios by choosing nest sites that will have particular thermal properties. This theoretical prediction has generated decades of empirical research, yet convincing evidence that sex-ratio selection is influencing nesting behaviours remains absent. Here, we provide the first experimental evidence from nature that sex-ratio selection, rather than only viability selection, is probably an important component of nest-site choice in a reptile with temperature-dependent sex determination. We compare painted turtle (*Chrysemys picta*) neonates from maternally selected nest sites with those from randomly selected nest sites, observing no substantive difference in hatching success or survival, but finding a profound difference in offspring sex ratio in the direction expected based on historical records. Additionally, we leverage long-term data to reconstruct our sex ratio results had the experiment been repeated in multiple years. As predicted by theory, our results suggest that sex-ratio selection has shaped nesting behaviour in ways likely to enhance maternal fitness.

1. Introduction

Fisher's [1] explanation of why dioecious organisms typically produce an equal number of sons and daughters is one of the most useful models in evolutionary biology [2,3]. The fact that every such diploid individual has a mother and a father ensures that males and females contribute equally to the next generation. When biased sex ratios exist, individuals of the rarer sex will have higher fitness. Thus, under many conditions, frequency-dependent selection favours parents that overproduce offspring of the rarer sex, thereby balancing the sex ratio [3] (i.e. sex-ratio selection). With genotypic sex determination (GSD), independent meiotic segregation of sex chromosomes usually results in a balanced primary sex ratio. However, maintaining an optimum sex ratio is more complex when environmental factors determine sex [4–6].

For many reptiles and some fish, temperature during early development irreversibly determines offspring sex. Several excellent studies on short-lived fish [7] and lizards [8,9] have provided evidence that temperature-dependent sex determination (TSD) is adaptive, and in such circumstances, the optimal sex ratio may vary slightly from 1:1 [10–12]. However, despite considerable effort, there is no definitive evidence that TSD is adaptive in any long-lived reptiles [6], thus a balanced or nearly balanced sex ratio is likely to be optimal in these organisms. As environments vary spatio-temporally, TSD may render populations susceptible to experiencing maladaptive sex-ratio skews. Yet many clades of animals with TSD have endured climatic warming and cooling events over millions of years, and currently exist across broad geographical (and, hence, thermal) ranges [13]. If sex is determined environmentally, then

how can these animals adapt to changing climates or to local conditions to maintain an appropriate sex ratio? Theory predicts that the two primary targets of sex-ratio selection are the thermal sensitivity of the sex determination pathway (hereafter, TSD reaction norm) and maternal nesting behaviour [5].

Decades of research have not convincingly demonstrated that sex-ratio selection shapes either the TSD reaction norm or nesting behaviour in wild reptiles. Multi-generation laboratory experiments with short-lived fish show that the TSD reaction norm can evolve as predicted when fish are maintained in constant, sex-ratio-biasing conditions [14], and comparisons of geographically distinct populations suggest that variation in this trait results from both sex-ratio selection [15] and other selective pressures [16]. In short-lived viviparous lizards, evolutionary transitions between GSD and TSD are apparent among populations inhabiting starkly different climates, yet this variation is likely to be driven by differing ecological selective pressures, rather than sex-ratio selection [9]. Comparisons of TSD reaction norms from reptile populations occupying thermally divergent regions have provided equivocal results; some studies have not detected significant variation [17], whereas in others the variation detected was not in either the direction or magnitude expected [18–20] under sex-ratio selection.

Nesting behaviours of several reptile species, however, do vary geographically in the direction predicted by Fisherian theory: mothers construct more exposed nests in relatively cool locales and more shaded nests in relatively warm locales [19,20]. Still, nest-site choice has important consequences beyond phenotypic modification, because this behaviour can also affect offspring survival and risk of maternal mortality [21]. Consequently, the ultimate causes of nesting behaviour are diverse. Empirical studies of nest-site choice in reptiles either suggest that nesting patterns are driven by selection to maximize embryonic survival [19,22] or have been unable to disentangle the effects of this survival selection from sex-ratio selection [20]. Thus, while some observed patterns accord with theory, we lack convincing evidence that nest-site choice is specifically influenced by sex-ratio selection.

We designed an experiment to examine the consequences of nest-site choice on offspring sex ratio and survival with the painted turtle (Chrysemys picta), a common aquatic chelonian with a broad geographical distribution in North America. At our study site in northwestern Illinois, females construct nests in early summer, and their eggs incubate and hatchlings hibernate within the nests until emerging the following spring [23]. The thermosensitive period (TSP; the period when sex is labile) [13] occurs approximately in the middle third of embryonic development. During this time, warmer temperatures produce females, cooler temperatures produce males and a narrow range of temperatures produces both sexes. Long-term research at our site has shown that there is typically a strong relationship between vegetation cover above the nest and the nest sex ratio; however, nest vegetation cover is not related to hatching success of the nest [24]. Calculations based on historical climate data estimate that the primary sex ratio produced at our site over the past 100 years is male biased, which thus would presently favour females nesting in relatively exposed, warmer nest sites. In this experiment, we split clutches to allow half the offspring to incubate in maternally selected nest sites and half to incubate in randomly selected nest sites. We subsequently redistributed hatchlings between these sites prior to hibernation. This design enabled us to quantify any microhabitat and thermal differences between the nest treatments. We also quantified the consequences of nest-site choice on offspring survival during both stages (incubation and hibernation) and on offspring sex ratio by comparing offspring from nests in maternally selected sites with offspring from nests in randomly selected sites. Finally, we used established annual relationships between nest vegetation cover and clutch sex ratios from long-term research to estimate sex ratio results from this experiment, had it been performed in previous years.

2. Material and methods

(a) Study species and site

The population of C. picta used for this experiment has been the focus of long-term research on reproductive ecology. This population resides in the backwaters of the Mississippi River and nests in the Thomson Causeway Recreation Area (TCRA) in Thomson, Illinois, USA. The TCRA is mostly deciduous forest, yet the nesting areas used by turtles are primarily short grass maintained by mowing on loamy soils. Female turtles dig nests from mid-May through June. After eggs hatch, the hatchlings typically remain in the natal nest until the following spring, when they emerge and disperse to aquatic habitats.

(b) Field methods

From 22 May to 17 June 2010, we monitored the TCRA for nesting activity. We excavated eggs from the top of a nest within approximately 12 h of oviposition, and placed eggs in containers filled with moist soil, and stored them in Styrofoam boxes. For each focal nest, T.S.M. (the first author) constructed two artificial nest chambers to identical dimensions (± 0.5 cm) to be the same depth as the original nest. We placed the nest in the 'maternal' treatment within 10 cm of the original nest location and the nest in the 'random' treatment in a randomly selected location within 30 m, the average distance from water to a natural C. picta nest at our site. We constructed both nests to control for any thermal effects induced by the structure of the natural nest; thus, any differences in nest temperature are attributable solely to differences in microhabitat. We chose the distance of the random nest from the maternal nest using a random number generator, bound to integer distances of 1–30 m. We determined the direction of the random nest from the maternal nest by tossing a spinning pencil in the air and placing the nest in the direction indicated by the pencil's tip when it landed [25]. If the specified random location was unquestionably unsuitable for turtle nesting (e.g. in the river), then we generated a new random distance and direction.

We weighed, marked with a felt tip pen and redistributed eggs such that half incubated in the maternally selected nest site and half incubated in a randomly selected nest site (figure 1). We added live eggs from other nests constructed on the same date to ensure that experimental nests all had clutch size = 10, but did not use these extra 'dummy' eggs in analyses. We programmed Thermocron iButtons (DS1921G, Embedded Data Systems), wrapped in latex and Parafilm (for waterproofing), to record hourly temperatures, and placed one in the centre of each nest among eggs. After inserting eggs, we filled nests with soil, protected them from predators with 1 cm-mesh aluminium hardware cloth secured with tent stakes, and mapped them for relocation. We measured canopy cover (% openness) and incident solar radiation (MJ m^{-2} per day) for each nest with hemispherical photography and Gap Light Analysis software [26]. We photographed the sky directly above each nest with a digital camera
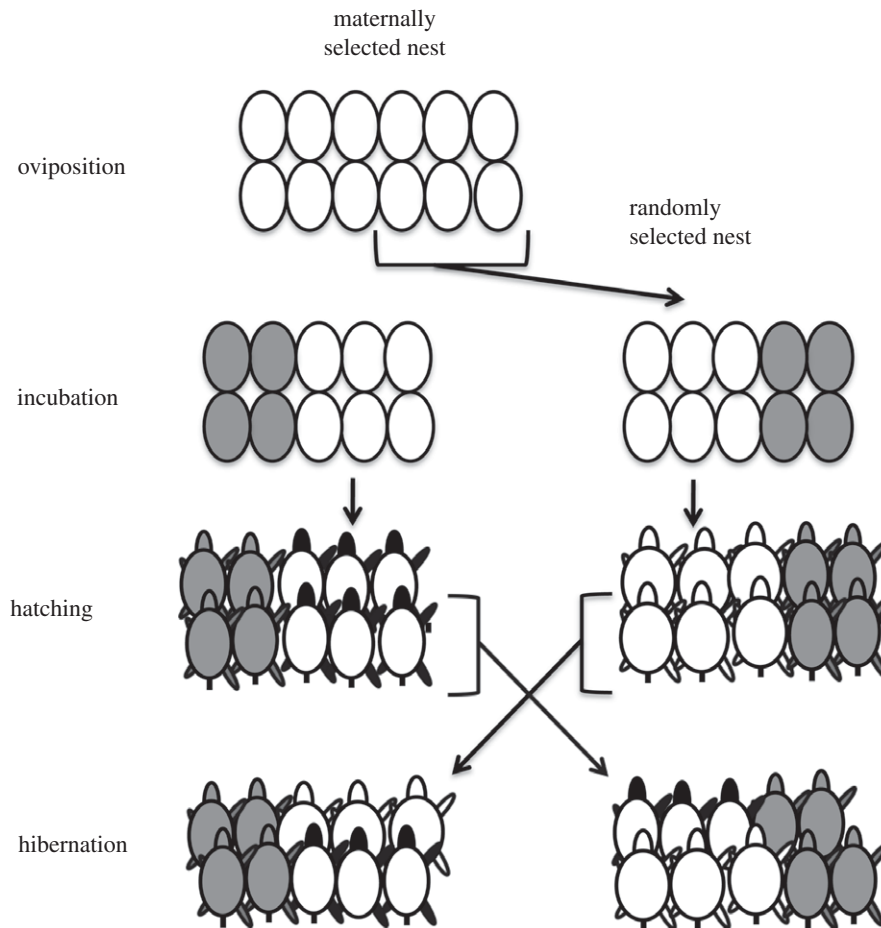
Figure 1. A schematic of the experimental design. All white eggs and hatchlings are siblings and represent the focal individuals. Shaded eggs and hatchlings were live 'dummies' not included in analyses. Hatchlings with black heads incubated in the maternal nest, whereas hatchlings with white heads incubated in the random nest. After hatching, we reorganized turtles again such that we had hatchlings that (i) incubated and hibernated in the maternal nest, (ii) incubated in the maternal nest, but hibernated in the random nest, (3) incubated the random nest, but hibernated in the maternal nest, and (iv) incubated and hibernated in the random nest.

fitted with a 180° fisheye lens. GAP LIGHT ANALYSIS software uses this photograph, latitude, longitude, elevation, day length and weather data to quantify canopy openness and total transmitted solar radiation during the time frame of interest.

On 20 and 21 July 2010, we excavated all nests, placed the nearly hatched eggs in Styrofoam boxes and transported them 315 km to Iowa State University (ISU). We packed each nest cavity with cotton-filled plastic bags to maintain cavital integrity until we returned hatchlings prior to winter. At ISU, we weighed eggs and placed them in plastic shoeboxes with moistened vermiculite (−150 kPa) in incubators maintained at 28.5°C. Eggs incubated at this temperature during the TSP produce both sexes, so even if eggs spent part of the TSP in the laboratory, it would only obscure treatment effects. We monitored eggs twice daily for pipping, at which point we placed a bottomless paper cup over the egg to ensure that we could identify which hatchling came from which egg. Eggs spent an average of 72% of the incubation period in the field (range: 52–96%), indicating that sex was determined for most clutches by the time we excavated eggs. Removing the few nests that spent less than two-thirds of the incubation period in the field did not qualitatively influence the results, so we included them in our analysis.

We photographed the unique plastron pattern of each hatchling on 8 October 2010 to ensure accurate identification. On 14 October, we redistributed hatchlings and returned them to nests for hibernation. We reorganized hatchlings such that half the hatchlings that incubated in the maternal nest would hibernate in the random nest, and half the hatchlings that incubated in the random nest would hibernate in the maternal nest (figure 1). Nest pairs that did not have enough hatchlings from

each nest were not included in this experimental manipulation. This design resulted in hatchlings from 20 mothers (20 maternal and 20 random nests) that spent (i) both the incubation and hibernation stage in the maternal nest, (ii) both the incubation and hibernation stage in the random nest, (iii) the incubation stage in the maternal nest, but the hibernation stage in the random nest, and (iv) the incubation stage in the random nest, but the hibernation stage in the maternal nest (figure 1). We placed hatchlings in the nests, along with C. picta eggshell fragments (as is the natural condition), and protected the nests as before. We buried an iButton 5 cm deep, 10 cm from each nest cavity, as placing one within a nest could introduce unnatural nuclei for ice formation during winter.

On 22 March 2011, we excavated nests and returned hatchlings to ISU in plastic cups filled with soil. We sacrificed only focal turtles, injecting an overdose of Sleepaway near the heart. F.J.J. scored hatchling sex by macroscopic examination of the gonads without knowing the treatment. If none of the focal offspring from a nest hatched, then we did not sex their siblings from the other treatment. Consequently, we acquired hatching success data for more mothers than we obtained sex-ratio data.

(c) Statistical analyses

We performed all statistical analyses in SAS. We used analysis of variance to compare microhabitat variables between the maternally selected nest sites and random nest sites. We used the MIXED procedure, with treatment modelled as a fixed factor, and mother (i.e. clutch) as a random factor. From the iButton dataloggers in the nests, we extracted mean daily mean, mean

Table 1. Comparison of microhabitat variables and thermal properties of maternally selected and randomly selected nest sites of painted turtles (*Chrysemys picta*). *n* is the number of nest pairs. The temperature statistics represent the field incubation period (approx. oviposition through nest excavation). Constant temperature equivalents, however, were calculated for the middle third of incubation.

variable	*n*	maternal nest sites LS mean $\pm$ s.e.	minimum	maximum	random nest sites LS mean $\pm$ s.e.	minimum	maximum	statistic
openness (%)	31	59.5 ± 2.7	30.4	93.9	51.1 ± 2.8	24.5	86.3	$F_{1,28} = 5.9$ $p = 0.021$
solar radiation (MJ m^{-2} d^{-1})	31	6.9 ± 0.3	3.2	9.5	5.1 ± 0.3	1.3	9.3	$F_{1,28} = 15.2$ $p < 0.001$
depth (cm)	31	8.2 ± 0.8	6.5	9.5	—	—	—	—
temperature (°C)								
mean	18	25.5 ± 0.4	23.4	28.5	23.6 ± 0.4	20.9	26.3	$F_{1,17} = 19.1$ $p < 0.001$
minimum	18	22.1 ± 0.2	20.8	24.1	21.2 ± 0.2	19.2	22.7	$F_{1,17} = 14.0$ $p = 0.001$
maximum	18	30.7 ± 0.7	26.4	34.5	27.0 ± 0.7	22.6	32.6	$F_{1,17} = 20.9$ $p < 0.001$
range	18	8.5 ± 0.5	5.4	11.3	5.8 ± 0.5	2.3	10.9	$F_{1,17} = 16.7$ $p < 0.001$
CTE (°C)	17	27.5 ± 0.4	25.0	28.8	25.8 ± 0.4	22.7	28.9	$F_{1,16} = 13.76$ $p = 0.002$

daily maximum, mean daily minimum and mean daily range of temperature from the entire incubation period. We also estimated the constant temperature equivalent (CTE; i.e. the median developmental temperature) [27] during the TSP for each nest. To do this, we split the total incubation period into thirds, and examined the temperature data from the middle third (which roughly approximates the TSP). For each day in the TSP, we estimated the CTE using a model parametrized for *C. picta* (see [28] for details). For some eggs, the latter part of the TSP occurred under constant laboratory incubation conditions after excavation. For these days, the CTE value was the constant temperature actually experienced (28.5°C). We then computed a mean CTE value by averaging the CTE values calculated for each day during the TSP. We then estimated the relationship between the mean CTE value and embryo sex ratio with a two-parameter sigmoid curve (equation: nest sex ratio = $1/(1 + \exp(-(\text{predictor} - \theta_1/\theta_2))))$) initiated at $\theta_1 = 25$ and $\theta_2 = -1$, and estimated iteratively using PROC NLIN. To analyse survival and sex-ratio data, we used generalized linear mixed models (PROC GLIMMIX) with a binomial distribution and a logit link function. Treatment was again modelled as a fixed factor, and mother as a random factor. Data are included in the electronic supplementary material.

Schwanz *et al.* [24] established equations for the annual functional relationship between nest vegetation cover and clutch sex ratio between 1990 and 2006 at our site. We use the same equations to estimate offspring sex ratios had our experiment been repeated over these 17 years. For this long-term dataset, nest vegetation had been quantified with a spherical densiometer, and the sum of south and west (S + W) vegetation cover used as the primary variable. Thus, we converted our data from hemispherical photographs and GAP LIGHT ANALYSIS into S + W densiometer readings with the equation S + W = 227.64 − (2.18 × % total radiation) (see [24]). These S + W values calculated for every nest are estimates of nest vegetation cover and are comparable with those from the long-term data. Substituting these nest vegetation cover values in the equations from [24], we estimate clutch sex ratio of each maternal and random nest for most years. For three particularly cool years, nest vegetation cover and sex ratio were unrelated, as these cold temperatures limited variance in sex ratio. In these three cases, we substituted the overall offspring sex ratio for a year for all nests in that year to yield conservative estimates. A flood destroyed all nests in 1993, so no data are available for that year.

3. Results

Maternally selected nest sites had relatively open canopies compared with the randomly selected nest sites. Consequently, they received more solar radiation, were warmer and had greater daily temperature ranges than random nests (table 1). Nests exposed to more solar radiation had higher mean nest temperatures during the approximate TSP ($r^2 = 0.46$, $p < 0.001$). Moreover, CTE calculated from nest temperature during the TSP covaried with offspring sex ratio (equation: nest sex ratio = $1/(1 + \exp(-(\text{temp} - 27.74/-1.80)))$), $p < 0.001$. While treatment did not affect hatching success during the incubation stage ($F_{1,27} = 0.70$, $p = 0.409$) nor offspring survival during hibernation ($F_{1,19} = 0.53$, $p = 0.474$; figure 2), it did influence offspring sex ratio ($F_{1,20} = 5.95$, $p = 0.024$). Maternally selected nests produced a nearly balanced sex ratio, whereas randomly selected nests were strongly male biased (figure 3).

Patterns of nest-site choice do not vary significantly among years at our site [29], so the maternally selected nests in our study are representative of usual nest locations. Additionally, mean air temperatures during the TSP (approx. July) were
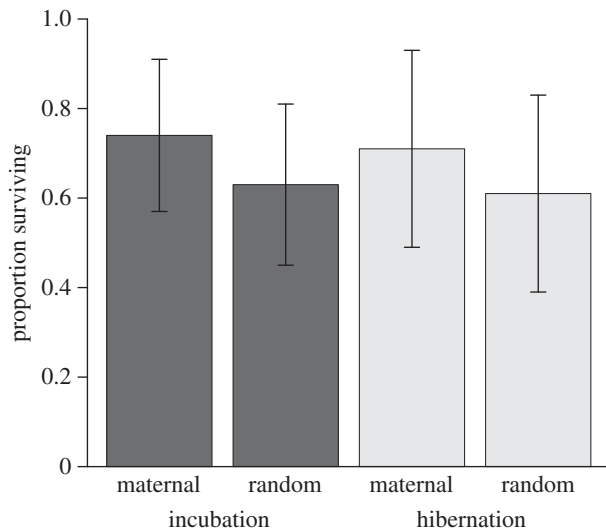
Figure 2. Hatching success of *C. picta* eggs incubated in $n = 28$ maternally and randomly selected nests, and survival of hatchlings hibernated in $n = 20$ maternally and randomly selected nests. Error bars are 95% CIs.
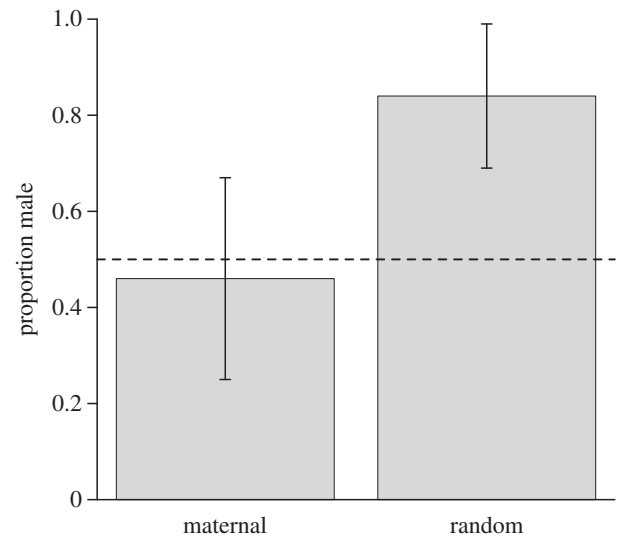
Figure 3. Sex ratio (proportion male) of *C. picta* hatchlings incubated in maternally and randomly selected nest sites ($n = 21$). The dashed line represents a balanced sex ratio. Error bars are 95% CIs.

within 1 s.d. of the 100-year mean at the nearest weather station (Clinton, IA, USA; National Climatic Data Center (www.ncdc. noaa.gov)), thus our sex ratio results should represent the outcome of the typical climatic situation. However, calculations based on the long-term data [24] suggest that the overall treatment sex ratios would have varied substantially among years (table 2). Regardless, maternal nests almost always yielded a less male-biased estimated sex ratio than did random nests. Averaging estimates across all years, the maternal treatment produced a 0.59 ± 0.30 offspring sex ratio and the random nests produced a 0.74 ± 0.27 offspring sex ratio (table 2).

4. Discussion

Theory predicts that nest-site choice is a trait that may respond to sex-ratio selection. Yet to detect such an evolutionary change in response to sex-ratio selection in the field is logistically challenging. To do so requires multi-generational studies, and many reptiles with TSD are characterized by long generation times. Moreover, this behavioural trait is not preserved in the fossil record, making palaeontological inferences difficult. Geographical comparisons have promise, but require intense fieldwork at multiple sites simultaneously across a broad geographical range. However, our novel experimental design allowed us to investigate the phenotypic and survival consequences for offspring of non-random maternal nest-site choice. Our results indicate that nesting behaviours had negligible consequences for survival, but substantial impacts on sex ratio, suggesting that the observed nesting patterns are likely to represent the signature of sex-ratio selection.

Our findings accord with long-term studies (15+ years) of nest-site choice, population dynamics and selective pressures in this population. Vegetation cover over natural nests does not substantially influence hatching success [24], but strongly affects offspring sex ratio [23,24]. Together with our results, these findings suggest that available nest sites for this population are generally suitable for embryonic development, but induce very different offspring sex ratios. While it is logistically unfeasible to accurately assess operational sex ratios in

our system, estimates derived from established relationships between annual offspring sex ratio and historical climate data suggest a male-biased (0.63) offspring sex ratio at our site over the past 100 years [24]. Thus, sex-ratio selection should presently favour mothers that nest in relatively open sites compared with what is available, which is the pattern that we observed. Still, how the trait will respond to such sex-ratio selection is debatable [30]. Interestingly, heritability of nest-site choice is higher following warmer winters ($h^2 = 0.19$) [31], suggesting that this trait could be a prime target for evolutionarily effective sex-ratio selection under warming climatic conditions. Nest-site choice thus warrants particular attention for research addressing response of reptiles with TSD, many of them already imperilled, to contemporary climate change [32].

Our historical calculations estimate sex ratios from both maternal and random treatments had the experiment been repeated annually over a recent 17-year period. Variation in weather during those years resulted in distinctive annual relationships between vegetation cover and nest sex ratio [24], and consequently the treatments would have yielded different sex ratios in different years. Even so, these retrospective results are notably congruent with findings from our field experiment. Averaging across all years suggested that maternally selected nests would have produced a 0.59 offspring sex ratio, strikingly similar to the long-term estimate from weather data for the past 100 years (0.63 [24]). Based on these calculations, had mothers been selecting nest sites randomly during these 17 years, the overall offspring sex ratio would have been considerably more male biased (0.74). Thus, historical calculations concur that maternal nest-site choice documented in our field experiment is in the direction expected by sex-ratio theory.

The evolution of nest-site choice is influenced by multiple factors, and thus identifying the ultimate cause(s) of the behaviour is complex. To adaptively modify offspring phenotype through nest-site choice, the mother must first select nests that are suitable for survival. Indeed, offspring survival is probably the primary factor driving nest-site choice in most oviparous organisms [21], and prior research on our

Table 2. Estimated maternal and random nest sex ratios had this experiment been repeated over 17 years. The equation describing the functional relationship between vegetation cover (veg) and nest sex ratio (sr) is obtained from long-term research, described in [24]. 'Veg' is the S + W densiometer readings of nest vegetation cover. Asterisk indicates where we used overall offspring sex ratio for that year for all nests.

year	equation (from [24])	n	sex ratio ± s.d.	
			maternal treatment	random treatment
1990	no relationship, mean sr = 0.92 ± 0.16	31	0.92 ± 0.0*	0.92 ± 0.0*
1991	sr = 1/(1 + exp(−(Veg − 96.1)/35.9))	31	0.35 ± 0.22	0.59 ± 0.27
1992	no relationship, mean sr = 1.0 ± 0.0	31	1.0 ± 0.0*	1.0 ± 0.0*
1993	no data, flood	31	—	—
1994	sr = 1/(1 + exp(−(Veg − 28.6)/49.9))	31	0.68 ± 0.14	0.80 ± 0.15
1995	sr = 1/(1 + exp(−(Veg − 99.3)/34.3))	31	0.33 ± 0.22	0.57 ± 0.27
1996	sr = 1/(1 + exp(−(Veg + 16.9)/54.6))	31	0.81 ± 0.09	0.89 ± 0.09
1997	sr = 1/(1 + exp(−(Veg − 12.2)/56.4))	31	0.72 ± 0.12	0.82 ± 0.12
1998	sr = 1/(1 + exp(−(Veg − 79.3)/47.1))	31	0.45 ± 0.18	0.64 ± 0.21
1999	sr = 1/(1 + exp(−(Veg − 106.7)/45.9))	31	0.33 ± 0.17	0.53 ± 0.22
2000	sr = 1/(1 + exp(−(Veg − 29.2)/27.4))	31	0.75 ± 0.19	0.88 ± 0.17
2001	sr = 1/(1 + exp(−(Veg − 73.1)/26.9))	31	0.47 ± 0.27	0.72 ± 0.28
2002	sr = 1/(1 + exp(−(Veg − 81.5)/37.9))	31	0.43 ± 0.21	0.65 ± 0.24
2003	sr = 1/(1 + exp(−(Veg − 7.9)/55.3))	31	0.74 ± 0.11	0.84 ± 0.12
2004	no relationship, mean sr = 0.93 ± 0.25	31	0.93 ± 0.0*	0.93 ± 0.0*
2005	sr = 1/(1 + exp(−(Veg − 134.9)/5.0))	31	0.07 ± 0.23	0.36 ± 0.44
2006	sr = 1/(1 + exp(−(Veg − 71.9)/36.4))	31	0.48 ± 0.22	0.70 ± 0.24
all years		527	0.59 ± 0.30	0.74 ± 0.27

population supports this broad-scale assessment [24]. Here, we have specifically shown that nesting behaviour is so finely tuned that, on the scale of our experiment, nest-site choice has negligible differential survival consequences within a year, but substantial impact on offspring sex ratio in the direction predicted by sex-ratio theory [5,30] and expected from long-term empirical work [24]. This study provides the first experimental evidence in the wild that sex-ratio selection under TSD is probably an important component of nest-site choice, validating the theoretical framework that has guided decades of research [5,6]. Indeed, sex-allocation theory [1–3] has been one of the most successful paradigms in evolutionary biology, and our empirical research is another prime example [15,33,34] of its profound utility and explanatory power.

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