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From the Field

Boosting Female Hatchling Production in Endangered, Male-biased Turtle Populations

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ABSTRACT In turtle species with temperature-dependent sex determination, hatchling sex ratios skewed toward males could threaten populations where females are vulnerable to harvest. We tested the efficiency of black plastic covers in producing more female hatchlings from nests of the six-tubercle Amazon River turtle (*Podocnemis sextuberculata*) at the Mamirauá Reserve, Amazonas, Brazil during September to November 2003. Covered nests produced 37% more females and midnest average temperatures were 1° C higher than control nests. Incubation period was 3 days shorter, but survivorship was 12% lower in nests with black plastic. This technique could be an inexpensive, local alternative for short-term sex-ratio manipulation in endangered male-biased populations with temperature-dependent sex determination.

KEY WORDS Amazon, management technique, *Podocnemis sextuberculata*, sex ratio manipulation, six-tubercle Amazon River turtle, survivorship, temperature sex determination.

Management of nesting beaches is a widespread conservation practice for endangered freshwater and sea turtle populations (Iverson 1991, Janzen and Paukstis 1991, Cantarelli et al. 2014, Revuelta et al. 2015). Management activities include protection of the nests against predators, control of the vegetation surrounding nests, and nest transplantation to safer areas (Souza and Vogt 1994, Yerli et al. 1997, Gomes and Ferreira-Júnior 2011, Jourdan and Fuentes 2015). Such activities can have a substantial impact on nest survivorship, sex ratio, and hatchling fitness. Thermal and hydric regimes during incubation can affect reptile hatchlings' morphological and behavioral phenotypes (e.g., body size, shape, locomotor performance; Shine et al. 1997, Booth 2006).

Nest microhabitat is a major determinant of hatching success, and incubation temperature is particularly important for species with temperature-dependent sex determination (Bull and Vogt 1979, Wood et al. 2014). Temperatures during the second third of the incubation period define the hatchling's sex for many species of reptiles (Bull 1980, Mrosovsky and Pieau 1991). Although temperature patterns that produce males or females vary among reptiles, in most turtles, hatchlings are predominantly male if incubated at lower temperatures and predominantly female if incubated at higher temperatures (Bull and Vogt 1979, Ewert et al. 1994). For example, the pivotal temperature (temp that results in 50% individuals of each sex) is typically near 32° C for Amazon River turtles from the family Podocnemididae (Alho et al. 1985, Souza and Vogt 1994, Páez et al. 2009).

Long-term changes in nesting habitat temperature caused by local factors (e.g., deforestation of surrounding vegetation, construction of dams and consequent change in water level patterns) and climate change may adversely affect temperature-dependent sex determination populations, causing skewing of hatchling sex ratios (Telemeco et al. 2013, Tomillo et al. 2015). Disproportionate production of one sex in temperature-dependent sex determination species may lead to an adult population with a biased sex ratio, decreasing effective population size and genetic diversity (Vogt 1980, Vogt and Bull 1984, Servan et al. 1989, Godley et al. 2001). The sex ratio of adult turtles can also be influenced by humans because females are preferentially harvested (Mrosovsky 1994, Souza and Vogt 1994, Aresco 2005, Gibbs and Steen 2005, Eisemberg et al. 2015). A primary sex ratio skewed toward males could be particularly problematic (Vogt 1980, Vogt and Bull 1984, Servan et al. 1989). Low numbers of females in a population can lead to decreased densities and eventual localized extinction (Vogt 1994, Wedekind 2012). However, an efficient and economical technique to obtain a higher percentage of females is lacking.

Vogt (1994) and Páez and Bock (1998) suggested using black plastic covers over turtle nests as an economical method to obtain a greater proportion of females. The black plastic potentially absorbs more solar radiation, thus warming the nest and producing more females. We tested the efficacy of black plastic covers on nests of the six-tubercle Amazon River turtle (*Podocnemis sextuberculata*). The study site was chosen because the adult population of *P. sextuberculata* was

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highly sex biased (2.05 M:1 F) because of uneven harvest pressure (Fachín-Téran et al. 2003). Furthermore, the only protected beach in the area was producing predominantly male hatchlings, with 75% of the nests comprised exclusively of males (Pezzuti and Vogt 1999).

STUDY AREA

We conducted the experiment at Pirapucú nesting beach, Japurá River, in the southeastern portion of the Mamirauá Sustainable Development Reserve (2°53′S, 64°51′W), Amazonas, Brazil. In 2003, Pirapucú was 2 km long and 600 m wide. The topography was irregular with fine-grained, light-yellow sand and sparse, low vegetation (Pezzuti and Vogt 1999). At the time, approximately half of the beach was protected by local residents from human predation of nesting females and their eggs.

METHODS

We used 30 recently constructed (within <12 hr) nests from 17 to 22 September 2003. We chose nests nonrandomly, and alternated the selection of black plastic and control nests to reduce the effect of nesting date on sex determination. We covered 15 nests were covered with black plastic and used 15 as controls. We used low-density polyethylene black plastic (0.1-mm thick) to cover an area of 2 m^2. We staked the black plastic sheet flat to the ground immediately over the nests, with the nest located in the middle of the plastic sheet (Fig. 1). The timing of nesting and proximity to water can affect nest development and mortality rate (Escalona and Fa 1998, Harms et al. 2005); hence, we recorded the date of oviposition and distance to shore in meters. We measured temperatures during incubation for 2 control nests and 6 covered nests. We excavated nests, counted and replaced eggs, and positioned data loggers (HOBO-TEMP; 0.05° C precision) in the middle of the nest (midpoint between bottom and top of the clutch) to monitor temperatures hourly. To decrease the effect of environmental variability among recorded nest temperatures, we chose a cluster of 8 nests in close proximity to each other for the installation of data loggers.

We removed the black plastic and protected the nest with a net to prevent hatchlings from escaping after 50 days of incubation. We defined the incubation period as the number of days from egg laying to eclosion of the first hatchling. We measured linear carapace length and carapace width with digital calipers (0.05-mm precision). We calculated within-nest egg apparent survivorship by dividing the number of live hatchlings by the total number of eggs in the clutch. We sacrificed 5 randomly selected hatchlings in each nest, and determined their sex by gonadal examination using a stereo dissecting microscope.

We used a simple linear regression to analyze the relationship between distance to shore and incubation period to test a possible influence of nest location on egg development. We tested differences between nests with and without black plastic using Student's t-test for incubation period, survivorship, distance to shore, and average morphometric measurements, and a chi-square test of independence for sex ratio (no. of males of the 5 dissected hatchlings). Before proceeding with the t-tests, normality and homogeneity of variances, we confirmed using Kolmogorov–Smirnov and Levene's tests. We performed statistical analyses using the open-source statistics Program R (R Core Team 2014, http://www.r-project.org) and Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). We followed ASIH guidelines for use of live amphibians and reptiles and reptiles in Field Research (ASIH 1987).

RESULTS

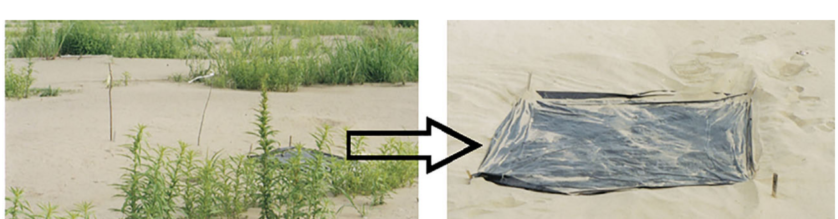
On average, the incubation period was 3 days longer and survivorship was 12% higher in nests without black plastic (Table 1). Intersexes were not observed in this study. Only one case of twins was registered in a nest with black plastic. During 26 September to 25 November, nest average temperature was 1° C higher in nests with black plastic (32° C) in comparison with nests without black plastic (31° C). However, temperature variation was similar (Table 1). Black plastic cover increased maximum temperatures during the day, except during 2 cloudy periods from 8 October to 21 October and 19 November to 25 November (Fig. 2).

We found no differences in linear carapace length or carapace width of hatchlings between nests with and without plastic (Table 1). Nests with and without plastic also did not differ in distance to shore (Table 1). There was a weak effect of distance from shore on incubation period ($F_{1,28} = 3.40$, $P = 0.06$, $r^2 = 0.13$). Covered nests hatched earlier and produced twice as many females as did nests without black plastic (Table 1). In total, 53 females (67.9%) were produced in covered nests while nests without black plastic produced 25 females (31.1%).

DISCUSSION

Effects on Sex Ratio

In natural turtle nests, the maximum temperature usually depends more on solar radiation than air temperature



COLOR

Figure 1. Black plastic placed on the top of *Podocnemis sextuberculata* nests from September to November 2003 at Pirapucú nesting beach, Amazon, Brazil.

Table 1. Mean, standard deviation, minimum, and maximum values for variables sampled in nests of *Podocnemis sextuberculata* with black plastic absent or present ($n=15$ nests each, except for temp) at Pirapucú nesting beach, Japurá River, on the southeast of the Mamirauá Sustainable Development Reserve, Amazonas, Brazil during 2003.

	Absent			Present				
Parameters	$\bar{x}$	±SD	Range	$\bar{x}$	±SD	Range		
Incubation period (days)	60.7	2.7	56.0–65.0	57.4	2.0	55.0–63.0	$t_{28}=3.86$	$P\leq 0.01$
Egg apparent survivorship (%)	0.92	0.15	0.46–1.00	0.80	0.12	0.61–1.00	$t_{28}=2.33$	$P<0.05$
Distance to shore (m)	88.6	41.2	16.6–170.8	121.1	75.5	20.9–260.0	$t_{28}=-1.47$	$P=0.15$
LCL (mm)[a]	39.7	3.1	34.0–45.1	40.7	2.4	35.6–43.7	$t_{28}=-0.96$	$P=0.35$
LCW (mm)[a]	36.0	23.0	29.9–41.3	36.21	1.78	32.64–38.74	$t_{28}=-0.19$	$P=0.85$
No. of males ($n=5$ hatchlings)	3.33	1.63	0.00–5.00	1.47	1.64	0.00–5.00	$\chi^2_1=10.89$	$P\leq 0.001$
Temperature (°C)[b]	31.0	3.7	24.4–40.8	32.0	3.8	24.9–42.4		

[a] LCL = Linear carapace length, LCW = Linear carapace width.
[b] Absent ($n=2$); present ($n=6$).

(Georges 1989). By increasing the amount of solar radiation absorbed by the sand, we increased production of female hatchlings, whereas uncovered nests produced predominantly males. Applying black plastic decreased the number of males on average from 69.9% to 32.1%. The sex ratio of black plastic treatment and control nests combined was 52% females, very close to 1:1. This is in contrast with a study by Pezzuti and Vogt (1999) from the same beach that recorded only 3% females. Our method succeeded in increasing the production of females, while still producing males.

Our technique is a simple, inexpensive alternative to more invasive sex-manipulation procedures, such as the use of estradiol, which may potentially induce gonadal morphological abnormalities (Raynaud and Pieau 1985, Pieau et al., 1994). Another common practice in turtle conservation programs, removing eggs from natural nests, is also not recommended. This practice can negatively affect egg and hatchling survivorship (Frazer 1992, Crouse 1999, Páez et al. 2015, Ahles and Milton 2016, Hart et al. 2016). However, the introduction of a large number of hatchling females (by artificially raising nest temperatures) may alter the genetic and ecological structure of the population (Mrosovsky and Godfrey 1995, Lovich 1996). This negative effect may be an issue in small, closed populations, but should not be noticeable in large, mobile populations such as *P. sextuberculata* in Mamirauá Reserve (1,124,000 ha; Girondot et al. 1998, Fachín-Téran et al. 2003). To achieve a balance between positive and negative effects and decisions on the scale of their female hatchling boosting program, wildlife managers should take into account species biology, local population size, and extent of distribution.

Turtles have a long reproductive lifespan, which means they are resilient to short-term high rates of hatchling mortality or skewed sex ratios (Janzen and Paukstis 1991, Hays et al. 2014). Reptiles with temperature-dependent sex determination can also evolve to avoid disproportional sex ratios (Bull et al. 1982, Janzen and Morjan 2001, Refsnider et al. 2014). Sexual bias could be corrected over decades by evolutionary and behavioral processes, such as changes in nesting timing and location, gradual change of the pivotal temperature and other temperature-dependent sex determination mechanisms (Mrosovsky 1994). However, many chelonian populations suffer from human harvest pressure, and consequently may have insufficient time or population variability to cope with a highly skewed sex ratio, leading ultimately to extinction. In such cases, as in the population described here, where adult males outnumber females, urgent actions are needed to reverse declines (Fachín-Téran et al. 2003).

As a result of community constraints, beaches in the Japurá area other than Pirapucú beach were not protected from harvest. Nests of *P. sextuberculata* are easy to find, and

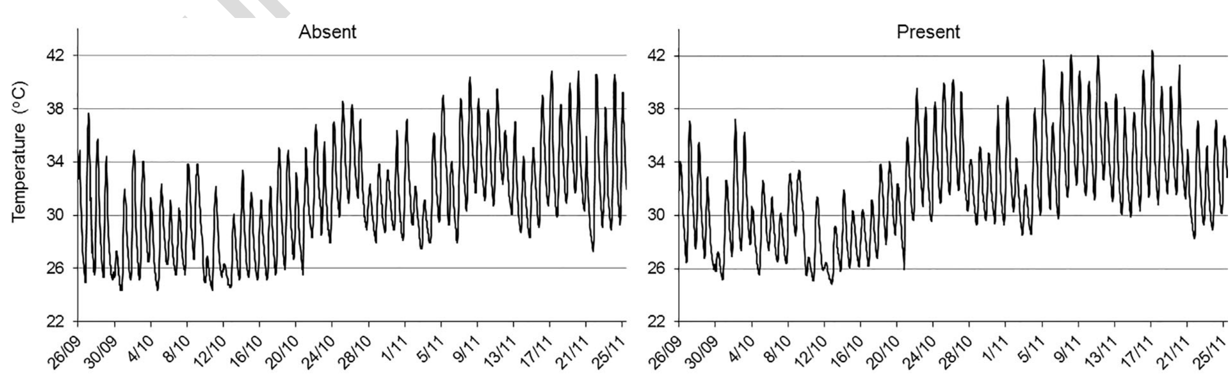


Figure 2. Hourly nest temperatures for *Podocnemis sextuberculata* nests with black plastic (present) or no cover (absent) from 26 September [20/09] to 25 November 2003 [25/11], at Pirapucú nesting beach, Amazon, Brazil.

mortality from human harvest was 100% outside the protected beach (Fachín-Téran et al. 2000). Protecting warmer beaches would potentially enhance production of females; however, this option was not feasible. In similar cases where management options are limited, the short-term manipulation of sex ratios could be a viable option (Wedekind 2012). This technique could also be useful where sandbanks are artificially shaded due to construction of houses or agricultural activities (Kolbe and Janzen 2002, Reid and Peery 2014).

In many cases, particularly with sea turtles, climate change might induce an opposite response to that reported here; that is, warmer temperatures producing populations highly skewed toward females (Hawkes et al. 2009, Tomillo et al. 2012, Booth et al. 2013, Marcovaldi et al. 2014). In such cases, males can be produced by cooling the substrate using water or shading the nest (Souza and Vogt 1994, Gomes and Ferreira-Júnior 2011, Jourdan and Fuentes 2015). Shading strategies can be applied for gregarious nesters, but providing artificial shade for dispersive nesters would require an excessively high level of resources and effort (Wood et al. 2014). Similarly, the effort involved in covering individual nests would only be justified in endangered populations with highly skewed sex ratios.

Effects on Nest Survivorship

We observed lower egg survivorship in covered nests. The main cause of increased nest mortality was probably maximum temperatures above the survivorship threshold. Dehydration may also be a factor because the black plastic may prevent rain from reaching the eggs, decreasing soil moisture and thereby increasing embryonic mortality rates (Hill et al. 2015). Turtle embryos in flexible-shelled eggs (such as *P. sextuberculata*) are particularly affected by hydric conditions during incubation (Bodensteiner et al. 2015). Embryos incubated in drier environments have lower rates of metabolism and growth (Janzen et al. 1990, Miller and Packard 1992), whereas well-hydrated hatchlings have greater survivorship (Packard 1999). However, the average apparent survivorship in covered nests was 80%, which is still relatively high in comparison with natural nest mortality reported for Amazonian riverine turtles in Mamirauá (Pezzuti and Vogt 1999) and other areas (Alho and Pádua 1982, Ferreira-Júnior and Castro 2010).

Although there are no data on survivorship of laboratory-incubated eggs for this species, we suggest that it is preferable to leave eggs *in situ*. It is likely that management techniques that involve egg manipulation will increase mortality. This is particularly true in remote areas (such as Mamirauá), where there are limited resources and personnel. Transport options are few and the nearest laboratory may be many hours or days away by boat. High rates of egg mortality is thus likely due to excessive disturbance. Wildlife managers should weigh the trade-offs between increased mortality and a greater production of females. In our particular case, females were increasingly rare in the population, and the use of black plastic would be justified. Regardless, nest temperature, egg mortality, and sex ratio should be monitored while using this technique.

On the other hand, increased nest temperature also accelerated embryonic development (Vogt and Bull 1982). Páez and Bock (1998) suggested the utilization of black plastic as a tactic to decrease the incubation period and consequently increase egg survivorship. Shortening the incubation period could be an important management tool in areas where the sudden rise of the water level is a common event and the main cause of egg mortality (Hildebrand et al. 1997, Eisemberg et al. 2016). Our results suggested that black plastic did accelerate embryonic development, reducing average incubation period by 3 days. However the effect of accelerating embryo development on hatchling fitness should be studied on the target species before this technique can be used to decrease incubation period (Booth 2006, 2017; Riley et al. 2014).

Turtle species vary in shapes and depths of nests (Morreale et al. 1982, Georges et al. 1994), which would influence the effectiveness of this technique. For example, egg mortality is likely to increase in shallow nests because heating is greater at the surface. On the other hand, sex ratio might not be affected in deep nests. Such nests have a temperature gradient, with warmer and variable temperatures near the surface, but cooler and more constant temperatures near the bottom (Valenzuela 2001).

We recognize the limitations of our study, which encompassed only one species in one area. However, these results were promising and suggest that this technique could be applied to other areas and species as a management tool for temperature-dependent sex determination species with male-biased populations. It is a relatively inexpensive method that is simple to implement and test experimentally. However, nest manipulation should not be seen as a long-term solution. The main priorities should be to prevent the major sources of decline, such as overharvest and habitat degradation.

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Summary for Online TOC: We tested a technique which could become an inexpensive, local alternative for short-term sex-ratio manipulation in endangered and male-biased populations of Amazon River turtles with temperature-dependent sex determination. *Podocnemis sextuberculata* nests covered with black plastic produced 37% more females, had a midnest average temperature 1° C higher and an incubation period 3 days shorter than control nests.