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Agricultural practices alter sex ratios in a reptile with environmental sex determination

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ABSTRACT

Anthropogenic changes to the environment have the opportunity to impact natural systems, particularly in organisms that exhibit phenotypic plasticity. Species with environmental sex determination (ESD) are uniquely susceptible to changes in the stimuli that affect sexual development, potentially leading to maladaptive sex ratios. We studied the factors affecting sex determination and sex ratios in the common snapping turtle, *Chelydra serpentina*, in an area heavily impacted by agricultural practices. We investigated the effects of soybean, corn, and sunflower planting on incubation temperatures, sex ratios, and depredation in naturally laid nests. We also identified and analyzed a novel mitochondrial microsatellite in order to examine the presence of natal homing and determine the likelihood that nest sites impacted by agricultural practices could be transmitted across generations. Females frequently chose to nest in agricultural fields over sand prairie sites, and offspring sex ratios and depredation rates were significantly influenced by crop planting. Despite detecting considerable genetic variation in our population, we found no relationship between relatedness and nesting location, suggesting that females are not transmitting nesting sites across generations. Our results suggest that agricultural practices can directly impact populations of animals with ESD, and will need to be considered in management decisions.

1. Introduction

In many animals, offspring sex is strongly influenced by environmental conditions experienced by the developing embryo, a phenomenon known as environmental sex determination (ESD). The common snapping turtle, *Chelydra serpentina*, exhibits temperature-dependent sex determination (TSD), whereby high and low temperatures result in females and intermediate temperatures produce males (Ewert and Nelson, 1991; Ewert et al., 1994). The sex of the embryo is determined during the middle third of the incubation period (Yntema, 1979), during which gonadagenesis is sensitive to changes in temperature. In turtles, the sensitivity of sex determination is impressive; a shift in constant incubation temperature as small as 0.3 °C can produce statistically detectable changes in offspring sex ratio (Bull et al., 1982).

The extreme sensitivity of turtle sex determination to environmental conditions has important implications for managing wild populations. Specifically, changes to the nesting environment could theoretically lead to biased population sex ratios, which in turn can lower effective population sizes. Female-biased sex ratios may also lead to higher rates of unfertilized clutches, and over time increase the potential for population declines. Models predict that the temperature increase expected to accompany global climate change may drive some populations of TSD turtles to extinction (Janzen, 1994; Hawkes et al., 2007). Sex ratios of turtles can also be altered by temperature changes resulting from more immediate human impacts such a landscaping (Kolbe and Janzen, 2002) and shade from buildings (Mrosovsky et al., 1995). ESD is taxonomically widely distributed in animals and characterizes many ecologically important species, including most turtles (Ewert and Nelson, 1991; Valenzuela 2004). Given that more than half of all assessed turtle species were officially listed as "globally threatened" on the 2009 IUCN Red List (Rhodin et al., 2009), understanding the effects of human impacts on the factors affecting their population survival will be critical to managing several vulnerable species.

Nest site choice in turtles may affect their ability to deal with anthropogenic influences on nesting habitats. Females appear to use overstory vegetational cover in determining where to construct a nest, a trait that ultimately has strong influences on nest temperatures and sex ratios (Janzen and Morjan, 2001). However, because female turtles abandon their nests once they lay their eggs, any changes in vegetational cover that occur after the nest has been constructed are not observed by the nesting female and cannot influence future nesting decisions. Agricultural sites often abut rivers, and thus turtles residing in rivers may commonly

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encounter agricultural fields when laying their nests on land. Because turtles in temperate regions generally lay their eggs in the spring, agricultural sites will initially appear to nesting turtles to be open and sparsely vegetated only to be covered by rapidly growing crops shortly after the nesting season commences. In addition, some turtles have been found to return to their natal nesting site to lay their eggs (Allard et al., 1994; Freedberg et al., 2005), and thus may transmit suboptimal nest sites to future generations. It is not clear if this natal homing behavior is a universal trait among turtles or is present only in some chelonian species.

To test for natal homing, many researchers have used genetic markers to determine if related females are nesting in close proximity to one another (Bowen et al., 1993; Allard et al., 1994; Bass et al., 1996; Dutton et al., 1999). Some studies examine genetic relatedness of nests using microsatellite analysis, i.e. polymorphisms of short tandem repeats located in the non-coding regions of DNA. Owing to their high mutation rates, microsatellites offer great intra-population variability (FitzSimmons et al., 1997; Freedberg et al., 2005), but are not ideal for investigating natal homing because the paternal genetic contribution may obscure any patterns of relatedness owing to maternal inheritance. Other studies examine variation in mitochondrial DNA (mtDNA) haplotypes, and therefore are valuable in that they are able to examine patterns arising only from maternal inheritance without the influence of paternal DNA (Allard et al., 1994). These studies are often limited by allelic diversity, as single nucleotide polymorphisms tend to show low variability on the population level. In addition, mtDNA evolves abnormally slowly in turtles relative to other animals (Avise et al., 1992), further limiting the utility of these markers. Although not previously attempted, using a mitochondrial microsatellite would be optimal for determining fine-scale relatedness of maternal lineages within a population. Mitochondrial microsatellites have been characterized in the Keeled box turtle (*Pyxidea mouhotii*; Zhang et al., 2008) and African helmeted turtle (*Pelomedusa subrufra*; Zardoya and Meyer, 1998). The microsatellite repeat is present in the control region in both species, although the microsatellite appears to be independently derived in the two unrelated lineages. Both species diverged from *Chelydra* at least 70 million years ago (Near et al., 2005), making the conservation of the microsatellite and its flanking primer sequences in snapping turtles unlikely.

The goal of the present study is to determine the factors affecting nest sex ratios in the snapping turtle, *C. serpentina*, in an area heavily impacted by agricultural practices. We assessed vegetational type and used global positioning system (GPS) to quantify the spatial distribution of nests. Combining these data with temperature and sex ratio data from each nest, we sought to determine how nest-site selection affected nest sex ratios in an agricultural/prairie landscape. We also identified a novel mitochondrial microsatellite that is both highly variable and maternally inherited, providing a uniquely powerful tool for examining natal homing. The results of the study may have important implications for managing turtle populations near agricultural sites.

2. Material and methods

In June of 2008, eighty snapping turtle nests were located and protected with wire cages in Kellogg, Minnesota. The nesting habitat consists of a 2 km stretch of agricultural fields and restored sand dune prairie adjacent to a backwater channel of the Mississippi River (Fig. 1). Each nest was monitored with a thermochron ibutton (Dallas Semiconductor), which was placed at the center of the top layer of eggs within each nest. The ibuttons were programmed to record temperatures every 30 min throughout the incubation period. After the initial pipping date for each nest was recorded, the temperature data from the middle third of the incubation period for each nest were used in further analyses. GPS coordinates were recorded for each nest using a Garmin handheld GPS unit. Just prior to the onset of the nesting season, three types of crops (soybean, corn, sunflower) were planted in separate fields in the nesting habitat (a small amount of sorghum was planted in the sunflower field as well; because sunflower was the predominant crop, we refer to this treatment as "sunflower"). The rest of the study site remained sand prairie and was characterized by a variety of prairie plants including little blue stem, big blue stem, goldenrod, brown headed bush clover, and purple cow vetch. Sixty days into the incubation period, after the thermosensitive period for sex determination had passed (Yntema, 1979), the eggs were collected and brought back to the lab to complete incubation at an average soil temperature of 25 °C. Five soil samples from ~15 cm beneath the soil surface were collected from each nesting site with a soil core sampler. Particle size was determined for each soil sample by sieve analysis at 600, 250, and 120 μm.

Three to four weeks after hatching, ten turtles from each clutch were randomly selected to be sacrificed and sexed macroscopically (In one nest, only eight turtles survived to hatch and all eight hatchlings were sexed.). Given the large size of snapping turtle clutches (see Section 3), the sacrificed turtles represent a small fraction of the turtles monitored, and underrepresent the number of turtles expected to succumb to depredation had nests not been protected from predation in this study. Using a dissecting microscope, females were confirmed by the presence of Müllerian ducts and ovaries with primary follicles; males all had testes and lacked Müllerian ducts. A subset of sexed turtles were blindly confirmed by another researcher. The remaining hatchlings were either released near their natal nests or were maintained in the turtle colony at St. Olaf College. Dissected turtles were stored in 95% denatured ethanol solution until DNA extraction. Up to three turtles from each nest were sampled for genetic analysis. DNA was extracted from tail tips using the Puregene Tissue Kit (Gentra Systems). DNA samples were stored at −20 °C prior to PCR (polymerase chain reaction).

2.1. DNA analysis

To measure genetic relatedness, primers for a ~400 base pair (bp) fragment from the mitochondrial control region were designed from *C. serpentina* sequences downloaded from Genbank. The fragment was chosen to capture a set of AT-rich short tandem repeats (STR) at the 3′ end of the control region, adjacent to the tRNA-Phe. The DNA fragment was amplified with PCR using the primers HVR-373F (5′-CCG AAT GGT CAC TTG CTT CT-3′) and HVR-748R (5′-ACT TCA GTG CCA TGC TTT GTA ATA-3′). The cycling thermal parameters used were as follows: 1 cycle at 94 °C (12 min) followed by 40 cycles at 94 °C (1 min), 51 °C (1 min), 72 °C (1 min), and a final cycle at 72 °C (4 min). Gel electrophoresis was used to confirm amplification. PCR product was sequenced at the University of Washington High-Throughput Genomics Unit in Seattle, Washington. Sequences for the first ~250 bp of the fragment were aligned and analyzed in Geneious (Biomatters Ltd.).

To examine repeat number in the HVR locus, the amplified fragment was also analyzed through fragment analysis. Fragment analysis was necessary because sequence chromatograms became unreadable after ~250 bp, and thus while sequencing revealed some variation in the sequence of the first several repeats, the total number of repeats could only be ascertained through fragment analysis. The DNA fragment was amplified with PCR using the primers HVR-373(Ned) and HVR-748R. PCR was run under the same protocol as above, except that the annealing temperature was raised to 51.5°. Samples were sent to the Plant Morphology Genomics Facility at Ohio State University in Columbus, OH for