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SHORT COMMUNICATION

Parentage assignment reveals multiple paternity in the critically-endangered Guatemalan beaded lizard (*Heloderma charlesbogerti*)

Brenna A. Levine[1,2] · Robert. L. Hill[3] · Joseph R. Mendelson III[3,4] · Warren Booth[1]

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Abstract
Within captive management programs for species of conservation concern, understanding the genetic mating system is of fundamental importance, given its role in generating and maintaining genetic diversity and promoting opportunities for sperm competition. If a goal of a conservation program is reintroduction, knowledge of the mating system may also inform prediction models aimed at understanding how genetic diversity may be spatially organized, thus informing decisions regarding where and which individuals should be released to maximize genetic diversity in the wild population. Within captive populations, such information may also influence how animals are maintained in order to promote natural behaviors. Here we investigate the genetic mating system of the Guatemalan beaded lizard, *Heloderma charlesbogerti*, a member of an entire clade lacking such information. A group of adult male and female *H. charlesbogerti* co-habited a large outdoor enclosure for five years during the species' perceived breeding season. Through genomic parentage analysis, 50% of clutches comprising multiple offspring were found to result from multiple paternity, with up to three males siring offspring within single clutches. Both males and females were observed to produce offspring with multiple partners within a given year. As such, within this captive environment, where opportunities existed for mating with multiple partners, the genetic mating system was found to be highly polygamous, with multiple paternity common within clutches. These findings are novel for the family Helodermatidae, and the results have broader implications about how reproductive opportunities should be managed within captive conservation programs.

Keywords Mating system · Polygamy · Conservation · Helodermatidae · Captive management

Introduction

Captive-breeding programs are crucial components of many species-conservation plans (Robert 2009), and the selection of individuals involved in breeding can have important consequences for the maintenance of genetic variation (e.g., inbreeding avoidance through pairing unrelated individuals; Frankham et al. 2010). Mating conditions for captive breeding can be informed by the wild behavior of the species in question, thereby improving the success of captive-breeding programs (Caro 1993) and minimizing mismatches in reproductive adaptations among captive and wild populations (Willoughby et al. 2015). However, the mating ecology of endangered species can be difficult to study in the wild, particularly if those species are also behaviorally cryptic.

The Guatemalan beaded lizard (*Heloderma charlesbogerti*, Campbell and Vannini 1988; Fig. 1) is endemic to the Motagua Valley in eastern Guatemala, but another population—potentially now extirpated—was known on the Pacific Versant of the country (Anzueto and Campbell 2010). The species is critically endangered as a result of illegal trade, local persecution, and habitat loss (Ariano 2006). However, it has not yet been evaluated by the IUCN Red List owing to their continued recognition of the taxon as a subspecies of the widespread *Heloderma horridum* (Reiserer et al. 2013).

✉ Warren Booth
warren-booth@utulsa.edu

1 Department of Biological Science, The University of Tulsa, Tulsa, OK, USA

2 Dorothy and George Hennings College of Science, Mathematics and Technology, Kean University, Union, NJ, USA

3 Zoo Atlanta, Department of Herpetology, 30312 Atlanta, GA, USA

4 School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA, USA

Fig. 1 The Guatemalan beaded lizard, *Heloderma charlsebogerti.* Photograph by Mike Kern

The taxonomy of Reiserer et al. (2013), adopted by Reptile Database (www.reptile-database.org), recognizes the species-level designation of *H. charlesbogerti*, and is followed here (See Douglas et al. 2010). The mating ecology of this species is poorly understudied due to its cryptic nature, where individuals live and likely mate in underground shelters (Ariano-Sanchez and Salazar 2015). In another helodermatid species, the Gila monster (*H.suspectum*), females have been observed visiting the shelters of multiple males during a single breeding season (typically April to June). Similarly, multiple males have been found to visit shelters used by lone females during a single breeding season (D. Beck, D. DeNardo, R. Repp, personal communications). Whether these visits result in successful copulations, and hence the impact of these on the parentage of resulting offspring, has not been documented (Beck 2005). Despite this, captive breeding programs rarely hold beaded lizards in group situations or introduce a female to multiple males during potential reproductive periods. Here, while reviewing the genetic relationships among members of the breeding colony of *H. charlesbogerti* maintained at Zoo Atlanta (Atlanta, Georgia, USA) in order to plan future pairings, we investigated parentage following the prolonged (~ 5 year) maintenance of this colony in a group setting.

Materials and methods

Captive rearing

Zoo Atlanta maintains a breeding colony of *H. charlesbogerti* that was founded by wild-caught individuals legally imported in the 1980 and 1990 s. From 2011 to 2016, a group of six adult males and five adult females (See Table 1;

Table 1 Parentage for 30 captive Guatemalan beaded lizard, *H. charlesbogerti*, offspring as assigned via genetic parentage analysis with 210 single nucleotide polymorphisms. Genetic maternity ('dam') and paternity ('sire') matched all known assignments, when available. Dashed lines indicate separations among clutches. Multiple paternity clutches identified in italics

Offspring	Year	Known Dam	Known Sire	Genetic Dam	Genetic Sire ID
GBL-11-R062	2006	GBL-A-06100	-	GBL-A-06100	GBL-A-06106
GBL-13-R027	2013	GBL-A-06100	-	GBL-A-06100	GBL-A-06109
GBL-13-R029	2013	GBL-A-06100	-	GBL-A-06100	GBL-A-06109
GBL-14-R023	2014	GBL-A-06100	-	GBL-A-06100	GBL-A-06109
GBL-16-R006	2016	GBL-A-06100	-	GBL-A-06100	GBL-A-06106
GBL-16-R008	2016	GBL-A-06100	-	GBL-A-06100	GBL-A-06106
GBL-17-R003	*2017*	*GBL-A-06100*	-	*GBL-A-06100*	*GBL-A-06104*
GBL-17-R004	*2017*	*GBL-A-06100*	-	*GBL-A-06100*	*GBL-A-06103*
GBL-17-R006	*2017*	*GBL-A-06100*	-	*GBL-A-06100*	*GBL-A-06104*
GBL-12-R009	2012	GBL-A-06105	-	GBL-A-06105	GBL-A-06104
GBL-16-R004	2016	GBL-A-06105	-	GBL-A-06105	GBL-A-06104
GBL-17-R008	*2017*	*GBL-A-06105*	-	*GBL-A-06105*	*GBL-A-06106*
GBL-17-R010	*2017*	*GBL-A-06105*	-	*GBL-A-06105*	*GBL-A-06106*
GBL-17-R011	*2017*	*GBL-A-06105*	-	*GBL-A-06105*	*GBL-A-06107*
GBL-17-R012	*2017*	*GBL-A-06105*	-	*GBL-A-06105*	*GBL-A-06103*
GBL-17-R013	*2017*	*GBL-A-06105*	-	*GBL-A-06105*	*GBL-A-06106*
GBL-18-R004	2018	GBL-A-06105	GBL-A-06106	GBL-A-06105	GBL-A-06106
GBL-18-R005	2018	GBL-A-06105	GBL-A-06106	GBL-A-06105	GBL-A-06106
GBL-18-R006	2018	GBL-A-06105	GBL-A-06106	GBL-A-06105	GBL-A-06106
GBL-A-96,101	2003	GBL-A-06110	GBL-A-06107	GBL-A-06110	GBL-A-06107
GBL-15-R046	2015	GBL-A-06110	-	GBL-A-06110	GBL-A-06106
GBL-16-R011	*2016*	*GBL-A-06110*	-	*GBL-A-06110*	*GBL-A-06109*
GBL-16-R012	*2016*	*GBL-A-06110*	-	*GBL-A-06110*	*GBL-A-06104*
GBL-17-R005	*2017*	*GBL-A-06110*	-	*GBL-A-06110*	*GBL-A-06104*
GBL-17-R007	*2017*	*GBL-A-06110*	-	*GBL-A-06110*	*GBL-A-06103*
GBL-17-R009	*2017*	*GBL-A-06110*	-	*GBL-A-06110*	*GBL-A-06104*
GBL-18-R007	2018	GBL-A-06110	GBL-A-06104	GBL-A-06110	GBL-A-06104
GBL-18-R008	2018	GBL-A-06110	GBL-A-06104	GBL-A-06110	GBL-A-06104
GBL-18-R009	2018	GBL-A-06110	GBL-A-06104	GBL-A-06110	GBL-A-06104
GBL-17-R015	2017	GBL-11-R062	-	GBL-11-R062	GBL-A-06109

note that male GBL-A-06108 and female GBL-A-96,101 did not produce offspring and are absent from the table) was maintained together in the Atlanta warm season (late April to early October) in an outdoor wood and mesh enclosure. Two of the adults, females GBL-11-R062 and GBL-A-96,101 were the mature offspring of other adults in the enclosure (Table 1). Beginning in 2017, the animals were placed in single male/female pairings for the outdoor summer season in separate smaller enclosures or not paired at all. The enclosure used between 2011 and 2016 was approximately 6 m x 5 m x 4 m (L x W x H) and contained multiple artificial burrows with buried plastic enclosures as refugia; the lizards additionally dug natural burrows. There were live plants, rotting logs, and branches to provide climbing structures. The enclosure was in nearly full sun throughout the season. Mating was occasionally observed; however, it was not possible to track all possible mating events among group members. Ample opportunities existed for females to mate with different males and males to mate with multiple females during this time. All animals were moved to individual indoor enclosures during the Atlanta cool season (late October through March). As oviposition occurred during this period, this allowed for unequivocal assignment of maternity for all offspring. Thirty offspring that hatched between 2003 and 2018 were included in this study – this number includes the 2 adult females that were the known offspring of other adults in the enclosure. Due to the nature of the group enclosure, paternity for most hatchlings was unknown except for 7 offspring for which paternity was known to result from single-mate pairings conducted in 2017 and 2018 (Table 1). Blood samples were collected from all individuals, including offspring resulting from matings between adults within the colony (30 offspring, including the two mature offspring of adults within the enclosure and nine adults).

Molecular methods and Bioinformatics

Whole genomic DNA was extracted from blood samples (N = 39) using a Qiagen DNeasy Blood and Tissue Kit (Qiagen), with concentrations of extracted DNA quantified using a Qubit 4 Fluorometer (Invitrogen™). DNA was prepared for high throughput, parallel sequencing via a ddRADseq library preparation protocol (Peterson et al. 2012), optimized for snakes by Levine et al. (2019).

FastQC (Andrews 2014) was used to inspect the raw fastq file for quality. The process_radtags module of program Stacks v. 2.41 (Catchen et al. 2011, 2013) was used to clean and demultiplexed the raw sequencing reads by barcode. Raw reads were clustered into loci using Stacks v. 2.41 following the *de novo* analysis pipeline described by Rochette and Catchen (2017), with core parameters optimized following identification of those at which the number of polymorphic loci shared by 80% of samples stabilized (i.e., $m = 3$, $M = 2$, $n = 2$). The populations module was then used to the retain the first single nucleotide polymorphism (SNP) at each locus (*--write_single_snp*) and present in all individuals ($r = 1.0$), and to produce an output file formatted for analysis with PLINK v. 1.9b (Purcell et al. 2007).

PLINK v. 1.9b (Purcell et al. 2007) was used to filter SNPs with departures from Hardy-Weinberg Equilibrium (*--hwe 0.05 midp*) and to minimize linkage disequilibrium (*--indep 50 5 2*) among the population founders (n = 9 adults, excluding 2 adult females from consideration as founders as they were mature offspring of other adults). Sequoia v. 1.3.3 (Huisman 2017) was then used to assign parentage to all offspring with the filtered SNP data set, with maximum number of sibship iterations (*MaxSibIter*) set to five and genotyping error rate (*Err*) set to 0.05. Importantly, Sequoia v. 1.3.3 facilitates multigenerational pedigree reconstruction and can therefore accommodate the fact that two females were the adult offspring of other adults in the enclosure but also had the potential to be parents themselves. To illustrate, this is the case for individual GBL-11-R062; this female is the offspring of other adults in the enclosure but produced offspring herself in 2017. Sequoia v. 1.3.3 accomplishes this by allowing the user to specify generations. In this sense, the original adults were classified as generation one, the offspring of adults that became adults themselves were classified as generation two, and all other offspring were classified as generation three. Although maternities of all offspring and paternities of seven offspring were known *a priori* (because they originated from known single male–single female pairings), genetic parentage assignment was performed blind to all known relationships. This allowed us to compare inferred assignments to known assignment to assay for errors and assess confidence in inferred parentage assignments.

Results

The populations module in Stacks v. 2.41 identified 3,094 SNPs present in each individual at a mean coverage = 30.6x. After filtering with PLINK v. 1.9b, 210 SNPs were retained that were optimal for parentage analysis with Sequoia v. 1.3.3. The reduction observed here is likely the result of having few founding individuals with which linkage disequilibrium and Hardy-Weinberg equilibrium could be calculated. A comparable reduction has been reported in other studies (e.g., Levine et al. 2019). Paternity and maternity were assigned to all offspring; known maternity and paternity matched genetic assignments in all cases (Table 1).

Of 15 sets of clutch-mates, 4 displayed multiple paternity (~27%; Table 1). The number of sires represented in these clutches ranged from 2 (in a clutch of 2 offspring) to 3 (in a clutch of 5 offspring) (See Table 1). When only considering those clutches for which multiple paternity was definitively possible (i.e., >1 offspring hatched, group mating environment; N = 6), ~67% displayed multiple paternity. Importantly, clutch size is defined here as the number of offspring that successfully developed and hatched from eggs and excludes any eggs that failed to hatch. Note that based on captive data, clutch size in *H. charlesbogerti* ranges from 1 to 11 (Otsuka et al. 2020). Therefore, the frequency of multiple paternity reported here may be an underestimate if males sired offspring that failed to hatch or offspring that died prior to sampling. Records of eggs that failed to hatch or offspring that died prior to sampling are not available. Females which produced clutches over several years were found to exhibit serial polyandry (i.e., mating with and producing offspring with multiple males). Two females were found to produce offspring with four males during the period of the group housing and one female produced offspring with five males (Table 1). Within single years, males were found to produce offspring with several females. Finally, several males produced offspring in multiple years.

Discussion

This study is the first to report multiple paternity in captive Guatemalan beaded lizards, *H. charlesbogerti*, and represents the first record in the family Helodermatidae. We show that females will produce offspring with multiple males across seasons, and that males will sire offspring from multiple females within and across years that were sampled. Hence, when housed as a group in a large outdoor enclosure, both sexes of *H. charlesbogerti* exhibit multiple mating behavior resulting in multiple paternity. Although novel, the capacity for multiple paternity in this species is unsurprising given the high incidence of multiple paternity in non-avian reptiles; indeed, multiple paternity has been reported across many non-avian reptile taxa (Schuett 1992; Uller and Olsson 2008; Jellen and Aldridge 2011) and is common outside of Reptilia (reviewed in Taylor et al. 2014]). Nonetheless, these findings have important consequences for the management of captive-breeding programs at zoological facilities and private collections, where mating among captive lizards is often promoted through the pairing of single males and females. However, by allowing multiple males to participate in reproduction, multiple paternity may elevate within-clutch genetic diversity among offspring, thereby contributing to the maintenance of genetic variation in this species (Uller and Olsson 2008; Taylor et al. 2014) and potentially allowing for cryptic female choice of sperm potentially with higher quality mates. Multiple paternity may also minimize the potentially deleterious effects of inbreeding (Tregenza and Wedell 2002). While females were observed to exhibit serial polyandry, females were also found to produce offspring with the same male across seasons, either in single- or multiple- paternity clutches. Here, it was not possible to determine whether these resulted from long-term sperm storage (LTSS) across seasons; however, LTSS has been reported in other reptiles, with the production of viable offspring resulting from the storage of sperm for up to six years (Schuett 1992; Booth and Schuett 2011; Levine et al. 2021). At Zoo Atlanta, eggs laid by female *H. charlesbogerti* that had been with males in previous years but not housed with males the following year have not provided evidence of LTSS, as in all cases eggs failed to show signs of development. Both within and across years, males mated with multiple females. Overall, under this captive environment, both male and female *H. charlesbogerti* mated with multiple partners and multiple paternity was common in clutches.

Regardless of its evolutionary significance in natural populations, multiple mating and multiple paternity within a captive environment reflects a previously unknown aspect of the breeding biology of *H. charlesbogerti*. Whether either should be promoted in captivity is subject to debate. When developing breeding programs for species of conservation concern, pairings should be strategically planned in order to minimize inbreeding and prevent a bias of offspring sired by single or a few males. Although multiple mating has not been documented in wild Helodermatid lizards (Beck 2005), anecdotal observations of a closely related species (*H. suspectum*) indicate the potential for it (D. Beck, D. DeNardo, R. Repp, personal communications). As such, efforts should be directed towards understanding the significance of multiple matings and multiple paternity within wild populations of Helodermatid lizards, including the Guatemalan beaded lizards, and address the potential implications for the conservation and management of natural populations and captive colonies. Furthermore, the possibility of LTSS should be studied within captive colonies given the significant implications for captive management.

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Authors' contribution WB, RLH, and JRM conceived the study. RLH and JRM maintained the animals and collected blood samples, BAL conducted the laboratory work and analyzed the data, BAL, RLH, JRM, and WB wrote the final manuscript. All authors were involved in writing and data interpretation and read and approved the final manu-

script.

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Availability of data and material Genomic data is available at XXXX.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval The Scientific Review Committee of Zoo Atlanta approved this study on 29 November 2018.

Consent to participate Not applicable.

Consent for publication Not applicable.

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