

Parentage Assignment Reveals Multiple Paternity in the Critically-Endangered Guatemalan Beaded Lizard (*Heloderma Charlesbogerti*)

Brenna A Levine

Kean University

Robert Hill

Zoo Atlanta

Joseph Mendelson

Zoo Atlanta

Warren Booth (✉ warren-booth@utulsa.edu)

University of Tulsa <https://orcid.org/0000-0003-2355-0702>

Short Report

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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 in order 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 a genus lacking such information. A group of adult male and female *H. charlesbogerti* were co-habited for five years during the species perceived breeding season. Through genomic parentage analysis, 50% of clutches comprising multiple offspring were found to result from polyandry, with up to three males siring offspring within single clutches. Furthermore, males were found to be polygamous both within and across seasons, and females would exhibit promiscuity across seasons. As such, within this captive environment, where opportunities existed for mating with multiple sexual partners, the genetic mating system was found to be highly promiscuous, 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.

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 considered to be 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). The taxonomy of Reiserer et al. (2013), which recognizes the species level designation of *H. charlesbogerti*, is followed here. 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). Among other

helodermatid species, females have been observed visiting the shelters of multiple males during a single breeding season, and multiple males have been found to visit the shelter of single females (D. Beck, D. DiNardo, personal communications), however 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 species 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 1980s and 1990s. From 2011–2016, a group of six adult males and five adult females was maintained together in the Atlanta warm season (late April to early October) in an outdoor wood and mesh enclosure. The enclosure was approximately 6m x 5m x 4m (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 a variety of branching 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 during this time. All animals were moved to individual indoor housing during the Atlanta cool season. This allowed the maternity of all offspring to be known as all eggs were laid during the cool season. Thirty offspring hatched between 2006 and 2018 were included in this study. Due to the nature of the group enclosure, paternity for hatchlings was unknown. Blood samples were collected from all hatchlings (N = 30) and adults (N = 11) in the Zoo Atlanta collection at the time of sampling.

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 ddRAD 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) cleaned 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., $m = 3$, $M = 2$, $m = 2$). The populations module was then used to 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 adults that 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. Although maternities of all offspring and paternities of seven offspring were known *a priori*, genetic parentage assignment was performed blind to all known relationships so as to assess confidence in Sequoia 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. Paternity and maternity were assigned to all offspring; known maternity and paternity matched genetic assignments in all cases (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-12-R009	2012	GBL-A-06105	-	GBL-A-06105	GBL-A-06104
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-15-R046	2015	GBL-A-06110	-	GBL-A-06110	GBL-A-06106
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-16-R004	2016	GBL-A-06105	-	GBL-A-06105	GBL-A-06104
<i>GBL-16-R011</i>	<i>2016</i>	<i>GBL-A-06110</i>	-	<i>GBL-A-06110</i>	<i>GBL-A-06109</i>
<i>GBL-16-R012</i>	<i>2016</i>	<i>GBL-A-06110</i>	-	<i>GBL-A-06110</i>	<i>GBL-A-06104</i>
GBL-17-R015	2017	GBL-11-R062	-	GBL-11-R062	GBL-A-06109
<i>GBL-17-R003</i>	<i>2017</i>	<i>GBL-A-06100</i>	-	<i>GBL-A-06100</i>	<i>GBL-A-06104</i>
<i>GBL-17-R004</i>	<i>2017</i>	<i>GBL-A-06100</i>	-	<i>GBL-A-06100</i>	<i>GBL-A-06103</i>
<i>GBL-17-R006</i>	<i>2017</i>	<i>GBL-A-06100</i>	-	<i>GBL-A-06100</i>	<i>GBL-A-06104</i>
<i>GBL-17-R008</i>	<i>2017</i>	<i>GBL-A-06105</i>	-	<i>GBL-A-06105</i>	<i>GBL-A-06106</i>
<i>GBL-17-R010</i>	<i>2017</i>	<i>GBL-A-06105</i>	-	<i>GBL-A-06105</i>	<i>GBL-A-06106</i>
<i>GBL-17-R011</i>	<i>2017</i>	<i>GBL-A-06105</i>	-	<i>GBL-A-06105</i>	<i>GBL-A-06107</i>
<i>GBL-17-R012</i>	<i>2017</i>	<i>GBL-A-06105</i>	-	<i>GBL-A-06105</i>	<i>GBL-A-06103</i>
<i>GBL-17-R013</i>	<i>2017</i>	<i>GBL-A-06105</i>	-	<i>GBL-A-06105</i>	<i>GBL-A-06106</i>
<i>GBL-17-R005</i>	<i>2017</i>	<i>GBL-A-06110</i>	-	<i>GBL-A-06110</i>	<i>GBL-A-06104</i>
<i>GBL-17-R007</i>	<i>2017</i>	<i>GBL-A-06110</i>	-	<i>GBL-A-06110</i>	<i>GBL-A-06103</i>
<i>GBL-17-R009</i>	<i>2017</i>	<i>GBL-A-06110</i>	-	<i>GBL-A-06110</i>	<i>GBL-A-06104</i>
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

Offspring	Year	Known Dam	Known Sire	Genetic Dam	Genetic Sire ID
GBL-18-R006	2018	GBL-A-06105	GBL-A-06106	GBL-A-06105	GBL-A-06106
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-A-96101	-	GBL-A-06110	GBL-A-06107	GBL-A-06110	GBL-A-06107

Of 15 sets of clutch-mates, 4 displayed multiple paternity (~ 27%; Table 1). The number of sires represented in these clutches ranged from two (in a clutch of two offspring) to three (in a clutch of five offspring). 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. 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. Females which produced clutches over several years were found to exhibit serial polyandry. 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. Within single years, males were found to be polygamous, with multiple males siring the offspring of several females.

Discussion

To our knowledge, this study is the first to report multiple paternity in the Guatemalan beaded lizard, *H. charlesbogerti*, and represents the first record in the family Helodermatidae. Furthermore, we show that females will produce offspring with multiple males across seasons, and that males will sire offspring from multiple females within and across seasons. Hence, the mating system of this species when housed in a group is highly promiscuous with within season polyandry. Although novel, the capacity for multiple paternity in this species is unsurprising given the high incidence of multiple paternity in reptiles; indeed, multiple paternity has been reported across many reptile taxa (Uller and Olsson 2008) 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 and allow for cryptic female choice of sperm potentially with higher genetic quality, thereby contributing to the maintenance of genetic variation in this species (Uller and Olsson 2008, Taylor et al. 2014). If indicative of the natural mating system, 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. It was not

possible here 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 (Booth & Schuett, 2011; Levine et al. 2021). Observations at Zoo Atlanta of eggs laid by females that had been with males in previous seasons but not housed with males in following seasons have not provided evidence of LTSS as in all cases eggs failed to show signs of development. Both within and across seasons, males were found to be polygamous. Overall, under this captive environment, *H. charlesbogerti* was found to be highly promiscuous, with multiple paternity common within clutches.

Regardless of the evolutionary significance, multiple paternity and a promiscuous mating system within a captive environment may reflect a previously unknown aspect of the natural mating system of this, and other helodermatid, species, that should be promoted in captivity. It should be cautioned however, that 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 never been observed in the wild in helodermatids (Beck 2005), anecdotal observations indicate the potential for it. As such, efforts should be directed towards understanding the significance of promiscuity 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, within captive colonies, the existence of long-term sperm storage should be studied, given the significant implications for captive management.

Declarations

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

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

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 manuscript.

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

Supplementary Information The online version contains supplementary material available at...

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Figures



Figure 1

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