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BRIEF REPORT

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Female long-term sperm storage results in viable offspring in the Himalayan Mountain Pitviper, *Ovophis monticola*

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Abstract

The ability of females to store sperm for extended periods in their reproductive tracts (termed long-term sperm storage, LTSS) has been reported across a diversity of vertebrate taxa. The evolutionary, ecological, and physiological significance of LTSS is wide-ranging and includes the ability to produce offspring when mates may be temporally scarce by way of decoupling copulation from ovulation, inbreeding avoidance, and the generation and maintenance of genetic diversity in progeny. Among vertebrate lineages, nonavian reptiles exhibit a remarkable capacity for LTSS, with the production of viable offspring reported after periods exceeding 6 years since prior contact with a potential mate. Given that female reptiles are able to store viable sperm for prolonged periods, it is important to disentangle LTSS from that of facultative parthenogenesis (FP), a reproductive trait which appears widespread among all reptile lineages. The implications of this distinction are particularly important in the context of the development and management of captive breeding programs. To accurately determine between the two reproductive strategies, genomic screening is highly recommended. Following a period of isolation for 13 months from a potential male mate, a female Himalayan Mountain Pitviper (*Ovophis monticola*) produced a clutch of three male offspring. Here, through genome-scale analyses of the female and her progeny, we document the first record of LTSS in this genus and exclude FP as the alternative hypothesis.

KEYWORDS

reproduction, reproductive strategy, snake, Viperidae

1 | INTRODUCTION

The production of offspring by female vertebrates following prolonged periods of isolation from potential mates has been documented across a variety of taxonomic groups (Bernal et al., 2014; Holt & Lloyd, 2010; Pearse, 2001; Schuett, 1992; Schuett & Gillingham, 1986). Mechanistically, this may be attributed to facultative parthenogenesis (FP) or to female long-term sperm storage (LTSS) (Booth & Schuett, 2011, 2016). Among vertebrates, the nonavian reptiles (i.e., Chelonia, Crocodylia, Rhynchocephalia, and Squamata) in particular show a remarkable proclivity for LTSS, with the production of viable offspring following nearly 6 years of isolation from potential mates documented in the Western Diamond-backed Rattlesnake, *Crotalus atrox* (Levine et al., 2021). This case, confirmed through DNA analyses, may represent the the upper-limit for LTSS in vertebrates. Understanding the mating system of a species has important implications for long-term captive management (Levine et al., 2022); thus, it is important to accurately disentangle LTSS from FP. Fortunately, this can be achieved with relative ease through genome-scale molecular sequencing and bioinformatic analyses.

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Here, we provide the first documentation of LTSS in a female Himalayan Mountain Pitviper, *Ovophis monticola*. Following isolation from a male in May 2015, a female *O. monticola* produced viable clutches in both 2015 and 2016. While the clutch in 2015 comprised both males and females, supporting sexual reproduction, the period of isolation from the male and the production of only male offspring in the 2016 clutch presented the potential for FP (e.g., Booth & Schuett, 2011, 2016; Schuett et al., 1997). Like other members of Viperidae (Graves, 2015), it is assumed that *O. monticola* exhibits ZZ|ZW sex chromosomes, consequently parthenogens will be male, ZZ (Booth & Schuett, 2016). Following high throughput reduced representation genomic sequencing, we identify heterozygosity in the offspring at loci homozygous in the mother, indicating the presence of paternal alleles and hence supporting LTSS and rejecting FP.

Research Highlights

- A female mountain pitviper produced three male offspring after 13 months of isolation.
- Genome-scale analyses confirm long-term sperm storage as the mechanism, rejecting the alternative strategy of facultative parthenogenesis.

2 | METHODS

2.1 | Study system

The Himalayan Mountain Pitviper (*O. monticola*) is a high-altitude, wide-ranging Asiatic pitviper (Figure 1). These stout-bodied snakes occupy terrestrial habitats and are primarily nocturnal. Diet of adults is chiefly small mammals. This species is sexually dimorphic with females larger. Males can attain a total length up to 49 cm, while the total length in females is up to 110 cm (Cox et al., 2018). Females are considered sexually mature at 3 years old. *O. monticola* is oviparous, with females producing 5–11 eggs per clutch which they guard for ~2 months until hatching (Orlov et al., 2002). It should be noted that within the collection at Denver Zoo, clutch sizes ranged from 4 to 6, based on six clutches from three different females.

2.2 | Captive rearing

At the Denver Zoo, adult *O. monticola* were housed individually in enclosures with 0.61 to 1.22 sq meters of floor space. Substrate comprised of a 50/50 mix of cypress mulch and sphagnum moss that was kept damp. Relative humidity within the enclosure ranged from 60% to 80%, and ambient temperature ranged from 16.7°C to 19.4°C. A basking site of ~37.8°C was offered. In late March 2015 after ~2.5 months of a cooling period at 10°C, the adult male was introduced into the enclosure of the adult female for breeding. The pairing lasted for 2 weeks followed by separation and reintroduction of the same male in May for a second 2-week period. The female was never exposed to additional males. Copulation was not observed and likely occurred during evening hours. Four eggs were laid on July 17, 2015. Following incubation at 22.2°C, three eggs hatched (one male, two females) after 57 days. The fourth egg contained a stillborn fetus

FIGURE 1 Adult Himalayan Mountain Pitviper, *Ovophis monticola*. Photo credit H. Creamer, Denver Zoo. [Color figure can be viewed at wileyonlinelibrary.com]

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(sex unknown). The adult female was not cooled the following winter (2016) nor introduced again to a male. On June 27, 2016 the female was found guarding three eggs. From these, three males hatched (OS-1, OS-2, and OS-3). Given that the female had not been introduced to a male since May 2015, and that all offspring were males, FP was considered a possible hypothesis to test. Blood from the mother and all progeny ($n = 3$) from 2016 was collected from the tail vein using a 1 mL heparinized syringe with a 25 g needle and immediately stored at −80°C until analysis.

2.3 | Molecular methods and bioinformatics

DNA was extracted from blood samples collected from the adult female and the three candidate parthenogenetic male offspring (OS-1–OS-3) using a Qiagen DNeasy Blood and Tissue Kit (Qiagen). DNA was then prepared for double-digest restriction site associated DNA sequencing (ddRADseq; Peterson et al., 2012) following the modified protocol of Levine et al. (2019). The ddRADseq library was 100-bp single-end sequenced by the University of Oregon (GC3F) on a single lane of an Ilumina HiSeq. 4000.

Fastq files were demultiplexed by index and barcode using program *Stacks v. 2.41* (Catchen et al., 2011). Core parameters for clustering raw reads into loci with Stacks v. 2.41 were selected by implementing the *r80* method described by Rochette and Catchen (2017). Loci were clustered using the optimized parameter settings ($m = 3$, $M = 3$, $n = 3$), and the *populations* module was executed to only retain the first SNP at each locus (*–write_single_snp*) that was present in the mother and all three male offspring (*–r = 1.0*). Further, the *populations* module tested these SNPs for departures from Hardy-Weinberg Equilibrium (*–hwe*). SNPs with significant deviations from HWE ($n = 588$) were written to a blacklist, and *populations* was re-run as before to exclude the blacklisted SNPs. SNPs ($n = 9697$) for each individual were exported as a *Structure* file.

The *Structure* file was converted to the input format for *ParthenoGenius* (Levine & Booth, 2023; https://github.com/brenna-levine/LEVINE_ParthenoGenius) by removing the *pop_flag* column and converting the text file to a comma-separated format. Briefly, *ParthenoGenius* tests for likely parthenogenetic origin of offspring by comparing the homozygous genotypes of the mother against the genotypes of the offspring at these SNPs, using a per-locus genotyping error rate to gauge whether offspring alleles that differ from the mother's alleles at maternal homozygous loci are more likely due to genotyping error or paternal contribution. The *PG_file_prep.sh* script was used to generate individual input files for each mother-offspring pair. The *PG_wrapper.sh* script was then run to execute *ParthenoGenius* on each input file, with the per-base genotyping error rate set to 0.01 (*–error 0.01*).

3 | RESULTS

Analytic output from ParthenoGenius (Levin & Booth, 2023) supported LTSS and not FP for the 2016 clutch of three male offspring. The mother was homozygous at 4087 of the 9697 SNP loci. The three male offspring displayed heterozygous genotypes, resulting from paternal alleles, at 31%–39% of these loci. This greatly exceeds the proportion of maternally homozygous loci expected to be heterozygous based on genotyping error alone (i.e., the proportion expected if these offspring were parthenogens; Table 1). Given these results, LTSS is therefore supported as the reproductive mechanism.

TABLE 1 *ParthenoGenius* did not support a parthenogenetic origin for three male Himalayan Mountain Pitviper, *Ovophis monticola* offspring of known maternity.

Offspring ID	# Offspring het loci (E)	# Offspring het loci (O)	Proportion offspring het loci (O)
OS-1	40.87	1272	0.31
OS-2	40.87	1601	0.39
OS-3	40.87	1529	0.37

Note: ID number of each male offspring = Offspring ID. Number of mother's homozygous SNPs at which the offspring was expected to display at least one allele different from the mother's given effects of genotyping error alone = # Offspring het loci (E). Number of mother's homozygous SNPs at which the offspring was observed to display at least one allele different from the mother's alleles = # Offspring het loci (O). Proportion of mother's homozygous SNPs at which the offspring was observed to display at least one allele different from the mother's alleles = Proportion offspring het loci (O).

4 | DISCUSSION

Following genomic screening, the results of our analyses robustly support LTSS as the mechanism responsible for the three male offspring produced in 2016. Isolation from the male was ca. 13 months. With the identification of heterozygosity in the offspring ranging from 31% to 39% at maternally homozygous loci, FP was unequivocally rejected.

Factors that supported LTSS over FP before genetic screening include the absence of large numbers of infertile ova and the viability of the male offspring produced. With ZZ | ZW systems, males are ZZ and females are ZW. As FP in snakes results from the fusion of the egg nucleus with the second polar body through the process of terminal fusion automixis (Card et al., 2021), the offspring will exhibit either ZZ or WW sex chromosomes. The latter combination is considered nonviable in snakes (Booth & Schuett, 2016) and is reflected in FP litters or clutches as infertile ova (Schuett et al., 1997). Given that we expect Z and W female gametes to be produced in equal ratios, we would therefore expect to see multiple infertile ova in an FP event (Booth et al., 2012). Additionally, in snakes with ZZ | ZW sex chromosomes, it is not uncommon for males produced through FP to be stillborn, and/or to exhibit developmental abnormalities which may include postcranial and brain case deformities (Booth & Schuett, 2016; Card et al., 2021; Reynolds et al., 2012; Schuett et al., 1997). In contrast, litters or clutches produced through

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LTSS will be indistinguishable from those that are sexually produced (Booth & Schuett, 2011; Levine et al., 2021).

Despite the fact that the duration of LTSS reported here is not as extensive as that reported in *Crotalus atrox* (Levine et al., 2021), the ability to store sperm over successive seasons may result in sperm competition and multiple paternity under captive conditions where females are paired and copulate with different males (Schuett & Gillingham, 1986). Consequently, unless molecular DNA testing is performed, designation of paternity of progeny is highly likely to be inaccurate. In captive breeding programs pairing females with different males over time is not an uncommon practice as it purports to maintain genetic diversity in captive colonies (Levine & Booth, 2023).

Evolutionarily, a female's ability to store and utilize sperm across multiple reproductive events offers numerous reproductive advantages that influence fitness. These include maximizing offspring genetic diversity (Booth et al., 2007; Calsbeek et al., 2007), decoupling the timing between copulation and ovulation (Shine, 2003), inbreeding avoidance (Tregenza & Wedell, 2002), and the promotion of opportunities for sperm competition (Birkhead, 1987; Clark et al., 2014; Smith, 2012). Given the theoretic and applied significance of LTSS, it is evident that captive management programs must consider the potential for this phenomenon when developing future breeding plans for species where it is known to be common. Furthermore, given its occurrence in multiple species of Old- and New World pitvipers, these taxa likely represent ideal model systems that should be explored when investigating the biological mechanisms which store viable sperm under noncryogenic conditions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw genomic data are available at request from Dr. Brenna Levine (brlevine@kean.edu). Input files for the *ParthenoGenius* run and all output files are available at https://github.com/brenna-levine/LEVINE_ParthenoGenius.

ETHICS STATEMENT

Samples were collected with the approval of Denver Zoo, protocol DZ#2018-006.

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