



Molecular genetic evidence for alternative reproductive strategies in North American pitvipers (Serpentes: Viperidae): long-term sperm storage and facultative parthenogenesis

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The first documentation of facultative parthenogenesis (FP) in non-avian reptiles (snakes) occurred in 1997, following the application of molecular genetic methods for parentage analysis, and since has been described in only four additional snake species. Here, in two species of live-bearing (viviparous) North American pitviper snakes, using microsatellite DNA fingerprinting, we describe the first record of a virgin birth by FP in the copperhead (*Agkistrodon contortrix*), and the first genetically confirmed case of long-term sperm storage (LTSS) of exceptional duration (5 years) in the eastern diamond-backed rattlesnake (*Crotalus adamanteus*). The capacity of female squamate reptiles (snakes, lizards, and amphisbaenians) to store viable sperm for extended periods of time is a competing hypothesis to FP, and it is often inferred when interpreting certain births where adult females have been isolated from adult male mates for prolonged periods. On reviewing the literature of certain cases of LTSS in snakes, we provide compelling support for the prevalence of FP, casting doubt over the widespread acceptance of LTSS. Accordingly, with the discovery of FP in squamates and the results of the present study, we advocate that in order to differentiate between LTSS and FP, especially under natural conditions, rigorous molecular testing will be required. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, 104, 934–942.

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INTRODUCTION

The ability of females to store viable spermatozoa within the reproductive tract that may be utilized months or even years after insemination has been reported in a variety of taxonomic groups (Pearse & Avise, 2001; Holt & Lloyd, 2010). Across these groups, durations over which sperm is stored prior to fertilization appear to vary greatly. In female mammals, this may range from a few hours to several days, but up to months in bats (Birkhead & Møller, 1993); however, in ectothermic vertebrates, such as reptiles (e.g. turtles, lizards, and snakes), this is significantly

prolonged, with reports ranging from months to years (reviewed by Saint Girons, 1975; Devine, 1984; Gist & Jones, 1987; Schuett, 1992; Olsson & Madsen, 1998; Sever & Hamlett, 2002; Holt & Lloyd, 2010). Despite numerous reports of such extreme storage durations, only in a handful of chelonian (turtles) species have genetic markers been applied to conclusively confirm the presence of a paternal genotype in the resulting offspring (Pearse & Avise, 2001; Pearse, Janzen & Avise, 2001; Roques, Diaz-Paniagua & Andreu, 2004; Johnston *et al.*, 2006; Roques, Diaz-Paniagua & Andreu, 2004).

A competing reproductive strategy to long-term sperm storage (LTSS) that explains the production of offspring, typically after prolonged periods in the

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absence of males, is facultative parthenogenesis (FP), which is the ability to produce offspring lacking any paternal genetic contribution (Olsen & Marsden, 1954; Mogie, 1986; Avise, 2008; Lampert, 2008). Following the application of molecular genetic analyses, FP has been documented in a variety of squamate reptiles (lizards, snakes, and amphisbaenians), but primarily in snakes (Schuett *et al.*, 1997, 1998; Groot, Bruins & Breeuwer, 2003; Booth *et al.*, 2011a, b) and varanid lizards (Lenk *et al.*, 2005; Watts *et al.*, 2006). Although only approximately 0.6% of squamates have been shown to be capable of parthenogenetic reproduction (Kearney *et al.*, 2009), in light of these recent findings it appears that parthenogenesis may, in fact, be an important aspect of vertebrate evolution and not the evolutionary novelty it was once considered (reviewed by Neaves & Baumann, 2011).

In the squamate reptiles, parthenogenesis has most frequently been described in snakes, and within this group, all of which exhibit ZZ:ZW genetic sex determination, both the parthenogenetic mode and the reproductive outcomes have proven exceptionally diverse. In advanced snakes (Caenophidia), a group containing the majority of extant snake species, all parthenogenetic litters have resulted in the production of males (ZZ) (Schuett *et al.*, 1997), presumably by way of automictic terminal fusion of the post-meiotic products (i.e. reduced ovum and second polar body). The resulting male offspring represent half-clones of the mother and are therefore not identical. In comparison, parthenogenesis in the Pythonidae has produced female (ZW) embryos that retained maternal heterozygosity (Groot *et al.*, 2003). The authors attributed this finding to the parthenogenetic mechanisms of pre-meiotic doubling, apomixes, or central fusion automixis, but were unable to determine which. Contrasting further, Booth *et al.* (2011a, b) identified multiple, viable female (WW) parthenogenetic offspring in two species of viviparous boid snakes, the boa constrictor (*Boa constrictor imperator*) and rainbow boa (*Epicrates maurus*). In these instances each female exhibited elevated homozygosity relative to the mother. These offspring, presumably produced through terminal fusion automixis, essentially confound decades of research on vertebrate reproductive systems that considered WW embryos to be non-viable (Olsen & Marsden, 1954; Schuett *et al.*, 1997; Groot *et al.*, 2003; Watts *et al.*, 2006). Avise (2008) and Lampert (2008) provide general reviews of parthenogenetic mechanisms in vertebrates.

As the phenomenon of FP (i.e. the ability of a species to reproduce both with and without males) has become better understood, the number of FP cases has increased, especially in light of technological advancement in molecular tools and their ease of application (Lampert, 2008; Neaves & Baumann,

2011). Of the molecular markers currently available, microsatellite DNA loci have proven highly suited to studies of parthenogenesis, parentage, and the determination of LTSS (Fitzsimmons, 1998; Pearse & Avise, 2001; Pearse *et al.*, 2001; Watts *et al.*, 2006; Booth *et al.*, 2007, 2011a, b; Chapman *et al.*, 2007; Chapman, Firchau & Shivji, 2008; Feldheim *et al.*, 2010).

Here, for the first time, following the application of microsatellite DNA fingerprinting, we provide molecular evidence for alternative reproductive strategies in two North American pitviper snakes: FP in a virgin copperhead (*Agkistrodon contortrix*) and LTSS in the eastern diamond-backed rattlesnake (*Crotalus adamanteus*). To our knowledge, the latter represents the first molecular genetic confirmation of LTSS in any snake species. Furthermore, females of both species are capable of LTSS (Schuett & Gillingham, 1986; Schuett, 1992; Hoss *et al.*, 2011), and in some populations it is an obligate component (Schuett, 1992; Smith *et al.*, 2009, 2010; Hoss *et al.*, 2011). In comparing the reproductive outcome of these two litters, as well as those of other species for which FP has been genetically confirmed, a number of characteristics/traits have become evident that cast doubt over certain widely cited cases of LTSS in snakes, and thus evaluation of these cases is required. Accordingly, we review cases of LTSS in snakes in which we suspect the alternative reproductive strategy of FP.

MATERIAL AND METHODS

STUDY SPECIES

Copperhead (A. contortrix)

A juvenile (174 g) female copperhead (AC-1) was collected in North Carolina in August 2004. The subject was placed on exhibit at the North Carolina Aquarium at Fort Fisher with a female (confirmed through veterinary examination) of the same species. The only male snake with which she has had contact with in the last five years was a corn snake (*Pantherophis guttatus*), a colubrine species with which hybridization is not considered likely or even possible. On 1 August 2009, AC-1 produced a litter consisting of four offspring that were outwardly normal in appearance, two of which were alive at birth, and 12 infertile ova. Shed skins were collected from the dam and one live offspring, whereas a tissue sample was recovered from one stillborn individual, for subsequent genetic analysis.

Eastern diamond-backed rattlesnake

(C. adamanteus)

On 2 January 2005, a juvenile female eastern diamond-backed rattlesnake was collected in

Clermont, Florida. The snake measured approximately 30 inches, indicating a potential birth date of August or September 2003. After collection, the female was housed in isolation from males at temperatures averaging 29.4 °C during the summer months, and 20 °C during the winter. On 25 August 2010, the female gave birth to a total of 19 offspring (ten females, nine males). No infertile ova were produced. For subsequent genetic analysis, approximately 200 µL of blood was collected from the dam, whereas shed skins were collected from each of the offspring.

MOLECULAR DNA FINGERPRINTING

DNA isolation followed the phenol-chloroform method outlined by Taggart *et al.* (1992) with minor modifications. All samples, both *A. contortrix* and *C. adamanteus*, were screened at 12 microsatellite loci, isolated but untested by Castoe *et al.* (2010) from an *A. contortrix* microsatellite library (Table 1). PCR reactions were carried out in 12 µL volumes, each containing 1× PCR buffer, 2.0 mM MgCl₂, 100 µM dNTPs, ~ 50 ng DNA template, 0.3 U Taq DNA Polymerase (Bioline), 1 pM of primer, and ddH₂O to 12 µL. The forward primer of each pair was end-labelled with a M13F-29 IRDye tag (Li-Cor, Inc., Lincoln, NE,

USA). PCR cycling conditions comprised an initial denaturation stage of 3 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, with a subsequent terminal extension at 72 °C for 2 min, carried out using ABI 2720 thermal cyclers (Applied Biosystems, Foster City, CA, USA). Following PCR, 4 µL of stop solution (95% formamide, 20 mM EDTA, bromophenol blue) was added to each 12-µL reaction. Reactions were subsequently denatured at 95 °C for 4 min, and ~ 1 µL was loaded onto 25 cm 6% 1× TBE polyacrylamide gels, mounted on a Li-Cor 4300 automated DNA sequencer. Loci were sized using a 50–350-bp standard (Li-Cor). Gels were run at a constant power of 40 W at 50 °C for 2 h. Results were analysed using GENEPROFILER software (Scanalytics, Inc., Rockville, MD, USA).

RESULTS

Of the *A. contortrix* samples, eight loci successfully amplified unambiguous products, with seven exhibiting heterozygosity in the maternal sample (Table 2). At these maternally heterozygous loci, homozygosity was observed in each of the offspring sampled, with differential alleles fixed at four of these loci

Table 1. Characteristics of 12 microsatellite loci developed by Castoe *et al.* (2010), employed in this study

| Locus | Repeat motif | Sequence | Accession number |
|--------|--------------|---|------------------|
| Ac4335 | (ATT)34 | F: ATCCTTCCCCAAGCCAAGG R: GCTGGAGACTGGAGAAGAGAGC | GQ193458 |
| Ac6790 | (ATT)22 | F: TTCAATGTGGCACAATTCCC R: CCTGGTGCCCTTTTGGTAGG | GQ185992 |
| Ac6349 | (ATC)21 | F: TTGAGGAATCCAATGAGGAGC R: ACAAGTACCACAGGGCACCG | GQ187373 |
| Ac7679 | (ATC)20 | F: GTGATAATGGGATGGGTGGC R: TGACGTAGATAACCTCCAAGGTCC | GQ191314 |
| Ac5793 | (ATT)20 | F: GAGGGCTGGACTTGATGACC R: ACCACTTCCGAGAAGATACATCC | GQ191888 |
| Ac3046 | (TCC)20 | F: TGGGGTTTGTGAGAAGGAGG R: ATGCCCTCGATAAGCGCC | GQ195812 |
| Ac3113 | (ATT)20 | F: GAGAAGGAATTGTCCGGTGG R: TAGGGTTTCCCTTGCCCTGAGC | GQ185412 |
| Ac8192 | (ATT)20 | F: GTGTGTCAGATCCAGCCAGG R: TAACAAGTGAGCCCTGTGGC | GQ195663 |
| Ac5683 | (TTCC)19 | F: TGATGATACTTCGGTTTCCAGC R: AGTCGGGCTGCTTATTCCACC | GQ192417 |
| Ac6673 | (AAAT)19 | F: ATTCTGCGATAAGAAAGGCG R: AAAATTGTGAAGAATGCTACATACG | GQ185689 |
| Ac6402 | (TTCC)19 | F: AAAAGGGAGGCAGAGAGGC R: GCATTCTAACCCCTTGAGTCTGC | GQ192257 |
| Ac7363 | (AAGT)19 | F: ACTTCGTATAAGCAGTCATATACATTGG R: CAGAGCATCAGTTCCCAAAGG | GQ196161 |

Table 2. Genotypes of the mother and offspring of *Agkistrodon contortrix* and *Crotalus adamanteus* screened at nine microsatellite loci

| Offspring sex | ID | Ac4335 | Ac6790 | Ac7679 | Ac3113 | Ac8192 | Ac5683 | Ac6673 | Ac6402 | Ac7363 |
|----------------------|-----------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| <i>A. contortrix</i> | | | | | | | | | | |
| Dam | | 190/208 | 219/237 | 213/213 | 210/219 | – | 226/230 | 148/152 | 150/154 | 210/222 |
| Male | OS1 | 208/208 | 219/219 | 213/213 | 219/219 | – | 226/226 | 148/148 | 150/150 | 210/210 |
| Male | OS2 | 208/208 | 219/219 | 213/213 | 210/210 | – | 230/230 | 152/152 | 154/154 | 210/210 |
| <i>C. adamanteus</i> | | | | | | | | | | |
| Dam | | 149/176 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 234/258 |
| Female | OS1 | 149/176 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 234/254 |
| Female | OS2 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/225 | 125/125 | – | 254/258 |
| Female | OS3 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 254/258 |
| Female | OS4 | 152/176 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 230/258 |
| Female | OS5 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 234/254 |
| Female | OS6 | 149/176 | – | 219/219 | 283/283 | 424/424 | 225/225 | 125/125 | – | 234/254 |
| Female | OS7 | 149/149 | – | 219/219 | 283/283 | 424/424 | 233/233 | 125/125 | – | 254/258 |
| Female | OS8 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 234/254 |
| Female | OS9 | 152/176 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 254/258 |
| Female | OS10 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 230/258 |
| Male | OS11 | 149/152 | – | 219/219 | 283/283 | 424/424 | 233/233 | 125/125 | – | 230/258 |
| Male | OS12 | 149/152 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 234/254 |
| Male | OS13 | 152/176 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 254/258 |
| Male | OS14 | 152/176 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 230/234 |
| Male | OS15 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/225 | 125/125 | – | 230/234 |
| Male | OS16 | 149/149 | – | 219/219 | 283/283 | 424/424 | 233/233 | 125/125 | – | 234/254 |
| Male | OS17 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 230/234 |
| Male | OS18 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/225 | 125/125 | – | 230/234 |
| Male | OS19 | 149/149 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 230/258 |
| | Paternal alleles (inferred) | 149/152 | – | 219/219 | 283/283 | 424/424 | 225/233 | 125/125 | – | 230/254 |

Inferred paternal alleles are provided for *C. adamanteus*.

(Table 2). Across the seven maternally heterozygous loci, the probability of an individual receiving identical alleles from a male, and hence the offspring resulting from LTSS for at least 5 years, is $P = 4.882 \times 10^{-4}$ [i.e. $(0.5)^3$ (assuming paternal homozygosity at 3 loci) $\times (0.25)^4$ (assuming paternal heterozygosity at 4 loci)]. The probability of both offspring receiving alternate alleles, but identical to one of the maternal alleles at the heterozygous loci, is $P = 1.526 \times 10^{-5}$ [i.e. $(4.882 \times 10^{-4}) \times (0.25)^4$]. This extremely low probability strongly supports the exclusion of LTSS as the reproductive mechanism involved in the production of this litter, and instead favours the alternative hypothesis of FP. Of the *C. adamanteus* samples, seven loci amplified unambiguous alleles, of which three proved polymorphic. In contrast to the findings observed in *A. contortrix*, paternal alleles were detected at each of the polymorphic loci (Table 2).

DISCUSSION

FACULTATIVE AUTOMIC TIC PARTHENOGENESIS

We provide several lines of evidence supporting the first documented occurrence of FP in the North American pitviper *A. contortrix*. First, the female was collected as a juvenile (less than 1 year old) and reared in captivity in strict isolation for 5 years from male *A. contortrix*. Second, the results of molecular analysis exclude, with high confidence, the possibility of a paternal genetic contribution. Third, only male offspring were produced, which is indicative of the model of automixis in all advanced snakes (Schuett *et al.*, 1997), owing to the ZZ:ZW sex determination system where males are homogametic ZZ.

Other models of reproductive development, such as strict hermaphroditism, can be ruled out for two reasons. First, we have no evidence of its occurrence in *A. contortrix* or any other species of snake. In the South American pitviper *Bothrops insularis*, hermaphroditic-like individuals are known (Almeida-Santos & Salomão, 2002); however, production of offspring via hermaphroditism has not been documented in snakes. In fact, FP is suspected in *B. insularis*, and a compelling case is presented for its congener *B. moojeni* (Almeida-Santos & Salomão, 2002). Secondly, such a mechanism would probably produce both male and female offspring in litters due to the ZZ:ZW sex determination system where females are ZW, unlike the model of automixis we invoke.

LONG-TERM SPERM STORAGE

In contrast to LTSS, a central prediction of FP is that litters of advanced snakes should be substantially smaller, mostly owing to developmental failure (e.g. presumptive loss of WW embryos, Schuett *et al.*, 1997,

1998). The litter (viable offspring) produced by the present female *A. contortrix* was small (Fitch, 1960; Campbell & Lamar, 2004), contained only males, and had multiple developmental failures (e.g. infertile ova), which is in agreement with the model of FP. In contrast, the litter produced by the female *C. adamanteus* was typical in size (Klauber, 1972; Timmerman & Martin, 2003; Campbell & Lamar, 2004), contained both males and females, and had no developmental failures, a result that supports a model of LTSS.

BENEFITS OF FP OVER LTSS

Facultative parthenogenesis in squamates has yet to be documented in naturally occurring populations; thus, the evolutionary significance of FP will not be fully realized until reproductive competence can be demonstrated under natural conditions. Presumably in nature, within lineages producing ZZ males via FP (i.e. advanced snakes and varanid lizards), parthenogenesis would represent an evolutionary mechanism promoting population establishment and survival when females become isolated from mates (e.g. following invasion on new habitats). To understand the circumstances in which FP might be adaptive, we need to know how often a female is in a situation where the small reproductive benefit (i.e. small litter size; developmental failure or stillbirth) outweighs the potentially detrimental effects of future reproductive events with parthenogenetic sons. While potentially detrimental due to the reduction of genetic diversity and thus the increased likelihood of the fixation of deleterious alleles and/or gene complexes, the purging of detrimental genetic variation is also possible. This alternative scenario can have a positive effect on an incipient population (Hedrick, 2007). For example, positive reproductive performance has been documented in parthenogenetic male turkeys (Cassar, John & Etches, 1998). Thus, through the process of captive propagation, these lines may have been purged of deleterious alleles and/or gene complexes, effectively elevating fitness relative to parthenogens derived from genetically diverse wild stock (Feldheim *et al.*, 2010).

INFORMATION ON LTSS REPRODUCTION IN SNAKES

We collected data on published cases of LTSS in snakes that following our identification of traits associated with FP and LTSS now appear suspect, and evaluated them against the hypothesis of FP (Table 3). The model of FP has specific predictions in both birds and non-avian reptiles (e.g. advanced snakes and varanid lizards) (Olsen & Marsden, 1954; Schuett *et al.*, 1997, 1998; Lenk *et al.*, 2005; Watts *et al.*, 2006), and include: (1) few viable (live) progeny

Table 3. Reported cases of LTSS in female snakes that are suspected to be the result of FP

| Species | MOP | Storage duration (months) | FP evidence | Authority |
|-------------------------------------|-----|---------------------------|-------------|------------------|
| Achrochordidae | | | | |
| <i>Acrochordus javanicus</i> * | V | 84 | 1, 2, 4, 5 | Magnusson (1979) |
| Colubroidea | | | | |
| Colubridae | | | | |
| <i>Ahaetulla nasuta</i> | V | 36 | 4, 5 | Wall (1905) |
| <i>Boiga dendrophila</i> | O | 15 | 1, 2, 4, 5 | Groves (1973) |
| <i>Leptodeira septentrionalis</i> † | O | 60 | 1, 2, 4, 5 | Haines (1940) |
| <i>Drymarchon couperi</i> | O | 52 | 1, 4, 5 | Carson (1945) |
| Natricinae | | | | |
| <i>Nerodia erythrogaster</i> ‡ | V | 25 | 1–5 | Conant (1965) |
| <i>Thamophis couchii</i> § | V | 53 | 1, 2, 4, 5 | Stewart (1972) |
| Viperidae | | | | |
| <i>Agkistrodon contortrix</i> | V | 24 | 1, 5 | Allen (1955) |
| <i>Agkistrodon piscivorus</i> | V | 60 | 4, 5 | Schuett (1992) |
| <i>Gloydus blomhoffii</i> ¶ | V | 53 | 1, 3–5 | Fukada (1986) |

*Dubach, Sajewicz & Pawley (1997) provide genetic molecular evidence for FP in the congener *A. arafurae*.

†Formerly *Leptodeira annulata polysticta*.

‡Formerly *Natrix erythrogaster*.

§Germano & Smith (2010) provide genetic molecular evidence for FP in a *T. couchii*. Litter size was not provided in either of two cases (2005, 2006).

¶Formerly *Agkistrodon blomhoffii*.

MOP, mode of parity; O, oviparous; V, viviparous. Evidence for FP in these cases includes: (1) few viable progeny (or embryos); (2) a high degree of abortive events and/or developmental failures (e.g. infertile ova); (3) production of only male embryos or offspring (but see Groot *et al.*, 2003; Booth *et al.*, 2011a, b); (4) duration of LTSS suspect; and (5) absence of adult male mates.

(or embryos); (2) a high degree of abortive events (yolk plugs) and/or developmental abnormalities, presumably owing to homozygous combinations of deleterious alleles and/or WW individuals (but see Booth *et al.*, 2011a, b); (3) production of only diploid males, ZZ (but see Groot *et al.*, 2003; Booth *et al.*, 2011a, b); (4) absence of paternal genes; and (5) elevated homozygosity and limited heterozygosity (but see Groot *et al.*, 2003).

Our analysis of certain cases of LTSS in snakes (Table 3) questions the validity based on the alternative hypothesis of FP, which offers greater support through the model attributes we describe herein. For example, Stewart (1972) reports the production of a single, non-viable embryo along with several hardened yolk masses by a wild collected female *Thamophis couchii* held in isolation from males for 4 years, and thus attribute this to LTSS. A recent study by Germano & Smith (2010), however, document FP in this species following a period of isolation of 5 years. Carson (1945) documents the production of five eggs by a female eastern indigo snake (*Drymarchon couperi*), captured as an adult but held in isolation from males for 52 months. In line with the expecta-

tion of FP in advanced snakes, of the five eggs produced, a single egg contained a viable embryo. The author rejected the potential for FP and instead favoured LTSS.

We do not question the importance of LTSS in reptiles (Schuett & Gillingham, 1986; Gist & Jones, 1987, 1989; Schuett, 1992; Sever & Hamlett, 2002; Uller & Olsson, 2008; Jellen & Aldridge, 2011), and there are cases that are well supported (e.g. Schuett & Gillingham, 1986; Olsson & Madsen, 1998; Aldridge & Duvall, 2002; Uller & Olsson, 2008). However, we contend that LTSS cannot necessarily ascribe to all instances where adult females, isolated for prolonged periods, produce viable (fertilized) eggs and/or offspring. Thus, it appears that the same levels of scrutiny used to analyse the presence or absence of paternal contributions in suspected cases of FP (e.g. genetic molecular methods) in reptiles and other vertebrates will be required, in many cases, to assess LTSS.

Our analysis of LTSS did not include other reptilian lineages, namely Chelonia (turtles), Crocodylia (e.g. alligators, crocodiles, gharials), and the monotypic tuatara (Sphenodontidae, *Sphenodon punctatus*).

Based on morphological and behavioural evidence, numerous turtle species are capable of LTSS (Gist & Jones, 1989; Sever & Hamlett, 2002), and in several cases LTSS has been corroborated by molecular genetic (microsatellite) analyses (Pearse *et al.*, 2001; Roques *et al.*, 2006; Refsnider, 2009). Nonetheless, at present, there is no evidence for FP in turtles. Recently, based on morphological evidence, females of the American alligator (Alligatoridae, *Alligator mississippiensis*) show the capacity for sperm storage (Gist *et al.*, 2008), although molecular evidence for LTSS (e.g. across reproductive seasons) has yet to be demonstrated (Lance *et al.*, 2009). With one possible exception (Davenport, 1995), there have been no cases where FP is suspected in crocodylians.

In conclusion, through the application of molecular genetic markers, we identified the first recorded case of FP in a copperhead snake (*A. contortrix*). The litter characteristics (i.e. small number of viable male offspring, large number of infertile ova) comply with numerous recorded cases in snakes previously attributed to LTSS. In the absence of molecular support for these previous studies, we propose FP as a more plausible scenario. Furthermore, we provide the first genetically verified record of LTSS in any snake species. To our knowledge, this record of LTSS for a period of at least 67 months may be the longest genetically confirmed record in any vertebrate species. The reproductive outcome of this litter (i.e. production of a large, healthy litter containing both males and females, and the identification of paternal contribution) lends supports to our review of suspect cases of LTSS in snakes, favouring the alternative of FP as the most plausible explanation. In concert with recent confirmation of FP in other snakes, birds, and sharks, it is clear that genetic molecular markers now offer researchers the potential to accurately differentiate between these competing forms of reproduction.

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