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Successive virgin births of viable male progeny in the checkered gartersnake, *Thamnophis marcianus*

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In recent years genotyping analysis using mini- and microsatellite markers has provided robust DNA-based support for facultative parthenogenesis (FP) in several lineages of squamate reptiles (snakes and lizards) and sharks. Rather than incidental cases of reproductive error, there is growing evidence that FP is an alternative reproductive strategy and an important mode of reproduction in these phylogenetically divergent vertebrate groups. Because documentation of FP in vertebrates is in its infancy, additional instances supported by molecular genetic methods provide insights that advance our general understanding of this phenomenon. Here, in a female checkered gartersnake (*Thamnophis marcianus*) reared in isolation since a juvenile, we describe five successive parthenogenetic litters produced over a 7-year period that resulted in several viable male progeny. Cross species microsatellite amplification was performed across 30 primer pairs derived from *Thamnophis* spp. and related natricines to the female and nine available progeny. Five loci proved heterozygous in the maternal sample with the progeny differentially homozygous at all but one locus. Combined with evidence pertaining to captive history and litter characteristics, our analysis supports a specific type of FP, terminal fusion automictic parthenogenesis, over the competing hypothesis of long-term sperm storage. Importantly, we document that a single individual was capable of producing successive litters composed of live parthenogens. In two cases, males achieved adulthood and showed the anatomical potential to demonstrate reproductive competence (normal looking hemipenes and testes). *Biological Journal of the Linnean Society*, 2012, **107**, 566–572.

ADDITIONAL KEYWORDS: asexual reproduction – automixis – facultative parthenogenesis – microsatellite DNA genotyping – Reptilia – Serpentes – ZW sex-determination.

INTRODUCTION

Among multi-cellular bisexual organisms the capacity to alternate between sexual and asexual reproductive modes is termed facultative parthenogenesis (FP) (reviewed by Mogie, 1986; Simon *et al*., 2003; Avise, 2008, 2012; Lampert, 2008; Neaves & Baumann, 2011). Full-term, viable progeny (i.e. hatched or live-birth) resulting from FP can occur without genetic manipulation (e.g. hybridization, genetic tools) in a variety of animal lineages (Avise, 2008; Lampert, 2008; Sinclair *et al*., 2009). In vertebrates, FP was first investigated in commercial turkeys and chickens in the early 1950s (reviewed by Olsen, 1975), with no additional examples for over 40 years. In 1997, FP was documented in multiple lineages of snakes

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(Dubach, Sajewicz & Pawley, 1997; Schuett *et al*., 1997, 1998). Subsequently, instances of FP resulting in live embryos or viable progeny have been reported in other species of snakes (Groot, Bruins & Breeuwer, 2003; Booth & Schuett, 2011; Booth *et al*., 2011a, b), several species of varanid lizards, including the endangered Komodo dragon, *Varanus komodoensis* (Lenk *et al*., 2005; Watts *et al*., 2006), and sharks (Chapman *et al*., 2007; Chapman, Firchau & Shivji, 2008; Feldheim *et al*., 2010; Robinson *et al*., 2011). In birds, FP has been recently described in embryos of the zebra finch, *Taeniopygia guttata* (Schut, Hemmings & Birkhead, 2008) and Chinese painted quail, *Coturnix chinensis* (Parker & McDaniel, 2009).

To date, naturally occurring and successful FP in mammals is unknown and attributed to genomic imprinting (Haig, 2002), a *cis*-acting mechanism that silences either the maternally or paternally inherited copy of a gene while allowing the other copy to be functional in the embryo (McGrath & Solter, 1984; Surani, Barton & Norris, 1984; Barlow *et al*., 1991; Ohlsson, Hall & Ritzen, 1995; Morrison, Ramsay & Spencer, 2005; Kono, 2006; Renfree *et al*., 2009). However, through genetic manipulations, laboratory strains of mice have produced viable parthenogens that can survive to adulthood and reproduce successfully (Kono *et al*., 2004; Kono, 2006; Kawahara & Kono, 2010). Although FP is not uncommon in invertebrates (Avise, 2008; Lampert, 2008; Buřič *et al*., 2011; Lehmann *et al*., 2011), it appears to be rare in vertebrates (Avise, 2008; Kearney, Fujita & Ridenour, 2009).

Nearly all suspected cases of FP in birds and squamates result in male offspring, which is attributable to the ZW sex determination system and specific mode of FP (Dubach *et al*., 1997; Schuett *et al*., 1997, 1998; Lampert, 2008; Booth & Schuett, 2011; Livernois, Graves & Waters, 2012). Accordingly, homogametic males (ZZ) are presumably produced by way of terminal fusion of post-meiotic products, i.e. reduced ovum and second polar body, which is termed automixis (reviewed by Mogie, 1986; Avise, 2008; Lampert, 2008). Specifically, this category of FP is terminal fusion automictic parthenogenesis (FAP) (Olsen, 1975; Schuett *et al*., 1997, 1998; Lampert, 2008). Nonetheless, in the Burmese python (*Python bivittatus*), Groot *et al*. (2003) provide evidence that parthenogenetic female embryos were heterogametic (ZW). In poultry, the combination of WW cells have long been considered to be nonviable (Olsen, 1975). However, recent analyses of FP in boid snakes has demonstrated WW cells to result in viable female progeny (Booth *et al*., 2011a, b). To date, we are unaware of any non-experimentally induced WW female parthenogens outside of boids. Progeny resulting from FAP are probably diploid, predominantly homozygous, and identical for approximately 50% of their genomes (Olsen, 1975; Schuett *et al*., 1997, 1998; Lampert, 2008). Unlike boids and pythonids (Groot *et al*., 2003; Booth *et al*., 2011a, b), FAP in advanced snakes (*Caenophidia*) results in few viable progeny and numerous underdeveloped ova, presumably some of which are homogametic WW (Olsen, 1975; Schuett *et al*., 1997; Booth & Schuett, 2011).

The checkered gartersnake (*Thamnophis marcianus*) is a common natricine (caenophidian) of western North America (Stebbins, 2003). The present female and progeny of her first litter (one live, two stillborn) were first discussed in Schuett *et al*. (1997). Briefly, zookeepers at the Phoenix Zoo collected her on 18 August 1992 as a juvenile (b. 1992) in Maricopa County, Arizona, where she was reared at the zoo to adulthood (1992–2000). Subsequently, we (G.W.S.) maintained her at Arizona State University and later Georgia State University (2000–2003). At no time was she exposed to any other snake. In the original description (Schuett *et al*., 1997), no molecular analyses were performed to provide robust support for parthenogenesis.

Here we used microsatellite genotyping to analyse the female *T. marcianus* and nine of her progeny from four of five litters produced from 1997 to 2003. No progeny from her last litter in 2003 were available for DNA-based analysis. Through our genetic analysis, FAP was detected in all offspring that were tested. Several of her litters contained viable male progeny, two of which survived to adulthood and exhibited the potential for sexual competence (i.e. the presence of hemipenes and testes). Assuming that such parthenogens are ultimately shown to be sexually competent, our current findings suggest that a single unmated female may have at least the potential to initiate a new population in the absence of other unrelated males, which extends the potential significance of this captive phenomenon.

MATERIAL AND METHODS

Microsatellite analysis

DNA samples consisted of ethanol-stored (95%) tissues (blood, liver, muscle) and air-dried shed skins that had been stored for 5–12 years at –20 °C. Whole genomic DNA was extracted using the DNeasy DNA extraction kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's protocol and stored at –20 °C. Because no polymorphic species-specific microsatellite markers have been published for *T. marcianus*, we used the polymerase chain reaction (PCR) to screen 30 microsatellite markers developed for other *Thamnophis* species and related natricine snakes following amplification profiles described by the respective authors. Te1Ca2, Te1Ca3, Te1Ca18,

Te1Ca50 (Garner *et al.*, 2004); Ts2a, Nsμ2b, Nsμ3a, Nsμ9d (Albright, 2001); TE051B, TS010 (Manier & Arnold, 2005); and Ts1, Ts3, Ts4 (McCracken, Burghardt & Houts, 1999) were genotyped on an EGene® multicapillary electrophoresis system (Irvine, CA, USA). This instrument uses disposable micro-channel cartridges containing sieving-gel matrix with EtBr dye to generate gel images and allele sizes. Internal 25-bp size markers where incorporated into each run. Peaks generated were cleaned and called using Biocalculator (Qiagen). TbuA01, TbuA03, TbuA04a, TbuA09, TbuA27, TbuA28, TbuA49, TbuA62, TbuA64, TbuA70, Tbu74, TbuA83, TbuA92, TbuA95, TbuB10, TbuB12, and TbuB38 (Sloss *et al.*, 2012) were genotyped on a LiCor 4300 (dual laser) DNA analyser (Li-Cor, Inc., Lincoln, NB, USA) with the forward primer of each end labelled with an M13F-29 IRDye tag (Li-Cor). Results were analysed using GENEPROFILER software (Scanalytics, Inc., Rockville, MD, USA). Prior to automated sequencing, products were visualized by agarose gel (2%) electrophoresis to determine whether successful amplification occurred. To check for reliability across PCR and genotyping machine runs, each locus was repeated from the PCR stage and genotyped a second time.

Table 1. Reproductive data for the female *Thamnophis marcianus* in our study

	Progeny		
Births	Live	Stillborn	Yolked ova
1997	1	2	6
1999	1	2	5–7
2000	1	2	3
2002	0	2	6
2003	0	3	3–5

The live progeny (males) produced in 1997 and 1999 were normal in appearance and survived to adulthood. In nearly all cases, stillborn progeny showed slight to severe developmental abnormalities.

Morphological examination of adult parthenogens

The female and two living male offspring died in transit while being air-shipped in 2003. These individuals were subsequently stored frozen (–20 °C). Examination of thawed tissues of the progeny revealed the presence of normal looking hemipenes and testes. While being examined, both testes were removed, and placed in 10% buffered formalin for 3 weeks. Subsequently, they were processed through various concentrations of EtOH and prepared for histological analyses. Once embedded in paraffin and sectioned (10 μm), all samples were stained with Erlich's haemotoxylin.

RESULTS

The reproductive history of the female we studied is presented in Table 1. She produced five litters from 1997 to 2003, which were composed of three viable (live and normal in appearance) and 11 non-viable (stillborn) progeny, as well as numerous yolked ova. Many of the stillborn progeny showed severe developmental abnormalities. Of the 30 microsatellite loci screened, 18 amplified unambiguous and repeatable products in the size range expected: Ts2a, Nsμ2b, Nsμ3a, Nsμ9d (Albright, 2001); and TbuA3, TbuA4, TbuA27, TbuA49, TbuA62, TbuA64, TbuA70, TbuA74, TbuA83, TbuA92, TbuA95, TbuB10, TbuB12, TbuB38 (Sloss *et al.*, 2012). Of these, five proved maternal heterozygous and were informative to our central hypothesis of FAP (Table 2). At four loci, differential homozygosity was observed in each of the

Table 2. Genotypes of the unmated (virgin) female *Thamnophis marcianus* and nine of her progeny for five maternally heterozygous microsatellite loci: note heterozygosity at locus TbuB10 in progeny 6–9

Snake ID	Litter year	TbuA3	TbuA62	TbuA64	TbuA83	TbuB10
Mother	Wild-collected	247/253	301/340	256/258	354/364	185/193
Progeny 1	1997	247/247	340/340	256/256	364/364	185/185
Progeny 2	1999	253/253	340/340	258/258	364/364	193/193
Progeny 3	1999	247/247	340/340	258/258	364/364	185/185
Progeny 4	1999	247/247	340/340	256/256	364/364	185/185
Progeny 5	2000	247/247	301/301	256/256	354/354	185/185
Progeny 6	2000	247/247	301/301	256/256	354/354	185/193
Progeny 7	2000	247/247	301/301	256/256	354/354	185/193
Progeny 8	2002	253/253	301/301	256/256	354/354	185/193
Progeny 9	2002	247/247	301/301	256/256	354/354	185/193

progeny, and at a single locus (TbuB10), four progeny (6–9) possessed identical genotypes to their mother. Gross morphological examination of the two male progeny that achieved adulthood – revealed normal-looking hemipenes and testes; however, histological analysis of the sectioned testes was inconclusive regarding the presence of spermatozoa.

DISCUSSION

Since Schuett *et al*. (1997, 1998) and Dubach *et al*. (1997), the number of cases of virgin births documented via genotyping analyses in squamates (Groot *et al*., 2003; Lenk *et al*., 2005; Watts *et al*., 2006; Booth & Schuett, 2011; Booth *et al*., 2011a, b) and sharks (Chapman *et al*., 2007, 2008; Feldheim *et al*., 2010; Robinson *et al*., 2011) has gradually increased. Here, in the checkered gartersnake (*T. marcianus*), we present multiple lines of evidence that successive litters were produced by FAP over a period of 7 years. The fact that the female we studied was isolated from all other snakes shortly after her birth is compelling evidence alone (i.e. without DNA-based analysis) of FP, especially because other modes of reproduction (e.g. hermaphroditism) are entirely unknown in snakes. But, through the use of microsatellite genotyping analysis, we provide specific and robust evidence for terminal FAP (Avise, 2008; Lampert, 2008).

FAP versus long-term sperm storage

In both squamates and sharks, the primary competing hypothesis to FAP is long-term sperm storage (LTSS), a phenomenon reported in a wide variety of vertebrates and invertebrates (Schuett, 1992; Hamlett & Koob, 1999; Holt & Lloyd, 2010). The longest genetically confirmed record of LTSS in a vertebrate is by the rattlesnake *Crotalus adamanteus*, which was captured as a young adult and ~60 months later produced a large ($N = 19$), healthy litter composed of both males ($N = 9$) and females ($N = 10$) (Booth & Schuett, 2011). In this study, we reject the alternative hypothesis of LTSS for three main reasons. First, for a male to have been a sire to each of the progeny, mating would have had occurred in the wild prior to the female's collection. But, owing to her age (~2–3 months old) and diminutive size at the time of her capture, successful mating is highly improbable. Second, no discernible paternal allele was detected in any of her progeny. For a male to have been a sire, he must share identical genotypes at each of the maternally heterozygous loci and contribute the identical maternal allele to each progeny. The probability of this result is infinitesimally small (probability of contributing identical maternal allele at four loci per progeny = 0.0039; combined probability across nine progeny = 2.087×10^{-22}). Furthermore, because FAP increases homozygosity across much of the genome (Pearcy, Hardy & Aron, 2011), the detection of progeny expressing identical heterozygous genotypes as the mother was not unexpected (Lampert, 2008). Whereas FAP in boids and pythonids results in female (WW or ZW) embryos or progeny (Groot *et al*., 2003; Booth *et al*., 2001a, b), caenophidian snakes, a group containing the majority of extant species, FAP has been characterized by only male progeny, as well as frequent developmental failures (e.g. WW) or developmental abnormalities (Schuett *et al*., 1997, 1998; Booth & Schuett, 2011), which we report here across each of the five successive litters. Presumably, FAP allows for the expression of lethal alleles as a result of elevated homozygosity (Hedrick, 2007). Although we report multiple stillborn progeny that exhibited slight to severe deformities, three individuals were normal in appearance, two of which survived to adulthood.

Evidence for sexual competence in male parthenogens

Our two adult male parthenogens that died in transit appeared to have the capacity to reproduce given that they had normal-looking hemipenes and testes. Although our results are evocative, reproductive competence of FP progeny remains to be demonstrated in both squamates and sharks (Lenk *et al*., 2005; Lampert, 2008). In support of the view that FP progeny in squamates and sharks can be reproductively competent, male parthenogen turkeys (*Meleagris gallopavo*) are capable of mounting hens and fertilizing eggs (Olsen, 1975; Cassar, John & Etches, 1998). Similarly, laboratory mice that have been genetically manipulated to undergo parthenogenesis can produce viable offspring that achieve adulthood and successfully reproduce (Kono *et al*., 2004; Kono, 2006; Kawahara & Kono, 2010).

Other cases of FAP in gartersnakes

Since FAP was discovered in gartersnakes (genus *Thamnophis*) using minisatellite analysis (Schuett *et al*., 1997), it has been inferred in two other cases. Murphy & Curry (2000) described two presumed virgin births, one year apart, by a 3-year-old plains gartersnake (*T. radix*), purchased as a 15-cm neonate and isolated from males from birth. Litter characteristics mirrored those described here, i.e. low numbers of viable male progeny and high numbers of developmental failures. A decade later, Germano & Smith (2010) described two instances of virgin births in a Sierra gartersnake (*T. couchii*). Although the age of the female was unknown, her mass at capture (38.3 g)

suggests she was obtained as a juvenile (adult females are ~200 g). Microsatellite genotyping was applied to the female and the only viable (male) progeny from her second litter. However, the finding of identical homozygous genotypes in both the mother and male progeny does not permit a definitive determination of FAP. Regardless, the captive history and litter characteristic in these two reports strongly suggest cases of FAP.

Successive virgins births in vertebrates

Successive virgin births (i.e. over separate reproductive seasons) resulting in viable progeny through FAP have been genetically confirmed in only a handful of cases. A female captive zebra shark (*Stegostoma fasciatum*) produced a total of 15 pups over a period of four consecutive years (Robinson *et al*., 2011). In snakes, Booth *et al*. (2011a, b) described cases of successive virgin births in two species of New World boids (*Boa constrictor*, *Epicrates maurus*). It therefore appears that successive virgin births are probably common in species exhibiting FAP and only through the application of informative molecular tools will the extent of this phenomenon be determined (Booth & Schuett, 2011).

Future research directions for FP

In captive squamates and sharks, only recently has FP been rigorously documented using DNA-based genotyping methods (Schuett *et al*., 1998; Lenk *et al*., 2005; Watts *et al*., 2006; Booth & Schuett, 2011; Booth *et al*., 2011a, b). Rather than incidental cases of reproductive error (Avise, 2008; Lampert, 2008), there is accumulating evidence that FP in squamates and sharks is an alternative reproductive strategy and a potentially important mode of reproduction in these groups of vertebrates (Booth *et al*., 2011a, b; Neaves & Baumann, 2011). Accordingly, nonhybrid occurrences (origins) of parthenogenesis may be more common than previously thought (Sinclair *et al*., 2009). Nonetheless, the evolutionary significance of FP (e.g. FAP) in squamates, sharks, and perhaps other vertebrates cannot be established until documentation is made in nature and the reproductive competence of parthenogens is established.

Beyond the role of hybridization (Sinclair *et al*., 2009; Lutes *et al*., 2011), the cues that trigger an individual to switch from sexual to asexual reproduction, such as FP, are unknown in squamates and sharks. Based on current knowledge, the absence of males is not a sufficient explanation (Booth & Schuett, 2011; Booth *et al*., 2011b). In poultry and quail, for example, viruses (live fowl pox) and genetic factors (e.g. selective breeding) can promote (increase) instances of FAP (Olsen, 1975; Parker *et al*., 2010). In a variety of invertebrates, infections caused by cytoplasmatically inherited endosymbionts (e.g. the bacterium *Wolbachia*) are common and can induce FP (Simon *et al*., 2003). Interestingly, in these cases, FP can be reversed (restoration of sexual reproduction) via antibiotic or thermal treatments (Lehmann *et al*., 2011). Thus, investigating the proximate mechanisms of FP (e.g. FAP) in squamates and sharks is a rich area for future research programmes (Neaves & Baumann, 2011).

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