

Sperm Storage in a Family-Living Lizard, the Tree Skink (*Egernia striolata*)

Julia L. Riley^{1,2,3*}, Adam Stow¹, Peri E. Bolton^{1,4}, Siobhan Dennison¹, Richard Byrne⁵, and Martin J. Whiting¹

¹ Department of Biological Sciences, Macquarie University, Sydney, New South Wales, Australia

² Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada

³ Department of Biology, Mount Allison University, Sackville, New Brunswick, Canada

⁴ Department of Biology, East Carolina University, Greenville, North Carolina, United States

⁵ School of Psychology and Neuroscience, University of St. Andrews, St. Andrews, Fife, United Kingdom

*Corresponding author email: julia.riley87@gmail.com

ORCIDs and Email Addresses:

JLR - 0000-0001-7691-6910, julia.riley87@gmail.com

AS - 0000-0002-6796-4854, adam.stow@mq.edu.au

PEB - 0000-0002-2057-1973, peri.bolton@gmail.com

SD - 0000-0002-3308-2810, siobhan.dennison@gmail.com

RWB 0000-0001-9862-9373, rwb@st-andrews.ac.uk

MJW - 0000-0002-4662-0227, martin.whiting@mq.edu.au

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Abstract

The ability to produce viable offspring without recently mating, either through sperm storage or parthenogenesis, can provide fitness advantages under a suite of challenging ecological scenarios. Using genetic analysis, we demonstrate that three wild-caught female Tree Skinks (*Egernia striolata*) reproduced in captivity with no access to males for over a year, and that this is best explained by sperm storage. To the best of our knowledge, this is the first time female sperm storage has been documented in any monogamous family-living reptile, including social Australian egerniine skinks (from the subfamily Egerniinae). Furthermore, by using paternal reconstruction of genotypes we show that captive-born offspring produced by the same females in the preceding year, presumably without sperm storage, were sired by different males. We qualitatively compared aspects of these females' mates and offspring between years. The parents of each litter were unrelated, but paternal and offspring genotypes from litters resulting from stored sperm were more heterozygous than those inferred to be from recent matings. Family-living egerniine skinks generally have low rates of multiple paternity, yet our study suggests that female sperm storage, potentially from outside social partners, offers the real possibility of benefits. Possible benefits include increasing genetic compatibility of mates and avoiding inbreeding depression via cryptic female choice. Sperm storage in Tree Skinks, a family-living lizard with a monogamous mating system, suggests that females may bet-hedge through extra-pair copulation with more heterozygous males, reinforcing the idea that females could have more control on reproductive outcomes than previously thought.

Keywords: DNA profiling, genetic bet-hedging, heterozygosity, multiple mating, paternity, sexual conflict

Introduction

There are costs and benefits to sexual and asexual reproduction, but in predominantly sexual taxa, such as most vertebrates, sometimes opportunities for mating are rare, sex can be costly (i.e., the “two-fold” cost of sex; Gibson, Delph, & Lively, 2017), and the act of mating can increase predation risk or conspecific competition (Lewis Jr, 1987). High costs of sexual reproduction and difficulties in encountering potential mates may be offset by two mechanisms for production of offspring without recent involvement of a mate: facultative parthenogenesis and sperm storage (Miller et al., 2019; Orr & Brennan, 2015). Parthenogenesis is the production of offspring without fertilization (Watts et al., 2006), with an embryo developing from a female gamete with no contribution from a male gamete (Lampert, 2008; Miller et al., 2019). In contrast, sperm storage is the maintenance of sperm inside a female’s reproductive tract for an extended period of time after mating (Friesen & Olsson, 2016; Orr & Zuk, 2012). Genetic analyses of mothers and offspring allow for unequivocal discrimination between parthenogenesis and sperm storage (Groot, Bruins, & Breeuwer, 2003; Miller et al., 2019). If offspring are produced by female sperm storage, then offspring genotypes would contain contributions from both the mother and father (except for mutations), whereas offspring that are the product of facultative parthenogenesis would lack a paternal contribution. Both processes are present across the animal kingdom - including in insects (Suomalainen, 1962), fishes (Dudgeon, Coulton, Bone, Ovenden, & Thomas, 2017; Feldheim et al., 2010; Robinson, Baverstock, Al-Jaru, Hyland, & Khazanehdari, 2011), non-avian reptiles (Orr & Zuk, 2012), and birds (Ramachandran & McDaniel, 2018).

Both processes outwardly present themselves in the same manner (i.e., successful production of offspring after a long period of isolation from potential mates: Booth & Schuett, 2011; Orr & Brennan, 2015; Orr & Zuk, 2012); indeed, if not tested genetically, facultative parthenogenesis can be mistaken for female sperm storage and vice versa. Yet, these two mechanisms have different benefits and costs. Both facilitate reproduction when individuals are scarce or isolated from one another, but

parthenogenesis has the potential to increase the rate of population growth more quickly than sexual reproduction tactics (Normark, 2013). Also, an aspect of parthenogenesis that can, potentially, be both positive and negative is genetic stability. Genetic stability is beneficial if an organism is adaptively suited to a stable environment; but can be costly in changing environments where genetic variation provides evolutionary potential and an improved chance of survival. In reptiles, facultative parthenogenesis is rarer than obligate parthenogenesis, which has been documented in one snake (Booth & Schuett, 2015) and in over 20 lizard species (Cosentino, Schooley, Bestelmeyer, Campos, & Burkett, 2019; Miller et al., 2019; Vrijenhoek, Dawley, Cole, & Bogart, 1989). Although rarer, facultative parthenogenesis has been documented in captive females from eight squamate families (Acrochordidae, Agamidae, Boidae, Colubridae, Elapidae, Pythonidae, Varanidae, and Viperidae; Allen, Sanders, & Thompson, 2008; Booth & Schuett, 2015; Dubach, Sajewicz, & Pawley, 1997; Groot et al., 2003; Kinney, Wack, Grahn, & Lyons, 2013; Miller et al., 2019; Schuett et al., 1997; Shibata, Sakata, Hirano, Nitasaka, & Sakabe, 2017; Watts et al., 2006), as well as in wild populations of copperheads (*Agkistrodon contortrix*) and cottonmouths (*Agkistrodon piscivorus*); demonstrating that, at least in snakes, facultative parthenogenesis can occur naturally (Booth et al., 2012).

Female sperm storage has been documented in all major reptilian taxa except amphisbaenians (Birkhead & Møller, 1993; Olsson & Madsen, 1998; Sever & Hamlett, 2002). Even sperm stored over long periods can be viable (Holt & Lloyd, 2010; Orr & Zuk, 2012; Uller & Olsson, 2008). For example, in captive female turtles, viable sperm has been stored for up to four years (Palmer, Rostal, Grumbles, & Mulvey, 1998; Pearse, Janzen, & Avise, 2001). In wild populations, clutch fertilization by stored sperm has a documented duration of one to two years in female Blanding's Turtles (*Emydoidea blandingii*; Anthonysamy, Dreslik, Douglas, Marioni, & Phillips, 2014) and six years in a female Western Diamondback Rattlesnake (*Crotalus atrox*; Levine, Schuett, & Booth, 2021). The record duration for female sperm storage in vertebrates is for Javan Wart Snakes, *Acrochordus javanicus*, that can store sperm for up to seven years (Magnusson, 1979). As with parthenogenesis, female sperm storage may be particularly adaptive in species where encounter rates between males

and females are low (Gist & Congdon, 1998; Orr & Zuk, 2012; Pearse & Avise, 2001; Uller & Olsson, 2008). However, support for this hypothesis is mixed, because female sperm storage has also been found in densely aggregating species. Female sperm storage may also be important for species with different life histories and/or reproductive cycles between sexes (Orr & Zuk, 2012). The possible advantages for females include decreased costs of mating, ensuring fertilization, increased possibility for sperm competition, mating with males whose sperm have high fertilizing efficiency which may be heritable and confer an advantage to their sons (i.e., the sexy-sperm hypothesis; Curtsinger 1991; Egan et al., 2015), and increased control of fertilization success and offspring fitness via cryptic female choice (Orr & Brennan, 2015; Friesen, Kahrl, & Olsson, 2020). For example, in highly polyandrous field crickets (*Gryllus bimaculatus*) stored sperm is more likely to be from unrelated males than siblings, which counteracts potential inbreeding depression (Bretman, Newcombe, & Tregenza, 2009). The benefits and costs of female sperm storage likely vary between species depending on their social and mating systems. Although female sperm storage is often assumed to be widespread in reptiles, it is yet to be documented in some taxa (e.g., social reptiles like egeriine skinks, Chapple 2003, and night lizards, Denburgh 2015), which limits our understanding of the trade-offs inherent in their sociality.

In reptiles, multiple mating is the norm (range of multiple paternity within a clutch or litter: 11 to 87%: Wapstra & Olsson, 2014; Uller & Olsson, 2008). This norm applies to skinks, like the highly promiscuous Grand Skink (*Oligosoma grande*; Berry, 2005) and Common Five-lined Skink (*Plestiodon fasciatus*; Bateson, Krenz, & Sorensen, 2011). In contrast, family-living egeriine skinks tend to have lower rates of multiple paternity (Chapple, 2003; While, Chapple, Gardner, Uller, & Whiting, 2015; Whiting & While, 2017); specifically, multiple paternity in egeriine skinks varies from 3 to 25% across species (Chapple, 2003; Chapple & Keogh, 2006; Gardner, Bull, & Cooper, 2002; Stow & Sunnucks, 2004; While, Uller, & Wapstra, 2009; Whiting & While, 2017; Riley, unpublished data). In these monogamous, social skinks there is less sexual conflict (i.e., a difference in the optimal fitness strategies between males and females; Parker, 2006) than in polygamous lizards

(Hosken, Garner, & Ward, 2001; Martin & Hosken, 2007), which is reflected in their lack of dramatic sexual dimorphism (Riley et al. 2021).

In captivity, we had three wild-caught female Tree Skinks (*Egernia striolata*) give birth during a year where they had no access to males. Using genetic parentage analyses, we tested the alternative hypotheses of whether these offspring were produced by parthenogenesis or by fertilization using sperm stored within the females' reproductive tracts. We show that these reproductive events are best explained by female sperm storage. This has implications for our understanding of the Tree Skinks' mating system and highlights an unexpected increase in potential for sexual conflict in a monogamous family-living species of lizard.

Materials and Methods

Study Species

The Tree Skink is a medium-sized (18 - 22 cm in total length: Cogger, 2014), viviparous lizard that lives across central and south-eastern Australia. Tree Skinks inhabit cracks, hollow limbs, and gaps under the bark of standing trees or fallen timber, and crevices in rock outcrops (Cogger, 2014). Parturition of 1-6 offspring is from January to March each year (Chapple, 2003). The lifespan of *E. striolata* is unknown, but it is estimated to be about 5-10 years based on data from similar species (Cogger, 2014). Generally, *E. striolata* lives in kin-based social groups ranging in size from 2-6 individuals (Bonnett, 1999; Bustard, 1970; Duckett, Morgan, & Stow, 2012; Riley, *unpublished data*). Although there is intra-population variability in this species' social behaviour, the strongest kin-based social associations are typically between full-siblings and parents and their offspring (i.e., nuclear family units; Riley, *unpublished data*). Tree Skinks are largely genetically and socially monogamous. There are high levels of female genetic monogamy: specifically, in a wild population in Albury, NSW, Australia only 13% of litters had two fathers, and no litters were shown to have more than two

fathers (Riley, *unpublished data*). The number of litters sired by one male varies from 1 to 3, with an average of 1.35 ± 0.64 (median and mode = 1; Riley, *unpublished data*). Further, within this wild population, the majority of identified mate pairs exhibited preferred social associations (i.e., they were socially associating twice as often as one would expect by chance; Riley, *unpublished data*). It is unknown whether multiple paternity is the result of females multiply mating during the breeding season or the use of stored sperm over the short- or long-term. Benefits that females gain from the low amount of multiple paternity within litters are unknown.

Lizard Collection, Housing, and Monitoring

In December 2013, we collected 27 gravid, female *E. striolata* by hand, lasso, or Elliot trap near Albury, NSW, Australia (35.98°S, 146.97°E) for a series of experimental studies investigating the effect of social environment on behavioral development (Riley et al., 2016; Riley et al., 2017, Riley et al., 2018a, Riley et al., 2018b). After capture, we uniquely marked each individual with a Passive Integrated Transponder (PIT) tag and took a tissue sample (removing less than 0.5 cm of the tip of the tail with scissors). We then transported lizards to Macquarie University, Sydney, NSW, Australia. Immediately after capture, we housed females in a climate-controlled room (24 °C) individually within opaque plastic tubs (350 mm W x 487 mm L x 260 mm H) to monitor births. We fed female lizards 3 adult house crickets (*Acheta domesticus*) dusted with calcium and vitamin powder twice a week, and puréed fruit (1.25 ml of Heinz® baby food: mango, apple, or pear) once weekly. From February to March 2014, we visually checked if females had given birth twice daily. Immediately after offspring were born, we uniquely marked each individual with a toe-clip and took a tail tissue sample for genetic analyses.

After all females had given birth in the summer of 2014, we transferred them from their indoor housing into larger, outdoor enclosures (6.2 m² plastic tubs lined with mulch that contained vegetation, tile refuges, and a water dish) that were within a predator-exclusion enclosure. Females

were housed in these outdoor enclosures throughout the autumn, winter, and spring of 2014. During this period no males were in our captive colony, and there were no wild populations of *E. striolata* nearby (Macquarie University is not within the geographic range of this species; Cogger, 2014). In the summer of 2015, we observed that three females appeared gravid and moved them indoors, housing them under the same conditions as in 2014, and monitored them daily from 17 January to 10 February 2015. When offspring were found they were processed using the same protocol as the year prior. All protocols in this study were approved by the Macquarie University Animal Ethics Committee (ARA # 2013/039) and research was approved by the New South Wales National Parks and Wildlife Service, Office of Environment and Heritage (License # SL101264).

Genetic Analysis

DNA extraction, single nucleotide polymorphisms (SNPs) sequencing, and bioinformatics

DNA was extracted from skin tissue samples using GenCatch™ Blood and Tissue Genomic Mini Prep Kits (Epoch Life Science, Inc., Sugarland, TX, USA) in accordance with manufacturer instructions. Aliquots of all DNA samples were electrophoresed on 0.8% agarose gels pre-stained with GelRed™ (Biotium, Inc., Fremont, CA, USA; Huang, Baum, & Wei-Ling, 2010) to confirm they contained high molecular weight DNA. After extraction, DNA samples were sent to a commercial genotyping service - Diversity Arrays Technology Pty. Ltd. (Canberra, ACT, Australia). This company has developed a widely-used technique called DArTseq™ that is used to identify SNPs. Detailed descriptions of the DArTseq™ process of SNP identification are provided in Jaccoud, Peng, Feinstein, and Andrzej (2001) and Sansaloni et al., (2011). Using this process, we obtained a dataset of approximately 15,188 SNPs with an average call and reproducibility rate of $95.16 \pm 11.52\%$ (mean \pm standard deviation) and $99.45 \pm 1.12\%$, respectively.

We filtered this SNP dataset using the R packages ‘*Radiator*’ (Gosselin, 2017) and ‘*adegenet*’ (Jombart, 2008; Paradis, 2010) in R version 3.4.4 (R Core Team, 2018), with the final filtered dataset consisting of 2105 SNPs. During the filtering process, we removed any sequence clusters that were monomorphic or duplicated. In cases where multiple polymorphisms were found within the same sequence read, a single SNP was selected at random and retained in order to avoid bias due to physical linkage (Lemay & Russello, 2015). We retained loci with a call rate $\geq 99\%$ and a reproducibility rate $\geq 95\%$. We screened the data for allele coverage and removed any SNPs displaying a read depth of less than 8 and greater than 30 (Lemay & Russello, 2015). We filtered SNPs for minor allele frequencies $< 2\%$, because low frequency SNPs can create biases in the data (Roesti, Salzburger, & Berner, 2012). We used the ‘*hw.test*’ function in the R package ‘*pegas*’ (Paradis, 2010) to assess if any SNPs significantly departed from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using data from adults from a wild population in Albury, NSW, Australia. Loci that significantly departed from HWE following correction for multiple comparisons were removed from the dataset. After our SNP data were finalized, we ensured data integrity by identifying duplicate individuals using a combination of a likelihood method via the program COLONY (Jones & Wang, 2010) and relatedness estimates using the program COANCESTRY (Wang, 2011), as well as correcting labelling errors as appropriate.

Sibship, Paternity, and Relatedness Analysis

We quantified the relatedness (including paternity and sibship) of three captive-born *E. striolata* litters, as well as two *E. striolata* litters from the same females immediately after they were wild-caught. We used COLONY to identify paternity of litters, sibship of juveniles within litters (i.e. half or full siblings), and verified these results using relatedness values generated in COANCESTRY (see details below). This approach used multiple methods of inference to bolster confidence in our analyses. Maternity was known in our study, because females were housed individually while

parturition was monitored. We also counted the number of heterozygous loci and calculated the observed heterozygosity (H_o), by dividing the number of heterozygous loci out of the total number of loci for each individual. At our study site, the average population-level H_o is 0.295 (standard error = 0.003, 95% confidence interval: 0.289, 0.302). We qualitatively compare our results within and between litters, and, unless otherwise specified, data are presented as mean \pm standard deviation.

We used the program COANCESTRY (Wang, 2011) to determine the appropriate relatedness estimator to use because performance relies on the underlying true population genetic structure (Van de Casteele, Galbusera, & Matthysen, 2001; Wang, 2011). So, we simulated multilocus genotype data, based on the allele frequencies of our observed dataset, for 100 individuals for each of these predefined relationships: unrelated ($R = 0$), first cousins ($R = 0.125$), half siblings/avuncular/grandparent-grandchild ($R = 0.25$), full siblings ($R = 0.5$), parent-offspring ($R = 0.5$), twins/clones ($R = 1$). We then calculated pairwise relatedness for this simulated data using seven relatedness estimators (two likelihood estimators: dyadic and triadic likelihood estimators from Milligan, 2003 and Wang, 2007 respectively, as well as five moment estimators: Li, Weeks, & Chakravarti, 1993; Lynch & Ritland, 1999; Ritland, 1996; Queller & Goodnight, 1989; Wang, 2002). We assessed correlation between relatedness estimates and true expected relatedness using Pearson correlation coefficients (R^2 using the R function 'cor': R Core Team, 2018). Based on this analysis, we selected the dyadic likelihood relatedness estimator because it yielded the strongest correlation between true and estimated values ($R^2 = 0.9986$). After this simulation, we calculated pairwise relatedness values for our observed dataset using the dyadic likelihood estimator (Milligan, 2003); these data were used in subsequent analyses to determine relatedness between siblings within the same litter, as well as offspring and parents.

Second, we used COLONY's maximum-likelihood method to assign parentage and sibship groups and to reconstruct genotypes of fathers (Jones & Wang, 2010). COLONY conducts simultaneous inference of multiple relationships among individuals and as such performs with greater statistical power compared to pairwise parentage analysis (Sieberts, Wijsman, & Thompson, 2002; Walling, Pemberton, Hadfield, & Kruuk, 2010). Additionally, COLONY assesses statistical confidence at an individual level established from the proportion of iterations that a specific relationship occurs and by the probability of configurations (Wang & Santure, 2009). Female and offspring genotypes, as well as known maternity of offspring, were entered into COLONY. Potential paternal genotypes were also entered into COLONY that were inclusive of all males we marked and sampled at a nearby long-term study site close to where the females in this study were collected. An estimate of genotyping error (for all loci = 0.0001) was included in the COLONY input, and the program was set to estimate and update allele frequencies throughout the analysis. The COLONY output clusters offspring into full- and half-sibling groups, as well as identifies fathers. The output from COLONY also contains reconstructed paternal genotypes, in the case that a father was not identified in the list of potential fathers, using the most-likely minimum-father combination (Phillips et al., 2013; Wang, 2004).

Results

Immediately post-capture in 2014, two of the three females within this study gave birth, presumably resulting from recent matings (Figure 1, Table 1). One litter consisted of two full siblings, and the other consisted of three full siblings (all probabilities of sibship > 0.99; *R* between offspring within each litter averaged 0.514 ± 0.025). Relatedness between mothers and offspring within each of these litters was 0.510 ± 0.020 (range of 0.491 to 0.537; Table 1). We compared the characteristics of these two litters from 2014 (presumed recent matings) to two litters produced in 2015 from the same females that, at this point, had not had contact with a male for over a year. In both cases, the litters had different fathers than the subsequent litters in 2015 (all probabilities of paternity > 0.99).

Relatedness between reconstructed paternal genotypes and offspring averaged 0.544 ± 0.06 (range 0.500 to 0.627; Table 1). Between litters delivered by the same female in 2014 and 2015, relatedness values averaged 0.227 ± 0.025 , which indicates that between-year juveniles from the same female are half-siblings (all probabilities of half-sibship > 0.99).

In 2015, one year after capture and the last time they could have contact with a male, a total of three females gave birth (Figure 1). Of these three females, two had also given birth in the preceding year (Figure 1). In 2015, two litters consisted of three full siblings, and one litter consisted of two full siblings (all probabilities of sibship > 0.99 ; R between offspring within each litter averaged 0.412 ± 0.08). Relatedness between mothers and offspring within each of these litters averaged 0.489 ± 0.019 (range of 0.435 to 0.496; Table 2). Relatedness between paternal genotypes, reconstructed using COLONY with an exclusion probability over all loci = 0.900 ± 0.140 , while offspring averaged 0.487 ± 0.02 (range 0.435 to 0.500; Table 2). From these relatedness values, it can be concluded that these three litters resulted from Tree Skinks storing sperm rather than parthenogenesis.

Interestingly, the heterozygosity of both paternal and offspring genotypes was lower in 2014 cohorts than in 2015 cohorts (from stored sperm) (Table 3). The number of heterozygous loci of the two fathers in 2014 was 595 ($H_o = 0.283$) and 464 ($H_o = 0.220$), whereas, the number of heterozygous loci of the three fathers for which sperm was stored were 679 ($H_o = 0.323$) and 601 ($H_o = 0.286$) (one male was identified to be the father for two litters from different females; Table 1). The offspring from 2015 cohorts (from stored sperm, $n = 8$) had, on average, 627 (standard deviation = 33, 95% confidence intervals = 576, 676) heterozygous loci, and an average H_o of 0.298 (standard deviation = 0.016, 95% confidence intervals = 0.274, 0.321) (Figure 1). In contrast, the offspring from 2014 cohorts (likely from recent matings, $n = 5$) had, on average, 583 (standard deviation = 44, 95% confidence intervals = 518, 622) heterozygous loci, and an average H_o of 0.277 (standard deviation = 0.021, 95% confidence intervals = 0.246, 0.295) (Figure 1). There is a small overlap between

confidence intervals for both groups, and, unfortunately, we are unable to test the difference between these groups with statistical confidence due to the low samples size. Yet, a qualitative comparison suggests that offspring produced from stored sperm were more heterozygous (Table 3).

Some final notes: None of the potential paternal genotypes that we included in COLONY were identified as fathers for any of the litters, which is not surprising as the females were not collected from the same study site as the wild-living males reside, and Tree Skinks are known to exhibit strong site fidelity (Riley, *unpublished data*). The relatedness between mother and fathers in the 2014 litters ($R = 0.084$ and 0.000) was similar to that in the 2015 litters ($R = 0$ in all cases). Lastly, 41% and 44% of loci were different between paternal genotypes of offspring from the same female in 2015 (stored sperm) and 2014 (presumed recent mating), clearly indicating unique paternal identity.

Discussion

Tree Skinks stored sperm for more than a year. To the best of our knowledge, this is the first time female sperm storage has been determined using molecular methods in any genetically and socially monogamous, family-living reptile. The presence of female sperm storage within the realm of monogamous, social reptiles is a novel phenomenon and it increases the likelihood of sexual conflict. In our study, three females gave birth after being isolated from males for one year. Two of these females had also given birth in the preceding year after capture from the wild; curiously there was no multiple paternity within any litter but there was between litters (i.e., the mate differed between years for the females that gave birth to two litters in this study). We cannot confirm from our data whether the fathers of the 2014 offspring were from a female's social partner, an extra-pair mating from that year (i.e., recent matings), or were a result of previously stored sperm. Relatedness between mating pairs was similar between years (i.e., unrelated in all cases), but the paternal and offspring genotypes from litters resulting from stored sperm were more heterozygous. Overall, this study identified female polyandry through sperm storage in Tree Skinks, which introduces the possibility for sexual conflict

in this and other egeriine skink's social and mating systems. Yet, it is important to note that our study is based on opportunistic observations in captivity, and it is unknown the rate of sperm storage and the extent of between-year multiple paternity that arises from this phenomenon in natural populations, which limits our ability to draw conclusions on any ecological implications.

Regardless, the costs and benefits of female sperm storage in Tree Skinks may differ from those of other reptiles but have similarities to other social vertebrate taxa. Tree Skinks live at relatively high densities in family groups (Riley, *unpublished data*), contrary to the hypothesis that sperm storage occurs in species with low encounter rates driven by low densities or large home ranges (Orr and Zuk 2012). An alternative explanation proposed for the evolution of female sperm storage is that it occurs in species with asynchronous life histories and/or contrasting dispersal rates between sexes (Orr and Zuk 2012). However, Tree Skink dispersal, life-history, and reproductive cycles are similar between the sexes (Chapple, 2003; Riley, *unpublished data*), thus female sperm storage cannot be explained by these hypotheses either. Sperm storage is common in lizards (Uller & Olsson, 2008), so in Tree Skinks it might be a result of phylogenetic conservatism. Yet, even in this context, female sperm storage arising from female multiple mating may be adaptive, either by indirectly increasing reproductive fitness through offspring benefits (e.g. increased genetic diversity) or by direct fitness benefits through the ability to reproduce at all.

The indirect fitness benefits of female sperm storage and polyandry are well-documented across vertebrates, including lizards (Wapstra & Olsson, 2014). For example, females that mate multiply may be genetically bet-hedging by reducing the probability of genetic incompatibility or inbreeding, and sperm storage can facilitate cryptic female choice. In Swedish Sand Lizards (*Lacerta agilis*), the chance of producing non-viable inbred offspring is reduced when females mate multiply and actively select sperm from distantly-related males (Olsson, Shine, Madsen, Gullberg, & Tegelström, 1996). In our study, mate pairings from recent litters and litters arising from stored sperm were equally

unrelated. However, Tree Skinks live in family groups that are clustered spatially (Riley, *unpublished data*); thus multiple mating outside the social or spatial ‘norm’, as well as storing that sperm, may be an act of inbreeding avoidance by females. Moreover, Tree Skinks can discriminate between kin and non-kin, either by using visual or chemosensory cues (Bull, Griffin, Bonnett, Gardner, & Cooper, 2001). Thus, in this system and within the current study, we did not find evidence for an indirect benefit of inbreeding avoidance.

In contrast, we observed that paternal and offspring genotypes for which female Tree Skinks stored sperm were qualitatively more heterozygous than those from recent matings. Offspring fitness can be positively enhanced by females choosing heterozygous males (Foerster, Delhey, Johnsen, Lifjeld, & Kempnaers, 2003; Fromhage, Kokko, & Reid, 2009; Hoffman, Forcada, Trathan, & Amos, 2007; Ryder, Tori, Blake, Loiselle, & Parker, 2010). Thus, female Tree Skinks may indirectly benefit from mating outside a social pair bond if their mate exhibits higher genotypic heterozygosity. Many animals have been found to select for mates that have high heterozygosity or greater dissimilarity within major histocompatibility complex (MHC) genes (i.e., Atlantic Salmon, *Salmon salar*: Landry, Garant, Duchesne, & Bernatchez, 2001; Brown Anoles, *Anolis sagrei*: Calsbeek, Bonneaud, Prabhu, Manoukis, & Smith, 2007; mice, *Mus musculus domesticus*: Potts, Manning, & Wakeland, 1991; Seychelles Warbler, *Acrocephalus sechellensis*: Richardson, Komdeur, Burke, & Von Schantz, 2005; reviewed in Kamiya, O'dwyer, Westerdahl, Senior, & Nakagawa, 2014). Further, a synergistic beneficial effect can arise when polyandry and mate genetic diversity is selected. For example, Tree Swallows (*Tachycineta bicolor*), which are socially monogamous, have high levels of extra-pair paternity in their clutches, and extra-pair offspring are consistently more heterozygous than their half-siblings (Stapleton, Kleven, Lifjeld, Robertson, 2007). Blue tit (*Cyanistes caeruleus*) offspring fitness is positively related to their heterozygosity (Foerster et al., 2003). Furthermore, offspring survival in other taxa (e.g., Decorated Crickets, *Grylloides sigillatus*: Ivy & Sakaluk, 2005; and Grey Foam Nest Treefrogs, *Chiromantis xerampelina*: Byrne & Whiting, 2011) has similarly been found to be higher in polyandrous matings. Although we didn't observe multiple paternity within litters, the paternity

differed between litters from the same females across years. Thus, it may be that females receive indirect benefits from multiple mating because male identity and genetic diversity differ between litters (those from stored sperm vs. those from presumed recent mating). This between-litter pattern of extra-pair mating generates many questions; for example, are mate-order effects, cryptic female choice, and/or sperm competition involved? And what are the proximate causes of sperm storage and this pattern of multiple paternity in Tree Skinks?

It is still largely unknown how low levels of polyandry through sperm storage, as we have uncovered in Tree Skinks, may affect the degree of sexual conflict within monogamous reptilian systems. Yet, insights can be gained from another family-living egegnine species, the Gidgee Skink (*Egernia stokesii*). In this species, mate choice is affected by both group membership and MHC genes (Pearson, Godfrey, Schwensow, Bull, & Gardner, 2017). Gidgee Skinks were most likely to mate with an individual from within their social group, and, in addition, females were more likely to mate with a male with higher MHC genotypic diversity and lower relatedness (Pearson et al., 2017). We believe similar factors may be involved in mate choice, female sperm storage, and the low occurrence of extra-pair mating in Tree Skinks. First, female Tree Skinks may prioritize mating with individuals within their social group (i.e., their social partner). But they may also bet-hedge through extra-pair mating (at a low frequency) and sperm storage, potentially favouring higher heterozygosity of male genotypes that then result in more heterozygous offspring. This hypothesis regarding Tree Skink mating behaviour, based on the few observations within this study, needs further testing. Additional research is needed to untangle the direct costs and benefits of sperm storage facilitating extra-pair mating.

Female sperm storage and polyandry, even at low levels, has the potential to directly increase female fitness at a cost to the male (i.e., sexual conflict). One direct benefit of sperm storage for females is that it ensures the availability of sperm for fertilization, which is also a known benefit of female

polyandry (fertilization insurance hypothesis: Briefer, Farrell, Hayden, & McElligott, 2013; Byrne & Whiting, 2008; Parker, 1970). In our study, females were collected from the wild and housed in captivity, so fertilization by stored sperm could have been triggered in response to this change and reproductive isolation. Further, documenting the co-occurrence of female sperm storage and polyandry in Tree Skinks has broad implications because social reptiles are largely monogamous. The role it may play in these species' social and mating systems is yet to be determined. For example, males' tolerance of offspring in the White's Skink (*Liopholis whitii*) depends on relatedness (While et al., 2009): offspring sired by males other than the social partner are not tolerated by the resident male and are restricted to associations with their mother. Thus, female polyandry similarly risks incurring costs in egegnine skinks; but sperm storage itself, especially if sperm can be stored for long periods, may directly reduce the costs of mating outside a social pairing. Sperm storage could reduce the number of times a female has to engage in risky extra-pair mating that could cause injury, and decrease the probability that their social mate may observe such extra-pair mating and thus have social consequences for her or her offspring. Yet, it is unknown whether chemosensory or observational cues influence male interactions within their social group in egegnine skinks. In other taxa, particularly mammalian species with altricial young (Blumstein, 2000; Hausfater & Hrdy, 2008), infanticide of unrelated offspring is widespread, for example in primates (Van Schaik & Kappeler, 1997) and carnivores (Bellemain, Swenson, & Taberlet, 2006; Packer & Pusey, 1983). Further, punishment by primate males against polyandrous females includes physical retribution and sequestration of females from their social group (Muller, Kahlenberg, & Wrangham, 2009). At this time, we can only speculate as to the direct consequences of social mates observing extra-pair mating in Tree Skinks or other egegnine skinks. Regardless, the benefits of extra-pair mating, potentially even via female sperm storage, must also be weighed against any social costs.

Overall, our demonstration of female sperm storage in Tree Skinks and the patterns of paternity and heterozygosity we have uncovered sets the stage for valuable future research. First steps include studying the structure of Tree Skink reproductive tract, which could uncover how sperm storage, as

well as the sequential nature of multiple paternity between litters, is facilitated. Also, we need to establish how widespread sperm storage is in social reptiles, and the degree to which females may be manipulating paternity and, by extension, enhancing their own fitness. We suggest that female sperm storage should be examined in a greater diversity of taxa and in variable social and mating systems in order to properly understand the selective pressures in regard to these traits. It may be that a low frequency extra-pair mating is all that is required to promote heterozygosity and increase female fitness, when sperm storage is possible. If this is the case, females may well have the upper hand in a world often dominated by sexual conflict.

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Data Accessibility

A view-only link for this study's data on the relatedness between litters and their parents, as well as genetic heterozygosity, is: https://osf.io/972wz/?view_only=f94f2c62c6be47b2a3cae86c6c2a9e3a

Please note, this will change to a unique DOI once the OSF repository is made public upon acceptance.

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Tables

Table 1. Litters from 2014 that were born immediately after females were captured from the wild. Fathers were assigned with 95% confidence using COLONY. Relatedness was calculated using the dyadic likelihood estimator (Milligan, 2003) in COANCESTRY, and father-offspring values were calculated using reconstructed male genotypes from COLONY.

Litter Number	Mother ID(s)	Juvenile ID	Father ID	Mother-Offspring Relatedness	Father-Offspring Relatedness
1	3371916	B0020	3	0.54	0.50
		B0025		0.52	0.50
		B0030		0.51	0.50
2	3366628	B0121	9	0.52	0.63
		B0130		0.49	0.59

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Table 2. Litters from 2015 that were born after females were in captivity for one year without exposure to males. Fathers were assigned with 95% confidence using COLONY. Relatedness was calculated using the dyadic likelihood estimator (Milligan, 2003) in COANCESTRY, and father-offspring values were calculated using reconstructed male genotypes from COLONY.

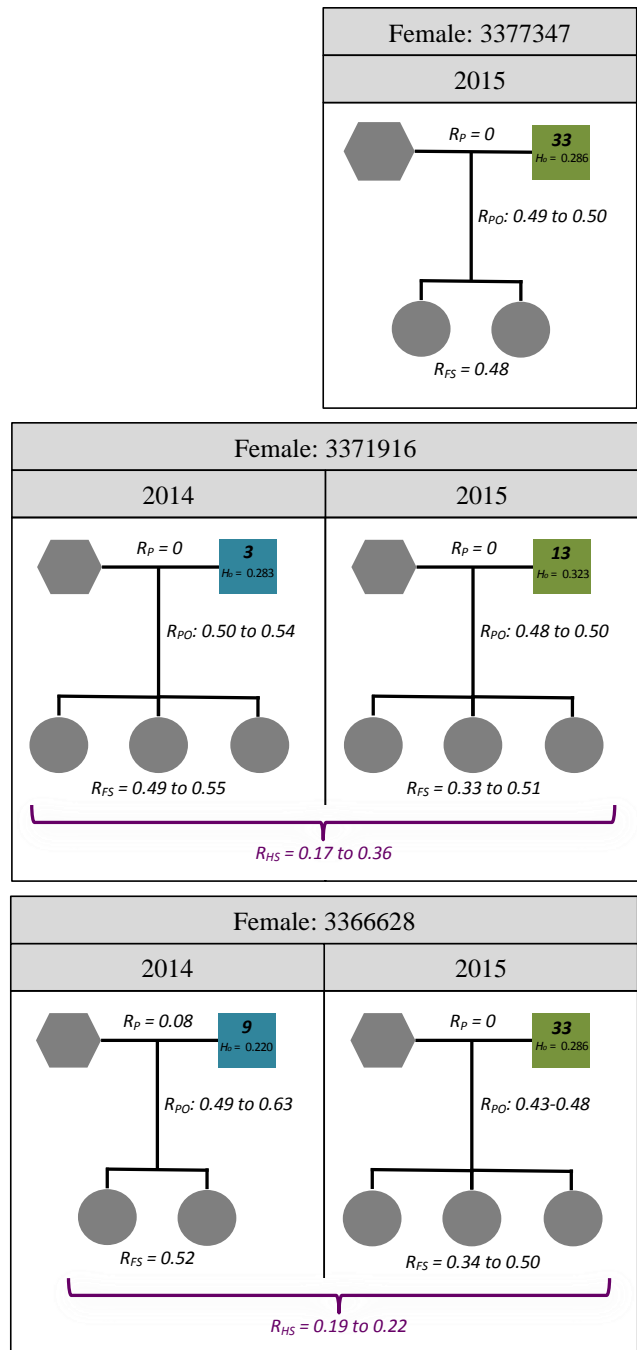
Litter Number	Mother ID(s)	Juvenile ID	Father ID	Mother-Offspring Relatedness	Father-Offspring Relatedness
1	3371916	B0055	13	0.48	0.50
		B0100		0.50	0.50
		B0102		0.49	0.50
2	3377347	B0103	33	0.49	0.50
		B1005		0.48	0.50
3	3366628	B1053	33	0.48	0.48
		B1054		0.44	0.43
		B1055		0.48	0.48

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Table 3. A summary of heterozygosity of Tree Skink (*Egernia striolata*) litters from 2014, which were inferred to be from recent mating, and from 2015, which arose from stored sperm. We present the number of heterozygous loci (n_{HL}) and observed heterozygosity (H_o) for each individual. For sires and dams, values for both measures of heterozygosity are presented in brackets after their identification numbers.

Mother ID(s)	Father ID	Juvenile ID	Number of Heterozygous Loci (n_{HL})	Observed Heterozygosity (H_o)
2014 Cohorts (Recent Mating)				
3371916 ($n_{HL} = 594, H_o = 0.282$)	3 ($n_{HL} = 595, H_o = 0.283$)	B0020	591	0.281
		B0025	624	0.296
		B0030	607	0.288
3366628 ($n_{HL} = 576, H_o = 0.274$)	9 ($n_{HL} = 464, H_o = 0.220$)	B0121	585	0.278
		B0130	510	0.242
Average \pm Standard Error			583 \pm 44	0.277 \pm 0.009
2015 Cohorts (Sperm Storage)				
3371916 ($n_{HL} = 594, H_o = 0.282$)	13 ($n_{HL} = 679, H_o = 0.323$)	B0055	655	0.311
		B0100	613	0.291
		B0102	636	0.302
3377347 ($n_{HL} = 651, H_o = 0.309$)	33 ($n_{HL} = 601, H_o = 0.286$)	B0103	680	0.323
		B1005	631	0.300
3366628 ($n_{HL} = 576, H_o = 0.274$)	33 ($n_{HL} = 601, H_o = 0.286$)	B1053	604	0.287
		B1054	570	0.271
		B1055	630	0.299
Average \pm Standard Error			627 \pm 33	0.298 \pm 0.006

Figures



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Figure 1. Litters in 2014 and 2015 of all female Tree Skinks (shown using a hexagon) in our study. Sires are shown using a square (green if litters resulted from stored sperm and blue if inferred to result from recent matings). The unique number reflecting the identity of the sire is within the square in bold, and the observed heterozygosity (H_o) of the sire has is also presented within the square. More detailed data on H_o can be found in Table 3. The offspring are shown using circles, and connections between half-siblings across years are shown using a purple bracket below the circles. Relatedness estimates, or their range in the case of multiple pairwise estimates, are shown between mothers and fathers (R_P), parents and offspring (R_{PO}), full-siblings (R_{FS}), and half-siblings (R_{HS}).

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