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Very low rate of multiple paternity detected in clutches of a wild agamid lizard

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Abstract. Genetic mating systems described for squamate reptiles range from primarily monogamous to completely polygynandrous. The presence of female multiple mating is almost ubiquitous among squamates and even occurs, albeit at a low rate, in socially monogamous species. Here we examine the genetic mating system of the territorial tawny dragon lizard (*Ctenophorus decresii*). Paternity was assigned to captive-born hatchlings using eight microsatellite loci, revealing a 4% rate of multiple paternity. One-quarter of males sired more than one clutch, although multiple mating by males is likely underestimated. The rate of multiple paternity in *C. decresii* represents one of the lowest among squamates and may be a result of successful male territoriality. However, the observed low rate of multiple paternity does not eliminate the possibility of widespread female multiple mating due to the potential for sperm storage and sperm competition. We conclude that the tawny dragon lizard employs a predominantly polygynous genetic mating system.

Additional keywords: *Ctenophorus decresii*, genetic mating system, polygyny.

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Introduction

The evolution of mating systems involves a delicate balance between the costs and benefits of mating to each sex (Birkhead and Møller 1998). Male multiple mating is expected in most taxa due to the direct benefits involved, whereas the benefits of multiple mating by females are often not immediately apparent (Barbosa *et al*. 2012; Uller and Olsson 2008). Male multiple mating has been detected to varying degrees in all squamate reptiles where male mating patterns have been examined (Table S2, Supplementary Material). Furthermore, the presence of female multiple mating is almost ubiquitous among squamates (Table S2, Supplementary Material). This is true even within socially monogamous species, although rates of male and female multiple mating are relatively low (Bull *et al*. 1998; Gardner *et al*. 2002). The only squamate species for which female multiple mating has not been detected is the great desert skink, *Liopholis kintorei* (McAlpin *et al*. 2011), some true sea snake species (Lukoschek and Avise 2011) and a species of garter snake (Wusterbarth *et al*. 2010). However, the studies investigating female multiple mating in snakes (Lukoschek and Avise 2011; Wusterbarth *et al*. 2010) comprised very small sample sizes, ranging from one to four individuals per species (Table S2, Supplementary Material) and that of *L. kintorei* did not include full clutches but wild-caught animals only. The high incidence of female multiple mating among squamates implies that females may benefit from multiple mating. Indeed, there are several examples of indirect benefits associated with female multiple mating (Eizaguirre *et al*. 2007; Frère *et al*. 2015; Madsen *et al*. 1992; Noble *et al*. 2013). However, the sexual conflict hypothesis predicts that this may not always be the case: the occurrence of female multiple mating is sometimes governed by the cost of mating versus the cost of resisting male sexual advances (Chapman *et al*. 2003; Keogh *et al*. 2013; McLean *et al*. 2010; York and Baird 2015).

Female multiple mating within and between reproductive cycles can lead to multiple paternity within clutches or litters in squamate reptiles (Uller and Olsson 2008). However, the relationship between multiple mating and multiple paternity is not straight-forward in lizards and snakes; the potential for cryptic female postcopulatory choice and sperm competition (Friesen *et al*. 2014; Tolley *et al*. 2014; Uller *et al*. 2013) means that multiple mating may not always result in multiple paternity. As a result, the genetic mating system cannot be used to infer the behavioural mating system in squamate reptiles. The rate of multiple paternity is highly variable among squamates, ranging from 2.6% in Cunningham's skinks (*Egernia cunninghami*) to 100% in Cape dwarf chameleons (*Bradypodion pumilum*) (Table S2, Supplementary Material). Uller and Olsson (2008) argue that the main driver of this variation is mate encounter rate. Factors that are likely to influence mate encounter rate, and hence rates of multiple mating, include population density (Jensen *et al*. 2006), social pair formation (Bull *et al*. 1998;

Gardner *et al*. 2002; Stow and Sunnucks 2004), territoriality (York and Baird 2015) and mate-seeking behaviours (Glass *et al*. 2016).

Here, we investigated the genetic mating system of a wild population of colour polymorphic tawny dragon lizards (*Ctenophorus decresii*), a small (<30 g) rock-obligate agamid lizard endemic to South Australia. We used a robust captive hatching method that ensured that all maternal relationships were known and all members of a clutch were sampled. Sampling of the male population allowed paternity assignment using a maximum-likelihood approach. We hypothesise that *C. decresii* exhibits a predominantly polygynous genetic mating system, with a relatively small portion of clutches containing multiple paternity, as is the case in other species of *Ctenophorus* (Lebas 2001; Olsson *et al*. 2007*a*). Defining the genetic mating system employed by *C. decresii* will provide a better understanding of the basic biology of the species and will direct future work investigating its reproductive biology and factors influencing mate choice.

Materials and methods

Sample collection

During spring and summer between 2013 and 2015 individual tawny dragons were collected from a single location near Hawker in the central Flinders Ranges, South Australia (31°57′12.0″S, 138°22′20.3″E). Males were released at the point of capture after a blood sample (≤50 µL) was taken. During the spring of 2014, 23 gravid females were captured from the Hawker field site and taken to Flinders University, Adelaide, to lay their eggs. Females were subsequently released at the point of capture at the Hawker field site after a blood sample (≤50 µL) was taken. Both males and females were marked with a unique number using a non-toxic paint pen to ensure they were not recaptured and could be identified by sight. Hatchlings were housed individually at the Flinders University Animal House. At 12 weeks of age, a blood sample (≤15 µL) was taken from hatchlings before release near their mothers' capture locations. Refer to supplementary material (Text S1, Supplementary Material) for future details on captive hatching methodology. Blood was taken from adults and hatchlings via venipuncture of the *sinus angularis*, located inside the mouth. Whole blood was preserved on Whatman® Classic FTA cards (GE Healthcare, Buckinghamshire, UK) and stored at room temperature until DNA extraction. Five hatchlings died of natural causes and were frozen at −20°C until a tissue sample (5 mm tail tip) was taken for DNA extraction.

Microsatellite amplification

Hatchling paternity was determined from the genotypes of eight microsatellite loci. All mothers ($n = 23$) and their offspring ($n = 139$), and all adult males ($n = 90$, >70 mm snout–vent length) within 100 m of mothers were genotyped using eight previously published microsatellite markers designed for *C. decresii* (Ctde03, Ctde05, Ctde08, Ctde12, Ctde21 and Ctde45: McLean *et al.* 2014) or the closely related *Ctenophorus pictus* (CP10 and CP11: Schwartz *et al*. 2007). DNA was extracted from blood stored on FTA cards according to Smith and Burgoyne (2004) and tissue DNA extraction was undertaken using a Gentra® Puregene® (Gentra Systems) method. Microsatellite amplification reaction protocols followed McLean *et al*. (2014). Briefly, all loci were amplified singly and PCR reactions were 12.5 µL in total volume, containing 1× MRT buffer (4 mM dNTP, 0.25 mg mL^{-1} BSA), 0.4 µM of forward and reverse primers, 0.5 U of Immolase enzyme and ~20 ng of template DNA. Thermal cycling conditions for Ctde03, Ctde21, Ctde45, CP10 and CP11 consisted of initial denaturisation at 95°C for 10 min followed by 42 cycles of 95°C for 30 s, 48°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. For the remaining loci (Ctde05, Ctde08 and Ctde12) thermal cycling conditions consisted of initial denaturisation at 95°C for 10 min followed by four different annealing steps. These annealing steps included two cycles each of 95°C for 30 s, annealing temperatures of 60°C, 55°C and 50°C for 30 s followed by 72°C for 45 s. The last annealing step consisted of 35 cycles of 95°C for 30 s, 45°C for 30 s followed by 72°C for 45 s. A final extension of 72°C for 10 min was used. After diluting and pooling per individual, PCR products were sent to the Australian Genome Research Facility for fragment visualisation. Fragment sizes were called using the GS500(–250)LIZ size standard in GeneMapper® 4 (Applied Biosystems).

Paternity assignment and determination of the genetic mating system

Relatedness values among all adults were estimated using Coancestry 1.0.1.5 (Wang 2007). All microsatellite loci were tested for deviation from Hardy–Weinberg equilibria (HWE) and the presence of linkage disequilibrium using Genepop 4.5.1 (Rousset 2008) based on a subset of unrelated adults (<0.25 relatedness value, $n = 23$). The number of alleles, number of individuals typed, the observed and expected heterozygosities, and polymorphic information content were calculated for each locus using Cervus 3.0.7 (Kalinowski *et al*. 2007), based on all adults ($n = 113$). Cervus was also used to determine locus-specific error rates (including null alleles) based on known mother–offspring mismatches, with the average probability of detecting a mismatch taken into account (Kalinowski *et al*. 2007).

To estimate the rate of multiple paternity within *C. decresii* clutches we counted the number of paternal alleles at each locus for each clutch. More than two paternal alleles at two or more loci within a clutch was considered evidence of multiple paternity (Fitzsimmons 1998; Gardner *et al*. 2002; Todd *et al*. 2012).

Paternity assignment was conducted using Colony 2.0.6.2 (Jones and Wang 2010). Colony takes a maximum likelihood approach to assigning both sibship and parentage relationships and deals well with deviations from HWE, null alleles and other stochastic errors (Jones and Wang 2010). Males captured in the season preceding (2013–14) the collection of gravid females were included as potential fathers in paternity analyses in addition to males captured during the same season as females (2014–15). Male *C. decresii* live for at least five years in this population (Yewers 2016) and some males may not have been captured, despite being present during the 2014–15 season, given the low recapture rate reported by Yewers (2016). Another reason for including males from the previous season is the potential for between-season female sperm storage. Between-season sperm storage has been reported for other lizards (Uller *et al*. 2010) and

sperm storage has been reported in congeners *C. fordi* and *C. pictus* (Olsson *et al*. 2007*b*; Olsson *et al*. 2009; Uller *et al*. 2013). However, in contrast to *C. decresii*, both *C. fordi* and *C. pictus* are short-lived and most individuals die after their first reproductive season (Olsson *et al*. 2007*a*; Olsson *et al*. 2009; Uller *et al*. 2013), limiting the ability to test for between-season sperm storage in these species. In a captive *C. decresii* population only a single case of within-season sperm storage and no cases of between-season sperm storage occurred in 25 clutches (23 sires and 17 dams) (Rankin *et al*. 2016). Hence, although between-season female sperm storage is unlikely to occur in *C. decresii* it cannot be discounted and remains a possibility.

COLONY requires information on the mating system employed (male and female multiple mating) when assigning paternity. We discovered evidence of multiple paternity based on the number of paternal alleles at loci within clutches. Therefore, to infer paternity for *C. decresii* we assumed male multiple mating and undertook two COLONY runs, one with the mating system set to polygyny (only male multiple mating) and the other set to polygynandry (both male and female multiple mating). For both runs the run length was set to 'very long', which included a high number of configurations in the simulated annealing algorithm used by COLONY (Wang 2017). All maternal sibship relationships were known, no males were excluded as fathers and the probability that a father was included among the candidates was set to 0.75. Relationships (sibship and paternity) with a probability value above 0.95 were accepted. Microsatellite error rates, including estimated null allele frequency, were taken into account during COLONY analyses (Wang 2004). The spatial position of fathers relative to mothers was plotted using ARCMAPS® (ArcGIS Ersi) and was used to validate paternity assignment.

Results

The polymorphic information content was high for all loci except for CP11 due to the low number of alleles at this locus (Table 1). However, this locus was retained as the error rate was acceptable and it did not deviate from HWE. The error rates for all loci were acceptable. For all loci, except CP10 and CP11, most or all of the mismatches were likely caused by null alleles (Table 1). Mismatches associated with CP10 and CP11 may be due to mistyping or mutations. None of the microsatellite loci pairs were in linkage disequilibrium, although three loci (Ctde03, Ctde05 and Ctde21) were found to deviate from HWE. The slight deviations from HWE for Ctde03 and Ctde21 were likely caused by null alleles (Pemberton *et al*. 1995). As there were no mismatches associated with Ctde05 the source of the deviation of this locus from HWE is unknown (Kalinowski *et al*. 2007). As COLONY analyses are error-tolerant and are able to deal with some loci deviating from HWE (Wang 2004), all eight microsatellite loci were considered suitable to use for paternity assignment. All candidate males and mothers had unique genotypes.

We uncovered evidence for multiple paternity in a single clutch (1 of 23). This clutch had three paternal alleles at four different loci. No other clutches had more than two paternal alleles at any loci (Table S3, Supplementary Material). The polygyny COLONY run assigned paternity with high probability values for 21 of the 23 clutches. Paternity probability values were greater than or equal to 0.95 for all but one clutch, which had a paternity probability of 0.83 (clutch of Female 987 and Male 700) (Table S3, Supplementary Material). This paternity assignment was accepted because the lower probability was likely due to the presence of null alleles and missing data in the male, and the assigned male was in close proximity to the mother (12 m). In total, 16 males were identified as fathers and a quarter of those males sired more than one clutch. Three fathers were captured only in the season prior to captive hatching, all of which were captured in autumn (March). The polygynandry COLONY run produced the same paternity assignments as the polygyny run but paternity probability values were slightly lower for some hatchlings, which was likely due to the presence of null alleles in those hatchlings (Jones and Wang 2010). For both the polygyny and polygynandry COLONY runs single paternity was assigned to the clutches that were previously identified as lacking multiple paternity ($\leq$2 alleles at loci) (Table S3, Supplementary Material). Paternity was not assigned to two clutches, one of which was the clutch that was sired by multiple males (three paternal alleles at four loci). It is therefore likely that the fathers contributing to that clutch were not sampled. The other clutch for which paternity was not assigned was likely sired by a single unsampled male. All fathers were within close proximity to mothers (mean = 28 m, range = 5–83 m) (Fig. 1). Overall, paternity was assigned with high confidence and a 4% (1 of 23) rate of multiple paternity was uncovered.

Table 1. Microsatellite characteristics

k, number of alleles; *N*, number of individuals typed; H_o, observed heterozygosity; H_e, expected heterozygosity; PIC, polymorphic information content; Exclusion prob., mean probability of excluding an incorrect parent, given one parent is known; Error rate, estimated on basis of known parent–offspring mismatches (null allele rate is provided in parentheses); HWE *P*, Hardy–Weinberg equilibrium exact test *P*-value. HWE calculations are based on a subset of unrelated individuals ($r < 0.25$, $n = 23$). *, statistically significant at $P < 0.05$

Locus	*k*	*N*	H_o	H_e	PIC	Exclusion prob.	Error rate	HWE *P*
Ctde03	20	103	0.699	0.890	0.875	0.773	0.025 (0.024)	0.045*
Ctde05	19	111	0.793	0.928	0.919	0.846	0.000 (0.000)	0.025*
Ctde08	15	111	0.775	0.899	0.886	0.789	0.033 (0.033)	0.517
Ctde12	18	112	0.893	0.907	0.896	0.806	0.000 (0.000)	0.374
Ctde21	13	105	0.571	0.866	0.847	0.723	0.063 (0.063)	0.043*
Ctde45	12	106	0.708	0.865	0.847	0.727	0.025 (0.025)	0.675
CP10	21	107	0.720	0.905	0.892	0.801	0.029 (0.000)	0.601
CP11	4	112	0.598	0.555	0.500	0.310	0.023 (0.000)	0.261

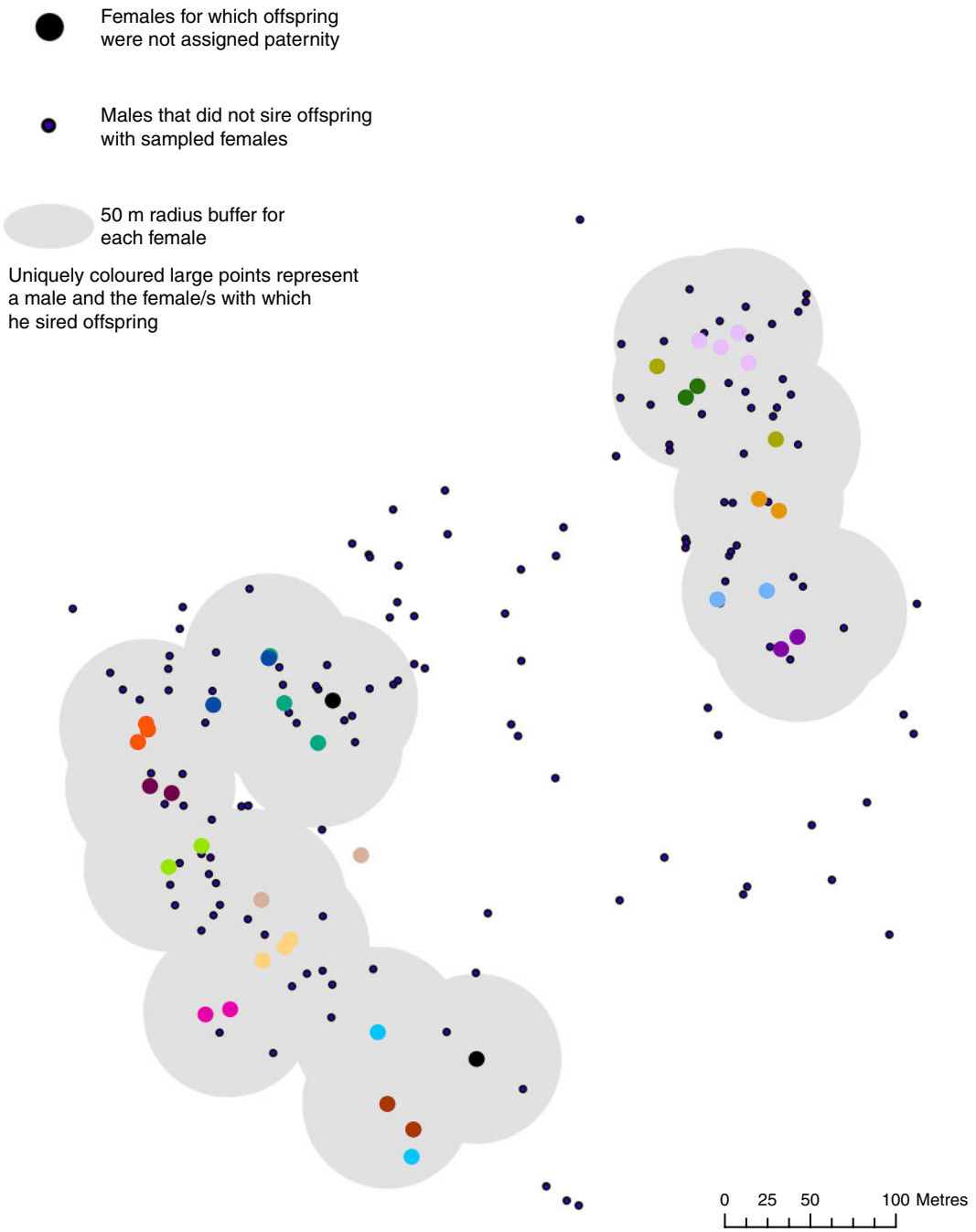

Fig. 1. Spatial position of females (mothers) and all adult males sampled. One-quarter of the males that sired offspring did so with more than one sampled female (range = 1–3) and most clutches (96%) were sired by a single male.

Discussion

Tawny dragons display a territorial social structure with males occupying home ranges that exclude other males (Yewers 2016) and are likely to encompass several females. We tested the hypothesis that *C. decresii* employs a predominantly polygynous genetic mating system, including instances of multiple paternity. Paternity assignment produced 21 complete family groups, with a single male siring each clutch. However, upon inspection of the two clutches not assigned paternity, one indicated that at least two males contributed to the clutch (multiple paternity). Hence, out of 23 clutches, one (4%) consisted of multiple paternities. A total of 16 sampled males contributed to the 21 clutches for which paternity was assigned and at least 18 males contributed to the total sample of 23 clutches. Of the sampled males, 25% (4 of 16) sired more than one clutch. However, male multiple mating is probably underestimated as it is likely that some males also mated with females that were not sampled. Consequently, we

accept our hypothesis that a predominantly polygynous genetic mating system is employed by *C. decresii*, with at least a quarter of fathers siring more than one clutch and a single case of multiple paternity detected. The extremely low rate of multiple paternity observed here is the third lowest reported for squamates; only Cunningham's skinks (*Egernia cunninghami*) (3%: Stow and Sunnucks 2004), the great desert skink (*Liopholis kintorei*) (0%: McAlpin *et al*. 2011) and potentially some snake species (0%: Lukoschek and Avise 2011; Wusterbarth *et al*. 2010) have lower reported rates of multiple paternity. However, it is possible that the rate of multiple paternity in these studies is underestimated. In the case of both *E. cunninghami* and *L. kintorei* multiple paternity was determined by sampling juveniles within the population. Therefore, some clutch members may have been missed during sampling. Furthermore, the very small sample sizes in the snake studies (1–4 individuals per species) limits the ability to detect cases of multiple paternity.

Uller and Olsson (2008) predicted that the low rates of multiple paternity in congeners *C. pictus* and *C. ornatus* (18% and 25%, respectively) are due to their highly territorial behaviour. However, in *C. ornatus*, Lebas (2001) found that 65% of clutches were sired completely or partially by a male that was not the main territory-holder, which may indicate that a mating strategy other than strict territoriality is employed, or territoriality is not an effective strategy for *C. ornatus*. Recent work suggests that male *C. decresii* are highly territorial and defend areas of 213 m^2, on average (Yewers 2016). Hence, the extremely low rate of multiple paternity observed for *C. decresii* may be associated with successful male territoriality. However, the factors that influence male territory size and the effectiveness of territoriality have not been investigated in *C. decresii*. Other factors may contribute to the lack of multiple paternity in most clutches. For instance, females may reject males other than the territory-holder or reject additional mates after the first mating (McLean *et al*. 2010). Sperm storage and sperm competition has been uncovered in congeners *C. pictus* (Olsson *et al*. 2009) and *C. fordii* (Uller *et al*. 2013). Therefore, another possibility is that female multiple mating in conjunction with cryptic postcopulatory mate choice or sperm competition results in single paternity clutches (Moore *et al*. 2009). The fact that some fathers were captured only in the season prior to captive hatching suggests that females may store sperm between seasons (from autumn to spring). However, another possibility is that these males mated with females in the same season as captive hatching but evaded capture.

A combination of behavioural and molecular data is required to gain a complete picture of the mating system employed by *C. decresii*. If successful male territoriality and/or female rejection tactics are the main drivers of the low rate of multiple paternity in *C. decresii*, the genetic mating system likely reflects the behavioural mating system. However, due to the potential for sperm storage and sperm competition, cryptic female multiple mating cannot be ruled out. The nature of both the genetic and behavioural mating system used by a species has important implications for several aspects of its biology. For example, mating systems and associated behaviours influence connectivity among individuals, which can affect rates of parasite transmission (van Schaik and Kerth 2017; White *et al*. 2011). Furthermore, polygyny associated with territoriality or harem structure can severely depress effective population size due to skew in male reproductive success within the population (Nunney 1993). Reduced effective population size can limit the evolutionary potential of populations to deal with environmental change (Franklin 1980), accelerate the rate of accumulation of deleterious mutations (Lande 1995), and inhibit the fixation of beneficial alleles (Whitlock and Bürger 2004).

The type of mating system employed by a species may differ spatially or temporally (Moore *et al*. 2009; Natoli *et al*. 2017; Phillips *et al*. 2013). Therefore, the genetic mating system described for this *C. decresii* population may not necessarily be employed throughout the entire season, across years or across all populations. For instance, the mating system employed by a tuatara population was examined over three years; in two of those years multiple paternity was observed but in one year there was no genetic evidence of female multiple mating (Moore *et al*. 2009). Population density is likely to influence mate-encounter rates and hence rates of multiple paternity. Therefore, spatial or seasonal variation in population density may result in varying rates of multiple paternity (Natoli *et al*. 2017). The population on which this study is based has a relatively high population density (C. McLean and D. Stuart-Fox, unpubl. data). Therefore, in the case of *C. decresii*, geographic variation in population density is unlikely to cause higher than observed levels of multiple paternity. However, further work is required to determine whether other factors influence rates of multiple paternity in *C. decresii*.

A predominantly polygynous genetic mating system was described for *C. decresii*, with a single clutch sired by more than one male. This result is consistent with those for other species of *Ctenophorus*, although the rate of multiple paternity was much lower than expected. This extremely low rate of multiple paternity may be due to mating behaviour such as male territoriality, or postcopulatory mechanisms such as sperm competition. Behavioural observations and experiments will provide further insights into the behavioural mating system employed by *C. decresii*, and wider spatial and temporal sampling will determine whether our results are representative of other *C. decresii* populations and whether there is seasonal variation in the genetic mating system employed by *C. decresii*. Our results provide a foundation for future work examining the factors influencing mate choice and reproductive success in the tawny dragon lizard.

Conflicts of interest

The authors declare no conflicts of interest.

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