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SHORT COMMUNICATION

Genetic evidence for polyandry in the threatened green and golden bell frog

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Abstract

Identifying which species exhibit polyandry may lead to further insights into evolutionary biology and social behaviour. However, confirming polyandry can be difficult. High-resolution genetics provides a useful means to gain evidence. Although the threatened Pelodryadid frog, the green and golden bell frog *Litoria aurea*, has been subject to numerous ecological studies, there is uncertainty surrounding its reproductive ecology. Polyandry has not been formally identified in *L. aurea* or any species within the Pelodryadidae family. We aimed to identify if there was genetic evidence of polyandry in a population occurring in a wetland complex on Kooragang Island, New South Wales. To accomplish this, we collected genetic samples of tadpoles within the same size cohort about 20–30 days after explosive breeding events. Genotypes of 14 females, nine males and 70 tadpoles were analysed with COLONY (1988 single nucleotide polymorphisms after filtering) to identify parentage, full-siblings and half-siblings. We found support for the hypothesis that *L. aurea* is polyandrous. Based on previous observations of multi-male matings and the narrow time periods that breeding occurred in, it is likely this species exhibits simultaneous polyandry. We discuss these results in regards to behavioural adaptive processes and avenues for further research.

Keywords Green and golden bell frog · Single-nucleotide polymorphisms · Amphibian evolution · COLONY · Mating system

Introduction

Polyandry, as defined as when a female reproduces with multiple males during a single reproductive cycle, has been a revolutionary field in evolutionary biology (Parker and Birkhead 2013). A preconceived assumption that only males benefit from reproducing with multiple partners has been over-turned in light of the discovery that an increasing number of species exhibit polyandry (Taylor et al. 2014). It is now understood that females can also obtain adaptive benefits from polyandrous behaviours (Hosken and Stockley 2003; Whittingham and Dunn 2010). Going beyond individual fitness, there are impacts of polyandry that extend to greater scales, including population, landscape, and species (Taylor et al. 2014). Identifying which species exhibit polyandry may lead to further insights into evolutionary biology and social behaviour (Byrne and Roberts 2012).

However, confirming polyandry in wild populations is difficult with field observations alone. Mating systems identified by judicious field observations have often been completely overhauled with insight from molecular techniques (Brooked et al. 1990; Avise et al. 2002). For example, molecular approaches have identified polyandry in many fish species that were long thought to be monogamous (Avise et al. 2002). Similarly, it was long thought that fairywrens (*Malurus* sp.) were monogamous, however allozyme analysis of clutches and potential parents revealed a high incidence of extra-pair copulation (Brooked et al. 1990). Molecular techniques are valuable for the investigation of reproductive behaviours in species that are difficult to observe in the wild.

Despite the importance of polyandry in understanding the evolution of anuran amphibians (Roberts 2020), there are a limited number of species that are confirmed to be polyandrous. There are many natural history accounts of anurans engaging in mating behaviours that may result in

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polyandry (Oda et al. 2021), yet there are few instances that have been confirmed with genetic paternity analysis or by means of experimental manipulation (Roberts and Byrne 2011). To date, polyandry has been confirmed via genetic means in seven families of frogs, including Bufonidae (Hase and Shimada 2014), Dendrobatidae (Summers and Amos 1997), Dicroglossidae (Wang et al. 2014), Myobatrachidae (Roberts et al. 1999; Byrne and Keogh 2009; Buzatto et al. 2017), Phyllomedusidae (d'Orgeix and Turner 1995) and Ranidae (Lodé and Lesbarrères 2004).

There are several key traits that are associated with polyandrous behaviours in anurans. A strong predictor is the testis mass relative to body mass, where > 1% is an initial indicator of polyandry (Liao et al. 2011; Zamudio et al. 2016). Sperm morphology is another indicator, where features that are likely to increase sperm movement are associated with probable polyandrous species (e.g. elongated sperm head, Salles et al. 2017). Similarly, the morphology of the Wolffian duct appears more complex in polyandrous species, which may confer advantages for sperm performance (Roberts and Byrne 2011; Roberts 2020).

Despite being the target of numerous long-term ecological investigations (Pyke et al. 2008; White and Pyke 2008; Pickett et al. 2014), there is limited knowledge on the mating system of the threatened Pelodryadid frog, the green and golden bell frog (*Litoria aurea*). This species exhibits behaviours that may lead to polyandry. For example, they often undergo explosive breeding events after heavy rainfall when ephemeral wetlands are replenished (Beranek et al. 2020a, in press). Additionally, there are ethological observations of multi-mating in captivity of this species (Ford 1986) and field observations of this behaviour in other Pelodryadid frogs, some which are close relatives of *L. aurea* (Byrne et al. 2002). It possesses some physiological traits typical of polyandrous anurans, including a relatively large testis mass compared to body size (0.8%) (Byrne et al. 2002).

Given that this species is threatened and requires conservation intervention, it is important to elucidate all aspects of its biology (Mahony et al. 2013). Hence, we aimed to confirm if there was genetic evidence for polyandry in *L. aurea*. Due to the traits this species possesses, we hypothesised that it would exhibit polyandry.

Methods

Study site

The field site is located on Kooragang Island (32° 50–54′ S, 151° 42–47′ E) situated ~ 14 km north-west of Newcastle, NSW, Australia. This site contains nine wetlands, two that have a permanent hydroperiod and eight that have varying ephemeral hydroperiod lengths. See Beranek et al. (2020b) and Beranek et al. (2021b) for more information on the site.

Experimental design

A combination of intensive field surveys and passive acoustic recorders were used in conjunction with genetic analysis to obtain evidence of polyandry in *L. aurea*. Since this species is threatened, collection of large numbers of eggs for genetic paternity analysis, which is typically used to determine polyandry, could not be justified. Hence, we obtained tadpole samples with an experimental design that enabled us to rule out other explanations of observed genetic paternity patterns. To do this, we targeted breeding events associated with late summer rainfall that occurred in ephemeral wetlands which were refilled after being previously dry. These breeding events are typically explosive and male choruses may only last 1–5 nights, which means that if there was genetic evidence of a female producing offspring with multiple males, it would be occurring within one reproductive cycle, and hence be designated as an instance of polyandry. The length of male chorusing was validated with weekly repeat field monitoring and in two instances with passive acoustic recorders. In total, nine late summer explosive breeding events were studied for evidence of polyandry, spread across seven wetlands (See Table 2 for summary).

Visual encounter surveys

Weekly visual encounter surveys (VES) were undertaken from 2016 to 2020 during the breeding season which includes austral spring, summer and autumn (September–April). These surveys were a part of a larger program for monitoring abundance of *L. aurea* (see, Beranek et al. 2021a, b, c). VES begun with 2 min of listening for any *L. aurea* calls, followed by 1 min of mimicking the call, and then another 2 min of listening for responses. After the auditory survey, active searching for frogs by 2–8 observers was undertaken, where the entire surface area of each wetland was covered. During these surveys, genetic samples of males and females were opportunistically taken. Surveys were conducted weekly after explosive breeding events to quantify the chorus period.

Passive acoustic monitoring

Song meters were used to determine the length of male chorusing. Song meters (SM2 +) were setup before impending heavy rainfall in ephemeral wetlands 4B and 1A while they were dry on the 29/03/2017. A large rainfall event replenished the wetlands on 1/03/2017. The song meters were set to record for the first five minutes of every hour. They were recollected during tadpole sampling about 20 days later.

The audio data collected from song meters were visually inspected for *L. aurea* calls using Kaleidoscope Pro 5 (Wildlife Acoustics, USA; www.wildlifeacoustics.com). This provided a more comprehensive analysis of chorus period.

Tadpole surveys

Fyke nets were used to capture tadpoles as they have been identified as the most effective survey method for detecting the tadpoles of the southern bell frog *L. raniformis*, a sister species of *L. aurea* (Wassens et al. 2017). The Fyke nets used in this study were 0.7 m in height, 5.7 m in length from wing to wing and possessed a central hoop of 0.7 m diameter, with a mesh size of 2 × 2 mm (stretched diagonal). Fyke nets were deployed 3–4 weeks after explosive breeding events to maximise chances of capturing tadpoles as *L. aurea* tadpoles can grow large in this amount of time (~25 mm SVL) and widely disperse through wetlands. Nets were placed with the entrance open towards emergent or submerged vegetation to optimise capture of *L. aurea* tadpoles (Klop-Toker et al. 2017). There was approximately one Fyke net set out for every one mega-litre of wetland water volume, and these were set out for 14–18 h overnight from late afternoon to early morning.

Genetics collection

A sample of 5–11 (mean = 8) tadpoles from each netting occasion had tissue extracted where surgical scissors were used to excise a ~5 mm segment of the end of the tail. Only large tadpoles from the same relative age-class were targeted for tissue extraction, from 25 to 35 mm snout-vent-length, to ensure they were produced in the same breeding event to enhance chances of detecting polyandry.

Extracted tissue was immediately immersed into 5 ml screw top conical tubes that contained 70% ethanol and were stored in this solution until genotyping. In total, tissue samples from 70 tadpoles were included in genetic analysis (see S1 for summary). Additionally, tissue samples from 15 adult female and nine males were included to identify instances of parentage to improve paternity and sib-ship assignment. Frog tissue was collected by using a single use sterile biopsy hole puncher (Kai Medical, diameter: 3 mm) to extract skin webbing from between the toes (hallux and calcaneus).

Sequencing

DNA was extracted following a modified glass fibre extraction protocol (Ivanova et al. 2006) (using 100 µl of lysis mix and 200 µl of Binding Mix) or a Gentra Puregene DNA (QIAGEN) protocol. DNA extraction and sequencing was conducted by Diversity Arrays Technology (DArT PL). Its patented next generation sequencing protocol, DArTseq, is a cost-effective option for generating high quality, high-throughput SNP datasets for non-model species. A description of the DArTseq protocol is available in Jaccoud et al. (2001).

Table 1 Number of loci remaining after each quality filtering step

Filtering step	No. of loci
Initial	41,168
Call rate (100%)	13,731
Reproducibility (100%)	13,731
MAF (> 10%)	8710
Secondaries	8669
Linkage disequilibrium (< 90%)	2480

The returned DArT dataset was further filtered for locus quality (see Table 1). Firstly, loci were filtered based on a 100% call rate and 100% reproducibility. Next the loci were filtered for a minimum minor allele frequency (MAF) of 5%, which allows better assignment of related individuals (Huisman 2017). Secondaries were also removed from the dataset. All previous filtering steps were performed in the R statistics package, *DArTR* (Gruber et al. 2018). Finally, linkage disequilibrium was filtered at threshold of < 90% using the R package *SNPRelate* (Zheng et al. 2012). See S2 for a visualisation of the improvement in SNP quality.

Relationship assignment

The program COLONY was used to assign relationships within tadpole cohorts (to full-sibling and half-sibling and unrelated) and between tadpoles and possible parents (Jones and Wang 2010). The dataset was further reduced from 2492 to 1988 randomly selected loci to permit ease in uploading into COLONY. Two analyses were undertaken to account for different generations, one containing parents and offspring collected from 2016 to 2018 and the other containing parents and offspring collected in 2019 to 2020. Adult genotypes collected in the latter period were included as possible offspring in the former period since they were likely conceived during this period as *L. aurea* have low survival rates and relatively short lifespans rarely exceeding two years (Beranek et al. 2021a). This also allowed the construction of a multi-generation pedigree using R package "kinship2" (Sinnwell et al. 2014). Genotyping error rate was set to 0.001 and COLONY analyses were set to "very long" which allows greater precision in estimates. The probability of sibling cluster, half-sibling cluster and maternity assignment was reported for each instance.

Table 2 Number of mating pairs determined per breeding event as estimated by COLONY

Site	Date replenish	Date collect	n	Method	Days call	Full-sib clust	Mated females	Assigned Parents	Multiple ♀ copulation	Multiple ♂ copulation
1A	1/03/2017	23/03/2017	9	VES and SM	6	2	6		1 (1.00)	1 (0.30)
2A	1/03/2017	23/03/2017	10	VES	8	2	4	**CB0093F** (CB0152T, CB0154T, P: 1.00) **CB0118F** (CB0153T, P: 1.00)		
2C	1/03/2017	23/03/2017	6	VES	2	2	2			
4B	1/03/2017	29/03/2017	9	VES and SM	8	2	4			
2C	27/02/2018	28/03/2018	5	VES	9	1	2		1 (1.00)	
4A	22/03/2018	24/04/2018	7	VES	1*	1	3			
4B	22/03/2018	24/04/2018	7	VES	7*	1	4		1 (0.59–1.00)	
4C	22/03/2018	24/04/2018	6	VES	7*	2	4			1 (0.69)
2A	09/02/2020	20/03/2020	11	VES	3	3	6	**EJ0011F** (TS0036, TS0037, TS0039 and TS0040, P: 1.00)	1 (1.00)^3	

Days call = the number of days the latest calls were detected after wetlands were replenished. Assigned parents bold = parent genotype code and brackets = offspring genotype code and probability of maternity assignment. Multiple paternity column brackets = half-sibling probability. Multiple copulation brackets = probability of cluster

^{3}Mating with three separate males

VES chorus length determined via visual encounter survey, *SM* chorus length determined via song meter

Result

There were 25 females and 27 males identified to have produced the 70 tadpoles included in genetic analysis. Two or more mating pairs reproduced in each breeding event. There were 16 clusters of full-siblings identified. Most of these were groups of two ($n = 8$), however some formed groups of three ($n = 2$), four ($n = 4$) and five ($n = 2$).

There were 3/16 (19%) females assigned as mothers of tadpoles (Table 2). None of nine male genotypes were assigned as fathers. Most of the instances in maternity prediction produced reliable probabilities (probability of assignment = 1.0).

There were four instances of polyandry observed (see Table 2). The first instance occurred in wetland 1A, where three tadpoles were assigned to a simulated mother genotype. Of these tadpoles, two were full-siblings (CB0167T and CB0173T) but they were both half-siblings to CB0170T. The second instance occurred in wetland 2C where two tadpoles shared the same simulated mother, but were half-siblings (CB0971T and CB0974T). The third instance occurred in wetland 4B, where four tadpoles were assigned as full-siblings to each other (CB1011T, CB1013T, CB1022T and CB1023T), yet were all assigned as half-siblings to tadpole genotype CB1012 and all five tadpoles shared the same simulated mother. Lastly, tadpoles TS0036, TS0037, TS0039 and TS0040 were assigned as offspring to mother EJ0011F, where tadpoles TS0036 and TS0040 had different simulated father genotypes to TS0037 and TS0039, which also did not share the same father. This meant that EJ0011F had produced offspring with three different males. The probability of assignment in instances of multiple paternity ranged from 0.59 to 1.00.

Males siring offspring with more than one female occurred in two breeding events, with the probability in assignment ranging from 0.30 to 0.69. In one of these instances, a male sired offspring with two different females, each in different but closely situated wetlands. In the other instance, the male sired offspring to two different females in the same wetland.

There was one instance where a multi-generation pedigree could be constructed (see Fig. 1). Female EJ0011F (collected in late 2019) was found to be a full-sibling to tadpole CB1006T, and therefore was conceived in wetland 4C in April 2018. After 646 days from when she was estimated to have metamorphosed (05/05/2018), this female successfully reproduced in explosive breeding events in wetland 2A on the 10/02/2020 where at least three different males sired her offspring.

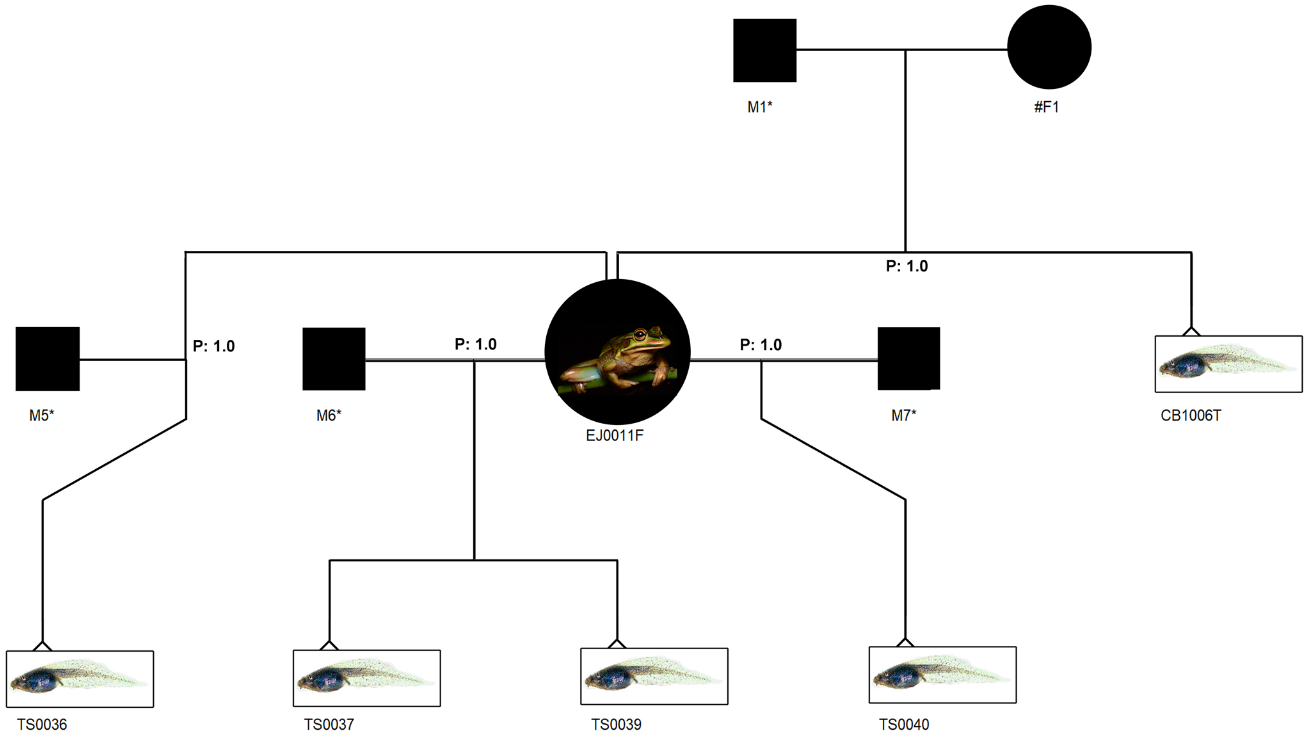

Fig. 1 Intergenerational pedigree demonstrating an instance of polyandry in *Litoria aurea*. Squares = male, circles = female, diamond = tadpole sample (unknown sex). *Simulated male genotype and $^{\#}$simulated female genotype. *P* probability of assignment

Discussion

Here we demonstrate with molecular analysis that *Litoria aurea* is polyandrous. This is the first time that polyandry has been confirmed in a species from the family Pelodryadidae (Byrne et al. 2002; Roberts and Byrne 2011), although there are numerous species in this group that possess traits and behaviours that are consistent with the occurrence of polyandry (Byrne et al. 2002; Anderson et al. 2010). We discuss these results in regards to behavioural adaptations and avenues for further research.

There are two broad categories of polyandry associated with external fertilising organisms, including amphibians, sequential and simultaneous (reviewed by Byrne and Roberts 2012). Simultaneous polyandry eventuates when more than one male releases sperm on or near eggs and can occur through multiple male amplexus or through clutch piracy (Vieites et al. 2004). Sequential polyandry occurs when a female mates with more than one male during a reproductive cycle, but not simultaneously (Byrne and Keogh 2009). The primary difference from an adaptive fitness perspective is that the success of male in the former is determined by sperm competition and cryptic female choice, where there is no risk of sperm competition in the latter instance because matings are spatially or temporally separated.

It is unknown which behaviours result in polyandry in *L. aurea*, but we can speculate based on observed behaviours. Field observations confirm that *L. aurea* forms chorus aggregations in explosive breeding events (Pyke and White 2001) and often occur in highly male-biased sex-ratios at breeding sites (Beranek et al. 2021a), which are features that appear linked with simultaneous polyandry (Roberts and Byrne 2011). This is in agreement with observations of multiple male amplexus in *L. aurea* and closely related taxa (Byrne et al. 2002; Ford 1986).

However, other more subtle male behaviours that result in polyandry cannot be ruled out. This includes sequential polyandry, sperm leakage and clutch piracy. There may be potential for sequential polyandry in this species. Christy (2001) provided unconfirmed evidence that females may withhold releasing all of a clutch during mating. If this is true, it could mean that *L. aurea* can also undergo sequential polyandry, but more research is needed to establish this. Clutch piracy involves secondary males fertilising the eggs deposited by a separate male–female pair (Vieites et al. 2004). There has been no observations as far as we are aware which may allude to the existence of clutch piracy in *L. aurea*. More ethological studies are needed on this species to confirm which behaviours mostly result in polyandry.

Conclusions

Despite many long-term field studies on *L. aurea* (Christy 2001; Patmore 2001; Goldingay and Newell 2005; Pickett et al. 2014; Goldingay et al. 2017), there are few published observations reported that would hint that this species is polyandrous (although see Ford 1986). We conclude that *L. aurea* exhibits polyandry, which is the first species confirmed to be polyandrous within the Pelodryadidae family. This highlights the utility of using molecular techniques to investigate reproductive ecology in frogs. Based on previous studies, it is likely that this species exhibits simultaneous polyandry. It is of value for future studies to identify if other frogs in the *L. aurea* complex such as *L. cyclorhyncha*, *L. moorei* and *L. raniformis* are also polyandrous. This would give further insight to evolutionary processes in this group and enhance our overall understanding of evolution in amphibians.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10709-021-00137-3.

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Data availability Summary of genotype specimens are included in the supplementary material. Data is accessible via Mendeley Data, including raw DArTseq data, R code for filtering and data used in COLONY. https://doi.org/10.17632/26j3gtbkgn.1.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval Field work and genetic specimen collection was conducted under approval from the University of Newcastle Animal Care and Ethics Committee (license number: ARA 2010-154) and a National Parks and Wildlife Services New South Wales scientific license (license number: SL100529).

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