

ORIGINAL RESEARCH

All in one: single-season multi-clutching in an ephemeral pool-breeding frog that is otherwise virtually semelparous

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abbreviate iteroparity; anuran; bet-hedging; ephemeral habitat; semelparity; *Lechriodus fletcheri*; reproductive strategies; breeding environment.

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Abstract

The sandpaper frog, *Lechriodus fletcheri*, is a temperate anuran that has previously been shown to be virtually semelparous, with adults overwhelmingly reproducing in a single year of life. Yet, this species almost exclusively oviposits in highly ephemeral pools where there is a high chance of total reproductive failure due to hydroperiods often being unpredictable and too short for their offspring to reach metamorphosis. We sought to understand how *L. fletcheri* copes reproductively in such a risky breeding environment in the absence of a classical iteroparous life history. We investigated aspects of reproduction in wild and laboratory-reared individuals to determine whether females are capable of intra-seasonal multi-clutching and/or clutch partitioning, and males of fertilizing multiple clutches. Direct field evidence was obtained that males participated in multiple mating events within season, while indirect evidence of this ability in females was obtained based on laboratory-held individuals that produced an additional batch of mature oocytes weeks after an initial release of eggs. Our findings suggest that both males and females likely have the capacity to participate in multiple reproductive events within season and, while most adults may not reproduce more than once, that they are abbreviate iteroparous rather than truly semelparous. Our findings provide evidence that short-lived anurans may exploit alternative bet-hedging strategies that mimic the fitness benefits of multi-year iteroparity.

Introduction

Species that have evolved to occupy temporary aquatic environments are faced with the risk of desiccation-induced mortality if these systems do not sustain water for sufficient periods of time (Aguilar-Alberola & Mesquita-Joanes, 2011; Chandler et al., 2017; Furness, 2016; Jeffries, 1994). This threat is apparent for amphibians that rely on ephemeral waterbodies to accommodate the aquatic stage of the biphasic lifecycle (Chandler et al., 2016; Pfennig et al., 1991; Wilbur, 1980). Having offspring occupy such niches confers advantages, such as fewer predators that are normally associated with permanent water (e.g. fish) and reduced competition from the offspring of sympatric species (Babbitt et al., 2003; Brönmark & Edenharn, 1994; Skelly, 1997). However, the possibility for extremely short and/or unpredictable hydroperiods may prevent offspring from successfully reaching metamorphosis and having the capacity to become independent of freestanding water before they dry (Denver et al., 1998; Gould, Clulow, & Clulow, 2022; Taylor et al., 2006), resulting

in catastrophic mortality of the tadpole population (Newman, 1992; Skelly, 1996).

Although some amphibians have evolved reproductive modes that negate the need for free-standing water altogether (e.g. direct development; Callery et al., 2001; Duellman & Trueb, 1986), a free-swimming tadpole stage has been retained by many amphibian species that exploit temporary environments (Baldwin et al., 2006; Pfennig et al., 1991). These species possess an array of parent and offspring-based strategies, such as adaptive oviposition site selection (Buxton & Sperry, 2016) and phenotypic plasticity in tadpole metamorphosis (Denver, 1997; Pfennig et al., 1991), which have been directly selected or confer a fitness advantage by reducing the risk of total reproductive failure per clutch. Producing offspring in multiple breeding periods, referred to as iteroparity, is a form of bet-hedging that can spread the risk of life-time reproductive failure by maximizing the chances of at least some offspring surviving, particularly in environments that are unpredictably variable within or between breeding periods (Benton & Grant, 1999; Goodman, 1984; Murphy, 1968). Yet,

this capacity is limited for short-lived species that are only available for reproducing in a few or single breeding years (Guarino *et al.*, 2010; Lehtinen & MacDonald, 2011; Lemckert & Shine, 1993; Trauth *et al.*, 2004).

A variety of other bet-hedging strategies related to reproduction are used among amphibians, including within-clutch egg variability (Crump, 1981; Lips, 2001), clutch partitioning (Altig & McDiarmid, 2007; Gopurenko *et al.*, 2006), multiple paternity (Gopurenko *et al.*, 2006), and intra-seasonal multi-clutching (Stamp, 1980; Wells, 1976; Wilbur, 1977). As with iteroparity, these strategies create variability among an individual's offspring, increasing the odds that some will develop under environmental conditions or with phenotypes and genetic combinations that will be advantageous for their survival. To this effect, they can potentially mimic the fitness benefits of breeding in multiple years and may be particularly important for species that exploit unpredictable systems under circumstances where most adults appear not to survive to breed in additional years.

The sandpaper frog, *Lechriodus fletcheri*, is a cool-temperate Australian anuran that almost exclusively breeds in highly ephemeral pools that form after summer rainfall (Gould, 2020). The timing of rainfall is unpredictable and these pools can have highly variable hydroperiod lengths, often drying out well before offspring can complete tadpole development (Gould, Clulow, & Clulow, 2022). We hypothesize that this system has driven the selection for short yet plastic developmental rates in this species (Anstis, 2017; Clulow & Mahony, 2008; Clulow & Swan, 2018), as well as adaptive oviposition site selection (Garnham, 2012) and the capacity for adults to respond rapidly to rainfall (Gould, Clulow, & Clulow, 2022). Despite these adaptations, total clutch failure is still a common occurrence as the unpredictability of rainfall prevents adults from being able to accurately assess the most optimal rain events to lay in, coupled with the requirement of multiple rain events for pools to maintain sufficient hydroperiods over the duration of tadpole development (Gould, Clulow, & Clulow, 2022). Under these circumstances, theory suggests that iteroparity is likely to evolve, whereby reproduction occurs over multiple years to improve the chances of at least some of an individual's offspring surviving (Benton & Grant, 1999; Goodman, 1984; Murphy, 1968). However, we have recently shown that this species, somewhat paradoxically, appears to be effectively semelparous, with adults in the wild overwhelmingly breeding in only a single year of their short lives (Gould, Clulow, Herb, & Clulow, 2022).

This unexpected finding of virtual semelparity raises questions on the nature of reproduction in *L. fletcheri* and, more widely, whether species in unpredictable breeding systems can exploit alternatives to classical iteroparity to improve their chance of reproductive success. In light of this, the current study investigated aspects of reproduction in wild and laboratory-reared *L. fletcheri* to determine whether (1) females are capable of intra-seasonal multi-clutching through repeated cycles of oogenesis, as well as clutch partitioning over different locations and/or time periods; (2) whether males have the capacity to fertilize multiple clutches within a single season, as

a condensed form of single-season iteroparity for a short-lived species.

Materials and methods

Study species and site

Lechriodus fletcheri is a medium-sized anuran (mean snout-vent lengths for adult males and females are 47 mm and 50 mm, respectively; Gould & Valdez, 2023) found in montane temperate forests along the east coast of Australia (Anstis, 2017). This species has a breeding season from September to March, with eggs laid in floating masses of frothed oviduct fluid (referred to as nests) in ephemeral pools that fill briefly during periods of rain (Gould, Clulow, & Clulow, 2022). This study was conducted on a population in the Watagan Mountains, NSW, Australia (33° 00' 30.6 S, 151° 23' 15.7 E). Pools used for reproduction at this site are relatively small (540–3 000 000 cm³; Gould *et al.*, 2021) and usually lacking in submerged or surrounding vegetation (Gould, Clulow, & Clulow, 2022), allowing for a near complete census of adults at pools across the breeding period.

Field surveys and animal collection

Eighty pools within the study site were routinely surveyed on nights during rain events over the 2015/16, 2016/17, and 2017/18 austral spring/summer breeding seasons (September to March) for the presence of adult individuals. Rain events were defined as days of consecutive rain above 0.2 mm, with gaps in rainfall of at least 2 days signifying the start or end of each event. Daily rainfall values below 0.2 mm were deemed too light to create a biologically significant difference in pool watering and subsequently considered as being equivalent to days of no rainfall. Rainfall data were obtained from Coorabong weather station (BOM ID: 061412) located approximately 8 kms south of the study site. Field surveys were initiated when rainfall was indicated for the study area and continued if there was evidence of rainfall (e.g. refilled pools). Adult *L. fletcheri* found during surveys were captured, and sexed using sexually dimorphic characteristics (e.g. size and nuptial pads). The location of each capture (i.e. pool) and whether individuals were found in amplexus were also recorded. Newly captured individuals were tagged with a passive integrated transponder (PIT), with recaptures identified with a Trovan radio frequency identification reader (Microchips Australia Pty Ltd., Keysborough, Vic, Australia). Each frog was released at the point of capture. Data on amplexing adults is presented in this study, while detailed information regarding all capture and recapture events can be found in Gould, Clulow, Herb, and Clulow (2022).

A small subset of adults were brought back to the laboratory for reproductive studies of their gonads to investigate potential reproductive strategies that have evolved in this species. Over three periods in one season (04-Dec-17, 20-Feb-2018, and 24-Mar-2018), 42 adult individuals were collected, including 13 female/male pairs found in amplexus, along with six females

and 10 males non-amplecting individuals that were assumed to have not recently participated in spawning given the absence of any fresh nests within the pools they were collected from. Animals were transported to the laboratory in $10 \times 15 \times 15$ cm containers with a small amount of pool water (~1 cm in depth), with each amplecting pair kept together and each non-amplecting individual kept separate. To minimize the impact this removal process might have had on the long-term sampled population, adults were only collected from pools outside of the long-term study site ($33^{\circ} 2' 14.75''$ S, $151^{\circ} 19' 7.86''$ E). All field and laboratory collected data can be found at Gould (2021).

Multi-clutching potential of females

After transport to the laboratory, non-amplecting individuals and amplecting pairs were moved to larger containers ($35 \times 21 \times 21$ cm) that included a small amount of damp forest leaf litter and kept over 48 h in a quiet room under a 12 h night/day cycle at room temperature (RT = 24°C); relative humidity was not recorded. Amplecting pairs were supplied with a small container ($17 \times 12 \times 7$ cm) filled with aged water (kept in an open container for at least 48 h to allow for chlorine evaporation) in order to facilitate oviposition. Although some amplecting pairs did separate during transportation, all returned to an amplecting state in the laboratory. Females were checked regularly for signs of oviposition for 48 h. All amplecting females oviposited a single clutch (i.e. nest) within 48 h of collection bar one. Nests were removed from containers and the number of eggs per clutch calculated based on a count of the number of tadpoles that hatched and the number of embryos that did not (possibly as a result of disease, halted development, or desiccation).

Of the 13 amplecting females that were collected, eight were immediately euthanized after ovipositing by immersion in 0.4% w/v ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma Aldrich, Missouri, United States; E10521) buffered with sodium bicarbonate, followed by excision of the heart. Ovaries were removed and weighed separately to the nearest 0.001 grams. Each ovary was placed in a small petri dish filled with Simplified Amphibian Ringer (SAR; 113 mM NaCl, 1 mM CaCl_2 , 2 mM KCl, 3.6 mM NaHCO_3 ; recipe from Browne *et al.*, 1998) for photographing. Sections were splayed out using forceps to form a single layer of eggs, which were photographed using a stereo-microscope mounted DAGE-MTI camera with Leica LAS EZ software V4.0.0 (Leica Microsystems) for egg counting and staging. Residual female body weight was measured after ovary excision. The remaining five amplecting females were removed from their male counterparts after ovipositing and maintained in fresh tanks at RT under a 12 h night/day cycle for a period of approximately 90 days prior to ovary collection. Females were fed crickets twice a week, assuming that this was sufficient to provide a non-limiting diet for ovarian recrudescence. This time interval was selected to determine whether females were able to recruit an additional cohort of mature ovarian oocytes within a realistic single season timeframe, given the breeding season lasts for

approximately 160 days (Anstis, 2017). Additionally, six non-amplecting females were euthanized and ovaries dissected within 48 h of collection using the same techniques as above. In effect, ovaries of females were analyzed at one of three different stages of the reproductive cycle (amplecting females dissected 0 day post-ovulation, amplecting females dissected 90 days post-ovulation, and non-amplecting females dissected while gravid but prior to ovulation).

Photographs of ovarian sections were analyzed in the program ImageJ® (Rueden *et al.*, 2017) to conduct egg counts. Oocytes were staged, as per the general appearance of eggs at different stages described by Dumont (1972), between stages II and VI, with stage V and stage VI oocytes both classed as stage V/VI due to the difficulties in differentiating between these later stages in this species. The distribution of oocyte stages between all female types was analyzed using a generalized linear mixed effects model with a poisson distribution and log link function. Count of total egg number was included as the response variable. Female type (amplecting females dissected 0 day post-ovulation, amplecting females dissected 90 days post-ovulation, and non-amplecting females dissected while gravid but prior to ovulation) and oocyte stage were included as explanatory variables, with an interaction term (female type * oocyte stage) included to determine whether egg count per stage differed between female types. We also included female individual as a random effect to account for the likely non-independence of repeat measurements for each individual. All analyses were performed using the lme4 (Bates *et al.*, 2015) and lmerTest (Kuznetsova *et al.*, 2017) R packages (R Team, 2018).

Relative ovary mass (ROM) was calculated for gravid females prior to ovulation and females 0 day post-ovulation by dividing wet mass of ovaries by total body mass, with the difference in ROM between these two female types providing a measure of *in situ* energy investment per spawning event. In addition, the number of eggs present in the laid clutches of females 0 day post-ovulation was compared to the number of mature oocytes present in the ovaries of gravid females prior to ovulation to determine whether females were ovipositing all mature eggs or partitioning egg cohorts across multiple nests (i.e. clutch partitioning). Each of these ovary or clutch data comparisons were made using Welch's two sample *t*-testing in R to account for unequal variance and/or sample sizes. Potential variation between samples was tested using the Fligner–Killeen test. Summary statistics are presented as means and standard deviations.

Multi-ejaculation potential of males

Amplecting and non-amplecting males were collected from pools during the same rain events as females. All amplecting males were immediately euthanized after their female counterparts had oviposited, as were non-amplecting males. Total body weight was measured to the nearest 0.001 grams, before testes were excised and weighed together. Testicular macerates were prepared by breaking apart the testes with forceps in 300 μL SAR. Sperm counts were undertaken in a

hemocytometer after 1:20 dilution of macerate. Two separate sperm counts were performed from the same macerate for each individual. When these two counts differed by more than 20 cells, an additional two separate counts were made from the same macerate. Relative testes mass (RTM) was calculated by dividing wet mass of testes by total body mass. The proportion of viable (live) sperm present within each macerate was determined by preparing a 1:1 dilution of testis macerate in 0.5% Eosin-Y in 0.9% NaCl; a stain used previously to differentiate live/dead amphibian sperm (O'Brien *et al.*, 2011). After approximately 30 s, 100–150 sperm cells were scored under a phase-contrast microscope, with pink stained sperm scored as non-viable. Sperm motility was assessed by activating macerates in a 1:6 dilution of distilled water. After 2 min, 100–150 cells were scored at random under a phase-contrast microscope, with any showing signs of flagella beating and/or forward progressive motion considered to be motile (Fitzsimmons *et al.*, 2007).

Differences in sperm motility and vitality between male types (amplected males post sperm release and non-amplected males pre sperm release) were analyzed using separate generalized linear mixed effects models in combination with a binomial distribution and link function. In these models, count of sperm motile versus non-motile or sperm vital versus non-vital were included as the binary response variable using the `cbind` function in R. Testes mass as a proportion of body mass (scaled by 100) was included as a predictor in these models, with male individual included as a random effect to account for differences in the total number of sperm measured between individual frogs. These analyses were performed using the `lme4` (Bates *et al.*, 2015) and `LmerTest` (Kuznetsova *et al.*, 2017) R packages. Additionally, differences in mean relative testes mass and sperm counts between male types were also analyzed using Welch's two sample *t*-testing, with differences in variation between samples tested using the Fligner–Killeen test. Summary statistics are presented as means and standard deviations.

Results

Animal captures

Although 58 amplexing pairs were found during the study period, no females were found in amplexus on more than one occasion. Nevertheless, one female was recaptured 77 days after being seen in amplexus during her first capture within the same season. Two males were found in amplexus on two occasions within season, with the time separating each amplexing event being 1 and 10 days, respectively. Seven males were also found in amplexus more than 40 days after first being captured (and non-amplectant) within the same season.

Multi-clutching potential of females

Although oocytes at various stages of maturation were found in the ovarian lobes of all individuals examined, the distribution of oocyte stages varied between female types (Fig. 1). We

found no significant differences in the number of early-stage eggs between female types, with the mean number of stage II and III eggs within ovaries across all females being 119.41 ± 51.10 eggs and 176.82 ± 70.13 eggs, respectively. In contrast, there were significant differences in the number of mature eggs found between female types. Females examined 90 days post-ovulation had more stage IV eggs (133.20 ± 22.07 eggs) compared to both females 0 day post-ovulation (65.50 ± 33.03 eggs) (Poisson GLMM: $Z = 9.64$, $P < 0.0001$) and gravid females pre-ovulation (43.50 ± 28.45 eggs) (Poisson GLMM: $Z = 7.68$, $P < 0.0001$). Females examined 90 days post-ovulation also had more stage V/VI eggs (73.00 ± 60.94 eggs) compared to females 0 day post-ovulation (17.25 ± 7.79 eggs) (Poisson GLMM: $Z = 13.19$, $P < 0.0001$), but less than gravid females pre-ovulation (536.25 ± 243.26 eggs) (Poisson GLMM: $Z = -35.34$, $P < 0.0001$). The intermediate size of ovaries derived from females held for 90 days post-ovulation was apparent (Fig. 2), including the greater number of mature stage V/VI oocytes that was similar to the ovaries of gravid females prior to ovulation, indicating repopulation of the post-ovulatory ovary with a cohort of freshly recruited, maturing oocytes within season.

There was a significant difference in ROM values between females pre- and post-ovulation (Fig. 3a; *T* Test: $T_{4,22} = -4.42$, $P < 0.05$), with ovarian tissue accounting for $11.40 \pm 4.36\%$ of total body weight in gravid females prior to ovulation and only $2.70 \pm 0.92\%$ in females immediately post-ovulation. Taking into consideration this difference, nearly three quarters of ovarian mass in gravid females was estimated to be comprised of stage V/VI eggs, accounting for approximately 8% of total gravid female body weight. There was no significant difference in variation in ROM values between female types (chi-squared = 2.84, $P = 0.09$).

There was no evidence of clutch partitioning because: (1) few mature oocytes remained in the ovaries of females examined 0 day post-ovulation (17.25 ± 7.80 cells) compared to gravid females pre-ovulation (536.25 ± 243.26 cells); (2) there was no significant difference in the number of mature oocytes present in the ovaries of gravid females pre-ovulation (536.25 ± 243.26 cells) compared to the number of mature eggs present in the clutches of females that had oviposited (301 ± 110 cells) (Fig. 3b; *T* Test: $T_{3,42} = -1.87$, $P = 0.15$). There was however a significant difference in variation in egg/oocyte numbers between female types (chi-squared = 6.89, $P < 0.01$).

Multi-ejaculation potential of males

Sperm motility did not significantly differ between male types (Fig. 4a; binomial GLMM: $Z = -0.49$, $P = 0.62$), with an overall mean across all males being $15.89 \pm 12.62\%$; however, three males post-amplexus had much higher proportions of motile sperm compared to all other males. Sperm vitality also did not differ between male types (Fig. 4b; binomial GLM: $Z = -1.09$, $P = 0.28$), with a mean of $94.78 \pm 5.95\%$ across male individuals. Relative testes mass was a significant positive predictor for both sperm motility (binomial GLM:

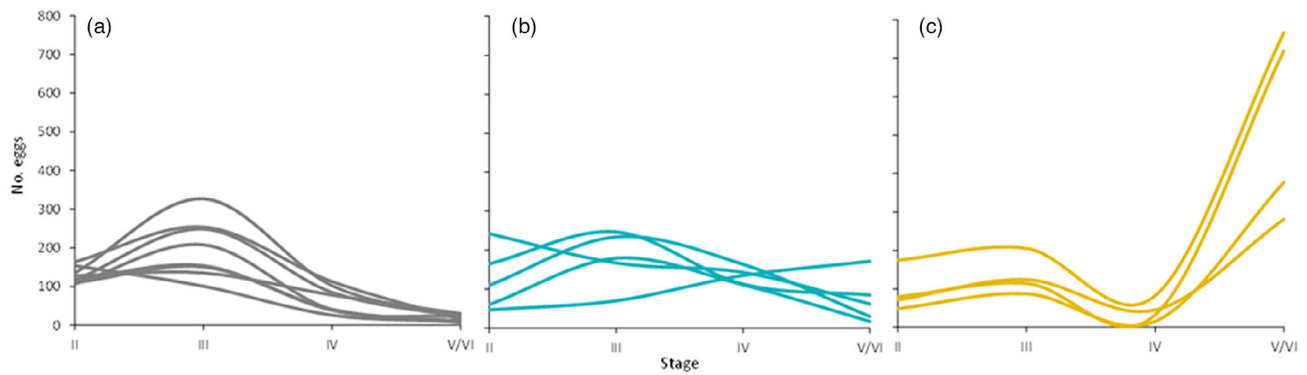


Figure 1 Ovarian contents of *Lechriodus fletcheri* females at different stages of ovulation. The total number of oocytes present at each stage of maturation are shown for females; (a) immediately post-ovulation ($N = 8$; grey), (b) 90 days post-ovulation ($N = 5$; blue), and (c) prior to ovulation ($N = 6$; orange). Ovaries were excised and oocytes staged according to the methodology of Dumont (1972), with stages including immature oocytes stages II through to mature stage V/VI oocytes. Adults were collected from pools within the Watagan Mountains, Australia, during the 2016/17 and 2017/18 breeding seasons.

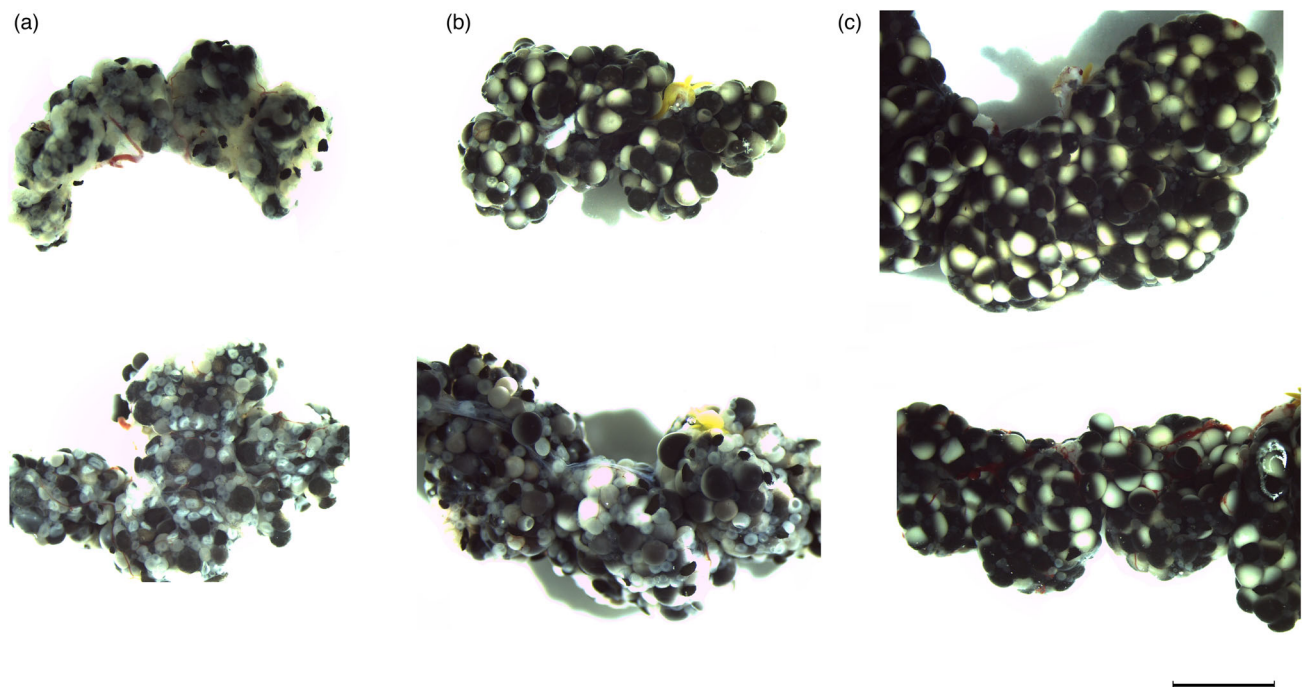


Figure 2 Excised ovaries from *Lechriodus fletcheri* adult females at various stages pre- and post-ovulation. Images show ovaries excised from females (a) immediately post-ovulation, (b) 90 days post-ovulation, and (c) gravid and prior to ovulation, indicating differences in ovarian mass and oocyte stages. One ovary from two different females is shown for each stage. Scale bar represents 5 mm.

$Z = 3.12$, $P < 0.01$) and vitality (binomial GLM: $Z = 2.24$, $P < 0.05$). However, there was no significant difference in relative testes mass between male types (Fig. 4c; T Test: $T_{11.67} = -0.88$, $P = 0.39$), with testes mass averaging around 15% of total body mass among all males, and no difference in variation between male types (chi-squared = 1.96, $P = 0.16$). There was also no significant difference in sperm count between male types (Fig. 4d; T Test: $T_{12.23} = -1.84$, $P = 0.09$), with a mean of $3.77 \times 10^7 \pm 2.51 \times 10^7$ cells per

mL obtained, but a significant difference in variation of counts between male types (chi-squared = 5.96, $P < 0.05$).

Discussion

The data presented in this study indicate that, although overwhelmingly restricted to breeding in a single season, *L. fletcheri* adults of both sexes may have the capacity to participate in multiple reproductive events. This is based on direct

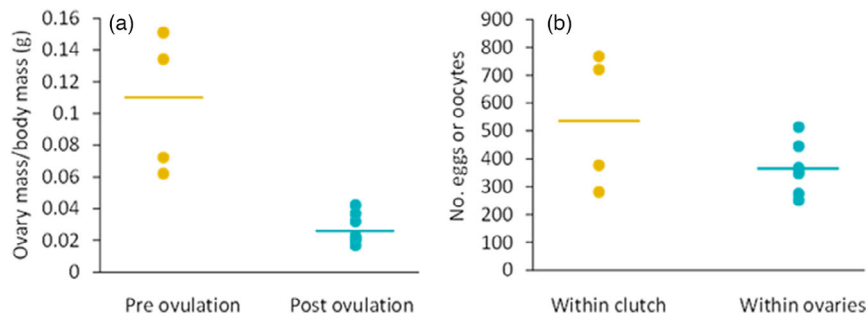


Figure 3 Comparison of eggs within the ovaries and laid clutches of *Lechriodus fletcheri* adult females at various stages pre- and post-ovulation. Shown include (a) the difference in ROM values between gravid females pre-ovulation and females 0 day post-ovulation, and (b) total number of eggs present in the first clutch of females 0 day post-ovulation compared to the total number of mature (type V/VI) oocytes present in the ovaries of gravid females pre-ovulation. Horizontal lines represent mean values. Adults were collected from pools within the Watagan Mountains, Australia, during the 2016/17 and 2017/18 breeding seasons.

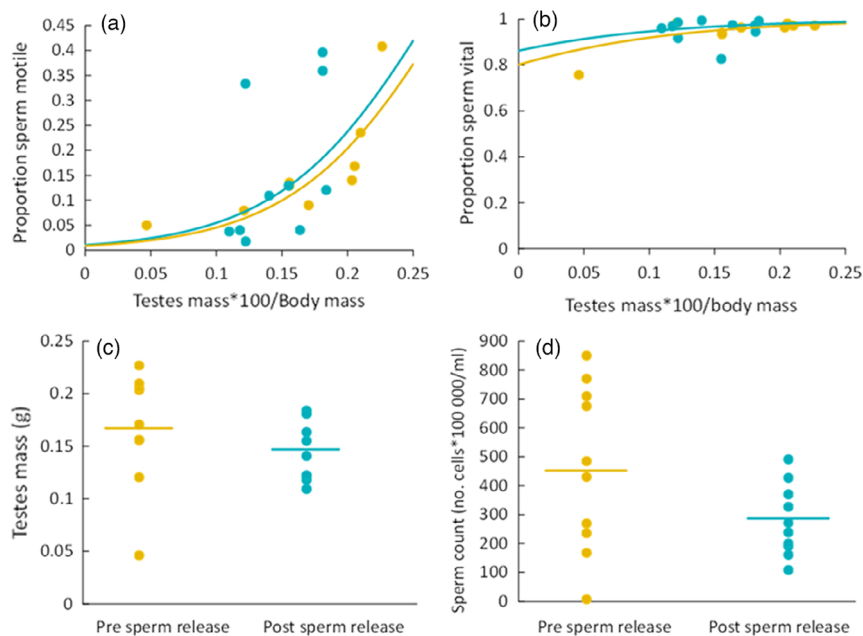


Figure 4 Comparison of *Lechriodus fletcheri* adult male testes and sperm. Shown include (a) regression line of proportion sperm motile against relative testes mass, (b) regression line of proportion sperm vital against relative testes mass, (c) relative testes mass with horizontal lines representing mean values, and (d) sperm counts with horizontal lines representing mean values, for non-amplected males pre-sperm release ($N = 10$; orange) and post-amplected males post-sperm release ($N = 13$; blue). Males were collected from pools in the Watagan Mountains, Australia, during the 2016/17 and 2017/18 breeding seasons on occasions when it was likely that individuals had not recently released sperm as part of a recent spawning event.

field evidence of males taking part in multiple mating events within season, and evidence of laboratory-held females producing an additional batch of mature oocytes weeks after an initial release of eggs. We are cautious of the lack of direct observations of multi-clutching in wild females except for evidence based on long recapture intervals between rain events. Nevertheless, we suggest that *L. fletcheri* possesses an intermediate life history that does not fit neatly within classical classifications; it may be defined as semelparity, as all reproductive effort is still confined to a single breeding year, or a

shallow form of iteroparity (Fritz *et al.*, 1982; Hughes, 2017). This reproductive pattern most closely resembles the abbreviated iteroparity reported for some species of goby (Daoulas *et al.*, 1993; Kevrekidis *et al.*, 1990; Miller, 1961), which is characterized by a long reproductive season and multiple spawning events over a short lifespan. Our findings provide evidence to the hypothesis that short-lived anurans may exploit a variety of bet-hedging strategies that mimic the fitness benefits of multi-year iteroparity by increasing the temporal and/or spatial variability of offspring development within

breeding seasons/years (Caldwell, 1987; Lemckert & Shine, 1993).

Single season multi-clutching appears to be a risky means of allocating lifetime reproductive energy for species exploiting ephemeral pools where total clutch failure is a common occurrence (Gould, Clulow, & Clulow, 2022). There are several potential reasons why this reproductive pattern allows *L. fletcheri* to maintain viable populations in the absence of multi-year iteroparity, which are related to the unique conditions under which reproduction occurs. This species experiences persistent uncertainty in breeding conditions as opposed to favorable/unfavorable years, with continuous drying and subsequent recharging of pools throughout a long breeding season (Gould, Clulow, & Clulow, 2022). Due to their small size, breeding pools require multiple rain events to remain charged for offspring to complete metamorphosis (Gould, Clulow, & Clulow, 2022). Yet, rainfall is unpredictable, limiting the ability of adults to determine optimal times to reproduce as they cannot predict if future rain events will replenish water levels based on the conditions experienced during egg deposition. Under these circumstances, there is no more advantage in reproducing once in multiple years as there is in reproducing multiple times in 1 year, as they will experience the same extent of unpredictability in breeding conditions regardless. However, the small size and relative abundance of ephemeral pools across the species' range allows for the mass spatial and temporal distribution of breeding events (Gould, Clulow, Herb, & Clulow, 2022). There is thus a high chance that there will be some windows and sites within any given season where clutches are laid under conditions that allow them to survive through to metamorphosis. This is supported by capture–mark recapture studies on this species that show relatively stable population sizes between years (Gould, Clulow, Herb, & Clulow, 2022).

Oocyte development and maturation in *L. fletcheri* females was found to be continuous and asynchronous, with oocytes at all stages of development present in all ovaries sampled. This observation does not exclude, however, the possibility of some acceleration or synchronization of stage V/VI oocyte growth and recruitment from the more advanced residual oocyte cohort following ovulation and oviposition. In this study, it was clear that *L. fletcheri* females were able to continue the progression of immature follicles to follicular maturation once an initial clutch had been oviposited, as indicated by the presence of stage V/VI oocytes in females held for 90 days and the increasing resemblance of their ovaries to those of gravid females just prior to ovulation. This secondary wave of egg development is typical of many pond breeding anurans (Wallace & Dumont, 1968), and likely to facilitate same-season multi-clutching in *L. fletcheri*. The comparable number of stage V/VI oocytes in the ovaries of one particular female in this study held for 90 days after oviposition is evidence for the capacity of females to generate a complete second cohort of mature oocytes within the time span of a season that extends for upwards of 160 days. This finding is of particular interest as multi-clutching has only been described in a few temperate zone anurans (Blair, 1960; Howard, 1978b; Humphries, 1979; Wells, 1976), but a strategy that is likely to be feasible for

many species with a prolonged breeding season. However, we caution that these findings were obtained for laboratory-held females exposed to optimal nutrients and may not reflect the speed of oocyte maturation found among wild females.

The small number of stage V/VI oocytes found in the ovaries of post-amplectic females shows that near complete ovulation of the full cohort of mature oocytes is occurring with each egg-laying event, strongly suggesting females do not participate in clutch partitioning. This is supported by instances of relatively long recapture intervals between rain events recorded within season for this species (Gould, Clulow, Herb, & Clulow, 2022), suggesting females are returning to pools to lay an additional clutch rather than due to failure in ovipositing an initial clutch or to clutch partition. Further, we have previously detected no decline in the size of frothed nests for this species over the length of the breeding season (Gould, Clulow, Herb, & Clulow, 2022), which if used as a proxy for clutch size (i.e. eggs per nest) could suggest that females replenish a second clutch that is not of a reduced size as reported in other multi-clutching species (Howard, 1978a; Lips, 2001).

Female *L. fletcheri* lost less than 10% of body weight per egg-laying event, yet clutch sizes were still relatively large (up to 500 eggs). This represents a relatively low investment per egg when compared to other multi-clutching species including *Crinia signifera*, whose females can lose a third of their body mass ovipositing clutches of around 88 eggs (Lemckert & Shine, 1993). This disparity could be due to *C. signifera*'s smaller size, as gonad investment is known to be negatively correlated with body size in amphibians (Crump, 1975). Based on the fundamental trade-offs that are known to exist between reproductive effort and adult survival (Sæther *et al.*, 1996), it could be hypothesized that *C. signifera* has selected for high investment in an initial clutch at the expense of lowering adult survival to subsequent breeding events, while *L. fletcheri* has selected for a comparatively lower investment per clutch in exchange for an improved probability to multi-clutch within season. Of course, multi-clutching is an energy demanding process with fitness consequences that many individuals may not complete. Indeed, we found variability in the maturation of eggs 90 days post-ovulation despite individuals being provided the same and continuous food supply, suggesting that only a portion of females in any given year will be able to produce a second clutch within seasonal time constraints. Further, the capacity to multi-clutch, coupled with the absence of mortality in females taken into the laboratory over the study period, indicates that the restriction of adults to a single season of breeding is not from reproduction-induced mortality as found in some truly semelparous vertebrates that have a suicidal reproductive pattern (Braithwaite & Lee, 1979; Holleley *et al.*, 2006).

Although there is no data on the rate of spermatogenesis in *L. fletcheri* (indeed, no data available for temperate Australian anurans generally), the small difference in sperm concentration between post-amplectic and non-amplectic males suggests that relatively few sperm (as a proportion of total testicular sperm reserves) are released for each clutch fertilized. This low reproductive output invested per spawn suggests that males are also capable of participating in multiple intra-

seasonal spawning alongside females. Indeed, males are often found to remain at pool sites for extended periods, with recaptures common both within and between rain events within season (Gould, Clulow, Herb, & Clulow, 2022), while two individuals recorded in amplexus with different females on separate nights in the current study adding further weight to this possibility. The lack of difference in the viability or motility of sperm collected from post-amplected and non-amplecting males further suggests that residual testicular sperm are mature and can be retained for a subsequent egg fertilization event. The primary physiological demand post-amplexus is presumably for an increase in sperm number through completion of additional rounds of the spermatogenic cycle to restore testicular sperm reserves. Nevertheless, it is unknown whether the pattern of the spermatogenic cycle (Ogielska & Bartmańska, 2009) in *L. fletcheri* involves continuous, asynchronous spermatogenesis predicted for amphibians from stable environments (Jørgensen, 1992), or seasonal waves of synchronous spermatogenesis predicted in fluctuating temperate environments (Jørgensen & Billeter, 1982; Lofts, 1974; Witschi, 1956). It is likely that reproductive success is much higher for males than females of this species but also more variable, given that the potential of males to reproduce several times within season will be subject to competition between males, while females are probably limited to a maximum of two reproductive events within season due to the length of time required for a new wave of mature eggs to form.

We provide direct evidence of intra-seasonal multi-clutch fertilization in *L. fletcheri* males, as well as evidence of the potential for multi-clutching among laboratory-held females. The capacity for both sexes to participate in multiple reproductive events within season may act as a form of bet-hedging, reducing the risk of total reproductive failure in a variable breeding system where total clutch loss from system drying is common and adult survival is low between seasons. As a rarely recorded life history pattern intermediate to both virtual semelparity and the shallowest depths of iteroparity, our finding of likely abbreviate iteroparity in *L. fletcheri* highlights the diversity of reproductive patterns that exist among amphibian. Future research is required to obtain direct observations of within-year multi-clutching among females in the wild, as well as to determine the occurrence of multi-clutching in amphibians in general and whether it is a trait that has evolved to directly accommodate a short lifespan in virtually semelparous species.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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