Genetic benefits of extreme sequential polyandry in a terrestrial-breeding frog

Sequential polyandry may evolve as an insurance mechanism to reduce the risk that females choose mates that are genetically inferior (intrinsic male quality hypothesis) or genetically incompatible (genetic incompatibility hypothesis). The prevalence of such indirect benefits remains controversial, however, because studies estimating the contributions of additive and non-additive sources of genetic variation to offspring fitness have been limited to a small number of taxonomic groups. Here, we use artificial fertilisation techniques combined with a cross-classified breeding design (North Carolina Type II) to simultaneously test the 'good genes hypothesis' and the 'genetic incompatibility hypothesis' in the brown toadlet (*Pseudophryne bibronii*); a terrestrial breeding species with extreme sequential polyandry. Our results revealed no significant additive or non-additive genetic effects on fertilisation success. Moreover, they revealed no significant additive genetic effects, but highly significant non-additive genetic effects (sire by dam interaction effects), on hatching success and larval survival to initial and complete metamorphosis. Taken together, these results indicate that offspring viability is significantly influenced by the combination of parental genotypes, and that negative interactions between parental genetic elements manifest during embryonic and larval development. More broadly, our findings provide quantitative genetic evidence that insurance against genetic incompatibility favours the evolution and maintenance of sequential polyandry.

KEY WORDS: Polyandry, compatible genes, good genes, external fertilisation

A long held notion in sexual selection theory has been that males, but not females, enhance their reproductive success by gaining multiple mates (Bateman 1948; Trivers 1972). However, in the last two decades advances in molecular techniques for assigning paternity have revealed that polyandry is prevalent in most animal taxa (Simmons 2005; Parker and Birkhead 2013). The reasons why females routinely mate with multiple males is now one of the most compelling, but least understood, questions in evolutionary biology (Simmons 2005; Slatyer et al. 2012; Parker and Birkhead 2013; Pizzari and Wedell 2013; Taylor et al. 2014). Copulation is potentially costly to females through time and energy expense (Sih et al. 1990), exposure to predators (Fairbairn 1993), increased risk of disease transmission (Thrall et al. 1997) and mechanical damage (Eberhard 1996). Therefore, unnecessary matings should be strongly selected against. In some species, females may be coerced into mating by promiscuous males and accept additional matings to reduce the costs of sexual harassment (Rice et al. 2006; Boulton et al. 2018). However, in cases where females actively solicit copulations with multiple males we can assume that polyandry is an adaptive female mating strategy (Byrne and Roberts 2012).

Arguments about the adaptive advantage of polyandry fall into two broad categories: direct 'material' benefits and indirect 'genetic' benefits. Material benefits may be attained if polyandry insures against male infertility (Walker 1980; Reding 2015), insures against nest site failure (Byrne and Keogh 2008), provides extra nutrients for progeny (Zeh and Smith 1985; Birkhead 1995), or secures additional paternal care (Griffith et al. 2002). Genetic benefits may also be attained in multiple ways. Early theoretical models centered on the idea that polyandry may increase the genetic diversity of a female's progeny as a buffer against disease, sibling competition and/or environmental perturbation (Yasui 1998; Oldroyd and Fewell 2007; Aguirre and Marshall 2012). More recently, theoretical models have focused on the idea that polyandry may improve the genetic quality of a female's progeny, either by

safeguarding against females mating with a male that is genetically inferior (intrinsic male quality hypothesis) or genetically incompatible (genetic incompatibility hypothesis)(Yasui 2001; Yasui and Garcia-Gonzalez 2016).

The 'intrinsic male quality hypothesis' (akin to the good genes model of mate choice) is based on the premise that variation in offspring viability is paternally inherited and predicts that polyandry (coupled with post-copulatory processes that enable differential fertilization, i.e. cryptic female choice and sperm competition) will facilitate the selection of intrinsically high quality alleles (good genes) (Yasui 1998; Fox and Rauter 2003; García-González and Simmons 2005). Selection for good genes is predicted to generate additive genetic variance in offspring fitness, whereby a male carrying good genes will produce offspring with higher fitness, independent of a female's genotype (Jennions 1997; Neff and Pitcher 2005). By contrast, the genetic incompatibility hypothesis postulates that offspring viability is determined by an interaction between male and female genotypes, and that polyandry increases the likelihood that females secure compatible genes. The central premise being that incompatibilities, resulting from allelic interactions within or between loci (dominance and epistasis) cause non-additive genetic variance in fitness, whereby only specific allelic combinations (male x female male crosses) elevate offspring viability (Neff and Pitcher 2005).

Across a diversity of taxa, numerous studies have reported positive correlations between polyandry and offspring viability (Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Fisher et al. 2006; Taylor et al. 2014). Nevertheless, the capacity for genetic mechanism to underpin polyandry remains strongly debated, largely because experimental evidence for widespread genetic benefits remains limited (Simmons 2005; Akçay and Roughgarden 2007; Slatyer et al. 2012; Taylor et al. 2014). One reason for this may be that studies attempting to comprehensively partition phenotypic variance in offspring traits into

components of additive and non-additive genetic variance have been limited to a small number of taxonomic groups. In recognition of this significant knowledge gap, there has been an emerging focus on using quantitative genetic breeding experiments to critically test the predictions of competing genetic-benefit hypotheses for the evolution of polyandry (García-González and Simmons 2005; Pitcher and Neff 2006; Evans et al. 2007; Marshall and Evans 2007; Pitcher and Neff 2007; Rodríguez-Muñoz and Tregenza 2008; Lumley et al. 2016).

Animal systems with external fertilisation provide un-rivalled opportunities to perform large-scale quantitative genetic breeding experiments required to accurately estimate sources of genetic variation in offspring fitness. Using artificial fertilisation (AF) technologies to manipulate and control parentage, it is possible to employ cross-classified breeding designs that restrict or eliminate non-genetic sources of variation (i.e. non genetic paternal or maternal effects). This is not possible in systems characterised by internal fertilisation, primarily because males can influence fertilisation outcomes by delivering variable amounts of sperm or seminal fluid (Bromfield et al. 2014), and females can alter offspring viability through post fertilisation provisioning (Gilbert et al. 2006). Critically, such effects can amplify or mask genetic processes (Kotiaho et al. 2003) and hinder accurate assessment of the relative importance of good genes versus compatible genes. One quantitative genetic approach increasingly being used (in combination with AF) to test for genetic benefits of polyandry in externally fertilising species is the North Carolina Type II breeding design; a cross-classified design whereby a set of sires and dams are crossed in every possible pairwise combination (Lynch and Walsh 1998). By splitting the clutch of each female between multiple males it is possible to hold maternal effects constant so that ‘half sibs’ differ only in paternally-inherited genes. Across multiple crosses (families), variance in offspring fitness can then be precisely partitioned among additive genetic effects (good genes), non-additive effects (compatible genes) and maternal effects (encompassing maternal

genetic effects and environmental effects) (Simmons 2005). Importantly, in this design the influence of environmental effects can be manipulated and minimised by raising half sibs in a controlled environment (Rudin-Bitterli et al. 2018).

A growing number of studies have used the North Carolina type II breeding design to test for genetic benefits of polyandry in species with external fertilisation, and there is emerging evidence that offspring fitness can be influenced by good genes (Marshall and Evans 2007; Kekäläinen et al. 2010), compatible genes (Rudolfsen et al. 2005; Dziminski et al. 2008; Rodríguez-Muñoz and Tregenza 2008), or combinations of the two (Wedekind et al. 2001; Pitcher and Neff 2006; Evans et al. 2007; Pitcher and Neff 2007). However, additional studies, spanning a diversity of externally fertilising polyandrous taxa, are urgently needed to enable an analysis of the relative importance of additive versus non additive genetic effects, and help elucidate interspecific variation in the magnitude of these effects. Because the type of genetic benefit afforded to a species can vary considerably depending on the type of fitness trait examined (Ivy 2007), there is a critical need for studies that evaluate genetic effects on multiple components of offspring performance across different life stages. Such work will help to pin point the types of traits influenced by additive and non-additive effects, and shed light on developmental points where genetic benefits begin to manifest. Ultimately, future work should also target model species where the genetic mating system has been resolved so that breeding experiments are designed to reflect natural rates of polyandry. This will ensure that experimental mating contexts (and resultant fitness consequences) are ecologically and evolutionary relevant (Lumley et al. 2016). There is also a critical need to test for genetic benefits in species with sequential polyandry. In these systems we can be sure that females have control over mating and that polyandry is an active female mating strategy.

Here, we use a North Carolina type II breeding design to test the intrinsic male quality hypothesis and the genetic compatibility hypothesis in the Australian terrestrial toadlet

Pseudophryne bibronii. A previous study quantifying the genetic mating system of this species revealed it has highest level of sequential polyandry of any vertebrate studied to date (Byrne and Keogh 2008). During a breeding season, all females are polyandrous, dividing their clutches between the nests of up to eight males (mean =5 males). Long-term field monitoring of nest sites has shown that polyandry provides a direct fitness benefit by insuring against nest failure (Byrne and Keogh 2008). However, we suspect that genetic benefits may strongly contribute to the evolution and maintenance of polyandry in this species because clutches containing inviable embryos are regularly observed in nature (Woodruff 1976b). The aim of our study was to test whether differences in offspring fitness are underpinned by good genes effects and/or compatible genes effects. If the acquisition of good genes is an important selective pressure favouring polyandry in *P. bibronii*, we expect to see significant differences in offspring fitness that depend on sire identity. Alternatively, if compatible genes are important we expect to see differences in offspring fitness that depend on sire by dam interactions. The relative importance of good genes and compatible genes to offspring fitness will be revealed by the amount of additive, and non-additive, genetic variance, respectively.

Methods

STUDY SYSTEM

Pseudophryne bibronii (Fig. 1) is a small terrestrial-breeding myobatrachid frog (22-36 mm snout-vent length) that is restricted to temperate regions of south-eastern Australia. Breeding occurs from March to June (austral Autumn to Winter) and typically occurs along ephemeral water courses that seasonally inundate (Woodruff 1976a). Males enter a breeding site before females and construct shallow nests in moist soil underneath leaf litter, logs or rocks (Woodruff 1976a; Mitchell 2001). Males remain at a nest site for the duration of a breeding season (typically 3-5 months) and use distinct calls to attract mates and deter rivals (Byrne

2008), though chemicals are also used in communication (Mitchell 2005; Byrne and Keogh 2007). Gravid females (those carrying mature oocytes) enter breeding sites after significant rain events (correlated with peaks in calling activity) and typically spend several days assessing multiple males before mating (Byrne unpublished data). Amplexus is inguinal and eggs are fertilised externally as females oviposit into the terrestrial nest. Embryo's develop quickly within gelatinous egg capsules until Gosner stage 26-28 (hind limb-buds developed) (Fig. 1A), at which point development is suspended (Woodruff 1976b; Bradford and Seymour 1985). Development resumes when heavy rainfall floods the nest and hypoxia triggers tadpoles to hatch into shallow pools. Tadpoles develop within these pools over winter and metamorphose between late austral spring and early summer when pools begin to dry (Woodruff 1976a; Bradford and Seymour 1985).

STUDY POPULATION AND ANIMAL COLLECTION

The study was conducted using frogs collected from a natural population located in an area of remnant Eucalypt, Banksia and Casurina bushland near Wrights Beach in Jervis Bay National Park, New South Wales, Australia. Breeding was restricted to an ephemeral creek line and drainage pan which was dry at the time of the study. Reproductively mature males and females were randomly gathered from the population. Reproductively mature males (Fig. 1C) were collected from their nests after haphazardly locating nest sites by tracking a resident male's advertisement call. Gravid non-amplexed females were collected from within male nests, with their presence revealed by the resident male calling at an elevated rate, and/or releasing courtship calls (Byrne 2008). Males (n=15) and females (n=14) were collected during three breeding episodes, corresponding with three experimental blocks (see below). Episode 1 took place between 14-17/4/2010, episode 2 between 23-27/4/10 and episode 3 from 2-4/5/10. For each experimental block, males and females were collected over two

nights and were used for artificial fertilisation trials that were conducted in a field station located approximately 1km from the study site.

BREEDING DESIGN

A North Carolina type II breeding design was used to control parentage and partition sources of genetic and phenotypic variance in offspring fitness (Lynch and Walsh 1998). This design allowed a simultaneous test of the intrinsic male quality hypothesis and the genetic incompatibility hypothesis (Fig. 2). A total of three experimental blocks were performed, hereafter referred to as block 1, block 2 and block 3. In block 1, five sires and four dams were mated in all 20 combinations (5 x 4 male-by-female factorial crosses). In block 2 and block 3, five sires and five dams were mated in all 25 combinations (5 x 5 male-by-female factorial crosses; Fig. 3). Each female was mated to a single male in each cell (i.e. fertilisations were non-competitive), and no males or females were used more than once. The final combined design for our genetic analysis generated 70 families of paternal and maternal half siblings. The body size range of frogs used in the experiment (male mass = 0.81 – 1.26g, mean ± SE = 1.03 ± 0.031g, n = 15; female mass = 1.18 – 3.39g, mean ± SE = 2.37 ± 0.164g, n=14) reflected variation in body size (and presumably age) observed in the study populations (Byrne, P.G. unpublished data). Therefore, on the assumption that body size (or age) and genetic quality are associated, we are confident that our design provided ample opportunity to detect intrinsic quality effects.

COLLECTION OF GAMETES AND ARTIFICIAL FERTILISATION (AF)

All crosses (matings) were performed using artificial fertilisation techniques previously developed for a closely related species, *Pseudophryne guentheri* (Silla 2013). Sperm

suspensions were prepared by removing and macerating the testes of euthanized (double-pithed) males. There is no evidence that anuran sperm obtained via testes macerates have lower viability (and hence potential to influence embryo viability) than sperm released as natural ejaculates. Testes were macerated in 200-300 µL of chilled 1:1 simplified amphibian ringer (SAR: 113 mM NaCL, 1 mM $CaCL^2$, 2 mM KCl, 3.6 mM $NaHCO_3$; 220 mOsmo kg^{-1}) in 1.5 mL Eppendorf tubes. The sperm concentration in each suspension was measured using an Improved Neubauer Haemocytometer chamber (0.1 mm depth; Bright Line, Optik Labor, Germany). A homogenised 1-µL sub-sample of each sperm suspension was diluted in 19 µL of SAR, homogenised and pipetted into the chamber, with the number of spermatozoa present in five quadrats recorded. Dilution and counting protocols were repeated twice per suspension, and sperm counts averaged. Sperm suspensions were refrigerated for ~12 h before use in fertilisation assays. Refrigerated storage of *Pseudophryne* sperm for this duration does not alter sperm performance or viability (Silla 2013). Sperm suspensions were pre-prepared in advance so that artificial fertilisations could be conducted immediately once oocytes were obtained from the females.

Oocytes were obtained from females according to non-invasive techniques described previously (Silla 2011, 2013). Briefly, females were hormonally induced to ovulate following the administration of synthetic gonadotropin-releasing hormone (GnRH-a, leuprorelin acetate, Lucrin, Abbott, Australia). GnRH-a was administered in two injections; a priming dose (0.4 µg g^{-1} bodyweight) followed by an ovulatory dose (2 µg g^{-1} bodyweight) twenty-six hours later. Each hormone injection was diluted in 100 µL SAR and administered subcutaneously into the dorsal lymph sac. Oocytes were obtained from each female 10-11 hours after the administration of the ovulatory dose. Expulsion of oocytes from the oviduct of each female was facilitated by holding the frog with legs unrestrained, and gently applying

pressure to the abdomen (a process termed stripping). Females that had ovulated expelled oocytes within 10-90 seconds of abdominal pressure being applied.

Oocytes from each female were evenly distributed among five dry plastic fertilisation trays (60 mm L x 60 mm W x 20 mm D), following a balanced fully factorial split clutch breeding design. Because females had different clutch sizes, the total number of eggs allocated to each family differed, though sub-clutches allocated to each sire were approximately equal in size. An aliquot of sperm suspension (at a pre-calculated volume) was added to the edge of each fertilisation tray (without contacting the oocytes). Chilled fertilisation medium (1: 4 SAR; 50 mOsm kg^{-1}) was then added, such that the final solution was exactly 200 µL with a fixed concentration of 1000 spermatozoa μL^{-1}. This procedure ensured that sperm concentrations were identical both within and among blocks (Evans et al. 2007). The fertilisation tray was agitated for exactly one minute by moving the tray back and forth between two markers spaced 5 cm apart. The order that a male's sperm was added to each fertilisation tray was randomised to avoid potential order effects, and trays were randomly allocated to a position on the experimental table to avoid potential spatial effects. The position of each fertilisation tray was changed daily to ameliorate potential room effects and fertilisation trays were covered in parafilm to reduce the risk of bacterial contamination.

FERTILISATION ASSAYS

Artificial fertilisations were conducted at 0900 h at room temperature (17.0 – 19.5°C). Developing embryos were supplied with 200 µL of 1: 4 SAR (50 mOsm kg–1) at 2, 4, 6 and 8 h after fertilisation. A further 200 µL was provided every 12–24 h until 168 h post fertilisation as required to maintain adequate hydration throughout early development. Room temperature during early development ranged from 11.0 – 25.5°C. Fertilisation success was determined as the proportion of embryos developing to Gosner Stage 13 (neural groove)

(Gosner 1960), achieved at approximately 72 h after fertilisation. After approximately 96 hours, once embryos reached Gosner stage 17 (tail bud) (Gosner 1960) any unfertilised eggs were removed using a plastic pipette. At 168 h post fertilisation, once embryos had reached Gosner stage 20 (tail elongation) (Gosner 1960) a piece of sponge (55 mm L x 55 mm W x 2 mm D) saturated with 3 ml of reverse osmosis water (Pureau, Australia) was added to each tray underneath the developing embryos to prevent them desiccating.

OFFSPRING REARING AND SURVIVAL ASSAYS

At 408 hours post-fertilisation, once embryos reached Gosner stage 27 (hind limb bud development) (Gosner 1960), eggs were transferred to individual plastic containers (one egg per container; 20 mm L x 20 mm W x 20 mm D) and flooded with 4 ml of reverse osmosis water to trigger hatching. For each cross, embryo viability was estimated by calculating the proportion of developing embryos that survived to hatching. Freshly hatched tadpoles were then transported to Monash University (Clayton, Victoria) where they were housed in a constant temperature room set to 20°C (range=18–21°C). The following day, tadpoles were moved to larger, cylindrical plastic rearing containers (one tadpole per container; 10cm D x 10.5cm H) holding 500ml of reverse osmosis water (Pureau, Australia). The rearing containers were positioned on a large flat shelf in a constant temperature room and containers were randomised with respect to family ID to avoid potential spatial effects. To prevent developmental disorders associated with UV deficiencies, UV-B lights, and reflectors (Reptisun 10.0 UVB 3600 bulb; Pet Pacific, Australia) were suspended approximately 30cm above each experimental container. Artificial lighting was maintained on an 11.5:12.5 light: dark cycle. Every second day, each rearing container received a 50% water change, and tadpoles were subsequently fed approximately 0.025 g dry mass of ground fish flakes (75:25mixtureofSeraFlora/SeraSans; SERA,Germany). This feeding regime ensured that food was provided *ad libitum*. To prevent water fouling, excess food and excrement was siphoned

from each rearing container once a week using a 30ml plastic syringe connected to a 15cm length of aquarium tubing (inner diameter = 3mm). At the point of forelimb emergence (Gosner stage 42), the water level in each rearing container was dropped by 75% of the original volume and the container was raised on one side to allow metamorphosing individuals to crawl from the water. In addition, a small piece of sponge (95×72×10 mm) was added to prevent recently metamorphosed individuals from drowning. From the point of forelimb emergence to full tail reabsorption (Gosner stages 42–46) tadpoles were not provided with any food because tadpoles stop feeding during this developmental stage (Hourdry, et al, 1996). For each family (cross), larval viability was calculated as the proportion of individuals that survived to i) initial metamorphosis (the point where an individual had left the water and begun breathing air) and ii) complete metamorphosis (the point where an individual had completely resorbed its tail).

STATISTICAL ANALYSIS

To estimate effects of sires, dams and their interaction on offspring fitness, Generalized Linear Mixed Effects Models (GLMM) with binomial error distribution, Laplace approximation logit link function were used to partition sources of phenotypic variation in four fitness-determining traits: 1) fertilisation success, 2) hatching success (embryo survival to hatching), 3) larval survival to initial metamorphosis and 4) larval survival to complete metamorphosis. For these analyses, we also implemented restricted maximum-likelihood methods (REML). Binary values were used for individual traits (e.g., 0 = dead; 1 = alive). GLMM models partitioned total variance as following:

$$Y_{ijklmn} = \mu + S_i + D_j + I_k + B_l + e_{ijklm}$$

where sire (S), dam (D), the interaction between sire and dam (I), and experimental blocks (B) were treated as random effects. Models were also compared using block as fixed effect.

The significance of each random effect was determined using Akaike information criterion (AIC) and the likelihood ratio tests (LRT) between the full model and a reduced model without the tested effect. Variance components were extracted from the GLMMs as VS (sire), VD (dam) and VI (sire x dam interaction). Confidence intervals (95%) for the variance components were produced using a bootstrap method. Assuming that epistasis is negligible, additive (VA), non-additive (VNA, including dominance) and maternal variance (VM) were calculated based on (Lynch and Walsh, 1998) as follows: VA = 4VS; VNA = 4VI; VM = VD – VS. The sire variance component (covariance among paternal half siblings) provided an estimate of additive genetic effects and the dam variance component (the covariance between maternal half siblings) provided an estimate of genetic and environmental maternal effects. The sire x dam interaction variance provided an estimate of the genetic variance due to non-additive nuclear gene action (dominance and extra nuclear interactions) (Evans et al. 2007). Prior to running the GLMM models, we used Linear Mixed Effects Models (LME) to test for any effect of egg number per sub-clutch (family) on fertilisation success, embryo viability and/or survival to initial or complete metamorphosis. In these models, sire and dam ID were included as random effects. These analyses revealed that number of eggs had a significant effect on fertilisation success (LME: $F_{1, 67} = 4.615$, $P = 0.035$), but this variable had no significant effect on either embryo viability (LME: $F_{1, 67} = 0.796$, $P = 0.375$), survival to initial metamorphosis (LME: $F_{1, 67} = 0.015$, $P = 0.900$) or survival to complete metamorphosis (LME: $F_{1, 67} = 1.507$, $P = 0.542$). Therefore, the number of eggs per sub-clutch was not included as a covariate in any of the GLMM models. Finally, we examined interrelationships between fertilisation success, hatching success (embryo viability) and survival to metamorphosis using Linear Mixed Effect (LME) Models. In these LME models, one response variable was treated as the predictor variable (fixed effect) while the other was treated as the response variable. Sire ID, Dam ID and Block number were treated as random

effects. Assuming that genetic effects manifest at fertilisation and carry across life stages, we predicted significant positive inter-relationships between all fitness measures. Analyses were performed using R (R Development Core Team, 2015) and JMP V11 (SAS Institute, USA). Mixed-effect models were performed with the lme4 package (Bates et al. 2015).

ETHICS STATEMENT

Research activities followed protocols approved by Monash University Animal Ethics Committee (protocol number BSCI/2007/14) in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 2013, and was authorised by New South Wales National Parks & Wildlife Service - Office of Environment and Heritage (licence number S12552).

Results

Crosses produced fertilised eggs in 98.5% of cases (69/70 crosses). Excluding the cross that did not produce fertilised eggs, mean ± SEM fertilisation success was 89.086 ± 0.018%. From these successful fertilisations, a mean of 83.043 ± 0.027% of individuals survived to hatching, of which a mean of 72.739 ± 0.033% survived to initial metamorphosis, and 57.550± 0.031 % survived to complete metamorphosis.

Across families, fertilisation success ranged from 0 -100%, and hatching success ranged from 20-100% (see supplementary information). For families with less than 100% hatching success, embryo death occurred anytime from the point that fertilisation was scored (Gosner stage 13-neaural plate formation) through to the point where development was suspended (Gosner stage 27-fully developed larvae). Larval survival to metamorphosis was also highly variable across families, with certain crosses producing 100% viable larvae that

340 successfully reached initial or complete metamorphosis, while others produced larvae that all
341 died before initial or complete metamorphosis.

342 The results of the quantitative genetic analysis (including parameter estimates and
343 coefficients of variation) are presented in Table 1. Our analysis of fertilisation success
344 showed a highly significant female-variance component (dam effect), though no significant
345 male-variance component (sire effect), and no interaction between these effects (sire x dam
346 effect). Our analysis of hatching success (embryo viability) also revealed a highly significant
347 female-variance component (dam effect) and no significant male variance component (sire
348 effect). By contrast, however, embryo viability was significantly influenced by the interaction
349 between sires and dams. Our analysis of survival to initial and final metamorphosis yielded
350 similar results. For both of these fitness traits there was no sire effect, though a significant
351 dam effect, and a highly significant sire x dam effect. These results indicate high levels of
352 non-additive genetic variance for embryo and larval viability and survival to initial and
353 complete metamorphosis. Block had a significant effect on fertilisation success, but did not
354 have a significant effect on any of the offspring fitness traits. Overall, additive genetic effects
355 explained less than 3.5% of the variance in fertilisation success, and less than 0.1% of the
356 variance in hatching success and larval survival to initial and complete metamorphosis (Table
357 2). By contrast, non-additive genetic effects explained between 45% and 91% of the observed
358 phenotypic variance in offspring fitness traits. Maternal effects explained between
359 approximately 8% and 22% of the phenotypic variance (Table 2).

360 We also examined the interrelationships between fertilisation success, embryo
361 viability (hatching success) and survival to metamorphosis. Fertilisation success was not
362 significantly interactive with either embryo viability (LME: $F_{1, 67} = 2.28$, $P = 0.135$), survival
363 to initial metamorphosis (LME: $F_{1, 67} = 0.004$, $P = 0.951$), or survival to complete
364 metamorphosis (LME: $F_{1, 67} = 1.122$, $P = 0.293$). However, there was a strong and significant

inter-relationship between embryo viability and survival to initial metamorphosis (LME: $F_{1, 67} = 9.284$, $P = 0.003$), but not with survival to final metamorphosis ($F_{1, 67} = 3.592$, $P = 0.062$). There was also a strong and significant inter-relationship between survival to initial metamorphosis and survival to complete metamorphosis (LME: $F_{1, 67} = 107.79$, $P < 0.001$). In all of these LME models, random effects (sire ID, dam ID and block number) were non-significant ($P < 0.05$).

Discussion

Despite a growing body of evidence demonstrating that polyandry improves offspring viability, adaptive genetic explanations remain conjectural. Here we use a North Carolina type II breeding design to experimentally investigate the potential for polyandrous brown toadlets to supply their offspring with genetic benefits, either by increasing the probability of procuring 'good genes' (intrinsic male quality hypothesis) or 'compatible genes'(genetic compatibility hypothesis). We found: i) no significant additive or non-additive genetic effects on fertilisation success, ii) no significant additive genetic effects, but highly significant non-additive genetic effects, on hatching success and larval survival to initial and complete metamorphosis, and iii) no relationship between fertilisation success and offspring viability, but strong positive correlations between embryo viability and larval viability. These findings provide no support for the 'good genes hypothesis', but support for the 'genetic compatibility hypothesis', and suggest that negative interactions between parental genetic elements manifest during early development. Taken together, our findings suggest that indirect genetic benefits may contribute to the evolution and maintenance of polyandry in terrestrial toadlets.

Non-additive genetic effects can arise due to a combination of dominance and epistatic effects (Lynch and Walsh 1998). However, based on our variance estimates, there is reason to suspect that epistatic effects are particularly important in *P. bibronii*. In the North

389 Carolina Type II design, models used to calculate genetic variance assume a negligible
390 amount of epistasis, and therefore overestimate genetic variance when it occurs (Lynch and
391 Walsh 1998; Pitcher and Neff 2007). Because the genetic effects we detected were so high
392 (variance estimates up to 99%), we can deduce that epistasis was an important factor in our
393 experiment. Similar results have come from quantitative genetic analysis of offspring
394 survivorship in several species of polyandrous fish (Wedekind et al. 2001; Rudolfsen et al.
395 2005; Pitcher and Neff 2007). In these studies, genetic and environmental effects exceeded
396 100%, and genetic estimates of non-additive effects were considered maximums (Pitcher and
397 Neff 2007). In accordance with this line of reasoning, it would be prudent to assume that our
398 estimates of non-additive effects in *P. bibronii* were inflated (either as an outcome of
399 epistasis or possibly other maternal genetic effects). Nevertheless, the highly significant
400 interactive effects we found, and the congruence in findings across our three measures of
401 offspring fitness, indicates that genetic incompatibility is an important component of the *P.*
402 *bibronii* breeding system (at least in our study population).

403 In other animal systems, genetic incompatibilities between parents have been linked
404 to inbreeding depression, outbreeding depression, selfish genetic elements, segregation
405 distortion and immunological effects (Zeh and Zeh 1996; Tregenza and Wedell 2000). While
406 further research will be required to ascertain the relative importance of these effects in *P.*
407 *bibronii*, we suspect that outbreeding depression may be particularly important. In general, a
408 major cause of outbreeding depression is the breakup of co-adapted gene complexes, which
409 are typically observed in locally adapted populations characterised by limited dispersal,
410 restricted gene flow and high levels of genetic differentiation (Templeton 1986; Keller and
411 Waller 2002). High levels of local adaptation can be expected in *P. bibronii* because both
412 sexes display extreme site fidelity (Byrne & Silla unpublished data) and phylogenetic work
413 has revealed pronounced genetic differentiation between neighbouring populations

(Donnellan, S.C. personal communication). Such genetic divergence is likely to create a high risk of genetic incompatibility in matings between non-kin. In support of this notion, crosses made between *P. bibronii* males from our study population and females from several neighbouring populations have produced families with extremely high proportions of inviable and/or deformed embryos and larvae (Byrne and Silla unpublished data). Furthermore, in a recent study using genomic tools to investigate mate choice in the red backed toadlet *P. coriacea*, a sister species to *P. bibronii*, females preferred to mate with more related males (O'Brien 2019). Interestingly, polyandry is extremely rare in *P. coriacea* (O'Brien et al. 2018), which raises the intriguing possibility that outbreeding depression has favoured the evolution of two alternative mating systems in toadlets: 1) monandry, whereby stringent mate choice facilitates genetically compatible pairings, and 2) sequential polyandry, whereby mating with multiple males ameliorates the costs of incompatible pairings.

Irrespective of the source of the incompatibilities observed, our detection of non-additive genetic effects across multiple life stages suggests that genetic benefits of polyandry may be substantial. For amphibians, mortality risk is usually highest during the embryonic and larval life stages (Matthews et al. 2013), and survival to metamorphosis is known to be an important predictor of population persistence (Biek et al. 2002). Therefore, elevated offspring survival during early development could strongly favour polyandrous behaviour. However, the role that genetic benefits have played in the evolution of polyandry in *P. bibronii* remains unclear because polyandry also provides a major direct benefit. In a prolonged field study that tracked offspring survival of an entire *P.bibronii* population, extreme polyandry was found to elevate female fitness by insuring against nest failure (Byrne and Keogh 2008). The primary cause of offspring mortality was the desiccation of embryos or larvae resulting from females depositing eggs in nests that failed to flood, or nests that flooded too early or too late in the breeding season (Byrne and Keogh 2008). Notably, Byrne

and Keogh (2008) reported that egg loss resulting from inviable embryos was low, suggesting that direct benefits contributed more to female fitness than genetic benefits. However, it is important to consider that genetic benefits in this system are likely to be context dependant, with non-additive genetic variance dependant on environmental context, and fitness gains only evident under specific environmental conditions (Marshall and Evans 2007; Rudin-Bitterli et al. 2018). For instance, during breeding seasons where rainfall patterns allow offspring to persist in nests for extended periods, there may be much stronger effects of compatible matings on female fitness. This notion is supported by a quantitative genetic study on the terrestrial toadlet *P. guentheri*, which showed that the magnitude of non-additive genetic effects for desiccation tolerance varied depending on the soil-moisture environment in which offspring developed (Eads et al. 2012). Assuming that *P. bibronii* experiences similar effects, a dynamic interplay between the hydrolic environment and direct and indirect benefits of polyandry may provide sustained fitness gains across breeding years and explain why polyandry in this species is so extreme.

Unexpectedly, we did not observe strong and significant parental interaction effects on fertilisation success, indicating that genetic incompatibilities do not manifest at the gametic level. This finding is in stark contrast with the results of previous quantitative genetic studies that have tested for genetic benefits of polyandry in external fertilisers. In various free spawning marine invertebrates, and a semi-aquatic frog species with simultaneous polyandry, highly significant male x female interaction effects on fertilisation success have been reported (Evans and Marshall 2005; Marshall and Evans 2005; Dziminski et al. 2008). In such systems, egg surface proteins have been implicated as mediating the binding of sperm to the egg surface, and it has been suggested that such gametic level interactions could provide a filtering mechanisms to ensure the combination of compatible genotypes (Dziminski et al. 2008). Selective fertilisation may not have evolved in *P. bibronii* simply because polyandry

464 is sequential rather than simultaneous. Considering that females only mate with one male at a
465 time (and the sperm of multiple males are not available to fertilise eggs), effective blocks to
466 incompatible sperm would result in entire clutches (or sub clutches) remaining unfertilised.
467 Undoubtedly, this would represent a much larger fitness cost to females than producing a
468 partial clutch of low quality offspring where some individuals might still survive and
469 reproduce. Given that both simultaneous and sequential polyandry are common in anuran
470 amphibians (Roberts and Byrne 2011; Byrne and Roberts 2012), this group provides
471 excellent opportunities for comparative research aimed at investigating how different forms
472 of polyandry influence the evolution and operation of gamete recognition systems for
473 selective fertilisation.

474 Although our findings provide support for the genetic incompatibility hypothesis, it is
475 important to recognize that our experimental approach may have contributed to the sire x dam
476 effect reported. Specifically, half sib families were reared as batches during embryo
477 development, so it is possible that 'batch specific' micro-environmental conditions affected
478 offspring survival, and inflated our estimates of non-additive genetic variance. Because the
479 position of fertilization trays was changed daily, it is highly unlikely that room effects created
480 batch specific differences in micro-environmental conditions. Moreover, because we found
481 no evidence for relationships between egg number and offspring fitness, we have no reason to
482 think that differences in sub-clutch size led to micro environmental differences. It is possible,
483 however, that different batches harbored distinct microbial communities that differentially
484 influenced offspring fitness. Past studies in fish have demonstrated that bacterial colonization
485 of egg surfaces during culture can significantly influence embryo and larval growth and
486 survival (Hansen and Olafsen 1999). Saying this, any inter-batch differences in microbial
487 activity are unlikely to have been significant. Our procedures were all conducted using sterile
488 equipment, fertilization mediums and rearing solutions, so there was little opportunity for

bacterial contamination. Furthermore, males were aseptically dissected to obtain sperm, and sperm suspensions obtained in this way are known to have very low bacterial abundance (Keogh et al. 2017). Microbes may have been vertically transmitted from mothers to eggs (and later tadpoles) (Walke et al. 2011), though any negative impacts on embryos are likely to have been negligible. Frog eggs, particularly those of terrestrial breeding species, are coated in extremely thick protective jelly coats that provide a physical barrier against bacterial and fungal infection, and they also contain proteins (lectins) and proteinase inhibitors with bacteriostatic activity known to play a defensive role in resistance to invasion of pathogens (Peavy et al. 2003; Fleming et al. 2009). Moreover, even if microbes were maternally transmitted, microbial communities on frog eggs tend to be stable, showing very similar assemblages among different maternal hosts (Hughey et al. 2017). Therefore, there is no logical reason to expect that impacts on offspring fitness would have manifested as non-additive effects (i.e. extremely low levels of paternally inherited microbiota and stable maternally inherited microbial communities would have restricted opportunities for significant interactions between parental biomes). Nevertheless, to exclude the possibility that micro-environmental variations among sub clutches might influence estimates of genetic variance, future studies should aim to separate embryos immediately after fertilization and rear offspring independently. This approach has been effectively employed by studies testing for additive and non-addive genetic effects on offspring survivorship and performance in various externally fertilizing fish species ((Jacob et al. 2007; Wedekind et al. 2008; Jacob et al. 2010; Clark et al. 2013a; Clark et al. 2013b; Pompini et al. 2013; Brazzola et al. 2014; Stelkens et al. 2014; da Cunha et al. 2018; da Cunha et al. 2019)).

In addition to finding significant non-additive genetic effects, we found significant maternal effects on both fertilisation success and offspring fitness. In the NCII design, maternal effects encompass additive genetic effects as well as non-genetic maternal effects

514 (indirect maternal influences on offspring phenotypes). Therefore, until additional
515 quantitative genetic experiments are conducted, the relative importance of genetic versus
516 environmental-maternal effects on offspring fitness in *P.bibronii* will remain uncertain.
517 Nevertheless, we expect that environmental-maternal effects may be substantial because they
518 are known to have strong effects on offspring performance in various amphibians (Merilä et
519 al. 2004; Eads et al. 2012; Rudin-Bitterli et al. 2018). Causes of environmental-maternal
520 effects are diverse, but they are typically related to variation in the degree to which females
521 invest in offspring (Wolf and Wade 2016). *Pseudophryne bibronii* has no maternal care, and
522 females in our study had no opportunity to interact with their offspring. Therefore, we can
523 eliminate differential maternal investment as contributing to variance in offspring viability.
524 However, because amphibians provision their eggs before fertilisation, there is considerable
525 opportunity for egg-mediated maternal effects. In various frog species, egg yolk volume has
526 been shown to have major effects on embryonic and larval survival (Dziminski and Roberts
527 2006; Dziminski et al. 2009; Rudin-Bitterli et al. 2018). Moreover, such effects have been
528 found in the terrestrial toadlet *P. guentheri* (Eads et al. 2012), and are expected to be common
529 in the *Pseudophryne* genus because prolonged terrestrial development has selected for
530 extremely large eggs with sizeable yolk reserves. Variation in yolk composition may also
531 explain the significant maternal effects we observed. Across various oviparous taxa there is a
532 rapidly growing body of evidence to suggest that offspring quality is impacted by the extent
533 to which yolk is provisioned with antioxidants, antibodies, steroids, fatty acids, and amino
534 acids (Schwabl 1996; Royle et al. 2001; Saino et al. 2003; Saino et al. 2005; Newcombe et al.
535 2015). Investigating how differential allocation of these compounds influences offspring
536 fitness in *P. bibronii* and other amphibians would be a fruitful area for future research.

537 Overall, the findings of our study advance our understanding of the importance of
538 genetic benefits as a driver of polyandry in anuran amphibians. Although polyandry is

widespread in anurans (Roberts and Byrne 2011; Byrne and Roberts 2012), and correlations between polyandry and offspring viability have implicated genetic benefits (Byrne and Whiting 2011), our study is only the third to dissect the genetic architecture of offspring fitness in a polyandrous frog. Using a similar experimental approach to ours, Dzminsiki *et al.* (2008) revealed significant non additive genetic effects on fertilisation success and offspring survival in the Western Australian quacking frog *Crinia georgiana*. In a more recent study in this species (also using a North Carolina II breeding design), Bitterli et al (2018) confirmed high levels of non-additive genetic variance for offspring fitness, and showed that levels of non-additive genetic variation were significantly higher when offspring were reared under stressful conditions. In quacking frogs, males force copulation and multiple male amplexus imposes large fitness costs to females through reduced fertilisation success (Byrne and Roberts 1999). Therefore, any genetic benefits that flow from multi-male spawning are likely to be compensatory (Byrne and Robert 2000). This contrasts with the mating system of *P.bibronii* where females' solicit matings and polyandry is likely to be an active female mating strategy. Such differences suggest that genetic incompatibility contributes to the evolution or maintenance of polyandry under various breeding contexts. This is not surprising because there is a growing body of evidence to suggest that genetic incompatibility (linked to genetic relatedness) plays a pervasive role in amphibian breeding biology. Controlled mating experiments have shown that females prefer more genetically similar males (Chandler and Zamudio 2008; Cayuela et al. 2017), artificial crosses within and between populations have provided evidence for high levels of genetic incompatibility (Sagvik et al. 2005; Eads et al. 2012), and sperm competition experiments have revealed that males who are more genetically similar to females achieve higher fertilisation success (Sherman et al. 2008). From an ecological perspective, it is logical to expect a strong role for genetic incompatibility in amphibian breeding because levels of genetic differentiation between amphibian meta

564 populations (and the potential for local adaptation) are higher than for any other vertebrate
565 class. Such fine-scale genetic structuring in amphibians, typically resulting from an uneven
566 distribution of breeding sites, strong site fidelity and low dispersal, is likely to create a high
567 risk of outbreeding depression. As more studies use quantitative genetic approaches to test
568 competing genetic benefit hypotheses in amphibians, it may become increasingly apparent
569 that insurance against genetic incompatibility contributes to the widespread occurrence of
570 polyandry in this vertebrate class.

571 More broadly, our results add to a growing list of studies demonstrating non additive
572 genetic effects on offspring viability in polyandrous species with external fertilisation. Such
573 effects have been demonstrated in diverse taxonomic groups (broadcast spawning marine
574 invertebrates, fishes, and anuran amphibians), and across a broad spectrum of fitness-
575 determining traits (growth and development, hatching success and survival)(Wedekind et al.
576 2001; Rudolfsen et al. 2005; Pitcher and Neff 2006; Evans et al. 2007; Pitcher and Neff 2007;
577 Dziminski et al. 2008; Rodríguez-Muñoz and Tregenza 2008). This emerging pattern
578 suggests that insurance against genetic incompatibility may be a widespread driver of
579 polyandry in external fertilisers. Saying this, the importance of genetic compatibility may
580 vary considerably within taxonomic groups. For example, in fish (where most quantitiative
581 genetic studies have been conducted) the genetic architecture of offspring survivorship
582 appears to be highly complex. In Chinook Salmon (*Oncorhynchus tshawytscha*) and Alpine
583 whitefish (*Coregonus* sp.), both additive and non-addive genetic variance have been shown to
584 contribute significantly to variance in offspring survivorship (Brazzola et al 2014; Pitcher and
585 Neff, 2006, 2007; Wedekind et al 2001; Wedekind et al2008; Clarke et al 2014). By contrast,
586 in a slow growing small type Alpine whitefish) (*C. albellus*) and brown trout (*Salmo trutta*),
587 offspring survival appears to be pre-dominantly influenced by additive genetic effects, with
588 no or negligible non-additive genetic effects reported (Brazzola et al 2014; Jacob et al 2010;

589 Marques da xunha 2018; Jacob et al 2007; Stelkens et al 2014). This differs from Atlantic
590 cod (*Gadus Morhua L.*), where, similar to our study, only non-additive effects appear to be
591 important (Rudolfsen et al 2005). Such variation is likely to reflect specific-specific
592 differences in population genetic structure resulting from a complex interplay between the
593 level of gene flow, the rate of genetic drift, and the strength and direction of various selective
594 processes (Shaw et al 2018).

595 With a view towards better understanding the relative importance of additive versus
596 non-additive genetic effects across taxa, and elucidating causes of interspecific variation, we
597 encourage studies in a greater diversity of species. Studies targeting species with sequential
598 polyandry, where females have pre-copulatory control over mating, would be particularly
599 valuable. Only by working with such systems can we confidently conclude that polyandry is
600 an active female mating strategy.. Moreover, given we know that genetic benefits can be
601 context dependent (Marshall and Evans 2007; Uller et al. 2011; Smith and Spence 2013;
602 Rudin-Bitterli et al. 2018), there is also a critical need for studies that test for spatial and
603 temporal variation in the magnitude of genetic benefits. Finally, based on the growing body
604 of evidence that polyandry supplies females with multiple benefits, we strongly support the
605 view that theoreticians and empiricists need to move away from the conventional approach of
606 attempting to identify a single mechanism driving polyandry and consider how benefits
607 obtained through combinations of mechanisms contribute to total fitness gains (Ivy 2007).
608 Such work will greatly improve our capacity to understand the adaptive significance of
609 polyandry.

610

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