ORIGINAL ARTICLE

Sperm competition and offspring viability at hybridization in Australian tree frogs, *Litoria peronii* and *L. tyleri*

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Hybridization between closely related species often leads to reduced viability or fertility of offspring. Complete failure of hybrid offspring (post-zygotic hybrid incompatibilities) may have an important role in maintaining the integrity of reproductive barriers between closely related species. We show elsewhere that in Peron's tree frog, *Litoria peronii*, males more closely related to a female sire more offspring in sperm competition with a less related rival male. Observations of rare 'phenotypic intermediate' males between *L. peronii* and the closely related *L. tyleri* made us suggest that these relatedness effects on siring success may be because of selection arising from risks of costly hybridization between the two species. Here, we test this hypothesis in an extensive sperm competition experiment, which shows that there is no effect of species identity on probability of fertilization in sperm

competition trials controlling for sperm concentration and sperm viability. Instead, there was a close agreement between a male's siring success in isolation with a female and his siring success with the same female in competition with a rival male regardless of species identity. Offspring viability and survival, however, were strongly influenced by species identity. Over a 14-day period, hybrid offspring suffered increasing mortality and developed more malformations and an obvious inability to swim and right themselves, leading to compromised probability of survival. Thus, hybridization in these sympatric tree frogs does not compromise fertilization but has a strong impact on offspring viability and opportunity for reinforcement selection on mate choice for conspecific partners.

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Introduction

The production of unfit offspring through hybridization of incipient species is predicted to result in selection for reproductive isolating barriers that reduce the risk of hybridization (Arnold, 1997; Coyne and Orr, 1998). Reproductive isolating barriers may arise at three different levels (1) premating (that is, mating/pollinator behavior, timing of gamete release/flowering times), (2) post-insemination/pollination (gamete recognition) and (3) post-fertilization (offspring viability, survivorship and sterility; Arnold, 1997). Isolation barriers that act between copulation and fertilization have until recently received little attention; however, there is growing evidence that they can have an important role in speciation (for example, Palumbi, 1994; Vacquier, 1998). The investigation of barriers to hybridization at a level of gametic compatibility was, however, for considerable time fraught with the limitation of being non-competitive (Arnold, 1997, 2006). In other words, crosses normally involved the fertilization of a female with a heterospecific male but not with a conspecific male. However, in

natural populations, females are expected to be exposed to sperm/pollen from both con- and heterospecific males (Arnold, 1997, 2006). More recently, experiments on heterospecific gamete competition, primarily in plants and insects, have revealed a variety of outcomes in staged trials in the laboratory. These experiments have resulted in full heterospecific gametic compatibility, assessed in terms of proportional fertilization success, when heterospecific gametes are brought together in isolation. However, when a female is mated with both a heterospecific and conspecific male (therefore allowing sperm competition between con- and heterospecific sperm), conspecific males often secure a greater proportion of fertilizations (Arnold, 1997, 2006 and references therein). For example, in two races of grasshoppers (*Podisma pedestris* spp.), there is sperm precedence for first males. However, when this is taken into account, conspecific males sire significantly more offspring (Hewitt *et al.*, 1989). In this case, the difference in siring success took place at the pre-fertilization level (Hewitt *et al.*, 1989). In another grasshopper crossing experiment, this time between *Chorthippus parallelus parallelus* and *C. p. erythropus*, there were significantly more pure progeny produced compared with hybrid progeny. There was, however, also evidence of second male sperm precedence, possibly due to sperm ageing or differential storage and use of sperm by the female (Bella *et al.*, 1992). Work on crickets (*Allonemobius* sp.) show similar results

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with poorer motility of heterospecific sperm stored in the spermathecae of females, resulting in lower fertilization success compared with conspecific males (Gregory and Howard, 1994). Work by Wade and Johnson (1994) and Wade *et al.* (1994) shows very similar conspecific dominance in siring success in *Tribolium* beetles, as do marine organisms (for example, sea urchins Geyer and Palumbi, 2005) and some plants (for example, Rieseberg *et al.*, 1995), although the underlying mechanisms differ (reviewed in Arnold, 1997, 2006).

When hybrid fertilization takes place, the next level of selection will be on the viability of the hybrid offspring. Historically, the views on the importance of successful hybridization in terms of evolutionary change have been all across the continuum from Lotsy (1916, 1931) arguing its dominating importance in evolution to Dobzhansky (1937, p. 231) claiming that 'no hybrids reach reproductive stage'. Clearly, the reality lies somewhere in between these views with successful introgression of genes occurring at least occasionally during hybridization between species and which generates greater fitness in hybrid offspring compared with purebred offspring. An example of this is (at least sometimes) the case of the fire-bellied toads (Vines *et al.*, 2003, but see Nurnberger *et al.*, 2005). Another set of examples come from hybridization resulting in new, successful species of Lake Malawi cichlids as a result of genetic mosaicism (Salzburger *et al.*, 2002; Seehausen *et al.*, 2003; Seehausen, 2004). Finally, one of the most detailed studies showed that hybridization effects on offspring viability can be sex-specific, consistent with Haldane's rule. Female (heterogametic) flycatchers resulting from crossings between *Ficedula hypoleuca* and *F. albicollis* showed sterility as a result of incompatibility between maternally transmitted mtDNA and paternal nuclear genes, whereas males remained fertile (Tegelström and Gelter, 1990; Saetre *et al.*, 2003). In this case, the sex chromosomes also contained genes that affect species recognition and concomitant compromised offspring viability as a result of 'poor' mate choice.

The current study builds on our previous observation of a fertilization advantage of males more closely related with a female in staged artificial fertilization trials in the Australian tree frog, *Litoria peronii* (Sherman *et al.*, 2008b). An implicit assumption in this study was that of compromised offspring viability in hybrid matings with the sympatric *L. tyleri*, much to the same effect as the recently described ongoing speciation between lineages of another Australian tree frog, *L. genimaculata* (Hoskin *et al.*, 2005). We test here the hypotheses that (i) hybridization compromises probability of paternity in sperm competition, (ii) hybrid embryos have lower hatching success than purebred embryos and (iii) post-hatching development and survival carries a cost at hybridization. We use the results from our laboratory trials, and observations of 'intermediate phenotypes' between *L. peronii* and *L. tyleri* in the wild, for making evolutionary inferences on reproductive barriers between the two species in the wild.

Materials and methods

Study species

The Australian tree frog, *L. peronii*, occurs throughout the coastal and inland regions of New South Wales (NSW),

southern Queensland and northern Victoria (Cogger, 2000; Griffiths, 2006). The range of *L. tyleri* is restricted to mainly coastal areas of NSW and southern Queensland within the range of *L. peronii* (Cogger, 2000; Griffiths, 2006). Both species are commonly found occupying the same breeding ponds, although one species is often numerically dominant (Sherman CDH personal observation). Morphologically, the two frogs are very similar but *L. peronii* can be distinguished from *L. tyleri* by more intense yellow and black mottling in its thighs and armpits. The vocal sack of *L. tyleri* is often a more intense yellow during the breeding season compared with that of *L. peronii* (Sherman CDH personal observation). The two species have distinct mating calls, which may be important in mate choice. *L. peronii* males are, however, indiscriminate choosers and have been observed amplexing males of the same species and even individuals of different species. Observations of matings and the success of naturally spawned clutches between these two species have not been observed directly; however, the presence of rare 'phenotypic intermediate' males between *L. peronii* and *L. tyleri* suggests that successful natural hybridization does occur, at least occasionally.

We collected adult male and female *L. peronii* from a single pond at Darkes Forest, NSW, Australia (34°13'S, 150°55'E). Male *L. tyleri* were collected from a nearby population (approximately 35 kilometers apart) at Yeramba Lagoon, Sydney (33°58'S, 151°00'E). Both species occur at both locations, although *L. peronii* is the numerically dominant species at Darkes Forest, whereas *L. tyleri* is the more common species at Yeramba Lagoon. Individuals of the two species were collected from different locations for logistic reasons, as only sufficient numbers of each species could be collected on the same night by targeting different locations. Only vocalizing males were captured, indicating that they were ready to mate and likely to be reproductively mature (Sherman *et al.*, 2008a, b). Gravid female *L. peronii* were collected during their migration from surrounding vegetation to the breeding pond. Insufficient females *L. tyleri* were found during the collections, limiting our crosses to *L. peronii* and *L. tyleri* males with *L. peronii* females. Frogs were transported to laboratory facilities at the University of Wollongong where we held males overnight at room temperature. Females were held at 10–12 °C to prevent the spontaneous release of gametes. For each frog we measured snout-vent length, to the nearest millimeter, and mass to the nearest 0.01 g. All frogs were used for *in vitro* fertilization experiments within 24 h of capture, and were released back to their place of capture within 72 h.

In vitro fertilizations and sperm competition trials

We followed the protocol for artificial fertilizations for *L. peronii* outlined by Sherman *et al.* (2008b). Briefly, 30 independent sperm competition and control fertilization trials were carried out using sperm collected from one randomly chosen male *L. peronii* and one randomly chosen male *L. tyleri* with a randomly chosen female *L. peronii* (Figure 1). We induced males to release sperm after a subcutaneous injection of luteinizing hormone releasing hormone (Sigma-Aldrich, Sydney, New South Wales, Australia). Approximately, 150 µl per 10 g body weight of a 5 mg per 100 ml luteinizing hormone

Figure 1 Experimental design of *in vitro* fertilization trials. Sperm competition trials contain equal numbers of sperm from *Litoria peronii* and *L. tyleri* males mixed with *L. peronii* eggs. Control fertilizations contain sperm from each male in isolation mixed with *L. peronii* eggs.

releasing hormone in isotonic saline solution (0.9% NaCl) was injected into the lymph sacs of the flanking region. Males were injected within 2 min of each other to ensure that longevity of sperm did not confound our experiments. Frogs were placed in conical flasks and kept in a dark room for 40 min before sperm, which is passively released from the frog's cloaca, was collected from the bottom of the conical flask. The conical flask was subsequently flushed with 2 ml of aged water and the resulting sperm suspension was collected using a sterile pipette. A small aliquot (100 μ l) of each male's sperm was taken to determine the proportion viable sperm using Live/Dead sperm viability kits as per the manufacturers instructions (Invitrogen, Molecular Probes, Mount Waverley, Victoria, Australia). Sperm concentration for each male was determined using a Hawksley hemocytometer (improved Neubauer, 0.1 mm in depth, Crown Scientific, Minto, New South Wales, Australia) with the sperm samples diluted to equal concentrations (sperm concentration varied between trials from 3.2×10^5 – 4.0×10^6 sperm cells per ml). This sperm concentration was within the range known to produce high rates of fertilization within this species, resulting in 1.9×10^3 – 2.4×10^5 sperm per egg. For each sperm competition trial, 3 ml of standardized sperm solution from each of the two males were mixed together (6 ml total) by gentle pipetting. This mixture was then divided between three replicate Petri dishes (2 ml of the mixed sperm solution per dish). A control Petri dish for each male received sperm from only one male (2 ml) and was used to assess each male's ability to fertilize a female's eggs in the absence of sperm competition (Figure 1). Approximately 100 eggs (mean 104 ± 2.2 s.e.) from a randomly chosen female were harvested directly into each Petri dish by gently squeezing her abdomen. After 2–3 min, the egg/sperm mixture was flooded with 100 ml of aged water. After 3 h, all eggs were transferred to a 750 ml plastic container with aged water and held at a constant temperature of 23 °C until hatching. Of the 30 trials, one trial failed completely, indicating unviable eggs and this trial was excluded from further analysis.

Assignment of paternity

From each of the three sperm competition replicates, 15 hatchlings were collected (mean number of hatchlings per trial 42 ± 4 s.d.) and preserved in 95% ethanol before DNA extraction. A toe clip from each adult in the sperm

competition trials was used for DNA extraction and the assignment of paternity. Genomic DNA was isolated from whole tadpoles using Qiagen DNAeasy Tissue Kits (Qiagen, Doncaster, Victoria, Australia) as per the manufacturer's instructions. All samples were amplified for nine polymorphic microsatellite loci (LP01, LP03, LP04, LP05, LP07, LP13, LP19, LP22 and LP23) originally designed for *L. peronii* (see Sherman and Olsson (2007) for PCR conditions). Of the nine loci, four (LP07, LP13, LP19 and LP22) successfully cross-amplified in *L. tyleri*, with no indication of null alleles detected from the progeny arrays. Paternity was unambiguously assigned to all offspring (1200 tadpoles) according to allele sharing between putative sires, dam and offspring using these four loci. The remaining five loci varied from partial amplification of some alleles (LP03 and LP05) to no amplification at all (LP01, LP04 and LP23) with *L. tyleri* sires.

Assessment of tadpole fitness

We compared fertilization success, hatching success, survivorship and viability of purebred *L. peronii* tadpoles and hybrid tadpoles (*L. peronii* eggs $\times$ *L. tyleri* sperm) from the control crosses. The proportion of eggs successfully fertilized for each replicate was assessed 48 h post fertilization when developing embryos could be distinguished from unfertilized eggs (stages 15–18; Gosner, 1960). Hatching success for both species was assessed 7 days after fertilization (Anstis, 2002) as the proportion of fertilized eggs that successfully hatched. Survivorship and viability was assessed at 9 and 14 days after hatching. Survivorship was assessed as the proportion of tadpoles alive at each sampling period. Tadpole viability was assessed as the proportion of tadpoles with normal morphology, swimming behavior and ability to right themselves in a resting position.

Statistical analyses

We assessed differences in fertilization and hatching success in the control and sperm competition trials using analysis of variance (One-Way ANOVA (analysis of variance)). All variables met the assumptions of normality and homogeneity of variances.

The difference in male siring success between the two heterospecific males (*L. peronii* and *L. tyleri*) in sperm competition was assessed using a General Linear Model (Proc GLM, SAS 9.1). Fertilization success in control Petri dishes, the proportion of viable sperm within each male's ejaculate and genetic similarity of each male relative to the female were included as main predictors in the model. All variables except the proportion of viable sperm within a male's ejaculate met the assumptions of normality and homogeneity of variances. After an arcsine square root transformation of the proportion of viable sperm the data met the assumptions of normality and equal variances.

Differences in survivorship and viability of purebred and hybrid tadpoles from the control fertilizations were analyzed using a General Linear Mixed Model (Proc MIXED, SAS 9.1). Female identity number was entered as a random factor and male species identity, and day (measurements taken at day 9 and 14) as fixed factors. Random effects were tested using likelihood ratio tests, fixed effects using F-tests, and the degrees of freedom were estimated using Satterthwaite's approximation (Littell et al., 1996).

Results

Fertilization and hatching success

Fertilization and hatching success in both the sperm competition and control trials were high (overall mean $\pm$ s.e. $73\% \pm 3\%$; Figures 2a and b). We found no significant difference in fertilization and hatching success between heterospecific ($L. peronii_{\text{eggs}} \times L. tyleri_{\text{sperm}}$) and

Figure 2 Fertilization (a) and hatching (b) success of male *Litoria peronii* and *L. tyleri* with female *L. peronii* in sperm competition and control trials. Controls contain sperm from each male in isolation.

conspecific ($L. peronii_{\text{eggs}} \times L. peronii_{\text{sperm}}$) fertilizations in the control or sperm competition trials (One-Way ANOVA fertilization success: $F_{2,82} = 1.31$, $P = 0.28$; hatching success: $F_{2,82} = 1.23$, $P = 0.30$; Figures 2a and b).

Siring success

Siring success in sperm competition trials was highly skewed ($>70\%$ siring success) toward one of the two competing males for 21 out of the 29 trials. For 10 of the sperm competition trials, fertilization success was skewed toward the conspecific male (*L. peronii*), whereas in 11 of the sperm competition trials fertilization success was skewed toward the heterospecific male (*L. tyleri*). For the remaining eight trials, siring success was approximately equal for both con- and heterospecific males (Figure 3).

Our GLM (general linear model) analysis showed that the only trait that significantly influenced a male's relative siring success in sperm competition was the fertilization success of that particular male in the control fertilizations (that is, when sperm from each male and species were allowed to fertilize eggs from a *L. peronii* female in isolation; Table 1). A pairwise *t*-test of a deviation from zero male difference in siring success within female identity number confirmed the result of no difference in siring success between the two species (mean = -0.045 , s.e. = 0.14 , $n = 29$, $P = 0.75$).

Survivorship and offspring viability

Survivorship and offspring viability was significantly lower for hybrid tadpoles compared with that for purebred tadpoles in the control fertilizations both at 9 and 14 days post hatching (Figures 4 and 5). The mixed model ANOVAs of the proportion of surviving tadpoles and their viability showed the strongest effects of hybridization, then day of assessment (with increasing severity toward the last day, 14, of the experiment, Table 2, Figures 4 and 5). Lastly, there was no effect of female identity on either of these response variables.

Discussion

Our results clearly indicate that the barrier to hybridization between these species lies at the level of developmental

Figure 3 The proportion of offspring sired by either conspecific male (hatched) or heterospecific male (black) in 29 sperm competition trials with standardized sperm numbers. Dotted line represents the 50:50 share of paternity expected under the null hypothesis of equal fertilization ability of competing sperm.

Table 1 Multiple regression analysis of predictors of siring success between a male *L. peronii* and *L. tyleri* in sperm competition trials

Predictors	Response variable: difference in siring success between <i>L. peronii</i> and <i>L. tyleri</i>			
	β	s.e.	<i>t</i>	P-value
Fertilization success in isolation	1.54	0.18	4.22	0.0003
Proportion alive sperm	0.40	0.72	0.55	0.585
Relatedness with female	−0.09	0.91	−0.10	0.919

Model $R^2 = 0.46$, $F_{3,24} = 6.90$, $P = 0.0016$. β , regression coefficient; s.e., standard error of regression coefficient.

The predictors are modeled as the difference in trait values between *L. peronii* and *L. tyleri* (i.e., as is the response variable siring success).

Figure 4 Proportion of purebred and hybrid tadpoles surviving to day 9 (a) and day 14 (b) post-hatching from control fertilizations. Significant difference between purebred and hybrid tadpoles indicated by scripts above standard error bars.

Figure 5 Proportion of viable purebred and hybrid tadpoles at day 9 (a) and day 14 (b) post-hatching from control fertilizations. Significant difference between purebred and hybrid tadpoles indicated by scripts above standard error bars.

stability and offspring survival rather than at fertilization and gametic fusion. Several hypotheses could explain these results. First, anuran amphibians communicate acoustically and, although unstudied in these species, potential mate choice based on acoustic quality is likely to be important. If so, there may have been limited selection for hybrid avoidance at the gametic level, and our laboratory trials may therefore represent an enforced fertilization process with the risk of overstating its importance in the wild. However, our observations of 'intermediate phenotype' males, that is males showing lack of typical body and throat coloration of either of the two species, suggests that occasionally hybridization may occur and that some male $\times$ female genetic interactions across the species may survive to adulthood.

Furthermore, the fact that *L. peronii* males more closely related with the female have a fertilization advantage also suggests that the risk of hybridization is real in the wild (Sherman *et al.*, 2008b). We were, however, surprised at the complete hybrid compatibility at the gametic level—especially in a situation of sperm competition with another species. Unlike our results, previous studies have shown a conspecific sire advantage (Bella *et al.*, 1992; Wade and Johnson, 1994; Wade *et al.*, 1994; Rieseberg *et al.*, 1995; Geyer and Palumbi, 2005). Previous work on amphibian cross-fertilization has also shown wide among-species compatibility at the fertilization level. However, in the toads *Bufo arenarum*, *B. paracnemis* and *Lepidactylus chaquensis*, this was followed by high levels of polyspermy and disturbed ontogenetic devel-

Table 2 Results from mixed model ANOVAs with species and day as fixed factors and female identity number as random factor using restricted maximum likelihood (REML)

Type 3 tests of fixed effects				
Effect	Num d.f.	Den d.f.	F	P-value
Response variable: proportion live tadpoles				
Species	1	85	133.2	<0.0001
Day	1	85	34.0	<0.0001
Random effect: female number, $\chi^2 = 1.08$, d.f. = 1, 0.1, $P > 0.5$				
Response variable: proportion viable tadpoles				
Species	1	84	648.5	<0.0001
Day	1	85	0.7	0.40
Random effect: female number, $\chi^2 = 1.1$, d.f. = 1, 0.1, $P > 0.5$				

The significance of the random factor was assessed using log likelihood ratio tests against a χ^2 distribution with d.f. = 1. Days = 9 or 14 days, Species = *L. peronii* or *L. tyleri*.

opment (Gómez and Cabada, 1994). Another well-characterized hybrid system is that of the European water frogs, *Rana lessonae* and *R. ridibunda*, resulting in the hybrid species *R. esculenta* (Berger, 1977; Abt and Reyer, 1993; Engeler and Reyer, 2001). A study by Reyer *et al.* (2003) showed no precedence for conspecific sperm over heterospecific sperm when sperm from the two parental species (*R. lessonae* and *R. ridibunda*) compete for fertilizations, consistent with the results from this study. However, hybrid *R. esculenta* males consistently have lower fertilization success in sperm competition trials with the two parental species, but this could not be attributed to the quality of sperm or a taxon-specific egg-sperm recognition system (Reyer *et al.*, 2003). Thus, being highly acoustic species, these observations agree with the hypothesis of a perhaps stronger (but unquantified) selection for pre-gametic hybrid avoidance and less opportunity for selection at a gametic compatibility level. In contrast, work on grasshoppers and crickets (reviewed in Arnold (1997, 2006)), which are equally acoustic species in terms of sexual communication, still show conspecific sperm precedence and, hence, this explanation does not apply to all taxa. Some of the fastest evolving and most sophisticated gamete recognition systems have been described in marine, sessile organisms, and which are known to have an important role in preventing hybridization or fertilization by incompatible partners (reviewed in Swanson and Vacquier (1998)). Our results provide no direct evidence for such taxon-specific gamete recognition system between these two species. Surprisingly, we also found no evidence that the proportion of viable sperm within a male's ejaculate affects siring success in sperm competition or in the single-species fertilizations (controls). This is consistent with our previous study on sperm competition in *L. peronii* (Sherman *et al.*, 2008b), which showed that the only significant predictor of siring success in sperm competition trials is the corresponding success of males in control fertilizations. This suggests that some unmeasured intrinsic property of a male's sperm (or semen) is important in determining a male's siring success at fertilization.

Owing to the logistic constraints, our study is limited to assessing the consequences of an asymmetric hybrid

breeding design, that is, between two different species of male frogs (*L. peronii* and *L. tyleri*) with females from only one of these species (*L. peronii*). There are numerous examples from the wild in which hybridization results in reduced fitness consequences in only one sex (for example, flycatchers, *Ficedula* sp.; Tegelström and Gelter, 1990). In our laboratory study, most hybrids were unviable after 14 days. This contrasts with our observations from the wild of phenodeviants that best can be described as adult intermediates between the two species and, hence, natural rates of hybrid viability may be higher if fertilization success and offspring viability had been investigated with a complete and balanced, symmetric breeding design. Such mortality effects of offspring from a given, but not opposite-sex partner, have been reported for several species of plants and animals (Arnold *et al.*, 1996; Coyne and Orr, 1998; Funk, 1998; Tiffin *et al.*, 2001; Coyer *et al.*, 2002) and may well explain a discrepancy in mortality between field and laboratory bred animals in the current study.

In conclusion, our study shows that gamete recognition and fertilization pose no barrier to hybridization between female *L. peronii* and male *L. tyleri*. However, selection against hybridization through offspring viability is strong and surviving young are unlikely to be sired by male *L. tyleri* and female *L. peronii*.

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