

Outbred embryos rescue inbred half-siblings in mixed-paternity broods of live-bearing females

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Females commonly mate with more than one male¹, and polyandry has been shown to increase reproductive success in many species^{2–4}. Insemination by multiple males shifts the arena for sexual selection from the external environment to the female reproductive tract, where sperm competition or female choice of sperm could bias fertilization against sperm from genetically inferior⁵ or genetically incompatible males^{6,7}. Evidence that polyandry can be a strategy for avoiding incompatibility comes from studies showing that inbreeding cost is reduced in some egg-laying species by postcopulatory mechanisms that favour fertilization by sperm from unrelated males^{8–10}. In viviparous (live-bearing) species, inbreeding not only reduces offspring genetic quality but might also disrupt feto-maternal interactions that are crucial for normal embryonic development^{11–13}. Here we show that polyandry in viviparous pseudoscorpions reduces inbreeding cost not through paternity-biasing mechanisms favouring outbred offspring, but rather because outbred embryos exert a rescuing effect on inbred half-siblings in mixed-paternity broods. The benefits of polyandry may thus be more complex for live-bearing females than for females that lay eggs.

Consanguineous mating is a significant source of fitness depression, and inbred offspring are more likely to be homozygous either for deleterious, recessive alleles or at loci with heterozygote advantage¹⁴. In live-bearing species, inbreeding can also disrupt the complex sequence of two-way, immunological interactions between fetal and maternal tissues¹⁵. Unfortunately, investigating inbreeding effects on fetal loss is hindered in most viviparous animals by the intrusive methods required to detect early stage, spontaneous abortion. Unlike most terrestrial arthropods, pseudoscorpions are viviparous. Embryos develop in an external, translucent brood sac and draw nutritive fluid from the mother's reproductive tract¹⁶. Previous research exploited this 'external-womb' form of viviparity to establish that polyandry in the pseudoscorpion, *Cordylochernes scorpioides*, significantly enhances female lifetime reproductive success by reducing the rate of spontaneous abortion of entire broods¹⁷. Here we investigate the effect of inbreeding on abortion rate and reproductive success in *C. scorpioides*, and assess whether females reduce this cost by mating with both a related and an unrelated male.

Virgin females were randomly assigned to one of five treatments, four involving a single mating with each of two males: two non-siblings of the female (NN), two full-siblings of the female (SS), a non-sibling first male and a full-sibling second male (NS), or vice versa (SN). Unlike the SS treatment, male pairs in NN replications were non-brothers, the usual situation for polyandrous females in nature¹⁸. For a conservative comparison, a fifth treatment included females that were mated twice to a single non-sibling (N; see Methods). Each mated female was monitored until she gave birth to a first brood of nymphs, spontaneously aborted her first brood, or failed to produce a brood in 45 d. All nymphs were counted at birth,

and DNA profiling¹⁹ was used to assign paternity for NS and SN nymphs. A second experiment assessed the effect of relatedness on number of sperm males allocated to spermatophores. Once-mated males were paired with a full-sibling (S female) or a non-relative (N female; $n = 12$ per treatment), the sperm packet was collected, and sperm were counted¹⁷.

A randomized block analysis of covariance (ANCOVA) showed a significant effect of mating treatment ($F_{4,187} = 8.45$, $P < 0.001$) and female body size ($F_{1,192} = 7.55$, $P = 0.007$) on reproductive success, that is, number of nymphs born. Comparison of treatment means (Fig. 1) showed that female reproductive success sorted into three levels, $SS < NS = SN = N < NN$, a grouping that was highly significant (contrast analysis²⁰, $F_{1,187} = 22.12$, $P < 0.001$), and accounted for 65% of the mating treatment effect. Underlying this pattern was a significant treatment effect on the frequency of spontaneous brood abortion (Fisher exact test, $P = 0.029$), with SS females suffering the highest abortion rate (40%; see Fig. 1). Mating treatment also significantly influenced the number of nymphs born to females that successfully carried broods to term ($F_{4,144} = 7.10$, $P < 0.001$); the $SS < NS = SN = N < NN$ pattern was again significant ($F_{1,144} = 13.93$, $P < 0.001$), accounting for 49% of the treatment effect. Thus, mating with a non-relative and a brother diminished cost of inbreeding by both reducing the risk of spontaneous brood abortion and increasing embryonic survival in successful broods. In the second experiment, relatedness had no effect on number of sperm allocated to spermatophores (S-female,

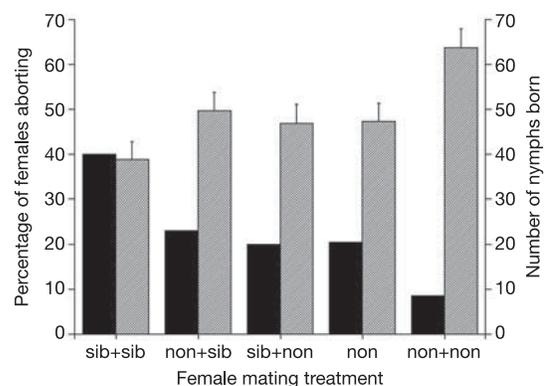


Figure 1 | Effect of mating treatment on brood production. Rates of whole-brood spontaneous abortion (filled bars) and number of nymphs (mean \pm s.e.m.) born to females carrying broods to term (hatched bars) for treatments in which females were mated to two full-siblings (sib + sib), to a non-relative followed by a full-sibling (non + sib), to a full-sibling followed by a non-relative (sib + non), to a single non-relative (non), or to two non-relatives (non + non).

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1,674 ± 147; N-female, 1,637 ± 151 (mean ± s.e.m.); $t_{22} = 0.18$, $P = 0.86$).

The SS < NS = SN = N < NN pattern of female reproductive success is consistent with the hypothesis that polyandry enables *C. scorpioides* females to bias paternity against sibling males. However, paternity assignment for NS and SN nymphs showed that this was not the case. Regardless of mating order, females gave birth to more inbred than outbred offspring. The proportion of NS and SN inbred nymphs was 0.66 ± 0.17 and 0.60 ± 0.24 (mean ± 95% confidence interval), respectively, and did not differ significantly between treatments ($F_{1,31} = 0.19$, $P = 0.668$). The pooled mean of 63% (±14%) suggests a sperm-competition advantage for sibling males. Because of higher mortality of inbred embryos, this at-birth value of 63% underestimates the actual fertilization success of sibling males. After correcting for embryonic survival differences (Supplementary Information), sibling-male fertilization success was more than twice that of non-relative males ($69\% \pm 14\%$ versus $31\% \pm 14\%$, $P = 0.011$). Although the mechanism underlying the sperm-competition advantage is unknown, genetic similarity could feasibly lower the risk for sibling-male sperm of being targeted by female immune defences.

This sibling-male, sperm-competition advantage can have deleterious, postfertilization consequences for female reproductive success. In 12 cases, females produced only inbred nymphs (Fig. 2). These females gave birth to significantly fewer nymphs (35.25 ± 4.96 ; mean ± s.e.m.) than females whose broods included at least one outbred nymph (54.90 ± 3.84 ; $t_{30} = 3.13$, $P = 0.004$). The high rate of spontaneous brood abortion and small brood size associated with inbreeding might result from males donating less sperm and/or accessory gland proteins (Acps) to related females²¹; however, the evidence available does not support this hypothesis. Males did not discriminate against sisters, either in propensity to produce a spermatophore or in number of sperm allocated to sperm packets. Adjustment of Acp quality and/or quantity independently of sperm number would therefore be required for Acp-mediated treatment effects on female reproductive success. Moreover, although seminal products stimulate ovulation in some live-bearing species,

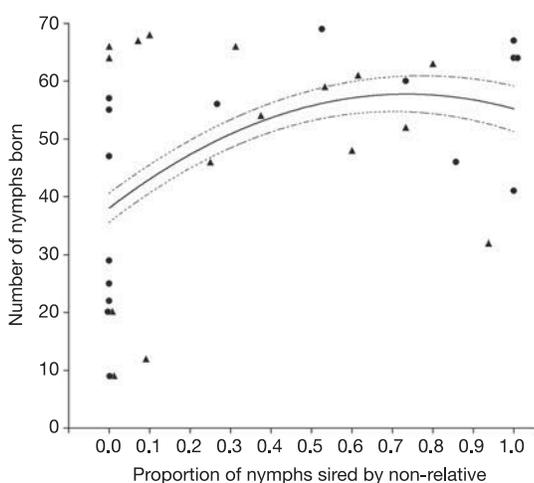


Figure 2 | Number of nymphs born versus proportion outbred in successful NS and SN broods. Cubic spline²⁷, standard and Gentleman–Givens regressions²⁶ all yielded a significant quadratic relationship between nymph number and proportion sired by the non-relative male in non+sib (triangles) and sib + non (circles) treatments. The unbroken line indicates the best-fit cubic spline solution. Broken lines indicate ±1 s.e.m., based on 1,000 bootstrap replicates. For standard, quadratic regression, $R^2 = 0.239$, $P = 0.019$. Neither linear nor quadratic regression was significant when exclusively inbred broods were excluded ($R^2 = 0.009$, $P = 0.688$ and $R^2 = 0.013$, $P = 0.897$, respectively).

mating treatment had no effect on whether *C. scorpioides* females produced a brood (Fisher exact test, $P = 0.2150$). Finally, with a mean ejaculate mass estimated at $1 \mu\text{g}$ and a mean brood mass of $8,800 \mu\text{g}$ just before birth, the material contribution made by males seems negligible.

Our findings show that polyandry can reduce the high rate of spontaneous brood abortion and the low reproductive success associated with inbreeding, if inbred embryos develop with outbred half-siblings. This rescuing effect can be quantified by considering reproductive success from a sibling-male perspective (Supplementary Information). A male that mates with his sister sires nearly twice as many inbred offspring that survive to birth if she also mates with an unrelated male (NS and SN treatments, 21.52 ± 2.28 ; mean ± s.e.m.), as compared with a second brother (SS treatment, 12.70 ± 2.55 ; Mann–Whitney test, $U = 1097.5$, $P = 0.021$). Indeed, despite losing about a third of fertilizations to the unrelated male, the reproductive success of the single, sibling male in NS and SN replications is statistically indistinguishable from the combined reproductive success (23.12 ± 3.86) of the two brothers in SS replications (Mann–Whitney test, $U = 1478$, $P = 0.990$), owing to the reduced rate of spontaneous brood abortion.

How might rescuing of inbred embryos occur? With inbreeding, homozygosity for a deleterious, recessive allele would result in intrinsically weak offspring that are unable to sequester adequate maternal resources. By their more vigorous activity, outbred embryos might draw sufficient nutrients into the communal brood sac to ensure development of the brood to birth. Alternatively, genetic similarity of inbred embryos to their mother may blur the self/non-self distinction essential for innate immune responses. Innate immunity, common to invertebrates and vertebrates²², has an important role in fetal loss, with perturbations of immune responses triggering spontaneous abortion²³. By establishing a non-self presence in mixed inbred/outbred broods and activating the normal cascade of fetomaternal interactions, outbred embryos might compensate for attenuation of interactions between mother and inbred concepti. Notably, both these hypotheses account for the variation in inbreeding cost apparent between sibling pairs in Figs 1 and 2. At any locus, whether it influences embryonic viability or is involved in recognition, mendelian genetics dictate that full-siblings may share one, both or no alleles. The fitness consequences of inbreeding will thus vary, depending on the multilocus genotypes of the particular brother and sister involved.

Our results are consistent with the view that parent–offspring conflict over maternal resource allocation drives the evolution of fetomaternal interactions, with resistance by the mother to manipulation of her reproductive physiology by the embryo creating tension that is crucial for normal embryonic development²⁴. Relaxation of this tension, through increased homozygosity or genetic similarity, could explain both the high rate of spontaneous inbred-brood abortion and the rescuing effect of outbred, half-siblings detected in our study.

So far, evidence that polyandry reduces inbreeding costs has been mixed, leading some to question the importance of genetic incompatibility avoidance in the evolution of polyandry²⁵. However, most counterevidence comes from egg-laying species, with females limited to pre-fertilization mechanisms for inbreeding avoidance. We have shown in a viviparous species that mixed paternity rather than paternity biasing reduces inbreeding cost. If mixed-paternity rescuing effects occur in other live-bearing species, reproductive mode may well be an important factor influencing not only the potential sources of incompatibility between paternal and maternal genomes⁶, but also the range of postcopulatory defences available to polyandrous females.

METHODS

Pseudoscorpions. Pseudoscorpions were laboratory-reared F_2 and F_3 descendants of 82 field-inseminated *C. scorpioides* females collected in Panama; nymphs

were reared individually to ensure virginity¹⁷. To generate full-sibling families, F₁ females were randomly mated to a single, unrelated male, and progeny were reared to adults. These F₂ individuals either were used in block 1 or were mated to provide F₃ individuals for block 2.

Polyandry and inbreeding experiment. Within blocks, a completely randomized mating design was used to maximize sample size. Before the experiment, each male was mated once to a non-experimental female. Each mating pair was placed in a 28-mm-diameter arena, interactions were videotaped for 30 min¹⁷, and pseudoscorpions were then returned to their vials. After 48 h, females were presented with the same male (N treatment) or a different male (NN, NS, SN and SS treatments). Only females that unambiguously accepted a sperm packet in both matings were retained for subsequent monitoring ($n = 41$ for the N treatment; $n = 39$ for NN; $n = 39$ for NS; $n = 36$ for SN, and $n = 44$ for SS).

Monitoring female reproductive success. Females were maintained in transparent vials containing *Ficus* frass, were fed nine *Tribolium confusum* larvae per week, and were checked every 3–4 d, increasing to daily at late stages of gestation. During gestation, females remain in a silken nest constructed on the vial wall, enabling non-intrusive monitoring of brood development¹⁷. After a 14-d developmental period, nymphs are born simultaneously and remain in the nest for 2–3 d. Within 24 h of birth, nymphs were counted, and NS and SN nymphs were frozen for paternity testing. Females were assigned a reproductive success score of 0 if they aborted their entire first brood. Females not producing a brood sac in 45 d ($n = 11$) were excluded from the reproductive success analyses, because such females do not become gravid¹⁷.

Statistical analyses were done with SAS²⁶. Cephalothorax length, the trait most strongly correlated with female lifetime reproductive success¹⁷, was used to control for body size in the ANCOVA analysis of female reproductive success (see text). For the analysis of the complete data set, a non-parametric ANCOVA was done on rank-transformed reproductive success data and residuals were normally distributed (Shapiro-Wilk test, $W = 0.989$, $P = 0.157$). For the analysis excluding cases of spontaneous abortion, data transformation was not required ($W = 0.990$, $P = 0.403$).

Minisatellite paternity assignment. Amplification by PCR of alleles at the *cCscMS23* minisatellite locus (heterozygosity = 0.99)¹⁹ was used to assign paternity for 465 nymphs from 16 replications of each of the NS and SN treatments. PCR products from the mother, the two putative sires and 8–20 offspring were run on agarose gels stained with ethidium bromide to visualize alleles. Paternity was assigned on the basis of the presence of unique paternal alleles in offspring. Only replications in which the putative sires shared no alleles were scored (32 out of 34 replications tested).

Several measures ensured amplification of both alleles from heterozygous individuals (Supplementary Information). First, a strict consensus primer pair was designed from a large sample of *cCscMS23* alleles ($n = 22$). Second, the high primer-pair T_m enabled a robust, two-step PCR reaction. Finally, PCR was done with a mixture of DNA polymerases capable of amplifying high molecular mass alleles (to 22 kb) from femtomolar quantities of DNA.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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