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Inbreeding effects on sperm production in clam shrimp (*Eulimnadia texana*)

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ABSTRACT

Hypothesis: Inbreeding depression is manifest in lower sperm production.

Organism: Freshwater crustaceans (clam shrimp – *Eulimnadia texana*), from the south-western United States, which have high levels of inbreeding.

Methods: Comparisons of semi-thin sections of the male gonad among selfed and outcrossed siblings from four families.

Results: There was a twofold reduction in sperm production in inbred relative to outcrossed males. Inbreeding depression in males was higher than previous estimates from hermaphrodites.

Conclusions: Inbreeding markedly reduces sperm production. The observed low levels of sperm production can explain both the low average outcrossing rates as well as the variation in these rates reported in previous studies of these crustaceans.

Keywords: androdioecy, branchiopod crustacean, inbreeding depression, mating system, Spinicaudata.

INTRODUCTION

The process of mating between close relatives (termed 'inbreeding') leads to reduced genetic diversity, both within individuals (i.e. reduced heterozygosity) and between individuals (Wright, 1969). It has long been recognized that inbreeding is associated with a reduction of fitness among the offspring resulting from the inbreeding event (Darwin, 1876; Schemske and Lande, 1985; Husband and Schemske, 1996; Crnokrak and Roff, 1999). Such fitness reduction affects all aspects of the life histories of both plants and animals, including hatching success, juvenile survival, ability to mate, gamete production, and adult survival (Crnokrak and Roff, 1999). Thus, inbreeding has often been suggested to be inferior to outcrossing: 'cross-fertilisation is generally beneficial, and self-fertilisation injurious' (Darwin, 1876).

Interestingly, in both plants and animals, reports on the effects of inbreeding on gamete production have been primarily limited to egg/ovule production (Byers and Waller, 1999; Crnokrak and Roff, 1999). In animals, this is likely due to the difficulty of documenting overall sperm

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production in males. Nevertheless, there have been a few studies of the effects of inbreeding on sperm production in animals, primarily mammals (Wildt *et al.*, 1987a; Roldan *et al.*, 1998; Margulis and Walsh, 2002; Fritzsche *et al.*, 2006; Gage *et al.*, 2006; Asa *et al.*, 2007) and a few insects (Schrempf *et al.*, 2006; Bernasconi *et al.*, 2007; Nakahara and Tsubaki, 2008). These experiments examined sperm quality (Wildt *et al.*, 1987a; Roldan *et al.*, 1998; Gage *et al.*, 2006; Asa *et al.*, 2007), sperm competitiveness (Bernasconi *et al.*, 2007), breeding success (Fritzsche *et al.*, 2006; Nakahara and Tsubaki, 2008), and sperm quantity (Wildt *et al.*, 1987a; Margulis and Walsh, 2002; Schrempf *et al.*, 2006). Apart from these few studies, the effects of inbreeding on sperm production have been largely overlooked.

Understanding the possible differential effects of inbreeding on male and female function can be important to understanding the evolution of inbreeding depression. Rausher and Chang (1999) note that 'estimation of inbreeding effects on only the female component of fitness . . . may lead to erroneous interpretations of the role of inbreeding depression in the evolution of mixed-mating systems' (p. 246). They note that differences in expression of inbreeding depression in male and female functions can explain stable mating systems of partial outcrossing and partial selfing. Unfortunately, as noted above, few studies have measured inbreeding depression in male function, and even fewer have measured inbreeding depression in both male and female function in the same species (Rausher and Chang, 1999). Until such data are amassed, we will be unable to assess the potential for differential effects of inbreeding on male relative to female function.

Herein we report the first quantification of the effects of inbreeding on sperm production in a crustacean, the clam shrimp *Eulimnadia texana*. We compare outcrossed with selfed males and note the general levels of sperm production in each. We note an approximate two-fold reduction in sperm production in selfed relative to outcrossed males. We compare this level of inbreeding depression to previous reports of inbreeding depression in female function for these shrimp. We then discuss the importance of these results to the evolution of the unique, androdioecious (mixtures of males and hermaphrodites) breeding system in these shrimp.

MATERIALS AND METHODS

Natural history of *Eulimnadia texana*

Eulimnadia texana is a small (5–10 mm total length) branchiopod crustacean with a mixed mating system [~30% outcrossing and ~70% selfing (Weeks *et al.*, 2004)]. These shrimp inhabit small, temporary pools in southwestern North America, Central America, and South America. Hermaphrodites are of two types: 'monogenics', which produce 100% hermaphrodites whether outcrossed or selfed, and 'amphigenics', which produce 25% males when selfed and 50% males when crossed with a male (Sassaman and Weeks, 1993). Hermaphrodites cannot fertilize one another, and thus eggs are either fertilized by males or self-fertilized. These desiccation-resistant eggs (or 'cysts') are then laid on (or in) the pond bottom and dry out before they hatch. Nauplius larvae hatch from these cysts after hydration by spring, summer or fall rainfall (Weeks *et al.*, 1997). After hatching, growth is rapid and sexual maturity can occur in as few as 4 days (Vidrine *et al.*, 1987). Hermaphrodites can produce one to two clutches of eggs per day, with each clutch consisting of 100–300 eggs (Weeks *et al.*, 1997). At sexual maturity, males are easily distinguished from hermaphrodites by a pair of claw-like thoracic appendages termed 'claspers', which are needed for mate guarding for successful outcrossing (Weeks and Benvenuto, 2008).

Rearing protocol for field-collected soil

Approximately 500 ml, by volume, of field-collected soil (collected from a dry pond outside of Portal, AZ, USA; location previously reported as the 'WAL' population) containing clam shrimp cysts was placed in the bottom of 38-litre aquaria and hydrated with filtered tap water in an environmentally controlled laboratory facility under constant light at 26–28°C, following the procedures outlined in Weeks *et al.* (1997). The aquaria were supplemented with 10 ml of an equal solution of baker's yeast (0.5 g per 100 ml water) and finely ground Tetramin® flake fish food for algae eaters (0.5 g per 100 ml water). Directly before sexual maturity, 100 shrimp were isolated in 500-ml plastic cups containing ~5 ml of cyst-free soil and filled with water from the above hatching tanks. As shrimp matured, males were paired with hermaphrodites, one pair per 500-ml cup. In total, 55 pairs were isolated. Isolated pairs were allowed to produce eggs for about 7 days and were then frozen for later electrophoretic typing. Eggs in the cups were dried, the cups were sealed with lids, and then placed in the dark for approximately 30 days.

Electrophoretic analyses

Electrophoretic analyses were performed with cellulose acetate gel electrophoresis using 'buffer C' from Richardson *et al.* (1986). Five allozyme loci were scored from both adults of each pair: Fum (fumarate hydratase, EC 4.2.1.2), Idh-1, Idh-2 (isocitrate dehydrogenase, EC 1.1.1.42), Mpi (mannose-phosphate isomerase, EC 5.3.1.8), and Pgm (phosophoglucomutase, EC 5.4.2.2). Appropriate pairs were chosen on the basis of having homozygous or heterozygous patterns that were distinct so that outcrossing could be definitively determined among any reared offspring. Of the original 55 pairs, 21 pairs had appropriate electrophoretic genotypes to allow outcrossing estimates.

Rearing of laboratory egg banks

Egg banks from the 21 pairs identified above were hydrated using methods outlined for the field-collected soil. The cups were checked daily for hatched nauplii, which were then maintained in family groups and transferred to individual 7-litre rearing tanks for each family. These tanks contained 100 ml of cyst-free soil and filtered tap water. Tanks were maintained under the standard conditions noted above. Upon sexual maturity (~7 days), the shrimp were sexed and frozen for electrophoretic typing. Only four of the 21 egg banks produced males. In total, 25 males were produced in these four families.

All males were processed in the following way. The heads were removed and immediately frozen for electrophoresis. Offspring were typed as the products of selfing or outcrossing on the basis of their electrophoretic scores on the definitive allozyme locus from their parents' scores as outlined above. The remaining bodies were fixed in 2.5% glutaraldehyde for 2 h and post-fixed in 2% OsO_4 for 1 h, both in 0.2 M sodium phosphate buffer. The samples were washed in a 50% acetone mixture followed by a mixture of 1% uranyl acetate in 70% acetone. They were then washed in graded acetone steps (90%, 95%, and $2 \times 100\%$). Lastly, the bodies were embedded in Durcupan® ACM Fluka resin. The embedded samples were then sent to the University of Bologna for sectioning.

Sectioning procedures

Transverse, semi-thin sections were cut starting from the posterior metameres, which are younger and likely richer in male gametes. The young age of the samples (8–9 days old) permitted us to analyse a fully functional gonad in both the 'selfed' and 'outcrossed' shrimp. To compare sperm production among males, we chose the sections where the male gonad was larger. It was impossible to define the same metamere for each male because after the OsO_4 fixation, such identification is impossible. A histological control confirmed the true sexuality of all samples, and in fact no female gamete appeared in any sections.

Statistical analyses

Because family groups were reared together in 7-litre rearing tanks, we used a blocked, one-way analysis of variance to compare percent degenerate sperm between outcrossed and selfed males. Residuals from this analysis were normally distributed. These analyses were performed using the statistical package JMP (SAS Institute, 2003).

RESULTS

The percentage of degenerate sperm ranged over an order of magnitude, from 9 to 90% (Table 1). Samples of the semi-thin sections used to quantify sperm production are shown in Fig. 1. The degenerated sperm are identified by their poor colouring, caused by cytoplasmic depletion, and their irregular outline. On the other hand, the round, dense-coloured cells are the normal sperm, classified as 'vital' sperm for our comparisons. The cross-sections

Table 1. Percentage of degenerate sperm by family

Mating			Mating		
Family	Mating type	% Degen.	Family	Mating type	% Degen.
A14	Outcrossed	19.3	A46	Outcrossed	66.5
A14	Outcrossed	24.8	A46	Outcrossed	60.7
A14	Outcrossed	15.9	A46	Outcrossed	23.0
A14	Outcrossed	8.7	A46	Selfed	67.1
A14	Selfed	46.1	A46	Selfed	72.4
A14	Selfed	76.5	A46	Selfed	90.3
A14	Selfed	66.1	A46	Selfed	83.1
A14	Selfed	55.2	A46	Selfed	80.9
A14	Selfed	68.3	A46	Selfed	78.0
A14	Selfed	78.0		**Average**	**69.1**
	Average	**45.9**			
A40	Outcrossed	58.2	C3	Selfed	70.4
A40	Selfed	62.0	C3	Selfed	9.5
A40	Selfed	89.9	C3	Selfed	53.6
	Average	**70.0**		**Average**	**44.5**

Note: % Degen. = average percentage of degenerate sperm per male.

clearly show the increased proportion of degenerate sperm in the selfed relative to the outcrossed offspring (Fig. 1).

The selfed males had over two-fold more degenerate sperm, on average, than the outcrossed males (Fig. 2). This difference was highly significant ($F_{1,20} = 24.75$; $P < 0.0001$). There was also significant variation among the four families in the average proportion of sperm that were degenerate ($F_{3,20} = 5.36$; $P = 0.0072$).

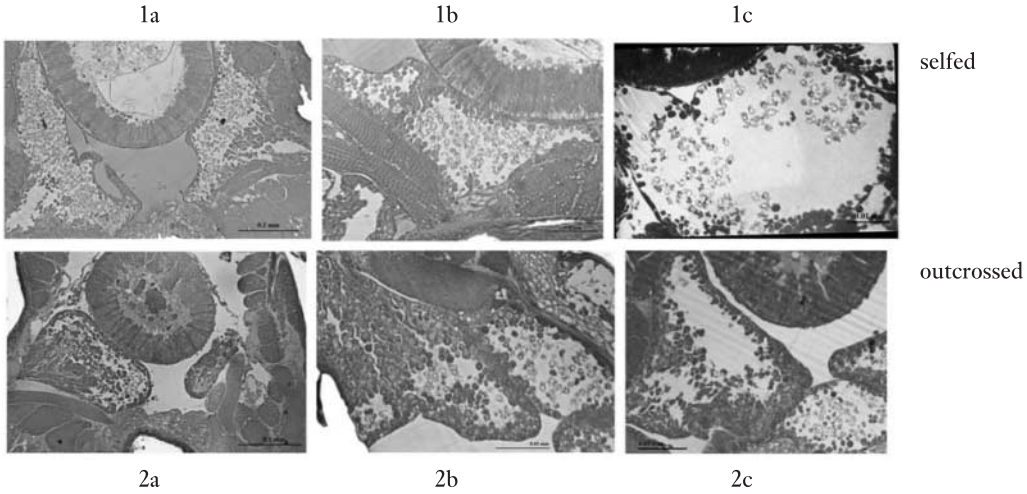

Fig. 1. The top row represents three magnifications of testis of a selfed male (1a, 1b, 1c). The second row represents three magnifications of testis of an outcrossed male (2a, 2b, 2c). 1a and 2a are general views of transverse sections of the body showing the midgut in the centre, the haemocoel cavity and the two male gonads strictly surrounding the midgut. 1b, 1c, 2b, and 2c represent the entire gonad in which the germ cells are easily recognizable.

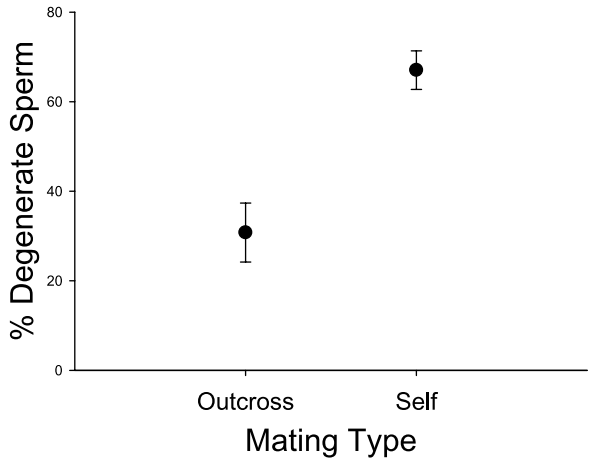

Fig. 2. Mean proportion of degenerate sperm in selfed relative to outcrossed males. Values represent least squared means adjusted for families. Error bars portray one standard error of the means.

DISCUSSION

The negative effects of inbreeding have been extensively documented, and range across all fitness-related traits in both animals and plants (Schemske and Lande, 1985; Jarne and Charlesworth, 1993; Husband and Schemske, 1996; Crnokrak and Roff, 1999; Roff, 2002; Jarne and Auld, 2006). However, the effects of inbreeding on male gamete production have not received a great deal of attention in either plants or animals (Byers and Waller, 1999; Crnokrak and Roff, 1999; Rausher and Chang, 1999). This oversight is likely due to the difficulty of measuring pollen (Carr and Dudash, 1995; Johannsson *et al.*, 1998; Good-Avila *et al.*, 2003) and sperm production/performance (Roldan *et al.*, 1998; Gage *et al.*, 2006; Asa *et al.*, 2007).

The few reports of the effects of inbreeding on male gamete production in animals have primarily come from mammalian species. Asa *et al.* (2007) noted significant increases in abnormal sperm and decreases in sperm motility with increased inbreeding coefficients in Mexican wolves. Similarly, in Cuvier's gazelles, inbreeding coefficients were negatively correlated with ejaculate volume, the number of viable sperm, and various measures of sperm functionality (Roldan *et al.*, 1998). Wild populations of cheetah, known to be highly inbred, show structural abnormalities in more than 70% of their sperm (Wildt *et al.*, 1987b). Asiatic lions that had been through a population bottleneck had lower ejaculate volume, lower sperm motility, and over 50% greater sperm abnormalities relative to lions that had not experienced a bottleneck (Wildt *et al.*, 1987a). European rabbits also show increased numbers of abnormal sperm with increased inbreeding (Gage *et al.*, 2006) and field mice (*Peromyscus polionotus*) produced significantly fewer sperm with increasing levels of inbreeding (Margulis and Walsh, 2002). The only published case in which inbred mammals did not have negative effects on sperm quality/quantity is in the golden hamster, where highly inbred laboratory strains of hamsters had similar levels of sperm density and sperm motility relative to their wild-caught counterparts (Fritzsche *et al.*, 2006). Thus, in mammals, sperm quality and quantity are commonly, but not universally, negatively affected by inbreeding.

Outside of mammals, very few reports of the effects of inbreeding on sperm quantity or quality can be found. Insects appear to be the only other animal taxon with information on the effects of inbreeding on male function. Ant colonies (*Cardiocondyla obscurior*) that are inbred have queens with low quantities of sperm, suggestive of low sperm production of the inbred males in the colony (Schrempf *et al.*, 2006). In damselflies, sperm quantity and quality did not differ between inbred and outcrossed individuals (Nakahara and Tsubaki, 2008). Sperm length (which is positively related to sperm competitiveness) tended to decrease in yellow dung flies (*Scathophaga stercoraria*) that were inbred for 15–16 generations (Bernasconi *et al.*, 2007). With the exception of the effects noted in plant populations (Carr and Dudash, 1995; Johannsson *et al.*, 1998; Willis, 1999; Good-Avila *et al.*, 2003; Ellmer and Andersson, 2004; Glaettli and Goudet, 2006), very little else is known about the effects of inbreeding on male function.

Herein we have endeavoured to measure the effects of inbreeding on sperm production in a mixed-mating crustacean, *Eulimnadia texana*. The results clearly demonstrate that inbreeding has a dramatic, negative effect on male gamete production: inbred males produced over twice the amount of degenerate sperm as did outcrossed males. Using relative sperm production as a sole estimate of inbreeding depression [i.e. $\delta = 1 -$ (viable sperm production of selfed males/viable sperm production of outcrossed males)], inbreeding depression is estimated as 0.52. This estimate is high relative to estimates derived from egg hatching ($\delta = 0.20$), juvenile survival ($\delta = 0.22$), age at maturity ($\delta = 0.06$) (Weeks *et al.*, 1999), and net reproductive rates in females ($\delta = 0.10$–0.42) (Weeks *et al.*, 2000a). The current data suggest that inbreeding may have particularly strong, negative effects on sperm production

in these shrimp that are disproportionate to the effects on other life-history traits, including estimates from hermaphrodites. To our knowledge, this is the first mixed-mating animal system in which the effects of inbreeding have been estimated on both male and female function. The current estimate of higher inbreeding depression for male relative to female gamete production may influence the stability of the mixed-mating system of these shrimp [selfing rates estimated between 0.44 and 1.0 (Weeks and Zucker, 1999)] by making such mixed mating more stable (Rausher and Chang, 1999). However, to fully determine the differential effects of inbreeding on gamete production in the two sexes, we would need to document the effects of inbreeding on hermaphroditic sperm production, which is logistically much more difficult because of the very small region of the ovotestes relegated to sperm production in hermaphrodites (Zucker *et al.*, 1997). Nonetheless, this would be an interesting and informative comparison for future studies.

The current data shed light on some heretofore enigmatic findings in these shrimp. Previous estimates of outcrossing rates have noted that males fertilize 30–45% of available eggs in two populations of *E. texana* (Crosser, 1999; Weeks *et al.*, 2000b, 2004). Although these low outcrossing values might be suggested to result from the inefficient pairing of males with hermaphrodites during the crucial period of egg extrusion [males can only fertilize eggs when they are first extruded from the gonopore into the brood chamber (Weeks and Benvenuto, 2008; Weeks *et al.*, 2004)], Weeks *et al.* (2004) assayed individual clutches collected after males were specifically observed fertilizing hermaphrodites and found similarly low proportions of outcrossed eggs (25–40%) within these clutches. Weeks *et al.* (2004) speculated that this low outcrossing rate must reflect greater competitive ability of self- relative to male-sperm or hermaphroditic preference for self- over male-sperm. However, this biased competition is difficult to accept given that hermaphrodites produce such a small quantity of sperm relative to males (Zucker *et al.*, 1997) and in other androdioecious species (nematodes in the genus *Caenorhabditis*) male sperm is competitively superior to hermaphrodite sperm (Lamunyon and Ward, 1995). Additionally, the benefits to outcrossing in this species (Weeks *et al.*, 1999, 2000a, 2001; Weeks, 2004) should strongly select against any preferential use of self over outcrossed sperm. Thus, these previous results have been problematic to interpret.

The current results allow an alternative interpretation of these low levels of outcrossing. If many males are inbred, then our results suggest that they will be producing low levels of viable sperm. Thus, even though competent (i.e. outcrossed) males produce much more sperm than hermaphrodites, when inbred males are paired with hermaphrodites they are likely to fertilize few eggs because they are producing few viable sperm. Therefore, the current findings could easily explain these earlier reports of low outcrossing in male/hermaphrodite pairs.

In fact, we should expect two categories of males in *E. texana* populations: competent, outcrossed males, which are the products of matings between males and amphigenic hermaphrodites, and sperm-limited inbred males, which are the products of selfing amphigenic hermaphrodites (Sassaman and Weeks, 1993). When comparing individual outcrossing success from previously published data (Crosser, 1999; Weeks *et al.*, 2000b, 2004), it would appear that this prediction holds true: the two most frequent outcrossing rates for males are 0 and 100%, with fewer males in between these end points (Fig. 3). Thus, the general prediction derived from the sex-determining system in these shrimp (Sassaman and Weeks, 1993) that there should be inbred and outcrossed males, combined with the current observation that these inbred males produce few viable sperm, can explain the previous observations of both low average outcrossing rates as well as the range of outcrossing rates documented in this species.

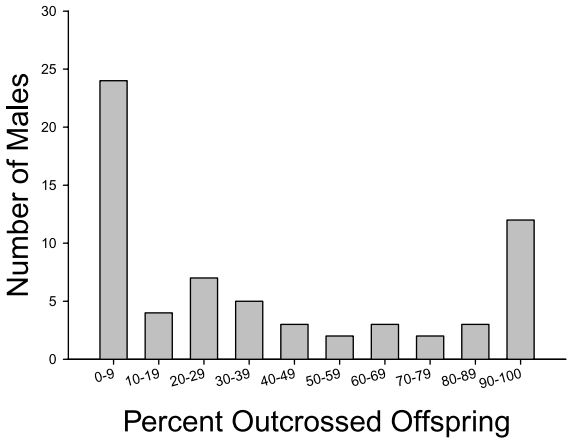

Fig. 3. Percent outcrossed offspring for males paired with single hermaphrodites. Data compiled from Crosser (1999), Weeks *et al.* (2000b, 2004).

Byers and Waller (1999) noted that 'successive inbreeding studies should pay particular attention to male fitness components' for a more complete understanding of the causes and effects of inbreeding depression and to better understand the stability of mixed-mating systems (Rausher and Chang, 1999). Unfortunately, there continues to be few studies of the effects of inbreeding on male fitness, and even fewer that examine inbreeding effects on both male and female function within the same species. The current study adds to this slowly growing documentation of such effects, and adds a new dimension to our understanding of the factors maintaining the androdioecious, mixed-mating system of this interesting freshwater crustacean.

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