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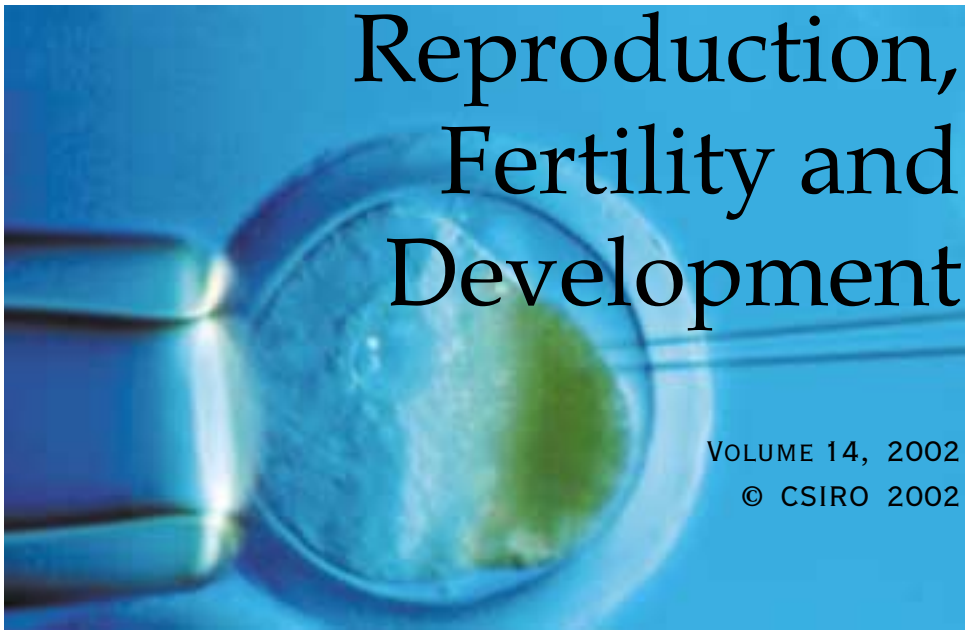

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The effects of inbreeding on testicular sperm concentration in *Peromyscus polionotus*

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Abstract. Inbreeding effects on fitness have most often been quantified via juvenile mortality. However, inbred adults may suffer from inbreeding depression if their fertility or fecundity is compromised as a consequence of inbreeding. Here, the effects of inbreeding on male fertility in oldfield mice, *Peromyscus polionotus*, were examined. Testicular sperm concentration was assessed in 93 males, 68 of which were paired for breeding. Forty of the 68 paired males failed to produce offspring. Total testicular sperm count, sperm count (g testis)$^{-1}$, and testis mass all declined significantly with increasing inbreeding coefficient. Sperm concentration did not significantly impact reproductive success. Although sperm concentration in males of most species can decline to low levels before reproductive impairment is detectable, the declines in testicular sperm concentration found here suggest that inbreeding can affect fertility in adult males. Furthermore, monitoring testicular sperm concentration could provide a mechanism to monitor potential declines in reproductive performance before population-level reproductive success is irreparably impaired. The implications for the management of small, captive and wild populations may be substantial.

Introduction

Inbreeding depression has been well studied in captive populations. The most common way of measuring inbreeding depression is via juvenile mortality in captive environments (Ralls *et al.* 1979; Ballou and Ralls 1982; Ralls and Ballou 1982*a*, 1982*b*; Brewer *et al.* 1990; Lacy 1993; Lacy *et al.* 1993, 1996). Such studies may underestimate the extent of inbreeding depression for two reasons. First, inbred individuals may not exhibit inbreeding-related deficits in a controlled environment, an environment that is largely invariant and non-stressful. Recent evidence suggests that inbreeding effects may be more significant in the wild (Chen 1993; Jiménez *et al.* 1994; Keller *et al.* 1994). Second, inbreeding may have impacts beyond the juvenile life history stage (Lynch 1977; Connor and Bellucci 1979; Van Noordwijk and Sharloo 1981; Bondari and Dunham 1987; Margulis 1996). The (typically unstated) assumption that an inbred individual that survives to maturity is not suffering from inbreeding depression may potentially underestimate the extent and scope of inbreeding depression by ignoring possible effects on fertility and fecundity, and by considering only outcomes in the present environment.

The potential consequences of inbreeding for adults have been measured primarily in the form of reduced fertility and fecundity. Most of this research has focused on females, in large part because it is easier to identify maternal effects on fitness and offspring survival than paternal effects. Inbreeding has been shown to have negative effects on female fertility in such species as the house mouse (*Mus musculus*, Lynch 1977; Connor and Bellucci 1979), the great tit (*Parus major*, Van Noordwijk and Sharloo 1981), and the oldfield mouse (*Peromyscus polionotus*, Margulis and Altmann 1997; Margulis 1998*a*). In studies of female oldfield mice, Margulis (1996, 1998*a*, 1998*b*) and Margulis and Altmann (1997) found that inbred adult females suffered from inbreeding depression in the form of delayed onset of reproduction, fewer young reared, and an increased prevalence of reproductive failure. Preliminary data suggested that normal patterns of cyclicity may be disrupted in inbred females (S. W. Margulis and K. K. Ryan, unpublished data). Male oldfield mice have been found to select mates based on extremely small differences in kinship (Ryan and Altmann 2001).

Abnormalities in male reproductive physiology have been documented both in wild (Wildt *et al.* 1987; Barone *et al.* 1994; O'Brien 1994) and captive populations (Roldan *et al.* 1998; Gomendio *et al.* 2000). For example, the small, remnant population of Florida panthers (*Felis concolor coryi*) is plagued by abnormalities in male reproductive physiology, including cryptorchidism, poor sperm motility and abnormal sperm morphology (Barone *et al.* 1994). Such abnormalities are not found in larger puma populations in which inbreeding is far less likely to occur, and may be linked to reduced fecundity (Barone *et al.* 1994). In their studies of captive populations of gazelle species (*Gazella dorcas*, *Gazella dama* and *Gazella cuvieri*), Gomendio *et al.* (2000) and Roldan *et al.* (1998) found variation in sperm quality both within and among species, based on the level of inbreeding. Congenital sperm and testicular abnormalities are also well documented for domestic species (Andersson

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and Mäkinen 1999). Case studies such as these may be limited in their generalizability owing to such factors as the lack of controlled study design, absence of non-inbred controls and small sample size.

Reproductive failure is a function of both male and female behavior and physiology. Studies on human infertility indicate that 30% of all cases of infertility are owing to sperm abnormalities in the male (Parazzini *et al.* 1998). However, even substantial declines in sperm count may not lead to a substantially greater increase in infertility (Bonde *et al.* 1999). Reductions in sperm count can, nevertheless, provide an indication of potential or future problems in fecundity, and thus may serve as an early warning that fertility may be compromised. This may be especially critical for small, captive or wild populations in which even minor changes in fecundity can greatly impact reproduction.

Here, we report on the results of an experimental study on the impact of inbreeding on male fertility in oldfield mice. This species has been used for a long-term investigation of the effects of inbreeding on reproduction in populations of varying size, and detailed information on reproductive history and relatedness is available for all subjects in the study population, making them an ideal species for our investigation. The findings from these long-term studies have shown that fecundity is lower in inbred mice (Brewer *et al.* 1990; Lacy *et al.* 1996; Margulis 1996, 1998*a*; Lacy and Ballou 1998). However, the possible mechanism by which reproductive success is impacted has not been satisfactorily established. We focused on possible inbreeding effects on male reproductive potential by examining testicular sperm counts in inbred and outbred male oldfield mice. We predicted that inbred males would show lower testicular sperm counts than non-inbred males. We further predicted that lower testicular sperm concentration would be correlated with reduced reproductive success for males.

Materials and methods

Animals

Male oldfield mice (*Peromyscus polionotus*) of two subspecies, *P. p. rhoadsi* and *P. p. subgriseus* were used in the study. These animals are part of a long-term study on the effects of inbreeding on small populations (e.g. Brewer *et al.* 1990; Lacy 1993; Lacy *et al.* 1996; Lacy and Ballou 1998). *Peromyscus polionotus* is monogamous and both parents contribute to care of the young (Foltz 1979, 1981; Margulis 1998*b*). Males appear to discriminate between very small differences in kinship with potential mates (Ryan and Altmann 2001; Ryan *et al.* 2002).

As part of a separate study (Margulis 1996), a total of 332 *P. polionotus* pairs were established for breeding. All pairs were housed in standard polycarbonate mouse cages, and received mouse chow and water *ad libitium*. The extensive pedigrees available for the population allowed us to identify both inbred and outbred adults for use in these pairings. The inbreeding coefficient (*f*) was used as a measure of degree of inbreeding, and is a measure of the probability that an individual has two copies of an allele that are identical by common descent (Hartl and Clark 1989). Animals with inbreeding coefficients ≥ 0.1 were considered inbred. All animals were at least 55 days old at the time of pairing. Pairs were housed together through the birth and rearing of up to three litters, or for 120 days (whichever came first). Pairs were separated at the weaning of the third litter. Females undergo a post-partum estrus and gestation is approximately 23–24 days (Smith 1966). Thus, most females were pregnant at this time. They went on to rear the fourth litter without the male being present.

Approximately 23% (n = 76) of these pairs failed to produce offspring. The reproductive tracts of both males (n = 40) and females (n = 46) were examined in over half of these non-reproductive pairs. In addition, the reproductive tracts of six mulitparous females were examined as a control, as well as the reproductive tracts of 28 breeding males and 25 males who had not yet been paired for reproduction. Data on females were inconclusive. We describe here only the results for the males.

Testicular sperm count protocol

Sperm were obtained from testicular tissue. The Animal Care and Use Committee of the Brookfield Zoo approved all protocols. Methods were based on standard sperm count procedures for humans (Lipshultz and Howards 1983) adapted for rodent testicular sperm counts at the University of Illinois at Chicago Department of Pharmacology Toxicology Laboratory. Testes were removed and weighed individually (right and left) immediately after the animals were killed. They were then placed individually in labeled cryo-vials for rapid freezing in liquid nitrogen. Testes were later thawed for less than 20 min at room temperature. Testes (including the tunica) were then homogenized in 8 mL of phosphate-buffered saline (pH = 7.3) (8.0 g NaCl, 0.2 g KH_2PO_4, 0.1 g $CaCl_2$, 0.2 g KCl, 2.1 g $Na_2HPO_47H_2O$, 0.1 g $MgCl_25H_2O$, dissolved in 950 mL of distilled deionized H_2O, adjusted to a final pH of 7.3 using 0.1 M HCl and brought to a final volume of 1000 mL), using a Potter Elvehjem 30-mL tissue grinder (catalog no. 2617242; Fisher Scientific). Homogenate samples were maintained in an ice bath until scoring. Before scoring, this suspension was vortexed using a Fisher Scientific Touch Mixer (model no. 231). A 3:1 mixture of 0.1% trypan blue stain (C.I. 23850, Direct Blue 14, lot no. 68F3670; Sigma Chemical Company, St Louis, MO, USA) and the suspension was prepared and vortexed once more. This suspension was then immediately loaded into both sides of a clean Neubauer Brightline hemacytometer (catalog no. 0267110; Fisher Scientific, Hanover Park, IL). A total of four tertiary squares, the bottom two tertiary squares from each side of the hemacytometer, were then manually counted, focusing in and out in order to count both layers of spermatid heads. All spermatid heads within the designated tertiary squares and those lying on the bottom and right sides of the tertiary squares were counted.

The total number of spermatid heads per gram of testis was then determined by first calculating the dilution factor and then dividing the number counted for each testis by the testis weight (g) and multiplying by the dilution factor.

Dilution factor = total volume of homogenate × 4 (1:3 dilution)/(no. of tertiary squares counted × volume occupied by a tertiary square)

No. spermatid heads (g testis)$^{-1}$ = (no. spermatid heads/testis weight) × dilution factor

Statistical analysis

Linear regression was used to examine the relationship between inbreeding and testicular sperm count (n = 93 males) (SAS REG Procedure, SAS Institute 1988). Logistic regression (Hosmer and Lemeshow 1989; SAS LOGISTIC procedure, SAS Institute 1990) was used to examine the effect of testicular sperm count and inbreeding on reproductive success, using only those males that had been paired (n = 68) (see Table 1). Thus, linear regression was used when inbreeding was measured as a continuous variable, and logistic regression was used when inbreeding was considered dichotomous, as either inbred or outbred.

Results

The average sperm count (g testis)$^{-1}$ (± SEM) was $18.25 \times 10^7 \pm 0.07 \times 10^7$ for outbred males (n = 51) and $16.41 \times 10^7 \pm 0.74 \times 10^7$ for inbred males (n = 42). Total testicular sperm count, sperm count (g testis)$^{-1}$, and testis mass all declined with increasing inbreeding coefficient (Fig. 1, linear regression; total testicular sperm count: $F_{1,88} = 6.24$, $P<0.01$; sperm count (g testis)$^{-1}$: $F_{1,88} = 3.75$, $P<0.056$; testis mass: $F_{1,91} = 5.18$, $P<0.025$). Testicular sperm counts were, on average, lower and more variable for inbred males than for outbred males (Fig. 2). Body mass of inbred and outbred males did not differ (15.13 ± 0.21 g for outbred males; 15.38 ± 0.31 for inbred males).

For those males that had mating opportunities, the average sperm count (g testis)$^{-1}$ (± SEM) was $18.1 \times 10^7 \pm 0.8 \times 10^7$ for successful breeders (n = 28) and $16.7 \times 10^7 \pm 0.66 \times 10^7$ for non-breeders (n = 40). The results of all logistic regression analyses were not significant: total testicular sperm count, sperm count (g testis)$^{-1}$, and testis mass did not affect reproductive performance. Inbreeding coefficient did not have a significant effect on reproductive outcome. Testicular sperm counts were generally lower for paired males whose mates failed to produce young than for successful breeding males, but among-subject variability was equally high for both successful and unsuccessful breeders (Fig. 3). Body mass of breeding and non-breeding males did not differ (15.14 ± 0.32 g for breeding males; 15.21 ± 1.26 for non-breeding males).

Discussion

Our findings suggest that inbreeding leads to a decline in testicular sperm count in mice. Retrospective studies have examined the effect of inbreeding on sperm production and morphology in a number of species (Wildt *et al.* 1987; Barone *et al.* 1994; Roldan *et al.* 1998; Gomendio *et al.* 2000). Such studies have not been experimental in nature and outbred controls were not always available. The current findings support the contention that inbreeding can have negative effects on fitness beyond the juvenile stage: inbred adult male oldfield mice may experience reductions in testicular sperm concentration when other variables (body size, age) are controlled for.

In the present study, our measure of testicular sperm concentration was actually one of sperm production potential, as we did not examine sperm in semen only. The

Table 1. Subsets of mice used for linear regression analysis to assess the relationship between testicular sperm count and inbreeding, and for logistic regression analysis on paired males to assess the relationships between testicular sperm count and reproductive success and testicular sperm count and inbreeding

Analysis	No. of animals
Linear regression: inbreeding and sperm count	93
Inbred	42
Outbred	51
Logistic regression: sperm count and reproductive success	68
Proven breeders	28
Paired, but no offspring	40
Logistic regression: sperm count and inbreeding	68
Inbred	36
Outbred	32

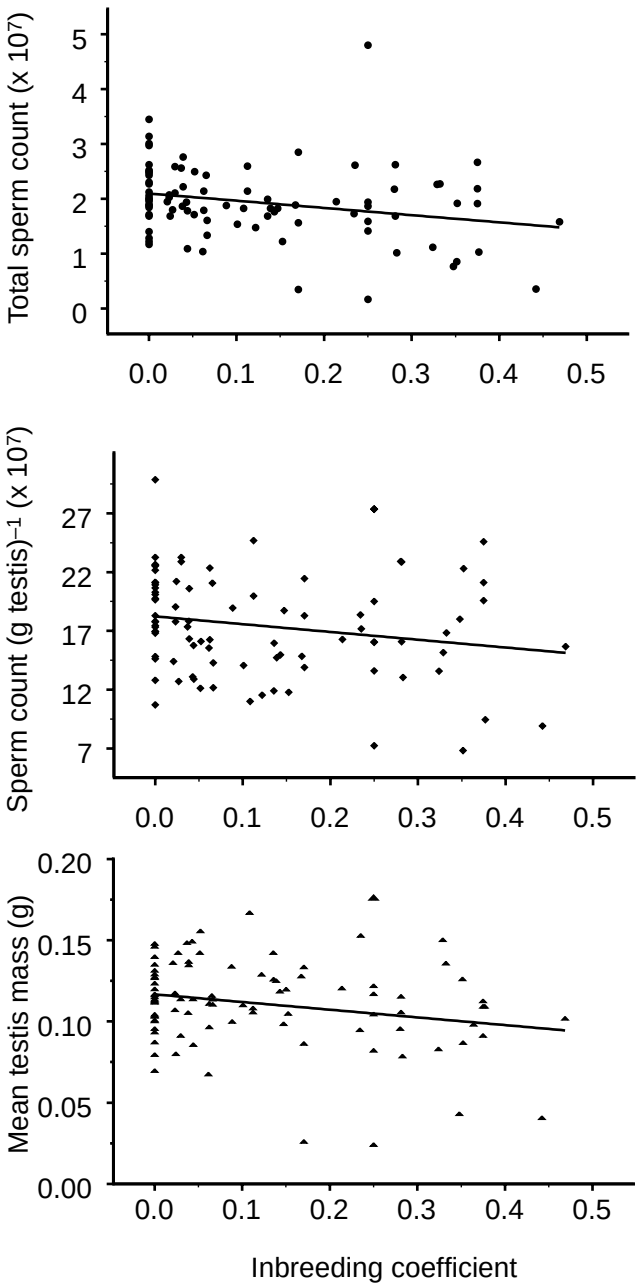

Fig. 1. Relationship between total sperm count, sperm count (g testis)$^{-1}$, and mean testis mass with inbreeding coefficient (n = 93 males).

possibility exists that inbreeding may be impacting spermatogenesis, although we were not able to investigate this intriguing possibility further. In addition, we did not evaluate other sperm characteristics that might influence fertility, nor did we examine more sensitive indicators of fertility such as counts of Sertoli cells in the testis or direct counts of sperm in the semen. Gomendio *et al.* (2000) reported a strong negative relationship between inbreeding and traits associated with sperm motility and morphology, but not testicular sperm concentration, in *Gazella cuvieri*. This is perhaps not surprising, given that there appears to be a fairly low threshold in testicular sperm concentration, below which fertility is compromised, but above which fertility is not markedly increased above normal. Therefore, one might expect to see a very large range of testicular sperm concentrations that all fall within the 'normal', 'fertile' range for a species (Bonde *et al.* 1998, 1999).

The observed decline in sperm count was not associated with decreased reproductive success. Previous research with this population has not found any deficiencies in sexual behavior among inbred males, although some deficits in paternal care were evident (Margulis 1998*b*). The most striking deficit found in relation to inbreeding was delayed onset of reproductive activity by inbred females (Margulis 1996; Margulis and Altmann 1997), which likely explains the differences in reproductive performance among pairs.

As populations of endangered animals decline, inbreeding becomes more likely. Small captive populations may similarly experience inbreeding. In both situations, inbreeding depression may impact fitness measures other than juvenile survival, such as reproductive performance. Although we did not find an association between testicular sperm concentration and fertility, our findings suggest that sperm count is likely to continue to decline as inbreeding increases. At some point, the fertility threshold may be crossed and fertility seriously compromised. Although we cannot assess how generally applicable our findings are to other species, it is likely that the same processes that may be influencing sperm production in *P. polionotus* are occurring more generally throughout sexually reproducing taxa. Our findings suggest that monitoring testicular sperm concentration in small, potentially inbred populations may provide insights into declines in fitness, and serve as an early warning of impending problems. Testicular sperm concentration may decline gradually with inbreeding without affecting fertility. At some point, however, inbreeding may be sufficiently severe to lead to detectable reductions in fertility and fecundity. Hence, careful monitoring of male reproductive performance in captive or small, isolated wild populations in which inbreeding processes may be occurring, could serve as a means of identifying and perhaps avoiding reproductive failure.

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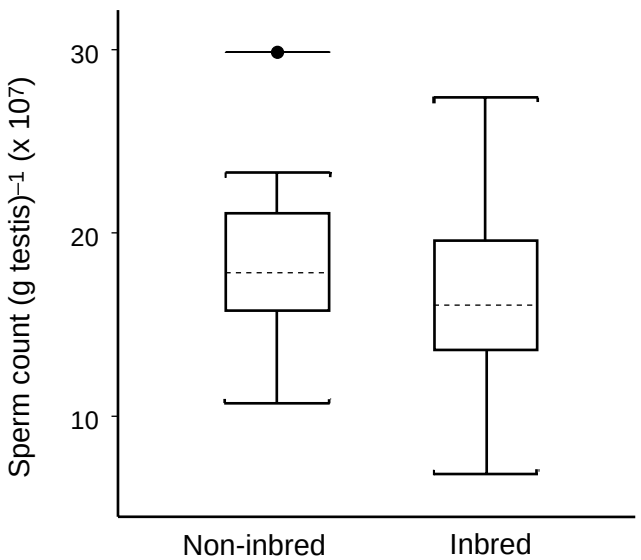

Fig. 2. Box plot comparison of sperm count (g testis)$^{-1}$ for non-inbred (n = 32) and inbred (n = 36) male *Peromyscus polionotus*. The dashed line in the center of each box represents the median; the upper and lower bounds of the box represent the first and third quartiles. The extent of the data beyond the quartiles (1.5 times the interquartile range) is indicated by the bars extending above and below the box. Outliers are indicated by a point and line.

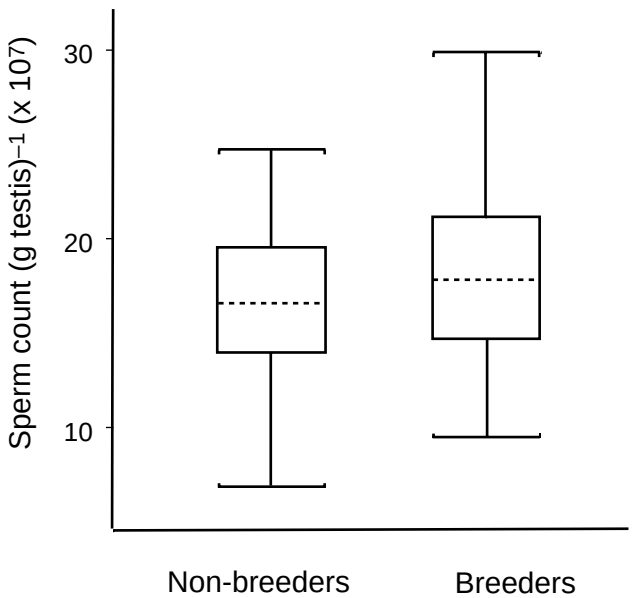

Fig. 3. Box plot comparison of sperm count (g testis)$^{-1}$ for non-breeding males (n = 40) (paired males whose mates failed to produce offspring) and breeding males (n = 28) (males who sired offspring). See Fig. 2 for description of graph.

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