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Experimental infection magnifies inbreeding depression in house mice

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Abstract

It is often assumed that inbreeding reduces resistance to pathogens, yet there are few experimental tests of this idea in vertebrates, and no tests for the effects of moderate levels of inbreeding more commonly found in nature. We mated wild-derived mice with siblings or first cousins and compared the resistance of their offspring to *Salmonella* infection with outbred controls under laboratory and seminatural conditions. In the laboratory, full-sib inbreeding reduced resistance to *Salmonella* and survivorship, whereas first-cousin inbreeding had no detectable effects. In competitive population enclosures, we found that first-cousin inbreeding reduced male fitness by 57% in infected vs. only 34% in noninfected control populations. Our study provides experimental evidence that inbreeding reduces resistance and ability to survive pathogenic infection, and moreover, it shows that even moderate inbreeding can cause significant fitness declines under naturalistic conditions of social stress, and especially with exposure to infectious agents.

Introduction

It is widely assumed that inbreeding reduces resistance to infectious diseases, and that exposure to infectious agents exacerbates the negative fitness consequences of inbreeding (i.e. inbreeding depression) (Coltman *et al.*, 1999; Keller & Waller, 2002). A number of observational studies are consistent with the idea that inbreeding reduces resistance to pathogens and parasites, yet there are surprisingly few experimental studies on inbreeding and disease resistance in vertebrates, and none with moderate levels of inbreeding. The goal of our study was to test experimentally whether close or moderate levels of inbreeding reduces resistance to infectious diseases and affects the fitness of infected individuals.

Several studies from wild and captive vertebrate species provide observational evidence that inbreeding reduces resistance to pathogens and parasites (Cassinello *et al.*, 2001; Acevedo-Whitehouse *et al.*, 2003, 2005, 2006; Valsecchi *et al.*, 2004; Whiteman *et al.*, 2006; Ross-Gillespie *et al.*, 2007), but experimental infections are needed to rule out other factors, such as variation in exposure to pathogens. Two recent studies in captivity manipulated infection and assessed inbreeding using microsatellite markers and found that increasing homozygosity resulted in higher host mortality from viral infection (Pearman & Garner, 2005) and increased disease severity from bacterial infection (Hawley *et al.*, 2005). A third study in the wild also manipulated infection by giving antihelminthic treatment to the subset of the host population (Coltman *et al.*, 1999). Increasing homozygosity was associated with increased parasite loads and resulted in increased mortality among nontreated hosts, but not in antihelminthic treated ones, providing experimental evidence that parasites magnify selection against more homozygous individuals. However, using microsatellite markers is problematic for measuring inbreeding and genome-wide homozygosity (Balloux *et al.*, 2004; Pemberton, 2004; Slate *et al.*, 2004; Bensch *et al.*, 2006), and more importantly, studies are needed that manipulate 'inbreeding', as well as infection.

Three studies have manipulated both inbreeding and infection (all in fish; two in heavily inbred lines: Wright's

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inbreeding coefficient $f > 0.70$ and $f = 0.86$–0.93, and one using offspring from full-sib matings: $f = 0.25$), and these provide mixed evidence for the idea that inbreeding reduces pathogen resistance (Hedrick *et al.*, 2001; Arkush *et al.*, 2002; Giese & Hedrick, 2003). To extrapolate to natural populations, however, studies are needed that examine inbreeding in wild (or outbred) organisms, and not only inbred lines. The study that provides the best evidence found that a higher proportion of full-sib inbred fish showed more severe disease symptoms than outbred ones after experimental infection with *Myxobolus cerebralis* (Arkush *et al.*, 2002). Full-sib inbreeding is rare compared to more moderate levels of inbreeding for natural vertebrate populations (Keller & Waller, 2002; Marshall *et al.*, 2002), which raises the question of whether moderate levels of inbreeding affect disease resistance (see also Reid *et al.*, 2003, 2007), particularly in light of data suggesting that moderate levels of inbreeding might be beneficial (Bateson, 1983). More importantly, it is unclear whether the disease symptoms are sufficient to affect fitness, so studies are needed that measure the evolutionary consequences of inbreeding. For this, it is critical to examine fitness effects under natural or seminatural ecological conditions where the consequences of inbreeding can be much higher than in the laboratory (Meagher *et al.*, 2000; Joron & Brakefield, 2003; Armbruster & Reed, 2005). So, although previous studies are consistent with the hypothesis that inbreeding increases susceptibility to infectious diseases, experimental studies are needed that manipulate both inbreeding and infection, and also examine the 'fitness' effects of moderate as well as close levels of inbreeding under more naturalistic conditions.

We conducted experiments using wild-derived house mice (*Mus musculus domesticus*) to determine how inbreeding affects resistance to *Salmonella enterica*. Mice were experimentally bred to produce inbred offspring at close (full-sib matings) and moderate (first-cousin matings) levels of inbreeding, and their resistance to experimental infection was compared to outbred controls. Resistance to infectious disease was determined by measuring pathogen loads and prevalence (infected or cleared), and the fitness consequences by measuring survival and reproductive success. We found that close inbreeding reduced resistance to infectious disease in the laboratory, unlike moderate inbreeding. However, moderate inbreeding caused a significant reduction in fitness and ability to clear *Salmonella* in seminatural enclosure populations, and *Salmonella* infection substantially magnified inbreeding depression.

Materials and methods

Experimental animals and laboratory conditions

The experimental mice were descendants of wild mice originally trapped near Gainesville, Florida. The progenitor mice were trapped in two locations (ca. 32 km apart) and then crossed between locations to produce uniformly outbred mice, and the F_2 descendants were used in a previous study on inbreeding depression (Meagher *et al.*, 2000). To prevent unintentional inbreeding caused by population bottlenecks, the number of breeding pairs was kept high in preceding generations (51–96 pairs per generation) and there were no matings between first-cousins or more close relatives. We used F_6 mice to produce experimentally full-sib inbred mice and their outbred controls, and F_7 mice to produce first-cousin inbred mice and their controls. We produced inbred experimental mice by mating sisters and brothers (full-sib inbred, $f = 0.25$) and outbred mice by breeding unrelated individuals ($f = 0.00$). We also mated first-cousins (first-cousin inbred, $f = 0.0625$), and outbred control mice by breeding unrelated individuals. The mice were bred and housed in standard laboratory conditions in $13 \times 18 \times 29$ cm clear acrylic cages under 12 h : 12 h dark/light cycle and provided water and food *ad libitum*.

Laboratory infection experiments

In the first laboratory infection experiment (exp. 1), we compared full-sib inbred individuals to outbred ones for resistance to *Salmonella* infection (bacterial clearance and ability to survive). *Salmonella enterica* serovar Typhimurium is an enteric mouse pathogen that becomes systemic by invading the intestinal mucosa and by replicating intracellularly within host macrophages (Santos *et al.*, 2001). Host resistance to *Salmonella* is under genetic control and influenced by major histocompatibility complex (MHC) and other resistance loci (Roy & Malo, 2002), and require both innate and acquired arms of the immune system (Ravindran & McSorley, 2005). We cultured the bacteria (stored as frozen stocks at –70 °C) in 25 mL of heart–brain infusion at 37 °C for 12 h while shaking. We diluted the overnight solution to the desired concentration with sterile phosphate-buffered saline (PBS) and verified the concentration of viable bacteria by quantitative plate counts in duplicates. We infected equal numbers of full-sib inbred ($n = 24$) and outbred mice ($n = 24$) by giving them an intraperitoneal injection with 200 μL [strain LT2 (Xu & Hsu, 1992), 10^4 colony forming units (cfu mL^{-1})]. All mice were fully mature and in both experimental groups half were males and half were females. Mice were housed singly and were euthanized 10-day post-inoculation. We dissected and homogenized the spleens of the mice in 1 mL of PBS under sterile conditions, cultured 50 μL of each homogenate on selective agar plates, and incubated the plates overnight (37 °C). We determined the *Salmonella* loads per spleen by calculating the number of cfu mL^{-1} of spleen homogenates on the plates (the mean of two replicate plates per mouse). We measured the *Salmonella* loads only from individuals that survived, and as it is probable (based on our previous experience) that the

mice that died harboured high loads, our estimates for *Salmonella* loads and prevalence are likely to be conservative. In a second experiment (exp. 2), we assessed the infection dynamics over time and examined the repeatability of our previous findings by infecting inbred mice from full-sib matings ($n = 24$) and outbred controls ($n = 24$) using the same protocol as before, except that we measured pathogen loads on days 6, 8 and 10 post-inoculation. In the third laboratory infection experiment (exp. 3), we compared *Salmonella* resistance of first-cousin inbred mice ($n = 36$) to outbred controls ($n = 36$). We used a protocol similar to the previous experiments (1 and 2), except that we infected the mice by giving them 30 μL (LT2, 10^8 cfu mL^{-1}) orally, which is a natural infection route for *Salmonella*, and euthanized them on day 14 post-inoculation. The mice were restricted from food and water 4 h prior to inoculation to rule out variation in systemic infection because of food in the gut.

Enclosure infection experiment

To assess the effects of first-cousin inbreeding on resistance and fitness under more naturalistic conditions, we released 240 mice into eight large seminatural population enclosures (ca. 22.2 m^2). Four of the populations were experimentally infected, whereas the other four served as sham controls. There were 10 males and 20 females within each population, half of them first-cousin inbred and half of them outbred. To avoid possible confounding effects of kin-based behaviours, males did not have any close relatives and females had no sibs in the same population. We provided mice with 12 nest-boxes within each enclosure and food and water *ad libitum*. Male house mice aggressively compete for territories; therefore, we increased environmental complexity by subdividing each enclosure into six equal sized subsections using hardware cloth fences. Mice could easily climb over these dividers, but they served two purposes: first, dominant males use them as territorial boundaries, and second, they reduce harassment of subdominant males by dominant males.

In experimentally infected populations, we infected the mice orally with 30 μL (10^8 cfu mL^{-1}) *Salmonella* just before releasing them, and thereafter re-captured and re-infected them in regular 4-week intervals. During each subsequent re-infection, we added a novel *Salmonella* strain into the inoculum, but kept the volume and concentration the same. We infected mice with the following *Salmonella* strains; LT2, PMAC45 (Charbit *et al.*, 1997), M525 (Hormaeche *et al.*, 1981), PMAC51 (Charbit *et al.*, 1997), χ4665 (Sukupolvi *et al.*, 1997), 628 (Hormaeche *et al.*, 1985) and ATCC 14028 (in the order added to the inoculum). We chose to increase sequentially the number of bacterial strains over time to imitate natural overlapping epidemics, where novel pathogen strains emerge, as a result of mutation or invasion. The mice in control populations were sham treated by giving them equal amounts of PBS in regular 4-week intervals. At the end of the 7-month long experiment, we euthanized the surviving mice, and determined *Salmonella* loads by using the same protocol as before.

We conducted weekly checks to monitor health and survival, to identify adults using unique ear-puncture marks, and to collect the new-born pups from their nests, which were euthanized to obtain tissue samples used for genetic analyses. We determined the reproductive success of first-cousin inbred and outbred mice by taking advantage of polymorphisms segregating in the wild-derived populations (Meagher *et al.*, 2000). For each enclosure population, we chose the founders such that first-cousin inbred and outbred mice carried distinct alleles for sex-specific markers. To distinguish whether male parents were inbred or outbred, we used PCR primers (5′-CAGGGTTTCTCTCTAGCACA and 5′-CAC AACTGGGCTTTGCACATTG) for a microsatellite marker near *Sry* on the Y chromosome (Gubbay *et al.*, 1992). To determine female parentage, we used a length variant in the control region of the mitochondrial genome (5′-TGGTTTCACGGAGGAGGATGGT and 5′-CACCAC CAGCACCCAAAGCT) (Meagher *et al.*, 2000). These markers allowed us to determine whether the mother was inbred or outbred (for all pups) and whether the father was inbred or outbred for the sons. To avoid confounding the effects of inbreeding with possible effects caused by markers or closely linked genes, we reversed the marker-treatment pairing in half of the populations. We could not determine mother's inbreeding status for 28 pups (1.9%) and father's inbreeding status for 65 pups (2.7%), because of poor quality DNA.

Statistical analyses

Because we used different infection methods (infection route, strains and dosage) in these experiments, we restrict our analyses to comparing inbred vs. outbred individuals 'within' each experiment. We had clear *a priori* predictions that: resistance and fitness would be lower in inbreds than in outbreds, fitness would be lower in experimentally infected vs. sham-infected animals, and infection would magnify inbreeding depression, and therefore used directed tests instead of one- or two-tailed tests (Rice & Gaines, 1994). We obtained the critical values for each directed test from the P-values from the corresponding one-tailed test, by using $\gamma/\alpha = 0.8$ as a pragmatic conventional value (Rice & Gaines, 1994). We always tested the data for assumptions of normality and equality of variances before conducting parametric tests. The *Salmonella* loads were $\log_{10}$-transformed to meet the requirements for parametric tests. We always statistically controlled for the effects of age, sex and duration of infection on *Salmonella* loads by including them in ANCOVA models either as a factor or covariates, but, for brevity, report only statistically significant covariate or sex effects. We used hierarchical log-linear models to

analyse *Salmonella* prevalence (infected or noninfected), and Cox regression to analyse survival in enclosure populations. We entered first the highest level interaction terms, and then chose the model with best fit by using backward (stepwise) model selection. In backward elimination, the effect whose removal results in the least significant change in the likelihood ratio χ^2 is eligible for elimination, provided that the observed significance level is larger than the criterion for remaining in the model (Norušis, 1993). We conducted all statistical tests using SPSS (Windows version 12.0), and report results as mean ± SEM.

Results

Laboratory experiments

In the first experiment, we found that a significantly higher proportion of full-sib inbred mice died within 10 days after infection compared to outbred controls (58% and 21% respectively; inbreeding: LR $\chi^2_1 = 7.3$, $P = 0.004$). Also, the surviving inbred mice had significantly higher *Salmonella* loads compared to outbred controls ($\log_{10}$ of cfu mL^{-1} ± SEM: 5.0 ± 0.38 and 3.7 ± 0.53 respectively; $F_{1,27} = 4.0$, $P = 0.03$). In the second experiment, we again found that full-sib inbred mice had significantly higher mean loads compared to outbred mice, and bacterial loads showed a nonsignificant tendency to increase with time in inbred mice and decrease in outbred ones (inbreeding: $F_{1,31} = 8.9$, $P = 0.004$; duration of infection: $F_{2,31} = 0.7$, $P = 0.51$; interaction inbreeding × duration of infection: $F_{2,31} = 2.5$, $P = 0.06$, Fig. 1). Furthermore, the full-sib inbred mice showed again higher mortality compared to outbred ones (33% and 13% respectively; inbreeding: LR $\chi^2_1 = 3.0$, $P = 0.05$). In the third experiment, we found that females showed lower *Salmonella* prevalence compared to males (67% and 92% respectively; sex: LR $\chi^2_1 = 7.2$, $P = 0.007$), but there were no significant differences between first-cousin inbred vs. outbred mice either in *Salmonella* prevalence (72% and 86% respectively; inbreeding: LR $\chi^2_1 = 2.1$, $P = 0.14$) or in mean pathogen loads (1.8 ± 0.21 and 2.2 ± 0.18 respectively; inbreeding: $F_{1,70} = 2.0$, $P = 0.16$).

Enclosure experiment

In the population enclosures, we found significant sex × inbreeding interaction effect on *Salmonella* prevalence (LR $\chi^2_1 = 4.6$, $P = 0.03$), but the main effects were statistically nonsignificant (inbreeding: LR $\chi^2_1 = 0.6$, $P = 0.29$; sex: LR $\chi^2_1 = 0.8$, $P = 0.37$). When testing the sexes separately, a significantly higher proportion of first-cousin inbred males were still infected at the end of the 7-month experiment compared to outbred males (Fisher's exact: $P = 0.04$; Fig. 2), but first-cousin inbreeding did not have any significant effects on females' ability to clear *Salmonella* ($\chi^2 = 0.2$, $P = 0.65$; Fig. 2).

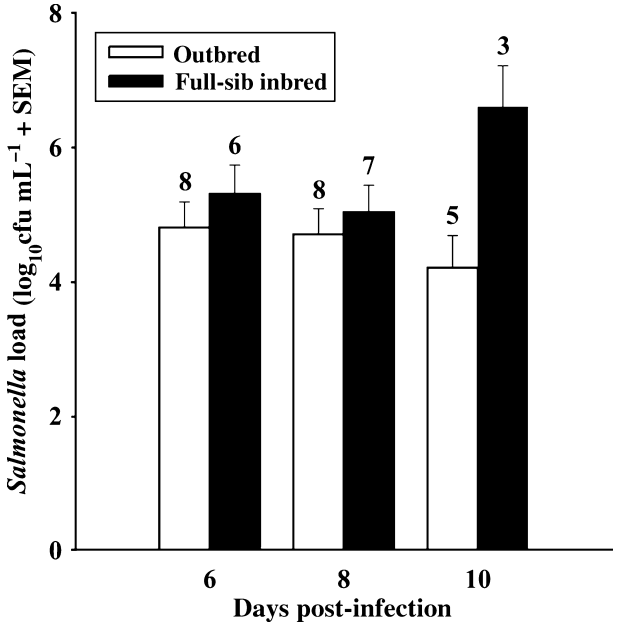

Fig. 1 Second laboratory infection experiment: the mean *Salmonella* loads of outbred and full-sib inbred mice on day 6, 8 and 10 post-infection. Sample sizes are indicated above the bars.

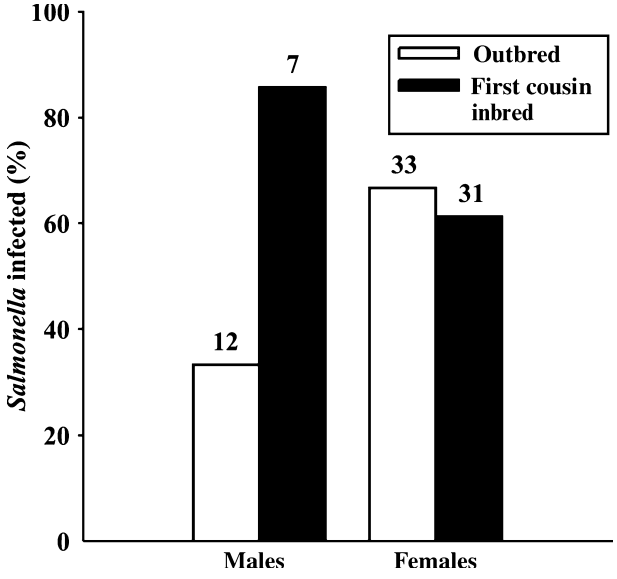

Fig. 2 The percentage of outbred and first-cousin inbred males and females that were still *Salmonella* infected at the end of 7-month enclosure experiment.

First-cousin inbred males had higher mortality rates compared to outbred ones (Cox regression; inbreeding: Wald$_1 = 6.6$, $P = 0.006$; Fig. 3a,b), and infected males

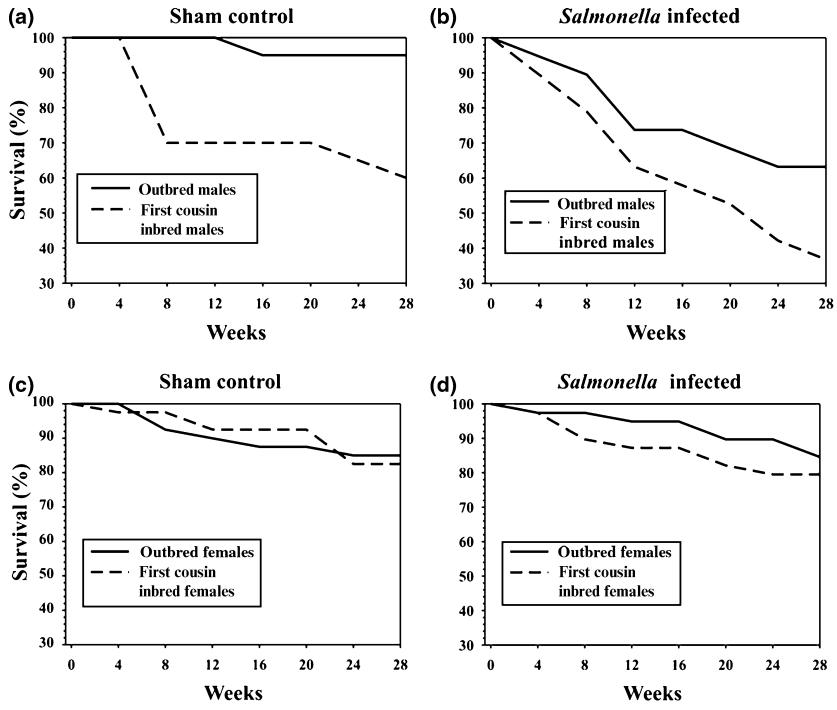

Fig. 3 Survival rates (percentage of individuals alive) in control and infected populations for outbred and first-cousin inbred males (a–b) and females (c–d).

had higher mortality rates compared to sham controls (treatment: $Wald_1 = 5.2$, $P = 0.01$), but infection did not result in increased difference in mortality rates between inbred and outbred males (inbreeding × treatment interaction: $Wald_1 = 5.8$, $P = 0.08$). Mortality rates in females were significantly lower than in males (sex: $Wald_1 = 11.0$, $P = 0.001$), but first-cousin inbreeding, infection or their interaction had no significant effects on female survival (inbreeding: $Wald_1 = 1.3$, $P = 0.16$; treatment: $Wald_1 = 0.2$, $P = 0.42$; inbreeding × treatment interaction: $Wald_1 = 0.5$, $P = 0.31$; Fig. 3c,d).

In all, 2452 pups were born in enclosure populations during the experiment. Reproductive success was significantly lower in *Salmonella* infected populations compared to sham-treated controls ($\chi^2_1 = 62.7$, $P < 0.001$). Compared to outbred controls, first-cousin inbred males sired 34% fewer offspring in uninfected control populations ($\chi^2_1 = 25.7$, $P < 0.001$) and 57% fewer offspring in experimentally infected populations ($\chi^2_1 = 74.2$, $P < 0.001$), and the reduction in reproductive success of inbred males was significantly greater in infected populations ($\chi^2_1 = 11.4$, $P < 0.001$; Fig. 4). First-cousin inbred females produced 11% fewer offspring than outbred ones in infected populations ($\chi^2_1 = 3.2$, $P = 0.046$), but there was no statistically significant differences in uninfected control populations ($\chi^2_1 = 0.0$, $P = 0.56$). The reduction in reproductive success of inbred females was somewhat greater in infected vs. sham-treated populations, but not quite statistically significant ($\chi^2_1 = 2.7$, $P = 0.06$; Fig. 4).

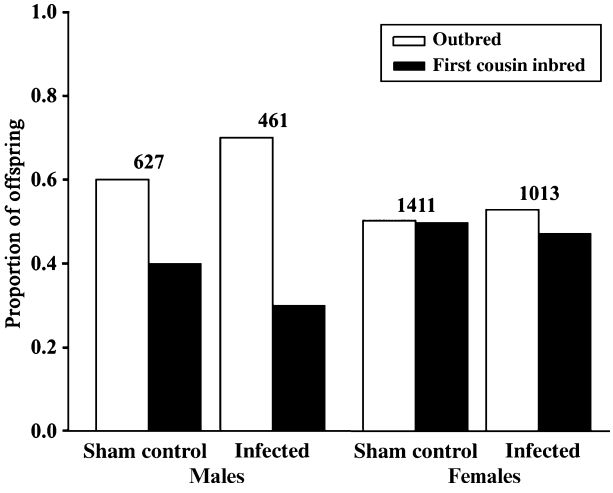

Fig. 4 Reproductive success of outbred and first-cousin inbred males and females in control and infected populations.

Discussion

In our experiments, we found that inbreeding negatively affected resistance to *Salmonella* infection both in the laboratory and enclosures, and we also found an impact from moderate inbreeding in the enclosures. In the laboratory, inbred mice from full-sib matings showed significantly higher mortality within 10-day post-*Salmonella* inoculation compared to outbred controls. We also found that the outbred mice were better able to control

Salmonella infection (pathogen loads in experiments 1 and 2), which indicates that the low survival of full-sib inbred mice was because of reduced pathogen defences [rather than endotoxin (lipopolysaccharide)-induced shock or other types of immunopathology]. This result argues against other possible explanations, such as mortality from differential susceptibility to handling stress, including injection *per se*. In the laboratory, we found no evidence that moderate inbreeding (first-cousin matings) reduced resistance to *Salmonella;* however, we cannot rule out the possibility that we might have found an effect by using another pathogen, strain, dosage, infectious route, or more stressful environmental conditions. Therefore, we conducted another experiment with first-cousin inbred mice in competitive population enclosures, where the fitness consequences of close inbreeding are more severe compared to laboratory conditions (Meagher *et al.*, 2000).

In the enclosures, we found that males from first-cousin inbreeding had reduced ability to clear *Salmonella* infection compared to outbred controls, and that moderate inbreeding increased male mortality and reduced reproductive success both in control and in infected populations, resulting in reduced fitness of 34% and 57% respectively. In other words, moderate inbreeding reduced fitness and experimental infection with *Salmonella* magnified inbreeding depression for males. There was also a significant reduction in reproductive success of infected inbred females, even though females did not show higher *Salmonella* loads or reduced survival from first-cousin inbreeding. Thus, contrary to inbred males, the inbred females were able to maintain immune defenses, but only at the cost of reduced investment to reproduction (Sheldon & Verhulst, 1996; Ilmonen *et al.*, 2000). Our results from the enclosure experiment show that even moderate inbreeding can substantially reduce fitness and the ability to control bacterial growth, and that inbreeding depression is magnified by infection. These results can help to explain why a previous study found that the effects of close inbreeding were magnified in seminatural population enclosures – where pathogens and parasites were naturally present – compared to the laboratory (Meagher *et al.*, 2000).

There are at least two potential reasons why moderate inbreeding reduced *Salmonella* resistance and fitness of experimentally infected mice in the enclosures, but not in the laboratory. First, as mentioned above, there were methodological differences between these experiments that could explain our results (single vs. repeated infections with multiple strains). Second, an alternative explanation is that examining resistance and fitness under more stressful competitive conditions is more effective at revealing the harmful effects of inbreeding than the benign laboratory conditions. Indeed, many other studies have found that inbreeding depression can be more pronounced under stressful conditions (Keller *et al.*, 1994, 2002; Meagher *et al.*, 2000; Armbruster & Reed, 2005) [though not all studies find such effects (Armbruster & Reed, 2005) and some stressors can even alleviate inbreeding depression (Kishony & Leibler, 2003; Armbruster & Reed, 2005; Killick *et al.*, 2006)]. Our results support the stress hypothesis by showing that inbreeding depression is magnified under experimental infection.

We found that the effects of moderate inbreeding on resistance to experimental infection in the enclosures were mainly on males rather than females, and this sex-effect was probably because of differences in competitive conditions and stress experienced by the sexes. Males were aggressively competing for territories and females, whereas in females there was no need to compete for any critical resources; food, water and nesting sites were in abundance. This finding corroborates results from a previous experiment, which found that fitness consequences of full-sib inbreeding were greatly magnified in enclosures, and especially pronounced in males (Meagher *et al.*, 2000). This result is also consistent with the idea that sexual selection via male-male competition can magnify selection against inbred individuals, and thus reduce mutational load in sexually reproducing populations (Peck & Waxman, 2000; Agrawal, 2001).

Although experimental infection magnified the negative impact of inbreeding on male reproductive success, it did not increase the difference in mortality between first-cousin inbred and outbred males. Furthermore, in females, the negative fitness consequences of first-cousin level inbreeding were seen only in reduced reproductive success under infection. These findings suggest that future experiments also need to measure reproductive success under infection, and not simply pathogen loads or even survival, to estimate inbreeding depression in its full extent. It is possible that we underestimated the extent to which inbreeding affected survival and reproductive success in the enclosures because of an (unavoidable) artificial aspect of our study. Infected and sham-infected individuals were separated into different populations to avoid transmission of *Salmonella* to sham controls, and thus they did not compete against each other. Therefore, intra-sexual competition was probably much lower in infected than control populations as infected individuals were only competing with infected and not uninfected ones. To assess the combined effects of inbreeding and pathogenic infections, future studies should examine fitness under common competitive conditions (see also Bedhomme *et al.*, 2005).

To summarize, our results show that inbreeding increases susceptibility to infectious diseases, and even a moderate level of inbreeding can have severe fitness consequences under stressful environmental conditions. Full-sib inbreeding reduced the ability of mice to clear and survive *Salmonella* infection even in benign laboratory conditions, whereas for first-cousin inbreeding, these negative effects were found only in competitive population conditions, and only in males. Furthermore,

first-cousin inbred males sired fewer offspring than outbred ones both in control and in infected populations, and this reproductive disadvantage was magnified in infected populations. Thus, the harmful effects of inbreeding in the wild may also be conditional depending upon exposure to pathogens (see also Coltman *et al.*, 1999; Spielman *et al.*, 2004) or perhaps other stressors. To the best of our knowledge, our results provide the first experimental evidence that inbreeding reduces resistance to infectious diseases in a mammal, and the first test of the effects from moderate levels of inbreeding in any vertebrate. Further studies are needed to test the generality of our results using other host-pathogen systems, and if possible, these should allow experimentally infected individuals to compete against sham controls when measuring the fitness consequences. More studies are also needed to determine the underlying proximate mechanisms that explain our results. Inbreeding depression is due to the expression of recessive deleterious alleles, loss of overdominance, or both (Charlesworth & Charlesworth, 1999; Crnokrak & Roff, 1999; Keller & Waller, 2002). Inbreeding could reduce resistance to pathogens by unmasking genome-wide recessive deleterious alleles or through the breakdown of overdominance in specific resistance loci, like MHC, although the latter is controversial and unresolved (Penn, 2002; Penn *et al.*, 2002; McClelland *et al.*, 2003; Ilmonen *et al.*, 2007). Thus, our results have important implications for several problems in evolutionary biology, such as the functions of inbreeding avoidance, outbreeding, and sexual reproduction (Lively & Howard, 1994; Coltman *et al.*, 1999), and also for applied topics, including human health (consanguineous marriages) (Bittles *et al.*, 1991) and endangered wildlife populations and species (O'Brien & Evermann, 1988; Altizer *et al.*, 2003; Frankham, 2005).

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References

Acevedo-Whitehouse, K., Gulland, F., Greig, D. & Amos, W. 2003. Disease susceptibility in California sea lions. *Nature* **422**: 35.

Acevedo-Whitehouse, K., Vicente, J., Gortazar, C., Hofle, U., Fernandez-De-Mera, I.G. & Amos, W. 2005. Genetic resistance to bovine tuberculosis in the Iberian wild boar. *Mol. Ecol.* **14**: 3209–3217.

Acevedo-Whitehouse, K., Spraker, T.R., Lyons, E., Melin, S.R., Gulland, F., Delong, R.L. & Amos, W. 2006. Contrasting effects of heterozygosity on survival and hookworm resistance in California sea lion pups. *Mol. Ecol.* **15**: 1973–1982.

Agrawal, A.F. 2001. Sexual selection and the maintenance of sexual reproduction. *Nature* **411**: 692–695.

Altizer, S., Harvell, D. & Friedle, E. 2003. Rapid evolutionary dynamics and disease threats to biodiversity. *Trends Ecol. Evol.* **18**: 589–596.

Arkush, K.D., Giese, A.R., Mendonca, H.L., McBride, A.M., Marty, G.D. & Hedrick, P.W. 2002. Resistance to three pathogens in the endangered winter-run chinook salmon (*Oncorhynchus tshawytscha*): effects of inbreeding and major histocompatibility complex genotypes. *Can. J. Fish. Aquat. Sci.* **59**: 966–975.

Armbruster, P. & Reed, D.H. 2005. Inbreeding depression in benign and stressful environments. *Heredity* **95**: 235–242.

Balloux, F., Amos, W. & Coulson, T. 2004. Does heterozygosity estimate inbreeding in real populations? *Mol. Ecol.* **13**: 3021–3031.

Bateson, P. (ed.) 1983. Optimal outbreeding. In: *Mate Choice*, pp. 257–277. Cambridge University Press, Cambridge.

Bedhomme, S., Agnew, P., Vital, Y., Sidobre, C. & Michalakis, Y. 2005. Prevalence-dependent costs of parasite virulence. *PLoS Biol.* **3**: e262.

Bensch, S., Andrén, H., Hansson, B., Pedersen, H.C., Sand, H., Sejberg, D., Wabakken, P., Åkesson, M. & Liberg, O. 2006. Selection for heterozygosity gives hope to a wild population of inbred wolves. *PLoS ONE* **1**: e72.

Bittles, A.H., Mason, W.M., Greene, J. & Rao, N.A. 1991. Reproductive behavior and health in consanguineous marriages. *Science* **252**: 789–794.

Cassinello, J., Gomendio, M. & Roldan, E.R.S. 2001. Relationship between coefficient of inbreeding and parasite burden in endangered gazelles. *Conserv. Biol.* **15**: 1171–1174.

Charbit, A., Newton, S.M., Klebba, P.E., Clement, J.M., Fayolle, C., Lo-Man, R., Leclerc, C. & Hofnung, M. 1997. Expression and immune response to foreign epitopes in bacteria. Perspectives for live vaccine development. *Behring Inst. Mitt.* **98**: 135–142.

Charlesworth, B. & Charlesworth, D. 1999. The genetic basis of inbreeding depression. *Genet. Res.* **74**: 329–340.

Coltman, D.W., Pilkington, J.G., Smith, J.A. & Pemberton, J.M. 1999. Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* **53**: 1259–1267.

Crnokrak, P. & Roff, D.A. 1999. Inbreeding depression in the wild. *Heredity* **83**(Pt 3): 260–270.

Frankham, R. 2005. Stress and adaptation in conservation genetics. *J. Evol. Biol.* **18**: 750–755.

Giese, A.R. & Hedrick, P.W. 2003. Genetic variation and resistance to a bacterial infection in the endangered Gila topminnow. *Anim. Conserv.* **6**: 369–377.

Gubbay, J., Vivian, N., Economou, A., Jackson, D., Goodfellow, P. & Lovell-Badge, R. 1992. Inverted repeat structure of the Sry locus in mice. *Proc. Natl Acad. Sci. U.S.A.* **89**: 7953–7957.

Hawley, D.M., Sydenstricker, K.V., Kollias, G.V. & Dhondt, A.A. 2005. Genetic diversity predicts pathogen resistance and cell-mediated immunocompetence in house finches. *Biol. Lett.* **1**: 326–329.

Hedrick, P.W., Kim, T.J. & Parker, K.M. 2001. Parasite resistance and genetic variation in the endangered Gila topminnow. *Anim. Conserv.* **4**: 103–109.

Hormaeche, C.E., Fahrenkrog, M.C., Pettifor, R.A. & Brock, J. 1981. Acquired immunity to *Salmonella typhimurium* and delayed (footpad) hypersensitivity in BALB/c mice. *Immunology* **43**: 547–554.

Hormaeche, C.E., Harrington, K.A. & Joysey, H.S. 1985. Natural resistance to salmonellae in mice: control by genes within the major histocompatibility complex. *J. Infect. Dis.* **152**: 1050–1056.

Ilmonen, P., Taarna, T. & Hasselquist, D. 2000. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proc. R. Soc. Lond. B Biol. Sci.* **267**: 665–670.

Ilmonen, P., Penn, D.J., Damjanovich, K., Morrison, L., Ghotbi, L. & Potts, W.K. 2007. Major histocompatibility complex heterozygosity reduces fitness in experimentally infected mice. *Genetics* **176**: 2501–2508.

Joron, M. & Brakefield, P.M. 2003. Captivity masks inbreeding effects on male mating success in butterflies. *Nature* **424**: 191–194.

Keller, L.F. & Waller, D.M. 2002. Inbreeding effects in wild populations. *Trends Ecol. Evol.* **17**: 230–241.

Keller, L.F., Arcese, P., Smith, J.N., Hochachka, W.M. & Stearns, S.C. 1994. Selection against inbred song sparrows during a natural population bottleneck. *Nature* **372**: 356–357.

Keller, L.F., Grant, P.R., Grant, B.R. & Petren, K. 2002. Environmental conditions affect the magnitude of inbreeding depression in survival of Darwin's finches. *Evolution Int. J. Org. Evolution* **56**: 1229–1239.

Killick, S.C., Carlsson, A.M., West, S.A. & Little, T.J. 2006. Testing the pluralist approach to sex: the influence of environment on synergistic interactions between mutation load and parasitism in *Daphnia magna*. *J. Evol. Biol.* **19**: 1603–1611.

Kishony, R. & Leibler, S. 2003. Environmental stresses can alleviate the average deleterious effect of mutations. *J. Biol.* **2**: 14.

Lively, C.M. & Howard, R.S. 1994. Selection by parasites for clonal diversity and mixed mating. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **346**: 271–280.

Marshall, T.C., Coltman, D.W., Pemberton, J.M., Slate, J., Spalton, J.A., Guinness, F.E., Smith, J.A., Pilkington, J.G. & Clutton-Brock, T.H. 2002. Estimating the prevalence of inbreeding from incomplete pedigrees. *Proc. R. Soc. Lond. B Biol. Sci.* **269**: 1533–1539.

McClelland, E.E., Penn, D.J. & Potts, W.K. 2003. Major histocompatibility complex heterozygote superiority during coinfection. *Infect. Immun.* **71**: 2079–2086.

Meagher, S., Penn, D.J. & Potts, W.K. 2000. Male-male competition magnifies inbreeding depression in wild house mice. *Proc. Natl Acad. Sci. U.S.A.* **97**: 3324–3329.

Norušis, M.J. 1993. *SPSS Advanced Statistics User's Guide*. SPSS, Chicago.

O'Brien, S.J. & Evermann, J.F. 1988. Interactive influence of infectious disease and genetic diversity in natural populations. *Trends Ecol. Evol.* **3**: 254–259.

Pearman, P.B. & Garner, T.W.J. 2005. Susceptibility of Italian agile frog populations to an emerging strain of Ranavirus parallels population genetic diversity. *Ecol. Lett.* **8**: 401–408.

Peck, J.R. & Waxman, D. 2000. Mutation and sex in a competitive world. *Nature* **406**: 399–404.

Pemberton, J. 2004. Measuring inbreeding depression in the wild: the old ways are the best. *Trends Ecol. Evol.* **19**: 613–615.

Penn, D.J. 2002. The scent of genetic compatibility: sexual selection and the major histocompatibility complex. *Ethology* **108**: 1–21.

Penn, D.J., Damjanovich, K. & Potts, W.K. 2002. MHC heterozygosity confers a selective advantage against multiple-strain infections. *Proc. Natl Acad. Sci. U.S.A.* **99**: 11260–11264.

Ravindran, R. & McSorley, S.J. 2005. Tracking the dynamics of T-cell activation in response to Salmonella infection. *Immunology* **114**: 450–458.

Reid, J.M., Arcese, P. & Keller, L.F. 2003. Inbreeding depresses immune response in song sparrows (*Melospiza melodia*): direct and inter-generational effects. *Proc. R. Soc. Lond. B Biol. Sci.* **270**: 2151–2157.

Reid, J.M., Arcese, P., Keller, L.F., Elliott, K.H., Sampson, L. & Hasselquist, D. 2007. Inbreeding effects on immune response in free-living song sparrows (*Melospiza melodia*). *Proc. R. Soc. Lond. B Biol. Sci.* **274**: 697–706.

Rice, W.R. & Gaines, S.D. 1994. 'Heads I win, tails you lose': testing directional alternative hypotheses in ecological and evolutionary research. *Trends Ecol. Evol.* **9**: 235–237.

Ross-Gillespie, A., O'Riain, M.J. & Keller, L.F. 2007. Viral epizootic reveals inbreeding depression in a habitually inbreeding mammal. *Evolution Int. J. Org. Evolution* **61**: 2268–2273.

Roy, M.F. & Malo, D. 2002. Genetic regulation of host responses to *Salmonella* infection in mice. *Genes Immun.* **3**: 381–393.

Santos, R.L., Zhang, S., Tsolis, R.M., Kingsley, R.A., Adams, L.G. & Baumler, A.J. 2001. Animal models of *Salmonella* infections: enteritis versus typhoid fever. *Microbes Infect.* **3**: 1335–1344.

Sheldon, B.C. & Verhulst, S. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**: 317–321.

Slate, J., David, P., Dodds, K.G., Veenvliet, B.A., Glass, B.C., Broad, T.E. & McEwan, J.C. 2004. Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity* **93**: 255–265.

Spielman, D., Brook, B.W., Briscoe, D.A. & Frankham, R. 2004. Does inbreeding and loss of genetic diversity decrease disease resistance? *Conserv. Genet.* **5**: 439–448.

Sukupolvi, S., Edelstein, A., Rhen, M., Normark, S.J. & Pfeifer, J.D. 1997. Development of a murine model of chronic *Salmonella* infection. *Infect. Immun.* **65**: 838–842.

Valsecchi, E., Amos, W., Raga, J.A., Podesta, M. & Sherwin, W. 2004. The effects of inbreeding on mortality during a morbillivirus outbreak in the Mediterranean striped dolphin (*Stenella coeruleoalba*). *Anim. Conserv.* **7**: 139–146.

Whiteman, N.K., Matson, K.D., Bollmer, J.L. & Parker, P.G. 2006. Disease ecology in the Galapagos Hawk (*Buteo galapagoensis*): host genetic diversity, parasite load and natural antibodies. *Proc. R. Soc. Lond. B Biol. Sci.* **273**: 797–804.

Xu, H.R. & Hsu, H.S. 1992. Dissemination and proliferation of *Salmonella typhimurium* in genetically resistant and susceptible mice. *J. Med. Microbiol.* **36**: 377–381.

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