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Intergenerational effects of inbreeding in *Nicrophorus vespilloides*: offspring suffer fitness costs when either they or their parents are inbred

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Abstract

Inbreeding depression is the reduction in fitness caused by mating between related individuals. Inbreeding is expected to cause a reduction in offspring fitness when the offspring themselves are inbred, but outbred individuals may also suffer a reduction in fitness when they depend on care from inbred parents. At present, little is known about the significance of such intergenerational effects of inbreeding. Here, we report two experiments on the burying beetle *Nicrophorus vespilloides*, an insect with elaborate parental care, in which we investigated inbreeding depression in offspring when either the offspring themselves or their parents were inbred. We found substantial inbreeding depression when offspring were inbred, including reductions in hatching success of inbred eggs and survival of inbred offspring. We also found substantial inbreeding depression when parents were inbred, including reductions in hatching success of eggs produced by inbred parents and survival of outbred offspring that received care from inbred parents. Our results suggest that intergenerational effects of inbreeding can have substantial fitness costs to offspring, and that future studies need to incorporate such costs to obtain accurate estimates of inbreeding depression.

Introduction

Inbreeding depression is the reduction in fitness of individuals resulting from matings between related mates, and is caused mainly by the expression of deleterious recessive alleles due to an increase in homozygosity (Falconer & Mackay, 1996; Lynch & Walsh, 1998; Charlesworth & Willis, 2009). The study of inbreeding depression is an important issue in evolutionary and conservation biology because it leads to selective pressures that shape mating systems and behaviours (Charlesworth & Willis, 2009), and because it poses a conservation concern to many wild populations (Ralls *et al.*, 1979; Crnokrak & Roff, 1999; Keller & Waller, 2002; Armbruster & Reed, 2005). There is ample evidence for significant costs of inbreeding from studies on a wide range of taxa, including birds, mammals, insects and plants. These studies show that the effects of inbreeding may have detrimental effects on a wide range of juvenile and adult traits, including hatching success, offspring survival and the number of eggs laid by adult females (Noordwijk & Scharloo, 1981; Keller, 1998; Slate *et al.*, 2000; Szulkin *et al.*, 2007). Due to these wide-ranging effects, accurate estimates of inbreeding need to be based on fitness consequences measured across the entire life cycle of an individual (Charlesworth & Charlesworth, 1987). The finding that inbreeding affects adult traits suggests that inbreeding in parents also could affect the fitness of outbred offspring. If so, accurate estimates of inbreeding depression need to account for intergenerational effects of inbreeding in parents on offspring fitness. Unfortunately, there has so far been little attention to the potential significance of intergenerational effects of inbreeding in species where offspring fitness is determined or influenced by their parents.

Current studies on intergenerational effects of inbreeding have focused mainly on plants, and have shown that the inbreeding coefficients of parents

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influence fitness traits of progeny (del Castillo, 1998; Riginos *et al.*, 2007). There are far fewer studies of intergenerational effects in inbreeding in animals, where the potential for such effects may be particularly important because many species have elaborate parental care (Clutton-Brock, 1991; Royle *et al.*, 2012). For example, in oldfield mice, inbreeding in parents led to a reduction in male (but not female) parental care, although inbreeding in parents had no effect on offspring fitness (Margulis, 1998). A recent study on red deer, *Cervus elaphus*, which compared the effects of inbreeding in offspring traits when either the offspring themselves or the female parents were inbred, found that inbreeding in offspring was associated with a reduction in birthweight and first-year survival, while the inbreeding in female parents had no effects on the same offspring traits. Thus, this study suggests that intergenerational effects of inbreeding are weak compared with the direct effects of inbreeding (Walling *et al.*, 2011). On the other hand, there is support for intergenerational effects of inbreeding from a study on song sparrows, which found that offspring of inbred female parents had a lower immune response than offspring of outbred parents (Reid *et al.*, 2003). Furthermore, in great tits, *Parus major*, offspring of inbred parents have lower fledging success, recruitment into the breeding population and lower reproductive success than offspring of outbred parents (Szulkin *et al.*, 2007). These findings suggest that there might be intergenerational effects of inbreeding resulting from detrimental effects of inbreeding on the amount of parental care provided to offspring.

To improve our understanding of the potential significance of intergenerational effects of inbreeding, it is now important to investigate inbreeding depression in offspring traits both when the offspring themselves are inbred and when the parents are inbred. Here, we report the results of experiments on the burying beetle *Nicrophorus vespilloides*; an insect with highly elaborate parental care, whereby one or both parents defend the carcass and the brood against conspecific competitors and microbial decomposers, and provide predigested carrion to the begging larvae (Eggert *et al.*, 1998; Smiseth *et al.*, 2003; Rozen *et al.*, 2008; Arce *et al.*, 2012). Parental care has a strong effect on offspring fitness by enhancing offspring survival and growth and speeding up the offspring's rate of development (Eggert *et al.*, 1998; Smiseth *et al.*, 2003). We conducted two controlled experiments where the inbreeding status of either the offspring or the parents were manipulated through experimental breeding, allowing us to estimate the effects of inbreeding on offspring traits when either the offspring themselves were inbred or when their parents were inbred. We monitored effects of inbreeding in offspring and parents on offspring traits during different stages of the life cycle, including hatching success of eggs, survival and growth of larvae during the period when they received care from their parents, and after independence. In the experiment on intergenerational effects of inbreeding, we manipulated inbreeding in male and female parents to test for potential sex differences in the effects of inbreeding in parents on offspring fitness.

Materials and methods

Study species

Like all members of the genus *Nicrophorus*, *N. vespilloides* breeds on carcasses of small vertebrates, which are buried underground, rolled into a ball and then prepared and maintained by both parents (Eggert & Muller, 1997; Scott, 1998). Females lay eggs in the soil around the carcass over a mean period of 30 h and the eggs hatch about 60 h after laying (Smiseth *et al.*, 2006). Once the larvae hatch, they crawl to the carcass, where they obtain food by self-feeding directly from the carcass and by begging for regurgitated food from the parents (Smiseth *et al.*, 2003). Parents provide elaborate care that includes provisioning of food to the larvae, protecting and defending the brood from predators and competitors and applying antimicrobials to defend the brood from microbes (Eggert *et al.*, 1998; Smiseth *et al.*, 2003; Rozen *et al.*, 2008; Arce *et al.*, 2012). Females are more involved in direct care of the larvae than males, and also provide care for a longer period of time (Smiseth & Moore, 2002; Eggert *et al.*, 2008). The larvae disperse from the carcass about 5–6 days after hatching, pupate about 10–12 days after dispersal and finally eclose as adults about 12 days after pupation.

General methodology

We used beetles from a large outbred laboratory population maintained at The University of Edinburgh. Inbreeding in the stock population was avoided by maintaining a large stock population and ensuring that breeding occurred among unrelated or distantly related individuals, defined as when two individuals did not share a common ancestor two generations or more back in time. The population comprised of fifth-generation beetles from lines originally collected at Corstorphine Hill, Edinburgh, and 16th-generation beetles from lines originally collected at Kennel Vale, Cornwall. Thus, we are confident that the stock population would not have been subject to any significant forms of inbreeding, purging or selection that would have biased the estimates of inbreeding depression in our experiments.

The beetles were maintained at 20 °C under constant light conditions in transparent plastic containers (12 cm × 8 cm and 2-cm high). Nonbreeding adults were fed organic beef twice a week. We only used virgin beetles for the experiments, and each individual was only mated once. Experimental pairs were bred in

transparent plastic boxes (17 cm × 12 cm and 6-cm high) by providing them with 1 cm of moist soil and a previously frozen mouse carcass with a mass of 20 ± 5g. The mice were supplied from Livefoods Direct Ltd, Sheffield, UK. The containers were checked for eggs on days 1–7 after pairing by noting the number of laid eggs and the number of unhatched eggs (i.e. decomposing eggs) that were visible through the base and sides of the transparent container. We used this method as it reliably estimates the number of eggs laid while at the same time reducing the risks of damaging eggs through handling, which might reduce hatching success (Monteith *et al.*, 2012). The containers were checked for dispersing larvae on days 10–12 after pairing by noting whether the larvae had left the carcass. Once the larvae had dispersed, we recorded the date, the number of larvae that were alive and the mass of the brood. The dispersed larvae were then placed in individual containers and left to pupate and eclose. The individual containers were again checked for eclosing adults 27–34 days after pairing.

Experimental design

Experiment 1: Inbreeding in offspring

To examine the effects of inbreeding when the offspring themselves were inbred, we used a block design similar to the one used by Fox *et al.* (2008). Each block comprised of eight beetles, four male and four female, derived from two unrelated families of the stock population (i.e. families that did not share a common grandparent). Each block produced two broods of inbred offspring by mating a female from each of the two families with her brother (i.e. full-sibling mating), and two broods of outbred offspring by mating a female from each of the two families with the unrelated male from the other family (Fig. 1a). Such a block design provides a powerful means for attributing variation in offspring trait values to inbreeding among offspring in a way that controls for potential confounding effects due to genetic differences among the different families used in the experiment (Fox *et al.*, 2008). Controlling for genetic differences among families is highly advantageous in studies of inbreeding depression because families share genes that may affect the mean values of the traits that are potentially affected by inbreeding (Fox, 2005). Thus, the use of a blocked design ensures that the same set of alleles contributes to both inbred and outbred broods, and potential effects due to genetic differences among families can therefore be excluded (Fox, 2005). This design also ensures that all parents used in the experiment are outbred, such that any effect due to inbreeding would be caused by inbreeding among the offspring.

We set up 15 blocks in this experiment, consisting of 10 families of 16th generation and 13 families of fifth generation beetles, yielding a total of 30 inbred broods and 30 outbred broods. Three inbred matings (in three separate blocks) were excluded because one of the parents died prior to egg laying. We removed all males from the breeding container 7 days after pairing because this experiment was designed to test for effects of inbreeding in the offspring and their subsequent fitness. Previous studies show that that the extent to which males stay with the brood to provide care or desert is highly variable in this species (Smiseth & Moore, 2002), and that the removal of the male has no effect on offspring fitness (Smiseth *et al.*, 2005). Thus, the male was removed to control for potential variation in male behaviour.

We measured effects of inbreeding in the offspring on the following traits: *(i) Number of eggs* recorded as

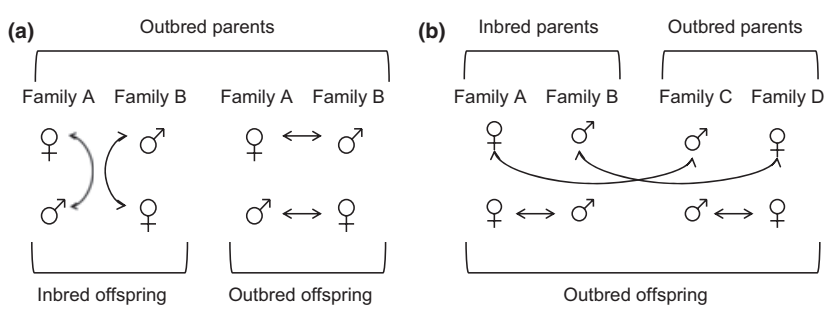

Fig. 1 The two block designs used to test for the effects of inbreeding in offspring and parents in *Nicrophorus vespilloides*. (a) In Experiment 1, each block comprised four males and four females derived from two unrelated families of the stock population. Each block produced two broods of inbred offspring by mating a female from each of the two families with her brother (i.e. full-sibling mating), and two broods of outbred offspring by mating a female from each of the two families with the unrelated male from the other family. (b) In Experiment 2, each block comprised four males (two outbred and two inbred) and four females (two outbred and two inbred) derived from four unrelated families. Mating within each block ensured that all offspring were outbred, and that these offspring were produced and cared for by one of the following four treatment combinations: an inbred female mated to an inbred male; an inbred female mated to an outbred male; an outbred female mated to an inbred male; and an outbred female mated to an outbred male.

the number of eggs laid for each brood; *(ii) Hatching success* recorded by counting the number of unhatched eggs (i.e. decomposing eggs) visible through the base and sides of the container, subtracting this number from the total number of eggs laid to give the number of successfully hatching eggs, and then calculated relative to the total number of eggs laid; *(iii) Time to dispersal* recorded as the number of days from when the parents were paired up to when larvae dispersed from the carcass; *(iv) Survival to dispersal* recorded by counting the number of larvae dispersing from the carcass, and then calculated relative to the total number of eggs laid; *(v) Size at dispersal* recorded by weighing the whole brood to the nearest 0.1 mg, and dividing the brood mass by the number of larvae in the brood to yield a mean body mass per larva in the brood; *(vi) Overall offspring survival* recorded as the number of larvae surviving to dispersal from the carcass, and then calculated relative to the number of eggs laid to for a given brood; *(vii) Survival to eclosion* recorded as the number of larvae successfully eclosing relative to the number of larvae dispersing from the carcass for a given brood. For measures of survival (hatching success, survival to dispersal, overall offspring survival and survival to eclosion), we calculated mean values for each treatment based on the proportion of surviving offspring for each brood, while we used the number of surviving and nonsurviving offspring as binomial variables in our statistical models (see below).

Experiment 2: Inbreeding in parents

To test for possible intergenerational effects of inbreeding in parents on the offspring's fitness, we used a novel block design based on the inbred and outbred individuals generated from Experiment 1. The design comprised of eight beetles derived from four unrelated families (i.e. families that did not share a common grandparent). Each block comprised two females from two different families (i.e. two sets of full-sibling sisters), one set of which was inbred and one of which was outbred, and two males from two additional families (i.e. two sets of full-sibling brothers), one of which was inbred and one of which was outbred (Fig. 1b). These beetles were mated to produce the following four treatments: (i) an inbred female mated to an inbred male; (ii) an inbred female mated to an outbred male; (iii) an outbred female mated to an inbred male; (iv) an outbred female mated to an outbred male (Fig. 1b). Thus, this set-up produced a two-by-two factorial design where parents of either sex were either inbred or outbred. The design allowed us to compare the effects of inbreeding in male and female parents on the offspring's fitness, while at the same time ensuring that all offspring were outbred (offspring were always outbred because all parents, inbred or outbred, were mated to an unrelated male or female; Falconer & Mackay, 1996; Frankham *et al.*, 2002). Thus, any effects of inbreeding on offspring fitness in this experiment would be due to inbreeding among parents. Furthermore, this factorial design also allowed us to detect any potential differences in the effects of inbreeding in females and males. Such effects might be found in this species because female parents are more involved in direct care for the larvae than males (Eggert & Muller, 1997; Smiseth & Moore, 2002). Because we were interested in testing for such a sex difference, we did not remove the male from the breeding container in this experiment, thus allowing both parents to provide parental care. We created 11 blocks, yielding a total of 11 broods of each treatment with the exception of the treatment comprising an inbred female and an outbred male, which was reduced to 10 broods because one adult died before mating. To determine whether inbreeding in parents had an effect on offspring traits, we estimated the same offspring and adult traits as in Experiment 1 (with the exception of time to dispersal and survival to eclosion, which were not recorded due to time limitations), following the same procedures as described above for Experiment 1.

Statistical analysis

All data was analysed using R version 2.15. We used linear mixed-effects models for traits that had a normal error distribution (the number of eggs laid and size at dispersal in both experiments), and we used generalized linear mixed models for traits with a Poisson error structure (time to dispersal), and for traits with a binomial error structure (hatching success, survival to dispersal, overall offspring survival and survival to eclosion). All analyses were based on family means (Fox *et al.*, 2008). In Experiment 1, the experimental treatment (i.e. whether offspring were outbred or inbred) was assigned as a fixed effect, whereas in Experiment 2, the experimental treatments (i.e. whether males or females were outbred or inbred) were added as two fixed effects (i.e. one for each sex). In all models, block was assigned as a random factor. In the models of three traits (size at dispersal, survival to dispersal and survival to eclosion), we included additional covariates in the initial statistical models because these covariates were predicted to have an effect on the trait in question. First, in the model of size at dispersal, we included brood size as an additional fixed effect because brood size is likely to determine the amount of resources available to each offspring and therefore the size of offspring (Smiseth *et al.*, 2007). Second, in the model of survival to dispersal, we included mouse mass as an additional fixed effect because survival may be influenced by the amount of resources available to the brood. Third, in the model of survival to eclosion, we added size at dispersal as an additional fixed effect because the size of the larvae may have a possible effect on the larvae's ability to eclose successfully. We based

decisions as to whether to include these covariates in the final model based on AIC model-selection criteria, according to which a covariate should be added if it significantly improves the fit of the model. In the analysis on survival to dispersal, which aimed to establish the effect of inbreeding on larval survival from hatching to the time of dispersal, we excluded two broods from Experiment 2 where no eggs hatched. In the analyses on inbreeding in parents (Experiment 2), we always first tested for a main effect of inbreeding depending on the sex of the parent before we generated separate models for the interaction between the inbreeding status of males and females. For all traits, we calculated inbreeding depression as a proportional change in mean fitness of inbred (w_i) and outbred (w_o) individuals using the equation as $\delta = w_o - w_i/w_o$ (Hedrick & Kalinowski, 2000). We note that the tables summarize the effect of inbreeding on each trait while controlling for the effects of block and other additional fixed effects listed above, while the figures show means and standard errors for each trait based on the raw data. Whenever there are apparent discrepancies between tables and figures, we always base inferences about the effects of inbreeding on the statistical analyses reported in the tables.

Results

Experiment 1: Inbreeding in offspring

We first examined the additional covariates in our initial models to determine whether to include these in the final models for the effects of inbreeding on size at dispersal, survival to dispersal and survival to eclosion. Although larger broods produced larvae that were significantly smaller at dispersal ($t_{40} = -5.491$, $P < 0.001$), AIC model selection suggested that brood size should not be included in the final model for size at dispersal. Given that these results indicate some ambiguity concerning the effect of brood size, we conducted two separate tests in which brood size was either included or excluded. These tests confirmed that the exclusion or inclusion of brood size had no effect on estimates of inbreeding depression, and we therefore report effects from a model in which brood size was excluded. Mouse mass had no significant effect on larval survival to dispersal (Z = 0.3, $P = 0.76$), and was not included in the final model on survival to dispersal based on AIC model selection. Finally, larvae that were larger at dispersal were more likely to survive to eclosion (Z = −2.826, $P = 0.005$), and size at dispersal was included in the final model for survival to eclosion based on AIC model selection.

There was no evidence that females mated to a full-sib male laid a different number of eggs than females mated to an unrelated male (Table 1; Fig. 2). Thus, there was no evidence suggesting that females adjusted their reproductive investment in response to the risk of inbreeding in their offspring.

Table 1 Direct effects of inbreeding on offspring traits in *Nicrophorus vespilloides*. For each trait, there is information on parameter estimates (Par) with standard error (SE), test statistics (*t*-value for number of eggs and size at dispersal, and Z-value for time to dispersal, hatching success, survival to dispersal, overall offspring survival and survival to eclosion) and *P* values based on comparisons between outbred and inbred offspring. All data are provided by linear mixed-effects models of egg number and size at dispersal and generalized linear mixed models for time to dispersal (fitted with Poisson error structure), hatching success, survival to dispersal, overall offspring survival and survival to eclosion (fitted with a binomial error structure), in which experimental block is assigned as a random factor in all cases.

Trait	Par	SE	t/Z value	*P* value
Number of eggs	2.83	2.1	1.35	0.184
Hatching success (%)	−0.821	0.22	−3.83	0.0001
Time to dispersal (days)	0.014	0.081	0.18	0.855
Survival to dispersal (%)	−0.43	0.126	−3.42	0.0006
Size at dispersal (g)	−0.004	0.008	−0.462	0.647
Overall offspring survival (%)	−0.535	0.116	−4.611	< 0.0001
Survival to eclosion (%)	−0.733	0.18	−4.12	< 0.0001

Inbred offspring suffered substantial inbreeding depression in a wide range of traits (Table 1, Fig. 2), including hatching success of eggs (reduced by 3.5%), survival to dispersal (reduced by 8.5%), overall offspring survival from hatching to dispersal (reduced by 11.2%) and survival to eclosion as adults (reduced by 8.7%; Table 3). Inbred offspring suffered a significant reduction in survival both during the period when larvae depend on parental care (i.e. from egg laying to dispersal from the carcass) and after independence (from dispersal to eclosion; Table 1). In contrast, there was no evidence of a difference between inbred and outbred offspring with respect to the time to dispersal and size at dispersal (Table 1), suggesting that inbreeding had no detectable effect on the duration of life-history stages or the body size of surviving offspring.

Experiment 2: Inbreeding in parents

At first, we examined the additional covariates in our initial models to determine whether these should be included in the final models on the effects of inbreeding on the size at dispersal and survival to dispersal. As in Experiment 1, larger broods produced significantly smaller larvae at dispersal ($t_{17} = -3.97$=, $P = 0.001$), but in this case, brood size was included in the final model based on AIC model selection. Mouse mass had no significant effect on the survival to dispersal (Z = −1.338, $P = 0.181$) and was not included in the final model of survival to dispersal based on AIC model selection.

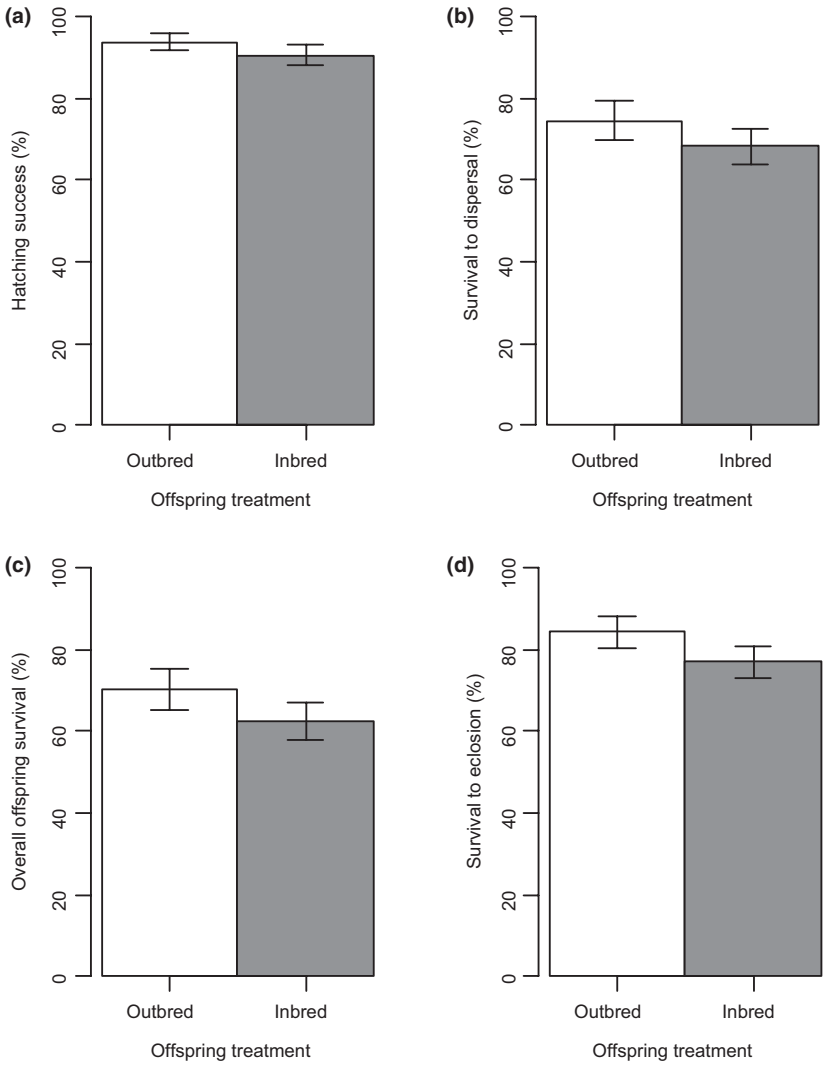

Fig. 2 Direct effects of inbreeding on offspring traits in *Nicrophorus vespilloides*. Comparisons of hatching success (a), survival to dispersal (b), overall offspring survival (c) and survival to eclosion (d) when offspring were outbred (white bars) or inbred (grey bars) (mean ± SE).

Outbred offspring suffered significant costs due to inbreeding in their male and/or female parents, causing a reduction in hatching success of eggs, survival to dispersal and overall offspring survival (Table 2). Our results indicate that the intergenerational effects of inbreeding in parents on offspring traits were often complex, involving in some cases, sex differences in the effects of inbreeding, and in other cases, interaction effects due to inbreeding in both sexes (Table 3). There was no evidence that inbred and outbred females laid a different number of eggs as indicated by the nonsignificant main effect of inbreeding in females. However, females laid significantly more eggs when mated to an inbred male as compared with when mated to one that was outbred (Table 2). The main effect of inbreeding in males was associated with a significant effect of the interaction between inbreeding in males and females (Table 3; Fig. 3a). Inbred females increased the number of eggs they laid by 70% when they were mated to an inbred male as opposed to when they were mated to an outbred male. In contrast, outbred females laid a similar number of eggs regardless of whether they were mated to an inbred or an outbred male.

There was no evidence that inbreeding in either male or female parents affected the hatching success of outbred eggs (Table 2). However, hatching success was significantly influenced by the interaction between inbreeding in male and female parents. Hatching success of eggs sired by inbred males was reduced by 12% when he was mated to an inbred female as opposed to when he was mated to an outbred female. In contrast, hatching success of eggs sired by outbred males appeared similar, regardless of whether they were mated to an inbred or an outbred female (Table 3). Inbreeding in female parents caused a significant reduc-

Table 2 Intergenerational effects of inbreeding in parents on the traits of outbred offspring in *Nicrophorus vespilloides*. For each trait, there is information on parameter estimates (Par) with standard error (SE), test statistics (*t*-value for number of eggs and size at dispersal, and Z-value for hatching success, survival to dispersal and overall offspring survival) and *P* values based on comparisons of outbred offspring produced by and receiving care from males that were outbred or inbred, females that were outbred or inbred and the effect of the interaction between inbreeding in males and females. All data are provided by linear mixed-effects models of egg number and size at dispersal, and generalized linear mixed effects models of hatching success, survival to dispersal and overall offspring survival (fitted with a binomial error structure), in which experimental block is assigned as a random factor in all cases.

	Male				Female				Interaction			
Trait	Par	SE	t/Z value	*P* value	Par	SE	t/Z value	*P* value	Par	SE	t/Z value	*P* value
Number of eggs	7.09	2.66	2.66	0.01	−0.64	2.66	−0.239	0.812	11.93	5.04	2.37	0.0248
Hatching success (%)	−0.304	0.16	−1.881	0.06	−0.17	0.16	−1.069	0.285	−1.51	0.34	−4.431	< 0.0001
Survival to dispersal (%)	−0.097	0.16	−0.61	0.541	−0.407	0.15	−2.67	0.0076	1.0	0.32	3.14	0.0017
Size at dispersal (g)	0.007	0.01	0.822	0.422	0.002	0.01	0.268	0.792	0.011	0.02	0.676	0.508
Overall offspring survival (%)	−0.219	0.13	−1.639	0.101	−0.311	0.13	−2.35	0.0187	0.088	0.27	0.328	0.743

Table 3 Inbreeding depression (δ) was calculated for all traits as $\delta = w_o - w_i / w_o$ such that trait values associated with an inbred individual is compared to that of an outbred individual. Offspring δ was estimated for the direct effects of inbreeding when the offspring themselves were inbred using data from Experiment 1. Male δ, female δ and both parents δ were estimated as intergenerational effects in outbred offspring due to effects of inbreeding in male parents, female parents and both parents using data from Experiment 2. Male δ was calculated separately for males mated to inbred and outbred females; that is, the column Male parent δ (inbred female) reports the reduction in offspring fitness due to inbreeding in males when males were mated to an inbred female. Likewise, female δ was calculated separately when females were mated to inbred and outbred males. Both parents δ was calculated by comparing trait values of the treatment in which both parents were inbred with the trait values when both the parents were outbred.

		Male parent δ		Female parent δ		
Trait	Offspring δ	Female outbred	Female inbred	Male outbred	Male inbred	Both parents δ
Number of eggs	−0.092	−0.049	−0.702	0.264	−0.193	−0.252
Hatching success (%)	0.035	−0.067	0.022	0.036	0.117	0.057
Time to dispersal (days)	−0.015	–	–	–	–	–
Survival to dispersal (%)	0.085	0.236	−0.218	0.386	0.022	0.253
Size at dispersal (g)	0.018	−0.035	−0.084	0.035	−0.011	−0.047
Overall offspring survival (%)	0.112	0.138	−0.3	0.4	0.095	0.221
Survival to eclosion (%)	0.087	–	–	–	–	–

tion in larval survival to dispersal, while there was no evidence that inbreeding in male parents had an effect on larval survival to dispersal (Table 2, Table 3). The main effect of inbreeding in females was associated with a significant effect of the interaction between inbreeding in males and females (Table 3). Inbreeding in females reduced larval survival to dispersal by 38% when females were mated to an outbred male, whereas there was no effect of inbreeding in females when females were mated to an inbred male (Table 3). Inbreeding in female parents reduced overall offspring survival by 40%, while inbreeding in male parents had no such effect (Fig 3; Table 3). However, there was no significant effect of the interaction between inbreeding in male and female parents on overall offspring survival (Table 2). There was no evidence that inbreeding in males and females, or the interaction between the two, caused a reduction in the size of larvae at dispersal (Table 2).

Discussion

Here, we report evidence from an experimental study on the burying beetle *N. vespilloides* showing that inbred offspring suffer significant fitness costs and that outbred offspring suffer significant fitness costs when their parents are inbred. Our results provide a valuable contribution to the understanding of inbreeding depression by demonstrating that offspring suffer the costs of inbreeding not only when they themselves are inbred but also when they are produced by and receive care from parents that are inbred. We argue that such intergenerational effects of inbreeding can be important in many species, particularly in those where parents provide elaborate care for their offspring as in birds and mammals, and we suggest that further work on inbreeding in these species need to consider the potential implications of intergenerational effects of inbreeding. On the basis of our results, we propose that the

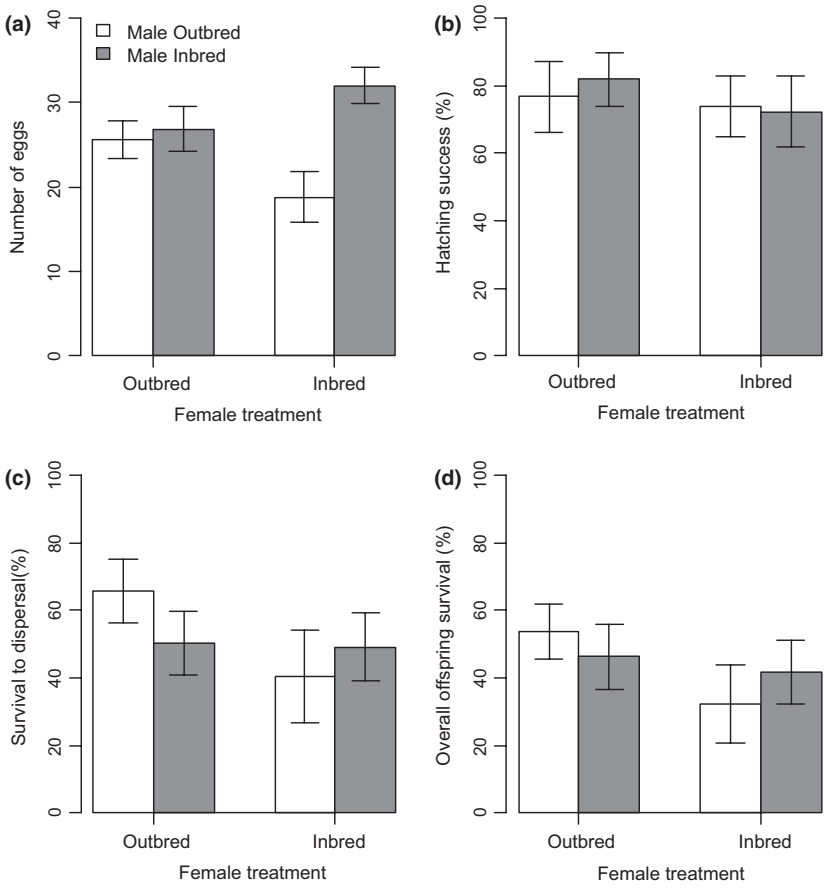

Fig. 3 Intergenerational effects of inbreeding in parents on the traits of outbred offspring in *Nicrophorus vespilloides*. Comparison of the number of eggs laid (a), hatching success (b), survival to dispersal (c) and overall offspring survival (d) when outbred offspring were produced by and received care from outbred or inbred female parents (female treatment) and outbred (white bars) or inbred (grey bars) male parents (mean ± SE).

costs of inbreeding might extend to individuals other than those that are inbred, and that such indirect effects of inbreeding may be important in a wide range of social contexts in which the fitness of an outbred individual depends on the behaviour of individuals that are inbred. Below, we provide a more detailed discussion of the major implications of our results.

The main aim of this study was to examine both direct effects of inbreeding when the offspring themselves were inbred, and intergenerational effects of inbreeding when outbred offspring were produced by and received care from inbred parents. Direct effects of inbreeding were associated with a reduction in overall offspring survival by 11%, whereas intergenerational effects of inbreeding were associated with a reduction in overall offspring survival by 40% when females were inbred (and males were outbred; Table 3). In Experiment 1, males were removed from the brood to remove potential effects due to variation in male desertion, while males were left with the brood in Experiment 2 to provide estimates on the relative importance of inbreeding in males and females. Because of this difference in the design between the two experiments, caution is required when comparing the estimates of inbreeding depression when the offspring themselves and their parents were inbred. For example, leaving males with the brood in Experiment 2 may have influenced offspring fitness either because males would have had the opportunity to provide additional care or because deserting males could have cannibalized some larvae (Scott & Gladstein, 1993). Nevertheless, our results suggest that intergenerational effects of inbreeding may be comparable in strength, or even stronger than the direct effects of inbreeding in *N. vespilloides*. This finding contrasts with a recent study on red deer, suggesting that the direct effects of inbreeding are much stronger than any intergenerational effects of inbreeding (Walling *et al.*, 2011).

The finding that inbreeding had severe fitness costs to offspring both when they themselves were inbred and when the parents were inbred is somewhat surprising because these effects must be based on different mechanisms. Although we did not specifically set out to investigate the mechanisms behind these effects, our results provide some suggestions for possible mechanisms based on observed reductions in survival during specific developmental stages. The observed reduction in hatching success and offspring mortality when the

offspring (eggs or larvae) were inbred is likely to reflect mechanisms taking place after fertilization, such as an increase in embryonic or larval mortality directly caused by the inheritance of rare homozygous deleterious recessive alleles. However, this mechanism cannot explain the reduction in hatching success or offspring survival when parents were inbred, because in this situation, the offspring (eggs or larvae) were outbred. Instead, the reduction in hatching success when parents were inbred must reflect mechanisms taking place prior to or during fertilization, such as reduced quality or viability of eggs and sperm. Similar results have been reported in a recent study on the seed beetle, *Stator limbatus*, in which eggs laid by inbred females were less likely to hatch than those laid by outbred females (Messina *et al.*, 2013). Finally, we note that the reduction in offspring survival from hatching to dispersal when parents were inbred is likely to reflect mechanisms taking place after hatching, such as reduced quantity or quality of care provided by inbred parents.

We found some evidence for a sex difference in the intergenerational effects of inbreeding. First, inbreeding in males was associated with an increase in the number of eggs laid by an inbred female, whereas inbreeding in females had no such effect. Although this finding is consistent with females attempting to compensate for an expected reduction in hatching success when mated to an inbred male, we believe that such a conclusion is not warranted because we have not been able to replicate this finding in later experiments based on the same design (S.N. Mattey & P.T. Smiseth, unpublished data). Second, inbreeding in females was associated with a reduction in larval survival from hatching to dispersal and overall offspring survival, whereas inbreeding in males had no such effect, a finding that we have replicated in later experiments (S.N. Mattey & P.T. Smiseth, unpublished data). These results are consistent with females being more involved in provisioning of food to larvae than males in *N. vespilloides* (Smiseth & Moore, 2002; Smiseth *et al.*, 2005). Thus, the finding that inbreeding in females may have had a stronger impact on larval survival from hatching to dispersal may simply reflect that females are more involved in the form of care that is likely to have the strongest impact on larval survival; that is, the provisioning of food to larvae (Eggert *et al.*, 1998; Smiseth *et al.*, 2003). The finding that inbreeding in males had no detectable effect on larval survival from hatching to dispersal suggests that inbreeding had no effect on how males left with the brood influenced their offspring's fitness via parental care or cannibalism.

We also found evidence for significant effects of the interaction between inbreeding in males and females with respect to the number of eggs laid, hatching success of eggs and larval survival from hatching to dispersal. These interaction effects suggest that the intergenerational effects of inbreeding in a parent of a given sex depend not only on its own inbreeding status but also on that of its partner. These interaction effects are likely to depend on different mechanisms. For example, the interaction effect on hatching success may reflect that successful fertilization depends on the quality of both sperm and eggs (both of which may be reduced by inbreeding), although further work is needed to confirm whether this is indeed the case. In contrast, the interaction effect on larval survival from hatching to dispersal occurs during the period where larvae receive care from their parents, and may potentially reflect that inbreeding alters the behavioural dynamics between male and female parents. Such effects might be expected in species with biparental care because each parent often adjusts its contribution towards care based on the amount of care provided by its partner (Houston & McNamara, 2005; Lessells & McNamara, 2012). These considerations suggest that intergenerational effects of inbreeding may alter the complex behavioural dynamics of interactions among family members, and there is now a need for further work to investigate this intriguing possibility.

Although the finding that outbred offspring suffered from reduced survival when cared for by inbred parents suggests that inbred parents provide less care than outbred parents, it is important to recognize that other mechanisms such as reductions in maternal immunity cannot be excluded (Reid *et al.*, 2007). Nevertheless, our study provides some indications that inbreeding may affect the amount of care provided by parents. First, inbreeding in parents had the strongest effect on offspring fitness during the period from hatching to dispersal. This represents the stage in the offspring's life cycle during which parents provide care for offspring (Eggert *et al.*, 1998; Smiseth *et al.*, 2003). Second, inbreeding in female parents was more detrimental for survival to dispersal than inbreeding in male parents, which is consistent with females being more engaged in direct parental care for larvae than males in this species (Smiseth & Moore, 2002). At present, surprisingly few studies have documented effects of inbreeding on parental care. A notable exception is a study on oldfield mice, which showed that inbreeding led to a reduction in the amount of care provided by males (but not by females), although in this case, inbreeding in parents had no effect on offspring survival (Margulis, 1998). Further work on a wide range of taxa is now needed to examine the potential effects of inbreeding on parental care.

The finding that intergenerational effects of inbreeding in parents can have strong and detrimental effects on the offspring's fitness has important implications for the study of inbreeding. First, if intergenerational costs of inbreeding are commonplace, ignoring such costs runs the risk of underestimating the true cost of inbreeding. This issue might be particularly problematic when attempting to estimate inbreeding depression in species with elaborate forms of parental care, such as birds and

mammals. Second, intergenerational effects of inbreeding may be part of a larger class of inbreeding effects that occur in a wide range of social contexts. These contexts include all instances where the fitness of an outbred individual depends on behavioural interactions with others and where inbreeding influences the behaviour of the individuals with which the outbred individual interacts. If so, such effects of inbreeding may be considered a form of indirect genetic effects, which occur when a trait expressed in a focal individual is influenced by genes in another individual with which the focal individual interacts (Moore *et al.*, 1997). However, it should be noted that, in the case of inbreeding, these indirect genetic effects are based on dominance effects rather than on additive genetic effects, as is the case for classical indirect genetic effects. Thus, we encourage further work on inbreeding to examine indirect effects in a wide range of social contexts, including competition, social dominance and aggressive interactions, and communal and cooperative breeding.

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