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Differential effects of offspring and maternal inbreeding on egg laying and offspring performance in the burying beetle *Nicrophorus vespilloides*

LUCY E. FORD (iD), KIRSTEN J. HENDERSON & PER T. SMISETH

Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh, UK

Abstract

We investigate the effect of offspring and maternal inbreeding on maternal and offspring traits associated with early offspring fitness in the burying beetle *Nicrophorus vespilloides*. We conducted two experiments. In the first experiment, we manipulated maternal inbreeding only (keeping offspring outbred) by generating mothers that were outbred, moderately inbred or highly inbred. Meanwhile, in the second experiment, we manipulated offspring inbreeding only (keeping females outbred) by generating offspring that were outbred, moderately inbred or highly inbred. In both experiments, we monitored subsequent effects on breeding success (number of larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, duration of larval development and average larval mass). Maternal inbreeding reduced breeding success, and this effect was mediated through lower hatching success and greater larval mortality. Furthermore, inbred mothers produced clutches where egg laying was less skewed towards the early part of laying than outbred females. This reduction in the skew in egg laying is beneficial for larval survival, suggesting that inbred females adjusted their laying patterns facultatively, thereby partially compensating for the detrimental effects of maternal inbreeding on offspring. Finally, we found evidence of a nonlinear effect of offspring inbreeding coefficient on number of larvae dispersing. Offspring inbreeding affected larval survival and larval development time but also unexpectedly affected maternal traits (clutch size and delay until laying), suggesting that females adjust clutch size and the delay until laying in response to being related to their mate.

Introduction

Inbreeding is the mating between related individuals (Wright, 1977), which often leads to a reduction in fitness referred to as inbreeding depression (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009). Inbreeding depression is due to a general loss of heterozygosity, which may reduce offspring fitness either by increasing the risk that recessive deleterious alleles are expressed (partial dominance hypothesis;

Charlesworth & Charlesworth, 1987, 1999) or by reducing the production of heterozygotes in situations where there is heterozygote advantage (overdominance hypothesis; Charlesworth & Charlesworth, 1987, 1999). Inbreeding depression has become widely recognized as a significant evolutionary force that may drive the evolution of mate choice (Blouin & Blouin, 1988) and mating systems (Charlesworth & Charlesworth, 1979; Lande & Schemske, 1985; Charlesworth *et al.*, 1990). Furthermore, inbreeding has important implications for agriculture because it can have detrimental effects on food production (Kristensen & Sørensen, 2005) and for conservation because it can increase the risk of extinction of local populations (Crnokrak & Roff, 1999; Keller & Waller, 2002). Therefore, understanding the mechanisms and consequences of inbreeding depression is a

Correspondence: Lucy E. Ford, Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Charlotte Auerbach Road, Edinburgh EH9 3FL, UK.
Tel.: 0131 651 7112; fax: 44 131 650 6564;
e-mail: L.E.Ford@sms.ed.ac.uk

vital subject area in evolutionary biology (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009).

For inbreeding depression to occur, there must be directional dominance; that is, the deleterious alleles causing inbreeding depression must be biased towards one side of the trait mean (Wright, 1977; Lynch & Walsh, 1998). When there is directional dominance, the mean value of a trait will change as a consequence of inbreeding (Lynch & Walsh, 1998). Inbreeding depression can affect traits across the entire life cycle of an organism (Charlesworth & Charlesworth, 1987). Thus, the detrimental effects of inbreeding can extend to later in life, such as when inbred individuals reproduce as adults. When this is the case, the fitness of an individual can be influenced by both its own inbreeding coefficient (offspring inbreeding) and the inbreeding coefficient of its mother (maternal inbreeding). Indeed, in some species, maternal inbreeding may have as large an influence on offspring fitness as offspring inbreeding (Mattey *et al.*, 2013). Offspring and maternal inbreeding may have differential effects on traits depending on whether they are under offspring or maternal control. Traits that are expressed in the offspring, such as offspring growth and survival, can be regarded as offspring traits, whereas traits that are under maternal control, such as the number and size of eggs, can be regarded as maternal traits (Wilson *et al.*, 2005). Offspring inbreeding is likely to influence offspring traits only given that offspring cannot influence the number and size of eggs produced by their mothers. In contrast, maternal inbreeding may affect both maternal and offspring traits, given the potential for maternal effects on offspring growth and survival (Mousseau & Fox, 1998). For example, maternal inbreeding could affect offspring growth and survival through its effects on maternally controlled traits, such as egg size, laying and hatching patterns and maternal care (e.g. McParland *et al.*, 2007). Despite their potential importance, few studies have investigated differential effects of offspring and maternal inbreeding on offspring and maternal traits associated with early offspring fitness (Walling *et al.*, 2011; Mattey *et al.*, 2013; Huisman *et al.*, 2016). Neglecting effects of inbreeding on these traits could lead to an underestimation of the fitness costs of inbreeding if they cause early mortality of poor quality inbred individuals.

Prior work on the effects of maternal inbreeding has focused on the offspring's performance after laying or birth, and little is known about how maternal and offspring inbreeding affects egg size (Szulkin *et al.*, 2007; Fox, 2013) and patterns of egg laying or hatching. This is unfortunate given that these traits often have profound effects on the offspring's subsequent performance, thereby potentially explaining the detrimental effects of maternal inbreeding. For example, if inbred females lay smaller eggs, this can have detrimental fitness effects given that egg size often is positively associated with offspring growth and survival (birds: Krist, 2011; fish: Heath & Blouw, 1998; arthropods: Fox & Czesak, 2000). Similarly, patterns of egg laying or hatching can affect offspring survival, time to independence and size at independence (Stoleson & Beissinger, 1995; Stenning, 1996; Ford & Smiseth, 2016), thereby exacerbating the severity of inbreeding depression in offspring (de Boer *et al.*, 2015). Thus, if inbred females lay clutches that hatch more asynchronously, this can also have detrimental effects on offspring performance.

Here, we investigate the effect of maternal and offspring inbreeding on breeding success and maternal and offspring traits associated with early offspring performance in the burying beetle *Nicrophorus vespilloides* (Herbst). In this species, offspring suffer significant fitness costs across their life cycle when either the offspring themselves or their mothers are inbred (Mattey *et al.*, 2013; Pilakouta *et al.*, 2015, 2016; Pilakouta & Smiseth, 2016). *Nicrophorus vespilloides* is an excellent system to study the effects of inbreeding on egg size and egg laying because the eggs can be measured accurately and the timing of egg laying can be reliably recorded (Ford & Smiseth, 2016, 2017). This species breeds on small vertebrate carcasses, which are buried underground (Scott, 1998). Females lay eggs in the surrounding soil (Pukowski, 1933), and the eggs hatch asynchronously over a period of 16–56 h (Müller & Eggert, 1990; Smiseth *et al.*, 2006). The larvae self-feed directly off the carcass but also beg for predigested carrion from the parents (Eggert *et al.*, 1998). The larvae reach independence around 6 days after hatching and disperse into the soil where they pupate and ultimately eclose as adults (Smiseth *et al.*, 2006). Here, we manipulate the level of inbreeding of females producing outbred offspring (maternal inbreeding) and the level of inbreeding of offspring produced by outbred mothers (offspring inbreeding). This design allows us to test for differential effects of maternal and offspring inbreeding on maternal and offspring traits associated with early offspring performance.

It has generally been assumed that inbreeding depression is a linear function of the inbreeding coefficient (Lynch & Walsh, 1998). However, inbreeding may have nonlinear effects that would only be detected if three or more groups of individuals with different inbreeding coefficients are examined. Few studies have experimentally tested for a nonlinear effect of inbreeding, and the majority of those that have done so have focused on domestic cattle (Hudson & Van Vleck, 1984; Miglior *et al.*, 1992; Thompson *et al.*, 2000; Biffani *et al.*, 2002; Sørensen *et al.*, 2006; Croquet *et al.*, 2007; Gulisija *et al.*, 2007). Here, we use three levels of inbreeding to test for a nonlinear relationship between the inbreeding coefficient and the magnitude of inbreeding depression: outbred (the focal individual's parents were unrelated at the grandparent level; $F \approx 0.00$), moderately inbred (the focal individual's parents were

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cousins; F ≈ 0.125) and highly inbred (the focal individual's parents were siblings; F ≈ 0.25). We measured effects of maternal and offspring inbreeding on breeding success (number of dispersing larvae), maternal traits (egg laying patterns and egg size) and offspring traits (i.e. larval survival and mass at independence). We expected both maternal and offspring inbreeding to lead to a reduction in breeding success. We expected maternal inbreeding to influence both maternal and offspring traits, given the potential for maternal effects on offspring growth and survival (Mousseau & Fox, 1998). Conversely, we expected offspring inbreeding to influence offspring traits only given that offspring cannot influence the number and size of eggs produced by their mothers.

Materials and methods

Study animals

The beetles used in this study were third- to sixth-generation beetles from an outbred laboratory population maintained at the University of Edinburgh. The stock population descended from wild beetles caught at Corstorphine Hill, Edinburgh, UK (55.9500° N, 3.2833° W). To ensure that we had full control over the pedigree of our stock population, we housed all beetles in individual transparent plastic boxes (124 mm × 82 mm × 22 mm) from the day that they eclosed as adults. Furthermore, when beetles were paired for breeding, we mated each female with a single male of known identity to prevent paternity uncertainty. We therefore knew the identity of the ancestors of every beetle in our laboratory population dating back to the wild-caught beetles (Mattey & Smiseth, 2015). Keeping beetles in individual boxes from dispersal ensured that all experimental beetles were virgins at the start of the experiments. We ensured that the laboratory population was outbred by maintaining a large population each generation (86–98 broods per generation), recruiting three offspring only from each family to the next generation, outcrossing the laboratory population with wild-caught beetles each summer, and never mating closely related males and females (i.e. siblings or cousins; Mattey & Smiseth, 2015; Mattey et al., in press). These practices ensured that the inbreeding coefficient, F, in our stock population was very low (mean for individuals in the sixth generation: F = 0.0002; Mattey et al., in press). Beetles were kept at 20 ± 2°C (mean ± range) under a 16:8 light:dark cycle and were fed small pieces of organic beef twice a week. All beetles were sexually mature, virgins and of prime reproductive age (10–28 days post-eclosion) at the start of the experiments.

Experimental procedures

For the experiment on the effects of maternal inbreeding, we generated experimental females with the following three levels of inbreeding: (1) outbred females, produced by mating a given female's mother with an unrelated male (i.e. a male with whom the mother did not share a common grandparent or a closer relative; F ≈ 0.00); (2) moderately inbred females, produced by mating a given female's mother with a cousin (i.e. a male with whom the mother shared two common grandparents; F ≈ 0.125); and (3) highly inbred females, produced by mating a given female's mother with a brother (i.e. a male with whom the mother shared both parents; F ≈ 0.25). We always mated experimental females to an unrelated outbred male (i.e. a male with whom the female had no shared grandparents). We did this to ensure that the offspring in this experiment always were outbred, such that any subsequent effects of the experimental treatments could be attributed to the inbreeding status of the experimental females.

For the experiment on the effects of offspring inbreeding, we generated experimental broods of offspring with the following three levels of inbreeding: (1) outbred broods, produced by mating the brood's mother with an unrelated male (i.e. a male with whom the mother did not share a common grandparent or a closer relative; F ≈ 0.00); (2) moderately inbred broods, produced by mating the brood's mother with a cousin (i.e. a male with whom the mother shared two common grandparents; F ≈ 0.125); and (3) highly inbred broods, produced by mating the brood's mother with a brother (i.e. a male with whom the mother shared both parents; F ≈ 0.25). In this experiment, we used outbred females only to ensure that any subsequent effects could be attributed to the inbreeding status of the offspring.

Different individuals were used in the maternal inbreeding and offspring inbreeding experiments. We carried out both experiments in batches of up to 20 broods at a time with the three levels of inbreeding represented in all batches to minimize any confounding effects due to potential differences between batches. At the start of the experiments, we placed the breeding female together with a male in a Petri dish (90 mm × 12 mm) for 4 h to allow them time to mate (Botterill-James et al., 2017). We then transferred each female to a clear breeding box (170 mm × 120 mm × 60 mm) containing 1 cm of moist compost and a previously frozen mouse carcass weighing 23.06 g ± 2.26 (mean ± SD, supplied from Livefoods Direct Ltd, Sheffield, UK). The male was removed at this stage because the presence or absence of the male does not affect offspring fitness under laboratory conditions (Smiseth et al., 2005). In *N. vespilloides*, eggs are visible at the bottom of the breeding box and can be seen on images obtained by placing the boxes on flatbed scanners (Canon CanoScan 9000F Mark II; Canon Inc., Tokyo, Japan; Ford & Smiseth, 2016). In the small amount of soil used, the number of eggs that are visible is strongly correlated with the actual clutch size (Monteith

et al., 2012). We scanned the breeding boxes every hour using VueScan professional edition software (Hamrick Software, Sunny Isles Beach, FL, USA). From the scanned images, we counted the number of new eggs laid each hour to determine laying spread and laying skew (see below) and clutch size (the number of eggs laid).

In N. vespilloides, females determine the hatching pattern through their timing of laying (Smiseth et al., 2006), and we can therefore use laying time as a proxy for hatching time as the former can be determined accurately from the scans. The laying pattern can be described in two ways: the time between the first and last egg being laid, which is termed 'laying spread' (Smiseth et al., 2006; Takata et al., 2015); and the extent to which laying is skewed towards the earlier part of the laying period, which is termed 'laying skew' (Smiseth et al., 2008). We calculated a laying skew index using the formula $\Sigma((t_i - t_m)/t_m)p_i$, where p_i is the proportion of the total clutch that is laid in a given scan, t_i is the time interval of a given scan in relation to the initiation of oviposition and t_m is the middle of the laying period (Smiseth et al., 2008; Ford & Smiseth, 2016). Prior work shows that this index is usually negative, indicating that egg laying is normally skewed towards the first half of the laying period. Values closer to −1 represent a more asymmetric laying skew where a larger proportion of eggs are laid early on, whereas values closer to 0 represent a more symmetrical laying skew. Additionally, we measured egg size of the first five eggs that were lying flat against the bottom of the box in each clutch using ImageJ (Abramoff et al., 2004). Eggs were measured from the second image on which they were present given that they appear to shrink somewhat during the first hour after being laid (personal observation). For each egg, we measured its length and width three times, and the mean length and width were used to calculate a prolate spheroid volume (V) using the equation $V = (1/6)\pi w^2 L$, where w is the width and L the length of the egg (Berrigan, 1991). We continued to check the scans after hatching started to gauge the number of unhatched eggs.

To monitor offspring performance, we checked the boxes for dispersal each day. At the time of dispersal, we counted the number of larvae in the brood and weighed the entire brood. After dispersal, we measured the pronotum width of each female with a Mitutoyo Digimatic Calliper. We confirm that there was no difference in the pronotum width of females depending on their inbreeding status (ANOVA: $F_2 = 1.00$, $P = 0.381$). We initially set up 178 pairs across our two experiments. However, 20 were excluded due to technical problems with the scanners; 23 were excluded because females did not lay any eggs; 37 were excluded from analyses of larval mass and development time because, although females laid eggs, no larvae reached dispersal; and one was excluded because the female started laying too late to allow measurement of laying spread, laying skew or clutch size. The final sample sizes (i.e. number of pairs) in each treatment were as follows: outbred offspring $n = 21$, moderately inbred offspring $n = 24$, highly inbred offspring $n = 26$, outbred mother $n = 26$, moderately inbred mother $n = 26$ and highly inbred mother $n = 28$.

Statistical analyses

We carried out the statistical analyses in R 3.3.1 (R Core Team, 2014). We first tested for effects of maternal and offspring inbreeding on breeding success; that is, the number of dispersing larvae, defined as the number of larvae in the brood at the time of dispersal. We then carried out analyses using the following nine traits (five maternal traits and four offspring traits): (1) Clutch size, defined as the total number of eggs laid before the first egg hatched (Müller et al., 1990). (2) Delay until the onset of laying, defined as the number of hours after the female was placed on the carcass until the first egg was laid. (3) Laying skew index as defined above. (4) Laying spread, defined as the number of hours between the first and last egg being laid. (5) Egg size, calculated as the average volume in mm^3 of five eggs in each clutch. (6) Hatching success, based on counts of the number of eggs hatching and the number of unhatched eggs. We did the analyses in two ways; that is, when including all clutches and when excluding clutches where no eggs hatched. (7) Larval survival, based on the number of larvae surviving until dispersal and the number of larvae dying between hatching and dispersal. We did the analyses in two ways; that is, when including all broods where eggs hatched and when excluding broods where eggs hatched but no larvae survived to dispersal. To ensure that all of the data were retained and the response was as informative as possible, we used the cbind function to bind the number of successes and failures for both hatching success and larval survival. That is, for hatching success, we used the number of eggs hatching vs. the number of unhatched eggs, whereas for larval survival, we used the number of larvae dispersing from the carcass vs. the number of larvae that died between hatching and dispersal. (8) Larval development time, calculated as the time in hours from when the first egg in the clutch was predicted to hatch until the larvae dispersed into the soil. We calculated the predicted time of hatching for the first egg by adding the average duration of egg development, which is 59 h (Smiseth et al., 2006), to the information on the onset of laying (see above). (9) Average larval mass, calculated as the mass of the brood at the time of dispersal divided by the number of larvae in the brood. We analysed data on the effects of maternal inbreeding and offspring inbreeding separately, using the same procedures for both. We carried out a separate generalized linear model (GLM) to test for an effect of maternal and offspring inbreeding status on each of the traits above. The model family used for each model is

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listed in Tables 1 and 2. For every GLM, we conducted a Fligner–Killeen test to check for homogeneity of variance. We also checked that the residuals were normally distributed and that there were no unduly influential observations (i.e. no Cook's distances >1). Because we carried out multiple tests, we applied a Benjamini–Hochberg procedure to the model results from each experiment. This did not lead to a change in the interpretation of any of the results.

Results

Maternal inbreeding

Maternal inbreeding had a significant negative effect on breeding success, reflecting that highly inbred females produced broods with a significantly smaller number of dispersing larvae than outbred females (Table 1). As

expected, there were significant effects of maternal inbreeding on both maternal and offspring traits. Firstly, maternal inbreeding had a significant effect on laying skew (Table 1). The majority of clutches had a negative laying skew index (92% for clutches laid by outbred females, 86% for moderately inbred females and 81% for highly inbred females), indicating that most of the eggs in a given clutch were laid during the first half of the laying period. This index was closer to 0 for clutches laid by highly inbred females than for clutches produced by outbred females (Table 1, Fig. 1a). Thus, highly inbred females produced clutches where the eggs were laid more symmetrically around the middle of the laying period than did outbred females. There was no evidence that maternal inbreeding had an effect on any of the other maternal traits (Table 1). Secondly, maternal inbreeding had a significant effect on hatching success when we excluded broods where all eggs failed to hatch, although

Table 1 Results of GLMs testing for effects of maternal inbreeding on breeding success (number of dispersing larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, larval development time and larval mass). Significant effects are shown in bold.

Trait	Model error distribution	Mean outbred (±SD)	Mean moderately inbred (±SD)	Mean highly inbred (±SD)	Overall effect of maternal inbreeding		Moderately inbred compared to outbred		Highly inbred compared to outbred	
					Test statistic	P	Test statistic	P	Test statistic	P
Number of dispersing larvae	Negative binomial	15.47 (8.83)	13.41 (6.73)	8.74 (6.23)	$X^2_{2,55} = 7.52$	**0.023**	$z = -0.70$	0.482	$z = -2.65$	**0.008**
Clutch size	Negative binomial	30.40 (9.45)	31.76 (8.21)	30.81 (8.78)	$X^2_{2,64} = 0.27$	0.872	$z = 0.52$	0.606	$z = 0.16$	0.875
Delay until laying (h)	Inverse Gaussian	18.72 (7.94)	15.76 (7.14)	17.76 (7.55)	$F_{2,64} = 0.74$	0.479	$t = 1.32$	0.193	$t = 0.40$	0.693
Laying skew	Gaussian	−0.35 (0.22)	−0.24 (0.26)	−0.14 (0.19)	$F_{2,64} = 4.88$	**0.011**	$t = 1.62$	0.110	$t = 3.12$	**0.003**
Laying spread (h)	Gaussian	51.40 (18.53)	51.67 (15.33)	41.43 (17.38)	$F_{2,64} = 2.48$	0.092	$t = 0.05$	0.958	$t = -1.96$	0.055
Egg size (mm³)	Inverse Gaussian	1.72 (0.20)	1.82 (0.19)	1.77 (0.28)	$F_{2,60} = 0.91$	0.409	$t = -1.34$	0.184	$t = -0.65$	0.520
Hatching success including failures	Quasibinomial	0.77 (0.40)	0.92 (0.11)	0.84 (0.14)	$X^2_{2,64} = 4.38$	0.112	$t = 1.96$	0.054	$t = 0.22$	0.825
Hatching success excluding failures	Quasibinomial	0.96 (0.07)	0.92 (0.11)	0.84 (0.14)	$X^2_{2,59} = 15.73$	**0.0004**	$t = -1.06$	0.295	$t = -3.64$	**0.0006**
Larval survival including failures	Quasibinomial	0.38 (0.31)	0.36 (0.27)	0.18 (0.21)	$X^2_{2,59} = 8.14$	**0.017**	$t = -0.19$	0.851	$t = -2.49$	**0.016**
Larval survival excluding failures	Quasibinomial	0.47 (0.27)	0.45 (0.22)	0.32 (0.18)	$X^2_{2,42} = 3.12$	0.210	$t = -0.44$	0.664	$t = -1.68$	0.100
Larval development time (h)	Inverse Gaussian	140.69 (17.82)	142.12 (15.34)	150.83 (11.61)	$F_{2,42} = 1.60$	0.213	$t = -0.27$	0.789	$t = -1.68$	0.101
Larval mass (g)	Gaussian	0.22 (0.06)	0.21 (0.05)	0.23 (0.12)	$F_{2,55} = 0.35$	0.707	$t = -0.66$	0.513	$t = 0.07$	0.944

Table 2 Results of GLMs testing for effects of offspring inbreeding on breeding success (number of dispersing larvae), maternal traits (clutch size, delay until laying, laying skew, laying spread and egg size) and offspring traits (hatching success, larval survival, larval development time and larval mass). Significant effects are shown in bold.

Trait	Model error distribution	Mean outbred (±SD)	Mean moderately inbred (±SD)	Mean highly inbred (±SD)	Overall effect of offspring inbreeding Test statistic	P	Moderately inbred compared to outbred Test statistic	P	Highly inbred compared to outbred Test statistic	P
Number of dispersing larvae	Negative binomial	7.94 (6.18)	12.47 (8.15)	8.89 (5.67)	$X^2_{2,51} = 4.33$	0.115	$z = 1.97$	**0.049**	$z = 0.50$	0.618
Clutch size	Negative binomial	37.94 (15.16)	31.04 (9.48)	31.08 (7.83)	$X^2_{2,64} = 6.10$	**0.047**	$z = -2.17$	**0.030**	$z = -2.17$	**0.030**
Delay until laying (h)	Inverse Gaussian	19.44 (8.37)	26.63 (23.65)	32.92 (23.99)	$F_{2,64} = 3.04$	0.055	$t = -1.78$	0.080	$t = -2.23$	**0.029**
Laying skew	Gaussian	−0.12 (0.26)	−0.17 (0.21)	−0.18 (0.28)	$F_{2,63} = 0.36$	0.696	$t = -0.67$	0.504	$t = -0.82$	0.418
Laying spread (h)	Inverse Gaussian	45.17 (27.39)	39.42 (16.56)	35.92 (16.94)	$F_{2,64} = 1.08$	0.347	$t = 0.87$	0.389	$t = 1.51$	0.137
Egg size (mm³)	Inverse Gaussian	1.94 (0.30)	1.97 (0.27)	2.03 (0.34)	$F_{2,61} = 0.42$	0.660	$t = -0.27$	0.789	$t = -0.85$	0.398
Hatching success including failures	Quasibinomial	0.85 (0.25)	0.89 (0.17)	0.83 (0.20)	$X^2_{2,64} = 0.61$	0.737	$t = 0.66$	0.515	$t = -0.03$	0.980
Hatching success excluding failures	Quasibinomial	0.90 (0.14)	0.89 (0.17)	0.83 (0.20)	$X^2_{2,63} = 1.64$	0.441	$t = -0.45$	0.657	$t = -1.24$	0.220
Larval survival including failures	Quasibinomial	0.25 (0.24)	0.33 (0.30)	0.24 (0.27)	$X^2_{2,63} = 2.26$	0.323	$t = 1.46$	0.148	$t = 0.67$	0.508
Larval survival excluding failures	Quasibinomial	0.28 (0.24)	0.46 (0.25)	0.36 (0.26)	$X^2_{2,47} = 6.00$	**0.049**	$t = 2.34$	**0.024**	$t = 0.82$	0.415
Larval development time (h)	Gaussian	158.00 (37.86)	132.47 (28.68)	137.28 (17.60)	$F_{2,47} = 3.53$	**0.037**	$t = -2.52$	**0.015**	$t = -2.07$	**0.044**
Larval mass (g)	Gaussian	0.23 (0.03)	0.21 (0.03)	0.20 (0.04)	$F_{2,50} = 1.86$	0.166	$t = -1.06$	0.292	$t = -1.93$	0.060

not when we included these broods (Table 1). When we excluded clutches with total hatching failure, highly inbred females produced eggs with a significantly lower hatching success than outbred females (Table 1, Fig. 1b). Maternal inbreeding significantly affected larval survival when we included broods where no larvae dispersed but not when we excluded these broods (Table 1). When we included broods where no larvae dispersed, fewer larvae survived in broods produced by highly inbred mothers compared to broods produced by outbred mothers. The lack of an effect of maternal inbreeding on larval survival when we excluded broods where no larvae dispersed suggests that this effect was driven by a greater number of total brood failures when the mother was highly inbred (no larvae reached dispersal in nine broods when the mother was highly inbred compared to four broods when the mother was moderately inbred and four when she was outbred). There were no effects of maternal inbreeding on any other offspring traits (Table 1). Thus, our results suggest that highly inbred females had a lower breeding success because their eggs had a lower hatching success and their larvae suffered greater mortality, rather than because they produced a smaller number of eggs.

Offspring inbreeding

Although there was no overall effect of offspring inbreeding on breeding success, there were a greater number of dispersing larvae when offspring were moderately inbred than when they were outbred (Table 2). Contrary to what we expected, there were significant effects of offspring inbreeding on both maternal and offspring traits. Firstly, offspring inbreeding had a significant effect on both clutch size and onset of egg laying (Table 2). Females that were mated to an unrelated male (i.e. females producing outbred eggs) laid a greater number of eggs than females that were mated to their brother or cousin (i.e. females producing highly inbred and moderately inbred eggs, respectively) (Table 2). Likewise, females that were mated to their brother or cousin took on average longer to begin laying eggs than females that were mated to an unrelated male (Table 2). Given that offspring cannot influence the number and size of eggs produced by their mothers, these findings suggest that females adjust decisions about how many eggs to lay and when to start laying based on whether they mated with a related or an unrelated male. There was no effect of offspring inbreeding on any of the other
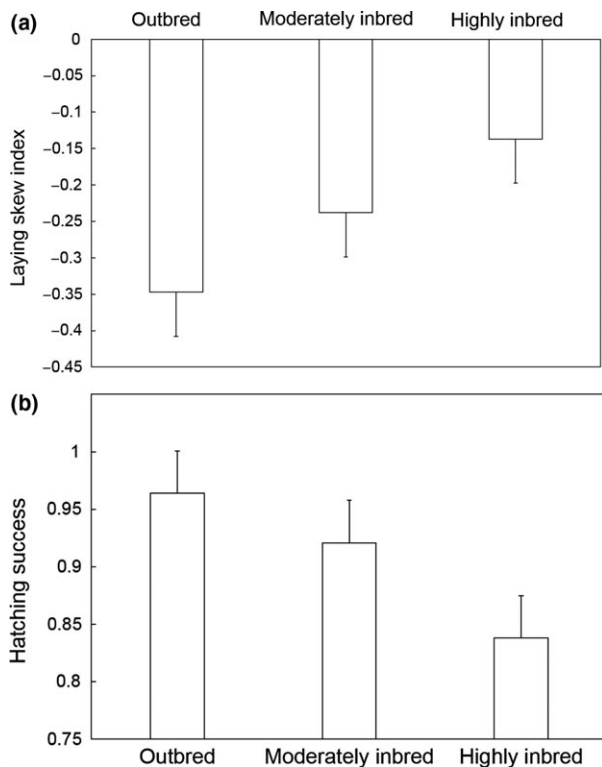
Fig. 1 The effect of maternal inbreeding on (a) laying skew index (an index calculated based on the extent to which laying is skewed towards the earlier part of the laying period) and (b) hatching success (the proportion of the clutch that hatched, excluding clutches where eggs were laid but none hatched). Error bars represent standard errors.

Fig. 2 The effect of offspring inbreeding status on (a) larval survival from hatching to dispersal (the proportion of the larvae hatching that survived to dispersal, excluding broods where larvae hatched but none dispersed) and (b) the duration of larval development (the number of hours from the estimated timing of the start of hatching until dispersal). Error bars represent standard errors.

maternal traits (Table 2). Secondly, offspring inbreeding had a significant effect on two offspring traits: larval survival from hatching to dispersal and larval development time (Table 2). Moderately inbred larvae had higher survival than outbred larvae when we excluded broods where no larvae dispersed (Table 2, Fig. 2a). Furthermore, moderately and highly inbred larvae developed significantly faster than outbred larvae (Table 2, Fig. 2b). There was no effect of offspring inbreeding on hatching success (Table 2). Thus, keeping in mind that females mated to an unrelated male (i.e. females producing outbred eggs) laid a greater number of eggs than females mated to a related male, our results suggest that the greater breeding success when offspring were moderately inbred was associated with high larval survival from hatching to dispersal (Table 2).

Discussion

We investigated the effects of both maternal and offspring inbreeding using an experimental design with three levels of maternal and offspring inbreeding (i.e. $F \approx 0.00$, $F \approx 0.125$ and $F \approx 0.25$). Firstly, we find
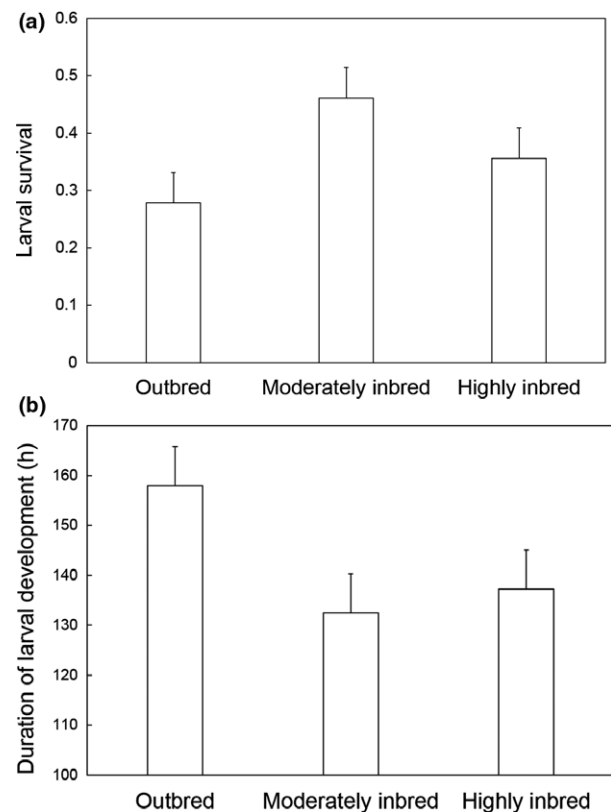
evidence that both maternal inbreeding and offspring inbreeding affected breeding success. As expected, highly inbred females produced broods with significantly fewer dispersing larvae than outbred females. In contrast, there was no overall effect of offspring inbreeding on breeding success, although there were a greater number of dispersing larvae when offspring were moderately inbred than when they were outbred. Secondly, we find evidence for differential effects of maternal inbreeding and offspring inbreeding on maternal and offspring traits. Maternal inbreeding affected laying skew, hatching success and larval survival, whereas offspring inbreeding affected clutch size, delay until onset of egg laying, larval survival and larval development time. Our results confirm that maternal inbreeding affected both maternal and offspring traits, which is in line with our expectations given that female parents can influence offspring traits through maternal effects. However, our results suggest that offspring inbreeding also affected both maternal and offspring

traits, which is surprising given that offspring cannot influence the number of eggs produced by their mothers or the timing of egg laying. Thus, this finding suggests that females alter their decisions about the number of eggs they lay and the timing of egg laying based on whether they are mated with a related or an unrelated male. Finally, we found evidence of a nonlinear effect of the level of offspring inbreeding on larval survival, with moderately inbred offspring experiencing the greatest survival from hatching to independence. Below, we discuss the wider implications of these results for our understanding of the effects of maternal and offspring inbreeding on breeding success and offspring performance.

We found that maternal inbreeding had a negative effect on breeding success with highly inbred females producing fewer dispersing larvae than outbred females. This finding is largely consistent with prior work reporting negative effects of maternal inbreeding on breeding success and early offspring performance in *N. vespilloides* (Mattey *et al.*, 2013) as well as in birds (Keller, 1998; Jamieson *et al.*, 2003; Reid *et al.*, 2003; Richardson *et al.*, 2004; Szulkin *et al.*, 2007) and mammals (Huisman *et al.*, 2016). We recorded effects of maternal inbreeding on maternal and offspring traits associated with early offspring performance, which allowed us to identify at least some of the potential mechanisms by which maternal inbreeding causes a reduction in breeding success. We found that maternal inbreeding affected laying skew, hatching success and larval survival. As discussed below, highly inbred females produced clutches that had a laying skew index that was closer to 0 (i.e. egg laying was less skewed towards the beginning of the laying period) than did outbred females. We also found that a laying skew index closer to 0 was associated with higher offspring survival, suggesting that the effect of maternal inbreeding on hatching skew cannot account for lower breeding success of highly inbred females. We found that highly inbred females laid eggs that had a significantly lower hatching success compared to eggs laid by outbred females when we excluded clutches where no eggs hatched. Furthermore, fewer larvae survived from hatching to dispersal in broods produced by highly inbred females compared to those produced by outbred females. In contrast, there was no evidence that maternal inbreeding affected clutch size, delay until laying, laying spread, egg size, larval growth or larval development time. Thus, our results suggest that the detrimental effects of maternal inbreeding on breeding success are mediated through a reduction in hatching success of eggs laid by highly inbred females and greater mortality of larvae produced by highly inbred mothers rather than a reduction in clutch size or egg size.

As mentioned above, we found that highly inbred females produced clutches that had a hatching skew index that was closer to 0 than outbred females. In other words, highly inbred females produced clutches where egg laying was less skewed towards the beginning of the laying period than did outbred females. This finding is contrary to what we anticipated if the detrimental effects of maternal inbreeding on breeding success were mediated through an effect on hatching skew. The reason for this is that a hatching skew index closer to 0 is assumed to be associated with increased rather than reduced offspring survival. We conducted a post hoc test of this assumption using data from the experiment on the effects of offspring inbreeding (we used these data because all mothers were outbred). We found that a more negative value of the laying skew index (i.e. when egg laying was more strongly skewed towards the beginning of the laying period) was associated with reduced larval survival (Pearson's correlation, $t_{46} = 3.07$, $P = 0.004$, $r = 0.41$) as well as with fewer dispersing larvae ($t_{47} = 2.74$, $P = 0.009$, $r = 0.37$). This effect is presumably mediated through an effect of laying skew on sibling competition. There is good evidence from studies on birds that asynchronous hatching is associated with asymmetric sibling competition (Magrath, 1990; Stoleson & Beissinger, 1995; Mock & Parker, 1997), where early-hatched offspring are competitively superior to offspring that hatch later (Lack, 1947; Stinson, 1979). Likewise, studies on *N. vespilloides* and the closely related *Nicrophorus quadripunctatus* find that late-hatched larvae have reduced growth rates and are less likely to survive to independence than early-hatched larvae (Smiseth *et al.*, 2007; Takata *et al.*, 2013). Thus, the finding that highly inbred females produce clutches where egg laying was less skewed towards the beginning of the laying period suggests that these females adjust their laying patterns facultatively and that by doing so, they partially mitigate some of the negative effects of maternal inbreeding on breeding success. However, we note that females cannot completely compensate for the detrimental effect of maternal inbreeding given that the number of larvae reaching dispersal is still lower for broods produced by highly inbred females.

Although there was no overall effect of offspring inbreeding on breeding success, we found that a greater number of larvae reached dispersal in broods where offspring were moderately inbred compared to broods where they were outbred. We also found that females that were mated to related males (and thus were producing inbred offspring) laid fewer eggs than those that were mated to unrelated males (and thus producing outbred offspring). This finding is consistent with a recent theoretical model predicting that parents producing inbred offspring should produce fewer offspring and invest more resources in individual offspring (Duthie *et al.*, 2016). Additionally, we found that females producing highly inbred offspring took longer to begin egg laying after encountering the carcass. It is unlikely that this would be beneficial for the offspring given that this

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delay would be associated with an increase in microbial growth over time, which reduces egg survival (Jacobs *et al.*, 2014). We found that inbred larvae developed more quickly from hatching to dispersal than outbred larvae but attained a similar average mass at dispersal. Highly inbred larvae dispersed around 21 h sooner after hatching than outbred larvae, which more than counteracted the 13-h delay in the onset of laying by females producing highly inbred offspring. Our study provides no information on the mechanism behind the shorter development time for highly inbred larvae. However, a recent study on the same species found that outbred females provide more direct care towards inbred larvae, resulting in inbred and outbred larvae attaining the same mass at dispersal (Mattey *et al.* in press). Thus, it is possible that an increase in direct care towards inbred larvae allows them to develop faster, which would be beneficial for the offspring because the carcass becomes increasingly inhospitable for larvae as decomposition occurs (Rozen *et al.*, 2008). If so, this could contribute to the greater larval survival that we observed in moderately inbred broods, leading to a greater number of larvae at dispersal.

Finally, our results provide evidence of a nonlinear effect of offspring inbreeding on breeding success. We found that a greater number of larvae reached dispersal in broods of moderately inbred larvae compared to broods of either outbred or highly inbred larvae. Likewise, moderately inbred larvae had greater survival than either outbred or highly inbred larvae when excluding broods where no larvae dispersed. Previous studies on this species have found evidence for an effect of offspring inbreeding on offspring survival (Mattey *et al.*, 2013; Pilakouta *et al.*, 2015, 2016; Pilakouta & Smiseth, 2016). We note that these studies also found detrimental effects of offspring inbreeding on other components of offspring performance, such as survival from dispersal to eclosion (Mattey *et al.*, 2013; Pilakouta *et al.*, 2015, 2016; Pilakouta & Smiseth, 2016), survival from hatching to eclosion (Pilakouta *et al.*, 2016) and adult lifespan (Pilakouta *et al.*, 2015; Pilakouta & Smiseth, 2016). Our design differs from that used in the majority of laboratory studies investigating inbreeding depression, which simply compare fitness-related traits of outbred individuals with experimentally generated inbred individuals (with the inbreeding coefficient of the inbred treatment varying between studies) (Lynch & Walsh, 1998). Meanwhile, studies on inbreeding depression in the field often determine inbreeding coefficients from a pedigree, often assuming that inbreeding depression is a linear function of the inbreeding coefficient (Lynch & Walsh, 1998). Nevertheless, there is some evidence for nonlinear effects of inbreeding from studies on domestic cattle (Hudson & Van Vleck, 1984; Miglior *et al.*, 1992; Thompson *et al.*, 2000; Biffani *et al.*, 2002; Sørensen *et al.*, 2006; Croquet *et al.*, 2007; Gulisija *et al.*, 2007),

and studies on mice find that offspring produced by intermediately related parents are larger than those produced by unrelated or closely related parents (Barnard & Fitzsimons, 1989; Keane, 1990). There is also some evidence from studies on humans that couples that are moderately related to each other have a greater number of children (Helgason *et al.*, 2008; Labouriau & Amorim, 2008). We suggest that such nonlinear effects of offspring inbreeding could arise as a consequence of maternal effects on offspring. There is evidence that maternal care buffers against the detrimental effect of offspring inbreeding in *N. vespilloides* (Pilakouta *et al.*, 2015). Furthermore, the smaller clutch sizes laid by females producing inbred offspring may lead to a lower initial number of larvae hatching, which would allow females to provide more care to each larva in inbred broods as predicted by a recent theoretical model (Duthie *et al.*, 2016). Thus, nonlinear effects of inbreeding could arise if females overcompensate for the detrimental effects of moderate offspring inbreeding by providing more care, resulting in higher larval survival for moderately inbred offspring compared to outbred offspring. Meanwhile, an increase in maternal care may just be sufficient to mitigate the detrimental effects of inbreeding when offspring are highly inbred, resulting in similar survival of highly inbred and outbred offspring (Mattey *et al.*, in press).

In conclusion, we found that both maternal inbreeding and offspring inbreeding affected breeding success, that maternal inbreeding and offspring inbreeding affected different traits associated with early offspring performance and that there were nonlinear effects of offspring inbreeding. Our results provide novel insights into inbreeding by suggesting that maternal inbreeding and offspring inbreeding have differential effects on maternal and offspring traits. Maternal inbreeding affected laying skew, hatching success and larval survival, whereas offspring inbreeding affected clutch size, delay until onset of egg laying, larval survival and larval development time. Furthermore, our results suggest that inbred females facultatively adjust their laying patterns to compensate for some of the detrimental effects of maternal inbreeding on offspring. In support of this, we found that inbred females lay clutches with hatching skew index that was closer to 0 (i.e. the eggs were laid more symmetrically around the middle of the laying period), which is associated with greater offspring survival. Finally, we found evidence of a nonlinear effect of offspring inbreeding coefficient on the number of larvae dispersing, with the greatest number of larvae dispersing in moderately inbred broods, reflecting that these broods experienced the greatest larval survival. This result highlights the importance of considering deviations from linearity when testing for an effect of inbreeding, and we recommend that future studies incorporate multiple inbreeding treatments where possible to increase our

understanding of the effects of inbreeding and to gain an insight into the potential mechanisms behind these effects.

Acknowledgments

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