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No maternal effects after stimulation of the melanization response in the yellow fever mosquito *Aedes aegypti*

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The costs and benefits of activating the immune system can reach across generations. Thus, in vertebrates and in several invertebrates, stimulating the immune system of a female can enhance immunity of her offspring or decrease offspring fitness. We evaluated the potential maternally transmitted costs and benefits of the melanization response, an innate immune response of insects that helps to protect mosquitoes from malaria parasites. We manipulated the maternal melanization response of the yellow fever mosquito *Aedes aegypti* by inoculating female mosquitoes with negatively charged sephadex beads or with immunologically inert glass beads; a control group was not inoculated. In the next generation, we assayed the melanization response and measured three other life-history traits: survival up to emergence, the age at emergence, and body size (estimated as wing length). We found no evidence of fitness costs or benefits for the offspring. A retrospective power analysis found that our experiment would have detected an effect size that is three times smaller than the maternal immune priming effects that have been reported in the literature. We did find a strong correlation between offspring wing length and melanization response. Overall, our findings indicate that trans-generational immune priming in invertebrates cannot be generalized, and that it may depend on the species, the immune challenge, and the environmental conditions.

Like any other fitness-related trait, the efficacy of immune responses is determined by the evolutionary pressures that balance their costs and benefits (Sheldon and Verhulst 1996). A powerful immune system provides the obvious advantage to minimize the negative effects of pathogens. These benefits are counter-acted by various evolutionary costs due to tradeoffs between immunity and other traits. For example, stimulating the immune system decreases the lifespan of the bumblebee *Bombus terrestris* (Moret and Schmid-Hempel 2000). Reproductive activity reduces the ability of the damselfly *Matrona basilaris japonica* (Siva-Jothy et al. 1998), and the fruitfly *Drosophila melanogaster* (McKean and Nunney 2001), to encapsulate foreign bodies or to clear an *Escherichia coli* infection, respectively. The ability to encapsulate parasitoid eggs decreases the competitive ability of *D. melanogaster* larvae (Kraaijeveld and Godfray 1997).

The evolution of immunity will also be affected by the indirect costs and benefits of an individual's immune response expressed in its offspring. Again, the potential benefits are clear: an individual whose immune system is stimulated can transfer aspects of its own immune response to its offspring to protect them, at least temporarily, against infection. Such maternal effects (which are called trans-generational immune-priming) are most obvious in vertebrates, where immune-challenged females transfer antibodies to their offspring (Grindstaff et al. 2003, 2006). Although acquired immune protection was long thought to be impossible in invertebrates, as their immune system lacks the mechanisms responsible for (vertebrate) immune memory (Gillespie et al. 1997), studies on the shrimp *Penaeus monodon* (Huang and Song 1999), the water flea *Daphnia magna* (Little et al. 2003), the bumblebee *B. terrestris* (Moret and Schmid-Hempel 2001, Sadd et al. 2005, Sadd and Schmid-Hempel 2007), and the mealworm beetle *Tenebrio molitor* (Moret 2006), have shown that an individual's experience with a pathogen can provide its descendants with enhanced immunity.

There is also evidence that activating a mother's immune response is associated with a maternally transmitted cost, i.e. that immunizing an individual decreases the quality of its offspring. Thus female flycatchers *Ficedula hypoleuca* immunized with a diphtheria–tetanus vaccine tend to feed their young less than control females, thereby producing fledglings in poor condition (lower body mass) (Ilmonen et al. 2000). Similarly, diphtheria–tetanus vaccination of female blue tits *Parus caeruleus* reduces their nestling feeding rate compared with that of saline-inoculated controls which affects the growth and survival of nestlings (Råberg et al. 2000). To our knowledge, no such maternally

transmitted costs of activating the maternal immune response have been documented in invertebrates. We have recently shown that *Aedes aegypti* mosquitoes infected with the microsporidian parasite *Vavraia culicis* produce smaller eggs than uninfected mothers, and this decrease in egg size is associated with delayed pupation (Fellous et al. unpubl.). However, whether this effect is a trans-generational cost of the immune response or due to, for example, resource depletion by the parasite remains unanswered.

Although some instances of trans-generational immune priming in invertebrates have been observed (above), the generality of maternal effects of immunity in invertebrates is unknown. To test for trans-generational immune priming and trans-generational immune costs we used the mosquito *Aedes aegypti* and its melanization response, an innate immune response in insects that detects and melanizes foreign bodies in the hemolymph (Christensen et al. 2005). The melanization response has attracted much interest in mosquitoes as it may be involved in mosquito resistance to malaria parasites (Collins et al. 1986, Paskewitz et al. 1988) and can be assayed by inoculating sephadex beads into the mosquito thorax (Paskewitz and Riehle 1994, Chun et al. 1995, Suwanchaichinda and Paskewitz 1998). We chose the melanization response in *A. aegypti* to investigate trans-generational immune priming and trans-generational immune costs because it is biologically relevant and because it has previously shown to be costly (Koella and Boete 2002, Schwartz and Koella 2004).

We investigated the possibilities of trans-generational immune priming and trans-generational costs of immunity by stimulating the melanization response of adult *A. aegypti* females and by comparing the melanization responses and several life-history traits of their offspring. We stimulated the melanization response by inoculating the thorax of mosquitoes with negatively charged C-25 sephadex beads (Chun et al. 1995). To control for the wounding of the inoculation procedure, we inoculated the thorax of other mosquitoes with glass beads, which are immunologically inert and do not stimulate the melanization response (Schwartz and Koella 2004). A control group was not inoculated. The measured life-history traits were: survival up to emergence, the age at emergence, and the adult size (estimated as wing length). The trans-generational immune priming hypothesis predicts that the offspring of sephadex bead-inoculated mothers should have a more effective melanization response than that of glass bead- and non-inoculated mothers. The trans-generational costs hypothesis predicts that the offspring of sephadex bead-inoculated mothers should have lower fitness than the offspring of glass bead- and non-inoculated mothers.

Material and methods

Mosquito rearing protocol

We used the F_1 and F_2 offspring from a mixture of ten isofemale lines of *A. aegypti* that had descended from a colony collected in Senegal and had been maintained for over 30 generations. Mosquitoes were kept in an insectary at 26°C, 70% humidity and 12:12 day:night cycle. Eggs were hydrated with de-ionised water and hatched in petri dishes in a vacuum chamber, which synchronizes larval hatching. To eliminate density-dependent variation in development, we reared larvae individually in 12-well tissue culture plates. They were fed 0.03, 0.04, 0.08, 0.16, 0.32 mg of crushed Tetramin on days 1, 2, 3, 4, 5 and 0.60 mg every day thereafter. Adults had constant access to cotton wool soaked in 8% sugar solution except for the 12 h before a blood meal.

Maternal generation

The maternal generation (the F_1 offspring of the isofemale mixture) was laid in mid-December 2005. We hatched the eggs two months later and selected a sample of 600 adults that emerged within 24 h of each other. Males and females were mated in four plexiglas 30-cm cubic cages and two days after emergence females were blood-fed for one h on the arms of MJV. We captured 200 blood-fed females (hereafter referred to as the mothers), placed them into individual 50-ml falcon tubes, and randomly assigned them to one of three treatments: sephadex bead inoculation ($n = 67$), glass bead inoculation ($n = 67$) and no inoculation ($n = 66$). Twenty-four h after the blood meal, we processed the 200 mothers in an alternating sequence of these three bead inoculation treatments (this took 14 h). We chose a 24-h interval between blood feeding and bead inoculation as previous work on fecundity costs of bead melanization in *A. aegypti* used this timing (Schwartz and Koella 2004). Two days after bead inoculation, mothers were transferred to oviposition cups, where they were allowed to lay eggs on moist filter paper for three days, after which the females were scored for their post-inoculation condition (below), and frozen for dissection the following day. Hence counting emergence as day 0, mothers were blood-fed on day 2, inoculated with beads on day 3, allowed to lay eggs on days 5 to 8, scored for their post inoculation condition on day 8, and frozen for dissection on day 9. For each mother we counted the number of eggs that she had laid and allowed the eggs (still attached to the filter paper) to dry in the insectary. For each mother we also dissected the abdomen to count the number of retained eggs (i.e. eggs that had not been laid). We removed the wings and measured their lengths using a light microscope with an ocular micrometer. We dissected the thorax of the sephadex bead-inoculated mothers to estimate the amount of melanin on the bead (below).

Offspring generation

The offspring generation (F_2 generation of the isofemale mixture) was laid two weeks after the mothers hatched. For each of the three maternal bead inoculation treatments about 40 mothers laid enough eggs to rear at least 20 offspring, giving a total of 123 full-sib families. To manage the workload, we randomly assigned these families of eggs to one of four temporal blocks, of which we hydrated the eggs 18, 24, 39 or 45 days after they had been laid (blocks 1, 2, 3 and 4, respectively). Each block had the three treatments with about 10 families per treatment for a total of 30 families per block. After adding water, we scanned each family every 12 h to determine the time that the eggs hatched. For each family, we selected up to 24 larvae and assigned them to one of two 12-well tissue culture plates. In the insectary, we stored the 60 family plates on a shelf and then randomized their

position every day. We transferred pupae into open 1.5 ml eppendorf tubes filled with water and placed these tubes into 50 ml falcon tubes that were covered with a fine mesh. We scanned the tubes every 12 h to determine the age of emergence. To assay the melanization response, we inoculated the female offspring with sephadex beads. We measured wing length for male and female offspring.

Melanization response

The melanization of sephadex beads depends on many factors. Time course experiments in *Anopheles gambiae* have shown that bead melanization reaches a maximum value by 24 h (Paskewitz and Riehle 1994, Chun et al. 1995, Warr et al. 2006) and a similar bead melanization plateau has been observed in *A. aegypti* (Schwartz and Koella unpubl.). The strength of the response declines with age (Chun et al. 1995) and depends on larval (Suwanchaichinda and Paskewitz 1998) and adult nutrition (Koella and Sorensen 2002). In *A. gambiae* (Chun et al. 1995, Schwartz and Koella 2002) and *A. stephensi* (Koella and Sorensen 2002) a blood meal increases the melanization response in females.

To minimize age-related variation in the melanization response, the mothers and the daughters were inoculated with beads 72 ($\pm$12) h after emergence and the mothers were inoculated within 24 ($\pm$6) h of their blood meal. CM C-25 sephadex and glass beads were hydrated in *Aedes* solution (0.76 g NaCl, 0.037 g KCl, 0.0147 g $CaCl_2$ in 1000 ml of distilled water) and dyed with 0.001% methyl green and 0.001% cresyl violet, respectively, to facilitate visibility during inoculation and dissection. We anaesthetized the mosquitoes by briefly chilling them on ice (2–8 min). We inoculated a single bead into the thoracic cavity of the mosquito with a micro-capillary tube pulled into a very fine tip ($\phi = 40$ μm) and about 0.1 μl of *Aedes* solution. Inoculated females were placed into 50-ml falcon tubes that were laid on their sides and contained moist filter paper and a sugar food source. Daughters (mothers) were assessed for their post-inoculation condition (see below) 3 to 4 days (5 days) after bead inoculation. Hence all females that were alive when their post-inoculation conditions were assessed had sufficient time to reach their bead melanization plateau. Mothers were assessed for their post-inoculation condition (dead or alive) by tapping their oviposition cups whereas daughters were released into plexiglass 30-cm cubic cages. Many of the live daughters were inactive so we prodded them with forceps to check if they were capable of walking, jumping or flying. After assessing their post-inoculation condition, all females were immediately frozen for dissection at -20°C. Over the next three months we dissected the thorax of all sephadex bead-inoculated females, searched for up to 15 min for a bead, and estimated the % melanin cover. For the glass bead-inoculated mothers, we dissected a sample of 11 individuals to confirm that these beads had not been melanized.

Statistical methods

Maternal wing length, egg production and bead melanization

In *A. aegypti*, stimulating the melanization response with sephadex beads reduces female egg production (Schwartz and Koella 2004). To test whether melanization was costly in the mothers, we used ANCOVA to model egg production (the number of laid eggs plus retained eggs) as a function of the bead inoculation treatment and wing length. We used egg production rather than the number of laid eggs to test for melanization costs in the mothers because *A. aegypti* females retain eggs for reasons that are not related to their immune status (e.g. no sperm, unsuitable oviposition sites).

For the sephadex beads that we managed to recover from the sephadex bead-inoculated mothers, we calculated the mean% melanin cover and tested whether % melanin cover was correlated with wing length or egg production.

Offspring fitness

For the offspring, we measured four fitness traits: (1) survival to adulthood, (2) emergence time, i.e. time taken for larva to emerge as an adult from the time of hatching (3) wing length, and (4) % melanin cover (for females only). To test for treatment, block and sex effects, we used three-way ANOVAs on the family means (weighted by the square root of the number of offspring per family). We could not estimate the effect of sex on survival, as we only sexed the adults. We were not interested in, and therefore did not include, the block $\times$ treatment and block $\times$ sex interaction terms.

Offspring bead melanization

As analyzing the family means does not allow us to include covariates (e.g. post-inoculation condition) that may affect an individual's melanization response, we used the lmer() function in R to model % melanin cover as a mixed effects model containing two fixed factors: bead inoculation treatment and block, three methodological covariates: age of inoculation, age at which post-inoculation condition was assessed, and post-inoculation condition (modeled as a ranked variable: dead = 0, walking = 1, jumping = 2, flying = 3), two life history covariates: emergence time, and wing length, and two random factors: family and plate. For model simplification, we first tried to remove the least interesting variables such as the random factor plate and the methodological covariates, followed by the life history covariates and last the fixed factors. We always retained a random effects term for family because the previous analyses of offspring fitness (above) used the variance among family means as the residual error term. We determined the statistical significance of terms via deletion tests between hierarchical models and retained all terms with a p-value < 0.05. Once we determined the minimal adequate model, we added the deleted terms one at a time to confirm that they were not statistically significant.

Heritability of traits

We used the lmer() function in R to partition the variance of six offspring traits – survival, bead melanization, male and female emergence time and male and female wing length – into four components: (1) block (2) family (3) plate and (4) individual where individuals are nested in plates, plates are nested in families, and families are nested in blocks. As before, we tested significance of random terms by comparing hierarchical models. We included block in

the variance component analysis to show that hydrating the eggs at different times (18, 24, 39 and 45 days after laying) made a negligible contribution to the variance in offspring phenotype. We used the variance components to estimate full-sib heritabilities (Falconer and Mackay 1996).

Power analysis

We conducted a retrospective power analysis for all six offspring traits: survival, melanization, female and male emergence, and female and male wing length, where we varied effect size (differences in means among treatments) but not sample size (number of full-sib families included in the study). For each trait, we obtained the mean (μ) and the standard deviation among family means (σ). Following Martinez-Abrain (2007), the difference between the sephadex and control treatments (δ) was expressed as a fraction of μ so that $\delta = 0.01\mu$, 0.02μ, 0.04μ, 0.06μ, 0.08μ, 0.10μ, 0.12μ, 0.16μ, 0.20μ and 0.24μ and the expected means for the control, glass and sephadex treatments (following the trans-generational immune priming hypothesis) were $\mu-\delta/2$, μ, and $\mu+\delta/2$, respectively (reverse the signs for the trans-generational costs hypothesis). For example, for bead melanin cover, the mean was 74.1%, the standard deviation among family means was 17.9% and for $\delta = 0.10\mu$ the expected means for the control, glass and sephadex treatments (following the trans-generational immune priming hypothesis) were 70.4%, 74.1% and 77.8%, respectively. We used the rnorm() function in R to generate a sample of 40 family means for each treatment. For example, for the control treatment of bead melanin cover we used rnorm(40, 70.4, 17.9). We used a one-way ANOVA on the family means to test for significant differences among the bead inoculation treatments. We replicated this randomization 1000 times for each effect size and power was calculated as the proportion of ANOVAs that returned a p-value <0.05. For each of the six offspring traits, we compared the observed value of δ (expressed as a fraction of μ) with the power curve for that trait.

Literature comparison

We compared the effect size in our study with four studies showing trans-generational immune priming in *T. molitor* and *B. terrestris* (Fig. 1 in Moret and Schmid-Hempel 2001, Fig. 1 in Sadd et al. 2005, Fig. 3a–b in Moret 2006, and Fig. 1 and 2 in Sadd and Schmid-Hempel 2007) and one study showing within-generation immune priming in *T. molitor* (Fig. 1a–b in Moret and Siva-Jothy 2003). These five studies induced an immune response in female insects by inoculating them with lipopolysaccharides (LPS) or heat-killed bacteria relative to non-inoculated controls or individuals inoculated with immunologically inert Ringer solution. The immune responses measured were phenoloxidase activity (V_{max} values) and the zone of inhibition diameter (mm). None of the studies, except the one by Sadd and Schmid-Hempel (2007), reported means and standard errors in the text so we used the figures mentioned above and a ruler to measure these statistics and convert them to the appropriate units (V_{max} units for phenoloxidase activity and mm for zone of inhibition assay). We are confident in this approach because our measured estimates were very similar

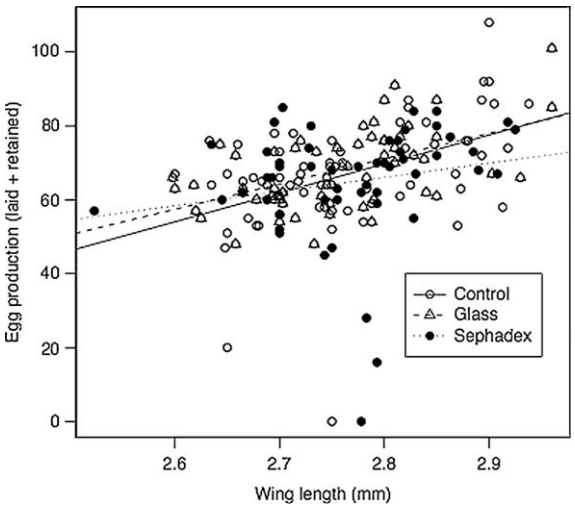

Figure 1. Egg production (number or eggs laid plus number of eggs retained in abdomen) increases with wing length in the control (n = 65), glass bead- (n = 60), and sephadex bead-inoculated mothers (n = 53). Shown are the lines of best fit for the three bead inoculation treatments.

to those given in Sadd and Schmid-Hempel (2007). For all four studies, we pooled the standard errors from the treatments to estimate the standard deviation within treatments (σ).

Similar to the power analysis, we calculated the effect size (δ) as the difference in the mean immune response between offspring from immune-primed and non-inoculated control mothers (we ignored offspring from the Ringer-inoculated procedural control mothers). Three of the five studies had an experimental design where the immune-priming and control treatments of interest were cross-factored with the levels of another factor that was not relevant to calculating δ. For these studies, we calculated the means for the immune-priming and control treatments by

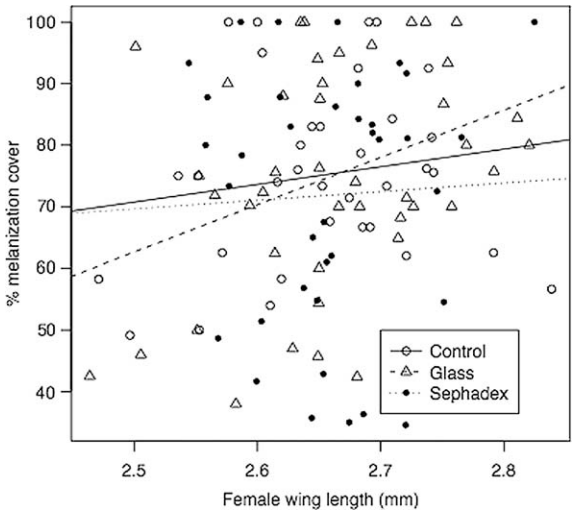

Figure 2. The % melanin cover of the sephadex bead increases with wing length for the daughters of the control (n = 34), glass bead- (n = 40), and sephadex bead-inoculated mothers (n = 37). Shown are the lines of best fit for the three bead inoculation treatments. The data are the means for each family (n = 111).

averaging the levels of the other, non-relevant factor. Hence in Moret (2006) we averaged the three F_1-immune treatments, in Moret and Siva-Jothy (2003) we averaged the 4 and 7 days post-challenge treatments, and in Sadd and Schmid-Hempel (2007) we averaged whole eggs and internal egg extracts and we averaged surrogate immune-primed and control mothers. Combining the data in this way also increased the sample sizes of the immune-priming and control treatments of interest.

We wanted to compare the mean effect size between the published literature and our study. In particular, we wanted to determine if our study contained sufficient replication to detect the effect sizes reported in the literature. To facilitate comparison among studies we standardized the effect size (δ) as a fraction of μ or σ. To compare the mean value of δ/μ (or δ/σ) between the literature and our study is further complicated because these values may be positive or negative. The trans-generational immune-priming hypothesis predicts positive values of δ whereas the trans-generational costs hypothesis predicts negative values of δ. Because we are interested in both hypotheses (i.e. we are interested in the magnitude of δ) we took the absolute value of δ/μ (or δ/σ) for each study and this allowed us to compare the mean effect size (regardless of its sign) between the literature and our study.

Results

Maternal treatment and sample sizes

The original sample sizes for the control, glass bead-, and sephadex bead-inoculated mothers were 66, 67 and 67, respectively. For eight sephadex mothers, the inoculation was not successful as the bead was subsequently spotted in the inoculation needle, so we excluded these females from the analysis. During the inoculation protocol, one control, two glass, and one sephadex mother escaped. Five days after inoculation, zero control, five glass and five sephadex, mothers had died. All 178 mothers that survived the five days after inoculation were capable of flight. We obtained egg production (the number of laid eggs plus the number of retained eggs) and wing length data for 65 control, 60 glass and 53 sephadex mothers. Of these 178 mothers, we selected $\sim$120 mothers that had laid enough eggs (>20) to produce reasonable numbers of offspring for measuring offspring fitness traits.

Maternal wing length, egg production and bead melanization

The mean ($\pm$SE) wing lengths of the control (2.76 ± 0.011 mm, $n = 65$), glass (2.75 ± 0.011 mm, $n = 60$), and sephadex mothers (2.77 ± 0.011 mm, $n = 53$) did not differ significantly ($F_{2,175} = 0.35$, $p = 0.706$). The mean ($\pm$SE) egg production (the number of laid eggs plus retained eggs) of the control (66.8 ± 1.92 eggs, $n = 65$), glass (68.1 ± 1.36 eggs, $n = 60$), and sephadex (64.8 ± 2.18 eggs, $n = 53$) mothers did not differ significantly ($F_{2,175} = 0.74$, $p = 0.478$). Similarly, an ANCOVA with wing length as a covariate found no effect of treatment on egg production (treatment $\times$ wing length interaction: $F_{2,172} = 0.93$, $p = 0.395$, treatment: $F_{2,172} = 0.86$, $p = 0.424$), although wing length itself was highly significant ($F_{1,172} = 29.58$, $p < 0.001$) with larger females producing more eggs (Fig. 1).

We recovered the bead and scored a melanization phenotype for 50 of the 53 sephadex bead-inoculated mothers and the mean ($\pm$SE) % melanin cover was $27 \pm 4.9\%$ and 38 of the 50 mothers (76%) melanized the bead at least a little. For the glass bead-inoculated females we recovered five glass beads from a sample of 11 individuals; as expected, none were melanized. For the sephadex-bead inoculated mothers, melanin cover correlated with neither wing length ($r = 0.01$, $t = 0.10$, $DF = 48$, $p = 0.921$) nor egg production ($r = 0.17$, $t = 1.19$, $DF = 48$, $p = 0.239$).

Summary information for the offspring

Of the 2098 hatched individuals, 1858 individuals (89%) emerged as adults with 877 females and 981 males. Offspring came from 120 different full-sib families with 1 to 20 offspring per family. We obtained wing length data for 825 females and 874 males. We scored a melanization phenotype for 713 females, of which 560 (79%) were still alive when we assessed them for their post-inoculation condition, and of which 477 were able to fly (67%). The mean ($\pm$SE) age of bead inoculation was 3.1 ± 0.01 days (range = 2.5 to 3.6 days) and the mean ($\pm$ SE) age at which females were assessed for their post-inoculation condition was 6.2 ± 0.01 days (range = 5.7 to 7.0 days).

Offspring fitness

The mean ($\pm$SE) % melanin cover in the daughters was not significantly different among the control ($75.1 \pm 2.52\%$), glass bead-inoculated ($75.2 \pm 2.82\%$), and sephadex bead-inoculated ($71.8 \pm 3.41\%$) mothers (Table 1, 2). None of the other offspring fitness traits were affected by the maternal bead inoculation treatment (Table 1, 2). Males emerged earlier (8.28 ± 0.032 days; mean $\pm$ SE) and at smaller body sizes (2.18 ± 0.006 mm) than females (8.98 ± 0.033 days, 2.66 ± 0.006 mm; Table 2). Block had a significant effect on all traits (except % melanin cover) when it was modeled as a nominal factor (Table 2), but there was no effect of block when it was modeled as a continuous covariate describing the age of the eggs when they were hydrated (i.e. 18, 24, 39 and 45 days after oviposition for blocks 1, 2 3 and 4, respectively).

Offspring bead melanization

We recovered the bead and scored a melanization phenotype for 713 offspring and the mean ($\pm$SE) % melanin cover was $73 \pm 1.3\%$, which was almost three times greater than that of the maternal generation; 685 of the 713 daughters (96%) melanized the bead at least a little. For % melanin cover, the minimal adequate model contained wing length ($\chi^2 = 5.76$, $DF = 1$, $p = 0.016$) and post-inoculation condition ($\chi^2 = 4.93$, $DF = 1$, $p = 0.026$). For the 153 daughters that died before their post-inoculation conditions were assessed, bead melanization may not have had enough

Table 1. Means ± SE for six offspring traits: survival (%), bead melanization (%), female and male emergence time (days), and female and male wing length (mm) for three bead inoculation treatments: control, glass and sephadex. The sample sizes (number of full-sib families) are shown in brackets.

Trait	Control	Glass bead	Sephadex bead
Survival	83.00 ± 3.671 (39)	86.14 ± 3.124 (40)	87.33 ± 3.370 (39)
Melanization	75.13 ± 2.520 (34)	75.18 ± 2.818 (40)	71.84 ± 3.414 (37)
Female emerge	9.01 ± 0.064 (37)	8.96 ± 0.071 (40)	8.96 ± 0.056 (37)
Male emerge	8.28 ± 0.047 (38)	8.26 ± 0.055 (38)	8.29 ± 0.063 (37)
Female wing	2.66 ± 0.014 (36)	2.66 ± 0.012 (40)	2.66 ± 0.011 (37)
Male wing	2.19 ± 0.008 (38)	2.18 ± 0.009 (38)	2.19 ± 0.008 (37)

time to reach its maximal value. After removing these 153 daughters, the effect of wing length was still significant ($\chi^2 = 5.28$, DF = 1, $p = 0.022$) but the post-inoculation effect was not (i.e. there was no difference in bead melanization between daughters capable of walking, jumping or flying). The % melanin cover increased with wing length (slope ± SE = 32.4 ± 14.08% melanin mm^{-1}; Fig. 2). The other terms – maternal bead inoculation treatment, block, plate, age at inoculation, age at which post-inoculation condition was assessed, and emergence time – had no effect on % melanin cover.

Heritability of traits

Family accounted for a significant portion of the phenotypic variance for all of the six offspring traits (Table 3). Block and tissue culture plates never accounted for more than 6% and 4%, respectively, of the phenotypic variance. The full-sib heritabilities (h^2) for survivorship and bead melanization were 0.39 and 0.20, respectively. For males and females, the full-sib heritabilities were similar for emergence time (0.22, 0.38) and wing length (0.72, 0.69). The mother–offspring heritabilities for wing length (Fig. 3) were also highly significant for sons ($h^2 = 0.64$, $F_{1,103} = 39.23$, $p < 0.0001$) and daughters ($h^2 = 0.72$, $F_{1,103} = 21.63$, $p < 0.0001$). For the sephadex bead-inoculated mothers, the mother–offspring heritability of % melanin cover ($h^2 = 0.23$) was similar to the full-sib heritability ($h^2 = 0.20$) but was not statistically significant ($F_{1,31} = 1.15$, $p = 0.291$).

Power analysis

For survival and bead melanization, the power analysis found that we would have had a >90% chance of getting a significant p-value ($p < 0.05$) if the difference between the sephadex and control treatments (δ) was 0.20μ, where μ is the trait mean (Fig. 4; note δ is written as 'Delta' in Fig. 4). For female and male emergence and female and male wing length, we would have had ~100% chance of detecting statistical significance even if δ was as small as 0.05μ (Fig. 4). For survival and bead melanization, the observed differences between the control and sephadex treatments (the triangles in Fig. 4) represent ~5% of the mean and our power analysis suggests that our experiment had a 10% chance of detecting statistical significance. For emergence time and wing length, the observed δ values represent <1% of the mean and our experiment had a 5% chance of detecting statistical significance (i.e. the probability of committing a type I error).

Literature comparison

After standardizing the absolute value of the effect size as a percentage of the mean, the average value of $100 \times |\delta|/\mu$ in the published literature (45.64%) is 26 times greater than the average of the six traits in our study (1.75%; Table 4). For the five studies that found a significant effect of immune-challenge on anti-bacterial activity using the zone of inhibition assay, the average value of $100 \times |\delta|/\mu$ was 66.23% (Table 4).

Table 2. Two or four-way ANOVA on the family means of four offspring fitness traits: survival to adulthood, time to emergence, wing length, and % melanin cover. Fixed factors (Factor) include bead inoculation treatment, block, full-sib family, and sex. Also shown are the number of family means (N), number of offspring per family mean (n), range of offspring per family mean (Range), the numerator (DF1) and denominator degrees of freedom (DF2) of the F-statistic (F), and the p-value (p). Significant results are given in bold-face.

Trait	N	n	Range	Factor	DF1	DF2	F	p
Survival to adulthood†	120	17.4	1–24	treat	2	114	0.42	0.658
				block	3	114	6.15	**<0.001**
Time to emergence‡	113	8.2	1–20	treat	2	108	0.38	0.541
				block	3	108	19.35	**<0.001**
				family	112	108	2.31	**<0.001**
				sex	1	108	408.69	**<0.001**
Wing length‡	113	7.5	1–20	treat	2	107	1.55	0.217
				block	3	107	22.44	**<0.001**
				family	112	107	6.10	**<0.001**
				sex	1	107	6948.14	**<0.001**
% Melanin cover‡	111	6.4	1–18	treat	2	105	0.80	0.452
				block	3	105	0.69	0.559

†family means weighted by number of offspring per family (n).
‡family means weighted by square root of n.

Table 3. Variance components analysis of six offspring fitness traits: survival, % melanin cover, female and male emergence time, and female and male wing length. The variance is partitioned into four components (Comp): blocks, full-sib families, tissue culture plates, and individuals. Shown are the variance components (Var), their % contribution to the total variance (%), the χ^2-statistic from comparing nested models, and the p-value (p). Significant results are given in bold face.

	Survival				% melanin cover			
Comp	Var	%	χ^2	p	Var	%	χ^2	p
Block	0.00395	3.8	6.32	**0.012**	0.00	0.0	0.00	1.00
Family	0.02038	19.6	62.35	**<0.001**	112.31	9.8	15.93	**<0.001**
Plate	0.00030	0.3	0.06	0.802	0.00	0.0	0.00	1.00
Individ	0.07939	76.3			1036.50	90.2		
Total	0.10403	100.0			1148.81	100.0		
	Female emergence time (days)				Male emergence time (days)			
Comp	Var	%	χ^2	p	Var	%	χ^2	p
Block	0.01203	2.9	2.66	0.103	0.02335	6.3	15.18	**<0.001**
Family	0.08010	19.2	33.80	**<0.001**	0.04087	11.1	19.77	**<0.001**
Plate	0.00000	0.0	0.00	1.000	0.00000	0.0	0.00	1.000
Individ	0.32433	77.9			0.30387	82.6		
Total	0.41646	100.0			0.36808	100.0		
	Female wing length (mm)				Male wing length (mm)			
Comp	Var	%	χ^2	p	Var	%	χ^2	p
Block	0.00061	5.2	3.70	0.054	0.00004	0.8	0.00	1.000
Family	0.00413	34.7	51.04	**<0.001**	0.00206	35.9	82.38	**<0.001**
Plate	0.00056	4.7	3.61	0.057	0.00000	0.0	0.00	1.000
Individ	0.00659	55.4			0.00363	63.4		
Total	0.01189	100.0			0.00573	100.0		

Discussion

We found no support for the main hypothesis of this study, namely, that manipulating the maternal immune environment would either enhance or reduce offspring fitness. Under the trans-generational immune priming hypothesis we expected daughters from sephadex bead inoculated mothers to have a stronger melanization response than daughters from glass bead inoculated and control mothers. Instead, the % melanin cover in daughters from sephadex mothers (72%) was slightly lower than that in daughters from glass (75%) and control mothers (75%). Under the trans-generational immune costs hypothesis, we expected offspring from sephadex mothers to have lower fitness than offspring from glass and control mothers. Our study therefore suggests that manipulating the maternal melanization response has no fitness costs or benefits for the offspring. Our measure of fitness cost measures only direct maternal effects on individuals and it is possible that costs might be expressed in more competitive environments.

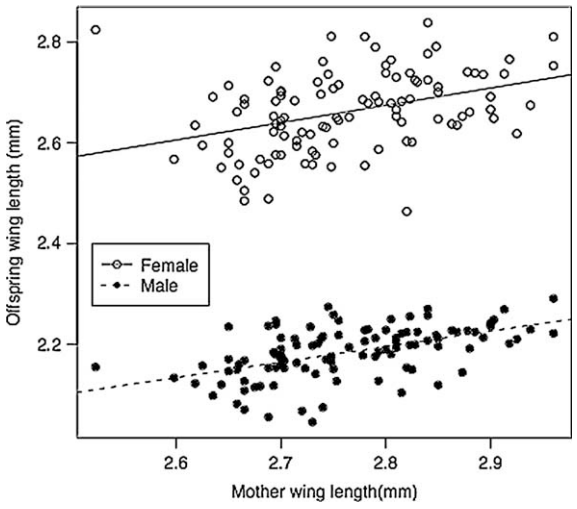

Figure 3. There is a strong positive relationship between a mother's wing length (mm) and that of her sons and daughters. The data for the offspring are the means for each family (n = 105).

In contrast, maternal exposure to heat-killed bacteria in the bumblebee *Bombus terrestris* (Sadd et al. 2005), or to bacterial lipopolysaccharides (LPS) in the mealworm beetle *Tenebrio molitor* (Moret 2006), increased the anti-bacterial activity of their offspring as measured by a zone of inhibition assay. These studies found strong evidence for adaptive trans-generational immune priming of antibacterial activity. In contrast, maternal exposure to LPS in *T. molitor* had no effect on the phenoloxidase (PO) activity of their offspring (Moret 2006) and a study testing within-generational immune priming in *T. molitor* (Moret and Siva-Jothy 2003) found that exposure to LPS increased anti-bacterial but not PO activity. Similarly in *B. terrestris*, inoculating female workers with LPS increased their anti-bacterial activity but actually decreased their PO activity (Moret and Schmid-Hempel 2001). These findings are relevant to the present study because PO plays a critical role in the innate immune response of insects including the melanization response that we assayed (Christensen et al. 2005). Perhaps the induction of the melanization response to foreign particles such as sephadex beads or parasites such as malaria is swift enough that there is little adaptive value to additional priming by an individual's mother. This conclusion is at odds with the demonstration in *B. terrestris* that males reared by female workers exposed to LPS increased their PO activity (Moret and Schmid-Hempel

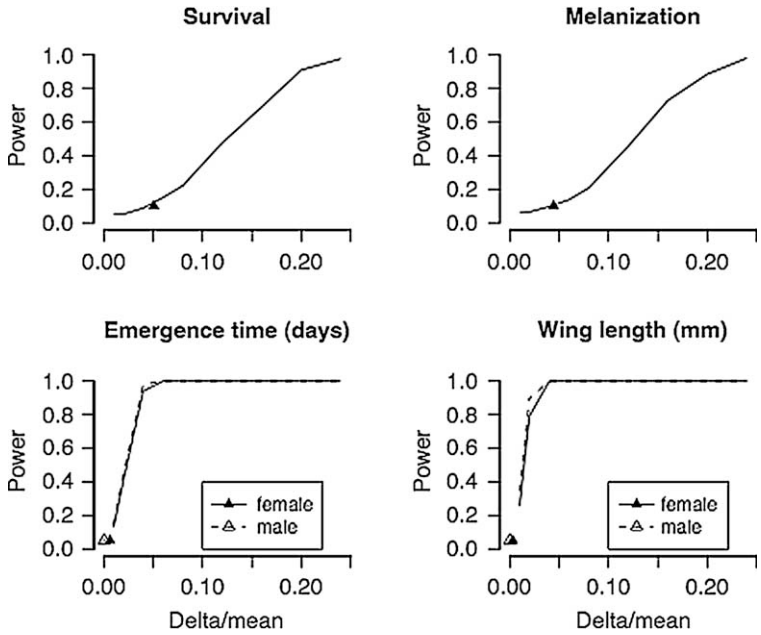

Figure 4. The power of our experiment to detect a statistically significant effect of manipulating the maternal immune response on each of the six offspring fitness traits: survival, bead melanization, male and female emergence time, and male and female wing length, depends on the difference in mean phenotype (Delta) between the sephadex bead and control treatments. Here Delta is expressed as a fraction of the mean. The triangles indicate the observed value of Delta/mean in our experiments.

2001) and with density dependent prophylaxis (DDP) in the Lepidoptera where larvae reared at high density invest more in immunity including PO activity (Wilson et al. 2001, Cotter et al. 2004). DDP is adaptive because density-dependent transmission of pathogens means that larvae at high density are more likely to encounter pathogens than larvae at low density (Wilson and Reeson 1998). Hence the *B. terrestris* example and the DDP literature suggest that it is adaptive to prime the melanization response before encountering parasites.

Table 4. A literature survey found that the mean effect size of immune priming in five published studies was much greater than that in our study. Studies 1A and 1B from Fig. 3a–b in Moret (2006), 2A and 2B from Fig. 1a–b in Moret and Siva Jothy (2003), 3A and 3C are male and 3B and 3D are female PO and antibacterial activity from Fig. 1 in Moret and Schmid-Hempel (2001), 4 from Fig. 1 in Sadd, Kleinlogel, Schmid-Hempel and Schmid-Hempel (2005), and 5A and 5B from Fig. 1 and 2 in Sadd and Schmid-Hempel (2007). The traits are abbreviated: PO = phenoloxidase activity, antibac = antibacterial activity, surv = % survival, melan = % melanization, f.emrg and m.emrg = female and male emergence time, f.wing and m.wing = female and male wing length. Shown are the samples sizes for the control (n_1) and immune-challenged treatments (n_2), the grand mean (μ), the standard deviation (σ), the difference between the immune-challenged and control means (δ), the absolute value of δ expressed as a percentage of the mean ($100 \times |\delta|/\mu$) or in SD ($|\delta|/\sigma$), and whether there was a significant effect of priming the immune response in the study (NS = not significant).

Study	Trait	n_1, n_2	μ	σ	δ	$100 \times \lvert\delta\rvert/\mu$	$\lvert\delta\rvert/\sigma$	p
1A	PO	18, 18	765.67	144.88	−48.67	6.36	0.34	NS
2A	PO	12, 12	364.55	138.98	−64.48	17.69	0.46	NS
3A	PO	11, 11	665.45	300.42†	370.91	55.74	1.23	**<0.02**
3B	PO	11, 11	954.55	349.96†	−221.82	23.24	0.63	**0.005**
1B	antibac	18, 18	9.67	6.15	5.72	59.17	0.93	**0.013**
2B	antibac	12, 12	3.27	2.43	4.71	144.18	1.94	**<0.001**
3C	antibac	11, 11	7.61	1.82†	0.13	1.67	0.07	NS
3D	antibac	11, 11	8.12	1.94†	3.18	39.19	1.64	**<0.02**
4	antibac	5, 5	11.60	2.65	5.13	44.23	1.94	**0.044**
5A	antibac	29, 28	8.03	3.17	5.42	67.47	1.71	**0.003††**
5B	antibac	10, 13	11.82	2.06	5.10	43.14	2.48	**<0.001**
Mean						45.64	1.22	
6	surv	39, 39	85.49	21.18	−4.34	5.07	0.20	NS
6	melan	34, 37	74.05	17.92	3.29	4.44	0.18	NS
6	f.emrg	37, 37	8.98	0.39	0.06	0.63	0.14	NS
6	m.emrg	38, 37	8.28	0.34	0.00	0.05	0.01	NS
6	f.wing	36, 37	2.66	0.08	−0.01	0.30	0.11	NS
6	m.wing	38, 37	2.18	0.05	0.00	0.01	0.01	NS
Mean						1.75	0.11	

† = σ is an underestimate because treatments had paired replicates.
†† = reported the least significant p-value between whole eggs ($p < 0.001$) and internal egg extracts ($p = 0.003$).

Adaptive trans-generational immune priming is expected to evolve when mothers and their offspring covary positively in the probability of encountering the same pathogen (Little and Kraaijeveld 2004). How does a sephadex bead mimic a pathogen and should we expect trans-generational immune priming? The melanization response to sephadex beads and the melanization response to malaria parasites share the same genetic mechanism (Gorman et al. 1996, Gorman and Paskewitz 1997). We therefore argue that we chose a biologically relevant immune response that is a suitable candidate for studying trans-generational immune priming. In some cases, acquired immunity in invertebrates is specific for the parasite strain (Little et al. 2003, Sadd and Schmid-Hempel 2006). How can a sephadex bead mimic the antigenic variety and specificity of a real parasite? Previous work on *Aedes aegypti* has shown that the strength of the melanization response depends on the type of bead used and that different beads can be thought of as different parasite antigens (Schwartz and Koella 2004). Furthermore acquired immunity in invertebrates can also provide broad-spectrum protection from pathogens. For example, *T. molitor* individuals exposed to *Escherichia coli*-derived LPS are also protected from subsequent fungal infections (Moret and Siva-Jothy 2003). Similarly, *T. molitor* individuals exposed to *E. coli*-derived LPS had offspring with reduced activity of *Arthrobacter globiformis* bacteria (Moret 2006). Hence acquired immunity and trans-generational immune priming involves both specific and more general immune responses.

The average magnitude of the difference in the mean immune response between the immune-challenged and control treatments for the six traits in our study was 26 times smaller than that in the published literature. For the five studies that found a significant effect of immune-challenge on anti-bacterial activity using the zone of inhibition assay, the average value of $100 \times |\delta|/\mu$ was 66.23% (Table 4). The power analysis found that our experiment had an 80% chance of detecting a statistically significant effect of maternal immune-challenge on offspring bead melanization when the difference between sephadex and control treatments was as little as 20% of the mean. In other words, if our experimental manipulation generated an effect size that is three times smaller than the published literature we would have detected a statistically significant effect of immune-challenge on bead melanization. Hence the absence of a significant treatment effect in our study is not due to a lack of replication, but rather to effect sizes that are exceedingly small compared to what has been reported in the literature (triangles in Fig. 4).

In our study, the percentage of mothers (76%) and daughters (96%) that melanized the C-25 sephadex bead was relatively high compared to other studies, where usually less than half of females did so (Boete et al. 2002, Koella and Boete 2002, Schwartz and Koella 2004). The mean % melanin cover of the daughters (73%) was almost three times higher than that of their mothers (27%). Previous studies on *A. gambiae* (Chun et al. 1995, Schwartz and Koella 2002) and *A. stephensi* (Koella and Sorensen 2002) showed that a blood meal increases the melanization response. There were no methodological differences in rearing the mothers and the daughters. Hence the only explanation that we have for the difference in bead melanization between the mothers and the daughters is that, in contrast to anopheline mosquitoes, a blood meal decreases the melanization response in *A. aegypti*. In *A. aegypti* the first blood meal can double a female's protein reserves (Briegel 1990b) and this may account for the differences between the mothers and daughters in survival (92% vs 79%) and flight recovery (92% vs 67%) following bead inoculation.

In a previous experiment, *A. aegypti* females that melanized a C-25 bead laid fewer eggs than those that did not melanize the bead (Schwartz and Koella 2004). Similarly in *A. gambiae*, melanization of sephadex C-25 or G-25 beads triggered follicular apoptosis and reduced fecundity relative to saline-inoculated controls (Ahmed and Hurd 2006). Both of these experiments suggest a fecundity cost of melanization. In our experiment, there was no evidence of such a cost in the mothers as egg production was similar in the three bead inoculation treatments (Fig. 1). There was also no evidence that the stress of bead inoculation or bead melanization in the mothers had any effect on the quality of their offspring (Table 2). Immunity costs are more likely to be detected in harsher environments where resources are limiting. Thus activating the immune system in *B. terrestris* with beads and/or LPS only reduces survival under starvation conditions (Moret and Schmid-Hempel 2000). Similarly and the costs of successful defense against parasitoids are more apparent in starved or dessicated *D. melanogaster* (Hoang 2001). Our protocol of rearing larvae individually rather than en masse and allowing females to blood feed for one hour rather than five minutes may have eliminated immunity costs.

After controlling for differences among the four temporal blocks, the variance among full-sib families was statistically significant for all six offspring traits (Table 3). Differences among full-sib families accounted for 10% of the phenotypic variance in bead melanin cover and male emergence time, 20% in female emergence time and survival, and 35% in male and female wing length (Table 3). The mother–offspring wing length heritability estimates (Fig. 3) for males ($h^2 = 0.64$) and females ($h^2 = 0.72$) were similar to the full-sib estimates ($h^2 = 0.72$ and 0.69, respectively) suggesting that dominance effects (included in full-sib but not mother–offspring estimates) are not important (Falconer and Mackay 1996). Life history traits such as emergence time and survival are closely related to fitness, are under strong selection and tend to have less genetic variation and lower heritability estimates than morphological traits such as wing length (Roff 1997), a pattern supported in this study. The low heritability of bead melanin cover is probably due to the fact that there is more 'measurement error' associated with scoring this phenotype than measuring phenotypes such as wing length or emergence time. Detecting significant heritability estimates for measurement error prone traits such as bead melanin cover further supports our contention that our experiment contained sufficient replication to detect the effect sizes reported in the literature.

We found evidence for a positive genetic correlation between the melanization response and female body size. A previous study suggested a similar relationship in *A. gambiae* although in that study variation in female body size was confounded with larval nutrition (Suwanchaichinda and

Paskewitz 1998). Likewise, a selection experiment on age at pupation in *A. aegypti* found that slowly developing lines emerged at larger body sizes and had a stronger melanization response than the smaller, fast-developing lines (Koella and Boete 2002). In *A. aegypti* and anopheline mosquitoes, teneral reserves are largely determined by body size at eclosion with larger females containing almost four times more protein than the smaller ones (Briegel 1990a, 1990b). Hence larger females may have had more available resources for mounting an immune response. Selection for *Plasmodium* resistance in *A. aegypti* found that refractory mosquitoes were significantly smaller than the susceptible ones (Yan et al. 1997). This study suggests that *Plasmodium*-mediated selection on the melanization response would select for larger females in our population of *A. aegypti*.

In conclusion, although our study was powerful enough to detect significant full-sib heritabilities for all six offspring traits: bead melanization, survival, male and female age at emergence and male and female wing length, we found that manipulating the maternal melanization response in female *A. aegypti* mosquitoes had no effect on the fitness of their offspring. A power analysis found that our study would have detected trans-generational immune priming even if the effect size was 1/3 of what has been reported in the literature. That trans- or within-generational effects have been reported for antibacterial activity (Moret and Siva-Jothy 2003, Sadd et al. 2005, Moret 2006) but not for phenoloxidase activity (Moret and Siva-Jothy 2003, Moret 2006) suggests that immune priming may be more important for some immune traits than others.

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