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Trans-generational immune priming is constrained by the maternal immune response in an insect

Jérôme Moreau, Guillaume Martinaud, Jean-Phillipe Troussard, Caroline Zanchi and Yannick Moret

J. Moreau, G. Martinaud, J.-P. Troussard, C. Zanchi and Y. Moret (yannick.moret@u-bourgogne.fr), Équipe Écologie Évolutive, UMR CNRS 6282 Biogéosciences, Université de Bourgogne, 6 Bd Gabriel, FR-21000 Dijon, France.

Immune-challenged vertebrate and invertebrate females can transfer immunity to their offspring. This trans-generational immune priming (TGIP) is beneficial for the offspring if the maternal infection risk persists across generations. However, because immunity is costly, fitness consequences of TGIP have been found in primed offspring. Furthermore, transferring immunity to offspring may be costly for immune-challenged females who are also carrying the costs of their immune response. A negative relationship between levels of immunity between mothers and offspring might therefore be expected. Consistent with this hypothesis, we show that in the insect, *Tenebrio molitor*, the magnitude of antibacterial immune response of immune-challenged females negatively correlates with levels of antibacterial activity of their eggs. This negative relationship was only present in small females that are inherently of lower quality. Furthermore, female body size did not affect immune responsiveness to the challenge, indicating that small females favoured their immunity at the expenses of that of their eggs.

Trans-generational immune priming (TGIP) corresponds to the plastic adjustment of offspring immunity as a result of maternal immune experience. When pathogens become prevalent in the maternal environment and offspring are likely to experience the same conditions, mothers will benefit from transferring levels of immunity to their offspring. TGIP has been demonstrated in both vertebrates (Hasselquist and Nilsson 2008) and invertebrates (Little et al. 2003, Sadd et al. 2005, Moret 2006, Sadd and Schmid-Hempel 2007, Roth et al. 2010, Zanchi et al. 2011). However, there are a few cases where TGIP in invertebrates has not been found (Vorburger et al. 2008, Linder and Promislow 2009), suggesting that this phenomenon cannot be generalised across host species and/or pathogens, probably because of its cost under specific ecological conditions.

While TGIP is beneficial when the maternal infection persists over the next generation (Roth et al. 2010), its inducible aspect suggests it is also costly. In the absence of costs, primed levels of immunity would be expected across all offspring, independently of the maternal experience. Since immunity is costly (Schmid-Hempel 2003), enhanced immunity in offspring through TGIP should have a selective disadvantage if infection risks do not persist over the maternal generation (Sadd and Schmid-Hempel 2009). In line with this, related fitness consequences of TGIP in insects have been found in primed offspring (Sadd and Schmid-Hempel 2009, Roth et al. 2010, Zanchi et al. 2011). In addition to paying the usual immune activation costs (Moret and Schmid-Hempel 2000), immune-challenged females are also expected to pay a cost to TGIP when producing and transferring immune products to the offspring. Yet, such a cost has never been demonstrated. The reason for this may come from the difficulty in distinguishing between the costs associated with the immune response and the costs associated with the maternal transfer of immunity to the offspring. In insects, maternal protection induced by maternal challenge is initiated as early on as the egg stage, with the transfer of maternal immune effectors to the eggs (Sadd and Schmid-Hempel 2007). If transferring immunity to the eggs is costly, then there will be a tradeoff between the female's immunity after infection and the immunity of the female's eggs.

Here, we used the yellow mealworm beetle, *Tenebrio molitor*, to investigate costs associated with the maternal transfer of immunity to the eggs. *T. molitor* is a stock pest insect characterised by overlapping generations and relatively low dispersal, which should favour persistence of infections across generations. In line with this, higher levels of immune activity have been shown as a trans-generational effect in the offspring of this species when parents received a bacterial immune challenge (Moret 2006, Zanchi et al. 2011). In this study, we first tested whether females exposed to a bacterial immune challenge transfer antibacterial protection to their eggs. We then examined the relationship

between the antibacterial activity in the haemolymph of mothers and that of her eggs. If the transfer of immunity to the eggs is costly, we then predict a negative relationship between antibacterial activity of the female and that of her eggs. If this tradeoff exists, we may also predict that its expression will depend on individual female quality (Reznick et al. 2000).

Methods

Experimental design

Age controlled virgin beetles (8 days ± 1 day post emergence) were obtained from pupae collected from stock cultures maintained at 25°C with ad libitum supply of food and water.

We mimicked a bacterial infection in virgin females by a single injection of 5 µl of Ringer's solution containing non-purified lipopolysaccharides (LPS: 0.5 mg ml^{-1}) extracted from *Escherichia coli* (Sigma: L8274). Non-purified LPS contain contaminating peptidoglycan fragments in addition of LPS (Haine et al. 2008a). Together, these molecules elicit a general and persistent production of antibacterial peptides over many days (Haine et al. 2008a) that is associated to microbial resistance (Haine et al. 2008b). A group of females were treated in the same way, but with the omission of LPS as a procedural control (control females). All injections were made through the pleural membrane between the second and the third abdominal tergites, using sterilized pulled glass capillaries after immobilisation of the insects on ice for 10 min.

Immediately after their immune treatment, females (30 per group treatment) were paired with a virgin and unchallenged male for four days in a Petri dish provided with bleach flour and ad libitum food and water under standard laboratory conditions (25°C, 70% RH, L12h:D12h). In general, the number of eggs laid during this first few days represents 50% of the total number of eggs laid by females during their adult life (unpubl.). On the 4th day post maternal challenge, eggs were searched by sieving the flour (Ø 600 µm) of the Petri dish, counted to estimate female fecundity, placed in pairs into micro-centrifuge tubes and stored at −80°C prior to measurement of their antibacterial activity (measured on two pairs of eggs taken at random). At the same time, each female provided a 5 µl sample of haemolymph flushed into a micro-centrifuge tube containing 25 µl of cold sodium cacodylate/$CaCl_2$ buffer (0.01 M sodium cacodilate, 0.005 M $CaCl_2$, pH 6.5, at 4°C) for measurement of its antibacterial activity (Moret 2006). Females were then freeze-killed and used to estimate their body size by measuring the length of the left elytra with a digital calliper (precision ± 0.02 mm) to have an indication of their individual quality since this parameter often predicts reproductive investment in insects (Thornhill and Alcock 1983).

Antibacterial activity of the haemolymph of females was measured on zone of inhibition plates seeded with *Arthrobacter globiformis* (Pasteur institute CIP 105365) as described in Moret (2006).

To measure antibacterial activity of the eggs, two random pairs of eggs per females were thawed on ice, suspended in 5 µl of sodium phosphate buffer (PBS: 8.74 g NaCl, 1.78 g Na_2HPO_4 $2H_2O$, 1000 ml distilled water, pH 6.5), homogenized using a pestle and subsequently centrifuged (10 000 rpm, 4°C, 5 min). Two µl of the supernatant were used to measure antibacterial activity using the antibacterial assay described in Moret (2006). The data point for each female corresponds to the mean of the zones of inhibition of two pairs of eggs. In this experiment we did not measured egg size. Therefore, antibacterial activity of the eggs measured as described above may either depend on the concentration of antibacterial products transferred to the eggs or egg size. However, preliminary data showed non-significant variation of egg volume according to maternal challenge and mother body size (Supplementary material Appendix 1). Therefore, variation in antibacterial activity measured in this experiment should be unrelated to egg size.

Statistics

Data on antibacterial activity of female's haemolymph and eggs and fecundity were appropriately transformed when necessary to homogenize the variance and analysed using ANCOVAs with maternal immune challenge as factor and female body size as a covariate.

To test for potential tradeoffs between immunity transferred to the eggs and immunity and/or fecundity of mothers, variation of antibacterial activity of the eggs was then further examined by including antibacterial activity and fecundity of mothers as covariates in addition of maternal immune challenge and female body size. Since both mother antibacterial activity and mother fecundity were significantly affected by the maternal immune treatment, their respective effect on egg antibacterial activity was investigated within the maternal immune treatment. This analysis used type III sum of square calculations and potential multicollinearity between covariates was checked using type I calculations of sum of square by alternating the entry of each covariate at first (Quinn and Keough 2002). Detection of outlier data points used a Grubbs's test (1969). For all analyses, the best statistical model was searched for using a stepwise backward procedure from an initial model that included all main effects and interactions. All data were analysed using SPSS 11 for Mac.

Results

One control and three immune-challenged females did not lay eggs and were consequently removed from the data analyses.

As expected, immune-challenged females had more antibacterial activity in their haemolymph than controls (Fig. 1, $F_{1,52} = 31.34$, $p < 0.001$) and there was no effect of female body size ($F_{1,52} = 0.17$, $p = 0.684$).

The strong positive correlation between female fecundity and body size (Fig. 2a, $F_{1,51} = 25.50$, $p < 0.001$) confirmed that the latter was a good estimator of female quality. The maternal immune challenge affected fecundity (Fig. 2a, $F_{1,51} = 5.18$, $p = 0.027$) in a size-dependent manner (Fig. 2a, $F_{1,51} = 5.89$, $p = 0.019$), suggesting a cost of the immune-challenge relatively larger for large females.

Eggs of immune-challenged females had higher levels of antibacterial activity than those of control mothers (Fig. 2b,

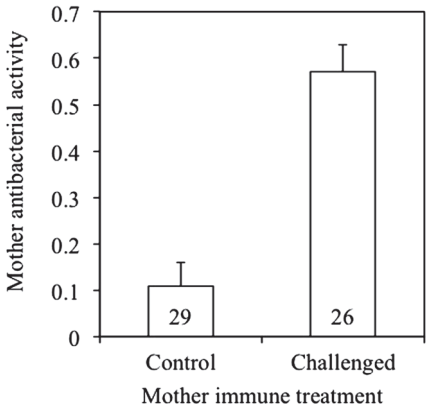

Figure 1. Antibacterial activity (mean + 1 SE) expressed as the natural logarithm value of the diameter (in cm) of a zone of bacterial growth inhibition of the haemolymph of control and immune-challenged mothers. Numbers inside bars represent the number of mothers assayed.

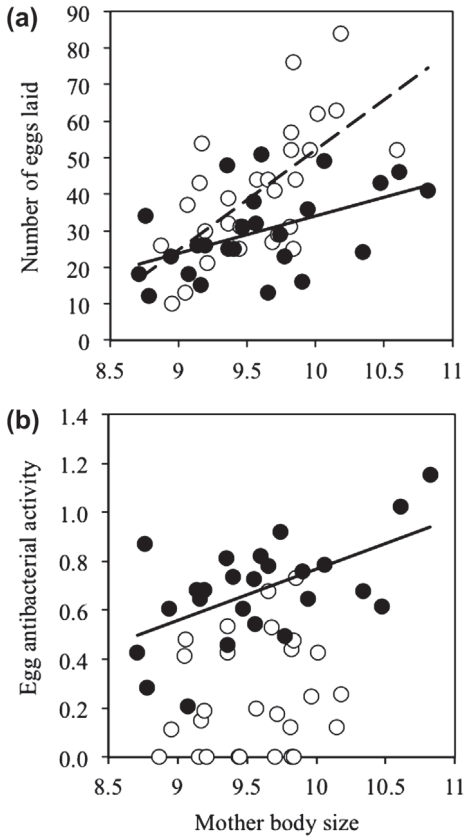

Figure 2. Variation of (a) female fecundity expressed as the number of eggs laid within the four days period by each female and (b) egg antibacterial activity expressed as the natural logarithm value of the diameter (in cm) of a zone of bacterial growth inhibition of 2 pairs of eggs as a function of body size estimated by the length of the left elytra (in mm) of control (open circles) and immune-challenged (filled circles) females. Fecundity positively covaries with both control (dashed line, $Y = -221.26 + 27.38 \times$ mother body size [mm], $R^2 = 0.41$, $F_{1,27} = 18.70$, $p < 0.001$) and immune-challenged mother body size (solid line, $Y = -61.40 + 9.61 \times$ mother body size [mm], $R^2 = 0.20$, $F_{1,24} = 5.88$, $p = 0.023$). Antibacterial activity of the eggs positively covaries with body size of mothers among immune-challenged mothers (solid line, $Y = -1.26 + 0.20 \times$ mother body size [mm], $R^2 = 0.30$, $F_{1,24} = 10.23$, $p = 0.004$), but not among control mothers ($F_{1,27} = 0.07$, $p = 0.788$).

ANCOVA $F_{1,52} = 59.26$, $p < 0.001$). Furthermore, large females transferred more antibacterial activity to their eggs than small ones (Fig. 2b, $F_{1,52} = 5.42$, $p = 0.024$).

Antibacterial activity of the eggs was significantly correlated with that of mothers within each maternal immune treatment group (Table 1) and this relationship was dependent on female body size as shown by the significant interaction term between female body size and maternal antibacterial activity (Table 1). For an illustrative purpose, we have artificially categorized large (> median 9.51 mm) and small (≤ median 9.51 mm) females to produce the Fig. 3. Among the control mother group, antibacterial activity of the eggs was positively dependent on that of mothers, especially for large mothers. This relationship is explained by the relatively low antibacterial activity of the eggs of control females except that of four relatively large females, which were also those having antibacterial activity in their haemolymph (Fig. 3a). Among the immune-challenged mother group, antibacterial activity of the eggs was negatively correlated on that of mothers (Fig. 3b, Table 1). However, as shown by the Fig. 3b, this tradeoff between the mothers's own immunity and the immunity of their eggs was present only in small females. Statistical analysis that used female body size as a discrete descriptive variable (instead of covariate) as define above provided similar results (results not shown).

Discussion

Using a bacterially based benign immune challenge, we found that *Tenebrio molitor* females transferred levels of antibacterial activity to their eggs, supporting previous results in another insect model (Sadd and Schmid-Hempel 2007). Transfer of immunity to the eggs was negatively correlated to the maternal immune response, suggesting that there is a tradeoff between the immunity of mothers and this of their eggs. However, the magnitude of this tradeoff was dependent on female body size. Indeed, the negative relationship between antibacterial activity of immune-challenged mothers and eggs was mainly significant in small females, suggesting that the cost of transferring antibacterial activity to the eggs was lower for large females. Furthermore, body

Table 1. Results of the ANCOVA examining variation of the antibacterial activity of the eggs as a function of the maternal immune challenge (Challenge – fixed factor), maternal antimicrobial activity in the haemolymph (Maternal response – covariate nested within Challenge), mother body size (Size – covariate) and fecundity (covariate nested within Challenged, which was not retained by the stepwise procedure).

Source	F	DF	p
Global model	22.10	6,48	< 0.001
Challenge	60.96	1,48	< 0.001
Size	0.79	1,48	0.379
Maternal response (Challenge)	7.23	2,48	0.002
Size × Maternal response (Challenge)	6.73	2,48	0.003

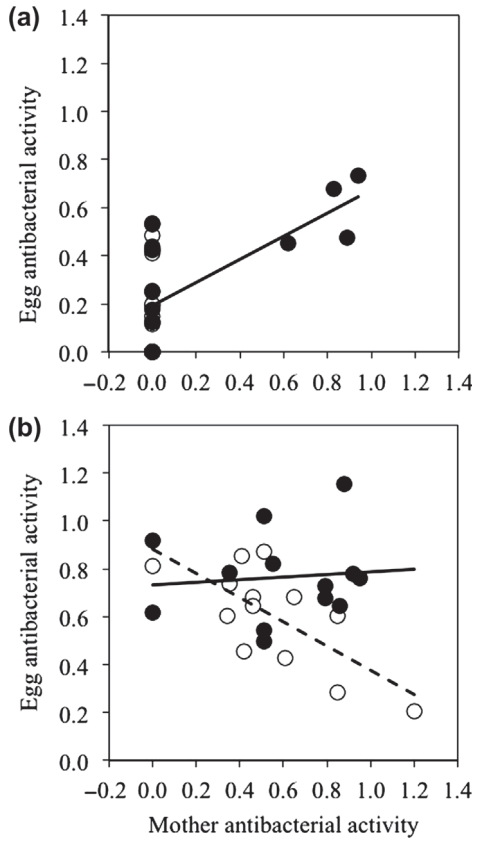

Figure 3. Relationships between the antibacterial activity expressed as the natural logarithm value of the diameter (in mm) of a zone of bacterial growth inhibition of the eggs and that of the haemolymph of large (> median 9.51 mm: filled circles) and small (≤ median 9.51 mm: open circle) (a) control and (b) immune-challenged mothers (see text for details). (a) Antibacterial activity of the eggs covaries positively with that of the haemolymph of large control mothers ($Y = 0.19 + 0.48 \times$ mother antibacterial activity [mm], $R^2 = 0.73$, $F_{1,15} = 17.20$, $p = 0.001$) mainly because of four large mothers that exhibited antibacterial activity in their haemolymph. (b) Antibacterial activity of the eggs negatively covaries with that of the haemolymph of small mothers ($Y = 0.88 - 0.51 \times$ mother antibacterial activity [mm], $R^2 = 0.72$, $F_{1,11} = 11.92$, $p = 0.005$), but not with that of large mothers (solid line for an illustrative purpose $Y = 0.73 + 0.05 \times$ mother antibacterial activity [mm], $R^2 = 0.01$, $F_{1,11} = 0.10$, $p = 0.755$). Grubb's tests for extreme values revealed no outlier data points ($G_{0.05} = 2.13$, $n = 26$, ns).

size had no effect of the immune responsiveness of females, suggesting that small immune-challenged females restricted their TGIP investment in favour of their own immunity. TGIP was previously found to bear fitness costs for the offspring in other insect models (Sadd and Schmid-Hempel 2009, Roth et al. 2010, Zanchi et al. 2011). Here we show that maternal transfer of immunity to the eggs is also costly, but the magnitude of the cost depends on female individual size.

Female fecundity was strongly correlated to body size, which is therefore a good indicator of female reproductive investment in *T. molitor* (Thornhill and Alcock 1983). Depending on body size, immune-challenged females produced fewer eggs than controls. Large females were relatively more affected by the cost of the immune challenge. The bacterial immune challenge did not increase the egg-laying rate as has previously been found in other insect species (Adamo 1999, Shoemaker et al. 2006, Cotter et al. 2010). This would be expected if the maternal immune challenge had induced a shift toward higher investment in current reproduction, consistent with the terminal investment hypothesis (Clutton-Brock 1984, Reaney and Knell 2010). Nevertheless, while laying no more eggs, these females invested more resources in their eggs, providing them with higher levels of antibacterial activity. In that case, size-dependent investment in antibacterial activity in the eggs of immune-challenged mothers could be explained by changes in female fecundity or/and egg size. Supplementary material Appendix 1 shows that there is no evidence for the latter. The statistical results in Table 1 show that antibacterial activity of the eggs was only explained by the maternal immune response in interaction with female body size (female fecundity was not retained by the stepwise procedure), suggesting that immunity of the eggs was not traded-off against mother fecundity.

Egg size was previously found to be unaffected by the maternal immune challenge or female body size (Supplementary material Appendix 1). Consequently, variation in antibacterial activity of the eggs by mothers according to their immune treatment and their body size is unlikely to reflect patterns of investment in egg size. Therefore, egg antibacterial activity did not result from a classic tradeoff between egg number and egg quality (Bascuñán-García et al. 2010).

A negative relationship between mother and egg immunity, as shown in this study, could be explained by a tradeoff between immune pathways of spatially separate physiological compartments (Siva-Jothy et al. 2001, Sadd and Schmid-Hempel 2009). Insects protect their eggs with an external coating of antimicrobial compounds secreted by the female reproductive tract and accessory glands (Marchini et al. 1997). In *Drosophila*, the regulation of antibacterial peptide genes upon infection is tissue-specific and includes the female reproductive tract too (Tzou et al. 2000). Such a local expression of antibacterial peptides in a tissue-specific manner supports the concept of a tradeoff between spatially separate physiological compartments (Siva-Jothy et al. 2001). In our study a similar conclusion can be drawn between the haemocoel and the female reproductive tract. As only small females exhibited a tradeoff between their antibacterial activity and that of their eggs, we propose that it results from a resource-based tradeoff between these compartments instead of conflicting regulation processes between immune pathways.

Our results may have implications beyond that of a better understanding of the evolution and maintenance of TGIP in insects as it may also highlight aspects related to sexual selection (Jokela 2010). In general, males are expected to choose female phenotypes associated with high fertility or reduced sperm competition (Carazo et al. 2004). When offspring are likely to suffer from the parental prevalent pathogenic threat, males may prefer females that will invest the most into the immune protection of their offspring. In many insects, female body size predicts female fecundity (Thornhill and Alcock 1983) and our results suggest that female body size also predict a better immune protection of the offspring through TGIP.

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Supplementary material (Appendix O19933 at < www.oikos office.lu.se/appendix >). Appendix 1.