

Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect

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Summary

1. When parasitized, both vertebrates and invertebrates can enhance the immune defence of their offspring, although this transfer of immunity is achieved by different mechanisms. In some insects, immune-challenged males can also initiate trans-generational immune priming (TGIP), but its expressions appear qualitatively different from the one induced by females similarly challenged.

2. The existence of male TGIP challenges the traditional view of the parental investment theory, which predicts that females should invest more into their progeny than males. However, sexual dimorphism in life-history strategies and the potential costs associated with TGIP may nevertheless lead to dissymmetric investment between males and females into the immune protection of the offspring.

3. Using the yellow mealworm beetle, *Tenebrio molitor*, we show that after parental exposure to a bacterial-like infection, maternal and paternal TGIP are associated with the enhancement of different immune effectors and different fitness costs in the offspring. While all the offspring produced by challenged mothers had enhanced immune defence, only those from early reproductive episodes were immune primed by challenged fathers.

4. Despite the fact that males and females may share a common interest in providing their offspring with an immune protection from the current pathogenic threat, they seem to have evolved different strategies concerning this investment.

Key-words: cost of immunity, host–parasite interaction, insect, maternal and paternal effects

Introduction

Among the factors that determine the phenotype of an organism, maternal effects by which females provide their offspring with nongenetic benefits can have an important impact on offspring fitness (Mousseau & Fox 1998). Maternal effects can also influence offspring's level of immunity, as in the case of trans-generational immune priming (TGIP), where maternal encounter with a pathogen can enhance offspring immunity. It is believed to improve offspring survival when the pathogenic threat persists over the next generation. Enhancement of offspring immunity as a result of maternal immune experience has been reported in both vertebrates (Grindstaff, Brodie & Ketterson 2003; Hasselquist & Nilsson 2008) and invertebrates (Little *et al.* 2003; Sadd *et al.* 2005; Moret 2006; Sadd & Schmid-Hempel 2007; Freitak, Heckel & Vogel 2009; Roth *et al.* 2010; Tidbury, Pedersen & Boots

2011). However, there are few cases where TGIP in invertebrates has not been found (Vorburger *et al.* 2008; Linder & Promislow 2009), suggesting that this phenomenon could not be generalised with regard to host species and/or pathogens.

TGIP is not restricted to maternal effects. Recently, paternally derived immune priming for offspring in the red flour beetle, *Tribolium castaneum*, has been demonstrated (Roth *et al.* 2010). Such a biparental-derived TGIP could have important implications for the understanding of many aspects of evolutionary biology including parental conflicts, the evolution of parental care, sexual selection, mate choice, the evolution of life-history traits and host–parasite co-evolution (see Jokela 2010 for review). In many species, males and females have sexually dimorphic life-history strategies, and the results by Roth *et al.* (2010) suggest that males and females invest differently in TGIP, at least qualitatively. In addition, previous work suggests that TGIP may be associated with costs for the offspring (Freitak, Heckel & Vogel 2009; Roth *et al.* 2010) and for the parents (J. Moreau, G. Martinaud,

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J.-P. Troussard, C. Zanchi & Y. Moret, unpublished data). If maternal and paternal TGIP stimulate different immune effectors in the offspring, the latter might suffer from different fitness costs. Because of these potential differences in fitness costs associated with maternal and paternal TGIP and of sexually dimorphic life-history strategies, we may expect fathers and mothers to show dissymmetric investments in TGIP. However, data on the relative investment of both parents in the immune protection of the offspring are scarce.

In this study, we examined the relative investment in TGIP by fathers and mothers through the measurement of the relative persistence of the maternal and paternal transfer of immunity to the offspring resulting from variable reproductive episodes from the parental challenge. To this purpose, adult males and females of a holometabolous coleopteran, the yellow mealworm beetle, *Tenebrio molitor*, were immune-challenged with lipopolysaccharides (LPS), important immunogens that characterize the surface of Gram-negative bacteria (Söderhäll & Cerenius 1998). The yellow mealworm beetle 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 antimicrobial activity have been shown as a trans-generational effect in larvae of this species when parents received a bacterial immune challenge at the larval stage (Moret 2006). Immediately after being challenged, males and females were given an immunologically naïve partner to produce offspring at different time intervals following the immune challenge. The resulting offspring were then assessed at the adult stage for several immune parameters before and after immune stimulation to reveal the occurrence of TGIP. Furthermore, potential costs associated with TGIP should affect investment into the immune protection of the offspring. Therefore, we examined the effects of the parental immune challenge on important offspring fitness-related traits such as survival to adulthood, larval developmental time, pupal mass and adult body size. Furthermore, as an individual immune response is dynamic over time (Haine *et al.* 2008a,b), parental transfer of immunity to the offspring could vary according to the time at which oviposition occurred after the parental immune challenge. Thus, we investigated the persistence of parental investment into the immune protection of the offspring along reproductive bouts from the parental challenge. Our results show that the challenge of the mothers or the fathers resulted in the stimulation of different immune effectors in the offspring. The duration of this TGIP along reproductive episodes also varies according to the sex of the stimulated parent. Finally, its associated costs for the offspring differed as well.

Materials and methods

INSECT CULTURING

Virgin adult beetles of controlled age were obtained from pupae taken at random from an outbred stock culture maintained in pathogen-free conditions at the University of Burgundy, Dijon, France.

We wanted to test for maternal and paternal effects on the immunity and life-history traits of the offspring separately. We therefore conducted at the same time and under the same conditions two experiments testing maternal effects and paternal effects, respectively. Both experiments followed exactly the same protocol where fathers and mothers were exposed to the same immune challenge.

We mimicked a bacterial infection in virgin females and virgin males (8 ± 1 day postemergence) by a single injection of a dose of LPS extracted from *Escherichia coli* in 5 μ L of Ringer's solution. LPS elicits a persistent response of production of antibacterial peptides over many days (Haine *et al.* 2008a,b). A group of control females and males were treated in the same way, but with the omission of LPS to control for the effect of the injection (control individuals). Immediately after their immune treatment, the females were paired with a virgin and naïve male of the same age and allowed to produce eggs in plastic boxes ($L \times l \times H$, $20 \times 12 \times 9.5$ cm) supplied with a mix of 60 g of bran flour and bleached flour (1 : 2 w : w) and an microcentrifuge tube of water in standard laboratory conditions (25 °C, 70% RH; light/dark 12h : 12h). Similarly, injected males were paired with a virgin and naïve female of the same age. As an individual's immune response is dynamic over time (Haine *et al.* 2008a,b), we can expect its transmission to vary across reproductive episodes following the parental challenge.

To test for this possibility, we allow each female to lay eggs at three different egg laying sequences following the maternal or paternal challenge. To do this, each couple was transferred into a new box every 4 days for 12 days following the parental immune challenge. The eggs of the resulting laying sequences (from day 0 to day 4, 4 to 8 and 8 to 12) were allowed to develop in the corresponding plastic box for 9 weeks. Four days after beginning of the experiment, challenged parental beetles were tested for the antimicrobial activity of their haemolymph. At the end of the last laying sequence, parental beetles were killed in alcohol and kept for body size measurements.

Nine weeks after egg laying, offspring larvae obtained from all the couples were counted. Six larvae per couple and per egg laying sequence were randomly taken and individually isolated into Petri dishes (diameter 9 cm) containing 10 g of a mix of bran flour and protein flour (4 : 1 w : w). A microcentrifuge tube of water and a piece of apple were weekly renewed. These larvae were maintained in standard laboratory conditions until adulthood. For each individual, larval developmental time (duration in days from hatching to pupae), pupal weight and adult size were recorded. When adult offspring beetles reached 10 days postemergence, we sampled 5 μ L of haemolymph to test for the concentration of haemocytes, the antibacterial activity and the maintenance and use of the prophenoloxidase system while they were unchallenged (corresponding to basal levels of these immune parameters). Immediately after this first sample of haemolymph, the beetles were immune challenged with LPS and tested again 3 days later, corresponding to the peak of the immune response (Haine *et al.* 2008a,b) for the concentration of haemocytes, the antibacterial and the phenoloxidase activities of their haemolymph while they were immune challenged.

IMMUNE TREATMENTS OF THE PARENTS AND THE OFFSPRING

Control adult parents received a single injection of 5 μ L of Ringer solution after being chilled on ice. Challenged parents received a 0.5 mg mL⁻¹ dose of nonpurified LPS extracted from *E. coli* (Sigma-Aldrich, St Louis, MO, USA, L8274) in 5 μ L Ringer solution after being chilled on ice. Similarly, adult offspring beetles received a same dose of LPS immediately after providing a first haemolymph sample.

Commercial LPS often contains contaminating peptidoglycan fragments (Haine *et al.* 2008b). Therefore, LPS injection in our experiments may not strictly mimic a Gram-negative bacterial infection as it may stimulate both the Imd and Toll pathways (Lemaitre, Reichhart & Hoffman 1997). Nonetheless, this should have little consequences for our study as both LPS and peptidoglycans are molecular signature of bacteria. All injections were made through the pleural membrane between the second and the third abdominal tergites, using sterilized pulled glass capillaries.

HAEMOLYMPH COLLECTION

Individual beetles were chilled on ice before 5 μL of haemolymph was collected from a wound in the beetle's neck and flushed into a microcentrifuge tube containing 25 μL of cold sodium cacodylate/ CaCl_2 buffer (0.01 M sodium cacodylate; 0.005 M CaCl_2 , pH 6.5, at 4 °C). For the offspring beetle, a 10- μL subsample was immediately used for the measurement of the concentration of haemocytes. Another 5- μL subsample was kept in an *N*-phenylthiourea (Sigma-Aldrich, St Louis, MO, USA, P7629)-coated microcentrifuge tube and stored at -80 °C until later examination for antibacterial activity. The remaining haemolymph solution was diluted with 15 μL of cold sodium cacodylate/ CaCl_2 buffer and immediately stored at -80 °C for later measurement of the phenoloxidase activity.

IMMUNE PARAMETERS

Concentration of haemocytes was measured using a Neubauer improved haemocytometer under a phase-contrast microscope (magnification $\times 400$).

Antimicrobial activity in the haemolymph was measured using a standard zone of inhibition assay (Moret 2006). Samples were thawed on ice, and 2 μL of the sample solution was used to measure antimicrobial activity on zone of inhibition plates seeded with *Arthrobacter globiformis* obtained from the Pasteur institute (CIP 105365). *Arthrobacter globiformis* from a single colony on a streak plate were incubated overnight at 30 °C in broth medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distilled water, pH 7.0). From this culture, bacteria were added to broth medium containing 1% agar to achieve a final density of 10^5 cells per mL. Six millilitres of this seeded medium was then poured into a Petri dish and allowed to solidify. Sample wells were made using a Pasteur pipette fitted with a ball pump. Two microlitres of sample solution was added to each well, and a positive control (Tetracycline: Sigma-Aldrich, St Louis, MO, USA, T3383) was included on each plate. Plates were then incubated for 48 h at 30 °C, after which the diameter of inhibition zones were measured for each sample.

For each individual haemolymph sample, the activity of naturally activated phenoloxidase (PO) enzymes only (hereafter PO activity) and the activity of the proenzymes (proPO) in addition to that of the PO (hereafter total-PO activity) were measured using a spectrophotometer. PO activity was quantified without further activation, while total activity required the activation of the proPO into PO with chymotrypsin. To this purpose, frozen haemolymph samples were thawed on ice and centrifuged (5000 r.p.m, 5 min, 4 °C). Five microlitres of supernatant was added to a microplate well containing 20 μL of PBS and either 140 μL of distilled water to measure PO activity only or 140 μL of chymotrypsin solution (Sigma-Aldrich, St Louis, MO, USA, C-7762, 0.07 mg mL⁻¹ of distilled water) to measure total activity. Then 20 μL of L-Dopa solution (Sigma-Aldrich, St Louis, MO, USA, D-9628, 4 mg mL⁻¹ of distilled water) was added to each well. The reaction was allowed to proceed at 30 °C in a microplate

reader (Versamax; Molecular Devices, Sunnyval, CA, USA) for 40 min. Readings were taken every 15 s at 490 nm and analysed using the software SOFT-Max[®]Pro 4.0 (Molecular Devices, Sunnyval, CA, USA). Enzyme activity was measured as the slope (V_{max} value: change in absorbance unit per min) of the reaction curve during the linear phase of the reaction and reported to the activity of 1 μL of pure haemolymph.

BODY MASS AND SIZE

Body mass of larvae and pupae were measured to the nearest 1 mg with a Sartorius Extend ED124S balance (Sartorius AG, Goettingen, Germany), and body size of adults was estimated by measuring the left elytra with a Mitutoyo digital callipers (Mitutoyo, Knagawa, Japan, precision ± 0.1 mm) (Moret 2006).

STATISTICS

Antimicrobial activity in the haemolymph of mothers or fathers was natural log transformed and analysed using a univariate analysis of variance (ANOVA) with maternal or paternal immune treatment as fixed factors and mother or father body size as covariates.

Variation in number of larvae along egg laying sequences was analysed using general linear models for repeated measures with maternal or paternal immune treatments as fixed factors and mother or father body size as covariates.

Survival of offspring larvae to adulthood was analysed using a chi-square test. Data on larval development time, pupae mass, adult body size, concentration of haemocytes, PO activity, total-PO activity and antibacterial activity of the offspring were analysed based on family means according to egg laying sequences and offspring gender allowing to test the effect of the parental treatment, offspring gender and egg laying sequences as fixed factors for all these dependent variables. In a first step, the data of both experiments were analysed as single data set by specifying in the statistical models whether the parental immune treatment was applied to mothers or fathers to test whether parental effects are gender specific (see Tables S1 and S2, Supporting information). If sex of the focal parent (e.g. to which the immune treatment was applied) significantly affects parental effects, either as main effect or as an interaction term, data of each experiment were analysed separately. In either case, mean changes in levels of immune defences upon the immune challenge of the offspring were analysed using general linear models for repeated measures. Mean variation in larval developmental time, pupae mass and adult body size was analysed using a multivariate analysis of variance (MANOVA).

For all parametric tests, the best statistical models were searched using a stepwise backward procedure from initial models that included all main effects and interactions. All the data were analysed using *SPSS 11* for Macintosh (SPSS Inc., Chicago, IL, USA).

Results

PARENTAL IMMUNE RESPONSE AND REPRODUCTIVE EFFORT

As expected from the treatment, LPS-treated mothers and fathers had higher antimicrobial activity in their haemolymph than control individuals (Fig. 1; ANOVAS mothers $F_{1,21} = 34.05$, $P < 0.001$; fathers: $F_{1,21} = 11.34$, $P = 0.003$). Body size of females or males did not affect the strength of their antimicrobial immune response

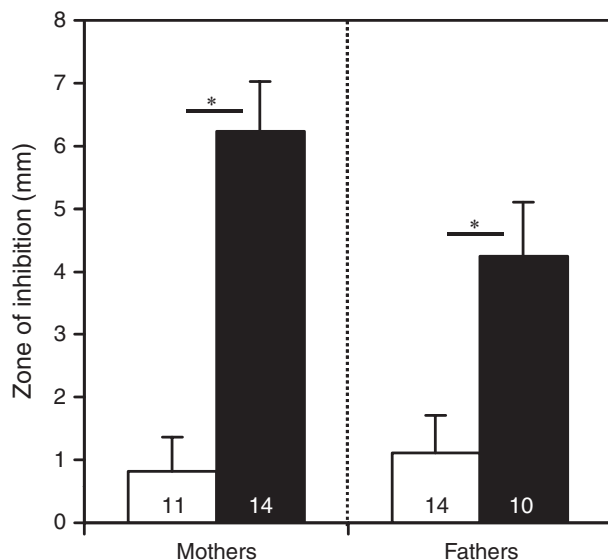


Fig. 1. Antibacterial activity expressed as the zone of inhibition diameter (mean \pm SE) in the haemolymph of parental females and males previously exposed to an injection with Ringer solution (control; open bars) and lipopolysaccharides solution (black bars) mimicking a bacterial infection. Numbers inside bars represent the number of females or males assayed and * denotes statistically significant difference ($P < 0.05$) between treatment groups.

(mothers $F_{1,21} = 3.21$, $P = 0.088$; fathers: $F_{1,21} = 0.01$, $P = 0.906$).

Nine weeks after the maternal immune treatment, control and LPS-treated mothers produced a similar total number of larvae (mean \pm SE control 24.9 ± 5.8 larvae; LPS 16.9 ± 5.1 larvae; ANOVA for repeated measures: between subject effects $F_{1,23} = 1.38$, $P = 0.522$) without significant variation along egg laying sequences (within subject effects: $F_{2,46} = 0.62$, $P = 0.544$; see Table S3, Supporting information).

Couples with control and LPS-treated fathers produced a similar total number of larvae (mean \pm SE control 17.4 ± 3.8 larvae; LPS 12.9 ± 4.4 larvae; ANOVA for repeated measures: between subject effects $F_{1,23} = 0.26$, $P = 0.614$). More larvae were produced during the last egg laying sequence (mean \pm SE day 0–4 = 3.9 ± 0.9 larvae; day 4–8 = 3.2 ± 0.9 larvae; day 8–12 = 8.4 ± 1.7 larvae; within subject effects: $F_{2,21} = 5.83$, $P = 0.010$), but this variation along egg laying sequences was independent of the paternal treatment (treatment \times laying rank: $F_{2,21} = 0.46$, $P = 0.636$).

PARENTAL EFFECT ON OFFSPRING IMMUNITY

Data analysis of both experiments as a single data set reveals a strong gender-specific effect of the parents exposed to the immune treatment on all the immune effectors of their offspring, either as a main effect or as an interaction term (see Table S1, Supporting information). The immune responses of the offspring to the LPS-challenge especially were dependent on both the parental immune treatment and the gender

of the focal parent (Table S1, Supporting information), suggesting that the immune challenge of mothers and fathers had different effects on offspring immunity. As a consequence, maternal and paternal effects were further analysed separately by comparing changes in immune effectors of the offspring of LPS-challenged mothers and fathers with those of control mothers and fathers, respectively (Table 1).

Maternal effect on offspring immunity

Overall, the offspring of LPS-treated mothers had a higher concentration of haemocytes than the offspring of control mothers (Fig. 2, Table 1), whereas levels of PO activity, total-PO activity and antibacterial activity were not affected by the maternal immune treatment (Table 1). While male and female offspring exhibited similar concentration of haemocytes and of PO activity, offspring males had more total-PO activity and higher antibacterial activity than offspring females (Table 1, mean of scores measured before and after immune challenge together \pm SE total-PO 52.35 ± 3.38 vs. 39.33 ± 3.15 od 10^2 min^{-1} , antibacterial activity 36.15 ± 3.18 vs. 25.74 ± 2.93 mm).

The LPS immune challenge of the offspring induced increased levels of all the immune parameters with a similar magnitude for the offspring of control and LPS-treated mothers (Table 1). Therefore, when immune challenged, the offspring of LPS-treated mothers still had a higher concentration of haemocytes in their haemolymph than the offspring of control mothers (Fig. 2). Change in antibacterial activity was dependent on the gender of the offspring (Table 1) because males mounted a stronger antibacterial immune response to the challenge than females (mean difference of scores measured before and after immune challenge \pm SE 62.96 ± 5.54 vs. 45.25 ± 5.91 mm).

Egg laying ranks did not affect the overall levels of immune defences of the offspring as well as the magnitude of their changes during the immune challenge (Table 1; Fig. 3a,b).

Paternal effect on offspring immunity

Overall, the paternal immune treatment had no main effect on levels of immune defences of the offspring (Table 1). Levels of immune defences were gender dependent (Table 1) as males exhibited higher scores than females for all the immune parameters (mean of scores measured before and after immune challenge together \pm SE haemocytes 83.81 ± 8.09 vs. 55.23 ± 8.10 cells per μL ; PO activity 33.13 ± 2.77 vs. 23.07 ± 2.73 od 10^2 mm^{-1} ; total-PO activity 66.99 ± 5.88 vs. 40.93 ± 5.81 od 10^2 mm^{-1} ; antibacterial activity 33.33 ± 3.44 vs. 22.39 ± 3.31 mm).

The LPS immune challenge of the offspring induced increased levels in all the immune parameters (Table 1). Furthermore, there was a significant interaction term between the paternal immune treatment and egg laying rank for increased levels of both PO and total PO activities (see Ch \times treat \times L-rank in Table 1). Indeed, upon the immune challenge, the offspring of LPS-treated fathers resulting from

Table 1. Results of the analyses for repeated measures testing changes in the concentration of haemocytes, activities of the PO enzymes (PO), the proenzymes in addition to that of the PO (total-PO) and antibacterial peptides (antibacterial) in the haemolymph of offspring 3 days after an immune challenge (Ch) according to maternal and paternal immune treatments (treat), the egg laying rank (L-rank) and sex

| Source | Maternal priming | | | Paternal priming | | | | |
|-----------------------------------|--|--|--|---|--|--|--|--|
| | Haemocyte | PO | Total-PO | Antibacterial | Haemocyte | PO | Total-PO | Antibacterial |
| <i>Between subjects</i> | | | | | | | | |
| Treat | $F_{1,74} = 4.59$ $P = \mathbf{0.035}$ | $F_{1,83} = 0.09$ $P = 0.760$ | $F_{1,83} = 1.11$ $P = 0.296$ | $F_{1,84} = 2.18$ $P = 0.143$ | $F_{1,64} = 2.30$ $P = 0.134$ | $F_{1,64} = 1.89$ $P = 0.174$ | $F_{1,64} = 2.43$ $P = 0.124$ | $F_{1,70} = 0.53$ $P = 0.471$ |
| Sex | $F_{1,74} = 1.99$ $P = 0.162$ | $F_{1,83} = 2.64$ $P = 0.108$ | $F_{1,83} = 72.99$ $P = \mathbf{0.006}$ | $F_{1,84} = 5.83$ $P = \mathbf{0.018}$ | $F_{1,64} = 6.42$ $P = \mathbf{0.014}$ | $F_{1,64} = 6.68$ $P = \mathbf{0.012}$ | $F_{1,64} = 10.01$ $P = \mathbf{0.002}$ | $F_{1,70} = 5.44$ $P = \mathbf{0.023}$ |
| L-rank | $F_{2,74} = 0.63$ $P = 0.538$ | $F_{2,83} = 0.08$ $P = 0.927$ | $F_{2,83} = 0.04$ $P = 0.963$ | $F_{2,84} = 1.01$ $P = 0.369$ | $F_{2,64} = 0.84$ $P = 0.434$ | $F_{2,64} = 1.51$ $P = 0.228$ | $F_{2,64} = 1.68$ $P = 0.194$ | $F_{2,70} = 0.03$ $P = 0.971$ |
| Treat \times L-rank | N. R. | N. R. | N. R. | N. R. | N. R. | $F_{2,64} = 2.48$ $P = 0.092$ | $F_{2,64} = 1.52$ $P = 0.227$ | N. R. |
| <i>Within subjects</i> | | | | | | | | |
| Ch | $F_{1,74} = 39.80$ $P < \mathbf{0.001}$ | $F_{1,83} = 49.64$ $P < \mathbf{0.001}$ | $F_{1,83} = 27.19$ $P < \mathbf{0.001}$ | $F_{1,84} = 173.15$ $P < \mathbf{0.001}$ | $F_{1,64} = 16.18$ $P < \mathbf{0.001}$ | $F_{1,64} = 49.27$ $P < \mathbf{0.001}$ | $F_{1,64} = 54.33$ $P < \mathbf{0.001}$ | $F_{1,70} = 68.66$ $P < \mathbf{0.001}$ |
| Ch \times treat | $F_{1,74} = 1.09$ $P = 0.299$ | $F_{1,83} = 0.72$ $P = 0.397$ | $F_{1,83} = 0.59$ $P = 0.446$ | $F_{1,84} = 1.15$ $P = 0.287$ | $F_{1,64} = 0.99$ $P = 0.322$ | $F_{1,64} = 2.65$ $P = 0.109$ | $F_{1,64} = 4.01$ $P = \mathbf{0.050}$ | $F_{1,70} = 0.90$ $P = 0.346$ |
| Ch \times sex | $F_{1,74} = 0.01$ $P = 0.950$ | $F_{1,83} = 0.24$ $P = 0.622$ | $F_{1,83} = 2.39$ $P = 0.126$ | $F_{1,84} = 4.64$ $P = \mathbf{0.034}$ | $F_{2,64} = 0.15$ $P = 0.702$ | $F_{2,64} = 4.65$ $P = \mathbf{0.035}$ | $F_{2,64} = 6.19$ $P = \mathbf{0.015}$ | $F_{2,70} = 6.92$ $P = \mathbf{0.010}$ |
| Ch \times L-rank | $F_{2,74} = 0.75$ $P = 0.476$ | $F_{2,83} = 0.17$ $P = 0.847$ | $F_{2,83} = 0.35$ $P = 0.704$ | $F_{2,84} = 1.62$ $P = 0.204$ | $F_{1,64} = 0.36$ $P = 0.699$ | $F_{2,64} = 0.31$ $P = 0.712$ | $F_{1,64} = 5.31$ $P = \mathbf{0.043}$ | $F_{1,70} = 0.05$ $P = 0.950$ |
| Ch \times treat \times L-rank | N. R. | N. R. | N. R. | N. R. | N. R. | $F_{2,64} = 4.75$ $P = \mathbf{0.012}$ | $F_{2,64} = 5.79$ $P = \mathbf{0.005}$ | N. R. |

N. R. refers to effects not retained by the stepwise procedure. Values $P \leq 0.05$ are given in bold.

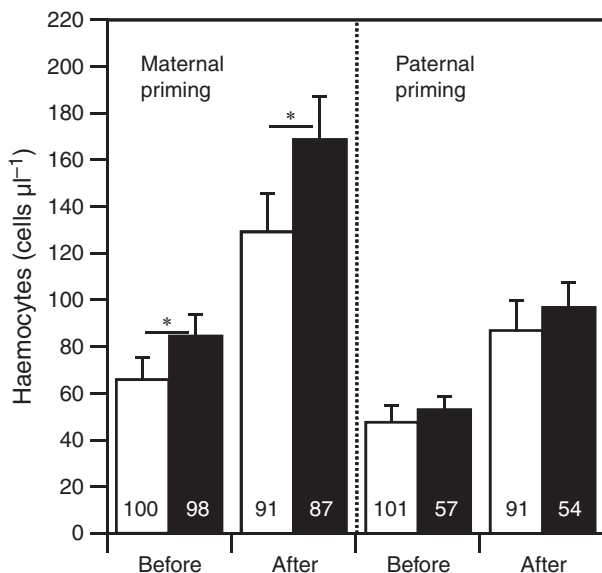


Fig. 2. Concentration of haemocytes (mean \pm SE) of adult offspring in relation to parental immune priming before and after immune stimulation with lipopolysaccharides (LPS). Open bars refer to the control parental immune treatment whereas black bars refer to the LPS immune treatment of the parents. Numbers inside bars represent the number of individual offspring assayed and * denotes statistically significant difference ($P < 0.05$) between treatment groups.

eggs laid early after the paternal immune treatment (day 0–4) exhibited higher PO activity and total-PO activity than those of control fathers (Fig. 3). This effect of the paternal immune

treatment disappeared for offspring derived from later egg laying sequences (day 4–8 and day 8–12; Fig. 3c,d).

Changes in PO activity, total activity and antibacterial activity were also gender dependent (Table 1) as increased levels for these immune parameters were more important in males than in females (mean difference of scores measured before and after immune challenge \pm SE PO activity 25.11 ± 4.69 vs. 12.75 ± 3.13 od 10^2 mm⁻¹; total-PO activity 46.24 ± 8.96 vs. 20.23 ± 5.71 od 10^2 mm⁻¹; antibacterial activity 54.98 ± 6.41 vs. 29.39 ± 6.97 mm).

PARENTAL EFFECT ON OFFSPRING LIFE HISTORY

Whether the immune treatment was applied to mother or father, mortality rates of the offspring to the pupal stage did not differ (maternal treatment 5.4% vs. paternal treatment 10.2%, $\chi^2 = 3.35$, d.f. = 1, $P = 0.067$). Furthermore, larvae of LPS-treated mothers or fathers had a similar mortality than those of control mothers or fathers (LPS parental groups 9.2% vs. control parental groups 6.5%, $\chi^2 = 1.08$, d.f. = 1, $P = 0.297$).

Data analysis of life-history parameters of the offspring of both experiments together reveals an overall effect of the immune treatment of the parents and of the gender of the offspring (Table S2, Supporting information). Only variation in pupae mass was explained by this statistical model and showed that pupae mass of the offspring of LPS-treated parents was lighter than this of offspring of control parents (Table S2, Supporting information; Fig. 4a). However, only pupae of LPS-treated fathers were significantly lighter than

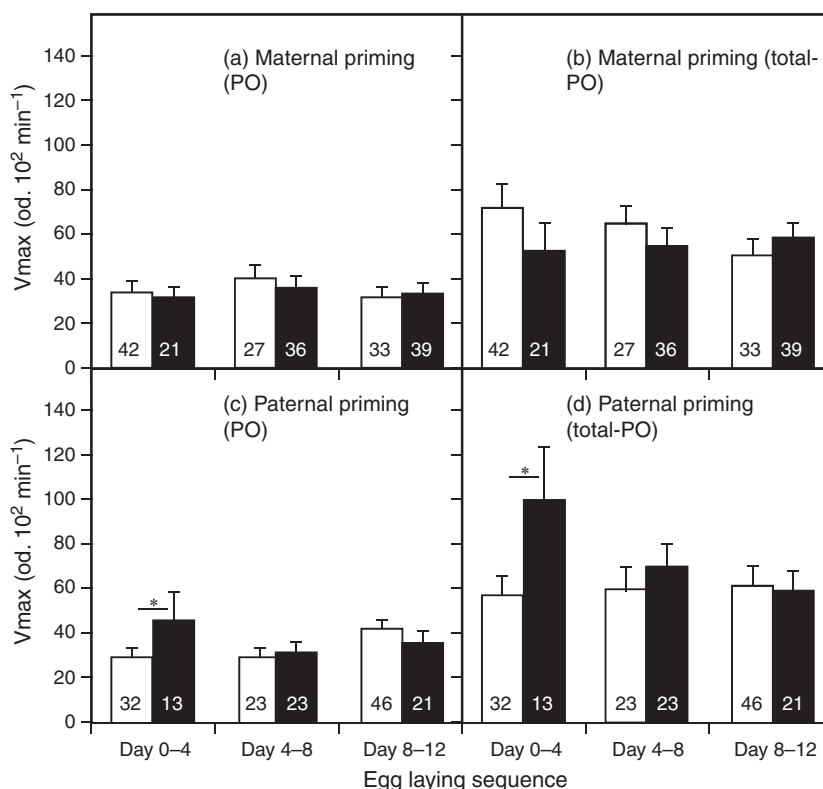


Fig. 3. Activity of naturally activated phenoloxidase enzymes (PO activity; mean \pm SE) and activity of the proenzymes in addition of the PO (total-PO; mean \pm SE) in the haemolymph of maternally (upper panels) and paternally (lower panels) immune primed offspring after being immune challenged according egg laying sequences from the parental challenge. Open bars refer to the control parental immune treatment whereas black bars refer to the lipopolysaccharides immune treatment of the parents. Numbers inside bars represent the number of individual offspring assayed and * denotes statistically significant difference ($P < 0.05$) between treatment groups.

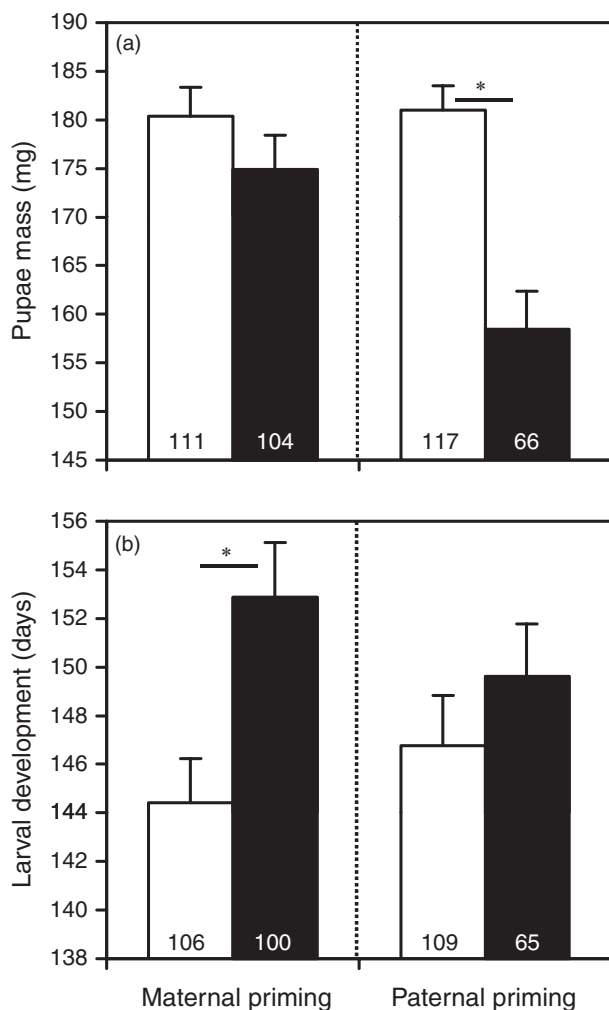


Fig. 4. (a) Pupae mass (mean \pm SE) of offspring in relation to parental immune priming and (b) larval developmental time from hatching of the eggs to the pupal stage (mean \pm SE). Open bars refer to the control parental immune treatment whereas black bars refer to the lipopolysaccharides immune challenge of the parents. Numbers inside bars represent the number of individual offspring assayed and * denotes statistically significant difference ($P < 0.05$) between treatment groups.

Table 2. Effects of the maternal and paternal immune treatments (Treat), sex and egg laying rank (L-rank) on larval development time (time to pupae), pupae mass, and adult body size of the offspring

| Source | Maternal priming | | | | Paternal priming | | | |
|--------------|---|---|----------------------------------|----------------------------------|---|----------------------------------|--|----------------------------------|
| | Multivariate test | Univariate tests | | | Multivariate test | Univariate tests | | |
| | Pillai's trace | Time to pupae | Pupae mass | Adult size | Pillai's trace | Time to pupae | Pupae mass | Adult size |
| Global model | | $F_{4,82} = 2.52$ $P = \mathbf{0.047}$ | $F_{4,82} = 1.34$ $P = 0.261$ | $F_{4,82} = 0.70$ $P = 0.596$ | | $F_{4,66} = 0.22$ $P = 0.927$ | $F_{4,66} = 5.33$ $P = \mathbf{0.001}$ | $F_{4,66} = 1.32$ $P = 0.273$ |
| Treat | $F_{3,80} = 4.09$ $P = \mathbf{0.009}$ | $F_{1,82} = 9.89$ $P = \mathbf{0.002}$ | $F_{1,82} = 0.87$ $P = 0.354$ | $F_{1,82} = 0.01$ $P = 0.917$ | $F_{3,64} = 5.97$ $P = \mathbf{0.001}$ | $F_{1,66} = 0.50$ $P = 0.483$ | $F_{1,66} = 14.65$ $P < \mathbf{0.001}$ | $F_{1,66} = 4.18$ $P = 0.045$ |
| Sex | $F_{3,80} = 3.36$ $P = \mathbf{0.023}$ | $F_{1,82} = 0.14$ $P = 0.708$ | $F_{1,82} = 3.13$ $P = 0.080$ | $F_{1,82} = 0.06$ $P = 0.802$ | $F_{3,64} = 1.33$ $P = 0.273$ | $F_{1,66} = 0.14$ $P = 0.704$ | $F_{1,66} = 1.42$ $P = 0.238$ | $F_{1,66} = 1.01$ $P = 0.319$ |
| L-rank | $F_{6,162} = 0.66$ $P = 0.683$ | $F_{2,82} = 0.23$ $P = 0.802$ | $F_{2,82} = 0.51$ $P = 0.600$ | $F_{2,82} = 1.30$ $P = 0.277$ | $F_{6,130} = 0.92$ $P = 0.482$ | $F_{2,66} = 0.08$ $P = 0.922$ | $F_{2,66} = 1.95$ $P = 0.150$ | $F_{2,66} = 0.04$ $P = 0.958$ |

Values $P \leq 0.05$ are given in bold.

those of control fathers, whereas there was no significant effect of the maternal immune treatment on pupae mass of their offspring (Table S2, Supporting information; Table S2, Fig. 4a). Overall, pupae that became males were heavier than those that became females (Table S2, Supporting information). The egg laying rank had no effect on any of the life-history parameters considered in this study (Table S2, Supporting information; Table 2). Separate analyses of the maternal and paternal immune treatment on life-history parameters of the offspring showed a significant effect of the maternal immune treatment on larval development time (Table 2). Indeed, it took significantly more time for larvae of LPS mothers to reach the pupal stage than those of control mothers (Fig. 4b).

Discussion

Our study provides evidence of both maternally and paternally derived immune priming in the mealworm beetle *T. molitor* as a result of a single bacterial-like immune challenge in the parental generation. As the parental treatment had no effect on survival of the parents and their offspring, enhanced immunity in offspring of immune-challenged parents could not be explained by selection. In this respect, our study confirms the occurrence of TGIP in insects (Rahman *et al.* 2003; Sadd *et al.* 2005; Sadd & Schmid-Hempel 2007; Freitag, Heckel & Vogel 2009; Roth *et al.* 2010) and more specifically in *T. molitor* (Moret 2006). The analysis of both experiments as a single data set suggests a differential expression of the maternal and paternal effects on immune and life-history parameters of the offspring of *T. molitor*. This might be expected because in each experiment the partners of focal parents, despite not immune treated, were of different sex. This difference is probably not neutral and justifies separate analyses of the data of each experiment to investigate maternal and paternal effects.

The adult offspring of bacterially immune-challenged mothers exhibited a higher concentration of haemocytes before and after immune stimulation through all the egg

laying sequences following maternal immune challenge. However, activity of the proPO system (PO and total PO) in the offspring was unaffected by the maternal immune treatment. Hence, enhanced immunity in maternally primed offspring was mainly achieved by maintaining an elevated basal concentration of immune cells instead of recruiting a larger number of haemocytes upon infection. As proliferation of haemocytes after infection is limited (Sorrentino, Carton & Govind 2002), the initial higher concentration of haemocytes may significantly improve the probability of success of the insect's immune response (Eslin & Prévost 1998).

By contrast, the concentration of haemocytes and the antibacterial activity in the haemolymph of the offspring of bacterially immune-challenged fathers and control fathers were unaffected. In fact, bacterially immune-challenged fathers transferred to their offspring, the ability to develop a stronger immune response mediated by the proPO system. This later result is in agreement with that of Roth *et al.* (2010) in another beetle species. However, only the offspring produced within the first 4 days that followed the paternal immune treatment were provided with this enhanced immunity mediated by the proPO system. Cessation of the paternal immune protection in late offspring is unlikely the result of costs of early reproductive effort in males or haemolymph collection at the end of the first reproductive episodes. Indeed, along egg laying sequences, life-history parameters of the offspring were not degraded and the production of offspring did not decrease, as it would be expected if early reproductive effort of males and haemolymph collection were costly. Furthermore, if early reproductive effort and haemolymph collection were costly, the dynamics of the maternal TGIP would be affected as well, which was not the case. Note that in the experiment investigating maternal TGIP, offspring production and their life-history parameters were not affected by egg laying rank either. Therefore, as opposed to the situation of maternal TGIP, the effect of the paternal immune challenge on offspring immunity was transient along father's reproductive episodes.

Our results contrast to those of Moret (2006) which showed that TGIP lead to increased levels of antimicrobial activity in the offspring, whereas activity of the proPO system was unaffected. However, in this previous experiment, parents were immune-challenged at the larval stage and the offspring were also assayed at the larval stage. This suggests that the expression of TGIP differs with regard to the developmental stage to which parents are challenged and the offspring assayed. As suggested by Freitak, Heckel & Vogel (2009), variation of immune defence in the offspring may involve complex mechanisms instead of a passive transmission of immune effectors from parents to their offspring. Furthermore, using a similar method, Sadd *et al.* (2005) showed a stronger antibacterial response in adult bumblebee workers of immune-challenged queens, indicating that TGIP takes different forms according to insect species and also pathogen types (Rahman *et al.* 2003; Roth *et al.* 2010).

Our results add up to those of Roth *et al.* (2010) who found both maternally and paternally derived TGIP in the

red flour beetle, *Tribolium castaneum*, an insect species that is phylogenetically and ecologically closely related to *T. molitor*. Roth *et al.* (2010) found that maternally derived TGIP was more pathogen specific than paternally derived TGIP. However, they were unable to determine which immune mediators could explain such a difference. Our results show that maternally derived TGIP is mainly mediated by haemocytes in *T. molitor*. In both *Drosophila melanogaster* (Pham *et al.* 2007) and in the woodlouse, *Porcellio scaber* (Roth & Kurtz 2009), haemocytes were shown to mediate specific immune priming in response to microbial challenges through phagocytosis. With regard to these studies, we may propose that immune priming within and across generations of insects may share common mechanisms and could explain difference in specificity between maternally and paternally derived TGIP. Testing this hypothesis would require to consider the involvement of haemocytes in maternal TGIP in *Tribolium castaneum* and to test pathogen specificity of maternal TGIP in *T. molitor*.

A striking result of our study is that challenged mothers transfer immunity to their offspring for a longer period than challenged males. Assuming that males and females are sharing the same interest in terms of offspring survival to pathogens, why do males not invest as much as females in the immunity of their offspring? Among all the hypotheses that could be proposed, males may disperse more than females for reproduction. Therefore, the infection of fathers is not a reliable long-term cue predicting the risk of infection of their offspring. Furthermore, as well as the cost of the infection to their immune response, transfer of immunity to the offspring is likely to be costly to the parents as it has been shown in *T. molitor* females (J. Moreau, G. Martinaud, J.-P. Troussard, C. Zanchi & Y. Moret, unpublished data). The facultative and transient nature of the paternal transfer of immunity suggests it bears some costs for the males as well. Costs for fathers could be larger than costs for mothers, explaining the shorter period of investment by males than females.

As maintaining and using enhanced levels of immune defences are costly (Moret & Schmid-Hempel 2000), enhanced levels of immune defence in primed offspring are expected to show trade-offs with other fitness-related traits (Schmid-Hempel 2005). In line with this, we found that offspring exhibited life-history costs related to the immune treatment of their parents. However, depending whether the parental challenge was maternal or paternal, life-history costs paid by the offspring were not expressed on the same traits. Maternally primed offspring had a prolonged developmental time, whereas pupal mass and adult body size were not affected by the maternal immune treatment. Prolonging the developmental time is likely to be costly in insects because it should translate into a low competitive ability for food under higher larval densities (Koella & Boëte 2002), and it should delay access to reproduction. Moreover, a fast development could reduce the probability of juvenile mortality (Bell 1980), especially in a species like *T. molitor* that exhibits cannibalism on juveniles (Ichikawa & Kurauchi 2009). Interestingly, enhancement of haemocyte concentration in maternally

primed offspring was associated with similar cost patterns found from selection experiments (Kraaijeveld, Limentani & Godfray 2000; Koella & Boëte 2002), suggesting that the relationship between haemocyte concentration and larval developmental time relies on the same basis whether it results from a maternal adjustment or selection.

Paternally primed offspring were lighter at the pupal stage, whereas developmental time and adult body size were not affected by the paternal immune treatment. In insects, pupal mass is often positively correlated with adult fecundity and/or fertility (Tammara, Esperk & Castellanos 2002), which determines its reproductive success. While the prolonged developmental time in maternally primed offspring could be attributable to a trade-off with enhanced immunity, it does not seem to be the case for the cost on pupal mass in offspring of paternally primed offspring. Indeed, among paternally primed offspring, only those from the first egg laying sequences had enhanced PO activity, whereas the cost on pupal mass is incurred across all egg laying sequences. Therefore, this may reflect a cost of the paternal immune challenge on the quality of the offspring. Nevertheless, it is still possible that the reduced pupal mass in paternally primed offspring could result from a trade-off with enhanced immune defences not measured in our experiment.

To conclude, our study demonstrated the existence of a maternally and paternally derived immune priming for offspring in the mealworm beetle, *T. molitor*. Enhancement of immunity in offspring of challenged mothers resulted in an increased concentration of haemocytes, which traded off against larval developmental time. In contrast, the paternal challenge induced an increased activity of the proPO system only in the offspring that hatched within the first 4 days from the paternal challenge. Our study comes in support of previous work with regard to the existence of paternally derived immune priming for offspring (Roth *et al.* 2010) and fitness associated costs in other insect species (Freitag, Heckel & Vogel 2009; Sadd & Schmid-Hempel 2009; Roth *et al.* 2010). However, our results highlight the difference in investment between males and females to the immune protection of their offspring in the context of TGIP. While fathers and mothers may have similar interests in terms of offspring survival to the prevalent pathogenic threat, they seem to have evolved different strategies to achieve the immune protection of their offspring. If TGIP raised numerous questions with regard to the mechanisms through which it is achieved, its differential expression when it is paternally or maternally originated is likely to have important implications in the evolution of life-history traits, parental investment and host–pathogen co-evolution (Jokela 2010).

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Authors' contributions

Y.M. and J.M. designed the experiments. Animals rearing and all experimental and laboratory work was performed by J-P.T., G.M. and C.Z. Y.M., J.M. C.Z. and J-P.T. analysed the data. C.Z., J.M. and Y.M. wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Results of the analysis for repeated measures testing changes in the concentration of haemocytes, activities of the PO

enzymes (PO), the proenzymes in addition to that of the PO (total-PO) and antibacterial peptides (Antibacterial) in the haemolymph of offspring 3 days after an immune challenge (Ch) of the two data sets together (parental: maternal vs. paternal treatment) according to maternal or paternal immune treatments (treat: challenge vs. control immune treatment), the egg laying rank (L-rank) and sex

Table S2. Results of the multivariate analysis (MANOVA) testing for changes on larval development time (time to pupae), pupae mass, and adult body size of the offspring in both data sets together (parental: maternal vs. paternal treatment) according to maternal or paternal immune treatments (treat: challenge vs. control immune treatment), the egg laying rank (L-rank) and sex

Table S3. Magnitude of the antibacterial immune response (expressed as the mean zone of inhibition diameter in mm) of parental females and males and their reproductive effort (number of offspring larvae) along the egg laying sequences following the immune treatment

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