Ecologie évolutive du transfert trans-générationnel d'immunité chez un insecte

Caroline Zanchi

UNIVERSITE DE BOURGOGNE

UMR 6282 Biogéosciences

THÈSE
Pour obtenir le grade de
Docteur de l'Université de Bourgogne
Discipline : Sciences Vie
Spécialité : Ecologie Evolutive

Par

Caroline Zanchi

Soutenance le 17 décembre 2012

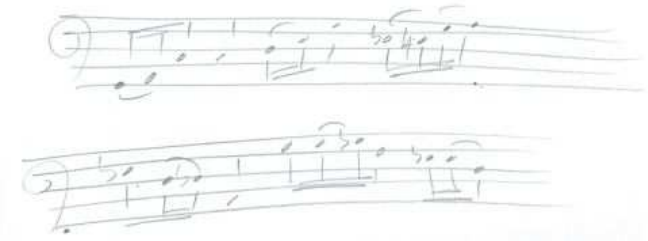
Ecologie évolutive du transfert trans-générationnel d'immunité chez un insecte.

Directeur de thèse : Yannick Moret

Co-directeur de thèse : Jérôme Moreau

JURY :

Benjamin GOURBAL	Maître de Conférences, Université de Perpignan	Examinateur
Yanis MICHALAKIS	Directeur de Recherche CNRS, Université de Montpellier II	Rapporteur
Jérôme MOREAU	Maître de Conférences, Université de Bourgogne	Co-directeur
Yannick MORET	Chargé de Recherche CNRS, Université deBourgogne	Directeur
Thierry RIGAUD	Directeur de Recherche CNRS, Université de Bourgogne	Examinateur
Paul SCHMID-HEMPEL	Professeur, ETH Zürich	Rapporteur



Michel Thévenard

Remerciements

Tout d'abord, je remercie Chef et Chef de m'avoir recrutée. C'était sympa! Merci donc à Yannick Moret et Jérôme Moreau de m'avoir donné cette opportunité, et de m'avoir encadrée pendant trois ans. Merci d'avoir organisé mes pensées désordonnées en raisonnements cohérents, et de d'avoir été aussi patients. Merci pour votre disponibilité et votre sympathie. Merci à Jean-Philippe Troussard d'être Jean-Philippe Troussard. Merci, Jipé, d'être indulgent envers les gens qui chantent moins bien que toi (vaut mieux remarque parce que ça fait beaucoup de gens), et d'avoir supporté mes vocalisations qui sortaient parfois du registre commun à l'espèce humaine. La salle Tenebrio résonne encore de nos duos magiques. Merci pour ta disponibilité, et sache que si tu as besoin de rien, je serai toujours là pour te rendre la pareille. J'y tiens. N'hésite pas à ne pas demander. Merci à Aurore Dubuffet pour nos discussions stimulantes, nos partages de biblio, pour avoir rendu mon manuscrit intelligible, et pour nos soirées. Et SURTOUT: Un grand merci à vous tous pour l'ambiance qui a régné au sein de l'équipe pendant ces années, j'aurais pas cru pouvoir me marrer autant au travail. Merci d'avoir pris la peine de rire à mes blagues (parfois), et de commenter la diversité, l'originalité, et l'assortiment des couleurs et des matières de mes tenues vestimentaires. J'imagine qu'on croise pas une équipe comme ça tous les jours, et vous allez tous beaucoup me manquer.

Merci à Thierry Rigaud pour ses conseils, son bon sens et son humour. Merci, Chef! Merci à François-Xavier Dechaume Moncharmont (j'avoue j'ai hésité à t'abréger) pour tous ses bon conseils en stats! C'est bien les Sagittaires, ça, toujours prêts à aider. Ou le contraire je sais plus. Merci à Rémy Wattier pour sa collection de CDs gravés (ho m**** je t'ai balancé, ha non ça va en fait tout le monde s'en fout maintenant des CDs. En plus personne lit les thèses). Merci à Maria Teixeira-Brandao, Nathalie Guichard et Sébastien Motreuil pour leur sympathie, leur disponibilité et leur sens de l'organisation qui profite à tout le monde dans le labo. Merci à Annie Marchand, Isabelle Santacroce et Cindy Pers d'avoir compensé mon manque d'organisation dans les démarches administratives par leur réactivité. ... et merci à toute l'équipe Ecologie Evolutive, d'avoir fait des pauses café un moment agréable où tout le monde rivalisait de créativité dans les blagues. Merci à Nicole Vitale pour nos conversations matinales et sa sympathie, et pour m'avoir ouvert chaque fois que j'oubliais mes clefs... ce qui était assez souvent. Merci à mes stagiaires d'avoir été aussi sympathiques et enthousiastes: Merci Benjamine, Karel, Marie, Adeline, Ambre et Coralie.

Merci à ma famille d'avoir été à mes côtés. Merci à ma mère, Bona, mon père et Myriam pour m'avoir autant soutenue, malgré mon manque de disponibilité. Merci Bona d'avoir lutté avec ardeur contre ma tendance à me laisser happer par la fainéantise, sans quoi je serais jamais arrivée jusque là. On va pouvoir aller faire le tour des caves bientôt! Merci Papanou et Mimi de m'avoir donné du recul, et de comprendre aussi bien les relations humaines. Merci de m'avoir aidé à relativiser! Et merci Kou de m'avoir toujours poussée à continuer et d'être fière de moi. Merci à Alex, et à mes oncles et tantes, c'est toujours un plaisir de vous voir en vacances. Merci à mes grands parents pour leur ouverture d'esprit et leur intelligence. J'ai hâte de pouvoir passer plus de temps avec vous tous.

Un grand merci à tous les gens qui ont mis de l'animation dans ma vie dijonnaise qui aurait été bien morne autrement. Merci à Christine Dubreuil de m'avoir supportée pendant tout ce temps. Depuis la première fois où je t'ai vue monter une poignée de porte à l'envers, on a fait pas mal de chemin! Une seule Chwistine vous manque, et tous les bars dijonnais sont

dépeuplés. Merci ma Chw, d'avoir toujours été là. Merci à Mark Gillingham d'être resté aussi stoïque face à mes fluctuations de moral, et de m'avoir sorti de la m*** aussi souvent. Merci de m'avoir fait profiter de ton expérience (en SCIENCES bien sûr esprit mal placé va!). Sans vous je me serais défenestrée plus d'une fois. Oui plus d'une, parce que j'habite pas très haut.

Merci à Matthias et Lucile d'avoir été des compagnons de galère, même si c'est un peu bateau comme formule (HA! JEU DE MOTS! galère/bateau... galère/bateau... c'est parce que la galère c'est un bateau en fait). Merci d'avoir égayé nos longues journées au boulot avec des calembours divers et variés. Et heureusement qu'il existe encore de par le monde trois/quatre énoooormes cerveaux comme les nôtres, bien éclairés, et que l'ordinateur n'est pas près de n***er ! Merci à Coraline pour sa tonne d'histoires nouvelles à chaque visite, et pour les soirées où on s'est entassés gaiement dans son appart à picoler largement plus que de raison. Merci à Emilie pour son dynamisme, ses fringues, ses plantes, et les petits gâteaux. Merci à Adrien, Juliette, Cécile, David, Romain, et à tous les gens non-titulaires que j'oublie certainement et qui ont contribué à mettre une bonne ambiance dans le labo. Oui je sais c'est décousu mais je vous retranscris ça pêle-mêle aussi!

Merci à Alouexe d'avoir organisé autant de soirées chez lui, d'avoir animé nos week-ends avec des matchs de basket, et d'avoir toujours une c****rie à dire. Merci à Aude pour son enthousiasme et les soirées picole et karaoké (bon mélange, très bon concept, je recommande). Merci à Aurélie pour les soirées films d'horreurs et jeux! Thanks to Tomasz and Tomasz for the wonderful vodka and the geeky conversations. Merci à Elise, Marion, Ronan et Cyril pour nos soirées sympathiques. Merci à Thomas pour les concerts et les soirées, et pour me laisser commenter ton énorme collection de CDs. Merci aussi à Jacques, Jean-Lou et Amélie. Merci aux Tanneries d'exister. Un merci tout particulier à Michel Thévenard, pour m'avoir fait attendre le mardi avec impatience.

Merci à Abdel, mon prof de maths du lycée pour les nombreuses heures qu'il a passées à m'enlever ma phobie des chiffres, sans lequel je n'aurais jamais continué dans la science. Merci à tous mes profs de collège et de lycée, d'ailleurs. Merci à l'Université de Picardie Jules Verne, où j'ai appris les trois-quarts de ce que je sais aujourd'hui. Merci à Geneviève Prévost, Aude Couty, Géraldine Doury, Philippe Giordanengo, Anas Cherqui, David Roger, Françoise Gillet, Jean-Philippe Morin, et plein d'autres enseignants dont j'ai oublié le nom mais auxquels je pense souvent. Merci d'avoir éveillé et entretenu ma curiosité. Merci aux professeurs de biochimie et biologie moléculaire de m'avoir torturée avec des voies métaboliques et des voies de transduction de signal horribles mais nécessaires, et que je n'aurais jamais apprises par moi-même.

Merci à mes amis picards, pour m'avoir offert des années universitaires qui rendraient beaucoup d'étudiants jaloux. Merci à Audrey et Rémi, pour les mojitos, les super soirées dans la campagne plate et pluvieuse, et pour tout leur soutien. Merci à Philippe, d'être égal à lui même, exigeant, de me connaître aussi bien. Merci pour tous ces liens stupides qui me faisaient marrer seule devant mon écran. Merci à Paul pour la danse de l'indien, la jeune mariée, et la puissance de la founk. Merci à O-DRE et Greg (et Alessa!) d'avoir toujours une geekerie à partager. Merci à vous tous de ne pas m'avoir oublié malgré la distance et la faible fréquence de nos soirées.

Et enfin, pour avoir su percevoir au delà des mots, pour avoir été présent sans être pressant, pour avoir écouté sans porter de jugement. Pour ta patience, ton courage, et ton soutien inconditionnel, merci, *Tenebrio molitor*.

Résumé

Le transfert trans-générationnel d'immunité (TTGI) est défini comme étant une élévation de l'immunocompétence de la descendance suite à la rencontre des femelles avec un organisme pathogène. Le TTGI est un phénomène bien connu chez les vertébrés, chez lesquels il se réalise par le transfert d'anticorps de la mère au jeune. Il n'a été décrit que récemment chez les invertébrés, chez lesquels le support de sa transmission est encore inconnu. Le TTGI apporte un bénéfice aux descendants lorsqu'ils rencontrent l'infection vécue par la mère, dans quel cas l'élévation de leur immunocompétence a un effet protecteur. Cependant, au-delà de ce bénéfice, plusieurs indices suggèrent que le TTGI est un phénomène coûteux pour les organismes. L'évolution du TTGI ne sera permise chez une espèce que lorsque les bénéfices qu'il représente en termes de protection des descendants surpasseront les coûts qu'il représente pour eux en termes de fitness. Ainsi, l'étude de ses coûts et de ses bénéfices nous renseigne sur les pressions de sélection qui ont conduit à son évolution chez les invertébrés. Au cours de cette thèse, j'ai associé l'expression du TTGI chez un insecte avec un certain nombre de coûts, tant pour les femelles qui le réalisent que pour les descendants qui l'expriment. Pour ce faire, j'ai utilisé comme organisme modèle le ver de farine, *Tenebrio molitor*.

Dans le premier chapitre, nous avons stimulé le système immunitaire des femelles adultes de *T. molitor* avec un immunogène non pathogène, et étudié divers aspects de la transmission d'activité antibactérienne aux œufs qui en résultait. Cela nous a permis de voir que la transmission d'activité antibactérienne interne aux œufs commençait deux jours après la stimulation du système immunitaire des femelles et cessait après dix jours. Enfin, nous avons pu mettre en évidence un coût pour les femelles à la protection de leurs œufs, en termes de fécondité.

Dans le second chapitre, nous stimulé le système immunitaire avec trois microorganismes différents tués par la chaleur, et exposé leurs jeunes larves à des microorganismes vivants. Nous n'avons pas réussi à mettre en évidence d'effet protecteur du TTGI sur les jeunes larves de *T. molitor*. Il s'avère cependant que l'exposition des jeunes larves à un champignon entomopathogène réduit le délai avant leur seconde mue larvaire.

Dans le troisième chapitre, nous avons stimulé soit le système immunitaire des femelles, soit celui des mâles de *T. molitor* avec un immunogène non pathogène, et observé différents paramètres de l'immunité de leurs descendants adultes. Cela nous a permis de mettre en évidence que le TTGI d'origine maternelle et paternelle n'affecte pas les mêmes effecteurs immunitaires chez les descendants, et que le TTGI d'origine maternelle comportait un coût pour eux en termes de temps de développement.

Ces coûts au TTGI suggèrent qu'il n'est pas seulement une conséquence de la stimulation du système immunitaire des femelles de la génération parentale, mais qu'il est bien un mécanisme qui a été sélectionné du fait des bénéfices qu'il représente pour les organismes dans certaines conditions écologiques.

Abstract

Trans-generational immune priming (TGIP) is defined as the plastic enhancement of offspring's immunocompetence following an immune challenge of the females of the parental generation. In vertebrates, this phenomenon is well described, and is achieved by the maternal transfer of antibodies. In invertebrates however, it has only recently been described. Since invertebrates do not possess antibodies, the mechanism of this transmission remains unknown. If the offspring is exposed to the maternal infection, an elevated immunocompetence can help it cope better with it. Nonetheless, apart from this benefit, several cues indicate that the TGIP bears some fitness costs for individuals. The evolution of TGIP will be favoured when its benefits outweigh its fitness costs. Thus, studying its costs and benefits can lead us to a better understanding of the selection pressures that lead to its evolution in invertebrates. During my thesis, I associated the occurrence of TGIP in an insect, the mealworm beetle *Tenebrio molitor*, to several fitness costs for the females transmitting it as well as for the offspring receiving it.

In the first chapter, we stimulated the adult female's immune system with a non pathogenic immunogene, and studied several aspects of the subsequent transfer of antibacterial activity to the eggs. We saw that the transmission of antibacterial activity inside the eggs started two days after the immune challenge, and stopped at ten. Then, we highlighted a cost for the females on their fecundity to this transmission.

In the second chapter, we stimulated the immune system of the females with three different heat-killed microorganisms, and exposed their larval progeny to living microorganisms. We did not see any benefit of the TGIP on the young larvae of *T. molitor*. However, we saw that the exposure of young larvae to an entomopathogenic fungus decreased the time-lap between the two first larval moults.

In the third chapter, we stimulated the immune system of either the adult females or the males of *T. molitor*, and we observed several immune parameters in their adult offspring. This allowed us to see that maternally and paternally-derived TGIP affected different immune effectors in the adult offspring, and that maternally-derived TGIP bear a cost on the developmental time of the offspring.

These fitness costs to the TGIP suggest that it is not just a side-effect of the immune reaction of the females, but rather an investment that has been selected because of the benefits it represents for the offspring in certain ecological conditions.

Table des matières

REMERCIEMENTS ..... **II**

RESUME ..... **IV**

ABSTRACT ..... **V**

TABLE DES MATIERES ..... **VI**

TABLE DES ILLUSTRATIONS ..... **IX**

INTRODUCTION GENERALE **2**

LES EFFETS PARENTAUX **2**
L'IMMUNITE DES INSECTES **6**
LE TRANSFERT TRANS-GENERATIONNEL D'IMMUNITE CHEZ LES INVERTEBRES **8**
OBJECTIF DE MA THESE **12**

MATERIEL ET METHODES GENERAL **16**

I. TENEBRIO MOLITOR, UN MODELE ADAPTE A L'ETUDE DU TRANSFERT TRANS-GENERATIONNEL D'IMMUNITE **16**
II. PARAMETRES OBSERVES ET TECHNIQUES EMPLOYEES **19**
II.1. STIMULATION DU SYSTEME IMMUNITAIRE DES FEMELLES 20
II.2. ESTIMATION DE LA CHARGE HEMOCYTAIRE 20
II.3. MESURE DE L'ACTIVITE ENZYMATIQUE DU SYSTEME PROPHENOLOXYDASE 21
II.4. MESURES D'ACTIVITE ANTIBACTERIENNE 22

CHAPITRE 1 : LE TRANSFERT TRANS-GENERATIONNEL D'IMMUNITE AUX UFS CHEZ T. MOLITOR : COUTS POUR LA FEMELLE, ET RECHERCHE D'UN EFFET PATERNEL **24**

PART 1 : RELATIONSHIP BETWEEN MATERNAL TRANSFER OF IMMUNITY AND MOTHER FECUNDITY IN AN INSECT **26**
I. INTRODUCTION 28
II. MATERIALS AND METHODS 31
II.1. Insect cultures, immune challenge and egg collection 31
II.1.1. Experiment 1: Temporal dynamics of the antibacterial immune response of immune-challenged females and of the transmission of antibacterial activity to their eggs 31
II.1.2. Experiment 2: Localisation of the antibacterial activity transferred to the eggs 32
II.1.3. Experiment 3: Testing the trade-off between mother fecundity and number of immune protected eggs 32
II.2. Analysing the antibacterial activity of the hemolymph and the eggs 33
II.2. Statistics 34
III. RESULTS 35
III.1. Experiment 1: Temporal dynamics of the antibacterial immune response of immune-challenged females and of the transmission of antibacterial activity to their eggs 35
III.2. Experiment 2: Localisation of the antibacterial activity transferred to the eggs 37

III.3. Experiment 3: Testing the trade-off between mother fecundity and number of immune protected eggs 37
IV. DISCUSSION 40
ACKNOWLEDGEMENTS 44
PART 2 : IN SEARCH OF A PATERNAL TRANSMISSION OF ANTIBACTERIAL ACTIVITY TO THE EGGS OF TENEBRIO MOLITOR. 45
I. INTRODUCTION 46
II. MATERIALS AND METHODS 48
II.1. Temporal dynamics of the transmission of antibacterial activity to their eggs by immune-challenged males 48
II.2. Analysing the antibacterial activity of the eggs 48
III. RESULTS 49
IV. DISCUSSION 49

CHAPITRE 2 : RECHERCHE D'UN TRANSFERT TRANS-GENERATIONNEL D'IMMUNITE AUX JEUNES LARVES DE *T. MOLITOR* ET DE SON DEGRE DE SPECIFICITE. 52

I. INTRODUCTION 55
II. MATERIALS AND METHODS 58
II.1. INSECT CULTURE 58
II.2. MICROORGANISMS USED 58
II.3. EXPERIMENTAL DESIGN 58
II.3.1. Experiment 1: specificity of the maternal immune priming on the young larvae of *T. molitor* 60
II.3.1.1. Maternal treatments 60
II.3.1.2. Larval treatments 60
II.3.2. Experiment 2: Existence of a maternal immune priming of the young larvae toward *M. anisopliae* in *T. molitor*, and effect of *M. anisopliae* on the duration of the first larval intermoult 61
II.3.2.1. Maternal treatments 61
II.3.2.2. Larval treatments 61
II.4. STATISTICAL ANALYSES 62
II.4.1. Experiment 1 62
II.4.2. Experiment 2 63
III. RESULTS 64
III.1. EXPERIMENT 1: SPECIFICITY OF THE MATERNAL IMMUNE PRIMING ON THE YOUNG LARVAE OF T. MOLITOR 64
III.2. EXPERIMENT 2: EXISTENCE OF A MATERNAL IMMUNE PRIMING OF THE YOUNG LARVAE TOWARD M. ANISOPLIAE IN T. MOLITOR, AND EFFECT OF M. ANISOPLIAE ON THE DURATION OF THE FIRST LARVAL INTERMOULT 64
III.2.1. Larval survival 64
III.2.2. Is the timing of moulting of *T. molitor* adjusted in response to a pathogenic threat? 65
IV. DISCUSSION 66

CHAPITRE 3 : RECHERCHE D'UN TRANSFERT TRANS-GENERATIONNEL D'IMMUNITE D'ORIGINE MATERNELLE ET PATERNELLE AUX ADULTES DE *T. MOLITOR* ET DE SES COUTS. 70

DIFFERENTIAL EXPRESSION AND COSTS BETWEEN MATERNALLY AND PATERNALLY DERIVED IMMUNE PRIMING FOR OFFSPRING IN AN INSECT 72

SUMMARY 73
I. INTRODUCTION 74
II. MATERIAL AND METHODS 76

II.1. INSECT CULTURING 76
II.2. IMMUNE TREATMENTS OF THE PARENTS AND THE OFFSPRING 77
II.3. HAEMOLYMPH COLLECTION 78
II.4. IMMUNE PARAMETERS 78
II.5. BODY MASS AND SIZE 79
II.6. STATISTICS 79
III. RESULTS 81
III.1. PARENTAL IMMUNE RESPONSE AND REPRODUCTIVE EFFORT 81
III.2. PARENTAL EFFECT OF OFFSPRING IMMUNITY 82
III.2.1. Maternal effect on offspring immunity 82
III.2.2. Paternal effect on offspring immunity 84
III.3. Parental effect on offspring life history 86
IV. DISCUSSION 88

DISCUSSION 97

LE TTGI : UN EFFET BIPARENTAL SUR L'IMMUNITE DE LA DESCENDANCE? 97
EFFET MATERNEL : SON EXPRESSION ET SES BENEFICES POTENTIELS. 100
COUTS & CONDITIONS D'EVOLUTION DU TTGI CHEZ LES INSECTES. 103
CONCLUSION 106

RÉFÉRENCES 108

RÉFÉRENCES 108

CHAPITRE 1 122

CHAPITRE 2 123

CHAPITRE 3 124

PROTOCOLES 125

TRANS-GENERATIONAL IMMUNE PRIMING IS CONSTRAINED BY THE MATERNAL IMMUNE RESPONSE IN AN INSECT. 127

Table des illustrations

***Figure 1**: Couple de T. molitor dans une boîte de Petri (Ø 9cm) contenant des œufs.* 16
***Figure 2** : Schéma représentant le cycle de vie de T. molitor, indiquant la durée des différents stades le composant.* 17
***Figure 3** : Photo d'un spermatophore de T. molitor dans une solution saline, une minute après expulsion.* 17
***Figure 4** : Illustration des conditions d'élevage de T. molitor dans notre laboratoire.* 18
***Figure 5** : Appareil copulatoire femelle **(a)** et mâle **(b)** dévaginés de T. molitor.* 19
***Figure 6** : Photo des hémocytes de T. molitor dans un hémocytomètre.* 21
***Figure 7** : Plaque 96 puits ayant servi au dosage de l'activité du système PO-PPO au bout de 40 minutes de suivi.* 21
***Figure 8** : Boîte de Petri ayant servi à des mesures d'activité antibactérienne, après 24h d'incubation.* 22
***Figure 9** : **a)** Antibacterial activity of the hemolymph of the females (mean diameter of the zone of inhibition in mm ± SE) and **b)** proportion of eggs protected (± SE) according to the time following the maternal immune challenge.* 36
***Figure 10** : Relationship between the amount of antibacterial activity allocated to each egg by control (dashed line) and LPS treated females (solid line) between the days 2 and 4 post immune-challenge according to the number of eggs protected in this egg laying sequence.* 38
***Figure 11**: Relationship between the current fecundity (between day 2 and day 4) and **a)** the number of eggs protected and **b)** the proportion of eggs protected by control (dashed line) and LPS-treated females (solid line) during this egg laying sequence.* 39
***Table 1**: Summary of the optimal GLM following stepwise-deletion of the number and proportion of eggs protected by females between day 2 and day 4 post immune-challenge,* 43
***Figure 12** : Schema synthesizing the experimental treatments realized in the parental and offspring generations for the test of the specificity of the TGIP on the young larvae of T. molitor.* 61
***Figure 13** : Schema synthesizing the experimental treatments realized in the parental and offspring generations for the test of the existence of a TGIP elicited by M. anisopliae on the young larvae of T. molitor, and the test of the effect of M. anisopliae on the duration of the first larval intermoult.* 62
***Figure 14** : Mean percentage of surviving larvae (± SE) at the end of the experiment, as a function of the "specificity" treatment (x axis).* 64
***Figure 15** : Percentage of surviving larvae after 30 days of experiment according to the larval treatment (x-axis).* 65
***Figure 16** : Mean duration of the first larval intermoult (in days ± SE) of the larvae from pricked and M. anisopliae-pricked females, according to the larval treatment (x-axis).* 65
***Figure 17** : Antibacterial activity (mean + SE) in the haemolymph of parental females and males previously exposed to an injection with Ringer solution (control; open bars) and LPS solution (black bars) mimicking a bacterial infection.* 81
***Figure 18** : Concentration of haemocytes (mean + SE) of adult offspring in relation to parental immune priming before and after immune stimulation with LPS.* 83
***Figure 19** : Activity of naturally activated phenoloxidase enzymes (PO activity; Mean + SE) and activity of the proenzymes in addition of the PO (total-PO; Mean + SE) in the haemolymph of maternally (upper panels) and paternally (lower panels) immune primed offspring after being immune challenged.* 85
***Figure 20** : **(a)** Larval developmental time from hatching of the eggs to imago (mean + SE) and **(b)** pupae mass (mean + SE) of offspring in relation to parental immune priming.* 87
***Table 2** : 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. N. R. refers to effects not retained by the stepwise procedure. Values $p \leq 0.05$ are given in bold.* 93
***Table 3** : 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.* 95
***Table 4** : Résumé de différentes caractéristiques des histoires de vies des espèces chez lesquelles le TTGI a été recherché.* 105
***Figure S 1** : Response curves of the GLM on the number of eggs protected by control (dashed line) and LPS-treated females (solid line) between the days 2 and 4 post immune challenge according to the past fecundity (between day 0 and day 2).* 122
***Figure S 2** : Response curves of the **(a)** GLM on the number and **(b)** the proportion of eggs protected by females from poor food condition (dashed line) and good food condition (solid line) between the days 2 and 4 post immune challenge according to the past fecundity (between day 0 and day 2).* 122

Figure S 3 *: Mean duration of the first larval intermoult (in days ± SE) of the larvae from Ringer, E. coli and B. thuringiensis-injected females, according to the larval treatment (x-axis).* *123*
Supplementary Table 1 *: 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.* *124*

Introduction générale

Introduction générale

Les effets parentaux

Le phénotype des organismes résulte de l'interaction entre leur génotype l'environnement dans lequel ils se sont développés. Au sein de cet environnement, les autres individus de la population contribuent à l'expression du phénotype d'un organisme. Les parents par exemple peuvent influencer l'expression des traits phénotypiques de leur progéniture au-delà des gènes qu'ils lui transmettent, par la transmission d'informations sur leur état physiologique ou leur environnement. Cette contribution parentale au phénotype de la progéniture peut s'exercer par des mécanismes variés, aussi bien physiologiques que comportementaux. Elle peut consister selon les espèces en la transmission de certaines modifications épigénétiques du génome des parents acquises au cours de leur vie sous l'effet de pressions environnementales (Jablonka & Raz, 2009), et peut aller jusqu'à l'existence de comportements de soins parentaux complexes (Rossiter, 1996).

Chez la plupart des espèces, les femelles sont le sexe qui a le plus d'influence sur le phénotype de la descendance. Elles abritent en effet les premiers stades de la vie de leurs descendants, et produisent les gamètes les plus riches en matériel non nucléotidique (Bernardo, 1996). Les effets maternels sont définis comme étant toute influence du génotype et de l'environnement de la femelle sur le phénotype de sa progéniture (Mousseau & Dingle, 1991). L'exemple le plus étudié d'effet maternel est celui du provisionnement des œufs en nutriments chez les espèces ovipares, facteur qui influence de nombreux aspects de la fitness de la progéniture comme la durée du développement embryonnaire (Benton et al. 2005) et la survie larvaire chez les insectes (Fox, 1993). Dans le cas particulier du provisionnement des œufs, l'effet maternel prend la forme d'un investissement coûteux : les nutriments approvisionnant les œufs proviennent de ressources énergétiques limitées chez les femelles, pour lesquels d'autres traits d'histoire de vie sont en compétition. Ainsi, le provisionnement des œufs se réaliserait aux dépends de l'expression chez les femelles d'autres traits phénotypiques coûteux (Stearns, 1992). La survie par exemple est un trait susceptible d'entrer en compétition avec la production d'œufs. En effet, chez *Drosophila melanogaster*, la

rétention d'œufs augmente la longévité des femelles (Partridge et al., 1987). La coccinelle *Epilachna niponica* quant à elle augmente sa longévité à la fin de la saison de reproduction en résorbant les œufs présents dans ses ovarioles (Ohgushi, 1996). D'autres exemples reportent l'existence d'un compromis entre le nombre d'œufs produits et leur taille, comme chez le coléoptère bruchidé *Stator limbatus* (Czesak & Fox, 2003). Ces deux exemples de compromis sont les plus étudiés, mais d'autres existent, tels qu'entre l'activité des muscles allaires lors du vol et la production d'œufs chez *Pararge aegeria* par exemple (Gibbs et al. 2010). Les femelles sont également souvent responsables du choix du site d'oviposition. À ce titre, elles peuvent percevoir certaines caractéristiques de l'environnement comme la composition et la disponibilité des ressources alimentaires ou encore son exposition aux pathogènes, parasites et prédateurs. Cet effet maternel conditionnera donc la survie la croissance et la reproduction des descendants à plus ou moins long terme (Thompson & Pellmyr, 1991 ; Desouhant, 1998).

Une dégradation générale de l'état physiologique des femelles peut affecter leur investissement dans ces différents aspects de la reproduction, et conduire à une diminution de la qualité de ses descendants. L'effet maternel aura donc des conséquences délétères sur la fitness des descendants et sera dit **négatif**. C'est le cas par exemple lorsque la diminution de la quantité de réserves énergétiques des femelles entraîne une diminution de la quantité de nutriments incorporés dans leurs œufs (Moya-Laraño, 2002; Gibbs et al. 2010). En revanche, certains effets maternels peuvent se traduire par une amélioration de la fitness de la descendance, lorsqu'ils permettent une adaptation du phénotype de celle-ci aux caractéristiques de leur environnement. Dans ce cas, l'effet maternel est dit **adaptatif** (Mousseau & Fox, 1998a). Ainsi, en réponse à une forte densité expérimentée avant l'oviposition, la femelle de l'hyménoptère parasitoïde *Copidosoma koehleri* engendre une progéniture dont le temps de développement larvaire est réduit (Morag et al. 2011). Ce processus de réduction du temps de développement larvaire est supposé conférer un avantage aux descendants dans une situation de forte compétition pour les ressources alimentaires provoquée par une haute densité de population.

Lors de l'évocation des effets parentaux, il est souvent fait référence aux effets maternels. Or, le phénotype paternel et son expérience de l'environnement peuvent également influencer le phénotype de la descendance. Les effets paternels les plus étudiés sont les soins post-nataux dispensés par les mâles chez certains animaux, y compris chez certaines espèces

d'insectes (Scott, 1989 ; Hunt & Simmons, 2007). À part les soins post-nataux, l'existence d'autres formes d'effets paternels a longtemps été sous-estimée (Falconer & Mckay, 1997). En effet, les effets paternels s'exprimant par l'intermédiaire des gamètes mâles peuvent sembler improbables du fait de la faible quantité de matériel non-nucléotidique qu'ils apportent au zygote en comparaison à celle du gamète femelle. Cependant, l'ADN des spermatozoïdes est susceptible de porter des modifications épigénétiques, telles que des méthylations d'ADN ou des modifications des histones (Jablonka & Lamb, 1995). Ainsi, l'existence d'effets paternels a été reportée même chez des espèces ne réalisant pas de soins paternels : chez le criquet *Schistocerca gregaria* par exemple, la phase (grégaire ou solitaire) des individus peut être déterminée par les conditions de copulation des mâles comme des femelles (Islam et al. 1994).

De manière générale, trois conditions sont nécessaires à l'existence d'effets parentaux adaptatifs: (1) il faut que la variation apparue dans les conditions environnementales de la génération parentale réduise potentiellement la fitness de la descendance, (2) que les parents puissent percevoir cette variation et prédire sa persistance dans l'environnement de la descendance et enfin (3) qu'ils soient capables d'adapter le phénotype de la descendance en conséquence. À cette dernière condition, les effets parentaux permettent l'adaptation de la progéniture à un changement intervenu dans la génération parentale au-delà de ce que ses potentialités génétiques seules lui permettent. Les effets parentaux adaptatifs sont ainsi supposés évoluer en réponse à la pression de sélection exercée par les environnements fluctuants (Mousseau & Fox, 1998b). Cependant, l'ajustement du phénotype de la progéniture aux conditions de l'environnement parental procurera un bénéfice à la descendance seulement si les conditions environnementales vécues par les parents persistent dans l'environnement de la prochaine génération. Cela suppose qu'ils évoluent dans les environnements dont les fluctuations ne sont pas trop fréquentes (Schuler & Orrock, 2012). Par conséquent, l'expression des effets parentaux adaptatifs devrait dépendre de la persistance des changements environnementaux expérimentés par les parents dans l'environnement de la descendance.

Parmi les fluctuations pouvant intervenir dans l'environnement, la rencontre avec les pathogènes et parasites est souvent imprévisible dans l'espace et dans le temps et est susceptible d'exercer une forte pression de sélection sur les populations hôtes (Loye & Zuk,

1991). Cette pression de sélection a conduit à l'évolution de divers effets parentaux permettant la protection de la progéniture, qui peuvent présenter des coûts pour différents aspects de la fitness des femelles. Chez le coléoptère *Mimosestes amicus*, les femelles sont capables de pondre des œufs non viables par-dessus leurs œufs viables, conférant à ces derniers une meilleure protection envers les attaques de l'hyménoptère parasitoïde *Uscana semifumipennis*. Elles sont également capables d'augmenter le nombre de ces œufs protecteurs en réponse à la présence de guêpes parasitoïdes sur le site d'oviposition. Cette protection s'accompagne d'un coût en termes de fécondité pour les femelles, puisque la production d'œufs protecteurs et non viables se fait au détriment de la production d'œufs viables (Deas & Hunter, 2012). Chez une autre espèce, la guêpe solitaire *Philantus triangulum*, les femelles protègent leurs larves d'une infection fongique en appliquant des hydrocarbures inhibant la croissance fongique sur les proies qui serviront à nourrir les jeunes larves. Cependant, la quantité de protection disponible au sein d'une femelle semble limitée, et la protection d'une portée se fait au détriment de la protection des portées ultérieures (Herzner, 2011).

Parmi l'ensemble des effets parentaux permettant d'augmenter la fitness des descendants en cas d'infection, certains animaux ont également évolué des effets maternels conduisant à la modification de l'immunocompétence de la descendance, et qui peuvent réduire l'impact d'une infection sur la fitness de cette dernière. Il s'agit du transfert trans-générationnel d'immunité.

Le **transfert trans-générationnel d'immunité** (**TTGI**) est un cas particulier d'effet maternel, dans lequel l'expérience immunitaire de la mère peut influencer le phénotype immunitaire de sa descendance. Il est défini comme une élévation de l'immunocompétence de la descendance suite à la rencontre des femelles avec un organisme pathogène (Little & Kraaijeveld, 2004). Son expression apporte donc un bénéfice si l'infection rencontrée par la mère persiste dans l'environnement de sa descendance, dans la mesure où la réponse immunitaire de cette dernière est plus efficace. Le TTGI est un phénomène bien connu chez les vertébrés, chez lesquels il se réalise par le transfert d'anticorps de la mère au jeune. Le transfert d'immunoglobulines spécifiques de l'organisme pathogène ayant été rencontré par la mère confère au jeune une protection précoce lorsque son système immunitaire n'est pas encore assez mature pour combattre une infection, et dirige sa maturation (Hasselquist &

Nilsson, 2009). Elle leur impose cependant certains coûts. Les auteurs supposent en effet une augmentation du risque d'infections intra-cellulaires chez le jeune suite au transfert d'anticorps par sa mère, et une diminution de sa production endogène d'anticorps plus tardivement dans sa vie (voir Grindstaff et al., 2003 pour revue). Les invertébrés ne présentent pas les effecteurs de l'immunité adaptative que l'on peut rencontrer chez les vertébrés, tels que des immunoglobulines pouvant être diversifiées par recombinaison somatique ou encore un système d'expansion clonale des lymphocytes (Klein, 1997). De ce fait, ils ont longtemps été considérés comme étant incapables de générer une réponse immunitaire adaptative face à l'intrusion d'un pathogène (Hoffman, 2003), et encore moins de la transmettre (Hauton & Smith, 2007). Cependant, en se plaçant d'un point de vue écologique plus que fonctionnel, l'absence d'effecteurs de l'immunité homologues à ceux des vertébrés chez les invertébrés n'enlève pas la possibilité d'observer chez eux des phénomènes analogues. On remarque ainsi que le TTGI affecte l'expression de différents effecteurs immunitaires chez la descendance des femelles invertébrées immunostimulées. Etant donné que j'ai étudié divers aspects du TTGI chez un modèle insecte, je ferai un bref rappel des principales composantes de leur immunité dans le paragraphe à venir.

L'immunité des insectes

Les réponses immunitaires des insectes sont activées suite à la reconnaissance d'un large spectre de motifs du non-soi, provenant de molécules bien caractéristiques de la surface cellulaire des agents pathogènes, appelées **Pathogen Associated Molecular Patterns** (**PAMPs**). Ces molécules peuvent être des peptidoglycanes exposés par les bactéries à Gram négatif, des ß-1,3 glucanes portés par les pathogènes fongiques, des lipopolysaccharides portés par des bactéries à Gram négatif et d'autres fragments de sucres (Theopold et al. 1999). Les protéines permettant la reconnaissance de ces déterminants antigéniques sont nommées **Pathogen Recognition Receptors** (**PRRs**). Elles sont principalement synthétisées par le corps gras, tissu adipeux réparti dans tous l'abdomen de l'insecte. En réponse au type de pathogène reconnu par ces PRRs, différentes voies immunitaires seront activées, conduisant au recrutement de l'effecteur approprié.

Les hémocytes circulants proviennent des tissus hématopoiétiques, dans lesquels ils se divisent et se différencient. Ils sont mobilisés dans les premières minutes suivant l'infection et

réalisent la phagocytose, la nodulation et l'encapsulation (Lavine & Strand, 2002). La réponse la plus précoce et la plus fréquente face aux pathogènes bactériens, fongiques ou viraux, est la **nodulation** (Horohov & Dunn, 1983). Elle consiste en une microagrégation d'hémocytes autour de plusieurs microorganismes infectieux suivie de la dégranulation de facteurs humoraux par ces derniers puis de leur mort, ce qui résulte en la formation d'un coagulum de débris cellulaires, de fibres et de protéines emprisonnant les agents infectieux et conduisant à leur élimination (Ratcliffe & Gegen, 1976).
La **phagocytose** consiste en l'internalisation par les hémocytes de petites particules étrangères, ce qui conduira à leur dégradation (Ratcliffe & Rowley, 1979).
Les insectes sont également susceptibles d'être parasités par des organismes trop volumineux pour être nodulés ou phagocytés, tels que des nématodes, des cestodes ou des œufs de parasitoïdes. On peut alors en observer l'**encapsulation** (Carton & Nappi, 1997). Lors de ce processus, la reconnaissance du parasite par les hémocytes conduira à leur formation autour du parasite d'une couche multicellulaire d'hémocytes morts (Pech & Strand, 1996), qui chez la plupart des espèces d'insectes est mélanisée par la suite sous l'action de la **phénoloxydase**.

La mélanisation intervient aussi bien dans l'élimination des bactéries et des protozoaires (Collins et al. 1986) que dans l'élimination des parasites plus volumineux par encapsulation. Bien que le rôle que joue la mélanisation en elle-même dans l'élimination du pathogène reste controversé (Leclerc et al. 2006, Schnitger et al. 2007), on sait que la **prophénoloxydase** et la cascade protéolytique conduisant à son activation en phénoloxydase sont un élément clef de la réponse immunitaire des insectes. En effet, des intermédiaires de la réaction (dopamine et 5,6-dihydroxyindole, quinones) présentent une activité antibactérienne (Zhao et al. 2007), et certaines enzymes impliquées dans la synthèse de mélanine ont un effet promoteur de la phagocytose et de l'agrégation cellulaire (Zhao et al. 2007, Sideri et al. 2008).

La synthèse de **peptides antibactériens** est induite dans le corps gras des insectes suite à la reconnaissance des PAMPs par les PRRs, mais leur expression sera plus tardive. Bien que leur cinétique d'induction varie, on observe chez *T. molitor* un pic d'activité antibactérienne dans les 24h suivant l'injection d'éliciteurs (Haine et al. 2008). Cette activité peut persister dans l'hémolymphe de cette espèce au moins sept jours après le challenge (Moret & Siva-Jothy, 2003).

Introduction générale

Le **lysozyme** est une protéine à activité antibactérienne principalement dirigée contre les bactéries à Gram positif qui agit en hydrolysant les peptidoglycanes constituant leurs parois. Il a également une action opsonisante facilitant la phagocytose (Hultmark, 1996). Des composés cytotoxiques tels que l'**oxyde nitrique** ou les **formes actives de l'oxygène** participent également à l'élimination des organismes parasites et pathogènes (voir Rivero, 2006 pour revue).

Le transfert trans-générationnel d'immunité chez les invertébrés

Chez les invertébrés, le TTGI a récemment été mis en évidence, principalement au travers d'études écologiques. Huang & Song (1999) ont constaté, chez la crevette d'élevage *Penaeus monodon*, que les descendants des femelles stimulées avec des β-1,3-1,6-glucanes dérivés de *Saccharomyces cerevisiae* montraient une meilleure résistance au virus responsable du « White Spot Syndrom ».

Le support de la transmission d'immunité des parents à leur progéniture est inconnu, mais des études ultérieures réalisées chez plusieurs espèces d'invertébrés ont permis de mettre en évidence la surexpression de certains effecteurs immunitaires dans la descendance des femelles immunostimulées. Chez les insectes, la première étude à le mettre en évidence est celle de Rahman et al. (2003) sur la pyrale de la farine, *Ephestia kuehniella*. Chez cette espèce, la stimulation du système immunitaire des femelles par une toxine conduit à l'élévation de l'activité du système PO dans l'hémolymphe des individus de la descendance. Cet effecteur est également affecté chez la descendance de *Trichoplusia ni* lorsque les femelles de la génération parentale ont été maintenues sur un milieu nutritif contaminé par des bactéries (Freitak et al., 2009). Une augmentation de l'activité antibactérienne induite dans l'hémolymphe des descendants a été observée suite à la stimulation des mères par des bactéries ou des immunogènes dérivés de bactéries, chez *Bombus terrestris*, *Tenebrio molitor* et *T. ni* (Sadd et al., 2005 ; Moret, 2006 ; Freitak et al., 2009). Freitak et al. (2009) ont également montré une surexpression de PRRs dans les hémocytes des descendants de femelles de *T. ni* immunostimulées. Une augmentation de l'activité antibactérienne à l'intérieur des œufs de *B. terrestris* suite à la stimulation des reines a également été reportée (Sadd & Schmid-Hempel, 2007). Un effet paternel au TTGI a été mis en évidence chez *T. ni* et *Tribolium castaneum* (Freitak et al., 2009; Roth et al. 2010). Chez *T. castaneum*, une meilleure protection des descendants face au pathogène ayant stimulé les femelles a été

montrée, alors que la stimulation paternelle résultait en une protection plus générale (Roth et al. 2010). Le but de cette thèse n'est pas d'explorer les aspects fonctionnels du TTGI. Cependant l'étude de ses caractéristiques générales et de son écologie peut donner des indications quant à son mode de transmission chez les invertébrés.

La mise en évidence d'un tel phénomène de TTGI chez des invertébrés soulève en effet un certain nombre de questions par rapport à son écologie et son évolution en plus des aspects fonctionnels par lesquels il se réalise. Si l'étude de Huang & Song (1999) souligne bien le caractère bénéfique du TTGI, il reste cependant à déterminer si ce phénomène est un phénomène adaptatif, en d'autre termes à savoir s'il a évolué en réponse à des contraintes sélectives imposées par les parasites et les pathogènes. Pour comprendre les conditions de l'évolution du TTGI et de son maintien dans les populations naturelles, il convient d'identifier les conditions de son expression et ses coûts.

Plusieurs indices suggèrent que le TTGI pourrait comporter des coûts. D'une part, le TTGI est induit par la stimulation du système immunitaire des femelles de la génération parentale. Or, le caractère inductible d'un mécanisme de défense est supposé évoluer lorsque celui-ci est coûteux pour les hôtes: il leur permet d'éviter ses coûts en l'absence de menace, tout en conservant leur capacité à induire une défense lorsque la menace est présente (Harvell, 1990). Ainsi, en l'absence de coûts, on s'attendrait à ce que le TTGI s'exprime chez tous les individus d'une population, indépendamment de l'expérience immunologique de la mère. D'autre part, on sait que l'évolution, le maintien et le déploiement de l'immunité comportent plusieurs types de coûts pour les organismes (Schmid-Hempel 2003). Un premier coût provient des corrélations génétiques négatives éventuelles existant entre un composant du système immunitaire et un autre trait affectant la fitness de l'organisme hôte : c'est le **coût évolutif** de l'immunité. Un second type de coût résulte de la consommation des ressources énergétiques occasionnée par le maintien et le déploiement de la réponse immunitaire par les hôtes : c'est le **coût énergétique** de l'immunité. Selon la théorie des traits d'histoire de vie, la quantité de ressources énergétiques disponibles au sein des organismes étant limitée (Stearns, 1992), la réponse immunitaire est susceptible d'entrer en compétition avec l'expression d'autres traits coûteux en énergie déterminant la fitness des individus. Il pourra alors en résulter des compromis entre l'expression de ces traits et l'expression de certains effecteurs de

l'immunité. Enfin, la mobilisation des effecteurs immunitaire peut endommager les tissus de l'hôte : c'est l'**autoréactivité**.

Ainsi, l'élévation de l'immunocompétence des descendants des femelles stimulées pourrait représenter pour eux le même type de coûts que ceux de la réponse immunitaire. Dans ce cas, si la menace rencontrée par la mère ne persistait pas dans l'environnement de la descendance, cette dernière pourrait ne subir que les coûts du TTGI et non ses bénéfices, et se trouver contre-sélectionnée. Il est donc probable que le TTGI ait évolué chez des espèces pour lesquelles les parents et leur progéniture partagent le même environnement, c'est-à-dire des espèces montrant peu de dispersion et un chevauchement des générations (Sadd et al. 2005). Ces caractéristiques augmentent la probabilité pour l'hôte de rencontrer l'infection de la mère, étant donné que la propagation des organismes parasites et pathogènes dépend de la persistance de la population hôte (Dugaw, 20005).

Cette hypothèse est confirmée par le fait que l'existence du TTGI a été reportée chez des espèces d'insectes grégaires, telles que *E. kuehniella* (Rahman et al. 2003), *T. molitor* (Moret, 2006), *T. ni* (Freitak et al. 2009), *T. castaneum* (Roth et al. 2010), *Plodia interpunctella* (Tidbury et al. 2011) voir même eusociales tel que *B. terrestris* (Sadd et al. 2005), et envers des microorganismes à fort taux de multiplication tels que des bactéries ou des virus. En revanche, le TTGI n'a pas été observé chez des couples hôte/pathogène ne présentant pas les conditions écologiques prédites comme étant nécessaires à la persistance directe d'une infection de la génération parentale dans l'environnement de la descendance. Les femelles du puceron *Myzus persicae* ayant combattu avec succès *Diaretiella rapae* ne produisaient pas une descendance capable de mieux éliminer ce parasitoïde (Vorburger et al. 2008). Chez le moustique *Aedes aegypti*, la stimulation de la réaction d'encapsulation chez les femelles par une bille de sephadex n'augmente pas la réaction de la descendance face à un même challenge (Voordouw et al. 2008). Enfin, chez *Drosophila melanogaster*, aucune amélioration de la survie chez les descendants n'a été observée suite à la stimulation des femelles avec *Lactococcus lactis* ou *Pseudomonas aeruginosa* (Linder & Promislow, 2009). Or, chez *A. aegypti*, les larves et les adultes ne partagent pas le même environnement, et les femelles adultes dispersent avant l'ovisposition (Reiter, 1996). *Drosophila melanogaster* montre aussi des comportements de dispersion avant oviposition (Mikasa, 1992). *Myzus persicae* quant à lui est grégaire et présente un grand chevauchement des générations, mais le

parasite employé dans l'étude de Vorburger et al. (2008) lui disperse (Nguyen-Huu et al., 2006) et la pression parasitaire qu'il exerce sur les populations hôtes est susceptible de ne pas persister directement d'une génération à l'autre.

Certains coûts associés au TTGI ont pu être mis en évidence chez certaines espèces insectes. Dans une étude menée sur *B. terrestris*, Sadd & Schmid-Hempel (2009) ont montré que l'injection d'*Arthrobacter globiformis* aux reines provoquait l'élévation de l'activité antibactérienne induite de leur descendance, et que cette stimulation s'accompagnait d'une susceptibilité accrue au trypanosome *Crithidia bombi*, un parasite intestinal. En revanche, aucun coût énergétique n'a été mis en évidence en cas de privation de nourriture, contrairement au coût de la réponse immunitaire chez la même espèce trouvé par Moret & Schmid-Hempel (2000). Les auteurs ont proposé que ce coût provenait d'un compromis entre les différents effecteurs mobilisés dans la lutte contre deux pathogènes distincts, ou d'un compromis entre la défense de l'un des deux compartiments au détriment de l'autre chez l'organisme hôte. Roth et al. (2010) ont remarqué que le priming des femelles de *T. castaneum* prolongeait légèrement le temps de développement de leur descendance alors que le priming des pères réduisait sa fécondité, mais les auteurs n'ont pas discuté ce résultat. Un effet de la stimulation paternelle, qui diminuait la survie jusqu'à l'âge adulte de la progéniture a également été démontré par Freitak et al. 2009 chez *T. ni*, mais aucun coût de la stimulation maternelle n'a été mis en évidence.

Les études ayant recherché formellement les coûts du TTGI sont assez rares. Aussi, dans le but de caractériser les conditions de l'évolution et du maintien du TTGI chez les invertébrés, mon travail de thèse s'est attaché à associer l'expression du TTGI chez un insecte à ses coûts et ses bénéfices, aussi bien pour les parents que pour la descendance. Le fait que le TTGI ait évolué chez les invertébrés malgré l'existence de tels coûts permettra de déterminer si ce phénomène est adaptatif. Pour ces travaux, le modèle biologique choisi est l'insecte *Tenebrio molitor* (Coleoptera : Tenebrionidae), pour lequel le TTGI a déjà été mis en évidence. En effet, lorsque la génération parentale de *T. molitor* reçoit une stimulation de son système immunitaire par des lipopolysaccharides (LPS) extrait de la bactérie *Escherichia coli*, les larves de la génération suivante présentent une plus forte activité antibactérienne. L'activité de leur système PO n'est par contre pas affectée par la stimulation parentale (Moret, 2006). Dans cette expérience, les larves de la génération parentale des deux sexes étaient

stimulées sans discrimination, et mises en couple avec des individus immunologiquement naïfs après émergence.

Moreau et al. (2012) ont récemment mis en évidence que les femelles de *T. molitor* préalablement immunisées avec du LPS sont également capables de transmettre une activité antibactérienne à leurs œufs. Cette protection semblait accompagnée d'un coût pour les femelles. En effet, la quantité d'activité antibactérienne présente dans les œufs était négativement corrélée à celle de l'hémolymphe de la mère seulement chez les femelles de petite taille. Cela suggère que la protection des œufs se réaliserait au détriment de la propre défense des mères lorsque celles-ci sont en mauvaise condition.

T. molitor est un modèle biologique idéal pour l'investigation du TTGI. Il semble effectivement remplir toutes les conditions écologiques supposées nécessaires à son évolution, à savoir peu de dispersion et des générations très chevauchantes. Sa taille relativement grande autorise des prélèvements d'importants volumes d'hémolymphe, rendant possible l'observation des éléments clefs de son système immunitaire. Les œufs pondus par les femelles sont suffisamment volumineux pour être récoltés et permettre des mesures individuelles d'activité antibactérienne.

Objectif de ma thèse

Le but de cette thèse est de caractériser l'expression du TTGI chez *T. molitor* et d'y associer des coûts, tant pour la descendance que pour les femelles transmettant leur immunité. L'investigation de ses coûts et de ses bénéfices nous renseigne sur les pressions de sélection ayant conduit à son évolution chez les insectes. Afin de caractériser le TTGI chez *T. molitor* et d'identifier les coûts et les bénéfices qui lui sont associés, je me suis fixé les trois objectifs suivants, qui font chacun l'objet d'un chapitre de mon manuscrit de thèse.

Dans le premier chapitre, nous avons exploré différents aspects de l'expression du TTGI au sein des œufs. Dans la première partie de ce chapitre, nous avons étudié la protection transmise par les femelles. Pour ce faire, nous avons réalisé une dynamique de transmission d'activité antibactérienne par les femelles à leurs œufs, en relation avec l'activité antibactérienne de leur hémolymphe. Nous avons également recherché où était localisée cette

activité au sein des œufs (à l'intérieur de l'œuf ou à sa surface), ce qui nous renseigne sur le mode de transmission de cette activité antibactérienne. Enfin, nous avons recherché un coût pour la femelle à la protection de ses œufs en mettant en évidence une relation entre sa fécondité et la quantité de protection transmise à ses œufs. Dans la seconde partie, nous avons recherché l'existence d'une transmission d'activité antibactérienne d'origine paternelle aux œufs.

Dans le second chapitre, nous avons cherché à mettre en évidence un bénéfice au TTGI d'origine maternel sur les jeunes larves immédiatement après éclosion. Nous avons également cherché à savoir si la protection conférée par les femelles protégeait leurs descendants spécifiquement contre le pathogène qui les avait stimulées. Dans une première expérience, nous avons exposé nos femelles expérimentales à deux bactéries différentes. Nous avons ensuite maintenu les larves de la descendance soit en présence de la même bactérie qui avait stimulé leurs mères, soit en présence de l'autre, soit en l'absence de bactéries. Nous avons suivi la survie des larves placées dans ces conditions. Etant donné que l'existence d'un TTGI envers un pathogène fongique n'avait jamais été recherchée, nous avons, dans une seconde expérience, stimulé le système immunitaire des femelles avec un champignon entomopathogène, puis placé les jeunes larves de leur descendance soit en présence de ce champignon, soit en des conditions contrôle. Enfin, la mobilisation des effecteurs immunitaires n'est pas le seul trait phénotypique plastique permettant une meilleure résistance aux pathogènes. En parallèle de ce suivi de survie en présence d'un pathogène fongique, nous avons recherché l'existence d'un effet maternel sur l'expression d'un autre trait phénotypique: le temps de développement. En effet, l'ajustement de ce dernier en réponse à une infection ou une forte probabilité d'infection est supposé procurer un bénéfice aux invertébrés.

Dans le troisième chapitre, nous avons recherché l'existence d'un TTGI d'origine maternelle et paternelle sur la descendance adulte de *T. molitor*, par la mesure de trois paramètres de son système immunitaire. Afin d'associer un éventuel coût à la surexpression d'effecteurs immunitaires chez les descendants des parents immunostimulés, nous avons observé différents aspects de leur fitness pendant leur développement larvaire. La progéniture des couples expérimentaux était divisée en trois rangs de ponte de quatre jours chacun, ce qui nous a permis de comparer la persistance au cours du temps des effets maternels et paternels résultant de la stimulation de l'un des deux sexes dans la génération parentale.

Mon manuscrit de thèse se termine par une discussion, dans laquelle je résume mes principaux résultats et discute les informations qu'ils nous donnent sur les conditions écologiques ayant mené à l'évolution du TTGI, ainsi que sur les mécanismes mis en œuvre dans sa réalisation chez *T. molitor*.

Matériel & Méthodes général

Matériel et Méthodes général

I. *Tenebrio molitor*, un modèle adapté à l'étude du transfert trans-générationnel d'immunité

Tenebrio molitor, communément appelé le ver de farine ou encore ténébrion meunier, est un coléoptère polyphage de l'ordre des Tenebrionidae. La larve vermiforme d'environ 2.5 cm de long au stade le plus avancé laissera place après la nymphose à un adulte mesurant entre 1.5 et 2 cm (**figure 1**).

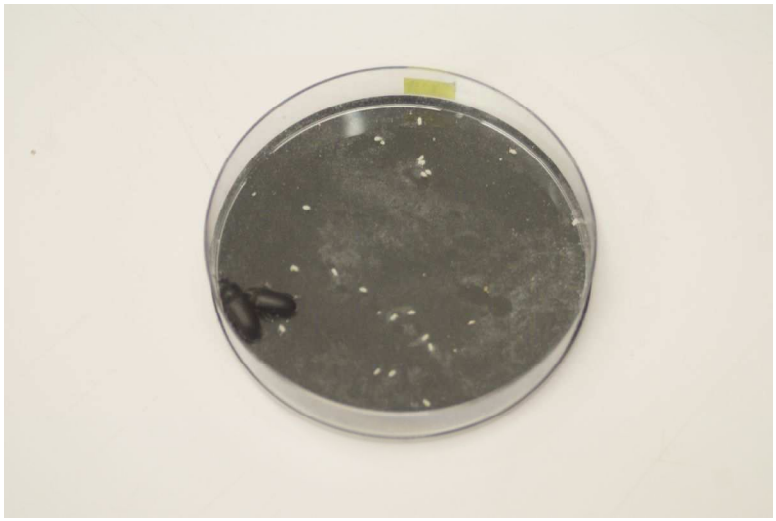


Figure 1: Couple de *T. molitor* dans une boîte de Petri (Ø 9cm) contenant des œufs.

Bien qu'il ne soit plus rencontré que dans les denrées stockées, les nombreuses adaptations à la dessiccation qu'il présente (Ramsay 1964 ; Slobodchikoff & Wismann, 1981) ainsi que sa proximité phylogénétique avec des espèces vivant dans les milieux désertiques (Ahearn, 1970) ont conduit les auteurs à penser que de telles adaptations auraient favorisé l'exploitation d'une niche écologique d'origine anthropique au détriment de sa niche écologique d'origine dans un écosystème désertique (Levinson & Levinson, 1994).

Son cycle de vie complet peut durer entre quatre mois en conditions optimales et deux ans en conditions défavorables. C'est une espèce relativement longévive, son cycle de vie et la durée respective des différents stades qui le composent sont représentés en **figure 2** (Howard, 1955). La plasticité dans la durée de son cycle de développement est permise par une grande variabilité dans le nombre de mues larvaires pouvant être effectuées par les individus (entre 10 et 25), qui est fonction des conditions environnementales telles que la température,

l'humidité, la disponibilité en dioxygène ou encore la densité en conspécifiques environnants (Punzo & Mutchmor 1980 ; Loudon 1988 ; Tschinkel & Wilson 1971).

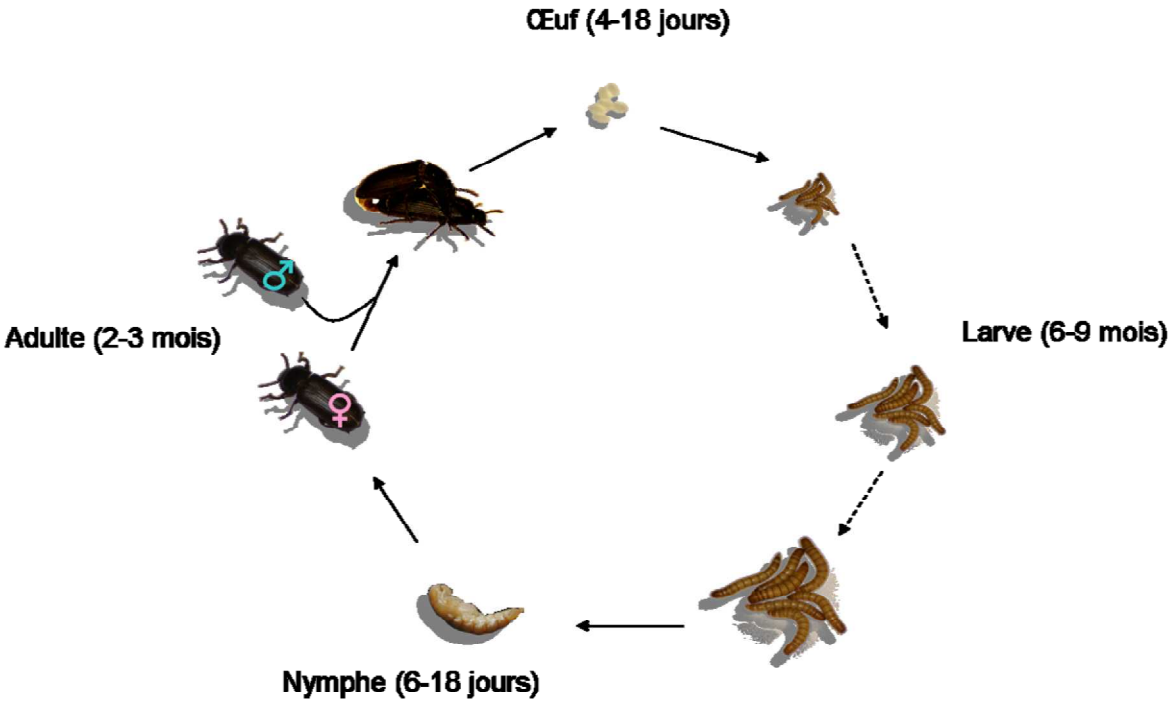


Figure 2 : Schéma représentant le cycle de vie de *T. molitor*, indiquant la durée des différents stades le composant.

Lors de la copulation, les mâles transfèrent le sperme aux femelles par l'intermédiaire d'un spermatophore (**figure 3**), qui est une structure protéique produite par les différentes paires de glandes accessoires de la reproduction du mâle.

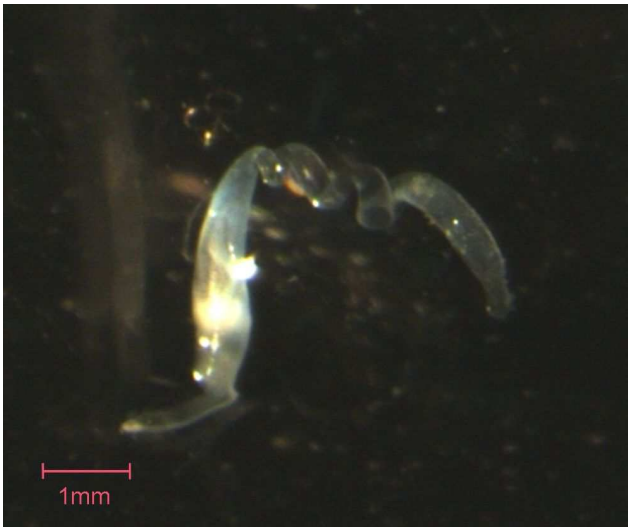


Figure 3 : Photo d'un spermatophore de *T. molitor* dans une solution saline, une minute après expulsion.

Cette espèce est caractérisée par un fort degré de polygamie et une haute fréquence de réaccouplements (Worden & Parker 2001). Les femelles pondent environ 300 œufs au cours de leur vie au cours de plusieurs épisodes de reproduction (Jacobs, 1988). En conditions « naturelles », *T. molitor* semble être exposé à une variété relativement faible de prédateurs : on peut cependant noter qu'il est l'hôte intermédiaire du cestode *Hymenolepis diminuta*, dont l'hôte définitif est un rongeur (généralement rat ou souris) contaminé par ingestion (Hurd & Fogo, 1991). Dans nos élevages cependant, la principale cause de mortalité est le cannibalisme, qui est exercé par tous les stades du développement des individus dès leur éclosion, et envers tous les stades dès l'oviposition, sauf l'adulte totalement mélanisé (Park et al. 1965).

T. molitor semble présenter tous les critères écologiques ayant conduit à l'évolution du TTGI chez les insectes, c'est-à-dire peu de dispersion et une stabilité des conditions environnementales d'une génération à la suivante (cf **introduction**). En effet, les ailes des individus de cette espèce ne semblent pas fonctionnelles, ainsi il semble que ses capacités de dispersion soient limitées. Les adultes et les larves exploitent le même habitat, qui dans le cas des denrées stockées, est très homogène aussi bien spatialement que temporellement. Les populations de cette espèce présentent un chevauchement de générations important, et tous les stades de développement se côtoient dans nos élevages (**figure 4**).

Figure 4 : Illustration des conditions d'élevage de *T. molitor* dans notre laboratoire.

Sa relativement grande taille permet des prélèvements de volumes d'hémolymphe suffisamment importants (jusqu'à 5µL environ) pour pouvoir y observer tous les paramètres

immunitaires décrits plus bas. Ses œufs sont suffisamment volumineux pour être récoltés après tamisage de la farine dans laquelle nous maintenons les couples, et pour permettre des mesures individuelles de leurs paramètres immunitaires (voir **figure 1**).

Les insectes utilisés au cours de ma thèse provenaient d'un élevage de masse maintenu au laboratoire à une température constante de 25°C, une humidité de 75% et une photopériode de 12 :12, dans des bacs en plastique (L/l/H : 40 x 30 x 21,4 cm) remplis jusqu'au tiers de leur hauteur avec du son de blé supplémenté avec des protéines animales (farine de porcelet), des morceaux de pomme, du pain, et des tubes Falcon remplis d'eau et bouchés avec du coton (**figure 4**). Tous les deux mois, les adultes morts sont retirés de l'élevage, et de nouveaux individus achetés chez notre fournisseur sont introduits afin d'éviter une trop grande consanguinité de nos individus expérimentaux. Dans ces conditions, le développement embryonnaire dure de sept à huit jours, le développement larvaire dure entre trois et quatre mois, et les adultes émergent de la nymphe au bout d'une semaine environ. Les adultes ne sont totalement mélanisés et sclérifiés qu'à partir de cinq jours après émergence. Ils peuvent être sexés à partir de cette date en exerçant une pression progressive de la tête jusqu'à l'abdomen, ce qui a pour effet de dévaginer leur appareil copulatoire (**figure 5)**.

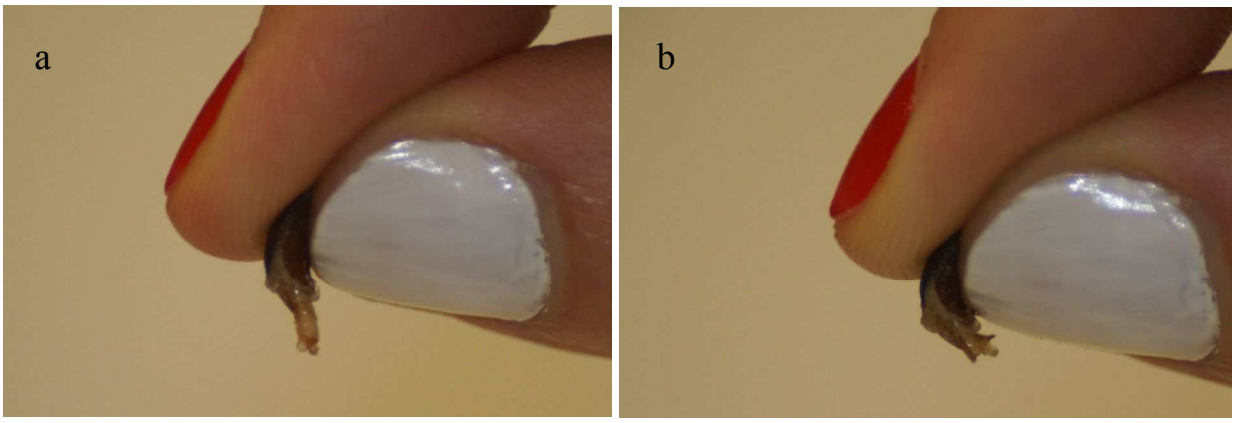


Figure 5 : Appareil copulatoire femelle (a) et mâle (b) dévaginés de *T. molitor*.

II. Paramètres observés et techniques employées

Au cours de ma thèse, j'ai mesuré l'immunité circulante dans l'hémolymphe des individus de *T. molitor*. J'ai estimé leur immunocompétence grâce à des techniques très simples me permettant d'observer les caractéristiques générales de trois éléments de leur

système immunitaire : les hémocytes, l'activité du système prophénoloxidase et l'activité antibactérienne présents dans leur hémolymphe. L'obtention, la préparation et le stockage des échantillons sont détaillés dans la section "**Materials and Methods**" de chaque chapitre, et seuls les avantages et inconvénients des techniques employées seront évoqués ici. Pour une description complète du protocole employé, le lecteur est invité à se référer aux **annexes**.

II.1. Stimulation du système immunitaire des femelles

Les injections étaient réalisées sur des insectes réfrigérés sur de la glace, à l'aide d'un capillaire effilé au travers de la membrane pleurale des insectes, sous les élytres. Nos élevages n'étant pas axéniques (exempts de germes), cette technique ne nous permet pas de réaliser des injections parfaitement stériles, mais nous permet d'injecter des volumes assez importants de solutions immunogènes. L'immunogène utilisé est le LPS (lipopolysaccharide), extrait d'*E. coli* et non purifié (Sigma : L8274). Par conséquent il est susceptible de contenir des peptidoglycanes. Il est connu pour éliciter une réponse immunitaire chez les insectes (Ratcliffe et al., 1991; Imler et al., 2000).

Chez *T. molitor*, l'encapsulation est couramment observée et peut être induite par l'insertion d'un filament de nylon dans la cavité cœlomique d'un individu (Vainikka et al., 2007). Le degré de mélanisation de cette capsule et son épaisseur sont souvent utilisés comme mesures d'immunocompétence (Boughton et al. 2011). Plutôt que d'observer le résultat de cette réaction, issue de processus cellulaires et humoraux, nous avons observé séparément l'activité du système phénoloxidase et l'immunité cellulaire. Un pic d'activité antibactérienne intervient dans les 24h suivant l'injection d'éliciteurs (Haine et al. 2008a). Cette activité peut persister dans l'hémolymphe de cette espèce au moins sept jours après le challenge (Moret & Siva-Jothy, 2003).

II.2. Estimation de la charge hémocytaire

Contrairement à la drosophile, chez laquelle des tissus hématopoiétiques n'ont pas été décrits chez l'adulte et ne sont présents que chez la larve, l'adulte de *T. molitor* possède des tissus hématopoïétiques qui ont été décrits par Chung & Moon (2004). Ils sont localisés dans l'abdomen, entre le diaphragme dorsal et la partie ventrale du vaisseau dorsal.

La concentration en hémocytes présente dans un prélèvement d'hémolymphe est déterminée par comptage du nombre total du nombre d'hémocytes présents dans les cellules de l'hémocytomètre (**figure 6**). Du fait de la difficulté d'identifier les hémocytes de *T. molitor* sans traitement du prélèvement, ce protocole ne permet pas de donner une idée du pourcentage de cellules à activité phagocytaire par exemple.

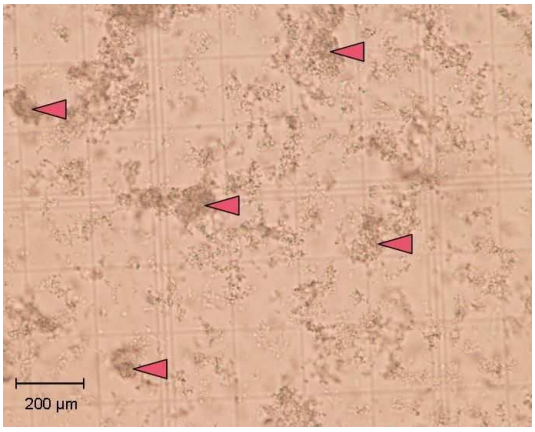


Figure 6 : Photo des hémocytes de *T. molitor* dans un hémocytomètre.
Les flèches rouges indiquent des hémocytes.

II.3. Mesure de l'activité enzymatique du système prophénoloxydase

L'activité de la phénoloxydase et de la prophénoloxydase sont estimées par le suivi de la dynamique de l'activité enzymatique de mélanisation dans une plaque 96 puits au spectrophotomètre après l'ajout d'un précurseur de la mélanine : la L-DOPA.

Nos prélèvements sont séparés en deux parties : la première partie nous sert à estimer la quantité de phénoloxydase active dans l'hémolymphe, alors que l'autre nous sert à estimer la quantité de son précurseur inactif, la prophénoloxydase, que nous détectons par ajout dans le puits de chymotrypsine, son activateur (**figure 7**).

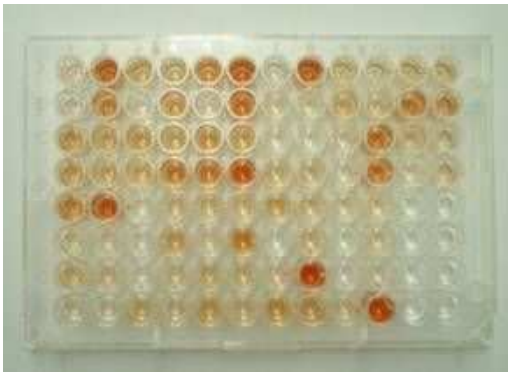


Figure 7 : Plaque 96 puits ayant servi au dosage de l'activité du système PO-PPO au bout de 40 minutes de suivi.

II.4. Mesures d'activité antibactérienne

Nous détectons l'activité antibactérienne de nos extraits en utilisant la bactérie *Arthrobacter globiformis* (Institut Pasteur, CIP 105365). Cette bactérie tellurique appartient à la famille des bactéries à Gram positif, mais est à Gram négatif dans les cultures jeunes, de moins de trente heures (Stevenson, 1961). Elle a de plus la particularité d'être très sensible aux antibiotiques. Elle nous permet ainsi de détecter une activité antibactérienne (même faible) présente dans notre extrait, de manière non spécifique. Le protocole que nous employons est celui de Haine et al. (2008a). La quantité d'activité antibactérienne est estimée par mesure du diamètre des zones d'inhibition de croissance d'*A. globiformis* provoquées par notre échantillon (**figure 8**).

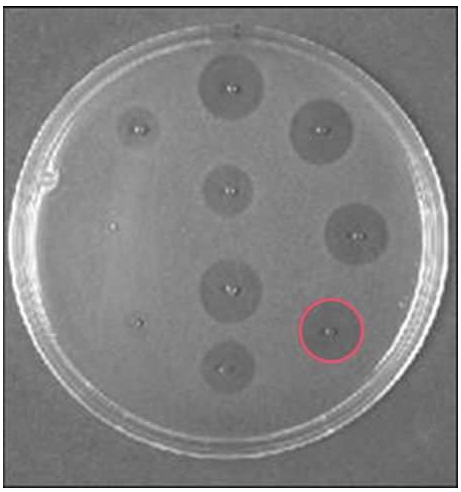


Figure 8 : Boîte de Petri ayant servi à des mesures d'activité antibactérienne, après 24h d'incubation. Le cercle rouge délimite une zone d'inhibition de croissance d'*A. globiformis*.

Chapitre 1

Chapitre 1 : Le transfert trans-générationnel d'immunité aux œufs chez *T. molitor* : coûts pour la femelle, et recherche d'un effet paternel.

L'existence d'un transfert d'activité antibactérienne aux œufs pouvant être induite par la stimulation du système immunitaire des femelles a été démontré chez *B. terrestris* (Sadd & Schmid-Hempel, 2007) ainsi que chez *T. molitor* (Moreau et al., 2012). Dans ce chapitre, nous avons voulu explorer différents aspects de cette transmission chez *T. molitor*.

Dans la **Partie 1** de ce chapitre, nous avons étudié la dynamique de transmission d'activité antibactérienne par la femelle à ses œufs suite à une stimulation au LPS. Nous avons ensuite recherché sa localisation au sein des œufs, afin de déterminer si l'activité antibactérienne était incorporée dans les œufs pendant l'ovogénèse ou plutôt apposée à la surface des œufs pendant leur passage dans le tractus génital des femelles.

Dans des expériences préliminaires, nous observions qu'un nombre plus ou moins élevé d'œufs d'un rang de ponte des femelles immunostimulées ne présentait pas d'activité antibactérienne. Une étude précédente a montré que la protection des œufs était coûteuse pour les femelles (Moreau et al., 2012). Nous avons donc émis l'hypothèse que cette variabilité dans la protection des œufs provenait d'un coût pour les femelles à la réaliser.

L'analyse des œufs pondus les 14 premiers jours, et récoltés par pas de temps de 2 jours, nous a permis de voir que les femelles stimulées au LPS commencent à transférer de l'activité antibactérienne à leurs œufs 2 jours après l'injection. Cette transmission reste maximale jusqu'à 8 jours après injection, et diminue à partir de 10 jours pour disparaître à 12 jours après stimulation. En comparant l'activité antibactérienne du contenu des œufs par rapport à celle présente à leur surface, nous avons vu que l'activité antimicrobienne était présente à l'intérieur des œufs, mais pas à leur surface. Sur la base de notre dynamique de transmission, nous avons décidé de baser la suite de nos expériences sur l'analyse de l'activité antibactérienne des œufs pondus entre les 2ème et 4ème jours après l'injection de LPS aux femelles. Au sein de ce pas de temps, nous avons trouvé une relation quadratique entre le nombre d'œufs protégés par les femelles au sein de leur ponte et le nombre d'œufs pondus.
Cette expérience a fait l'objet d'un article publié.

Dans la **Partie 2** de ce chapitre, nous avons recherché l'existence d'une transmission d'activité antibactérienne aux □ufs par les mâles. Nous l'avons cherchée au travers de l□établissement d□une dynamique de transmission similaire à celle que nous avons réalisée dans la première partie du chapitre, sauf que nous n'avons pas prélevé l'hémolymphe des mâles stimulés. La récolte des □ufs des mâles stimulés était effectuée tous les 2 jours pendant 14 jours. Avec ce protocole, nous n'avons pas mis en évidence de transfert d'activité antibactérienne dans les □ufs résultant de la stimulation du système immunitaire des mâles.

Chapitre 1

Part 1 : Relationship between maternal transfer of immunity and mother fecundity in an insect

Authors: C. Zanchi, J.-P. Troussard, J. Moreau, Y. Moret

Published : August 2012 in Proceeding of the Royal Society of the royal Society B.

Chapitre 1

Abstract

Trans-generational immune priming (TGIP) corresponds to the plastic adjustment of offspring immunity as a result of maternal immune experience. TGIP is expected to improve mother's fitness by improving offspring individual performance in an environment where parasitism becomes more prevalent. However, it was recently demonstrated that maternal transfer of immunity to the offspring is costly for immune-challenged female insects. Thus, these females might not provide immune protection to all their offspring because of the inherent cost of other fitness related traits. Females are therefore expected to adjust their investment to individual offspring immune protection in ways that maximise their fitness. In this study, we investigated how bacterially immune-challenged females of the mealworm beetle, *Tenebrio molitor*, provision their eggs with immune protection according to egg production. We found that immune-challenged females provide a variable number of their eggs with internal antibacterial activity along egg-laying bouts. Furthermore, within the first immune protected egg-laying bout (2-4 days after the maternal immune challenge), the number of eggs protected was strongly dependent on the number of eggs produced. Immune-challenged females might therefore adjust their investment into TGIP and fecundity according of their individual perception of the risk of dying from the infection and the expected parasitic conditions for the offspring.

Chapitre 1

I. Introduction

Maternal effects play a key role in offspring fitness by modulating its phenotype in accordance to the maternal experience of the environment (Mousseau & Fox, 1998b). They can even affect population dynamics when variation in offspring provisioning exists (Benton et al. 2005). Trans-generational immune priming (TGIP) is a parental effect on offspring immunity. It is defined as the transmission of an elevated immunocompetence to the offspring following an immune challenge in the parental generation, improving its resistance to further pathogen encounter (Little et al., 2003; Grindstaff et al., 2003). This transmission of an amplified immunocompetence to offspring is well documented in vertebrates, where it is achieved through maternal transfer of antibodies that confer to the progeny an early protection before the maturation of its own immune system (Zanchi et al., 2011). In invertebrates, this phenomenon has been shown to occur mainly through phenomenological studies. The underlying mechanisms of this transmission remain unknown, but the effects of the TGIP in the progeny can be found across all the life-stages of the protected progeny: since oviposition (Sadd & Schmid-Hempel, 2007; Moreau, 2012), during the larval development (Moret, 2006; Tidbury et al., 2011; Rahman et al., 2003; Freitak et al., 2009) and even persist unto the adult stage (Sadd & Scmhid-Hempel, 2009; Roth et al., 2010; Zanchi et al., 2011). TGIP has been shown to confer the offspring an enhanced protection in the case of persistence of the maternal infection in its environment (Huang & Song, 1999; Little et al., 2003; Tidbury et al., 2011; Rahman et al., 2003; Roth et al., 2010).

Maintaining and using immune defences is costly for the organisms (Siva-Jothy et al., 2005; Schmid-Hempel, 2003). It is therefore unsurprising that this elevated immunity comes at several costs for the offspring. In the bumblebee *Bombus terrestris*, the stimulation of the females with a bacterial pathogen decreases the survival to a heterologous parasitic challenge in the offspring Sadd & Schmid-Hempel, 2009). In the mealworm beetle *T. molitor*, the maternal challenge elevates the haemocyte load of the adult offspring at the expense of a prolonged developmental time (Zanchi et al., 2011). Because of these costs, the main condition for its adaptiveness in invertebrates is believed to be the persistence of the infection risk encountered by mothers to the next generation. Thus, it is assumed that generation overlap and/or gregarism would favour the evolution of TGIP in response to pathogens that could persist from one host generation to the next in the environment. In this case, the maternal infection becomes a reliable cue predicting the risk of infection of the progeny. As females synthesize and transmit effectors and/or elicitors of immunity to their offspring, we

could also expect this transmission to be costly for them, in addition of paying the usual costs of the immune activation.

After being immune-challenged, the females of the mealworm beetle *Tenebrio molitor* can provide their eggs with an antimicrobial activity (Moreau et al., 2012). This could result either from an imbuement of the eggs with immune substances within the female reproductive tract or from the incorporation of these immune substances into the eggs during ovogenesis: the first would suggest that the eggs are protected from a pathogen intrusion, whereas an internal localisation would rather protect the young larvae at hatching. Interestingly, immune-challenged females do not transfer antibacterial activity to all of their eggs (Zanchi, personal observation). The variability of this investment could indicate the existence of a cost for the females to egg protection. Recently, immune-challenged females of *T. molitor* were shown to trade-off their immunity with that transferred to their eggs (Moreau et al., 2012), thus, TGIP may respond to the same constraints as other costly maternal investments which affect progeny quality, such as egg provisioning (Messina & Fox, 2001; Roff, 1992; Stearns, 1992). As a result, mothers are expected to differentially invest in the immune protection of the offspring according to the number of offspring produced to maximize fitness in an environment where infection risk for the offspring is high. Furthermore, the maternal transfer of immunity to the eggs is expected to cease with the disappearance of the pathogenic threat.

In this study, we investigated how long the females of *T. molitor* transfer antibacterial activity to their eggs following an immune challenge, and the localisation of this protection (whether antibacterial substances are provided internally or on the surface of the eggs). Finally, because of the inherent cost of the maternal transfer of immunity to the offspring and reproduction, we examined the relationship between transfer of immunity to eggs and fecundity in immune challenged females. To this end, we assessed the antimicrobial activity of all the eggs from the first immune protected clutch laid by *T. molitor* females following their immune challenge. Here, we make the hypothesis that maternal transfer of immunity to the eggs is constrained by the availability of antibacterial substances produced by their mother. In that case, females may use two different strategies to protect their eggs according to the number of eggs produced. First, an immune-challenged female may transfer immune substances to all her eggs with the risk of providing an insufficient amount of theses immune substances to efficiently protect each egg. Second, an immune-challenged female may not protect all her eggs, but ensure the transfer of a sufficient amount of immune substances to each egg that received the maternal immune protection. Furthermore, because both egg

production and egg protection are costly, a negative relationship between the number of eggs laid and the number of eggs protected is expected. In order to accentuate a potential existing trade-off between the two traits, we manipulated the energetic reserves contained in adult females and their size by limiting their food supply during their larval development.

We show that bacterially immune-challenged females of *T. molitor* provide some of their eggs with internal antimicrobial activity, and that this transmission is transient along egg laying sequences. All the eggs are not protected within one egg laying sequence, and the number and the proportion of immune protected eggs is significantly associated with female fecundity.

II. Materials and methods

II.1. Insect cultures, immune challenge and egg collection

All mealworm beetles used in this study originated from an outbred stock culture maintained in our laboratory in bran flour added with *ad libitum* access to water and regularly added with proteins (piglet flour), apple and bread. Pupae were then collected from these stock cultures and adults were maintained individually after emergence in a Petri dish supplied with bran flour a piece of apple and water for ten days, except for Experiment 3 where insects were isolated from this stock culture as young (1cm) larvae then reared in good or poor food conditions (see **Experiment 3** below).

II.1.1. Experiment 1: Temporal dynamics of the antibacterial immune response of immune-challenged females and of the transmission of antibacterial activity to their eggs

We have previously shown that bacterially immune-challenged *T. molitor* females transferred levels of antibacterial activity to their eggs (Moreau et al., 2012). Here we wanted to assess in more details how the number of eggs protected by the mothers changes across the 14 days of the antimicrobial response of their haemolymph (Haine et al., 2008a). To do this, 140 age controlled (10 days old) virgin adult females were weighted to the nearest 1 mg and immune challenged by a single injection of 5 µl of Ringer's solution containing non-purified lipopolysaccharides (LPS: 0.5 mg/ml) extracted from *Escherichia coli* (Sigma: L8274). This commercial LPS may contain peptidoglycan contaminants (Leulier et al., 2003). 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 (Lemaître et al., 1997). Immediately after their immune challenge, females were paired with a virgin and unchallenged male of the same age and allowed to produce eggs in a Petri dish provided with bleach flour and *ad libitum* food and water under standard laboratory conditions (25°C, 70% RH, L12h:D12h). Random couples were sacrificed each 2 days and provided a haemolymph sample and 3 eggs which were stored at -20°C for later examination of their antibacterial activity. The remaining couples were transferred into a new Petri dish every second day following the maternal immune challenge, until the last remaining couples had their clutches separated into 7 egg

laying sequences. When the female or the male died before the hemolymph collection of the female, the couple was removed from the experiment. Thirty couples were thus removed, resulting in 13 couples used in the first egg-laying sequence, 15 in the second, 18 in the third, then 14, 19, 17 and 14 in the last one. The presence or absence of a zone of inhibition in their eggs was recorded.

II.1.2. Experiment 2: Localisation of the antibacterial activity transferred to the eggs

Antibacterial activity transferred to the eggs may either result from the mother secreting antibacterial factors onto the egg surface and/or into the eggs. Here we wanted to examine these possibilities by testing the antibacterial activity of both the surface and the inside of eggs. To this purpose, 10 virgin females (10 days old) were immune challenged, paired with a virgin and unchallenged male of the same age and then allowed to lay eggs as described above. Based on the results of **Experiment 1**, we assessed the antibacterial activity of the eggs laid by each female between day 2 and day 4 following the maternal immune challenge (see results). Five random eggs per female were used to test for the presence of antibacterial activity on both the surface and the inside of the eggs among the eggs laid. Since antibacterial activity at the surface of one single egg might be difficult to detect (Marchini et al., 1997), the 5 eggs collected were put together in a microcentrifuge tube containing 20 µL of cold phosphate-buffered saline (PBS: 100 mM) and then gently agitated for 5 minutes to suspend potential antibacterial factors present on their surface. Eggs were then removed and the suspension was immediately stored at -20°C for later antibacterial test. For internal egg antibacterial activity, the internal fluid of each previously washed egg was collected using a pulled glass micro-capillary and flushed into a microcentrifuge tube containing 10 µL of cold PBS and then stored at -20°C until antibacterial test.

II.1.3. Experiment 3: Testing the trade-off between mother fecundity and number of immune protected eggs

In this experiment, insects were either reared on good food or restricted food conditions (poor food). In the good food condition, 1cm-long larvae were isolated from the stock culture and then supplied with *ad libitum* bran flour supplemented with proteins (piglet flour), apple and water. In the poor food condition, 1cm-long larvae isolated from the stock

culture were supplied with *ad libitum* bran flour and water but without protein or apple supplementation. The latter food condition was used to generate adult insects of smaller size than those raised in good food condition.

Based on the results of **Experiment 1**, the influence of female fecundity on number of immune protected eggs was examined on the second egg-laying sequence that is between day 2 and day 4 after the maternal immune treatment. Age controlled females (10 days old), from good (n = 54) and poor (n = 41) food conditions were weighted to the nearest 1 mg and either injected with LPS solution as describe above or with Ringer solution only as procedural control. Females were paired with a virgin unchallenged male from good food condition and allowed to produce eggs along 2 egg laying sequences of 2 days each (from day 0 to day 2: past fecundity, and from day 2 to day 4: current fecundity). The number of eggs laid during each egg laying sequence was counted and those from the second egg laying sequences were all assessed for their antibacterial activity. We recorded the presence or absence of a zone of inhibition in these eggs, as well as the size of the zone of inhibition, which indicates the amount of antibacterial activity transmitted by the mothers to their eggs. Thirty-three females did not lay eggs and were removed from the experiment. Therefore, the analyses of the data were performed on a total of 62 females (18 good food/Ringer, 17 good food/LPS, 13 poor food/Ringer, 14 poor food/LPS).

II.2. Analysing the antibacterial activity of the hemolymph and the eggs

Antibacterial activity of the hemolymph 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, individual eggs were thawed on ice, suspended in 2µL of PBS and homogenized using a pestle, except in the second experiment, in which case egg content and egg surface were isolated before freezing. Antibacterial activity of all the samples was measured from 2 µl of extract using the antibacterial assay described in (Moret, 2006).

II.2. Statistics

For **Experiment 1**, the antimicrobial activity of the female's haemolymph according to time was analyzed using a generalized linear model (GLM) fitted with a Poisson distribution corrected for overdispersion (dispersion parameter = 4.12), with the female body mass as covariate. The temporal dynamics of transmission of antibacterial activity to the eggs was analysed as the proportion of eggs found protected according to their egg laying sequence using a general linearized mixed model (GLMM) fitted with a binomial distribution (presence/absence of protection in the eggs of each laying sequence), with the female's body mass as covariate and female identity as a random factor. Differences between each egg laying sequence were analysed using a Tukey's post-hoc test ($P < 0.05$).

For **Experiment 3**, the body masses of females were normally distributed within each rearing condition, the effect of larval food conditions on female body mass was therefore examined using a Student's t test. Variation in female fecundity was analysed using a GLMM fitted with a Poisson distribution, according to the maternal treatment, female larval food condition and egg laying sequences as factors. Since there were two egg-laying sequences, female's ID was repeated twice and thus included in the model as a random factor.

The size of the zone of inhibition of each egg according to the number of eggs protected by the females, their larval food condition and their immune treatment was analysed with a GLMM with a Gaussian distribution, with the female's ID as a random factor.

In this **Experiment 3**, we analysed both the number and the proportion of eggs protected by the females in their current clutch (laid between day 2 and day 4) according to this clutch size (current fecundity = number of eggs laid between day 2 and day 4), in order to highlight both the absolute investment in egg protection and the relative investment into egg protection compared to egg production in these females.

Initial data exploration revealed that, within the first immune protected egg laying sequence (between day 2 and day 4 after the immune challenge), the relationship between either the number or the proportion of immune protected eggs and the current number of eggs produced was quadratic. Therefore, the number of eggs protected in this sequence was analysed using a GLM, with a Poisson distribution corrected for overdispersion (dispersion parameter = 1.42). The initial model used female immune treatment and female larval food condition as factors, current fecundity (the number of eggs laid during this sequence) as a quadratic term and past fecundity (number of eggs laid before the production of the first

immune protected egg-laying sequence, between day 0 and day 2 after the immune challenge) as covariates. The proportion of eggs protected by a female during this sequence was analysed using a GLM with a binomial distribution (presence/absence of protection in the eggs) using the same explanatory variables as above.

All the data were analysed using R software (R development Core Team, 2011). The GLMMs were performed with the add-on R package lme4 (Bates et al., 2010).

Model selection was achieved using a stepwise backward deletion procedure with Akaike's information criterion (AIC) whereby initial models included all main effects and two-way interactions (Zuur et al., 2009).

III. Results

III.1. Experiment 1: Temporal dynamics of the antibacterial immune response of immune-challenged females and of the transmission of antibacterial activity to their eggs

As expected, the immune challenge elicited an antimicrobial immune response in the haemolymph of the females and affected the antimicrobial activity of their eggs. The antibacterial activity of the females varied over time ($F_{6,\ 104} = 87.3$, $P < 0.001$), was the highest 2 days after the immune challenge and then kept declining to day 14 (**figure 9a**). The proportion of eggs found protected also varies significantly over time ($\chi^2_{6,\ 104} = 59.1$, $P < 0.01$ **figure 9b**). Between day 0 and day 2, the occurrence of a zone of inhibition in the eggs was sparse. We detected a substantial transmission of antibacterial activity to the eggs at day 2, while the antimicrobial activity of the females was declining. The proportion of eggs found protected at each laying sequence remained at the same level between day 4 and day 8. From days 8 to 10 and 10 to 12, the proportion of protected eggs returns to a similar level as that of day 0 to day 2. After 12 days, no eggs were found protected. Even in the most protected egg laying sequences, the proportion of eggs protected never equalled 100%.

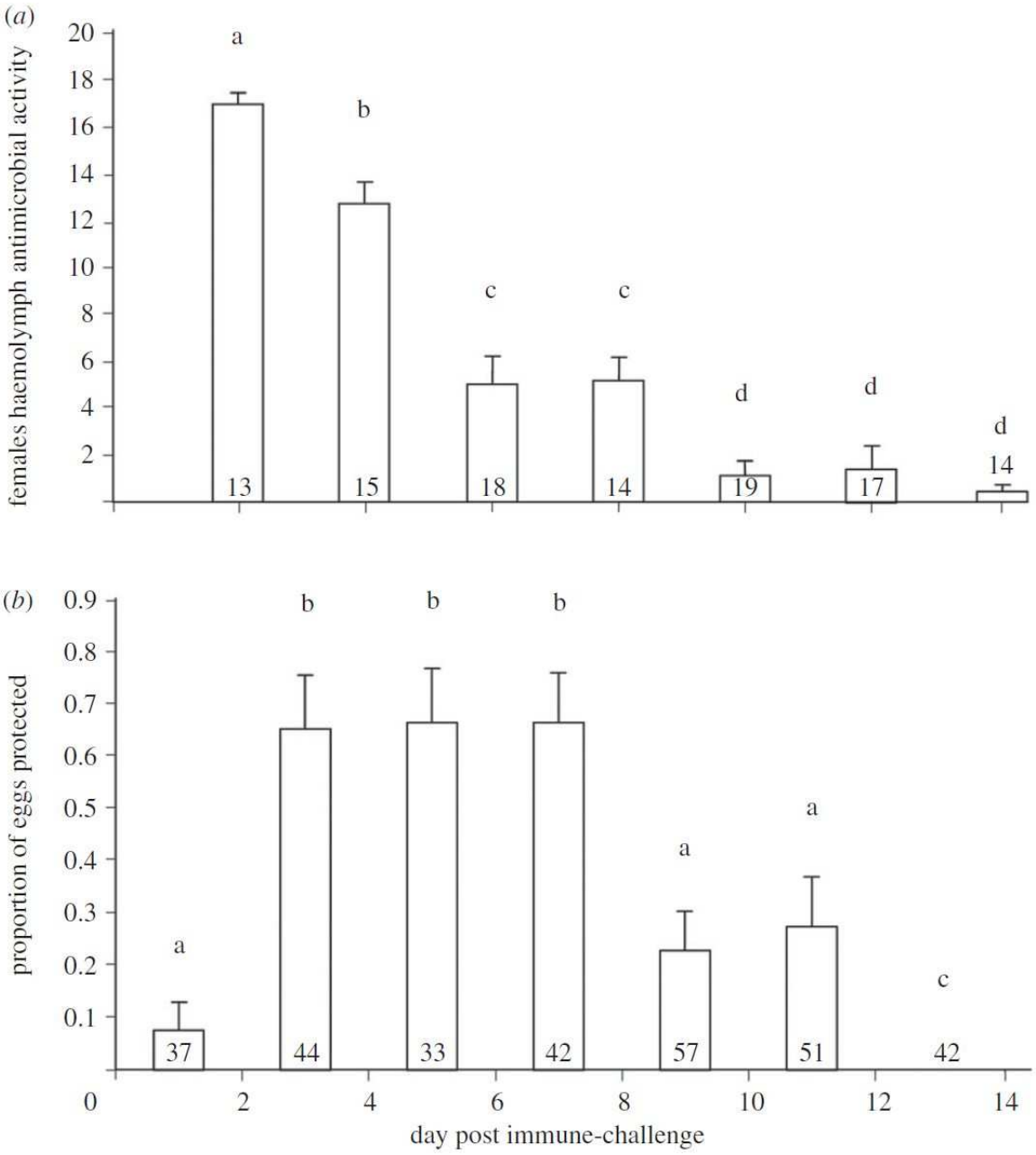


Figure 9 : a) Antibacterial activity of the hemolymph of the females (mean diameter of the zone of inhibition in mm ± SE) and b) proportion of eggs protected (± SE) according to the time following the maternal immune challenge.
Time laps with the same letter show no significant difference for the antibacterial activity of the females haemolymph or in the proportion of eggs protected (Tukey's post-hoc test, $P < 0.05$). Numbers inside the bars indicate the number of females or eggs assayed at each egg laying sequence.

III.2. Experiment 2: Localisation of the antibacterial activity transferred to the eggs

We pooled 5 eggs of each 10 females tested for analyse of antibacterial activity. Antibacterial activity was never found at the surface of these eggs (N = 10). We then analysed the internal extracts of each egg separately. 100% of the eggs showed an internal activity (N = 50).

III.3. Experiment 3: Testing the trade-off between mother fecundity and number of immune protected eggs

Larval food manipulation succeeded in producing adult females of different body masses. Females obtained from poor food conditions were significantly lighter than those obtained from good food conditions (mean ± SE: poor = 97.07 ± 22.47 mg; good = 128.20 ± 39.79 mg; t = 7.19 df = 60, P < 0.001).

Past fecundity and current fecundity were both independent of the maternal immune treatment ($\chi^2_{1, 58}$ = 0.68, P = 0.4) and body mass ($\chi^2_{1, 58}$ = 1.37, P = 0.24). Current fecundity was significantly higher than past fecundity ($\chi^2_{1, 58}$ = 85.28, P < 0.001, current fecundity = 13.56 ± 8.52; past fecundity = 8.21 ± 7.50; $F_{1, 60}$ = 18.66, P < 0.001).

There was no trade-off between the number of eggs protected and the amount of protection allocated per egg in the first protected egg laying sequence. Instead, the number of eggs protected correlated positively with the amount of protection they received (F1, 51 = 6.54, P = 0.014; **figure 10**). As expected, LPS-treated mothers provide their eggs with higher levels of antimicrobial activity (F1, 51 = 14.14, P < 0.001; **figure 10**). The amount of protection allocated per egg was independent of the larval food condition of females (F 1, 49 = 0.01, P = 0.92).

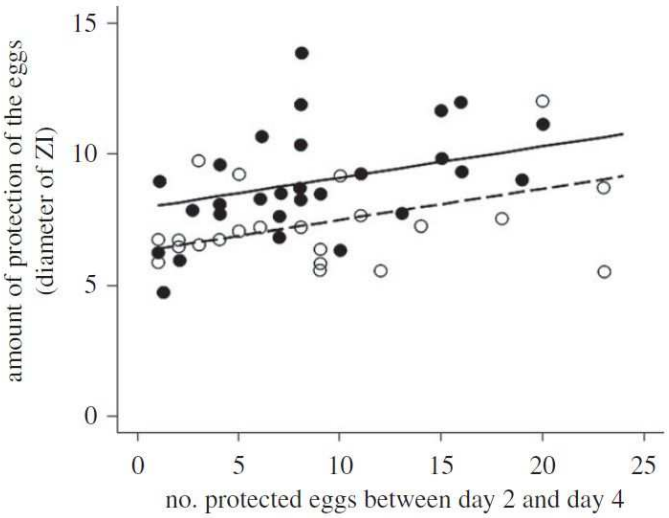


Figure 10 : Relationship between the amount of antibacterial activity allocated to each egg by control (dashed line) and LPS treated females (solid line) between the days 2 and 4 post immune-challenge according to the number of eggs protected in this egg laying sequence. Open and plain circles represent the mean antibacterial activity of all the eggs protected by each control and LPS-treated mother, respectively.

There was a significant relationship between the number of eggs protected in the current clutch (between day 2 and day 4 after the immune challenge) and the size of this clutch (current fecundity in interaction with the maternal immune treatment in **table 2**). In control females, the number of eggs protected correlated positively with current fecundity whereas for LPS-treated mothers, the relationship was quadratic (**figure 11a**). There was a significant interaction between past fecundity (between day 0 and day 2) and maternal immune treatment on the number of eggs protected between day 2 and day 4 (**table 1**). In control mothers, the number of eggs protected correlated positively with past fecundity but not in LPS treated-mothers (see **figure S1** in **supplementary material**). There was also a significant interaction between the larval food condition of mothers and their past fecundity on the number of eggs currently protected (**table** 1). In females from good food condition, the number of eggs protected correlated positively with their past fecundity but not in females from poor food condition (see **figure S2a** in **supplementary material**). Similarly to the number of eggs protected, the proportion of protected eggs was significantly associated to current fecundity in interaction with the maternal immune treatment (**table 1**). In control females, the proportion of eggs protected was not related to current fecundity, whereas for LPS-treated mothers, the relationship was significant and quadratic (**figure 11b**). The

proportion of protected eggs was associated to past fecundity in interaction with mother larval food condition (**table 1**). In females from good food condition, the proportion of protected eggs correlated positively with their past fecundity but not in females from poor food condition (see **figure S2b** in **supplementary material**). However, variation in the proportion of protected eggs could not be explained by past fecundity in interaction with the maternal immune treatment.

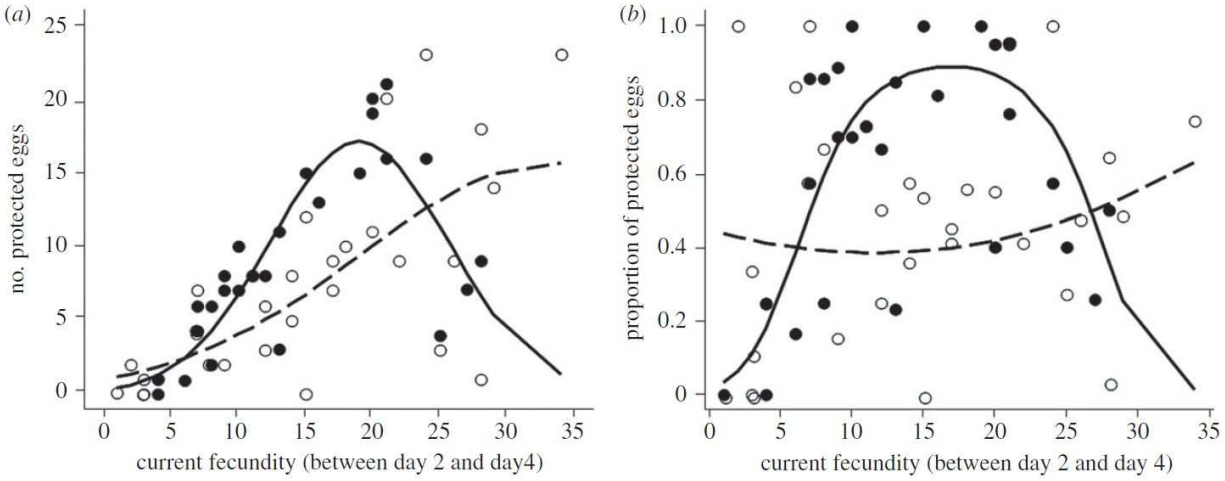


Figure 11: Relationship between the current fecundity (between day 2 and day 4) and a) the number of eggs protected and b) the proportion of eggs protected by control (dashed line) and LPS-treated females (solid line) during this egg laying sequence.
Open and plain circles represent the numbers and proportions of eggs protected by control and LPS-treated mothers respectively.

IV. Discussion

This study provides evidence of a transient maternal transfer of immune protection to the eggs after a bacterially based benign immune challenge of females of the mealworm beetle, *T. molitor*. As previously found in another insect model (Sadd & Schmid-Hempel, 2007), the maternal transfer of immunity to the eggs of *T. molitor* was achieved through the provision of antibacterial substances inside the eggs rather than an imbuement of the surface of the eggs with immune substances within the female reproductive tract (Marchini et al., 1997; Blackmeer et al., 1994; Eisner et al., 1996). During the vitellogenesis, the main components of the eggs are released in the female's haemolymph by the fat body and then recruited inside the eggs (reviewed in Valle, 1993). Since the fat body is also the main organ responsible for the synthesis of antimicrobial peptides following an immune challenge (Hoffman, 1995), this organ may also provide the antimicrobial substances incorporated inside the eggs. Thus, a certain amount of the antimicrobial peptides dedicated to the mother's own defence could be directed to the ovaries and imbued to the eggs.

More importantly, our data reveal that a large number of eggs were not protected, even for the egg-laying sequences where the maternal transfer of immunity was peaking. This result provides further evidence that maternal transfer of immunity in this species is costly and suggests that the immune protection of the eggs is constrained by the availability of antibacterial substances produced by immune challenged mothers. As a result, immune-challenged females seem to favour the immune protection of a limited number of eggs with a sufficient amount of immune substances per egg to efficiently protect them, instead of supplying equally each egg of the clutch, which might result in an inefficient protection. Indeed, the size of the zone of inhibition of the eggs was repeatable within females ($r = 0.577$ from **Experiment 3**). Furthermore, we did not found any trade-off between the amount of immune protection allocated per egg and the number of eggs protected, as it would be expected if females would share equally their immune resource to their eggs. In contrast, we found a positive relationship between these variables.

As female body condition only had a weak effect on the amount of protection allocated per egg, the cost of the maternal transfer of immunity to the eggs may not result from an energy restriction, but rather from a limited amount of antimicrobial peptides that could be transferred to the eggs at a given time. Alternatively, the fact that our model species is able to feed at the adult stage may allow it to compensate for a reduced energy stock at

emergence. Therefore, the amount of protection per egg and the number of eggs protected may reflect the quality of the female and/or individual level of investment into TGIP.

As the maternal transfer of immunity is costly for females (Moreau et al., 2012), a negative relationship is expected with other costly fitness traits such as fecundity. However, this relationship may not be necessarily linear (van Noordwijk & de Jong, 1986) as often observed for the relationship between offspring size and offspring number in iteroparous species (Fox & Czesak, 2000). In *T. molitor*, the number of eggs laid at each reproductive event is highly variable within and between individuals (from Experiment 1: mean fecundity ± SE between day 0 and day 2 = 12 ± 10.15; day 2 and day 4 = 10.54 ± 6.73; day 4 and day 6 = 9.59 ± 8.8; day 6 and day 8 = 16.28 ± 9.65; day 8 and day 10 = 16.84 ± 6.44; day 10 and day 12 = 15.47 ± 6.84; day 12 and day 14 = 8.93 ± 6.11). Among immune challenged females, we found a quadratic relationship between their current fecundity and number and proportion of eggs protected. The benefits to the protection of the eggs by challenged females remain to be tested. Assuming that an increase in the eggs immunocompetence would translate in a better resistance to pathogens (Abdel-latief & Hilker, 2008), such a relationship would reveal three main situations in response to the maternal immune challenge, which may have different implications for the fitness of mothers depending whether the infection persists over the maternal generation. First, some females did not invest either in egg production or egg protection (see left side of the bell-shaped curve in Fig. 3). The relative success of this clutch will be low whether the maternal infection persists or not. These females may have intended to postpone their reproductive effort to the next egg-laying sequences (Marshall & Uller, 2007). Alternatively, these females may have laid the eggs that had matured before the immune challenge and which were therefore not provided with immune protection, as suggested by the absence of protection in the first egg laying rank observed in Fig 1b. Second, some females exhibited an intermediate current fecundity but optimized the protection of their clutch (top of the bell-shaped curve in Fig. 3). The relative success of this situation will be maximal when the maternal infection is persistent in the next generation. Third, some females exhibited a relatively high fecundity but protected a low number of their eggs (right side of the bell-shaped curve in Fig. 3). They may gain from producing diverse offspring in a sceptic environment (Marshall & Uller, 2007), but their relative success will be maximal when the maternal infection does not persist in the next generation. Therefore, the expression of the trade-off between current fecundity and TGIP of the eggs should be maintained by the variation in the persistence of pathogens between generations.

Because of the trade-off between current egg production and egg protection, TGIP might be expected in iteroparous species rather than in semelparous ones. In line with this, TGIP has been evidenced in iteroparous arthropods (Huang & Song, 1999; Little et al., 2003; Tidbury et al., 2011; Rahman et al., 2003; Freitak et al., 2009; Sadd & Schmid-Hempel, 2009; Roth et al., 2010, Zanchi et al., 2011; Sadd et al., 2005) and not in semelparous ones (Voordouw et al., 2008; Linder & Promislow, 2009). Iteroparity could allow females to adjust their relative investment into egg protection compared to egg production in accordance to the risk of infection of the progeny and their own risk of dying from the infection. Indeed, iteroparous females may gain from saving immune substances when they are needed for their own defence by delaying investment into egg protection until the pathogenic threat is overcome.

We demonstrate in this study a cost to the inducible transmission of antibacterial activity to the eggs following the immune challenge of the mothers. The existence of such a cost suggests that this transmission might not just be a side effect accompanying the immune reaction of the mothers following an immune challenge, but rather an investment that has been selected.

Table 1: Summary of the optimal GLM following stepwise-deletion of the number and proportion of eggs protected by females between day 2 and day 4 post immune-challenge, fitted with the effects of the past fecundity (between day 0 and day 2), the current fecundity (between day 2 and day 4), the maternal immune treatment (Treatment) and the rearing condition of mothers (Condition). Number of eggs protected: Nfemales=59; deviance explained = 77.20%. Proportion of eggs protected: Nfemales = 59, deviance explained = 56.91%.

	Number of eggs protected		Proportion of eggs protected	
Source	LR $\chi^2_{1, 49}$	P	LR $\chi^2_{1, 50}$	P
Condition	14.14	< 0.001	46.17	< 0.001
Treatment	2.74	0.098	7.44	0.006
Past fecundity	4.07	0.044	97.66	< 0.001
Current fecundity	2.05	< 0.001	36.42	< 0.001
Current fecundity²	28.36	< 0.001	36.74	< 0.001
Condition x past fecundity	6.98	0.008	25.73	< 0.001
Treatment x past fecundity	9.10	0.003	-	-
Treatment x current fecundity	11.06	< 0.001	19.79	< 0.001
Treatment x current fecundity²	13.09	< 0.001	20.91	< 0.001

Acknowledgements

We thank M. Oudin for technical support; and M.A.F. Gillingham and F-X. Dechaume-Moncharmont for statistical advice and valuable comments on the manuscript. Funding was provided by the CNRS and the ANR (ANR-07-JCJC-0134 and ANR-08-JCJC-0006).

Part 2 : In search of a paternal transmission of antibacterial activity to the eggs of *Tenebrio molitor*.

Chapitre 1

I. Introduction

Theory predicts that a costly paternal investment into progeny's fitness is not favoured by selection in polyandrous species, because the occurrence of paternal care should be related to certainty of paternity (Westneat & Sherman, 1993). Moreover, the importance of paternal effects on progeny's fitness in insects is often dismissed, because of the higher amount of non-nucleotidic material provided to the zygote by the mothers compared to the fathers (reviewed in Galloway, 2000 and Rossiter, 1996). However, empirical studies have demonstrated the occurrence of a transmission of male-derived substances, which increase offspring performance in several polyandrous insect species (Boggs & Gilbert, 1979; Simmons, 1990; Molleman et al. 2004).

In some species, the males produces a spermatophore which package the sperm and can serve as a vector for the transmission of such compounds. This nuptial gift is believed to have evolved in polyandrous species to favour copulation rather than to benefit the offspring, but since they do, controversy still exists on whether this should be considered as paternal care or not (Zeh & Smith, 1985; Thornhill & Alcock, 1983). Within the spermatophore for example, substances that prevent the remating of females can be transmitted (Schlechter-Helas et al., 2011), and the structure of the spermatophore in itself can serve as a mechanical barrier inhibiting the copulation with subsequent males (Simmons, 2001). These mechanisms, plus behavioural adaptations such as post-copulatory mate guarding (Alcock, 1994), are ways to assure the males' paternity over the eggs sired, and could favour the evolution of costly paternal investments even in polyandrous species.

Indeed, a transfer of male-derived defensive substances to the eggs has been reported. These substances often consist in chemicals showing deterrent properties for predators and showing an antibacterial activity. In some meloid and pyrochroid beetles, cantharidin is transferred by the males to the females at mating, and is later redistributed by her to the eggs. This nuptial gift has been shown to protect both the female and the eggs from predators. A similar phenomenon has been shown to occur in arctiid and nymphalid moths with pyrrolizidin alkaloids, and in chrysomelid beetles with cucurbitacins (see Eisner et al., 2002 for review).

Paternal effects influencing offspring fitness can also emerge from indirect-paternal effects, if an investment of the female into a clutch is elicited by a paternal cue (Harris &

Uller, 2009). The only example concerning egg defence is the synthesis of ceratotoxins in *Ceratitis capitata*. The synthesis of this antibacterial peptide in the female's accessory glands can be elicited by the copulation (Marchini et al., 1995), and is also found at the surface of the eggs, which are coated with it in the female's genital tract (Marchini et al. 1997).

We have previously shown that after stimulation of their immune system, females of *T. molitor* could provide their eggs with an internal antibacterial activity. In this species, the male produces a spermatophore, which could serve as a vector for the transmission of a paternal effect. *T. molitor* females are also known to assess the immune state of the males prior to copulation (Worden et al. 1997), which could be a reliable cue indicating the presence of a pathogen into the maternal and offspring's environment. In this experiment, we investigated the existence of a paternally-derived antibacterial activity in the eggs of *T. molitor* following an immune challenge in the males of the parental generation. Since the maternal protection of the eggs was transitory, we could expect the paternal protection to vary along time according to the persistence of the pathogenic cue as well (i.e. persistence or not of the infection in the male). We therefore searched for a paternally-derived protection in the eggs through a transmission dynamics of seven laying ranks along fourteen days.

II. Materials and methods

II.1. Temporal dynamics of the transmission of antibacterial activity to their eggs by immune-challenged males

All mealworm beetles used in this study originated from an outbred stock culture maintained in our laboratory in bran flour added with *ad libitum* access to water and regularly added with proteins (piglet flour), apple and bread. Pupae were then collected from these stock cultures and adults were maintained individually after emergence in a Petri dish supplied with bran flour a piece of apple and water for ten days.

To do this, 10 age controlled (10 days old) virgin adult males were weighted to the nearest 1 mg and immune challenged by a single injection of 5 µl of Ringer's solution containing non-purified lipopolysaccharides (LPS: 0.5 mg/ml) extracted from *Escherichia coli* (Sigma: L8274). Immediately after their immune challenge, males were paired with a virgin and unchallenged female of the same age. The couple were allowed to produce eggs in a Petri dish provided with bleach flour and *ad libitum* food and water under standard laboratory conditions (25°C, 70% RH, L12h:D12h), and transferred into a new Petri dish every second day following the paternal immune challenge, until the couples had their clutches separated into 7 egg laying sequences. Three eggs were collected in each egg laying sequence and stored at -20°C for later examination of their antibacterial activity. The presence or absence of a zone of inhibition in their eggs was recorded.

II.2. Analysing the antibacterial activity of the eggs

To measure antibacterial activity of the eggs, individual eggs were thawed on ice, suspended in 2µL of PBS and homogenized using a pestle, except in the second experiment, in which case egg content and egg surface were isolated before freezing. Antibacterial activity of all the samples was measured from 2 µl of extract using the antibacterial assay described in Moret (2006).

III. Results

We found no antibacterial activity in the extracts of the eggs collected in all the laying ranks of the transmission dynamics.

IV. Discussion

We did not found any paternally-derived transmission of antibacterial activity to the eggs.

The direct transmission of paternal defensive substances to the eggs has been reported in some polyandrous insect species. However, in these species, the degree of polyandry was relatively low compared to the one observed in *T. molitor*. Indeed in the pyrochroid beetle *Neopyrochroa flabellata* for example, females are unable to receive another spermatophore for several days after mating, increasing the male's chance to protect the eggs he fertilized (Eisner et al. 1996b). In the arctiid moth *Utetheisa ornatrix*, the females mate multiply but only up to thirteen matings have been recorded in the wild (LaMunyon, 1994). Moreover, the male have means to bias the paternity to their advantage by producing larger spermatophores (LaMunyon & Eisner, 1994). In the danaine butterfly *Danaus gilippus*, in which male also transfer pyrrolizidine alkaloids to the females at mating, up to fifteen matings only have been observed in the wild (Pliske, 1973).

In comparison, the certainty of paternity in *T. molitor* is very low, since females are receptive throughout the whole of their adult life (Drnevitch et al. 2001). In this species, Dnervitch et al. found that complete second male precedence frequently occurred through the inhibition of the sperm release from the first spermatophore by the subsequent male (Drnevitch 2002).

In this respect, a direct transmission of immune effectors from the male to the female through the spermatophore seems unlikely.

Nonetheless, we could have expected an indirect paternal effect, either through an induction of antibacterial compounds by the males in the females, or by an adjustment of the protection of their progeny by the females in response to an immune challenge in the male.
The induction of antibacterial substances synthesis has been shown in the highly polyandrous medfly *Ceratitis capitata* (Bertin et al. 2010). In *C. capitata*, the synthesis of ceratotoxins applied onto the egg surface can be elicited in the female by the male at mating (Marchini et

al. 1995). Concerning the plastic adjustment of the maternal investment in offspring production by the females according to the phenotype of the males, it has been shown to occur in several insect species (Kotiaho et al. 2003, Wedell, 1996). *T. molitor* females are able to perceive an infection in their mate (Worden et al. 1999, Worden et al., 2005), but do not seem to adjust their investment into egg protection in consequence.

Chapitre 2

Chapitre 2 : Recherche d'un transfert trans-générationnel d'immunité aux jeunes larves de *T. molitor* et de son degré de spécificité.

Dans ce chapitre, nous avions deux objectifs principaux. Le premier était de tester si la stimulation du système immunitaire des femelles procurait une protection à leurs jeunes larves, et si cette protection était améliorée lorsque la larve était en présence du pathogène ayant stimulé la femelle. Le second était de tester si le transfert trans-générationnel d'immunité (TTGI) aux jeunes larves pouvait être élicité par la stimulation des femelles avec un champignon entomopathogène. Enfin, nous avons testé si les jeunes larves étaient capables d'ajuster leur temps de développement en présence de ce champignon pathogène, et si un effet maternel existait sur le temps de développement des jeunes larves.

I. Bénéfice au transfert trans-générationnel d'immunité pour les jeunes larves de *T. molitor* et spécificité.

Relativement peu d'études ont mis en évidence un bénéfice direct au TTGI. Trois études ont observé une meilleure résistance face à un agent pathogène qui avait stimulé les femelles de la génération parentale (Rahman et al., 2003; Roth et al., 2010, Tidbury et al., 2011). Dans ces expériences, le gain en fitness dû au TTGI était estimé chez la descendance adulte.

Cependant, les premiers stades larvaires semblent être les moins immunocompétents chez les insectes (Srygley, 2012). On peut donc s'attendre à ce qu'un effet maternel protecteur s'exerçant sur ces stades précoces ait des conséquences importantes sur la survie des individus (Rossiter, 1996). Dans ce chapitre, nous avons recherché si la stimulation des femelles de la génération parentale se traduisait par une augmentation de la survie de leurs jeunes larves maintenues en présence de microorganismes.

De plus, l'existence d'une spécificité du TTGI a été suggérée chez *T. castaneum* (Roth et al., 2010) et *Daphnia magna* (Little et al. 2003). L'existence d'une spécificité du TTGI serait une preuve supplémentaire de son caractère adaptatif. Dans un second temps, nous avons cherché à savoir si la stimulation du système immunitaire des femelles avec un pathogène conférait à leur descendance une meilleure protection face à ce pathogène en particulier.

Afin de répondre à ces questions, nous avons stimulé le système immunitaire des femelles avec deux microorganismes : une bactérie entomopathogène *Bacillus thuringiensis* et une bactérie opportuniste: *Escherichia coli*.

Nous avons ensuite exposé les larves de leur descendance soit au même microorganisme qui avait servi à stimuler le système immunitaire des femelles (combinaison homologue : les larves issues de femelles ayant reçu une injection d'*E. coli* étaient placées en présence d'*E. coli*, les larves issues de femelles ayant reçu une injection de *B. thuringiensis* étaient placées en présence de *B. thuringiensis*) soit à l'autre microorganismes (combinaison hétérologue : les larves issues de femelles ayant reçu une injection d'*E. coli* étaient placées en présence de *B. thuringiensis*, les larves issues de femelles ayant reçu une injection de *B. thuringiensis* étaient placées en présence d'*E. coli*), soit à des conditions contrôles.
Nous avons suivi leur survie quotidiennement pendant 30 jours.

II. Existence d'un TTGI envers un champignon entomopathogène, et ajustement du temps de développement des jeunes larves en fonction de leur infection ou de celle de leur mère

Le TTGI confère une protection à la descendance envers des virus (Huang & Song, 1999 ; Tidbury et al. 2011) et des bactéries (Rahman et al., 2003 ; Roth et al., 2010). Ces organismes présentent un taux de multiplication rapide et sont susceptibles de persister directement dans l'environnement de la descendance, condition prédite comme étant nécessaire à l'évolution d'un TTGI envers eux. Les champignons entomopathogènes remplissent également cette condition, cependant, l'occurrence d'un TTGI envers eux n'a jamais été recherchée. Dans une seconde expérience, nous avons stimulé le système immunitaire des femelles avec le champignon entomopathogène, *Metarhizium anisopliae*, et comparé la survie de leurs jeunes larves en présence et en absence du champignon.

D'autres traits phénotypiques que le système immunitaire sont susceptibles d'être plastiquement ajustés par les hôtes en réponse à la présence d'organismes pathogènes dans leur environnement. Par exemple, une modification du temps de développement des hôtes exposés peut réduire les conséquences négatives d'une infection (Agnew, 2000). Dans un premier temps, nous avons vérifié si ce trait était plastiquement ajusté chez les larves de *T. molitor* en réponse à la présence d'un microorganisme dans leur milieu dans un premier temps. Puis, dans un second temps, nous avons recherché l'existence d'un effet maternel

résultant de la stimulation immunitaire maternelle sur le temps de développement de leurs jeunes larves.

Ces expériences ne nous ont pas permis de mettre en évidence d'effet protecteur du TTGI sur les jeunes larves de *T. molitor*. Cependant, nous avons pu mettre en évidence la capacité des jeunes larves à ajuster leur temps de développement en réponse à la présence d'un microorganisme dans leur environnement. Ce trait n'était pas influencé par l'expérience immunologique maternelle.

Ces expériences ne feront pas l'objet d'un article.

Chapitre 2

I. Introduction

In insects, the first larval instars are the less immunocompetent ones (Rheins & Karp, 1985; Srygley, 2012), and are the target of several parasites and pathogens (Benrey & Denno, 1997). Indeed, because juvenile stages are the most vulnerable, maternal effects acting early in progeny's development are likely to have important fitness consequences (Rossiter, 1996). Therefore, maternal effects acting on offspring's immunity early in their development are expected to have important fitness consequences. Maternal effects on offspring's resistance to pathogen in invertebrates might result from the maternal exposure to various stressful conditions, such as food deprivation (Mitchell & Read, 2005; Boots & Roberts, 2012) or population density (Miller et al. 2009). These effects could be attributable to an adaptive increase in female's investment in the general quality of their offspring, which would in turn result in increased immunocompetence, and thus resistance to pathogens and parasites

Trans-generational immune priming (TGIP) is a special case of maternal effect, in which the maternal encounter with a pathogen leads to an increase in the immuncompetence of the offspring (Little & Kraaijeveld, 2004). TGIP has been shown to have an incidence on several developmental stages in vertebrates. In birds for example, maternal antibodies can be incorporated inside the eggs. They will confer the young chick an early protection before the maturation of its own immune system, enhancing its survival during the early part of life. The effects of the maternal antibodies on offspring immunity will persist into the adult stage (reviewed in Hasselquist & Nilsson, 2009), and the protection they confer to the offspring can show a high degree of specificity towards the pathogen that previously challenged the mothers (Richter et al., 2005).

In insects, TGIP has been shown to occur in the eggs (Sadd & Schimd-Hempel, 2007; Zanchi et al., 2012) as well as in late larval instars (Huang & Song, 1999; Moret, 2006; Freitak et al., 2009, Tidbury et al., 2011), and in the adult stage (Rahman et al., 2007, Roth et al. 2009; Zanchi et al. 2011). Its benefits however have only been shown in late larval instars (Tidbury et al., 2011) and adult stage (Rahman et al., 2003; Roth et al., 2010). Whether TGIP confers the same protective role to the most vulnerable larval instars of insects, in a similar fashion as the transfer of antibodies in young jawed vertebrates, remains to be tested. Furthermore, an increase in protection towards a specific pathogen that previously stimulated the mother would indicate an adjustment of the offspring's phenotype to the maternal

pathogenic environment, and confirm the adaptive nature of TGIP. In invertebrates however, this phenomenon has been has only been demonstrated in two species, *Daphnia magna* (Little et al. 2003) and *Tribolium castaneum* (Roth et al., 2010). Therefore, investigations about specificity of TGIP may need to be extended to other invertebrate model systems to draw general conclusions.

Moreover, TGIP has only been demonstrated to be elicited by bacterial (Rahman et al., 2003; Sadd & Schmid-Hempel, 2005; Moret, 2006; Freitak et al., 2009; Roth et al., 2010) or viral (Huang & Song, 1999; Tidbury et al., 2011) cues. These microorganisms have a high multiplication rate, and are likely to persist from one generation to another (Anderson, 1981). Fungal pathogens are transmitted by contact between individuals (Scholte et al., 2004), and their spores can resist adverse conditions for long periods while remaining infectious when the opportunity to infect a host occurs (Fargues et al., 1985). Therefore, we can expect them to persist from one generation to another in gregarious insect populations. However, TGIP towards a fungal pathogen has never been investigated.

Although the immune system provides an efficient way for organisms to fight infections once they occurred, there are some other ways to increase the host's fitness in a sceptic environment. The adjustment of several life-history traits in accordance to a pathogenic threat is another physiological adaptation that has evolved in some host organisms (Minchella 1985; Agnew et al., 2000). Fecundity compensation for example consists in an increased investment into early reproductive events following an infection (often at the expense of growth). It allows the host to partially catch-up with the loss of future fecundity caused by an early death (Agnew et al., 2000). Another example is the modification of the developmental time of the host in response to an infection. Indeed, by reducing the duration of the most vulnerable stage, or the pre-reproductive life-span, the host can efficiently reduce the impact of the infection (Michalakis & Hochberg, 1994). Moreover, reducing the time-lap between two moults may confer a benefit to individuals in a sceptic environment, considering that shedding the infected tegument at moulting can prevent parasite intrusion (Duneau & Ebhert, 2012). Interestingly, it is also a trait that can be influenced by the maternal experience with a stressful environment in some species. For instance in the seed beetle *Stator pruininus*, females that experienced a high density in conspecifics (and thus competition) during the larval stage produce offspring that takes longer to develop (Fox et al. 1999). On the contrary, females of the parasitoid wasp *Copidosoma koehleri* that experienced a high larval rearing

density produce male progeny that has a shorter developmental time (Morag et al. 2011). However, to our knowledge, the developmental time of the offspring has never been shown to be influenced by the maternal encounter with a pathogen in an adaptive way.

In order to answer these questions, we performed two separate experiments. In the first experiment, we investigated the occurrence of a protective effect of the maternal immune priming on the young larvae, and its specificity. To this purpose, we stimulated the immune system of adult females of *T. molitor* with two different heat-killed microorganisms: *Echerischia coli* and *Bacillus thuringiensis* and allowed them to produce offspring. We then exposed the larvae of their offspring either to the same microorganism that stimulated their mothers before or to a different one, and recorded their survival and moulting events daily during thirty days.

In a second experiment, we investigated whether maternal encounter with an entomopathogen that infect the host through the external integument had a protective effect on the young larvae after a subsequent exposure to this pathogen. Then, we investigated if moulting could be elicited by such a pathogen, either in the larval or the maternal environment. As shedding the old tegument provides the young larvae a mean to prevent fungal intrusion into the haemocoel, we expected that adaptive moulting can be elicited by a pathogen that infects its host by penetrating its cuticle. We therefore stimulated both the females and their larvae with the fungal pathogen *Metarhizium anisopliae*.

II. Materials and methods

II.1. Insect culture

Nymphs were isolated from a massive outbred stock culture (as described in **Matériel et Méthodes général**) and maintained in the dark at 25°C and 70%RH. Adults were individualised at emergence in Petri dishes containing bran flour, a pierced microcentrifuge tube filled with water and a piece of apple.

II.2. Microorganisms used

Microorganisms were obtained from the Pasteur Institute (*Metarhizium anisopliae* IP 1693.87 ; *Echerischia coli* : 1034.70: *Bacillus thuringiensis* : CIP53.1) *M. anisopliae* was cultured on Potato Dextrose Agar (agar: 15 g/L; dextrose: 20 g/L; potato extract: 4 g/L; pH= 5.6) and incubated one week at 27°C in order to obtain enough conidiospores for the experiment. Spores used for maternal immune stimulation were collected on the day of utilisation and heat-killed at 120°C during 5 minutes. Spores used for larval exposition were collected at the time of utilisation.

E. coli and *B. thuringiensis* were cultivated in liquid Broth medium (10 g bactotryptone, 5 g yeast extract, 10 g NaCl in 1000 mL of distillate water, pH = 7.5) during 48 and 72h respectively. Cultures used for maternal exposure were centrifugated three times at 10 000 rpm during 10 minutes. After each centrifugation, the Broth medium was removed and replaced by sterile insect's Ringer solution (128 mM $NaCl_2$; 10 mM $CaCl_2$; 1.3 mM KCl; 2.3 mM $NaHCO_3$). Bacterial concentration in the resulting solutions was estimated with a hemocytometer (Neubauer improved) and adjusted at 10^4 bacteria/µL, before being heat-killed at 120°C during 5 minutes.

II.3. Experimental design

Metarhizium anisopliae is an entomopathogenic fungus which invades the host's hemocoele by the production of hyphaes that penetrate its cuticle (Vey et al. 1982). Because of the hydrophobic nature of *M. anisopliae*'s conidiospores, we could not obtain a saline

solution of conidiospores in a known concentration for injection. We chose to prick our experimental females with a sterile needle dipped into heat-killed *M. anisopliae* conidiospores. We first confirmed that this mode of exposition elicits the immune system of *T. molitor* and induces a strong mortality when the spores are alive (on 10 females: 100% mortality in 3 days).

Bacillus thuringiensis contamination naturally occurs through the food source of insects (Chiang et al., 1986). The toxins of *B. thuringiensis* induce a lysis of the midgut epithelial cells, allowing the bacteria to cross the midgut barrier and invade host's hemocoele (Vey et al., 1982). *E. coli* is an ubiquitous and opportunistic bacteria which can induce a mortality by hemoceolic injection (personal observations). These heat-killed bacteria were injected in 5µL of insect Ringer's solution through a pulled capillary. Virgin females were immune-stimulated 10 days after emergence and immediately placed with a virgin and immunologically naïve male of the same age. Each couple was maintained in a Petri dish containing sterile bleach flour, a pierced microcentrifuge tube filled with water and a piece of apple.

Eggs of the first egg laying rank (between the days 0 and 2 after stimulation) were removed from the experiment, because we previously found that transfer of antibacterial activity to the eggs at this egg laying rank was very rare (see **Chapitre 1**). We thus assumed that this egg laying rank might be free of TGIP in general. Eggs laid between days 4 and 6 were allowed to hatch and the resulting larvae exposed experimentally either to one the above microorganisms or maintained in control conditions.

Since experimental larvae were too small to receive an injection or being pricked, the living microorganisms were provided to the larvae by mixing them to the larval medium. This procedure has the advantage of mimicking the natural route of infection of the larvae by the microorganisms. *M. anisopliae* infect its hosts by contact, *B. thurigiensis* and *E. coli* by being ingested from the food source. For this purpose, larvae were maintained individually in wells of a 96-well plate at the bottom of which the microorganisms were applied with a sterile brush. For the Ma treatment, the brush was dipped into a culture of *M. anisopliae* and thus covered with conidispores, and applied at the bottom of the well. For the bacterial treatments, a bacterial suspension was centrifugated (5000 rpm, 10 min, 4°C) to remove the Broth supernatant. The brush was then dipped into the bacterial precipitate and applied at the bottom

of the well. Sterile bleach flour, which was the nutritive medium of the larvae, was then poured on top of the microorganisms before the larva was placed into the well. For control condition « C », each larva had sterile wheat meal only in the well.

II.3.1. Experiment 1: specificity of the maternal immune priming on the young larvae of *T. molitor*

II.3.1.1. Maternal treatments

We performed 2 maternal treatments and 1 procedural control:

- the *E. coli* or « **Ec** »female group injected with insect Ringer solution containing heat-killed *E. coli* (n = 15),

-the *B. thurigiensis* or « **Bt** »female group injected with insect Ringer solution containing heat-killed *B. thuringiensis* (n = 20),

- Ringer or « **R** » female group injected with insect Ringer's solution only and used as a procedural control, for the potential effect of the injection (n = 13).

II.3.1.2. Larval treatments

Eight days after oviposition, most of the larvae of each clutch had hatched. Before their first larval moult occurs, the clutch of each female was divided in 3 groups of equal numbers of larvae. Each group of larvae was then randomly allocated to one of the infectious larval treatments in which larvae were maintained with either living *B. thuringiensis* « **Bt** »or *E. coli* « **Ec** » or to control conditions (without micro-organisms). As such, the offspring of each mother was exposed to either the same microbial agent she was previously challenged with (homologous combinations) or to a different one (heterologous combinations). This experimental design is represented in **figure 12**.

The number of dead larvae was recorded every day during 30 days.

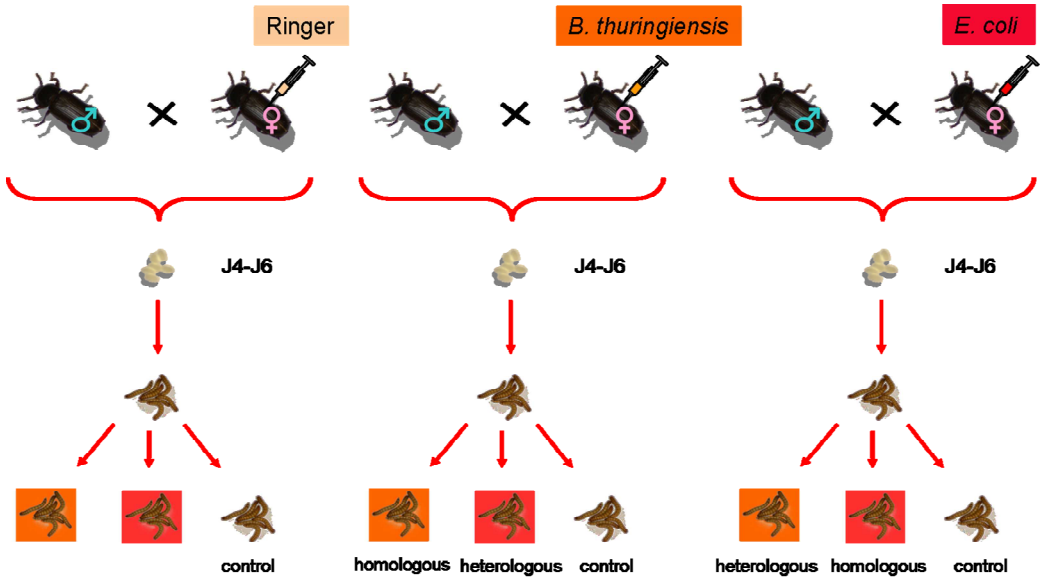


Figure 12 : Schema synthesizing the experimental treatments realized in the parental and offspring generations for the test of the specificity of the TGIP on the young larvae of *T. molitor*.

II.3.2. Experiment 2: Existence of a maternal immune priming of the young larvae toward *M. anisopliae* in *T. molitor*, and effect of *M. anisopliae* on the duration of the first larval intermoult

II.3.2.1. Maternal treatments

We performed 1 maternal treatment and 1 procedural control:

- the *M. anisopliae* or **Ma** female group pricked with a sterile needle dipped into heat-killed *M. anisopliae* conidiospores (n = 15),
- the pricked or **P** female group pricked with a sterile needle only and used as procedural control for the potential effect of pricking (n = 20),

II.3.2.2. Larval treatments

Eight days after oviposition, and before the first larval moult occurred, the clutch of each female was divided in 2 equal numbers of larvae. Each group of larvae was then randomly allocated to one of the two larval treatments: maintained with either living *M.*

anisopliae (**Ma**) or kept in control conditions (without micro-organisms). This experimental design is represented in **figure 13**.

The number of dead larvae was recorded every day during 30 days. The presence of a moult was recorded at the same time.

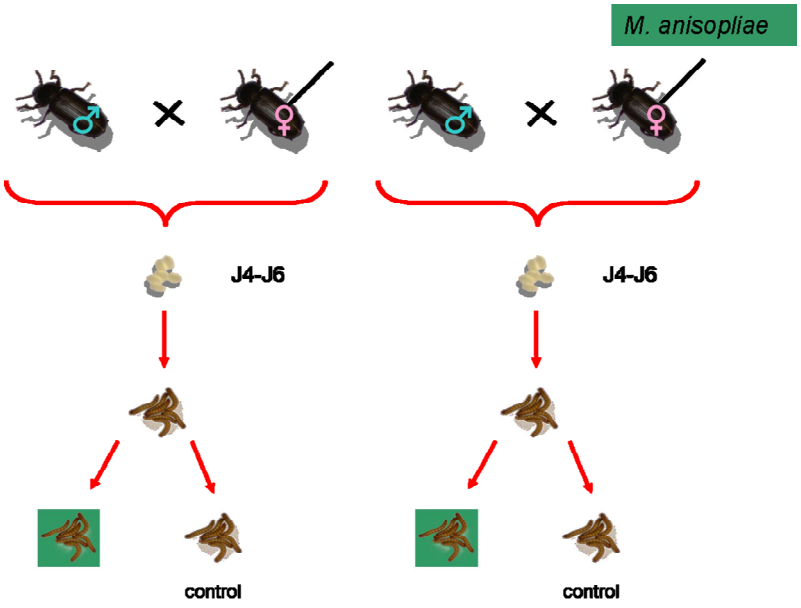


Figure 13 : Schema synthesizing the experimental treatments realized in the parental and offspring generations for the test of the existence of a TGIP elicited by *M. anisopliae* on the young larvae of *T. molitor*, and the test of the effect of *M. anisopliae* on the duration of the first larval intermoult.

II.4. Statistical analyses

The analyses were performed with R (R Development core team, 2011).

II.4.1. Experiment 1

We examined whether maternal priming provides the offspring with a specific protection, that is to say an enhanced protection in the microorganism that had challenged the females of the parental generation. We therefore created a factor named "specificity", combining the maternal and the larval treatments. The factor "specificity" had 4 levels: "heterologous" when mothers and larvae were exposed to the same bacterium, "homologous" when mothers and larvae were exposed to different bacteria, the "Ringer-microorganisms" control (or R-M) when the progeny of control mothers was exposed to bacteria, and a full control (R-C) when the progeny of control mothers were kept unexposed to bacteria. A Mixed Effects Cox Model was used to analyse the survival of the larvae of each modality, with the

specificity treatment as a fixed factor, and the female's ID as a random effect (coxme package: Therneau, 2012). Unfortunately, the coxme package does not allow to plot effects, and using Cox models without mixed effects yielded different (and therefore incorrect) results. Therefore, we plotted the proportion of surviving larvae after 30 days of experiment as a graphical illustration.

II.4.2. Experiment 2

We first tested if the maternal treatment with *M. anisopliae* had a protective effect on the young larvae of *T. molitor* when maintained with this fungus. We used a Mixed Effects Cox Model to analyse the survival of the larvae, with the maternal treatment (*M. anisopliae* or pricked control) as a fixed factor, the larval treatment as a fixed factor, their interaction, and the female's ID as a random effect.

We then examined whether the duration of the first intermoult of the larvae was affected by contact with *M. anisopliae* and the treatment of their mothers. For this purpose, we analysed the duration of the first intermoult with a Linear Mixed Model (LMM, nlme package: Pinheiro et al., 2012) with larval treatment, the maternal treatment and their interaction as fixed factors. The female's ID was included as a random factor.

III. Results

III.1. Experiment 1: specificity of the maternal immune priming on the young larvae of T. molitor

We found no effect of the "specificity" treatment on larval survival ($\chi^2_{4,93}$ = 2.49 ; p = 0.48, **figure 14**). Contrary to our expectations, larvae kept in presence of bacteria had an increased survival compared to controls, which suggests a positive effect of the exposure of the larvae to bacteria, even if this difference was not significant.

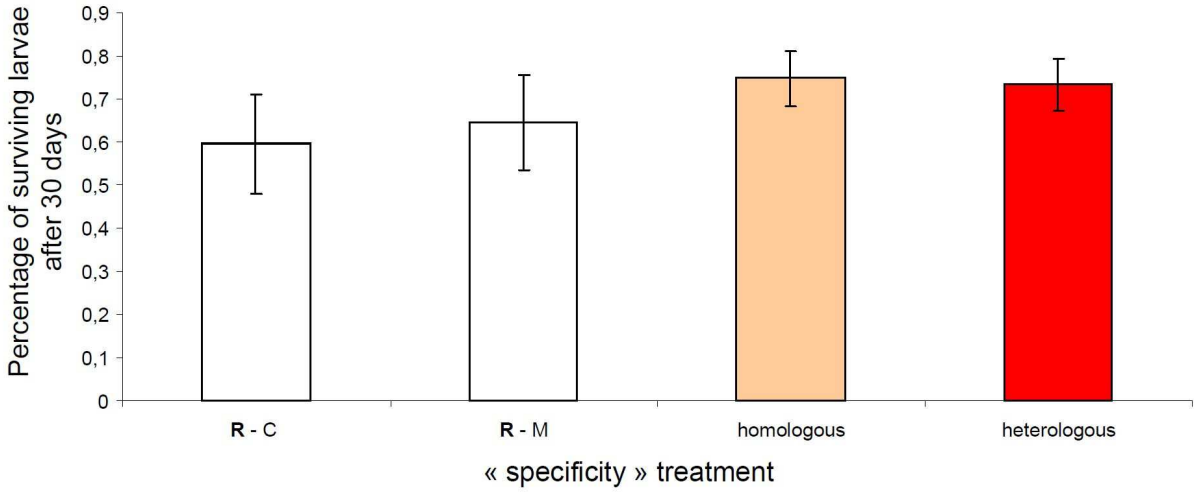


Figure 14 : Mean percentage of surviving larvae (± SE) at the end of the experiment, as a function of the "specificity" treatment (x axis).
The first 2 bars correspond to the mean survival of the larvae from control females. The maternal treatment is indicated in bald character, followed by the larval treatment (control larval treatment "C" exposed to microorganisms "M", both Ec and Bt).

III.2. Experiment 2: Existence of a maternal immune priming of the young larvae toward M. anisopliae in T. molitor, and effect of M. anisopliae on the duration of the first larval intermoult

III.2.1. Larval survival

We found no significant effect of the maternal treatment in interaction with the larval treatment on larval survival ($\chi^2_{1,61}$ = 0.16 ; p = 0.69). There was no significant effect of the maternal treatment on larval survival either ($\chi^2_{1,63}$ = 0.35 ; p = 0.55). As expected, the

presence of *M. anisopliae* in the larval environment decreased larval survival ($\chi^2_{1,63}$ = 3.86 ; p = 0.049) (**figure 15**).

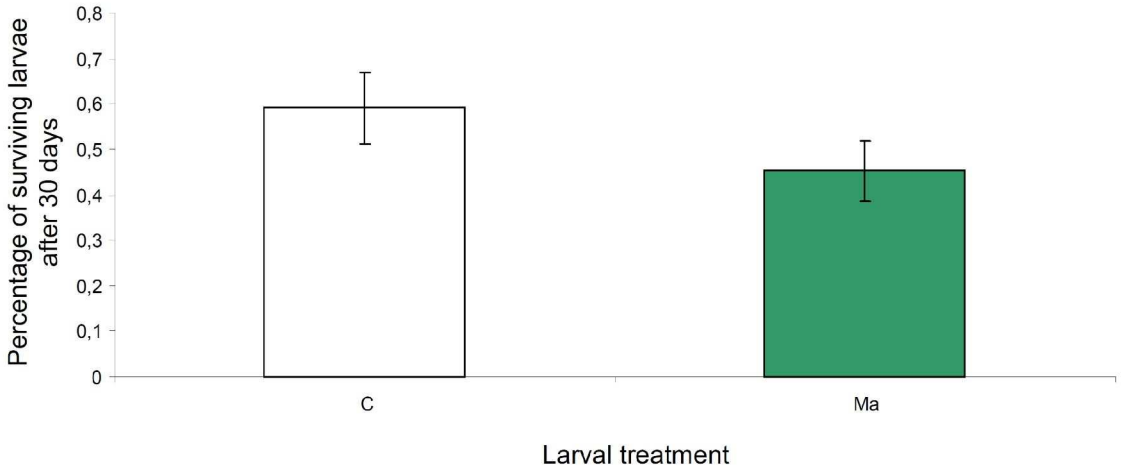


Figure 15 : Percentage of surviving larvae after 30 days of experiment according to the larval treatment (x-axis).

III.2.2. Is the timing of moulting of *T. molitor* adjusted in response to a pathogenic threat?

The presence of *M. anisopliae* in the larval environment significantly decreases the duration of the first larval intermoult (L ratio$_{4,30}$ = 10.94 ; p < 0.001) (**Figure 16**). There was no significant maternal effect on the duration of the larval intermoult (L ratio$_{5,30}$ = 2.16 ; p = 0.14). Finally, the interaction between the maternal and the larval treatments was not significant (L-ratio$_{5,34}$ = 0.18 ; p = 0.67). These latter two results suggest that the timing of moulting is not adjusted by the maternal immune experience.

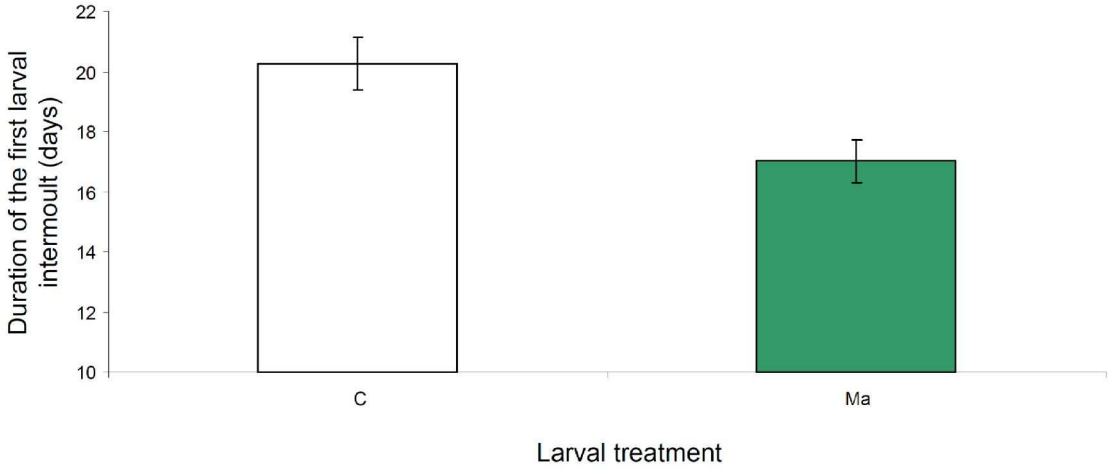


Figure 16 : Mean duration of the first larval intermoult (in days ± SE) of the larvae from pricked and *M. anisopliae*-pricked females, according to the larval treatment (x-axis).

IV. Discussion

This study had three main objectives. First, it tested whether maternal immune priming provides a survival benefit to the offspring, by examining whether the immune protection provided to the offspring by the maternal immune priming was specifically directed towards the microorganism that challenged the mother. Second, it investigated the existence of a TGIP towards *M. anisopliae*. Finally, it searched for potential changes in larval development of *T. molitor* in response to the pathogenic threat imposed by a fungal pathogen, and in response to the maternal stimulation with this pathogen.

For the first objective, we failed to demonstrate any benefit or specificity of the maternal priming on the survival of the larvae. Survival was not different between heterologous and homologous treatments. Hence, our results do not corroborate those of Little et al. (2003) and Roth et al. (2010). In *Daphnia magna*, Little et al. (2003) show a specific benefit of the maternal priming with two *Pasteuria ramosa* strains, but not in terms of survival in the offspring. They show that the fecundity of the offspring exposed to *P. ramosa* was increased if the females of the parental generation had been previously primed with this pathogen. They also shown that this benefit was greater if the offspring was exposed to the same strain (homolgous challenge) than to a different one (heterologous challenge). Roth et al. (2010) however found a specific benefit of the TGIP in terms of enhanced survival in the adults of *T. castaneum*, but did not include the female's ID as a random effect, which is less conservative. Therefore, convincing evidence of specificity to the TGIP is still lacking. However, our experimental protocol seemed to be flawed since larval survival was slightly enhanced by the presence of bacteria in the larval environment. The larvae maintained in bacteria also had a shorter intermoult compared to bacteria maintained in control conditions (see **supplementary material**, **figure S3**). The plausible explanation for this is that larvae fed on bacteria, which were not pathogenic enough to have a lethal effect on them. To confirm this hypothesis, we froze the surviving larvae at the end of the experiment for later measurements of their size. Stimulation of the insect's immune system by mixing pathogens to the food source or by cuticular application is often used in the literature (Rosengaus et al., 2007; Freitak et al., 2009). Still, it was proven insufficient to induce a significant mortality in our larvae. Despite the high relative humidity in which the experimental larvae were maintained during this experiment (see **Materials & Methods**), and despite the fact that

larvae started feeding immediately after the inoculation, the bacteria might have died in the wells before an infection could occur. Therefore, we cannot conclude about the existence of a specific TGIP in *T. molitor*. An immune stimulation of the young larvae by pricking or injection is difficult because of their small size. Challenging the adult offspring of *T. molitor* by intra-hemocoelic injection would surely have induced a significant mortality compared to a Ringer injection, and would have allowed us to see differential mortality according to the maternal immune challenge.

For the second objective, we did not found any maternal effect on the survival of the young larvae towards *M. anisopliae*. We found that the presence of *M. anisopliae* in the larval environment decreased larval survival. Maternal priming with *M. anisopliae* did not enhance larval survival in presence of this pathogen, so we found no evidence of a TGIP elicited by *M. anisopliae*. Several explanations could be proposed to explain this negative result. First, the young larvae of *T. molitor* might not be protected by a maternal immune challenge in general. Second, the treatment with *M. anisopliae* might impose a too strong mortality on the larvae, which could hide any potential protective effect of the maternal immune stimulation. This is unlikely, since the difference in larval survival between control and *M. anisopliae* larval treatment was only about 20%. Finally, TGIP might not have evolved in response to this pathogen. In order to elucidate this, we should prime the females of the parental generation with *M. anisopliae* and assess several immune parameters of their offspring at later stages, as well as their survival in presence of this fungus.

For the third objective of this study, we found that the first larval intermoult was reduced by the presence of a fungal pathogen in the larval environment. However, there was no evidence that maternal priming might have an effect on larval moulting. In presence of *M. anisopliae*, *T. molitor* larvae were able to speed-up the first stages of their development. This result is consistent with what was found by Roth & Kurtz (2008) on *T. castaneum*, in which the larval developmental time was reduced after an immune challenge with *E. coli* or *B. thuringiensis*. A change in the developmental rate in the presence of a pathogenic threat in the environment has been suggested to reduce the consequences of parasitism on survival and reproductive success (reviewed in Michalakis & Hochberg, 1994). In the case of *T. molitor* an *M. anisopliae* for example, the reduction of the time spent as larvae could allow the beetles to reach the less vulnerable adult stage whose thick cuticle is more efficient at preventing pathogenic intrusions (Siva-Jothy *et al.* 2005). Moreover, teguments renewal during moulting

has been proven to be an efficient way of decreasing the probability of hyphal penetration during the larval stage in the Colorado potato beetle, *Leptinotarsa decemlineata* (Vey, 1977). It can also have a protective effect against bacteria in *D. magna* (Duneau & Ebert, 2012). However, these studies did not report a plastic adjustment of the timing of moulting in accordance to the presence of a pathogen in the environment. Since the number of larval moults is a highly plastic phenotypic trait in *T. molitor*, it would not be surprising if this species was able to adjust its timing of moulting to avoid a fungal infection. Unfortunately, we were unable to correlate this reduction in the developmental time to an improved survival in the larvae of *T. molitor*. Since entomopathogenic fungi can cause delayed damages later in larval life, checking the survival for longer than 30 days might have enabled us to correlate a late mortality to the timing of the two first larval moults.

Our results suggest that there is no maternal effect on the timing of moulting. However, considering the potential benefit of an adjustment of the developmental time in a pathogenic environment, and the fact that maternal stimulation has been shown to induce a modification of the developmental time in *T. molitor* (see **Chapitre 3**), this possibility deserves to be explored further.

Chapitre 3

Chapitre 3 : Recherche d'un transfert trans-générationnel d'immunité d'origine maternelle et paternelle aux adultes de *T. molitor* et de ses coûts.

En réponse à la stimulation de leur système immunitaire, les femelles invertébrées peuvent transmettre une meilleure immunocompétence à leur descendance, montrant ainsi des phénomènes analogues à ceux décrits chez les vertébrés (Grindstaff et al., 2003 ; Huang & Song, 1999). Chez les invertébrés, la stimulation paternelle s'avère avoir également un effet sur l'immunité de la progéniture (Freitak et al., 2009 ; Roth et al., 2010). Cependant dans ces deux études, son expression apparaît qualitativement différente de celle induite par une même infection chez la mère. L'existence de ce TTGI d'origine paternelle remet en question la vision traditionnelle de la théorie de l'investissement parental, qui prédit que les femelles devraient investir davantage à la descendance que les mâles (Bateman, 1948 ; Trivers, 1972). Les différences de stratégies d'histoire de vie entre mâles et femelles et les coûts potentiels associés au TTGI peuvent néanmoins conduire à des investissements dissymétriques chez les mâles et les femelles dans la protection immunitaire de la descendance.

Dans ce chapitre, nous avons recherché sur quels effecteurs s'exprimait le TTGI d'origine maternelle ou paternelle chez la descendance de *Tenebrio molitor*. Pour cela, nous avons estimé l'immunocompétence de la descendance adulte issue de parents immunostimulés par l'observation de la charge hémocytaire, de l'activité antibactérienne induite et de l'activité du système PO-PPO présentes dans leur hémolymphe. Nous avons recherché les coûts associés à une éventuelle surexpression d'effecteurs immunitaires chez les descendants par l'observation de plusieurs de leurs traits d'histoire de vie : leur temps de développement, l'évolution de leur masse larvaire, leur masse nymphale et leur taille adulte. Ces mesures ont été réalisées sur la descendance issue de 3 séquences de ponte successives de 4 jours chacune après l'immunisation maternelle ou paternelle afin d'examiner la persistance des effets parentaux sur les paramètres considérés.

Afin de comparer les effets des simulations maternelle et paternelle, deux expériences ont été réalisées dans ce chapitre. Dans la première, les paramètres du système immunitaire et les traits d'histoire de vie de la descendance de couples dont les femelles avaient reçu une

injection de LPS ont été observés. Dans la seconde, les mêmes paramètres ont été observés chez la descendance de couples dont les mâles avaient reçu l'injection.

Nous avons montré que le TTGI, selon si celui-ci est d'origine maternel ou paternel, affecte différents effecteurs immunitaire chez la descendance et impose différents coûts sur cette dernière. En effet, la descendance des mères immunisées montrait une élévation de la charge hémocytaire basale associée à une croissance larvaire plus lente pour l'ensemble des séquences de ponte considérées. Par contre, la descendance des pères immunisés montrait une élévation de l'activité du système prophénoloxydase uniquement chez les descendants issus de la première séquence de ponte qui a suivi l'immunisation paternelle. Le challenge paternel s'accompagne d'une réduction de la masse nymphale de la descendance pour l'ensemble des séquences de ponte considérées, suggérant qu'elle n'est pas associée au transfert d'immunité d'origine paternelle.

Ainsi, en dépit d'un intérêt commun entre les mâles et les femelles pour la survie de leur descendance vis-à-vis de l'environnement pathogène, ceux-ci semblent avoir développé des stratégies différentes d'investissement dans le TTGI.

Ce chapitre a fait l'objet d'un article publié dans *Journal of Animal Ecology*.

Chapitre 3

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

Authors: Caroline Zanchi*, Jean-Philippe Troussard, Guillaume Martinaud, Jérôme Moreau and Yannick Moret

Published: November 2011 in Journal of Animal Ecology

Chapitre 3

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 appears 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 to 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.

Chapitre 3

I. Introduction

Among the factors that determine the phenotype of an organism, maternal effects by which females provide their offspring with non-genetic 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 the 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 et al. 2003; Hasselquist & Nilsson 2008) and invertebrates (Little et al. 2003, Sadd et al. 2005, Moret 2006, Sadd & Schmid-Hempel 2007; Freitak et al. 2009; Roth et al. 2010; Tidbury et al. 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 regards 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 coevolution (see Jokela 2010 for review). In many species, males and females have sexually dimorphic life-history strategies, and the results of 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 et al. 2009; Roth et al. 2010) and for the parents (Moreau et al 2012). 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 to maternal and paternal TGIP and of sexually dimorphic life-history strategies, we may expect that 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.

Chapitre 3

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 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 expression of maternal and paternal TGIP in *T. molitor* differs in the immune defences stimulated in the offspring. Its persistence over reproductive episodes following the parental challenge and the associated costs for the offspring are also different whether TGIP is from paternal or maternal origin.

II. Material and methods

II.1. 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 days ± 1 day post emergence) by a single injection of a dose of lipopolysaccharides (LPS) extracted from *Escherichia coli* in 5 µl 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 x l x H, 20 x 12 x 9.5 cm) supplied with a mix of 60 g of bran flour and bleached flour (1:2 w:w) and an micro-centrifuge 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 immune response is dynamic over time (Haine et al. 2008a, b), we can expect the transfer of immunity to the next generation to exhibit a dynamics over the time separating the parental challenge and oviposition. 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 following the immune treatment of the female 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 micro-centrifuge tube of water and a piece of apple weekly renewed. These larvae were maintained in standard laboratory conditions (see above) 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 ten days post emergence, we sampled 5 µl 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 then immune challenged with LPS and then tested again three days later, corresponding for 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.

II.2. Immune treatments of the parents and the offspring

Control adult parents received a single injection of 5 µl of Ringer solution after being chilled on ice. Challenged parents received a 0.5 $mg.ml^{-1}$ dose of non purified LPS extracted from *E. coli* (Sigma: L8274) in 5µl 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 et al 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.

II.3. Haemolymph collection

Individual beetles were chilled on ice before 5µl of haemolymph was collected from a wound in the beetle's neck and 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 the offspring beetle, a 10 µl sub-sample was immediately used for the measurement of the concentration of haemocytes. Another 5µl sub-sample was kept in a N-Phenylthiourea (Sigma P7629) coated micro-centrifuge 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.

II.4. Immune parameters

Concentration of haemocytes was measured using a Neubauer improved haemocytometer under a phase contrast microscope (magnification x 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 were used to measure antimicrobial activity on zone of inhibition plates seeded with *Arthrobacter globiformis* obtained from the Pasteur institute (CIP 105365). *A. globiformis* from a single colony on a streak plate were incubated overnight at 30°C in both 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/ml. Six millilitres of this seeded medium were 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 were added to each well, and a positive control (Tetracycline: sigma T3383) was included on each plate. Plates were then incubated for 48 hours 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, centrifuge (6500 r.p.m., 15 min, 4°C). Five µl of supernatant were 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 C-7762, 0.07 mg.ml-1 of distilled water) to measure total activity. Then 20 µl of L-Dopa solution (Sigma 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) for 40 min. Readings were taken every 15 seconds at 490 nm and analysed using the software SOFT-Max®Pro 4.0 (Molecular Devices). Enzyme activity was measured as the slope (Vmax value: change in absorbance unit.min-1) of the reaction curve during the linear phase of the reaction and reported to the activity of 1 µl of pure haemolymph.

II.5. Body mass and size

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

II.6. 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 were 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 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 **supplementary**

tables 1 and 2). 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 analyzed using a multivariate analyse 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.

III. Results

III.1. 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 (**figure17**; 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 (mothers $F_{1, 21} = 3.21$, $P = 0.088$; fathers: $F_{1, 21} = 0.01$, $P = 0.906$).

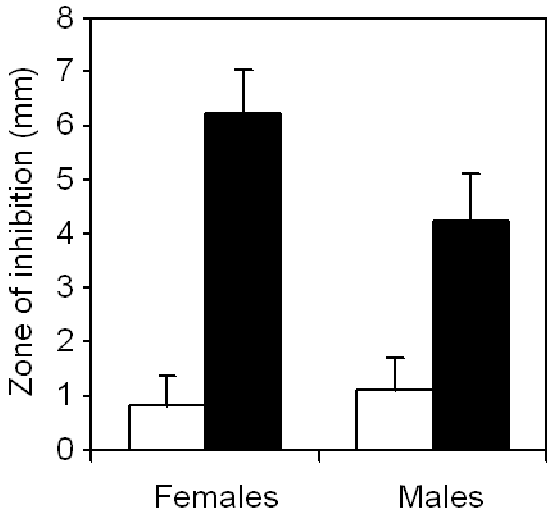


Figure 17 : Antibacterial activity (mean + SE) in the haemolymph of parental females and males previously exposed to an injection with Ringer solution (control; open bars) and LPS solution (black bars) mimicking a bacterial infection.

Nine weeks after the maternal immune treatment, control and LPS treated mothers produced a similar total number of larvae (mean ± SE control 24.9 ± 5.8 larvae; LPS 16.9 ± 5.1 larvae; ANOVA for repeated measures: between subject effects F1, 23 = 1.38, P = 0.522) without significant variation along egg laying sequences (within subject effects: F2, 46 = 0.62, P = 0.544; see **supplementary table 3**).

Couples with control and LPS treated fathers produced a similar total number of larvae (mean ± SE control 17.4 ± 3.8 larvae; LPS 12.9 ± 4.4 larvae; ANOVA for repeated measures: between subject effects F1, 23 = 0.26, P = 0.614). More larvae were produced during the last egg laying sequence (mean ± 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: F2, 21 = 5.83, P = 0.010) but this variation along egg laying sequences was independent of the paternal treatment (treatment x laying rank: F2, 21 = 0.46, P = 0.636).

III.2. Parental effect of 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 **supplementary table 1**). Especially, the immune responses of the offspring to the LPS-challenge were dependent on both the parental immune treatment and the gender of the focal parent (**supplementary table 1**), 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 2**).

III.2.1. Maternal effect on offspring immunity

Overall, the offspring of LPS-treated mothers had a higher concentration of haemocytes than the offspring of control mothers (**figure 18**, **table 2**), whereas levels of PO activity, total-PO activity and antibacterial activity were not affected by the maternal immune treatment (**table 2**). 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 ± s.e. total-PO 52.35 ± 3.38 od.10^2.min^{-1} vs 39.33 ± 3.15 od.10^2.min^{-1}, antibacterial activity 36.15 ± 3.18 mm 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 2**). 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

(**figure 18**). Change in antibacterial activity was dependent on the gender of the offspring (**table 2**) because males mounted a stronger antibacterial immune response to the challenge than females (mean difference of scores measured before and after immune challenge ± s.e. 62.96 ± 5.54 mm vs 45.25 ± 5.91 mm).

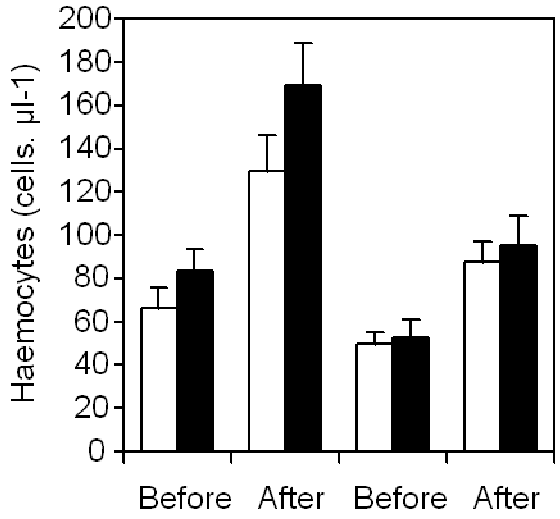


Figure 18 : Concentration of haemocytes (mean + SE) of adult offspring in relation to parental immune priming before and after immune stimulation with LPS.
Open bars refer to the control parental immune treatment whereas black bars refer to the LPS immune treatment of the parents.

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 2**; **figure 19a** and **b**).

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 2**). 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 (**figure 18**). Change in antibacterial activity was dependent on the gender of the offspring (**table 2**) because males mounted a stronger antibacterial immune response to the challenge than females (mean difference of scores measured before and after immune challenge ± s.e. 62.96 ± 5.54 mm 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 2**; **figure 19a** and **b**).

III.2.2. Paternal effect on offspring immunity

Overall, the paternal immune treatment had no main effect on levels of immune defences of the offspring (**table 2**). Levels of immune defences were gender-dependent (**table 2**) as males exhibited higher scores than females for all the immune parameters (mean of scores measured before and after immune challenge together ± s.e. Haemocytes 83.81 ± 8.09 vs 55.23 ± 8.10 cells.μl^{-1}; 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 2**). 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*Treat*L-rank in **table 2**). Indeed, upon the immune challenge, the offspring of LPS-treated fathers resulting from eggs laid early after the paternal immune treatment (day 0-4), exhibited higher PO activity and total-PO activity than those of control fathers (**figure 19**). This effect of the paternal immune treatment disappeared for offspring derived from later egg laying sequences (day 4-8 and day 8-12; **figure 19c** and **d**).

Changes in PO-activity, total-activity and antibacterial activity were also gender-dependent (**table 2**) 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 ± 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).

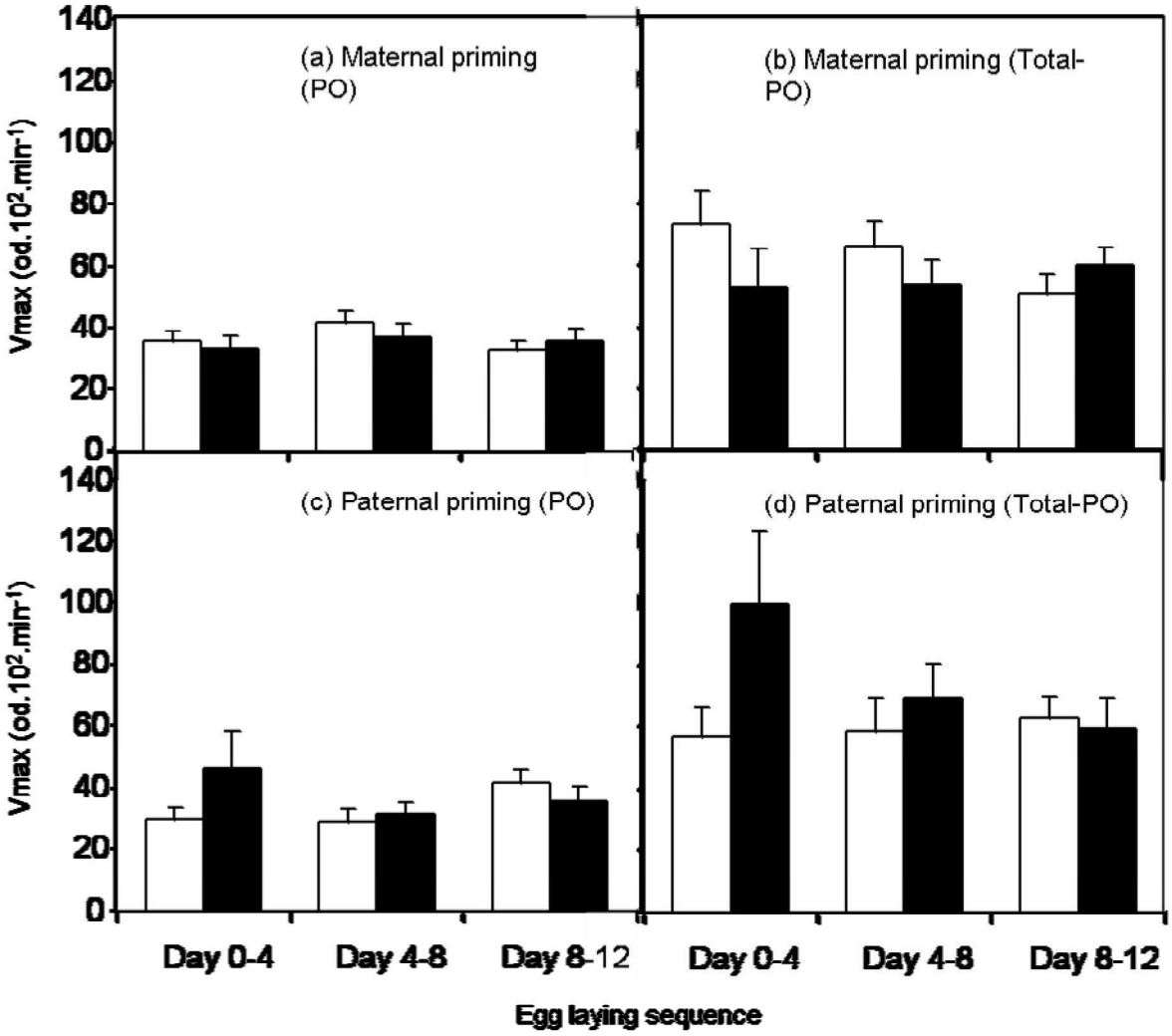


Figure 19 : Activity of naturally activated phenoloxidase enzymes (PO activity; Mean + SE) and activity of the proenzymes in addition of the PO (total-PO; Mean + SE) in the haemolymph of maternally (upper panels) and paternally (lower panels) immune primed offspring after being immune challenged.
Open bars refer to the control parental immune treatment whereas black bars refer to the LPS immune treatment of the parents.

III.3. 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 % versus paternal treatment 10.2 %, $\chi^2 = 3.35$, DF = 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 % versus control parental groups 6.5 %, $\chi^2 = 1.08$, DF = 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 (**supplementary table 2**). 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 (**supplementary table 2**, **figure 20a**). However, only pupae of LPS-treated fathers were significantly lighter than those of control fathers, whereas there was no significant effect of the maternal immune treatment on pupae mass of their offspring (**supplementary table 2**, **table 3**, **figure 20a**). Overall, pupae that became males were heavier than those that became females (**supplementary table 2**). The egg laying rank had no effect on any of the life history parameters considered in this study (**supplementary table 2**, **table 3**). 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 3**). Indeed, it took significantly more time for larvae of LPS mothers to reach the pupal stage than those of control mothers (**figure 20b**).

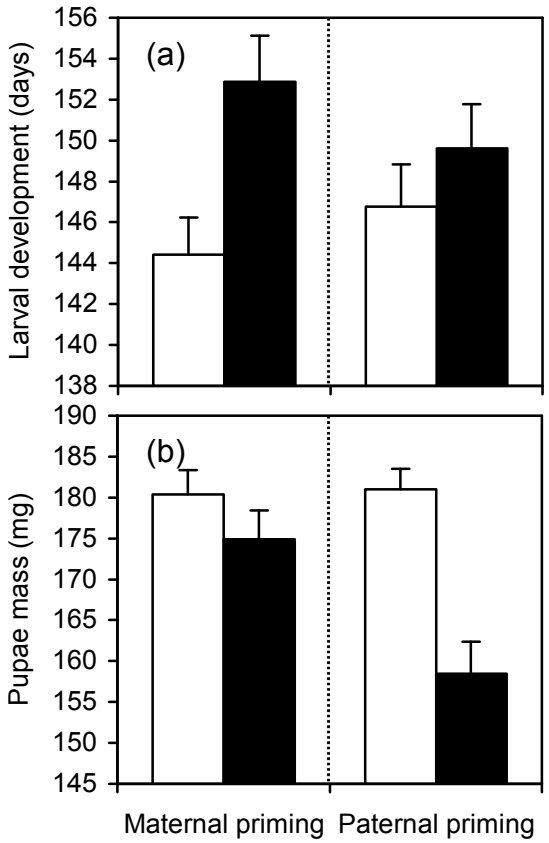


Figure 20 : (a) Larval developmental time from hatching of the eggs to imago (mean + SE) and (b) pupae mass (mean + SE) of offspring in relation to parental immune priming.
Open bars refer to the control parental immune treatment whereas black bars refer to the LPS-immune challenge of the parents.

IV. 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; Freitak et al. 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 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. Since proliferation of haemocytes after infection is limited (Sorrentino et al. 2002), the initial higher concentration of haemocytes may significantly improve the probability of success of the insect□s immune response (Eslin & Prevost, 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 those of Roth et al. (2010) in another beetle species. However, only the offspring produced within the first four 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 regards to the developmental stage to which parents are challenged and the offspring assayed. As suggested by Freitak et al. (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 and Schmid-Hempel (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 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 regards 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 *T. 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 (Moreau et al. 2012). 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 to similar cost patterns found from selection experiments (Kraaijeveld et al. 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 (Tammaru et al. 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 regards to the existence of paternally derived immune priming for offspring (Roth et al. 2010) and fitness associated costs in other insect species (Sadd & Schmid-Hempel 2009; Freitak et al. 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 regards 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).

Chapitre 3

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.

Acknowledgments

We thank B. Bois, C. Bonnet, A. Duroux, A. Pernat, B. Quaglietti, C. Saciat and F. Vogelweith for technical assistance and T. Rigaud for comments. This study was supported by the CNRS and grants from the ANR (ANR-07-JCJC-0134 and ANR-08-JCJC-0006).

Table 2 : 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. N. R. refers to effects not retained by the stepwise procedure. Values $p \leq 0.05$ are given in bold.

	Maternal priming				Paternal priming			
Source	Haemocyte	PO	Total-PO	Antibacterial	Haemocyte	PO	Total-PO	Antibacterial
	Between subjects							
Treat	$F_{1, 74} = 4.59$	$F_{1, 83} = 0.09$	$F_{1, 83} = 1.11$	$F_{1, 84} = 2.18$	$F_{1, 64} = 2.30$	$F_{1, 64} = 1.89$	$F_{1, 64} = 2.43$	$F_{1, 70} = 0.53$
	$\mathbf{p = 0.035}$	$p = 0.760$	$p = 0.296$	$p = 0.143$	$p = 0.134$	$p = 0.174$	$p = 0.124$	$p = 0.471$
Sex	$F_{1, 74} = 1.99$	$F_{1, 83} = 2.64$	$F_{1, 83} = 72.99$	$F_{1, 84} = 5.83$	$F_{1, 64} = 6.42$	$F_{1, 64} = 6.68$	$F_{1, 64} = 10.01$	$F_{1, 70} = 5.44$
	$p = 0.162$	$p = 0.108$	$\mathbf{p = 0.006}$	$\mathbf{p = 0.018}$	$\mathbf{p = 0.014}$	$\mathbf{p = 0.012}$	$\mathbf{p = 0.002}$	$\mathbf{p = 0.023}$
L-rank	$F_{2, 74} = 0.63$	$F_{2, 83} = 0.08$	$F_{2, 83} = 0.04$	$F_{2, 84} = 1.01$	$F_{2, 64} = 0.84$	$F_{2, 64} = 1.51$	$F_{2, 64} = 1.68$	$F_{2, 70} = 0.03$
	$p = 0.538$	$p = 0.927$	$p = 0.963$	$p = 0.369$	$p = 0.434$	$p = 0.228$	$p = 0.194$	$p = 0.971$
Treat *L-rank	N. R.	N. R.	N. R.	N. R.	N. R.	$F_{2, 64} = 2.48$	$F_{2, 64} = 1.52$	N. R.
						$p = 0.092$	$p = 0.227$	

	Within subjects							
Ch	$F_{1, 74} = 39.80$	$F_{1, 83} = 49.64$	$F_{1, 83} = 27.19$	$F_{1, 84} = 173.15$	$F_{1, 64} = 16.18$	$F_{1, 64} = 49.27$	$F_{1, 64} = 54.33$	$F_{1, 70} = 68.66$
	***p* < 0.001**	***p* < 0.001**	***p* < 0.001**	***p* < 0.001**	***p* < 0.001**	***p* < 0.001**	***p* < 0.001**	***p* < 0.001**
Ch*Treat	$F_{1, 74} = 1.09$	$F_{1, 83} = 0.72$	$F_{1, 83} = 0.59$	$F_{1, 84} = 1.15$	$F_{1, 64} = 0.99$	$F_{1, 64} = 2.65$	$F_{1, 64} = 4.01$	$F_{1, 70} = 0.90$
	$p = 0.299$	$p = 0.397$	$p = 0.446$	$p = 0.287$	$p = 0.322$	$p = 0.109$	***p* = 0.050**	$p = 0.346$
Ch*Sex	$F_{1, 74} = 0.01$	$F_{1, 83} = 0.24$	$F_{1, 83} = 2.39$	$F_{1, 84} = 4.64$	$F_{2, 64} = 0.15$	$F_{2, 64} = 4.65$	$F_{2 ,64} = 6.19$	$F_{2, 70} = 6.92$
	$p = 0.950$	$p = 0.622$	$p = 0.126$	***p* = 0.034**	$p = 0.702$	***p* = 0.035**	***p* = 0.015**	***p* = 0.010**
Ch*L-rank	$F_{2, 74} = 0.75$	$F_{2, 83} = 0.17$	$F_{2, 83} = 0.35$	$F_{2, 84} = 1.62$	$F_{1, 64} = 0.36$	$F_{2, 64} = 0.31$	$F_{1, 64} = 5.31$	$F_{1, 70} = 0.05$
	$p = 0.476$	$p = 0.847$	$p = 0.704$	$p = 0.204$	$p = 0.699$	$p = 0.712$	***p* = 0.043**	$p = 0.950$
Ch*Treat*L-rank	N. R.	N. R.	N. R.	N. R.	N. R.	$F_{2, 64} = 4.75$	$F_{2, 64} = 5.79$	N. R.
						***p* = 0.012**	***p* = 0.005**	

Table 3 : 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.
Values $p \leq 0.05$ are given in bold.

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

Discussion

Discussion

Le but de cette thèse était d'identifier l'expression du TTGI à différents stades du développement de *T. molitor*, ainsi que les coûts et les bénéfices qui y étaient associés. Nous avons mis en évidence que la stimulation immunitaire des femelles de la génération parentale induisait le transfert d'activité antibactérienne à leurs œufs, et augmentait la charge hémocytaire basale des descendants adultes. Nous avons vu que la transmission d'activité antibactérienne aux œufs s'accompagnait d'un coût pour les femelles. Nous avons également vu que la stimulation du système immunitaire des mâles de la génération parentale n'induisait pas de transfert d'activité antibactérienne aux œufs, mais induisait une augmentation de l'activité du système PO des descendants adultes. Cependant, cet effet ne persistait pas au-delà des premiers rangs de ponte.

Dans un premier temps, je discuterai de l'existence d'une composante paternelle au TTGI et les mécanismes potentiellement mobilisés lors de sa réalisation. Dans un second temps, je discuterai la nature de l'effet maternel sur la protection des œufs et de la progéniture adulte. Pour terminer, je réaliserai une synthèse de ce que les coûts connus du TTGI nous enseignent sur les pressions de sélection ayant mené à son évolution chez les invertébrés.

Le TTGI : un effet biparental sur l'immunité de la descendance?

Nous avons mis en évidence que la stimulation du système immunitaire des femelles induisait une transmission d'activité antibactérienne aux œufs (**chapitre 1**) et une augmentation de la charge hémocytaire constitutive de la descendance adulte persistant dans tous les rangs de ponte que nous avons observés (**chapitre 3**). En revanche, nous n'avons pas mis en évidence d'effet paternel sur la protection des œufs résultant de la stimulation du système immunitaire des mâles avec du LPS (**chapitre 1**). Par contre, cette même stimulation résultait en l'augmentation de l'activité PO inductible des individus issus des premiers rangs de ponte après la stimulation des mâles (**chapitre 3**). Ces deux chapitres révèlent donc un dimorphisme sexuel de l'investissement dans la protection de la descendance dans un contexte de TTGI.

Discussion

Dans une étude sur *Tribolium castaneum*, Roth et al. (2010) ont montré que la stimulation du système immunitaire des mâles résultait en une protection de leur descendance envers les deux espèces de bactéries qu'ils avaient employées. La protection transmise par les femelles quant à elle était plus efficace envers l'espèce de bactérie qui avait stimulé leur système immunitaire. Dans le **chapitre 3**, nous avions suggéré que ces différences dans l'investissement des deux sexes dans le TTGI puissent provenir du dimorphisme dans les histoires de vie des deux sexes. Plus précisément, nous avions proposé que les mâles puissent avoir plus tendance à disperser que les femelles. En effet, des exemples de dimorphisme sexuel dans le comportement de dispersion existent chez les coléoptères, en faveur des femelles (Dubois et al., 2010) ou des mâles (Lagisz et al., 2010). Si chez *T. molitor*, les mâles sont effectivement le sexe qui disperse le plus, leur infection pourrait ne pas constituer un indice fiable permettant de prédire le risque présent dans l'environnement de leur descendance. Il me semble important de nuancer cette hypothèse, étant donné que *T. molitor* est incapable de voler, ce qui doit limiter ses capacités de dispersion. De plus, la seule étude que j'ai trouvée relatant les capacités de dispersion de *T. molit*or n'a pas reporté de différence entre la performance de dispersion des mâles et des femelles (Jopp, 2006). Il semblerait plutôt que les mâles et les femelles partagent le même environnement que leur progéniture, dans quel cas l'infection des mâles est un indice tout aussi fiable que celle des femelles permettant de prédire la prévalence en pathogènes dans l'environnement de la descendance. Ainsi, on peut se demander pourquoi les mâles n'ont pas évolué un investissement équivalent à celui des femelles en dépit des contraintes physiologiques imposées par l'anisogamie. En effet, des exemples de transmission de substances défensives aux femelles par l'intermédiaire du spermatophore et pouvant être par la suite allouées aux œufs existent (Rossini et al., 2001)

D'autres aspects de l'écologie de *T. molitor* pourraient expliquer le dimorphisme sexuel de l'investissement dans le TTGI. La théorie de la sélection sexuelle prédit que les mâles de *T. molitor* auraient un intérêt supérieur à multiplier leur nombre de partenaires qu'à investir dans la qualité de la progéniture d'une seule femelle (Bateman, 1948 ; Trivers, 1972). De plus, les investissements paternels coûteux ne sont supposés être sélectionnés que chez les espèces dont la paternité du mâle investissant sur la descendance concernée est certaine (Westneat & Sherman, 1993). Du fait des phénomènes de « last-male sperm precedence » et du taux de réaccouplement des femelles chez *T. molitor*, cette certitude est faible (Drnevitch et al. 2001, 2002). Un effet passant par l'intermédiaire du spermatophore serait donc susceptible de profiter à la descendance d'un autre mâle. Ces caractéristiques suggèrent que

l'évolution d'un investissement paternel moins coûteux qu'une transmission directe d'effecteurs immunitaires par l'intermédiaire du spermatophore.

Le TTGI d'origine paternelle pourrait résulter d'une induction par les mâles d'une transmission d'immunité de la part des femelles. Chez certaines espèces, les mâles peuvent provoquer un investissement supérieur de la part des femelles dans un évènement reproducteur par le transfert d'éliciteurs dans le spermatophore (Chapman, 2001). Cet investissement peut sembler moins coûteux qu'une transmission directe d'effecteurs par le mâle, mais serait tout de même susceptible de profiter à la descendance d'un autre mâle du fait de la promiscuité dans laquelle vivent les individus de *T. molitor*. En revanche, la transmission paternelle directe d'une immunocompétence plus élevée aux descendants, au travers d'un effet épigénétique dans l'ADN du sperme par exemple (Jablonka & Lamb, 1995), n'impliquerait pas de synthèse d'éliciteurs ou d'effecteurs coûteuse pour les mâles, et profiterait seulement à la descendance qu'il féconde.

Enfin, l'effet de la stimulation paternelle sur l'immunité de la descendance pourrait provenir d'un ajustement par la femelle de la qualité de sa progéniture en réponse à la perception de l'état d'infection du mâle (Wedell & Karlsson, 2003). En effet, les femelles de *T. molitor* peuvent percevoir l'altération suite à un challenge immunitaire (Worden & Parker, 2005 ; Sadd et al. 2006). Dans ce dernier cas, le caractère transitoire de cet effet paternel indirect s'expliquerait plutôt par la disparition des indices permettant à la femelle de percevoir une infection chez le mâle, étant donné que la plupart des agents infectieux sont éliminés de l'hémocoele de l'hôte dans les trente minutes suivant l'infection (Ochiai & Ashira, 1998 ; Haine et al. 2008b).

Notre protocole ne nous a pas permis de faire la distinction entre ces possibilités. L'injection aux femelles d'extraits de spermatophores de mâles immunostimulés par exemple, couplée à l'observation de l'immunocompétence de leur descendance fécondée par un mâle sain, pourrait nous renseigner sur l'existence d'un éliciteur intégré au spermatophore.

Discussion

Effet maternel : son expression et ses bénéfices potentiels.

Dans le **chapitre 1**, nous avons montré que la stimulation des femelles adultes de *T. molitor* induisait un transfert d'activité antibactérienne interne à leurs œufs. Dans le **chapitre 3**, nous avons montré que cette même stimulation augmentait la charge hémocytaire constitutive de leur descendance adulte. Nous n'avons cependant pas mis en évidence de bénéfice au TTGI chez les jeunes larves dans le **chapitre 2**. Ces études mettent en évidence l'implication d'effecteurs immunitaires différents à deux stades distincts du développement de la progéniture des femelles immunostimulées.

Concernant le TTGI des œufs, le résultat du **chapitre 1** est cohérent avec celui d'une étude précédente ayant reporté un transfert d'activité antibactérienne interne aux œufs de *Bombus terrestris* (Sadd & Schmid-Hempel, 2007). Plusieurs mécanismes pourraient être responsables de cette activité antibactérienne. La femelle pourrait par exemple incorporer des éliciteurs immunitaires dans ses œufs, induisant la synthèse de peptides antibactériens chez l'embryon. Ce mécanisme mobiliserait les ressources de l'embryon même en l'absence de menace pathogénique. La transmission maternelle d'effecteurs immunitaires par contre, tels que des peptides antibactériens ou du lysozyme, aurait l'avantage de conférer une protection précoce à la progéniture tout en évitant les coûts de la mobilisation du système immunitaire à un stade précoce de son développement. Les œufs présents dans les ovaires disséqués de femelles ayant reçu une injection de LPS présentent une activité antibactérienne (Dubuffet, résultats non publiés), ce qui indique que cette dernière ne provient pas d'une synthèse d'effecteurs par l'embryon lui-même, et que le second mécanisme proposé est effectivement celui mobilisé lors du TTGI aux œufs de *T. molitor*. Ainsi, il est probable que cette protection précoce ne représente pas de coût pour la progéniture elle-même, et que seule la femelle subisse un coût à la transmission d'effecteurs qu'elle a synthétisés. Pour confirmer cette hypothèse, il serait tout de même judicieux de rechercher des coûts pour les œufs à leur protection, par exemple en termes de succès à l'éclosion. Pour ce faire, l'emploi d'un moyen non-invasif pour estimer le niveau de protection des œufs est nécessaire, afin de pouvoir le corréler à des mesures de fitness ultérieures, telles que le succès à l'éclosion par exemple.

On peut également se demander si le transfert d'activité antibactérienne aux œufs par les femelles de *T. molitor* les protège de l'intrusion d'un pathogène, ou protège la jeune larve

à l'éclosion. Nos tentatives d'application de *B. thuringiensis* et d'*E. coli* sur des œufs se sont soldées par un échec : le succès à l'éclosion des œufs issus de femelles immunologiquement naïves était aussi élevé qu'ils aient reçu une application de bactéries ou non. Ceci pourrait suggérer que le chorion de l'œuf (très épais chez *T. molitor*, certainement du fait d'une adaptation à la dessication) suffit à empêcher l'intrusion des bactéries. Une application de *M. anisopliae* ou *Beauveria bassiana*, deux champignons entomopathogènes étant capables de franchir le chorion des œufs d'insectes (Gindin et al., 2006) s'est également avérée infructueuse, puisque le succès à l'éclosion des œufs de femelles naïves comme immunostimulées était quasi nul et montrait très peu de variabilité (observations personnelles). Cependant, l'inoculation de bactéries aux œufs semble être une piste à explorer pour metre en évidence un bénéfice à la transmission d'activité antibactérienne interne aux œufs par les femelles immunostimulées. En effet, Gorman et al. (2004) ont démontré que différents paramètres de l'immunité interne aux jeunes œufs *Manduca sexta* pouvait être élicitée par l'inoculation interne de bactéries. Les auteurs ont proposé que cette réaction avait évolué en réponse à la transmission trans-ovarienne de pathogènes, mais elle pourrait également indiquer que les intrusions extérieures de bactéries dans les œufs existent et ont conduit à l'évolution d'un mécanisme de défense interne aux œufs.

Les résultats obtenus dans le **chapitre 2** ne nous ont pas permis de savoir si la protection transmise à l'intérieur des œufs profitait à la jeune larve dès l'éclosion, cependant l'investigation de l'évolution de la quantité d'activité antibactérienne présente dans les œufs après l'ovisposition pourrait nous renseigner sur sa présence dans les jeunes larves.

Le TTGI à la progéniture adulte a également été reporté chez plusieurs espèces (Rahman et al., 2003; Roth et al., 2010), mais l'implication des hémocytes dans son expression n'avait pas été recherchée, à l'exception de l'étude réalisée Freitak et al. (2009). Leurs résultats ont permis de mettre en évidence la surexpression de PRRs (plus précisément, des protéines de reconnaissance des β1-3 glucanes) dans les hémocytes de *T. ni* suite à la stimulation des femelles de la génération parentale. Dans le **chapitre 3**, nous avons mis en évidence la présence d'un TTGI à l'état adulte chez *T. molitor* associé à un temps de développement plus long. La durée de vie des effecteurs maternels incorporés dans les œufs ne doit cependant pas être suffisamment longue pour persister au cours de tous les stades de développement de la progéniture. Il est donc probable que les mécanismes responsables de la protection des œufs et de la protection des descendants à l'âge adulte soient dissociés. Le

résultat du **chapitre 3** suggère une modification des traits d'histoire de vie de la progéniture à long terme, résultant en une plus forte immunocompétence des adultes. Ici encore, plusieurs mécanismes peuvent être proposés pour expliquer ce phénomène.

Un premier mécanisme pourrait provenir de la diminution de l'investissement des femelles dans le provisionnement des œufs par exemple. En effet, chez le coléoptère *Stator pruininus*, les larves écloses d'œufs plus petits se développent plus lentement pour atteindre une taille adulte similaire aux larves écloses d'œufs plus gros (Fox et al., 1999). Nous avons testé cette hypothèse en mesurant les œufs des femelles du **chapitre 1**. Pour chacune de ces femelles, nous avons mesuré huit œufs pondus dans les quatre premiers jours après stimulation. Le traitement maternel n'avait pas d'effet sur le volume des œufs ($\chi^2_{3,62} = 0.41$; $p = 0.52$). Ainsi, la modification des traits d'histoire de vie observée chez les descendants des femelles stimulées ne semble pas attribuable à une diminution de leur investissement dans la qualité générale de leur progéniture.

Un autre mécanisme pourrait provenir d'autres types d'effets maternels susceptibles d'influencer le temps de développement des descendants, tels qu'une modification de l'expression de gènes à effet maternels par exemple. En effet, le développement embryonnaire des insectes est sous contrôle de nombreux gènes à effet maternel, dont les produits d'expression peuvent être incorporés dans les œufs sous forme d'ARN ou de facteurs de transcription (Manseau & Schüpbach, 1989). Certains d'entre eux influencent la durée de développement embryonnaire et post-embryonnaire (Wong et al., 1994). Par ailleurs, le développement embryonnaire et l'immunité empruntent les mêmes récepteurs et voies de transduction de signal (Stein et al., 1998). Il est donc possible que la modification du temps de développement de la progéniture et l'augmentation de son immunocompétence résultent d'un mécanisme commun. Le coût observé en termes de temps de développement de la descendance pourrait donc résulter de la pléiotropie des gènes de l'immunité.

Discussion

Coûts & conditions d'évolution du TTGI chez les insectes.

Le **chapitre 1** suggère que le TTGI aux œufs s'accompagne d'un coût sur la fécondité des femelles. Les femelles subissent donc en plus du coût de la réponse immunitaire (Schmid-Hempel, 2003) le coût de sa transmission. Le **chapitre 3** quant à lui montre un coût pour les descendants à recevoir le TTGI, qui se traduit par une augmentation de son temps de développement. Ces deux résultats montrent un cout à au TTGI subit aussi bien par les femelles que par les descendants. Les coûts recensés au cours des différentes études nous permettent de nous interroger sur les conditions de l'évolution du TTGI chez les insectes.

Dans le **chapitre 1**, nous proposons que l'itéroparité est une condition nécessaire à l'évolution du TTGI, voire même que son occurrence chez une espèce peut favoriser l'évolution de son caractère itéropare. En effet, si un coût énergétique existe à la synthèse (Povey et al. 2009) et/ou à la transmission d'effecteurs immunitaires aux œufs, on pourrait s'attendre à ce que les femelles subissant un fort coût à chaque évènement d'ovisposition (les plus semelpares) ne puissent pas se permettre de subir en plus le coût de la protection de la ponte. Si au contraire le TTGI n'était pas coûteux en énergie, mais plutôt du fait que les substances immunitaires disponibles au sein des femelles soient en quantités limitantes au cours d'un évènement d'oviposition, les femelles les plus semelpares ne protègeraient qu'un très faible nombre d'œufs sur toute leur ponte. Il est donc plus probable que des femelles semelpares aient évolué d'autres effets maternels que le TTGI qui maximisent la fitness de leurs descendants. Ainsi la dispersion vers un site ayant une prévalence en pathogènes moindre pourrait être une méthode alternative au TTGI chez de telles femelles (Meyling & Pell, 2006). Les femelles itéropares quant à elles répartissent leur effort reproducteur sur des épisodes de reproduction plus nombreux, ce qui peut leur permet de subir un coût énergétique moindre à chacun d'entre eux (Miller et al., 2012). Dans le cas où la limitation de la transmission ne provienne pas du coût énergétique lié à la synthèse et/ou à la transmission d'effecteurs ou d'éliciteurs immunitaires, mais plutôt d'une capacité limitée à réaliser cette synthèse au cours d'un évènement d'ovisposition, l'itéroparité leur permettrait également de reconstituer un stock de substances pouvant être transmises aux œufs entre chaque épisode d'oviposition. Cette hypothèse est cohérente avec les connaissances actuelles du TTGI, puisque parmi toutes les espèces d'invertébrés chez lesquelles il a été recherché, il n'a pas été reporté chez les plus semelpares d'entre elles. Les femelles de *A. aegypti* par exemple réalisent

des reproductions relativement « explosives » après la prise alimentaire (entre 100 et 200 œufs à chaque ponte), alors que celles de *T. molitor* pondent entre 5 et 30 œufs tous les deux jours (**tableau 4**).

Dans le **chapitre 3**, le coût révélé pour la descendance à l'expression du TTGI indique qu'il ne procure pas que des bénéfices à la descendance, et qu'il n'évoluera que lorsque ces derniers surpasseront ses coûts. Ce même coût en termes de temps de développement a été retrouvé chez *T. castaneum* après stimulation des femelles avec *E. coli* (Roth et al., 2010). De plus, nous avons mis en évidence dans ce chapitre un investissement supérieur de la descendance dans la part constitutive de l'immunité cellulaire, et non dans la part inductible de l'immunité cellulaire et humorale. Or, l'investissement dans les défenses constitutives en général est supposé être favorisé au cours de l'évolution lorsque la pression exercée par les attaques est constante dans l'environnement de l'espèce hôte (Adler & Karban, 1994). Dans le cas de l'évolution du système immunitaire, la constance de la pression exercée par les pathogènes et les parasites au cours des générations devrait sélectionner un investissement dans l'immunité constitutive plutôt qu'inductible (Dupas et al. 2004). Si ces conditions s'appliquent à l'ajustement plastique de l'immunocompétence d'une génération à une autre, ces arguments confirment que la condition principale ayant conduit à l'évolution du TTGI est la persistance de l'infection vécue par la génération parentale dans l'environnement de la descendance, comme proposé par Sadd & Schmid-Hempel (2005). Encore une fois, cette hypothèse est cohérente avec les connaissances actuelles du TTGI, puisqu'il n'a été trouvé que chez des espèces à générations chevauchantes et envers des pathogènes bactériens ou viraux, c'est-à-dire ayant un taux de réplication rapide et étant susceptibles de persister directement dans l'environnement de a descendance. Son existence n'a par exemple pas été reportée chez *D. melanogaster*, qui disperse après la ponte, alors que *B. terrestris* dont les reines partagent l'habitat de leur progéniture adulte, réalise le TTGI (**tableau 4**).

Discussion

Table 4 : Résumé de différentes caractéristiques des histoires de vies des espèces chez lesquelles le TTGI a été recherché.

Espèce hôte	Type de pathogène/ parasite	Chevauchement des générations de l'espèce hôte	Stabilité de l'environnement	Degré d'itéroparité	Présence de TTGI
Penaeus monodon	virus	+++	+++ (domestique)	+++ (3 à 50 ufs par semaine)	oui (Huang & Song, 1999)
Daphnia magna	bactérie	++	+	+++ (10 larves/ponte)	Oui (Little et al. 2003)
Bombus terrestris	bactérie	+++	+++ (eusocial)	+++ 1000 ufs /6 mois	Oui (Sadd et al., 2005)
Tenebrio molitor	bactérie	++	+++ (domestique)	+++ (300 ufs/1 à 3 mois)	Oui Moret, 2006
Ephestia kuehniella	toxine bactérienne	++	+++ (domestique)	++ (100 à 200 ufs/10 jours)	Oui (Rahman et al., 2003)
Trichoplusia ni	bactérie	+++ (6 à 7 générations sur une plante hôte)	++	+++ (300 à 600 ufs/1 mois)	Oui (Freitak et al., 2009)
Drosophila melanogaster	bactérie	- (disperse)	-	- (Environ 100 ufs/jour)	Non (Linder & Promislow, 2009)
Aedes aegypti	billes de Sephadex	-- (larve aquatique, adulte aérien)	-	-- (100 à 200 ufs/ponte, jusqu'à 5 pontes)	Non (Voordouw et al.,2008)
Tribolium castaneum	bactérie	+++	+++ (domestique)	+++ (400 ufs/5 à 8 mois)	Oui (Roth et al., 2010)
Plodia interpunctella	virus	++	+++ (domestique)	+ (400 ufs/30 jours)	Oui (Tidbury et al., 2011)
Myzus persicae	guêpe parasitoide	+++	++	+++ (80 larves/15 jours)	Non (Vorburger et al., 2008)

Conclusion

Dans le **chapitre 1**, j'ai proposé que le coût existant pour la femelle à la protection de ses œufs restreigne l'expression du TTGI aux espèces itéropares. Or, j'ai proposé dans la partie précédente de la discussion que la protection des œufs ne résultait pas des mêmes mécanismes qui conduisent à la protection de la progéniture à l'état adulte. Dans ce cas, si seul le TTGI aux œufs est coûteux pour les femelles, l'absence de protection des œufs chez les espèces semelpares ne devrait pas empêcher l'expression d'un TTGI chez les adultes de leur descendance. Cependant, les espèces semelpares ne présentent pas une histoire de vie compatible avec l'expression du TTGI en général. En effet, les espèces semelpares subissent un fort coût à la reproduction à chaque évènement reproducteur (Partridge et al., 1987 ; Dao et al., 2010), qui compromet leur longévité par rapport aux espèces plus itéropares (Hautekèete et al. 2001). Ainsi, leurs chances de partager l'environnement de leur descendance sont réduites, et l'infection des femelles semelpares pourrait ne pas être un bon indicateur du risque d'infection existant dans l'environnement de la descendance. L'expression du TTGI ne semble donc pas associée à des effecteurs définis, tels que les anticorps des vertébrés gnathostomes. Son évolution est plutôt associée à un ensemble de traits d'histoire de vie interdépendants, qui favorisent la persistance du risque infectieux de l'environnement de la génération parentale dans l'environnement de la descendance, et l'émergence d'investissements maternels coûteux.

Références

Références

Abdel-latief, M., Hilker, M. 2008 Innate immunity: eggs of *Manduca sexta* are able to respond to parasitism by *Trichogramma evanescens*. *Insect Biochemistry and Molecular Biology* **38**, 136-145.

Adler, F.R., Karban, R. 1994 Defended fortresses or moving targets? Another model of inducible defenses inspired by military metaphors. *American Naturalist* **144**, 813-832.

Agnew, P., Koella, J.C., Michalakis, Y. 2000 Host life-history responses to parasitism. *Microbes and Infection* **2**, 891-896.

Ahearn, G.A. 1970 The control of water loss in desert Tenebrionid beetles. *Journal of Experimental Biology* **53**, 573-595.

Alcock, J. 1994 Postinsemination associations between males and females in insects: the mate-guarding hypothesis. *Annual Review of Entomology* **39**, 1-21.

Anderson, R.M., May, R.M. 1981 The population dynamics of microparasites and their invertebrate hosts. *Philosophical Transactions of the Royal Society B*. **314**,533-570.

Bateman, 1948 Intra-sexual selection in *Drosophila*. *Heredity* **2**, 359-368.

Bates, D., Maechler, M. 2010 lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-34.

Bell, G. 1980 The costs of reproduction and their consequences. *The American Naturalist* **116**, 45-76.

Benton, T.G., Plaistow, S.J., Beckerman, A.P., Lapsley, C.T., Litlejohns, S. 2005 Changes in maternal investment in eggs can affect population dynamics. *Proceedings of the Royal Society of London B*. **272**, 1351-1356.

Benrey, B., Denno, R.F. 1997 The slow-growth-high-mortality hypothesis: a test using the cabbage butterfly. *Ecology* **78**, 987-999.

Bernardo, J. 1996 Maternal effects in animal ecology. *Integrative and Comparative Biology* **36**, 83-105.

Bertin, S., Scolari, F., Guglielmino, C.R., Bonizzoni, M., Bonomi, A., Marchini, D., Gomulski, L.M., Gasperi, G., Malacrida, A.R., Matessi, C. 2010 Sperm storage and use in polyandrous females of the globally invasive fruitfly, *Ceratitis capitata*. *Journal of Insect Physiology* **56**, 1542-1551.

Blaakmeer, A. Stork, A., van Veldhuizen, A., van Beek, T.A., de Groot, A. 1994 Isolation, identification, and synthesis of miriamids, new hostmarkers from eggs of *Pieris brassicae*. *Journal of Natural* Products **57**, 90-99

Boggs, C.L., Gilbert, L.E. 1979 Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. *Science* **206**, 83-84.

Boots, M., Begon, M. 1993 Trade-offs with resistance to a granulosis-virus in the Indian meal moth, examined by a laboratory evolution experiment. *Functional Ecology* **7**, 528-534.

Carton, Y., Nappi, A.J. 1997 Drosophila cellular immunity against paraitoids. *Parasitology Today* **13**, 218-227.

Chapman, T. 2001 Seminal-fluid mediated fitness traits in *Drosophila*. *Heredity* **87**, 511-521.

Chiang, A.S., Yen, D.F., Peng, W.K. 1986 Defense reaction of midgut epithelial cells int he rice moth larva (*Corcyra cephalonica*) infected with *Bacillus thuringiensis*. *Journal of Invertebrate Pathology* **47**, 333-339.

Collins, F.H., Sakai, R.K., Vernick, K.D., Paskewitz, S., Seeley, D.C., Miller, L.H., Collins, W.E., Campbell, C.C., Gwadz, R.W. 1986 Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science* **234**, 607-610.

Czesak, M.E., Fox, C.W. 2003 Evolutionary ecology of egg size and number in a deed beetle: genetic trade-off differs between environments. *Evolution* **57**, 1121-1132.

Dao, A., Kassogue, Y., Adamou, A., Diallo, M., Yaro, A.S., Traore, S.F., Lehmann, T. 2010 Reproduction-longevity trade-off in *Anopheles gambiae* (Diptera: Culicidae). *Journal of Medical Entomology* **47**, 769-777.

Deas, J.B., Hunter, M.S. 2012 Mothers modify eggs into shields to protect offspring from parasitism. *Proceedings of the Royal Society B*. **279**, 847-853.

Desouhant, 1998 Selection of fruits for ovisposition by the chestnut weevil *Curculio elephas*. *Entomologia Experimentalis et Applicata* **86**, 71-78.

Drnevitch, J.M., Papke, R.S., Rauser, C.L., Rutowski, R.L. 2001 Material benefits from multiple matings in female mealworm beetle (*Tenebrio molitor* L.). *Journal of Insect Behavior* **14**, 215-230.

Drnevitch, J.M. 2002 Number of mating males and mating interval affects last-male sperm precedence in *Tenebrio molitor* L. *Animal Behaviour* **66**, 349-357.

Dubois, G.F., Le Gouar, P.J., Delettre, Y.R., Brustel, H., Vernon, P. 2010 Sex-biased and condition dependent dispersal capacity in the endangered saprophytic beetle *Osmoderma eremite* (Coleoptera: Cetoniidae). *Journal of Insect Conservation* **14**, 679-687.

Dugaw, C.J. 2004 Seasonally limited host supply generates microparasite populaiton cycles. *Bulletin of Mathematical Biology* **66**, 583-594.

Duneau, D., Ebert, D. 2012 The role of moulting in parasite defense. *Proceedings of the Royal Society B*. **279,** 3049-3054.

Dupas, S., Morand, S., Eslin, P. 2004 Evolution of hemocyte concentration in the *melanogaster* subgroup species. *Comptes Rendus Biologies* **327**, 139-147.

Eisner, T., Attygale, A.B., Conner, W.E., Eisner, M., MacLeod, E., Meinwald, J. 1996 Chemical egg defence in a green lacewing (*Ceraeochrysa smithi*). *Proceedings of the National Academy of Sciences of the United States of America* **93**, 3280-3283.

Eisner, T., Rossini, C., Gonzàlez, A., Iyengar, V.K., Siegler, M.V.S., Smedley, S. 2002 Parental investment in egg defense. In *Chemoecology of insect eggs and egg deposition*. Ed: Hilker, M., Meiners, T. Blackwell Publishing pp 91-116.

Eslin, P. & Prévost, G. 1998 Hemocyte load and immune resistance to *Asobara tabida* are correlated in species of the *Drosophila melanogaster* subgroup. *Journal of Insect Physiology* **44**, 807□816.

Falconer, D.S., Mackay, T.F.C. 1996 *Introduction to Quantitative Genetics*. Longman Group Ltd, Essex, UK.

Fargues, J., Robert, P-H., Vermeil de Conchard, H. 1985 Persistance des conidiospores des hyphomycètes entomopathogènes *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metsch.) Sor., *Nomuraea rileyi* (F.) Samson et *Paecilomyces fumoso-roseus* Wize dans le sol, en conditions contrôlées. *Agronomie* **5**, 73-80.

Fellowes, M. D. E., Kraaijeveld, A. R. & Godfray, H. C. J. 1999 Association between feeding rate and defence against parasitoids in Drosophila melanogaster. *Evolution* **53**, 1302-1305.

Fox, C.W. 1993 The influence of maternal age and mating frequency on egg size and offspring performance in *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Oecologia* **96**, 139-146.

Fox, C.W., Czesak, M.E., Savalli, U.M. 1999 Environmentally based maternal effects on developmental time in the seed beetle *Stator pruininus* (Coleoptera: Bruchidae): consequences of larval density. *Environmental Entomology* **28**, 217-223.

Fox, C.W., Czesak, M.E. 2000 Evolutionary ecology of progeny size in arthropods. *Annual Review of Entomology* **45**, 341-369.

Freitak, D., Heckel, D.G., Vogel, H. 2009 Dietary-dependent trans-generational immune priming in an insect herbivore. *Proceedings of the Royal Society B*. **276**, 2617-2624.

Galloway, L.F. 2000 The effect of maternal and paternal environments on seed characters in the herbaceous plant *Campanula americana* (Campanulacae). *American Journal of Botany* **88**, 832-840.

Gibbs, M., Breuker, C.J., Van Dyck, H. 2010 Flight during oviposition reduces maternal egg provisioning and influences offspring development in *Pararge aegeria (L.)*. *Physiological Entomology* **35**, 29-39.

Gindin, G., Levski, S., Glazer, I., Soroker, V. 2006 Evaluation of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* against the Red Palm Weevil *Rhynchophorus ferrugineus*. *Phytoparasitica* **34**, 370-379.

Gorman, M.J., Kankanala, P., Kanost, M.R. 2004 Bacterial challenge stimulates innate immune responses in extra-embryonic tissues of tobacco hornworm eggs. *Insect Molecular Biology* **13**, 19-24.

Grindstaff, J.L., Brodie III, E.D., Ketterson, E.D. 2003 Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proceedings of the Royal Society B.* **270**, 2309-2319.

Haine, E.R., Pollitt L., Moret Y., Siva-Jothy M.T., Rolff J. 2008a Temporal patterns in immune responses to a range of microbial insults (*Tenebrio molitor*). *Journal of Insect Physiology* **54**, 1090-1097.

Haine, E. R., Moret, Y., Siva-Jothy, M. T. & Rolff, J. 2008b Antimicrobial defense and persistent infection in Insects. *Science* **322**, 1257 □1259.

Harris, W.E., Uller, T. 2009 Reproductive investment when mate quality varies: differential allocation versus fecundity compensation. *Proceedings of the Royal Society B*. **364**, 1039-1048.

Harvell, C.D. The ecology and evolution of inducible defenses. *Quarterly Review of Biology* **65**, 323-340.

Hasselquist, D., Nilsson, J. 2009 Maternal transfert of antibodies in vertebrates: trans-generational effects on offspring immunity. *Philosophical Transactions of the Royal Society B*. **364**, 51-60.

Hautekeète, N-C., Piquot, Y., Van Dijk, H. 2001 Investment in survival and reproduction along a semelparity-iteroparity gradient in the *Beta* species complex. *Journal of Evolutionary Biology* **14**-795-804.

Hauton, C., Smith, V.J. 2007 Adaptive immunity in invertebrates: a straw house without a mechanistic foundation. *BioEssays* **29**, 1138-1146.

Herzner, G., Engl, T., Strohm, E. Cryptic combat against competing microbes is a costly component of parental care in a digger wasp. *Animal Behaviour* **82**, 321-328.

Hoffmann, J.A. 1995 Innate immunity of insects. *Current Opinion in Immunology* **7**, 4-10.

Hoffman, J.A. 2003 The immune response of *Drosophila*. *Nature* **426**, 33-38.

Holz, A., Bossinger, B., Strasser, T., Janning, W. & Klapper, R. 2003 The two origins of hemocytes in *Drosophila*. *Development* **130**, 4955-4962.

Horohov, D.W., Dunn, P.E. 1983 Phagocytosis and nodule formation by hemocytes of *Manduca sexta* following injection of *Pseudomonas aeruginosa*. *Journal of Invertebrate Pathology* **41**, 203-213.

Howard, R.S. 1955 The biology of the grain beetle *Tenebrio molitor* with particular reference to its behaviour. *Ecology* **36**, 262-269.

Huang, C.C, Song, Y.L. 1999 Maternal transmission of immunity to white-spot syndrome associated virus (WSSV) in shrimp (*Penaeus monodon*). *Developmental and Comparative Immunology* **27**, 545-552.

Hultmark, D. 1996 Insect lysozymes. In *Lysozymes: Model Enzymes in Biochemistry and Biology*. Jolès, P. (Ed.)

Hunt, J., Simmons, L.W. 2007 Maternal and paternal effects on offspring phenotype in the dung beetle *Ontaphagus taurus*. *Evolution* **54**, 936-941.

Hurd, H., Fogo, S. 1991 Changes induced by *Hymenolepis diminuta* (Cestoda) in the behaviour of the intermediate host *Tenebrio molitor* (Coleoptera). *Canadian Journal of Zoology* **69**, 2291-2294.

Imler, J.L., Tauszig, S., Jouanguy, E., Forestier, C., Hoffman, J.A. 2000 LPS-induced innate immune response in *Drosophila*. *Journal of Endotoxin Research* **6**, 459-462.

Ichikawa, T. & Kurauchi, T. 2009 Larval cannibalism and pupal defense against cannibalism in two species of Tenebrionid beetles. *Zoological Science* **26**,525-529.

Islam, M.S., Roessingh, P., Simpson, S.J., McCaffery, A.R. 1994 Parental effects on the behaviour and colouration of nymphs of the desert locust *Schistocerca gregaria*. *Journal of Insect Physiology* **40**, 173-181.

Jablonka, E., Raz, G. 2009 Transgenerational epigenetic inheritance : prevalence, mechanisms, and implications for the study of heredity and evolution. *Quarterly Review of Biology* **84**, 131-176.

Jacobs, S.B. 1988 Entomological notes of the Department of Entomology of the Penn State University.

Jokela, J. 2010 Trangenerational immune priming as a cryptic parental care. *Journal of Animal Ecology* **79**, 305-307.

Jopp, F. 2006 The impact of local spatial resistance on the movement behaviour of *Tenebrio molitor* L. *Central European Journal of Biology* **3**, 412-429.

Kato, Y., Motoi, Y., Taniai, K., Kadono-Okuda, K., Hiramatsu, M. & Yamakawa, M. 1994 Clearance of lipopolysaccharide in the haemolymph of *Bombyx mori*: its role in the termination of cecropin mRNA induction. *Insect Biochemistry and Molecular Biology* **24**, 539-545.

Klein, J. 1997 Homology between immune responses in vertebrates and invertebrates: does it exist? *Scandinavian Journal of immunology* **46**, 558-564.

Koella, J. C. & Boëte, C. 2002 A genetic correlation between age at pupation and melanization immune response of the yellow fever mosquito *Aedes aegypti*. *Evolution* **56**, 1074-1079.

Kotiaho, J.S., Simmons, L.W., Hunt, J., Tomkins, J.L. 2003 Males influence maternal effects that promote sexual selection: a quantitative genetics experiment with dung beetles *Ontophagus taurus*. *The American Naturalist* **161**, 852-859.

Kraaijeveld, A. R., Limentani, E. C. & Godfray, H. C. J. 2000 Basis of the trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. *Proceedings of the Royal Society B*. **268**, 259-261.

Lagisz, M., Wolff, K., Sanderson, R.A., Laskowski, R. 2010 Genetic population structure of the ground beetle, *Pterostichus oblongopunctatus*, inhabiting a fragmented and polluted landscape: Evidence for sex-biased dispersal. *Journal of Insect Science* **10**:105

LaMunyon, C.W. 1994 Paternity in naturally-occurring *Utetheisa ornatrix* (Lepidoptera : Arctiidae) as estimated using enzyme polymorphism. *Behavioral Ecology and Sociobiology* **34**, 403-408.

LaMunyon, C.W., Eisner, T. 1994 Spermatophore size as determinant of paternity in an arctiid moth (*Utetheisa ornatrix*). *Proceedings of the National Academy of Sciences of the United States of America* **91**, 7081-7084.

Lavine, M.D., Strand, M.R. 2002 Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology* **32**, 1295-1309.

Leclerc, V., Pelte, N., El Chamy, L., Martinelli, C., Lygoxygakis, P., Hoffman, J.A., Reichhart, J. 2006 Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila melanogaster*. *EMBO reports* **7**, 231-235.

Lemaître, B., Reichhart, J.-M., Hoffman, J.A. 1997 *Drosophila* host defense, differential induction of antimicrobial peptides genes after infection by various classes of microorganisms. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 14614□14619.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., Lee, W.J., Mengin-Lecreulx, D., Lemaitre B. 2003 The Drosophila immune system detects bacteria through specific peptidoglycan recognition. *Nature Immunology* **4**, 478-484.

Levinson, H., Levinson, A. 1994 Origin of grain storage species consuming dessicated food. *Anzeiger für Schlädlingskunde* **67**, 47-59.

Linder, J.E., Promislow, D.E.L. 2009 Cross-generational fitness effects of infection in *Drosophila melanogaster*. *Fly* **3**, 143-150.

Little, T. J., Kraaijeveld, A. R. 2004 Ecological and evolutionary implications of immunological priming in invertebrates. *Trends in Ecology and Evolution* **19**, 58-60.

Loudon, C. 1988 Development of *Tenebrio molitor* in low oxygen levels. *Journal of Insect Physiology* **34**, 97-103.

Loye, J.E., Zuk, M. 1991 *Bird-parasite interactions*. *Ecology, evolution and behaviour*. Oxford university Press, Oxford.

Manseau, L.J., Schüpbach, T 1989 Cappuccino and spire: two unique maternal-effect loci required for both the anteroposterior and the dorsoventral patterns of the *Drosophila* embryo. *Genes and Development* **3**, 1437-1452.

Marchini, D., Manetti, A.G., Rosetto, M., Bernini, L.F., Telford, J.L., Baldari, C.T., Dallai, R. 1995 cDNA sequence and expression of the ceratotoxin gene encoding an antibacterial sex-specific peptide from the medfly *Ceratitis capitata* (Diptera). *Journal of Biological Chemistry* **270**, 6199-6204.

Marchini, D., Marri, L., Rosetto, M., Manetti, A.G.O., Dallai, R. 1997 Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata*. *Biochemical and Biophysical Research Communications* **240**,657-663.

Marshall, D.J., Uller, T. 2007 When is a maternal effect adaptive? *Oikos* **116**, 1957-1963.

Messina, F.J.**,** Fox, C.W. 2001 Offspring size and number. In: *Evolutionary Ecology: Concepts and Case Studies*. Eds. Fox, C.W., Roff, D.A., Fairbairn, D.J. pp. 113-127. *Oxford University Press*, New York.

Meyling, N.V., Pell, J.K. 2006 Detection and avoidance of an entomopathogenic fungus by a generalist insect predator. *Ecological Entomology* **31**, 162-171.

Michalakis, Y. Hochberg, M.E. 1994 Parasitic effects on host life-history traits - a review of recent studies. *Parasite* **1**, 291-294.

Mikasa, K. 1992 Quantitative genetic-study on sexual difference in emigration behavior of *Drosophila melanogaster* in a natural population. *Japanese Journal of Genetics* **67**, 463□472.

Miller, G.A., Pell, J.K., Simpson, S.J. 2009 Crowded locusts produce hatchlings vulnerable to a fungal attack. *Biology Letters* **5**, 845-848.

Miller, T.E.X., Williams, J.L., Jongejans, E., Brys, R., Jacquemyn, H. 2010 Evolutionary demography of iteroparous plants: incorporating non-lethal costs of reproduction into integral projection models. *Proceedings of the Royal Society B*. doi: 10.1098/rspb.2012.0326

Minchella, D.J., 1985 Host life-history variation in response to parasitism. *Parasitology* **90**, 205-216.

Mitchell, S.E., Read, A.F. 2005 Poor maternal environment enhances offspring resistance in an invertebrate. *Proceedings of the Royal Society B*. **272**, 2601-2607.

Molleman, F., Zwaan, B.J., Brakerfield, P.M. 2004 The effect of male sodium diet and mating history on female reproduction in the puddling squinting bush brown *Bicyclus anynana* (Lepidoptera). *Behavioural Ecology and Sociobiology* **56**, 404-411.

Morag, N. Keasar, T., Harari, A., Bouskila, A. 2011 Trans-generational effects of maternal rearing density on offspring development time in a parasitoid wasp. *Physiological Entomology* **36**, 294-298.

Moreau, J., Martinaud, G., Troussard, J-P., Zanchi, C., Moret, Y. 2012 Trans-generational immune priming is constrained by the maternal immune response in an insect. *Oikos* **121**, 1828-1832.

Moret, Y., Schmid-Hempel, P. 2000 Survival for immunity: Activation of the immune system has a price for bumblebee workers. *Science* **290**, 1166-1168.

Moret, Y., Siva-Jothy, M.T. 2003 Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. *Proceedings of the Royal society B*. **270**, 2475-2480

Moret, Y. 2006 Trans-generational immune priming specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*. *Proceedings of the Royal Society B*. **273**, 1399-1405.

Mousseau, T.A., Dingle, H. 1991 Maternal effects in insect life histories. *Annual Review of Entomology* **36**, 511-534.

Mousseau, T.A., Fox, C.W. 1998a The adaptive significance of maternal effects. *Trends in Ecology and Evolution* **13**, 403-407.

Mousseau, T. A. & Fox, C. W. 1998b *Maternal effects as adaptations*. Oxford University Press, New York.

Moya-Laraño, J. 2002 Senescence and food limitation in a slowly ageing spider. *Functional Ecology* **16**, 734-741.

Nguyen-Huu, T., Lett, C., Auger, P., Poggiale, J-C. 2006 Spatial synchrony in host-parasitoïd models using aggregation of variables. *Mathematical Biology* **203**, 204-221.

van Noordwijk, A.J. & de Jong, G. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. *American Naturalist* **128**, 137-142.

Ohgushi, T. 1996 A reproductive effort in a herbivorous lady beetle: egg resorption and female survival. *Oecologia* **106**, 345-351.

Park, T., Mertz, D.B., Grodzinsky, W., Prus, T. 1965 Cannibalistic predation in populations of flour beetles. *Physiological Zoology* **38**, 289-321.

Partridge L, Green A, Fowler K. 1987 Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. *Journal of Insect Physiology* **33,**745 749.

Pech, L.L., Strand, M.R. 1996 Granular cells are required for encapsulation of foreign targets by insect haemocytes. *Journal of Cell Science* **109**, 2053-2060.

Pinheiro, J., Bates,D., DebRoy, S., Sarkar, D. 2012 Linear and Nonlinear Mixed Effects Models. R package version 3.1-105.

Pham, N. L., Dionne, M. S. Shirazu-Hiza, M. & Schneider, D. S. 2007 A specific primed immune response in *Drosophila* is dependent on phagocytes. *PLoS Pathogens* **3** : 26.

Pliske, T.E. 1973 Factors determining mating frequecies in some New World butterflies and skippers. *Annals of the Entomological Society of America* **66**, 164-169.

Povey, S., Cotter, S.C., Simpson, S.J., Lee, K.P., Wilson, K. 2009 Can the protein costs of bacterial resistance be offset by altered feeding behaviour? *Journal of Animal Ecology* **78**, 437-446.

Punzo, F., Mutchmor, J.A. Effects of temperature, reltive humidity and period of exposure on the survival capacity of Tenebrio molitor (Coleoptera: Tenebrionidae).*Journal of the Kansas Entomological Society* **53**, 260-270.

Rahman, M.M., Roberts, H.L.S, Sarjan, M., Asgari, S., Schmidt, O. 2003 Induction and transmission of *Bacillus thuringiensis* tolerance in the flour moth *Ephestia kuehniella*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 2696-2699.

Ramsay, J.A. 1964 The rectal complex of the mealworm *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). *Philosophical Transactions of the Royal Society of London B* **248**, 279-314.

Ratcliffe, N.A., Gegen, S.J. 1976 Cellular defense reactions of insect hemocytes in vivo: nodule formation and development in *Galleria melonella* and *Pieris brassicae* larvae. *Journal of Invertbrate Pathology* **28**, 376-382.

Ratcliffe, N.A., Rowley, A.F. 1979 Role of haemocytes in defense against biological agents. In: *Insect Haemocytes: Development, Forms, Functions and Techniques*. Ed: Gupta, A.P. Cambridge University Press, Cambridge, pp. 331-414.

Ratcliffe, N.A., Brookman, J.L., Rowley, A.F. 1991 Activation of the phenoloxidase cascade and initiation of nuodule formation in locusts by bacterial lipopolysaccharides. *Developmental and Comparative Immunology* **15**, 33-39.

R Development Core Team. 2011. R: A language and environment for statistical computing. Vienna, Austria.

Reiter, P 1996 Oviposition and dispersion of *Aedes aegypti* in an urban environment. *Bulletin de la Société de Pathologie Exotique* 89, 120-122.

Rheins, L.A., Karp, R. D. 1985 Effect of gender on the inducible humoral response to honey-bee venom in the American cockroach (*Periplaneta americana*). *Developmental and Comparative Immunology* **9**, 41-49.

Richter, M.Y., Jakobsen, H., Haeuw, J-F., Power, U.F., Jonsdottir, I. 2005 Protective levels of polysaccharide-specific maternal antibodies may enhance the immune response elicited by pneumococcal conjugates in neonatal and infant mice. *Infection and Immunity* **73**, 956-964.

Rivero, A. 2006 Nitric oxide: an antiparasitic molecule of invertebrates. *Trends in Parasitology* **22**, 219-225.

Roff, D.A. 1992 The Evolution of Life Histories. *Chapman and Hall*, New York.

Rosengaus, R.B., Cornelisse, T., Guschanski, K., Traniello, J.F.A. 2007 Inducible immune proteins in the dampwood termite *Zootermopsis angusticollis*. *Naturwissenschaften* **94**, 25-33.

Rossini, C., González, A., Eisner, T. 2001 Fate of an alkaloidal nuptial gift in the moth *Utetheisa ornatrix*: systemic allocation for defense of self by the receiving male. *Journal of Insect Physiology* **47**, 639-647.

Rossiter, M.C. 1996 Incidence and consequences of inherited environmental effects. *Annual Review of Ecology and Systematics* **27**, 451–476.

Roth, O., Kurtz, J. 2008. The stimulation of immune defence accelerates development in the red flour beetle (*Tribolium castaneum*). *Journal of Evolutionary Biology* **21**, 1703–1710.

Roth, O., Kurtz, J. 2009 Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse *Porcellio scaber* (Crustacea: Isopoda). *Developmental and Comparative Immunology* **33**, 1151-1155.

Roth, O., Joop, G., Eggert, H., Hilbert, J., Daniel, J., Schmid-Hempel, P., Kurtz, J. 2010 Paternally derived immune priming for offspring in the red flour beetle, *Tribolium castaneum*. *Journal of Animal Ecology* **79**, 403-413.

Sadd, B.M., Kleinlogel, Y., Schmid-Hempel, R., Schmid-Hempel, P. 2005 Trans-generational immune priming in a social insect. *Biology Letters* **1**,386-388.

Sadd, B.M., Schmid-Hempel, P. 2007 Facultative but persistent trans-generational immunity via the mother's eggs in bumblebees. *Current Biology* **17**, R1046-1047

Sadd, B.M., Schmid-Hempel, P. 2009 A distinct infection cost associated with trans-generational immune priming of antibacterial immunity in bumblebees. *Biology Letters* **5**, 798-801.

Schnitger, A.K.D., Kafatos, F.C., Osta, M.A. 2007 The melanisation reaction is not required for survival of *Anopheles gambiae* mosquitoes after bacterial infections. *The Journal of Biochemical Chemistry* **282**, 21884-21888.

Scott, M. 1989 Male parental care and reproductive success in the burying beetle, *Nicrophorus orbicollis*. *Journal of Insect Behavior* **2**, 133-137.

Schlechter-Helas, J., Schmitt, T., Peschke, H. 2011 A contact anti-aphrodisiac pheromone supplied by the spermatophore in the rove beetle *Aleochara curtula*: mode of transfer and evolutionary significance. *Naturwissenschaften* **10**, 855-862.

Schmid-Hempel, P. 2003 Variation in immune defence as a question of evolutionary ecology. *Proceedings of the Royal Society B.* **270**, 357-366.

Scholte, E-J., Knols, B.G.J., Takken, W. 2004 Autodissemination of the entomopathogenic fungus *Metarhizium anisopliae* amongst adults of the malaria vector *Anopheles gambia*e s.s. *Malaria Journal* **3**: 45.

Schuler, M.S., Orrock, J.L. 2012 The maladaptive significance of maternal effects for plants in anthropogenically modified environments. *Evolutionary Ecology* **26**, 475-481.

Sideri, M., Tsakas, S., Markoutsa, E., Lampropoulou, M., Marmaras, V.J. 2008 Innate immunity in insects : surface-associated dopa decarboxylase-dependent pathways regulate phagocytosis, nodulation and melanisation in medfly haemocytes. *Immunology* **123**, 528-537.

Simmons, L.W. 1990 Nuptial feeding in tettigoniids: male costs and rates of fecundity increase. *Behavioural Ecology and Socibiology* **27**, 43-47.

Simmons, L.W. 2001 *Sperm competition and its evolutionary consequences in the insects*. Princeton University Press.

Siva-Jothy, M.T., Moret, Y., Rolff, J. 2005 Insect immunity: an evolutionary ecology perspective. *Advances in Insect Physiology* **32**, 1-48.

Slobodchikoff, C.N., Wismann, K. 1981 A function of the subelytral chamber of Tenebrionid beetles. *Journal of Experimental Biology* **90**, 109-114.

Söderhäll, K., Cerenius L. 1998 Role of the prophenoloxidase-activating system in invertebrate immunity. *Curent Opinion in Immunology* **10**, 23-28.

Sorrentino, R. P., Carton, Y. & Govind, S. 2002 Cellular immune response to parasite infection in the Drosophila lymph gland is developmentally regulated. *Developmental Biology* **243**, 65-80.

Stearns, S.C. 1992 *The evolution of life histories*. Oxford University Press, Oxford.

Stein, D., Goltz, J.S., Jurcsak, J., Stevens, L. 1998 The Dorsal-related immunity factor (Dif) can define the dorsal-ventral polarity in the *Drosophila* embryo. *Development* **125**, 2159-2169.

Stevenson, I.L. 1961 Growth studies on *Arthrobacter globiformis*. *Canadian Journal of Microbiology* **7**, 569-575.

Srygley, R.B. 2012 Ontogenic changes in immunity and susceptibility to fungal infection in Mormon crickets *Anabrus simplex*. *Journal of Insect Physiology* **58**, 342-347.

Tammaru, T., Esperk, T. & Castellanos, I. 2002 No evidence for costs of being large in females of *Orgyia* spp. (Lepidoptera, Lymantriidae): larger is always better. *Oecologia* 133, 430-438.

Theopold, U., Rissler, M., Fabbri, M., Schmidt, O., Natori, S. 1999 Insect glycobiology: a lectin multigene family in *Drosophila melanogaster*. *Biochemical and Biophysical Research Communications* **261**, 923-927.

Therneau, T. 2012 Mixed Effects Cox Models. R package version 2. 2-3.

Thompson, J.N. and Pellmyr, O. 1991. Evolution of oviposition behaviour and host preference in Lepidoptera. *Annual Review of Entomology* **36**, 65□89.

Thornhill, R., Alcock, J. 1983 *The evolution of insect mating systems*. Harvard Press pp 63-67.

Tidbury, H.J., Pedersen, A.B., Boots, M. 2011 Within and trans-generational immune priming in an insect to a DNA virus. *Proceedings of the royal Society B*. **278**, 871-876.

Trivers, 1972 Parental investment and sexual selection. In *Sexual selection and the descent of man*, pp. 136–179. Ed: B. Campbell, Chicago, IL.

Tschinkel, W.R., Wilson, C.D. 1971 Inhibition of pupation due to crowding in some Tenebrionid beetles. *Journal of Experimental Zoology* **176**, 137-145.

Valle, D. 1993 Vitellogenesis in insects and other groups: a review. *Memoirs of the Institute Oswaldo Cruz* **88**, 1-26.

Voordouw, M.J., Lambrechts, L., Koella, J. 2008 No maternal effects after stimulation of the melanisation response in the yellow fever mosquito *Aedes aegypti*. *Oikos* **117**, 1269-1279.

Vey, A. 1977 Histological and ultrastructural studies of *Beauveria bassiana* infection in *Leptinotarsa decemlineta* larvae during ecdysis. *Journal of Invertebrate Pathology* **30**, 207-215.

Vey, A., Fargues, J., Robert, P. 1982 Histological and ultrastructural studies of factors determining the specificity of pathotypes of the fungus *Metarhizium anisopliae* for scarabeid larvae. *Entomophaga* **27**, 387-397.

Voordouw, M.J., Lambrechts, L., Koella, J. 2008 No maternal effects after stimulation of the melanisation response in the yellow fever mosquito *Aedes aegypti*. *Oikos* **117**, 1269-1279.

Vorburger, C., Gegenschatz, S.E., Ranieri, G., Rodriguez, P. 2008 Limited scope for maternal effects in aphid defence against parasitoids. *Ecological Entomology* **33**, 189-196.

Wedell, N. 1996 Male quality affects reproductive effort in a paternally investing species. *The American Naturalist* **148**, 1075–1088.

Westneat, D.F., Sherman, P.W. 1993 Parentage and the evolution of parental behavior. *Behavioural Ecology* **4**, 66-77.

Wong, A., Boutis, P., Hekimi, S. 1994 Mutations in the *clk-1* gene of *Caenorhabditis elegans* affect developmental and behavioural timing. *Genetics* **139**, 1247-1259.

Worden, B.D., Parker, P.G., Pappas, P.W. 1999 Parasites reduce attractiveness in grain beetles. *Animal Behaviour* **59**, 543-550.

Worden, B.D., Parker, P.G. 2001 Polyandry in grain beetles, *Tenebrio molitor*, leads to greater reproductive success: material or genetic benefits? *Behavioral Ecology* **12**, 761-767.

Worden, B.D., Parker, P.G. 2005 Females prefer noninfected males as mates in the grain beetle *Tenebrio molitor*: evidence in pre- and postcopulatory behaviours. *Animal Behaviour* **70**, 1047-1053.

Zanchi, C., Troussard, J.P., Martinaud, G., Moreau, J., Moret, Y. 2011 Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. *Journal of Animal Ecology* **80**, 1174-1183.

Zeh, D.W., Smith, R.L. 1985 Paternal investment by terrestrial arthropods. *American Zoologist* **25**, 785-805.

Zhao, P., Li, J., Wang, Y., Jiang, H. 2007 Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by *Manduca sexta* phenoloxidase. *Insect Biochemistry and Molecular Biology* **37**, 952-959.

Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M. 2009 Mixed Effects Models and Extensions in Ecology with R. *Springer*, New York.

Annexes

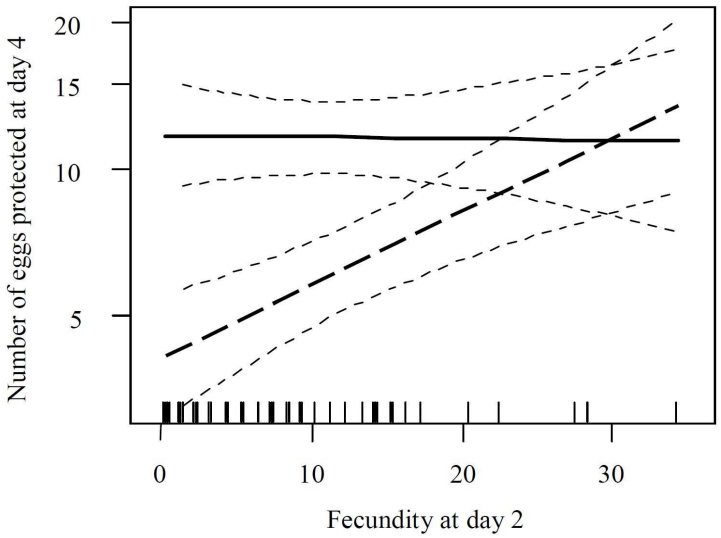


Figure S 1 : Response curves of the GLM on the number of eggs protected by control (dashed line) and LPS-treated females (solid line) between the days 2 and 4 post immune challenge according to the past fecundity (between day 0 and day 2).
The thin broken lines correspond to 95% Bayesian confidence limits for control and LPS-treated females. Ticks on the x-axis indicate the locations of the observations.

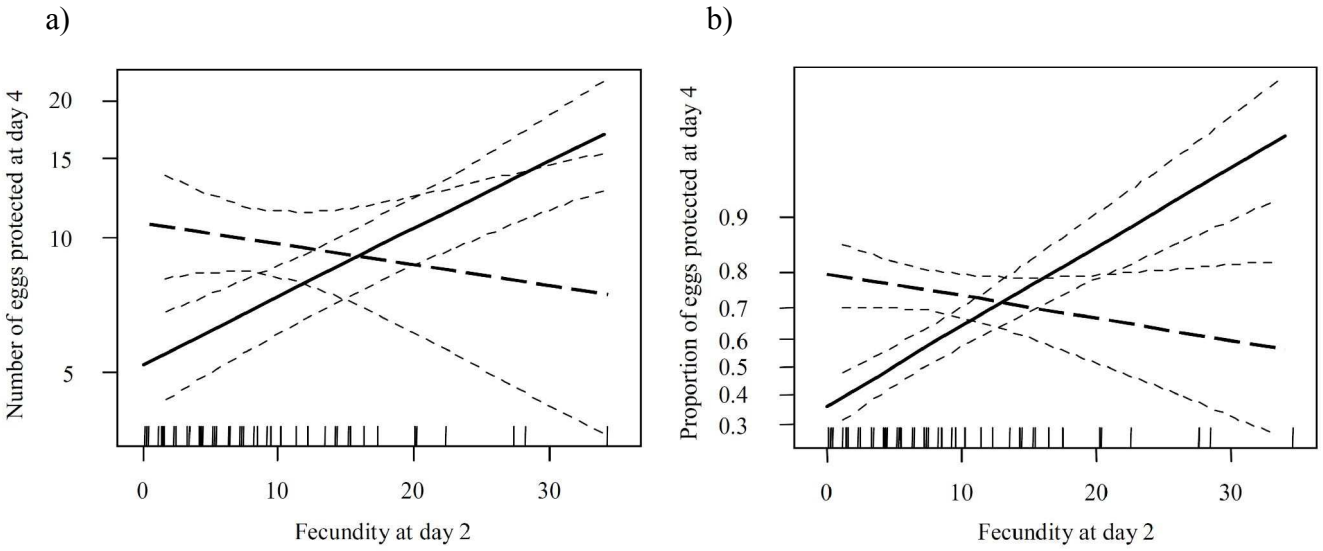


Figure S 2 : Response curves of the (a) GLM on the number and (b) the proportion of eggs protected by females from poor food condition (dashed line) and good food condition (solid line) between the days 2 and 4 post immune challenge according to the past fecundity (between day 0 and day 2).
The black broken lines correspond to 95% Bayesian confidence limits for females from poor and good food conditions. Ticks on the x-axis indicate the locations of the observations.

Chapitre 2

The presence of bacteria in the larval medium increased larval survival (see **chapter 2**) and decreased the duration of the first larval intermoult, suggesting a nutritive effect of bacteria on the young larvae.

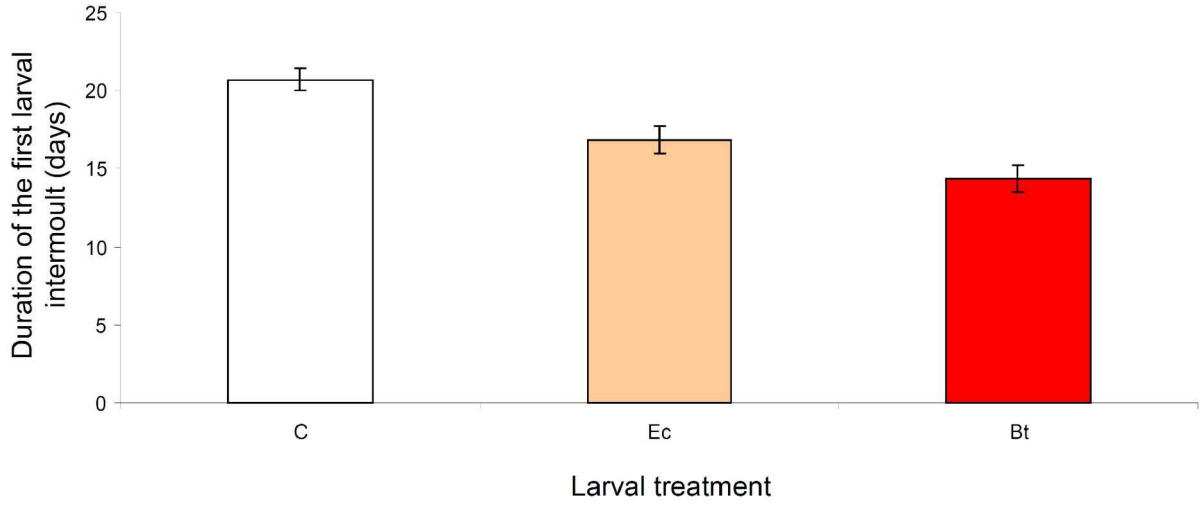


Figure S 3 : Mean duration of the first larval intermoult (in days ± SE) of the larvae from Ringer, *E. coli* and *B. thuringiensis*-injected females, according to the larval treatment (x-axis).

Chapitre 3

Supplementary Table 1 : 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.

	Maternal priming				Paternal priming			
	Control		LPS		Control		LPS	
	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.
Antibacterial	0.82	0.55	6.23	0.8	1.11	0.6	4.25	0.86
Larvae day 0-4	10.3	3.1	5.4	2.4	4.3	1.3	3.4	1.5
Larvae day 4-8	6	2.1	4.6	1.6	3.2	1.4	3.3	1.2
Larvae day 8-12	8.6	3.2	6.9	1.9	9.9	2.3	6.2	2.4
Larvae total	24.9	5.8	16.9	5.1	17.4	3.8	12.9	4.4

Protocoles

Mesures d'activité antibactérienne

Préparation des boîtes de Petri et de la gélose inoculée

1- Réaliser un inoculum d'*Arthrobacter globiformis* dans du milieu Broth stérile (10g de bactotryptone, 5g d'extrait de levure, 10g NaCl, 1000 mL d'eau distillée, pH = 7.5), et l'incuber avec agitation pendant 24h à 28°C.

2- Après 24h, estimer la concentration en bactéries de l'inoculum à l'aide d'un hémocytomètre.

3- Réaliser une solution de Broth + agar à 1g/L et l'autoclaver (120°C pendant 20 min).

4- A la fin du cycle d'autoclave, placer la solution de Broth + agar au bain marie à 45°C afin d'éviter sa gélification. Attendre environ 2h.

5- Ajuster la concentration en *A. globiformis* de manière à obtenir une concentration finale d'environ 10^5 bactéries/mL.

6- Couler 6mL de solution de Broth + agar + *A. globiformis* dans chaque boîte de Petri.

Les boîtes peuvent être conservées au réfrigérateur pendant 1 semaine.

Réalisation du test

1- Décongeler les échantillons sur de la glace.

2- Réaliser des puits de 2mm de diamètre à l'aide d'une pipette pasteur (stérilisée par flambage) surmontée d'une poire à pipeter.

3- Déposer 2µL de l'échantillon à tester par puits.

4- Entourer les boîtes de Petri de film plastique pour éviter leur déshydratation, et les incuber pendant 24h à 28°C.

5- 24h après, noter la présence (ou pas) d'une zone d'inhibition et mesurer son diamètre.

Mesures d'activité phénoloxydase et prophénoloxydase

Solutions
Sodium Cacodylate (0.01M cacodylate de sodium, 0.005 $CaCl_2$, pH = 6.5)
PBS (8.74g NaCl, 1.78g Na2HPO42H2O, 1000 mL d'eaeu distillée, pH = 6.5)
L-Dopa (Sigma D 9628 □4 mg/mL d'eau distillée passée au bac à ultrasons et filtrée)
Chemotrypsine (Sigma C 7762 □0.07 mg/mL d'eau distillée)

Matériel
Microplaques Elisa 96 puits
Spectrophotomètre Versamax (lecture de la densité optique à 492 nm)
Centrifugeuse (centrifugation à 1300 g pendant 5 minutes à 4°C)
Filtre (pores de 0.2 µm)

Méthode
1- Décongeler les échantillons sur de la glace

2- Centrifuger les échantillons (4°C pendant 5min, 5000 rpm)

3- Prélever 5 µL de l'échantillon et le déposer dans un puits d'une plaque Elisa maintenue au frais sur de la glace

4- Pour doser la PO, déposer dans chaque puits 140 µL d'eau distillée et 20 µL de PBS. Pour doser la PPO, remplacer l'eau distillée par la solution de chemotrypsine.

5- Déposer 20 µL par puits de solution de L-Dopa

6- Lire la densité optique au spectrophotomètre à 490 nm et à 30°C, à raison d'une mesure toutes les 15 secondes pendant 40 minutes, avec agitation avant et après chaque lecture.

7- L'activité enzymatique dans chaque puits correspond à la phase linéaire de la réaction (R^2>0.7).

Oikos 121: 1828–1832, 2012
doi: 10.1111/j.1600-0706.2011.19933.x

Subject Editor: Hamish McCallum. Accepted 7 December 2011

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,

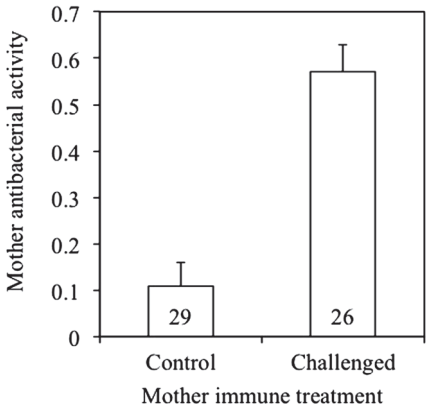


Figure 1. Antibacterial activity (mean + 1 SE) expressed as the natural logarithm value of the diameter (in cm) of a zone of bacterial growth inhibition of the haemolymph of control and immune-challenged mothers. Numbers inside bars represent the number of mothers assayed.

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

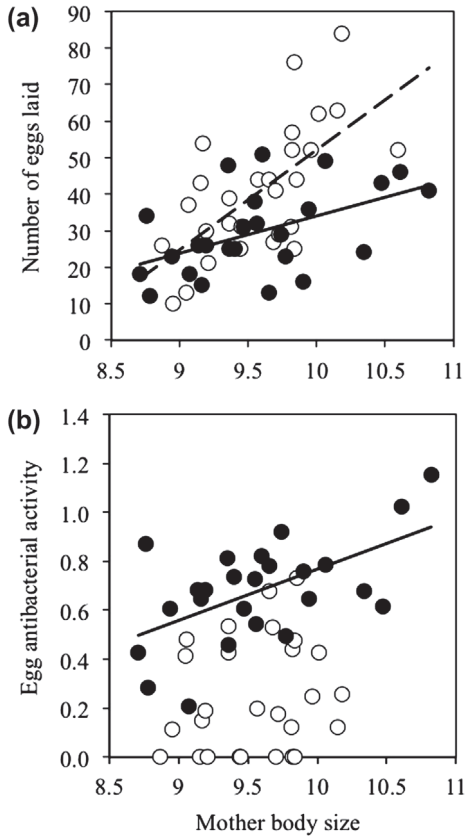


Figure 2. Variation of (a) female fecundity expressed as the number of eggs laid within the four days period by each female and (b) egg antibacterial activity expressed as the natural logarithm value of the diameter (in cm) of a zone of bacterial growth inhibition of 2 pairs of eggs as a function of 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 ($F_{1,27} = 0.07$, $p = 0.788$).

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

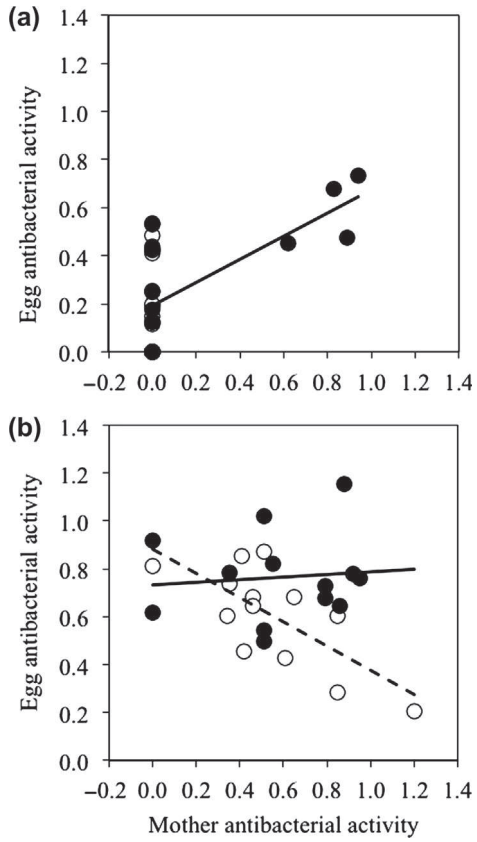


Figure 3. Relationships between the antibacterial activity expressed as the natural logarithm value of the diameter (in mm) of a zone of bacterial growth inhibition of the eggs and that of the haemolymph of large (> median 9.51 mm: filled circles) and small (≤ median 9.51 mm: open circle) (a) control 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.

Acknowledgements – We thank M. A. F. Gillingham, M. J. F. Brown, T. Rigaud and H. McCallum for valuable comments on previous versions of this manuscript. Funding was provided by the CNRS and the ANR (ANR-07-JCJC-0134 and ANR-08-JCJC-0006).

References

Adamo, S. A. 1999. Evidence for adaptive changes in egg laying crickets exposed to bacteria, and parasites. – Anim. Behav. 57: 117–124.

Bascuñán-García, A. P. et al. 2010. Immune investment impairs growth, female reproduction and survival in the house cricket, *Acheta domesticus*. – J. Insect Physiol. 56: 204–211.

Carazo, P. et al. 2004. Chemosensory cues allow male *Tenebrio molitor* beetles to assess the reproductive status of potential mates. – Anim. Behav. 68: 123–129.

Clutton-Brock, T. H. 1984. Reproductive effort and terminal investment in iteroparous animals. – Am. Nat. 123: 212–229.

Cotter, S. C. et al. 2010. Age-specific reproductive investment in female burying beetles: independent effects of state and risk of death. – Funct. Ecol. 25: 652–660.

Grubbs, F. E. 1969. Procedures to for detecting outlying observations in samples. – Technometrics 11: 1–21.

Haine, E. R. et al. 2008a. Temporal patterns in immune responses to a range of microbial insults (*Tenebrio molitor*). – J. Insect Physiol. 55: 1090–1097.

Haine, E. R. et al. 2008b. Antimicrobial defense and persistent infection in insects. – Science 322: 1257–1259.

Hasselquist, D. and Nilsson, J.-Å. 2008. Maternal transfer of antibodies in vertebrates: trans-generational effects on offspring immunity. – Phil. Trans. R. Soc. B 364: 51–60.

Jokela, J. 2010. Trangenerational immune priming as a cryptic parental care. – J. Anim. Ecol. 79: 305–307.

Linder, J. E. and Promislow, D. E. L. 2009. Cross-generational fitness effects of infection in *Drosophila melanogaster*. – Fly 3: 143–150.

Little, T. J. et al. 2003. Maternal transfer of strain-specific immunity in an invertebrate. – Curr. Biol. 13: 489–492.

Marchini, D. et al. 1997. Presence of antibacterial peptides on the laid egg chorion of the medfly *Ceratitis capitata.* – Biochem. Biophys. Res. Commun. 240: 657–663.

Moret, Y. 2006. 'Trans-generational immune priming': specific enhancement of the antimicrobial immune response in the mealworm *Tenebrio molitor*. – Proc. R. Soc. B 273: 1399–1405.

Moret, Y. and Schmid-Hempel, P. 2000. Survival for immunity: the price of immune system activation for bumblebee workers. – Science 290: 1166–1168.

Quinn, G. P. and Keough, M. J. 2002. Experimental design and data analysis for biologists. – Cambridge Univ. Press.

Reaney, L. T. and Knell, R. J. 2010. Immune activation but not male quality affects female current reproductive investment in a dung beetle. – Behav. Ecol. 21: 1367–1372.

Reznick, D. et al. 2000. Big houses, big cars, superfleas and the cost of reproduction. – Trends Ecol. Evol. 15: 421–425.

Roth, O. et al. 2010. Paternally derived immune priming for offspring in the red flour beetle, *Tribolium castaneum.* – J. Anim. Ecol. 79: 403–413.

Sadd, B. M. and Schmid-Hempel, P. 2007. Facultative but persistent trans-generational immunity via the mother's eggs in bumblebees. – Curr. Biol. 17: R1046–R1047.

Sadd, B. M. and Schmid-Hempel, P. 2009. A distinct infection cost associated with trans-generational immune priming of antibacterial immunity in bumblebees. – Biol. Lett. 5: 798–801.

Sadd, B. M. et al. 2005. Transgenerational-immune priming in a social insect. – Biol. Lett. 1: 386–388.

Schmid-Hempel, P. 2003. Variation in immune defence as a question of evolutionary ecology. – Proc. R. Soc. B 273: 2571–2574.

Shoemaker, K. L. et al. 2006. Egg-laying behaviour following infection in the cricket *Gryllus texensis*. – Can. J. Zool. 84: 412–418.

Siva-Jothy, M. T. et al. 2001. Investment in immune function under chronic and acute immune challenge in an insect. – Physiol. Entomol. 26: 1–5.

Thornhill, R. and Alcock, J. 1983. The evolution of insect mating systems. – Harvard Univ. Press.

Tzou, P. et al. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia. – Immunity 13: 737–748.

Vorburger, C. et al. 2008. Limited scope for maternal effects in aphid defence against parasitoids. – Ecol. Entomol. 33: 189–196.

Zanchi, C. et al. Differential expression and costs between maternally and paternally derived immune priming for offspring in an insect. – J. Anim. Ecol. 80: 1174–1183.

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